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REVIEW

Microbial chassis design and engineering for production of amino acids used in food industry

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Abstract

Rational microbial chassis design and engineering for improving production of amino acids have attracted a considerable attention. L-glutamate, L-lysine, L-threonine and L-tryptophan are the main amino acids demanded in the food industry. Systems metabolic engineering and synthetic biology engineering generally are believed as the comprehensive engineering approaches to obtain rationally designed strains and construct high-performance platforms for amino acids. The strategies focus on microbial chassis characterization optimization, precise metabolic engineering such as promoter engineering, modular pathway engineering, transporter engineering, and dynamic switch systems application, and global genome streamline engineering to reduce cell burden. In this review, we summarized the efficient engineering strategies to optimize *Corynebacterium glutamicum* and *Escherichia coli* cell factories for improving the production of L-glutamate, L-lysine, L-threonine, and L-tryptophan.

Keywords Microbial chassis · Amino acids · Food industries · Synthetic biology · Metabolic engineering · *Escherichia coli* · *Corynebacterium glutamicum* · L-Glutamate · L-Lysine · L-Threonine · L-Tryptophan

Abbreviations

PTS	Phosphotransferase system
Glc	Glucose
PP pathway	Pentose phosphate pathway
TCA cycle	Tricarboxylic acid cycle
ATP	Adenosine triphosphate
G6P	Glucose 6-phosphate
R5P	Ribulose 5-phosphate
F6P	Fructose 6-phosphate
F1P	Fructose 1-phosphate
FBP	Fructose 1,6-bisphosphate
E4P	Erithrose 4-phosphate
G3P	Glucose 3-phosphate
Acetyl-CoA (AcCoA)	Acetyl-coenzyme A
α-KG	α-Ketoglutaric acid
Ser	Serine
Gly	Glycine
Cys	Cysteine
Trp	Tryptophan
Phe	Phenylalanine
Tyr	Tyrosine
Val	Valine
Leu	Leucine
Lys	Lysine
Thr	Threonine
Ile	Isoleucine
Gln	Glutamine
Glu	Glutamate
Arg	Arginine
Pro	Proline
GABA	γ-Amino butyric acid
GMCM	Glucose monocorynomycolate
Keto-GMCM	Keto-glucose monocorynomycolate
MA	Mycolic acid

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L-Asp	L-aspartic acid
L-Aspartyl-P	L-aspartyl-phosphate
PEP	Phosphoenol pyruvate
OAA	Oxaloacetate
Pyr	Pyruvate
PHB	Poly-3-hydroxybutyrate
DAHP	3-Deoxy-darabino-heptulosonate 7-phosphate
GAP	Glyceraldehyde 3-phosphate
DHQ	3-Dehydroquinate
DHS	3-Dehydoshikimate
SA	Shikimic acid
S3P	Shikimate 3-phosphate
SHK	Shikimic acid kinase
EPSP	5-Enolpyruvateshikimate 3-phosphate
CHA	Chorismic acid
ANT	Anthranilate
PRA	Phosphoribosyl-anthranilate
CDRP	Carboxyphenylamino deoxyribulose phosphate
IGP	Indole glycerol phosphate
HPr	Histidine phosphorylatable protein
EI	Enzyme I of PTS
EII	Enzyme II of PTS

Introduction

Amino acids in food industrial have numerous applications, and the market demand for them is likely to grow. L-glutamate, L-lysine, L-threonine, and L-tryptophan are the main food industry fermented amino acids in the market demand [1]. These amino acids are the main precursors for many other important chemical products. For examples, and L-glutamate is the precursor for poly-(L-glutamic acid) (PLGA) which is a hydrogel material that is hydrophilic, biocompatible, biodegradable, and non-immunogenic [2], and L-glutamate is the direct precursor of γ-amino butyric acid (GABA) which is also the monomer of nylon 4 [3]; L-lysine can be further converted into valuable chemicals such as poly(L-lysine)-based structures as novel antimicrobials for diabetic foot infections [4], 1,5-diamino-2-hydroxy-pentane as a new type of aliphatic amino alcohol [5], GABAb antagonist 5-aminovalleric acid (5-AVA) [6], glutaric acid [7, 8], *cis*-3-hydroxypipecolic acid as a key structural component of tetrapeptide antibiotic GE81112 [9], and so on; the L-threonine could be converted to 2-hydroxybutyrate, one of the important monomers of polyhydroxyalkanoates (PHAs), through sequential reactions of several enzymes [10–12]; the L-tryptophan can be a potential precursor for melanin synthesis in life forms [13]. Therefore, optimizing the production of L-glutamate, L-lysine, L-threonine, and L-tryptophan are crucial.

The microbial chassis fermentation is the main efficient approach for amino acids production [14–16], because excellent microbial cell factories own economical and environmentally friendly. Therefore, development of rational design and efficiently engineering strategies increasingly attracting a considerable attention [15, 17]. Exploiting the inherent cellular synthetic capacity of a microorganism is an efficient strategy for achieving amino acids production. To enhance the synthetic capacity of microorganisms, systems metabolic engineering and synthetic biology approaches have been successfully applied in the microbial production of amino acids, including microbial chassis characterization optimization, precise metabolic engineering such as promoter engineering, modular pathway engineering, transporter engineering, and dynamic switch systems application to maximize metabolic flux to target amino acid, and global genome streamline to reduce cell burden. Although these engineering strategies are highly effective, improving the synthetic capacity remains a major challenge, due to the lack of adequate understanding of complex cellular regulations and difficulties for maximization of conversion rate from substrates. In fact, more and more high-performance microbial cell factories have been constructed. In this review, recent technical advances improving the production of food amino acids are discussed. In addition, existing challenges and potential strategies for increasing the output of these amino acids are described [14].

The microbial production of amino acids used in food industries is a large area where synthetic biology and systems metabolic engineering strategies have been successfully applied, mainly in two important producing microorganisms: *Corynebacterium glutamicum* and *Escherichia coli*. According to the market demand, the amino acids produced by fermentation and used in the food industries mainly include L-glutamate (3,210,000 tons), L-lysine (2,600,000 tons), L-threonine (700,000 tons), L-tryptophan (41,000 tons), L-arginine (1200 tons), L-valine (500 tons), L-leucine (500 tons), and L-isoleucine (400 tons) [1]. L-Glutamate is the major bulk amino acid covering nearly two thirds of the amino acid market. Then, the market demand of L-lysine ranks just next to L-glutamate and is mainly produced by microbial fermentation employing mutant strains of bacteria, such as *Corynebacterium* sp. and *Escherichia* sp. [1]. The L-threonine is currently the third amino acid produced by microbial fermentation second only to L-glutamate and L-lysine. Unlike the L-glutamate and L-lysine produced by fermentation of *C. glutamicum*, L-threonine and L-tryptophan are mainly produced by *E. coli* [18]. While, the production

of L-arginine, L-valine, L-leucine, and L-isoleucine by *C. glutamicum* or *E. coli* fermentation is still at a low level.

Systems metabolic engineering and synthetic biology generally are believed as the comprehensive engineering approaches to obtain rationally designed strains and construct high-performance platforms for amino acids. Synthetic biology emphasizes design and redesign to build new biological systems, which often brings unexpected effects. Combining the advantages of different approaches, it is promising to dig out the maximum potentials of microbial cell factories. Therefore, understanding the reported new strategies are important for establishing new design ideas and efficient approaches to optimize microbial cell factories. In this review, engineering strategies for the main amino acids including L-glutamate, L-lysine, L-threonine, and L-tryptophan used in food industries are summarized, mainly focusing on the two major industrial production microorganisms: *C. glutamicum* and *E. coli*.

Brief summary of microbial chassis design and engineering strategies for amino acids production

Amino acid production by fermentation is a success story of biotechnology. In *C. glutamicum* and *E. coli*, amino acids are generally synthesized from intermediate metabolites derived from glycolysis, pentose phosphate pathway and TCA cycle (Fig. 1). As shown Fig. 1, the intermediate oxaloacetate (OAA), α-ketoglutarate (α-KG), and 4-phosphate-erythrose (E4P) are the precursors of L-lysine and L-threonine, L-glutamate and L-tryptophan, respectively. Therefore, microbial chassis design and engineering is an emerging discipline that combines the concepts of synthetic engineering, systems' metabolic engineering and evolutionary engineering [17]. With the development of high-throughput technologies, synthetic biology has become much more mature and applicable, and has already manifested its giant potential in providing genome-wide information and clues for engineering. Various strain breeding approaches have been developed, whilst genetically defined metabolic strategies have gradually taken the place of the conventional random mutagenesis-selection method and become the mainstream.

Recent reports have described metabolic engineering methods for the rational design of amino acid-producing host cells [16]. Common strategies for the design of amino acid-producing strains are (i) enhancement of biosynthesis pathway enzymes for the target amino acids, (ii) enhancement of the precursors synthesis for target amino acid and reduction of the precursors consumption, (iii) reduction of by-products formation, (iv) release of feedback regulation of key enzymes by the target amino acid, (v) increased or replaced the supply of reducing equivalents such as NADPH and ATP, (vi) optimize the uptake of carbon source, (vii) increased export

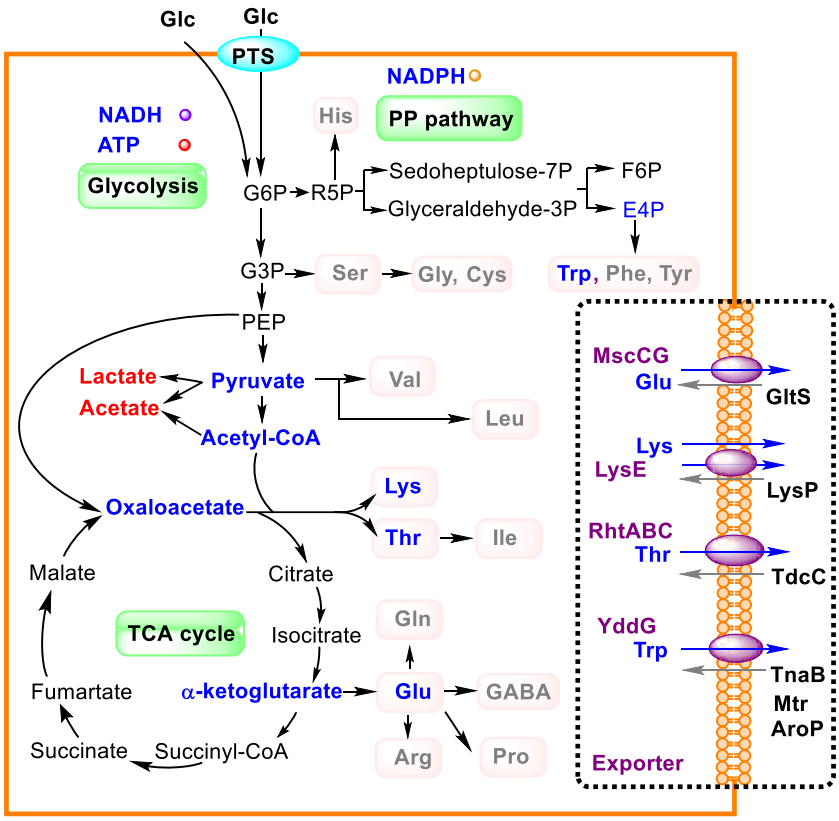

Fig. 1 The biosynthesis of amino acids L-glutamate, L-lysine, L-threonine, and L-tryptophan in *C. glutamicum* and *E. coli*

of target amino acids out of the cells. However, because of cellular metabolic complexity, metabolic engineering modifications often resulted in unbalance between cell growth and chemicals production, or unbalanced distribution of metabolic flux, which increasingly become the major limitation for further improving amino acids production. To dissolve this, researchers created rational dynamic switch regulation systems aimed at realizing the production efficiency maximum of the target amino acids from glucose or other substrates [19]. In addition, genome reduction has been applied to *C. glutamicum* and *E. coli* to improve amino acids [15], e.g. for lysine production strain *C. glutamicum* GRLys1 [20, 21]; and for L-threonine production strains *E. coli* MGF-01 [22] and *E. coli* MDS42 [23]. In addition, our recent studies suggested that cell envelope simplification also influence intracellular metabolism, which showed potential advantages for amino acids production [24–26], especially for the L-glutamate [27] and L-threonine [28, 29]. Omics-based metabolic engineering techniques and various evolution approaches when no obvious target genes are known also inspire us to design new engineering strategies [26, 27, 30]. Furthermore, several studies suggested that the introduction of gene cluster *phaCAB* for poly-3-hydroxybutyrate (PHB) biosynthesis from acetyl-CoA facilitates the amino acids production including L-glutamate [31], L-threonine [32], and L-tryptophan [33], which suggested that stimulating cellular carbon flux to acetyl-CoA benefits amino acids biosynthesis. In this review, the detailed rational design and engineering strategies of microbial chassis for L-glutamate, L-lysine, L-threonine, and L-tryptophan production are analyzed and discussed below.

The amino acids derived from the intermediates of glycolysis, TCA cycle and PP pathway. L-Glutamate derives from α-ketoglutarate and exported by McsCG, L-lysine and L-threonine derive from OAA, and exported by LysE and RhtABC, respectively, and L-tryptophan derives from E4P and exported by YddG. PTS: phosphotransferase system; Glc: glucose; PP Pathway: pentose phosphate pathway; TCA cycle: tricarboxylic acid cycle; ATP: adenosine triphosphate; G6P: glucose 6-phosphate; R5P: ribulose 5-phosphate; F6P: fructose 6-phosphate; F1P: fructose 1-phosphate; FBP: fructose 1,6-bisphosphate; E4P: erithrose 4-phosphate; G3P: glucose 3-phosphate; Acetyl-CoA (AcCoA), acetyl-coenzyme A; PEP: phosphoenol pyruvate; Ser: serine; Gly: glycine; Cys: cysteine; Trp: tryptophan; Phe: phenylalanine; Tyr: tyrosine; Val: valine; Leu: leucine; Lys: lysine; Thr: threonine; Ile: isoleucine; Gln: glutamine; Glu: glutamate; Arg: arginine; Pro: proline; GABA: γ-amino butyric acid; L-Asp: L-aspartic acid; L-Aspartyl-P: L-aspartyl-phosphate.

Microbial chassis design and engineering for production of L-glutamate

L-Glutamate, as the most demanded in the global market, is one of the most intensely studied food ingredients in the food supply and has been found safe [34]. The production of L-glutamate is mainly based on the fermentation of *C. glutamicum* and reaches up to 130 g/L [35, 36]. In *C. glutamicum*, L-glutamate derives from 2-oxoglutarate of TCA cycle under the catalysis of glutamate dehydrogenase (GDH). For synthesis of 1 mol of L-glutamate from 1 mol of glucose, 2 mol of NADH are generated and 2 mol of NADPH are consumed. Current findings are correlated with the change of intracellular metabolism [17, 37] and the structural and functional variation of cell envelope [27, 38, 39] (Fig. 2). To improve L-glutamate production, metabolic engineering strategies including metabolic pathway engineering, regulatory engineering, transport system engineering and in *C. glutamicum* were established [17, 40] (Fig. 2 and Table 1). Meanwhile, glycerol-auxotrophic, fatty acid-auxotrophic, temperature-sensitive and mycolate defective *C. glutamicum* strains have been constructed [27, 41, 42].

For intracellular metabolic pathway engineering, the enhancement of the anaplerotic pathway of the PEP-pyruvate-OAA node has been proved to be an effective approach to increase the carbon flux for L-glutamate. As PEP is needed for the anaplerotic pathway, the replacement of the PEP-dependent phosphotransferase system (PTS) with non-PTS could save more PEP. The introduction of the key non-oxidative glycolytic (NOG) pathway enzyme, phosphoketolase (PKT) with (T2A/I6T/H260) with improved specific activity, into heterologous *C. glutamicum* Z188 resulted in 16.67% improvement in L-glutamate titer, compared with the wild-type BA-PKT [43]. Then, the overexpression of *pyc* gene encoding PEP carboxylase and pyruvate carboxylase (PCx) in L-glutamate producer strains increased the supply of OAA for L-glutamate synthesis and decreased byproduct excretion at the pyruvate node [44]. Meanwhile, Yao et al. reported that double disruption of *dtsR1* (encoding a subunit of acetyl-CoA carboxylase complex) and *pyc* (encoding pyruvate carboxylase) caused increased activity of phosphoenolpyruvate carboxylase (PEPC) encoded by *ppc*, then facilitated efficient overproduction of L-glutamate in *C. glutamicum* ATCC13032 [45]. The above two strategies suggested that the increased OAA benefit L-glutamate production. Besides, the decrease in the 2-oxoglutarate dehydrogenase complex (ODHC, encoded by *odhA*) activity and the simultaneous increase of GDH (encoded by *gltAB*) activity is crucial and essential for the L-glutamate production [37]. In addition, the deletion of gene *ldhA*

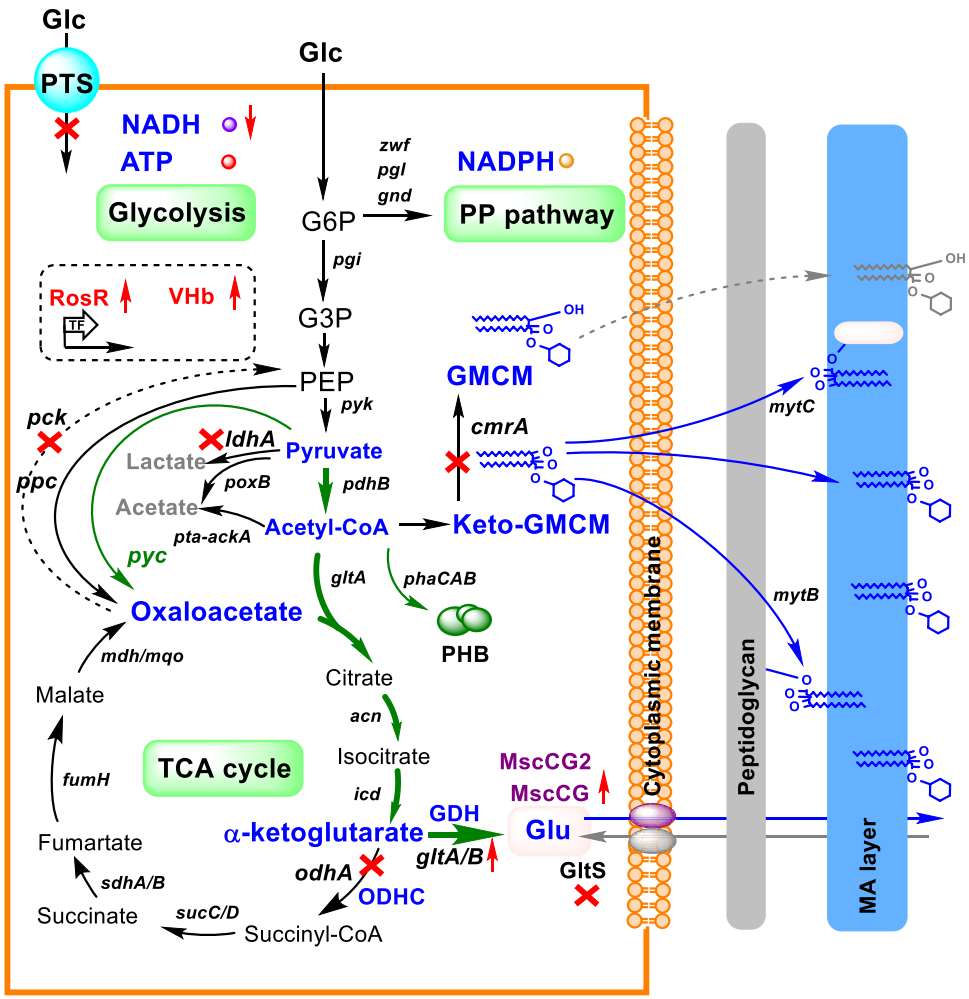

Fig. 2 The design and engineering strategies for L-glutamate production in *C. glutamicum*

responsible for the accumulation of the byproduct L-lactate made 11.61% higher L-glutamate (70.7 g/L) and 58.50% lower L-alanine production in *C. glutamicum* [46].

Additionally, rational engineering regulation factors could efficiently improve L-glutamate. The *gdh* and *gltBD* genes encoding L-glutamate dehydrogenase and L-glutamate synthase, respectively, are negatively regulated by ArgR, the expression of *gdh* is controlled by the transcription regulator FarR (fatty acyl-responsive regulator), accordingly, it was suggested that the ArgR protein in combination with FarR of *C. glutamicum* contributes to the transcriptional control of L-glutamate biosynthesis pathways [15]. Li et al. identified a crucial transcription factor RosR in *C. glutamicum* G01 and demonstrated that RosR regulated L-glutamate metabolic network by binding to the promoters of *glnA*, *pqo*, *ilvB*, *ilvN*, *ilvC*, *ldhA*, *odhA*, *dstr1*, *fas*, *argJ*, *ak*, and *pta*, then overexpression of RosR in G01 resulted in significantly decreased by-products yield and improved L-glutamate titer (130.6 g/L) and yield (0.541 g/g from glucose) in fed-batch fermentation [36], which provided a good reference not only for other *C. glutamicum* platforms to further improve L-glutamate production but also for optimizing other amino acid biosynthesis in *C. glutamicum* strains.

For transport system engineering, L-glutamate synthesized intracellularly is exported through mechanosensitive transmembrane channel proteins (MscCG and MscCG2, encoded by *mscCG* and *mscCG2*) activated by the force-from-lipids [47, 48], and the sodium-coupled secondary L-glutamate uptake system encoded by *gltS* has also been defined [49]; the studies showed that the overexpression of *mscCG* or *mscCG2* but deletion of *gltS* could all increase L-glutamate [47–49]. Notably, a high capacity export system capable of expelling the surplus of synthesized L-glutamate could be activated by higher temperature to 39 °C [41]. The higher temperature results in a rapid attenuation

Table 1 Strategies for improving L-glutamate production in *C. glutamicum*

Strains	Strategies	L-glutamate production	Refs.
C. glutamicum GDK-9Δ*ldhA* (GDK-9 is an L-glutamate-producing strain)	Deletion of gene *ldhA* responsible for the accumulation of the byproduct L-lactate	120 g/L	[46]
C. glutamicum Z188Δ*pfk*/PKT(T2A/I6T/H260) (Z188 is an L-glutamate-producing strain)	Establishing a growth-coupled evolution strategy for the enrichment and selection of phosphoketolases (PKT) mutants with improved specific activity in *C. glutamicum* hosts with defective 6-phosphofructokinase (PFK)	18 g/L (24-deep-well plates); 0.36 g/(g glucose)	[43]
C. glutamicum G01/RosR (G01 is an L-glutamate producer and isolated from soil)	Overexpression of RosR in G01	130.6 g/L; 0.541 g/(g glucose)	[36]
C. glutamicum CN1021/pXMJ19*pyc*(CN1021 is an L-glutamate producer strain triggered by a temperature shock)	Introducing a temperature shock, and overexpressing *pyc*	128 g/L; 0.51 g/(g glucose)	[44]
C. glutamicum PHB	Introducing polyhydroxybutyrate (PHB) synthesis genes, *phbCAB* from *Ralstonia eutropha* under the *Ptrc* promoter	37 g/L	[31]
C. glutamicum ATCC13032 Δ*dtsR1*Δ*pyc*	Double disruption of *dtsR1* (encoding a subunit of acetyl-CoA carboxylase complex) and *pyc* (encoding pyruvate carboxylase)	14.0 g/L (flask fermentation); 0.63 g/(g glucose)	[45]
C. glutamicum ATCC13869 Δ*cmrA*	Deleting the gene *cmrA* encoding the ketoacyl reductase in the biosynthetic pathway of mycolate	13.14 g/L (flask fermentation)	[27]

in oxoglutarate dehydrogenase complex (ODHC) activity and an increase from 28% to more than 90% of the isocitrate dehydrogenase flux split toward glutamate synthesis [41]. Therefore, a mutation in the *C. glutamicum ltsA* gene responsible for temperature-sensitive growth facilitated higher L-glutamate production at higher temperatures [42]. Further study suggested that the fluidity of the *C. glutamicum* mycomembrane plays an important role in glutamate excretion during the temperature-triggered process [50].

Notably, researchers established several efficient strategies based on synthetic biology approaches to improve L-glutamate production. Lin et al. established an efficient and stable microfluidic artificial photosynthetic system for reduced nicotinamide adenine dinucleotide (NADH) regeneration and L-glutamate synthesis under visible light, resulting that the NADH regeneration rate of the system reached 56.03%, the maximum production rate of L-glutamate was 98.3% [51]. Chen et al. found that the L-glutamate production could be increased 39–68% by the expression of PHB synthesis genes in *C. glutamicum*, with less intermediate metabolites or by-products including α-ketoglutarate, L-glutamine and lactate [31]. The overexpression of *vgb* gene encoding vitreoscilla hemoglobin (VHb) in *C. glutamicum* promoted 23% more L-glutamate production and 30% more cell density [52]. Heterologous expression of the *araBAD* operon from *E. coli* in the wild-type and in an L-lysine producing strain of *C. glutamicum* was shown to enable production of L-glutamate and L-lysine, respectively, from arabinose as sole carbon source [53].

Our recent study suggested that engineering the envelope of *C. glutamicum* could improve the L-glutamate production [27]. *C. glutamicum* has a specific mycolate outer membrane containing mycolic acid (MA) [54]. In *C. glutamicum*, L-glutamate production can be induced by biotin limitation [55], addition of β-lactam antibiotics [38, 56], and addition of fatty acid ester surfactants [57]. These conditions could all lead to decreased mycolic acid (MA) formation, suggesting that MA is related to L-glutamate production. In *C. glutamicum*, the lack of Pks13, which catalyzes the formation of MA, results to MA lack [58]. MA can be attached to glucose, forming keto glucose monoketo-corynomycolate (keto-GMCM), and the keto group of keto-GMCM is then reduced by ketoacyl reductase CmrA (encoded by *cmrA*) to form GMCM (Fig. 2). The *cmrA* mutant reduced the level of MA by 80% [59]. We found that the deletion of *cmrA* in *C. glutamicum* ATCC13869 promoted 10.77-fold more L-glutamate production than the control ATCC13869 [27]. Further transcriptomic analysis showed that the mutant *cmrA* causes the up-regulation of *mscCG* and the down-regulation of the genes relevant to L-arginine biosynthesis. However, the complete block of MA by deleting Pks13 resulted in the hindered cell growth and defective cell separation [58], which is not advisable. It suggested that the reasonable

membrane engineering is a new synthetic biology strategy to design and optimize the *C. glutamicum* chassis.

As shown in Fig. 2 and Table 1, the efficient engineering strategies for L-glutamate focused not only on intracellular metabolism but also on the cell envelope, especially for the enhancement of the anaplerotic pathway of the PEP–pyruvate–OAA node, and the reduction of MA in cell envelope. However, there is still no report so far on a system biology engineering approach to combine these effective strategies for optimizing L-glutamate production in *C. glutamicum*.

The L-glutamate is derived from the intermediate α-ketoglutarate of TCA cycle and exported by McsCG or McsCG2, but uptake by GltS. PTS: phosphotransferase system; Glc: glucose; PP Pathway: pentose phosphate pathway; TCA cycle: tricarboxylic acid cycle; ATP: adenosine triphosphate; G6P: glucose 6-phosphate; G3P: glucose 3-phosphate; Acetyl-CoA (AcCoA), acetyl-coenzyme A; PEP: phosphoenol pyruvate; Glu: glutamate. The gay font and red X represent the interrupted routes. The green arrow represents the enhanced reactions.

Microbial chassis design and engineering for bioproduction of L-lysine

L-Lysine is mainly produced by *C. glutamicum*, and the reported highest production reaches 220 g/L by systematically engineered *C. glutamicum* [60]. Meanwhile, engineered *E. coli* MG1655 mutant could produce 125 g/L of L-lysine in a recent study report [61]. It suggested that microbial fermentation is an excellent approach for L-lysine production in food industry. L-Lysine is derived from OAA of TCA cycle, and OAA is mainly from PEP or pyruvate of glycolysis. One lysine synthesis needs 4 NADPH and 1 ATP, and the supply of succinyl-CoA and L-glutamate, then efflux or exported by LysE to extracellular (Fig. 3). Rational metabolic engineering strategies have been successfully applied in the breeding of L-lysine high-producing strains (Fig. 3 and Table 2). Metabolic engineering strategies to enhance carbon flux to L-lysine biosynthesis pathway are efficient to improve L-lysine production. Oxaloacetate (OAA) and L-glutamate are essential precursors for the biosynthesis of L-lysine. The sufficient biomass, OAA, L-glutamate, succinyl-CoA, and

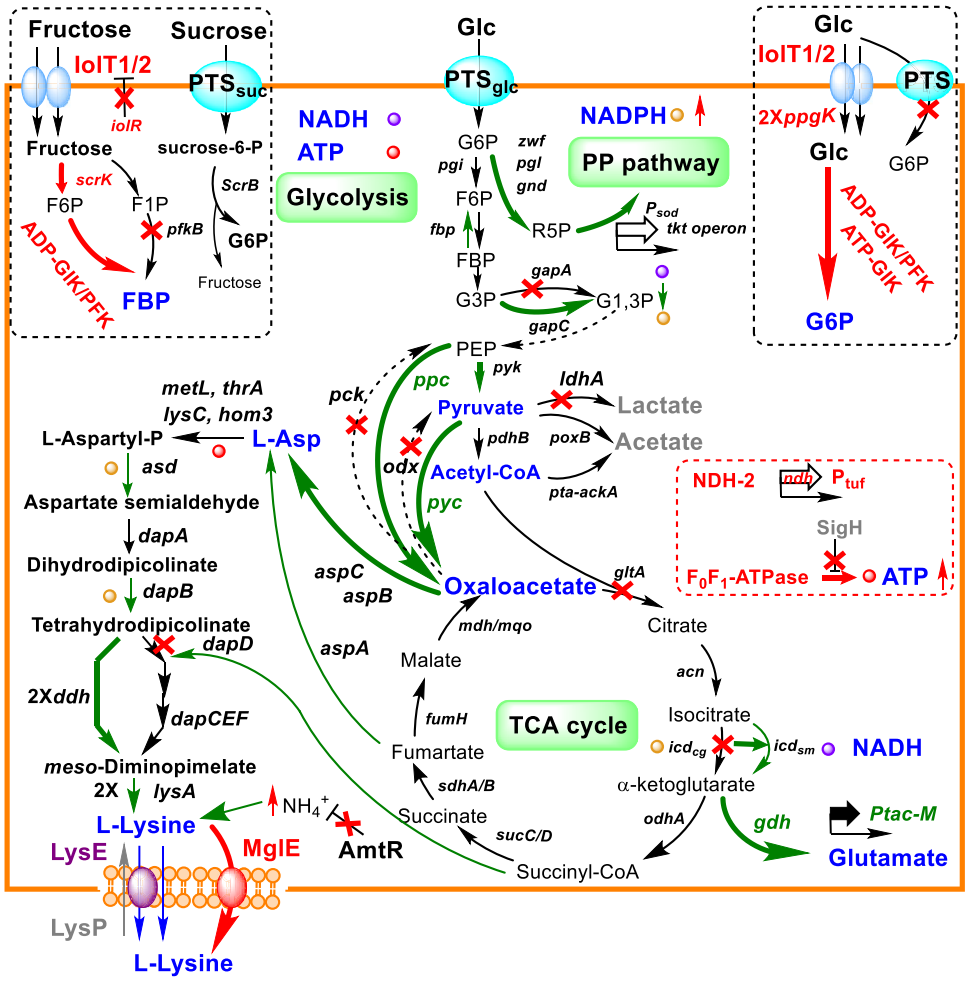

Fig. 3 The design and engineering strategies for L-lysine production in *C. glutamicum*

Table 2 Strategies for improving L-lysine production in *C. glutamicum* and *E. coli*

Strains	Strategies	L-lysine production	Refs.
C. glutamicum LYS-12 (Lysine-producing strain with 12 genome-based modifications: $lysC_{T311I}$ 2*ddh*Δ*pck* P_{sod}-*dapB* 2*lysA* P_{sod}-*lysC* hom_{V59A} P_{sod}-pyc_{P485S} $icd_{GTG-ATG}$ P_{eftu}-*fbp* P_{sod}-*tkt-operon*)	Enhancing the L-lysine biosynthesis pathway, the anaplerotic carboxylation, and the pentose phosphate pathway; and decreasing the flux of counteracting decarboxylating reactions, TCA cycle and the entire anabolism: exchanging *lysC*, *hom*, *pyc*, *icd*, *fbp*, *tkt-operon*, enhancing *ddh*, *dapB*, *lysA*, *lysC*, *pyc*, *fbp*, *tkt-operon*, and deleting *pck*	120 g/L; 0.55 g/(g glucose); 4.0 g/L/h	[64]
C. glutamicum SEA-7 (derived from *C. glutamicum* LYS-12): LYS-12Δ*atlR,* $mtlD_{D75A,}$ *crtEb*:: $mak_{EC(GTG-ATG)}$, *crtB*::$pntAB_{EC}$, *crtI2*:: *gapN* (SMU_676)	Lacking arabitol repressor AtlR and owns engineered L-lysine pathway; redirection of NADH toward NADPH: overexpression of the membrane-bound transhydrogenase *pntAB* together with codon-optimized *gapN*, encoding NADPH-dependent glyceraldehyde 3-phosphate dehydrogenase, and *mak*, encoding fructokinase from *E. coli*	76 g/L L-lysine from mannitol; 2.1 g/L/h; 0.26 mol/mol	[69]
C. glutamicum XQ-5-W4 (derived from XQ-5, and XQ-5 is an lysine producing strain)	Deletion of AmtR to reduce the requirement of NH_4^+, blocking the *dapD* and overexpressing the *ddh* gene	189 g/L; 0.35 g/(g glucose)	[66]
C. glutamicum RGI (derived from strain JL6, which is *C. glutamicum* AECr SDr FPs Metl from CICIM and derived from strain ATCC13032)	Engineering glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and isocitrate dehydrogenase (IDH) to switch the nucleotide-cofactor specificity: modification of the native GAPDH by nonnative NADP-GAPDH; introduction of non-native NAD-IDH, i.e., replacement of the natural *gapA* gene and *icdCg* gene with the Ptac-*gapC-rrnBT1T2* cassette and Ptac-icd_{Sm}-*rrnBT1T2* cassette in strain JL-6 chromosome, respectively	121.4 g/L (85.6 g/L for control); 0.46 g/(g glucose)	[68]
C. glutamicum JL-69Ptac-M *gdh*: *C. glutamicum* JL-6Δ*ppc*::*pck*Δ*pyc*::*odx*ΔP1-*gltA* Ptac-M *gdh*	Enhancing the L-lysine biosynthesis pathway: the genes *ppc* and *pyc* were inserted in the genes *pck* and *odx* loci, the P1 promoter of gene *gltA* was deleted, and the nature promoter of gene *gdh* was replaced by Ptac-M promoter; then suitable addition of 2.4 mg L^{-1} biotin with four times	181.5 g/L; 0.73 g/(g glucose); 3.78 g/L/h	[65]
C. glutamicum ZL-92 ATCC13032P_{iolT1}(113 (A→G) and 112 (C→G)P_{tuf}*iolT2*-2*ppgK*	Rational modification of glucose uptake systems: increasing the participation of non-PTS(Glc) in glucose utilization, increasing the expression level of *iolT1*, *iolT2* and *ppgK*: mutations 113 (A→G) and 112 (C→G) in the promoter of *iolT1*, replacement of natural promoter of genes *iolT2* and *ppgK* by *tuf* promoter, two copies of *ppgK* gene	201.6 g/L; 0.65 g/(g glucose); 5.04 g/L/h	[78]
C. glutamicum strain K-8 (derived from the *C. glutamicum* JL-6Δ*pck::ppc*Δ*odx::pyc*ΔP1*gltA*/Ptac-M *gdh*)	Modification of sugar uptake systems and cofactors supply: Replacement of phosphoenolpyruvate-dependent glucose and fructose uptake system (PTSGlc and PTSFru) by inositol permeases (IolT1 and IolT2) and ATP-dependent glucokinase (ATP-GlK); co-express bifunctional ADP-dependent glucokinase (ADP-GlK/PFK) and NADH dehydrogenase (NDH-2) as well as to inactivate SigmaH factor (SigH)	221.3 g/L L-lysine; 0.71 g/(g glucose); 5.53 g/L/h	[60]

Table 2 (continued)

Strains	Strategies	L-lysine production	Refs.
E. coli LATR11/pWG-$DC^{SM}A^{SM}BH_{c.g}LP$ (LATR11 is an L-lysine producer *E. coli* AEC^{hr} Thr^{-} Rif^{r}, derived from *E. coli* MG1655)	Enhancing the L-lysine biosynthesis pathway: overexpression of *ppc*, $lysC_{T344M}$, *asd*, $dapA_{H56K}$, *dapB*, and *lysA* combined with heterologous expression of *C. glutamicum ddh*	125.6 g/L; 0.59 g/(g glucose); 3.14 g/L/h	[61]
E. coli ec_iML1515 (derived from *E. coli* CCTCCM2019435)	Optimizing the expression of the 20 top-demanded proteins, then adjusting $NH_4^{(+)}$ and dissolved oxygen levels. TN culture solution (L-threonine 10.0 g/L, H_3PO_4 6.6 mL), glucose mother liquor 915 g/L, $(NH_4)_2SO_4$ mother liquor 0.5 g/L	193.6 g/L; 0.74 g/(g glucose)	[81, 83]

fed-batch biotin are important to improve L-lysine production [62, 63], and recent reports further confirmed that the related strategies could effectively improve L-lysine production [60, 61, 64, 65], such as overexpression of the PEP-Pyr-OAA node's genes, decrease of TCA cycle and OAA consumption, decrease in the activity of isocitrate dehydrogenase and citrate synthase, decrease of the pyruvate dehydrogenase complex (PDHC) activity. Reconstructing the diaminopimelic acid (DAP) pathway by deleting the nitrogen source regulatory protein AmtR is also functional for improvement of L-lysine [66]. These studies suggested that promoter engineering is efficient for rational enhancement or weaken of target genes. Here, we summarized L-lysine high-producing strains and their engineering strategies in Fig. 3 and Table 2.

The lysine biosynthetic pathways include two NADPH-dependent reactions that are catalyzed by aspartic semialdehyde dehydrogenase (encoded by *asd*) and dihydrodipicolinate reductase (encoded by *dapB*) [16]. Therefore, rationally optimizing the NADPH supply pathway is another efficient strategy for improving L-lysine. Besides traditionally enhancing PP pathway and NADPH regeneration reactions to improve NADPH supply [64, 67], researchers constructed or introduced some novel reactions to resolve the problem of NADPH limit, for examples, deleting the negative regulator *Cgl2680* for NADPH regeneration [67]; replacing NAD^+-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with $NADP^+$-dependent GAPDH from *Streptococcus* mutans [60, 64, 68]; overexpressing the membrane-bound transhydrogenase *pntAB* together with codon-optimized *gapN*, encoding NADPH-dependent glyceraldehyde 3-phosphate dehydrogenase, and *mak*, encoding fructokinase [69].

Apart from the pathway engineering, researchers found that rationally engineering glucose uptake systems could also functionally improve L-lysine production. The deletion of arabitol repressor AtlR made the L-lysine biosynthesis from mannitol in *C. glutamicum* [69]. Optimizing the expression of Pt dehydrogenase in the *exeR* genome locus with a competitive advantage over common contaminating microbes when grown on media containing Pt as a phosphorus source instead of phosphate could efficiently utilize xenobiotic phosphite (Pt) and establish a more robust fermentation process in *C. glutamicum*, resulting in 41.00 g/L L-lysine under nonsterile conditions [70]. *C. glutamicum* with starch utilization could be constructed by temperature-induced adaptive evolution, optimizing amylases expression, especially co-expression of alpha-amylase (AA) and glucoamylase (GA), and introducing AA-GA fusion protein, resulting 23.9 g/L L-lysine [71]. Rational modification of glucose uptake systems could improve the efficiency of L-lysine production through increasing the participation of non-PTS_{Glc} in glucose utilization [72, 73]. In *C. glutamicum*,

PTS transport systems for glucose, fructose and sucrose are different [74], due to the presence of two additional EII genes *scrB* and NCgl1861 for sucrose and fructose besides that for glucose, respectively [75, 76]. Furthermore, overexpression of inositol permeases (IolT1 and IolT2, encoded by *iolT1*, *iolT2*) [77] and *ppgK* resulted 201.6 g/L of L-lysine production with a productivity of 5.04 g/L/h and carbon yield of 0.65 g/g glucose in fed-batch culture [78]. Then introducing fructokinase from *Clostridium acetobutylicum* for a functional metabolic pathway of sucrose and fructose [75], as well as the replacement of phosphoenolpyruvate-dependent glucose and fructose uptake system including PTS_{Glc} and PTS_{Fru} by inositol permeases (IolT1 and IolT2) with the deletion of negative regulator *iolR*, further resulted a significant improvement of L-lysine production [60].

In addition, the L-lysine production could be enhanced by engineering transport system in *C. glutamicum.* The yield of L-lysine was increased by 9.0%, 12.3%, and 10.0% after the deletion of three amino acid transmembrane transporters, namely, GluE, BrnE/BrnF, and LysP, respectively, in *C. glutamicum* 23,604 [79]. The yield, titer, and the specific production of L-lysine in an industrial *C. glutamicum* strain were enhanced by 7.8%, 9.5%, and 12% by introduction of a novel exporter MglE defined by *Sailesh Malla* based on the construction of Cow fecal metagenomic DNA library [80].

Here, we summarized three typical L-lysine high-production cell platforms *C. glutamicum* LYS-12, *C. glutamicum* strain K-8 and *E. coli* LATR11/pWG-$DC^{SM}A^{SM}BH_{c.g}LP$ to study the rational design and engineering strategies for optimization of cell factories. J. Becker et al. constructed a genetically defined strain *C. glutamicum* LYS-12 hyperproducing 120 g/L L-lysine by systems metabolic engineering, with implementation of only 12 defined genome-based changes in genes encoding central metabolic enzymes redirected major carbon fluxes as desired toward the optimal pathway usage predicted by in silico modeling [64]. The engineered *C. glutamicum* strain K-8 produced highest L-lysine production of 221.3 g/L, with the highest productivity of 5.53 g/L/h and carbon yield of 0.71 g/g glucose in fed-batch fermentation [60]. The K-8 was constructed through four main engineering steps: enhancing the L-lysine biosynthesis pathway with the insertion of genes *ppc* and *pyc* in the genes *pck* and *odx* loci, deletion of the P1 promoter of gene *gltA*, and the introduction of Ptac-M promoter for gene *gdh*; introduction of fructokinase from *Clostridium acetobutylicum* for a functional metabolic pathway of sucrose and fructose; replacement of phosphoenolpyruvate-dependent glucose and fructose uptake system (PTS(Glc) and PTS(Fru)) by inositol permeases (IolT1 and IolT2) and ATP-dependent glucokinase (ATP-GlK); co-expression of bifunctional ADP-dependent glucokinase (ADP-GlK/PFK) and NADH dehydrogenase (NDH-2) as well as to inactivate SigmaH factor (SigH) to reduce the consumption of ATP and increasing ATP regeneration [60].

In addition to *C. glutamicum*, *E. coli* is another important producer for L-lysine, the metabolic engineering strategies in *E. coli* were similar to that in *C. glutamicum*. An L-lysine high-yielding strain was developed from *E. coli* LATR11 via overexpression of *ppc*, $lysC_{T344M}$, *asd*, $dapA_{H56K}$, *dapB*, and *lysA* combined with heterologous expression of *C. glutamicum ddh*, resulting 125.6 g/L of L-lysine with QP of 3.14 g/L/h and glucose conversion rate of 58.97% [61]. Another L-lysine high-producing *E. coli* ec_iML1515 model was constructed by optimizing the expression of the 20 top-demanded proteins, and then adjusting NH4 (+) and dissolved oxygen levels to regulate the synthesis rate of energy metabolism-related proteins, causing lysine titers and glucose yields to increase to 193.6 g/L and 0.74 g/g, respectively. The ec_iML1515 model provides insight into how enzymes required for the biosynthesis of certain products are distributed between and within metabolic pathways [81]. Moreover, using a newly developed high-throughput screening method (fluorescence-activated cell sorting from a 10-million-mutant library generated from a L-lysine high-producing *E. coli* strain) also evolved two L-lysine high-producing mutants MU-1 and MU-2 with 136.51 and 133.2 9 g/L of lysine, respectively [82]. We found that the efficient strategies for designing and constructing excellent microbial chassis for L-lysine mainly were developed in recent reports, which brought a good reference for microbial chassis design and construction for other amino acids production.

The L-lysine is derived from the intermediate OAA of TCA cycle, and exported by diffusion or LysE, but uptake by LysP. PTS: phosphotransferase system; Glc: glucose; PP Pathway: pentose phosphate pathway; TCA cycle: tricarboxylic acid cycle; ATP: adenosine triphosphate; G6P: glucose 6-phosphate; G3P: glucose 3-phosphate; R5P: ribulose 5-phosphate; F6P: fructose 6-phosphate; F1P: fructose 1-phosphate; FBP: fructose 1,6-bisphosphate; E4P: erithrose 4-phosphate; G3P: glucose 3-phosphate; Acetyl-CoA (AcCoA), acetyl-coenzyme A; PEP: phosphoenol pyruvate. The red font and lines represent the extrinsic routes, whereas the gay font and red X represent the interrupted routes. The green arrow represents the enhanced reactions. The blue font represents the crucial immediate for L-lysine production.

Microbial chassis design and engineering for bioproduction of L-threonine

L-Threonine, as a typical oxaloacetate derivative, is an essential amino acid and is produced by *E. coli* and *C. glutamicum* [84]. Rationally engineered *E. coli* producers could produce L-threonine up to 130 g/L, while the *C. glutamicum* mutants produce much lower production, only 11 g/L. L-Threonine

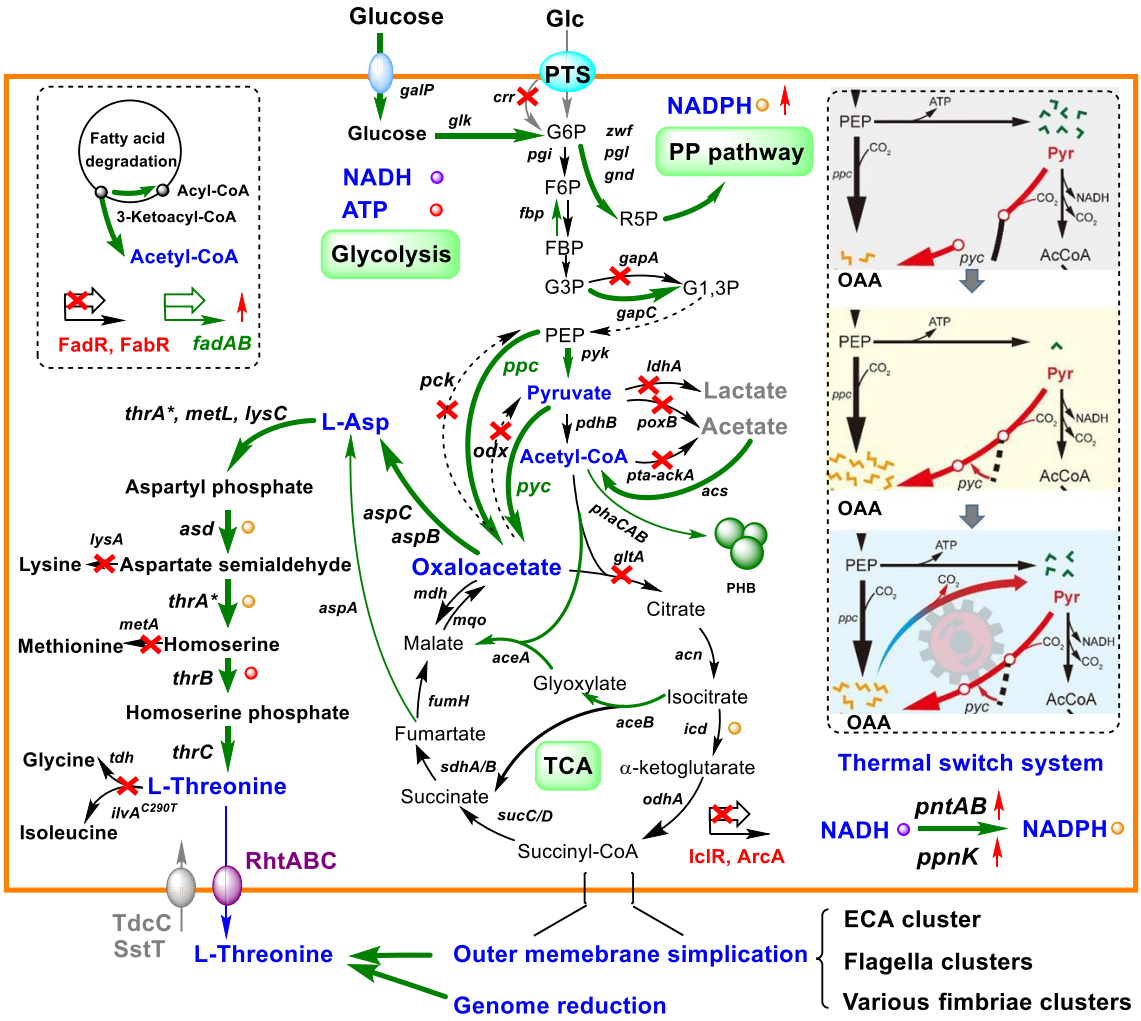

Fig. 4 The design and engineering strategies for L-threonine production in *E. coli*

is biosynthesized from OAA of TCA cycle (Fig. 4), and requires multiple cofactors (NADPH and ATP) [85]. To date, there have been a lot of efforts to improve L-threonine productivity in engineered microbes [86–89].

In *E. coli*, to optimize L-threonine production, researchers tried many efforts on the entire metabolic pathways and regulatory systems, and obtained many excellent L-threonine high producers (Table 3 and Fig. 4). As like the construction of L-lysine producer, developing an L-threonine-producing strain from wild-type *E. coli* generally is focused on modifying the glucose uptake, glyoxylate shunt, L-threonine biosynthetic pathway, regulators, transport system and cofactors supply [90]. Lee et al. constructed the first genetically defined producing strain from the basal strain *E. coli* WL3110, a *lacI*-mutant strain of W3110 [91]. They rewired regulatory and metabolic circuits for the development of an initial threonine producer TH07 (pBRThrABC) by removing the feedback inhibition for *thrA*, *metL*, and blocking the competing pathways of L-threonine by deleting *lysA* and *tdh*, mutant $ilvA_{C290T}$, then overexpressing *ppc* (phosphoenolpyruvate carboxylase), enhancing L-threonine exporter system and the glyoxylate shunt; then reducing the by-products acetic acid and overexpressing the *acs* gene to synthesize more acetyl-CoA, resulting in a final L-threonine producing strain TH28C with an L-threonine titer of 82.4 g/L [91]. The L-threonine production could be improved by elimination of transporters ProP and ProVWX in *E. coli* [92]. In addition, the betaine supplementation also benefits to improve L-threonine production [92, 93].

As description above, metabolic engineering cooperated with metabolic regulation has been developed to maximize production of natural chemicals [94], especially large-volume amino acid products [14, 16]. However, unbalanced cellular metabolic flux distributions between cell growth and target products have long limited product yield and cell productivity [95]. Traditional techniques, such as blocking the bypass pathways and overexpressing the key genes of

Table 3 The strains and strategies for L-threonine production in *E. coli*

Strains	Strategies	L-threonine production	Refs.
E. coli TH28C (pBRThrABCR3): W3110 *DlacI*, *thrA*^{C1034T}, *lysC*^{C1055T}, P*thr*::P*tac*, D*lysA*, D*metA*, *ilvA*^{C290T}, D*tdh*, D*iclR*, P*ppc*::P*trc,* D*tdcC,* P*acs*::Cm^{R}-P*trc*	Enhancing L-threonine production: removing the feedback inhibition for *thrA*, *metL*, blocking the competing pathways of L-threonine by deleting *lysA* and *tdh*, and mutanting *ilvA*$_{C290T}$, then overexpressing *ppc* (phosphoenolpyruvate carboxylase), enhancing L-threonine exporter system and the glyoxylate shunt; then reducing the by-products acetic acid and overexpressing the *acs* gene encoding acetyl-CoA	82.4 g/L; 0.458 g/(g glucose); 1.648 g/L/h	[91]
E. coli TWF001*/pFW01-phaCAB* (TWF001 is an original L-threonine producer)	Overexpressing the *phaCAB* gene operon in the L-threonine producer	133.5 g/L	[32]
E. coli JLTHR: (ILE^{L-}, AHV^{r}) containing pTHR101-*thrA*BC*	Betaine supplementation: fermentation fed with the glucose solution containing 2 g/L betaine hydrochloride	127.3 g/L; 0.581 g/(g glucose); 4.243 g/L/h	[93]
E. coli EC125: *E. coli* TRFC (ILEL, AHVr)Δ*ilvA*	During the growth phase, the levels of L-isoleucine were accurately optimized by balancing cell growth and production with Pontryagin's maximum principle, basing on the relationship between the specific growth rate mu and specific production rate rho (The initial fermentation medium contains 0.1 g/L L-isoleucine, and 10 mL/L trace element solution). Furthermore, the depletion of L-isoleucine and phosphate at the end of the growth phase favored the synthesis of L-threonine in the subsequent non-growth phase (exponential feeding were 3 g/L L-isoleucine and 15 g/L KH_2PO_4)	105.3 g/L; 0.405 g/(g glucose); 2.194 (g/L/h)	[101]
E. coli TRFC: (*ILE^{L-}, AHV^{r}*) containing pTHR101-*thrA*BC*	Two combined feeding strategies were applied to L-threonine fed-batch fermentation: exponential feeding and pH-stat feeding, and another strategy of combined pseudo-exponential feeding and glucose-stat feeding. 70 g/L of sucrose with residual glucose at 0.15 g/L	124.57 g/L from sucrose; 3.114 g/L/h	[102]
E. coli THPE5 (derived from THRD, TCCC 11825, containing pRSFDuet1-C with pBBaJ23117-*pycA-pckA*; pETDuet1-C with placUV5-*fdh-aspC-gdhA-pntAB*-placUV5-*tetO-citA*	Switch system process: 0–12 h, expressing *citA*, *pycA*, and *pckA*; at 10 h, switching off the *citA* expression and inducing the expression of *aspC*, *fdh*, *gdhA* and *pntAB* for directing the carbon flux into L-threonine biosynthetic pathway	70.8 g/L; 0.404 g/(g glucose); 1.77 g/L/h	[103]
E. coli TWF106/pFT24rp, TWF106: TWF001 Δ*poxB*Δ*pflB*Δ*ldhA*Δ*adhE*Δ*tdcC*, pFT24rp: PRL::*tet*R, *rhtC*, *pyc*mt, PL*tetO*1::*MCS*2	Reducing by-products and L-threonine back-transporter: Δ*poxB*Δ*pflB*Δ*ldhA*Δ*adhE*Δ*tdcC*; then thermal switch system application: switching off the gene *alaT* to block L-alanine synthesis pathway	25.85 g/L (shake flask); 0.820 g/(g glucose)	[19]
C. glutamicum ATCC21799/pGC42 (ATCC21799 is a Lysine-producing strain), *hom^{r}, Ptac-thrB*	Enhancing the biosynthesis pathway: expressing *hom^{r}*, and expressing *thrB* under control of *tac* promoter	11.8 g/L	[99]
C. glutamicum MH20-22B-(*hom^{r}-thrB*) (pEC-T18mob2-*thrE*) (MH20-22B is a lysine producer)	Enhancing L-threonine biosynthesis pathway by expressing *hom^{r}-thrB* and L-threonine efflux gene *thrE* in MH20-22B	8.1 g/L	[100]
C. glutamicum IDW103 (*C. glutamicum* ATCC13869Δ*ddh*Δ*lysE*/pDXW-8-*lysC1-hom1-thrB1-ilvA1*)	Deletion of *ddh* and *lysE*, and overexpressing *lysC1-hom1-thrB1-ilvA1* encoding a feed-back-resistant TD mutant	7.27 g/L	[98]

metabolic pathways, cannot address the challenges associated with more complex carbon distribution [96], for which it requires multiple cofactors to participate in product synthesis [97]. Recently, researchers designed and constructed dynamic regulation systems to balance the cell growth and L-threonine production, resulting the excellent L-threonine producers *E. coli* EC125 (105.3 g/L), *E. coli* TRFC (124.57 g/L from sucrose), *E. coli* THPE5 (70.8 g/L), and *E. coli* TWF106/pFT24rp, as shown in Table 2. Especially for the TWF106/pFT24rp, a maximized conversion efficiency of 82% from glucose was reported [19]. To reduce metabolic burden on the host during cell growth stage, a thermal switch system was designed and applied to divide the whole fermentation process into two stages: growth and production [19], redistributing metabolic intermediate ratio between pyruvate and oxaloacetate through cooperating thermal decarboxylation of oxaloacetate with overexpression of pyruvate carboxylase [19]. The thermal switch system was then employed to switch off the L-alanine synthesis pathway, resulting in the highest L-threonine yield of 124.03%, which exceeds the best reported yield (87.88%) and the maximum available theoretical value of L-threonine production (122.47%) [19].

In addition to the metabolic engineering strategies, synthetic biology-based engineering approaches also efficiently improve L-threonine production. Wang et al. found that the overexpression of gene cluster *phaCAB* from *R. eutropha* in L-threonine-producing *E. coli* TFW001, the resulting strain TWF001/pFW01-*phaCAB* could produce 96.4-g/L L-threonine in 3-L fermenter and 133.5-g/L L-threonine in 10-L fermenter, respectively [32]. The genome reduction is also functional in optimizing L-threonine production. The genome-reduced *E. coli* strain MDS42 which lacks about 700 genes or 14.3% of its genome as compared to the wild-type *E. coli* strain MG1655, produced 40 g/L of L-threonine with a yield of 0.4 g/g glucose, increasing about 80% than the mutant strain derived from the wild-type strain MG1655 by the same metabolic engineering strategy [23]. Notably, our latest studies showed that outer membrane simplification benefits the L-threonine production. The 12 genes responsible for the biosynthesis of the enterobacterial common antigen and 50 genes responsible for flagellar biosynthesis in outer membrane were deleted in *E. coli* MG1655, resulting in WQM022 [28], and all 12 chaperone-usher operons including 64 genes were deleted in MG1655, resulting in the fimbria-lacking strain WQM026 [29]. Mutants WQM022 and WQM026 grew better and could synthesize more L-threonine than MG1655. The knowledge gathered from this study may be applied to the development of superior chassis microorganisms.

We found that genetically defined *E. coli* chassis produced high production of L-threonine, while *C. glutamicum* produce much lower L-threonine. In *C. glutamicum* ATCC13869, the L-threonine production could be improved by blocking L-lysine production and efflux (deleting *ddh* enconding diaminopimelate dehydrogenase and *lysE*), and overexpressing *lysC*, *hom* and *thrB*, while the resulting strain IDW103 only produced 7.7 g/L L-threonine [98]. In addition, strain *C. glutamicum* ATCC21799/pGC42 produced 11.8 g/L L-threonine after enhancing biosynthesis pathway [99], and strain *C. glutamicum* MH20-22B-(*hom*^{r}-*thrB*) (pEC-T18mob2-*thrE*) produce 8.1 g/L L-threonine after enhancing L-threonine biosynthesis pathway and L-threonine efflux in MH20-22B [100]. Notably, the two engineered *C. glutamicum* strains for L-threonine were both derived from L-lysine producers. As we know, the production of L-lysine by *C. glutamicum* could reach above 220 g/L, while the L-threonine production of *C. glutamicum* showed much lower level, which suggested that there are still many difficulties in designing and engineering *C. glutamicum* chassis for improving L-threonine production. Although it is promising to make better L-threonine *C. glutamicum* chassis by taking more advantages of the current excellent lysine producers as the start strains. The complex and unbalanced metabolic regulation and low efficiency of efflux in *C. glutamicum* are the major limitation for L-threonine biosynthesis. Maybe, the dynamic metabolic regulation system to dissolve the unbalance and improve the efflux efficiency would be functional for improving L-threonine production in *C. glutamicum*.

The L-threonine is derived from the intermediate OAA of TCA cycle and exported by RhtABC, but uptake by TdcC or SstT. PTS: phosphotransferase system; Glc: glucose; PP Pathway: pentose phosphate pathway; TCA cycle: tricarboxylic acid cycle; ATP: adenosine triphosphate; G6P: glucose 6-phosphate; G3P: glucose 3-phosphate; R5P: ribulose 5-phosphate; F6P: fructose 6-phosphate; F1P: fructose 1-phosphate; FBP: fructose 1,6-bisphosphate; E4P: erithrose 4-phosphate; G3P: glucose 3-phosphate; Acetyl-CoA (AcCoA), acetyl-coenzyme A; PEP: phosphoenol pyruvate. The red font and lines represent the extrinsic routes, whereas the gay font and red X represent the interrupted routes. The green arrow represents the enhanced reactions. The blue font represents the crucial immediate for L-threonine production.

Microbial chassis design and engineering for production of L-tryptophan

As one of the three important aromatic amino acids, L-tryptophan has been widely used in food ingredients. Besides *Bacillus subtilis* [104] and yeast [105], *E. coli* and *C. glutamicum* are mainly used as chassis bacteria for L-tryptophan production [106, 107]. The highest L-tryptophan titer of 58 g/L was achieved after 80 h fed-batch fermentation by a recombinant *C. glutamicum* strain with increasing

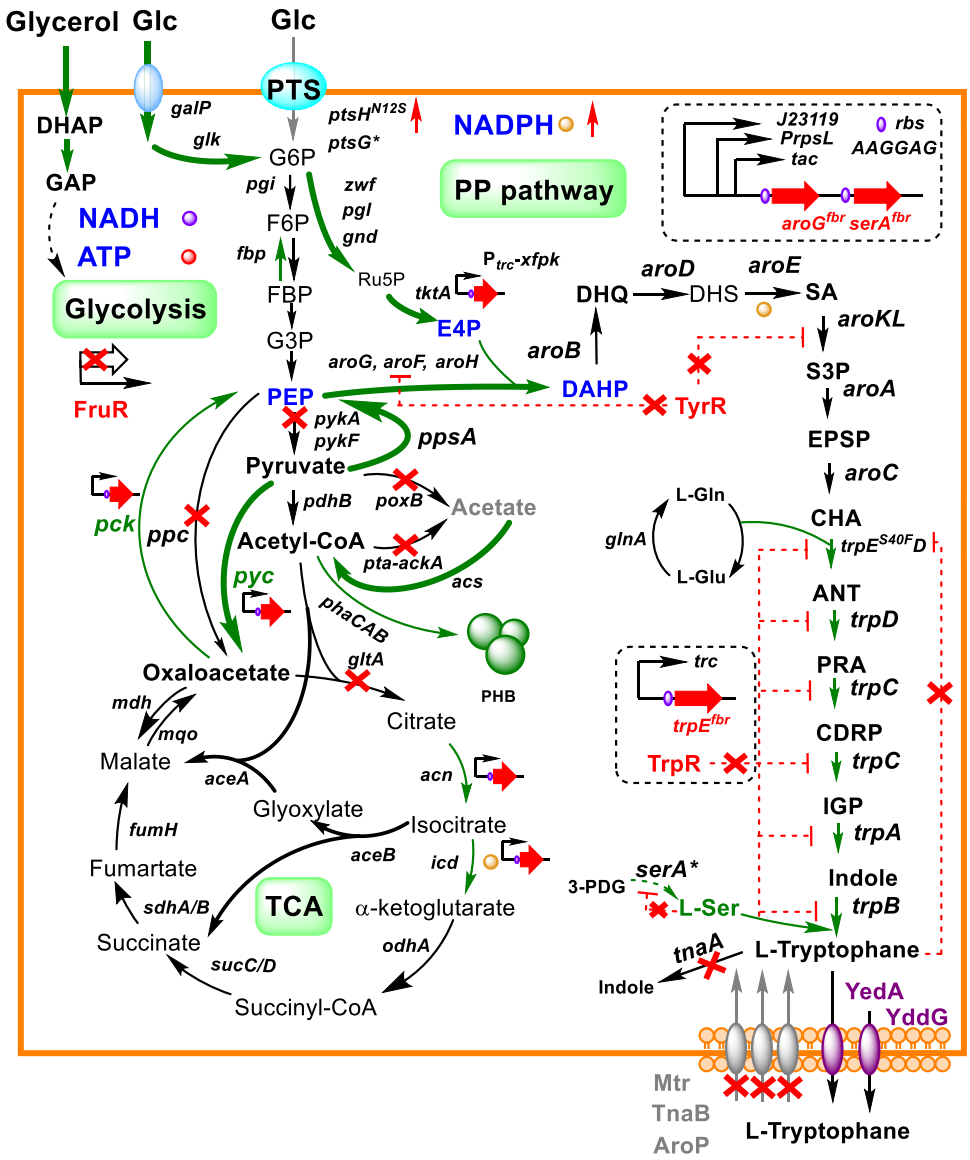

Fig. 5 Engineering strategies to improve L-tryptophan production in *E. coli*

transketolase activity [107]. Recently, most engineered high-producing microbial cell factories for L-tryptophan are genetically defined *E. coli* mutants (Fig. 5 and Table 4), because *E. coli* owns favorable characteristics, such as genetic tractability, metabolic plasticity as well as various existing and on-going tools for genetic engineering [106].

Genetically defined *E. coli* mutants are the general microbial platforms for L-tryptophan production, the rational design and engineering strategies facilitated L-tryptophan production to 40–55 g/L with conversion efficiency of 18–23% from glucose. E4P of PP pathway and PEP of glycolysis are the important precursors of L-tryptophan; meanwhile, additional precursors such as L-serine, PRPP, and glutamine are required (Fig. 5). In order to avoid carbon loss and increase the E4P supply, the gene *zwf* (encoding glucose 6-phosphate dehydrogenase) was deleted while the gene *tktA* (encoding transketolase) was overexpressed [108].

As shown in Fig. 5, the feedback inhibition steps exist in the biosynthesis pathway of L-tryptophan in *E. coli*. Feedback inhibition by L-tryptophan is exerted on DAHP synthase (encoded by *aroH*) and anthranillate synthase, AS (encoded by *trpED*) in *E. coli*. Furthermore, the genes of *trpLEDCBA* operon are repressed by TrpR, another regulation is made by the attenuation through *trpL* [108]. Strategies based on the regulatory mechanisms in L-tryptophan biosynthetic pathway for increasing precursor pools, relieving the feedback inhibition, repression and attenuation, trimming the competing and degradation pathways are functional to improve L-tryptophan production [106, 108, 109]. Metabolic engineering approaches have been attempted so far (Fig. 5 and Table 3). In the L-tryptophan biosynthesis, the overexpression of $aroG^{fbr}$, $aroF^{fbr}$, and $trpE^{fbr}$ [110, 111], as well as the tryptophan synthetic pathway genes, *trpEDCBA* must be considered [110, 111]. Furthermore, the deletion of *trpR* and

Table 4 The strategies for improving L-tryptophan production in *E. coli*

Strains	Strategies	L-tryptophan production	Refs.
E. coli GPT1002: W3110 (Δ*trpR*::FRT, Δ*tnaA*::FRT, Δ*ptsG*::FRT)/pTAT, and pTAT: pCL1920-*trpE*$^{FR}_{Met293Thr}$ and *aroG*$^{FR}_{Pro150Leu}$-*tktA*	One step tryptophan attenuator inactivation and promoter swapping strategy: overexpressing the *tktA*, mutated *trpE* and *aroG* genes and inactivating a series of competitive steps	10.15 g/L	[112]
E. coli GPT1002/PHB	Co-producing polyhydroxybutyrate (PHB) by expressing *phaCAB* operon genes from *R. eutropha*, and adding xylose as co-substrate (glucose and xylose concentration was 16 and 4 g/L, respectively)	14.4 g/L L-tryptophan from xylose	[33]
E. coli GPT1017: W3110(Δ*trpR*::FRT, Δ*tnaA*::FRT, Δ*ptsG*::FRTΔ*aroP*::FRT, Δ*tnaB*::FRT, Δ*mtr*::FRT)/pTAT, and pTAT: pCL1920-*trpE*$^{FR}_{Met293Thr}$ and *aroG*$^{FR}_{Pro150Leu}$-*tktA*	Deleting three permeases, Mtr, TnaB, and AroP in *E. coli* GPT1002	16.3 g/L	[116]
E. coli TRTH0709/pMEL03 (derived from *E. coli* MG1655)	Deletion of *trpR*, *tnaA*, *pta*, *mtr* and overexpression of *yddG*, *aroG*^{fbr}*trpE*^{fbr} DCBA, *serA*, *tet*^{R}, *tktA*, and *ppsA*	48.68 g/L; 0.2187 g/(g glucose)	[117]
E. coli FB-04(*pta1*) (FB-04:W3110Δ*trpR*Δ*tnaA*Δ*pheA*Δ*tyrA*)	The gene *pta* was replaced with a *pta* variant (*pta1*) from *E. coli* CCTCC M 2,016,009	44.0 g/L	[114]
E. coli TRTHBPA (*trp*EDCBA + Tet^{R}, Δ*tnaA*Δ*gltB*Δ*pta*Δ*ackA*Δ*poxB*)	Enhancing the biosynthesis pathway of L-tryptophan, then deleting the key genes underlying acetate biosynthesis and *gltB* encoding glutamate synthase; further cell recycling: concentrating cell solution: clear solution as 1: 1, and cell recycling within 24–30 h	47.18 g/L; 0.178 g/(g glucose); 55.12 g/L; 0.197 g/(g glucose)	[115]
E. coli NT367/pF112- *aroFBL-kan* (*E. coli LJ110*Δ*trpE-trpD-trpC-trpB-trpA*Δ*lac::Ptac-aroFBL*Δ*xyl::Ptac-serAFBR(T372D)*Δ*sdaB*Δ*tnaA*Δ*trpR*Δ*trpL*/pF112-*trpE* ^{fbr},*aroFBL-FRT-kan-FRT*, *serA*^{fbr})	System metabolic engineering: feedback-resistant enzyme variants (*trpE*^{fbr}, *aroFBL*, and *serA*^{fbr}), deletions of enzymatic steps for the degradation of precursors or the product L-tryptophan (*sdaB* and *tnaA*), and alterations in the regulation of L-tryptophan metabolism (deletion of *trpL* and *trpR*); Fed-batch with glycerol (800 g/L)	12.5 g/L	[110]
E. coli TRP07 (*E. coli* W3110Δ*lacI*Δ*tnaA*, Δ*mtr*, P_{trc}-*trpE*::trpLE*, P_{trc}-*aroG*::tyrR*Δ*pykA*, Δ*ppc*, P_{pck}-*pck::ycjV*, P_{citT}-*citT::poxB*, P_{lac}-*acnBA-icD::yghx*, P_{lac}-*pyc::yjiV*)	Central metabolic pathway modification (down-regulation of the tryptophan degradation pathway and transport system, enhancement of the branch acid synthesis pathway, up-regulation of the shikimic acid pathway, down-regulation of the PEP metabolic pathway, enhancement of the PEP synthesis pathway, enhancement of citric acid transport and TCA cycle): W3110Δ*lacI*Δ*tnaA*, Δ*mtrPtrc-trpE*::trpLE Ptrc-aroG*::tyrR* Δ*pykA*Δ*ppc* P_{pck}-*pck*::*ycjV PcitT-citT::poxB* P_{lac}-*acnB-acnA-icd::ygh Plac-pyc::yjiV*	49 g/L; 0.186 g/(g glucose)	[113]
E. coli SX11: MG1655, Δ*tnaAB*, *tyrR*::Ptrc-*aroG*$_{S180F\text{-}serAH344A,N364A}$,*trpE*::Ptrc-*trpE*$_{S40F}$, *yjiV*::Ptrc-*trpBA*, *yghX*::Ptrc-*xfpk* (*Bifidobacterium adolescentis*), *ptsG*::PM1-12-*glf*(*Zymomonas mobilis*) Δ*pykF*, *ylbE*::P*pck-pck*, *mbhA*::Ptrc-*pyc*$_{P458S}$(*C. glutamicum*)	System metabolic engineering: the flux redistribution of central carbon metabolism to maintain sufficient supply of PEP and E4P	41.7 g/L; 0.227 g/(g glucose)	[111]

trpL genes, and the block of by-products including acetate and indole accumulation are effective [33, 110–115]. Moreover, engineering transporters Mtr, TnaB, AroP, and YddG to increase L-tryptophan secretion into extracellular [108, 116, 117].

Wang et al. reported that the genetically defined *E. coli* TRTH0709/pMEL03 produced 48.86 g/L L-tryptophan with a highest glucose conversion rate of 0.2187 g/g [117], this high producing strain chassis was rationally designed and engineered from *E. coli* MG1655 by deleting *trpR*, *tnaA*, *pta*, *mtr* and overexpression of *yddG*, $aroG^{fbr}trpE^{fbr}$ DCBA, *serA*, *tktA*, and *ppsA* [117]. For another example, Du et al. rationally designed a "bottom-up" metabolic engineering strategy and gradually modified the L-L-tryptophan biosynthetic pathway, the central metabolic pathway and citric acid transport system with methods of CRISPR–CAS9 and promoter engineering in *E. coli* W3110, to obtain an L-tryptophan high-producing strain *E. coli* TRP07 (W3110Δ*lacI*Δ*tnaA*Δ*mtrPtrc-trpE*::trpLE Ptrc-aroG*::tyrR* Δ*pykA*Δ*ppc* P_{pck}*-pck::ycjV PcitT-citT::poxB* P_{lac}*-acnB-acnA-icd::ygh Plac-pyc::yjiV*)), which produces 49 g/L L-tryptophan with glucose conversion efficiency of 0.186 g/g glucose [113]. In strain *E. coli* TRP07, the tryptophan degradation pathway and transport system were down-regulated by deleting *tnaA* and *mtr*, then the branch acid synthesis pathway was enhanced by integrating P_{trc}*-trpE* (S40F) into the *trpLE* locus, then the shikimic acid pathway was up-regulated by integrating *Ptrc-aroG* (S211F) was into the *tyrR* locus, then the PEP metabolic pathway was down-regulated by deleting *pykA* and *ppc*, but the PEP synthesis pathway was enhanced by up-regulated *pck* and *pps*, at last, the citric acid transport and TCA cycle were enhanced by up-regulating *citT*, *acnAB*, *icd*, and *pyc* and deleting *ycjV*, *poxB*, and *yjiV* [113]. In addition, Liu et al. pointed out that modification of phosphoenolpyruvate glucose phosphotransferase system increases the conversion rate between glucose and L-L-tryptophan in *E. coli* [118]. Moreover, similar to L-glutamate and L-threonine, the co-production of PHB by introducing *phaCAB* operon in *E. coli* is also functional on improving L-tryptophan [33].

However, the effect of the reported studies are still far from the theoretical maximum, which means that there is still much room to improve the yield of L-tryptophan by balancing the ratio of biomass formation and L-tryptophan production. The development of dynamic regulation system to balance cell growth and metabolic flux to produce L-tryptophan would be a promising strategy in the near future study. In addition, neither *B. subtilis* nor *C. glutamicum* owns favorable characteristics, such as genetic tractability, metabolic plasticity as well as various existing and on-going tools for genetic engineering [106]. Therefore, how to improve the robustness and streamline of strains is very important for strain breeding and fermentation process controlling. Moreover, unlike L-threonine, there have been few reports on genome reduction to construct L-tryptophan high-producing strain. In addition, the *C. glutamicum* is a food-safety microbial cell factory for producing L-tryptophan, while the *C. glutamicum* KY9218/pIK9960 is the only reported genetically defined *C. glutamicum*. To promote the L-tryptophan production in food industry, the better optimized and metabolic engineered *C. glutamicum* chassis should be designed and developed in the future.

L-tryptophan is derived from the intermediates PEP of glycolysis and E4P of PP pathway. In addition, additional precursors such as PRPP, L-glutamine, and L-serine are required. L-tryptophan is exported by YddG and YedA, but uptake to intracellular by Mtr, TnaB, and AroP. The biosynthesis pathway of L-tryptophan could be regulated by many inhibition regulators, and the inhibition steps were marked as red dotted lines. PTS: phosphotransferase system; Glc: glucose; PP Pathway: pentose phosphate pathway; TCA cycle: tricarboxylic acid cycle; ATP: adenosine triphosphate; G6P: glucose 6-phosphate; R5P: ribulose 5-phosphate; F6P: fructose 6-phosphate;F1P: fructose 1-phosphate; FBP: fructose 1,6-bisphosphate; E4P: erithrose 4-phosphate; G3P: glucose 3-phosphate; Acetyl-CoA (AcCoA), acetyl-coenzyme A; PEP: phosphoenol pyruvate; Ser: serine; Trp: tryptophan; Gln: glutamine; Glu: glutamate; PHB: poly-3-hydroxybutyrate; DAHP: 3-deoxy-darabino-heptulosonate 7-phosphate; GAP: glyceraldehyde 3-phosphate; DHQ: 3-dehydroquinate; DHS: 3-dehydoshikimate; SA: shikimic acid; S3P: shikimate 3-phosphate; SHK: shikimic acid kinase; EPSP: 5-enolpyruvateshikimate 3-phosphate; CHA: chorismic acid; ANT: anthranilate; PRA: phosphoribosyl-anthranilate; CDRP: carboxyphenylamino deoxyribulose phosphate; IGP: indole glycerol phosphate. In *C. glutamicum*, two types of DAHPSs exist. One is an L-tyrosine sensitive, while another one (Type II) is L-phenylalanine and L-tyrosine sensitive. Others are similar as stated above for *E. coli*. The red font and lines represent the feedback inhibition regulators and steps, whereas the gay font and red X represent the interrupted routes. The green arrow represents the enhanced reactions. The blue font represents the crucial immediate for L-threonine production.

Perspectives

Systems metabolic engineering and synthetic biology engineering have become efficient and necessary ways to perform strain improvement and bioproduction of amino acids. With the development of advanced techniques of systems biology and synthetic biology, more excellent strains will be undoubtedly generated in the future. However, it is far from ideal productivity for many amino acids fermentation according to the current studies. Moreover, there are still

many challenges for amino acids production by *C. glutamicum* and *E. coli*, such as the low productivity for L-threonine and L-tryptophan by *C. glutamicum* platforms; lack of a systematic approach combining different engineering strategies established in different studies; understanding limitations of global and useful information such as omics data or unknown regulation networks, leading to poor integration of useful information into engineering strategies; and food-safety hazards existed in the amino acids production by *E. coli*. Therefore, we still need lots of efforts to overcome these difficulties for optimizing the production of amino acids used in food industries in the future.

We believed that engineering strategies for improving different amino acids can be learned from each other. For example, some engineering strategies for optimizing *C. glutamicum* to produce L-lysine can be used in the construction of *C. glutamicum* producers to synthesize L-threonine or L-tryptophan; in addition, some rational design and efficient approaches based on novel technologies developed in *E. coli* should be rationally designed and developed in *C. glutamicum*, such as dynamic switch systems or promoter regulation systems, which could better realize the balance between cell growth and chemicals production or the balance of metabolic flux distribution. Notably, it should be considered to try our best to combine the advantages of different efficient engineering strategies and integrated them into a genetically defined and excellent microbial chassis. For example, researchers have found that rational genome-scale optimization [22, 23], simplification of cell envelope or cell membranes [24–26, 28, 29, 119], engineering on cell lifespan [120] and so on, have significant positive influences on cellular metabolism including the amino acids biosynthesis, thus the potential and efficient engineering strategies should be attempted when designing and engineering the microbial chassis for amino acids production. Moreover, engineering strategies for optimizing substrate sugars utilization could also be considered for the production of different amino acids. Carbohydrate uptake and phosphorylation are mainly executed by phosphoenolpyruvate-carbohydrate PTS consisting of 1 membrane-bound carbohydrate-specific EIIABC component (EII) and 2 cytoplasmic components (i.e., enzyme I (EI) and histidine protein (HPr). Studies suggested that enhance PTS-independent carbohydrate uptake systems (i.e., non-PTS) also benefit for cell growth and production of target amino acids; i.e., introduction of fructokinase from *Clostridium acetobutylicum* for a functional metabolic pathway of sucrose and fructose, and replacement of the phosphoenolpyruvate-dependent glucose and fructose uptake system PTS_{Glc} and PTS_{Fru} by inositol permeases IolT1 and IolT2, could both realize the utility of other substrates sucrose and fructose to form amino acids [60]. Until now, biosensor has also been developed to control target gene expression, export system on amino acid, and amino acid-producing strain screening. For example, the utility of biosensor-enabled high-throughput screening facilitated higher production of beta-alanine [121], which suggested that biosensor engineering will be a perspective strategy. In addition, process engineering strategies such as optimization of substrate spectrum and process robustness by rational and evolutive strategies have been applied to efficiently increase amino acid titers, yields, and productivities [122]. Notably, as Gram-negative bacteria, *E. coli* owns less safety comparing with *C. glutamicum*. Therefore, we should pay attention to how to minimize the potential safety risk of *E. coli* to produce amino acids used in food industries. Notably, in our previously published review or paper, it was specifically pointed out that the safety hazards can be reduced by modifying the outer membrane of *E. coli* [119, 123], including biofilm, multiple antibiotic resistance, pathogenicity or toxicity of lipopolysaccharides, common antigen, pili, virulence factors or peptides and capsular polysaccharides. Therefore, rational cell envelope modification strategies, including simplification of lipopolysaccharide to the minimal structure Kdo_2-lipid A, and deletion of other above membrane structures, are expected to be carried out in future modified strains. With all these efforts, systems metabolic engineering and synthetic biology engineering will contribute more to developing excellent microbial chassis for the production of amino acids used in food industries.

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Declarations

Conflict of interest On behalf of all the authors, the corresponding author states that there is no conflict of interest.

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