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Metabolic engineering strategies for L-Homoserine production in *Escherichia coli*

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Abstract
L-Homoserine, serves as a non-essential precursor for the essential amino acids derived from L-aspartate in both animals and humans. It finds widespread applications across the food, cosmetics, pharmaceutical, and animal feed industries. Microbial fermentation, primarily utilizing *Escherichia coli*, is the dominant approach for L-Homoserine production. However, despite recent advancements in fermentative processes employing *E. coli* strains, low production efficiency remains a significant barrier to its commercial viability. This review explores the biosynthesis, secretion, and regulatory mechanisms of L-Homoserine in *E. coli* while assessing various metabolic engineering strategies aimed at improving production efficiency.

Keywords *Escherichia coli*, L-Homoserine, Metabolic engineering, Microbial fermentation, L-Homoserine biosynthesis

Background

L-Homoserine, a non-essential amino acid precursor to the essential amino acids methionine and threonine [1, 2], plays a significant role in various industries, including food, medicine, cosmetics, agriculture, and animal feed [3]. Additionally, it is recognized for its benefits in promoting the growth of young chicks and enhancing plant resistance to diseases [4, 5]. *Escherichia coli* and *Corynebacterium glutamicum* are well-established amino acid producers capable of achieving high titers on an industrial scale [6, 7]. However, current research consistently demonstrates that *E. coli* is more efficient in L-Homoserine production, particularly when utilizing metabolic engineering strategies, compared to *C. glutamicum* [8–11].

E. coli has emerged as one of the most widely used host organisms for metabolic engineering and synthetic biology, owing to its rapid growth, ease of cultivation, metabolic versatility, extensive biochemical and physiological understanding, and the availability of advanced genetic and genomic tools [12]. In the absence of natural producers, *E. coli* is often the host of choice for metabolic engineering applications. Beyond its role as a proof-of-concept model organism, *E. coli* has demonstrated commercial success as an industrial producer, exemplified by the successful large-scale production of L-threonine [13, 14], L-lysine [15], and 1,4-butanediol [16, 17].

In contrast, *E. coli* exhibits a significantly higher capacity for L-Homoserine production [8], highlighting the potential and necessity for further optimization through metabolic engineering to enhance production yields and reduce associated costs. This review synthesizes recent advancements in the biosynthesis, metabolic regulation, and metabolic engineering of L-Homoserine in *E. coli*.

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Biosynthetic pathways and metabolic regulation of L-Homoserine

Biosynthetic pathways of L-Homoserine

The metabolic pathway responsible for converting glucose to L-Homoserine in *E. coli* primarily involves the Embden-Meyerhof (EMP) pathway, the tricarboxylic acid (TCA) cycle, and the L-aspartate pathway (Fig. 1). Several key enzymes within these pathways require NADPH as a cofactor. L-Homoserine is synthesized from L-aspartate in three enzymatic steps (Fig. 1): first, aspartate is phosphorylated to aspartyl 4-phosphate by aspartokinase (encoded by *thrA/metL/lysC*); second, aspartyl 4-phosphate is converted to aspartate semialdehyde by aspartate semialdehyde dehydrogenase (encoded by *asd*); third, aspartate semialdehyde is reduced to L-Homoserine by homoserine dehydrogenase (encoded by *thrA/metL*). Additionally, L-Homoserine serves as a common precursor in the biosynthesis of L-threonine and L-methionine, while aspartate semialdehyde functions as an intermediate in the synthesis of L-lysine and L-Homoserine [18]. The synthesis of L-Homoserine is tightly regulated through feedback inhibition at several critical enzymatic steps within these microbial metabolic pathways.

Export control

An efficient transport system that facilitates product efflux while preventing reabsorption is crucial for enhancing product accumulation. In *E. coli*, RhtA (encoded by *rhtA*) and RhtB (encoded by *rhtB*) have been identified as effective exporters of L-Homoserine [10, 19–22]. The rhtA23 mutation, characterized by an A-to-G substitution at position −1 relative to the ATG start codon, has been shown to increase the expression level of the *rhtA* gene [23, 24]. Notably, it was the first report to demonstrate that the RhtA protein functions as a transport protein for both L-threonine and L-Homoserine and also plays a role in the export of 5-aminolevulinic acid [25]. This highlights the broad substrate specificity of RhtA and its potential to enhance product efflux, making it a promising candidate for metabolic engineering applications. While RhtB is also exports L-Homoserine, RhtA is more commonly employed due to its higher

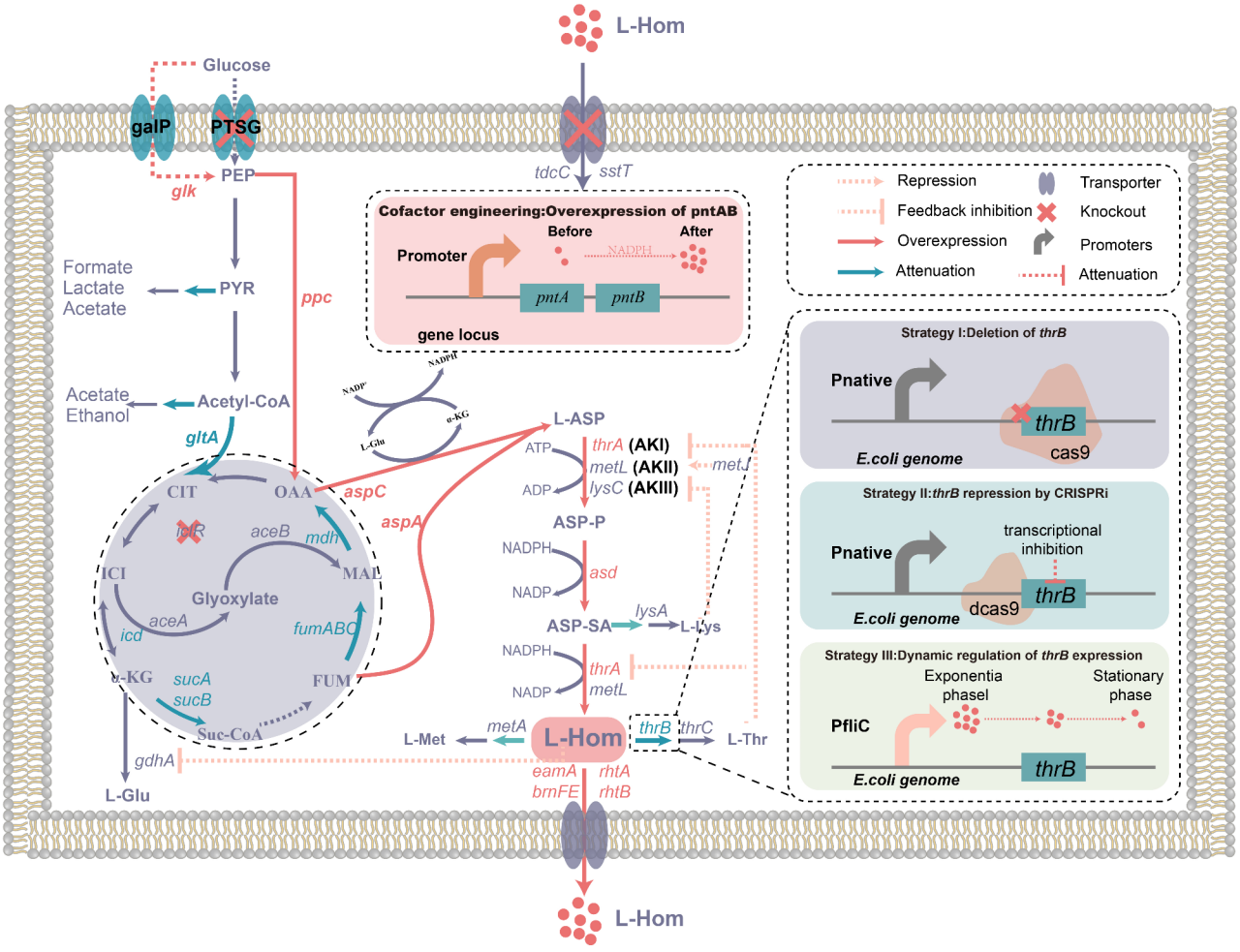

Fig. 1 The biosynthetic pathway of L-Homoserine in *E. coli* and the feedback inhibition on key enzymes

transport efficiency [26]. In addition to RhtA and RhtB, the two-component export system BrnFE encoded by the *brnFE* genes from *C. glutamicum*, has been tested for its ability to export intracellular L-Homoserine into the external environment. Overexpression of BrnFE has been shown to effectively enhance L-Homoserine production in *C. glutamicum* [19]. EamA was initially identified as a transporter for cysteine and O-acetyl-L-serine, and its overexpression has been shown to enhance resistance to toxic compounds [27]. Overexpressing the *eamA* gene has been employed to increase the export capacity of L-Homoserine, thereby improving its production and alleviating the growth burden on L-Homoserine-producing strains. However, the export capacity of L-Homoserine appears to have an upper limit. Studies have reported that enhancing the expression of *rhtB*, either through its overexpression or the addition of the exogenous exporter *brnFE*, failed to further improve L-Homoserine production efficiency [21]. These findings indicate that adding additional copies of *rhtB* is sufficient to meet the demand for L-Homoserine efflux. However, further increases in the expression level of the exporter do not enhance L-Homoserine production once the transport system reaches saturation. This highlights the necessity of exploring alternative metabolic engineering strategies to overcome this limitation and further improve production efficiency.

Additionally, the genes *tdcC* and *sstT* are involved in the transport of extracellular L-threonine into cells [28]. L-Homoserine, being a structural analog of L-threonine, benefits from the deletion of the *tdcC* gene, which has been shown to promote L-Homoserine production [8]. However, knockout of the *sstT* gene does not significantly affect L-Homoserine accumulation [29]. Thus, modifying the transport system to enhance L-Homoserine efflux remains a critical strategy for improving production efficiency [29].

Feedback regulation

L-Homoserine is a valuable non-proteinogenic amino acid, serving as a precursor in the biosynthesis of L-threonine and L-methionine. Additionally, the L-Homoserine biosynthetic pathway competes with the L-lysine biosynthetic pathway. In *E. coli*, L-Homoserine is synthesized from L-aspartate in three steps (Fig. 1): first, aspartate is phosphorylated to aspartyl-4-phosphate by aspartokinase; second, aspartyl-4-phosphate is converted to aspartate-semialdehyde by aspartate-semialdehyde dehydrogenase; and third, aspartate-semialdehyde is reduced to L-Homoserine by homoserine dehydrogenase [9]. The enzymes AKI, AKII, and AKIII (encoded by *thrA/metL/lysC*) catalyze key reactions in the L-Homoserine biosynthetic pathway, regulating the distribution of carbon flux in *E. coli*. These enzymes are subject to feedback inhibition. The aspartate kinase (AK) family consists of three isoenzymes: AKI, AKII, and AKIII. Both AKI and AKII are bifunctional enzymes, possessing both aspartate kinase and homoserine dehydrogenase activities. AKI and AKIII are feedback-inhibited by threonine and lysine, respectively. Additionally, the nicotinamide adenine dinucleotide phosphate ($NADP^+$)-specific glutamate dehydrogenase, encoded by *gdhA* is feedback-inhibited by homoserine.

AKI catalyzes the two-step conversion of aspartic acid to aspartic acid phosphate and aspartic acid semialdehyde to homoserine. As a precursor of threonine, homoserine is biosynthesized through the actions of homoserine kinases and threonine synthetases, encoded by the *thrB* and *thrC* genes, respectively. However, threonine feedback inhibits AKI. Therefore, reducing the threonine branch can minimize homoserine consumption and alleviate the feedback inhibition exerted by threonine on the genes involved in the L-Homoserine biosynthesis pathway.

Aspartate-semialdehyde is a direct precursor of homoserine. In the competitive biosynthetic pathway, diaminophosphate decarboxylase, encoded by *lysA*, catalyzes the production of lysine, which in turn exerts feedback inhibition on AKIII. By limiting the lysine biosynthetic pathway, the availability of aspartate-semialdehyde as a precursor for L-Homoserine is preserved, while reducing feedback inhibition on AKIII.

Transcriptional regulation

In addition to feedback inhibition of critical enzyme activities, L-threonine biosynthesis in *E. coli* can be regulated at the transcriptional level of key genes.

The *thrLABC* operon consists of four genes—*thrL*, *thrA*, *thrB*, and *thrC* (Fig. 2)—which encode the *thr* operon precursor peptide, AKI, homoserine kinase, and threonine synthetase, respectively. These genes are involved in both the biosynthesis and degradation of homoserine. The ThrL precursor peptide is a 21-amino acid peptide that plays a key role in regulating the expression of the *thrLABC* operon through attenuation, with 8 threonine and 4 isoleucine residues serving as regulatory points [30]. The expression of the *thrLABC* operon, and consequently L-threonine biosynthesis, is controlled by the *thrL* gene [31]. Additionally, *thrA*, a critical gene for L-Homoserine biosynthesis, is also repressed by the transcription of *thrL*. A study isolated mutant strains capable of inhibiting and desensitizing L-Homoserine toxicity through adaptive laboratory evolution (ALE), leading to the identification of a $ThrL^*$ allele. Notably, the substitution of the *thrL* with $thrL^*$ in the model strain MG1655 mitigated the inhibitory effects of L-Homoserine. This loss of toxicity is likely attributable to the enhanced conversion of the $thrL^*$ transcriptional activator, which

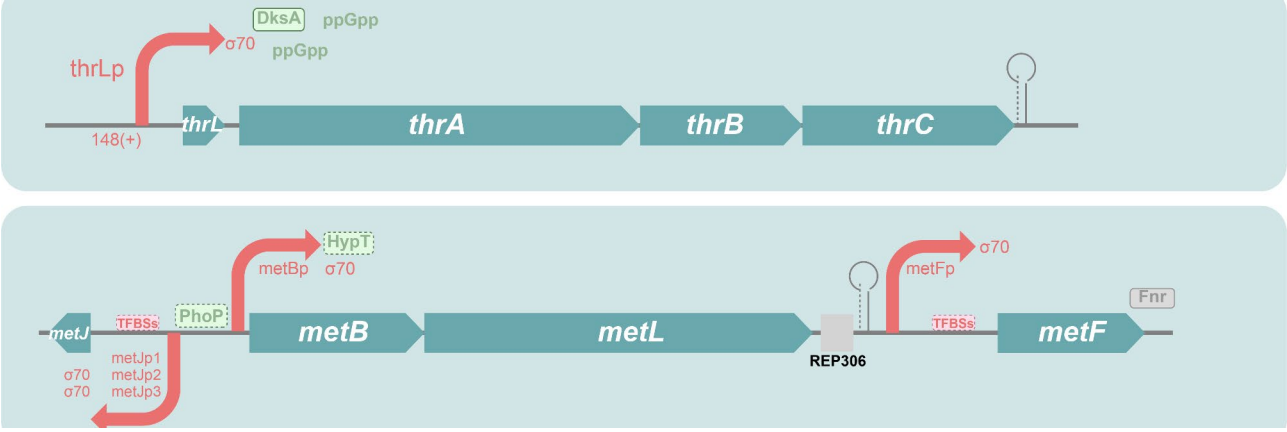

Fig. 2 The *thrABC* operon and *metL* are controlled by the transcriptional regulation of *thrL* and *metJ*, respectively

regulates the *thrABC* operon, thus promoting the biosynthesis of L-threonine.

In addition to its role in threonine biosynthesis, homoserine serves as a precursor for methionine biosynthesis, catalyzed by homoserine O-succinyltransferase encoded by *metA*. The expression of the *metL* gene, involved in methionine biosynthesis, is tightly regulated by the DNA-binding transcription inhibitor MetJ and the methionine-mediated nuclear repressor S-adenosylmethionine (Fig. 2) [32]. Therefore, reducing the metabolic flux through the methionine biosynthetic pathway can benefit L-Homoserine accumulation by both enhancing the availability of L-Homoserine precursors and alleviating the transcriptional repression of AKII.

Metabolic engineering strategies for L-Homoserine producing strains

Various strategies for enhancing L-Homoserine production can be categorized into six key approaches: (1) relieving feedback inhibition of L-Homoserine; (2) restoration of glucose uptake; (3) transformation of critical nodes in metabolic pathways; (4) balancing redox pathway for L-Homoserine production; (5) modification of the transport system; (6) large-scale target recognition to increase L-Homoserine production.

A major bottleneck in the production of L-aspartic acid family amino acids (AFAAs), including L-Homoserine, is the availability of oxaloacetate (OAA). Several strategies have been developed to enhance OAA supply [33, 34].

Relieving feedback inhibition of L-Homoserine

The feedback inhibition of AKI by L-threonine can be alleviated through point mutations, such as $thrA^{fbr}$ ($thrA^{C1034T}$) [35–37]. In one study, the critical gene *thrA*, located within the *thrABC* operon, was attenuated by a self-regulating promoter, resulting in no detectable accumulation of L-Homoserine in the modified strain. To overcome this, Cai et al. increased the number of Ptrc-$thrA^{fbr}$ copies to three, significantly upregulating the *thrA* transcription level and boosting L-Homoserine synthesis flux [29]. To determine which bifunctional enzyme would be more effective in boosting production, the Ptrc expression system with trc99a $thrA^{fbr}$ and *metL* (pTrc99a $thrA^{fbr}$ and pTrc99a *metL*) was introduced into *E. coli* W3110, forming the HP1 and HP2 strains, respectively. The HP1 strain, containing pTrc99a $thrA^{fbr}$, accumulated 7.18 g/L of L-Homoserine within 24 h, a 27.8% increase over the HP2 strain (5.62 g/L) [29]. These results suggest that $ThrA^{fbr}$ is more effective for constructing L-Homoserine-producing strains. Additionally, feedback inhibition of AKIII by lysine can be mitigated by the point mutation $lysC^{fbr}$ (C1055T) [36]. A strain expressing both *lysC* and $lysC^{fbr}$ accumulated 0.4 g/L and 0.35 g/L L-Homoserine after 44 h of culture [38], though these results were not as expected. Further overexpression of bifunctional enzymes demonstrated that the production capacity of HM4 strains carrying pBR$thrA$ or pBR$thrA^{fbr}$ was nearly threefold higher than that of HM strains carrying pBR$lysC$ or HM pBR$lysC^{fbr}$. These findings indicate that *thrA* exhibits superior performance compared to *lysC*, underscoring the reduction of aspartic acid semialdehyde to homoserine as a key rate-limiting step in the overproduction of L-Homoserine. Deletion of the *thrB* gene effectively decreases the metabolic flux from L-Homoserine to L-threonine. Similarly, removal of the *lysA* gene reduces L-Homoserine precursor consumption, alleviating competition for metabolic intermediates. These modifications also diminish feedback inhibition on AKI and AKIII.

Restoration of glucose uptake

In *E. coli*, glucose is primarily transported through the phosphotransferase system (PTS), which utilizes one molecule of phosphoenolpyruvate (PEP) — 50% of the available PEP — to phosphorylate glucose, thereby converting it to pyruvate [39, 40]. This system efficiently

couples glucose transport and glycolysis but limits the direct conversion of PEP to OAA. Deletion of the *ptsG* gene significantly reduces glucose consumption, cell growth, and L-Homoserine production. However, overexpression of the *galP* gene completely restores glucose consumption to normal levels [38], thereby enabling efficient L-Homoserine production. Modifying non-PtsG glucose transporters to restore glucose uptake enhances the availability of PEP, while maintaining effective glucose transport, which significantly improves L-Homoserine production. Among various L-Homoserine-producing strains, the non-PtsG modification strategy increased L-Homoserine productions by approximately 19.0% (6.27 g/L) and 400% (4.29 g/L) in different strains, suggesting that substituting PtsG with GalP is highly beneficial for L-Homoserine production.

Transformation of critical nodes in metabolic pathways

Overexpression of key genes in the L-Homoserine biosynthetic pathway

Overexpression of key genes in the L-Homoserine biosynthetic pathway is both a necessary and straightforward strategy for improving productions. The metabolic pathways for L-Homoserine biosynthesis can be divided into two main modules: PEP-OAA/FUM-ASP and ASP-HOM. Key genes involved in the PEP-OAA/FUM-ASP module include *ppc*, *aspA*, and *aspC*, while *thrA* and *asd* are central to the ASP-HOM module. AspA is involved in the degradation of aspartate to fumarate and ammonia, but this is under ammonia-limiting conditions [41, 42]. AspA catalyzes the reversible reaction of aspartic acid-fumaric acid [42]. When aspartate is used as a nitrogen source, AspA catalyzes the degradation of aspartate to fumarate and ammonia [41]. Ammonia is the preferred nitrogen source, and ammonia inhibits the use of L-aspartate as a nitrogen source [43]. Therefore, when ammonia is sufficient, AspA catalyzes the fumarate-aspartate process. Overexpression of AspA is a common strategy for the production of L-aspartic family amino acids (AFAAs) such as L-homoserine [11, 21, 24, 29, 37, 38]. Several studies have focused on the overexpression of these critical genes, with chromosomal integration providing a more stable and reliable gene expression method compared to plasmid-based overexpression [44, 45].

To increase the OAA pool, a strong Ptrc promoter was used to replace the natural promoter of the phosphoenolpyruvate carboxylase coding gene (*ppc*) in the chromosome, leading to an L-Homoserine titer of 7.02 g/L in the H06 strain, an 11.8% increase over the control [29]. To enhance the availability of L-aspartic acid, essential for L-Homoserine production, genomic integration of Ptrc-driven copies of the *aspC* (encoding aspartate aminotransferase) and *aspA* (encoding aspartate aminolyase) genes resulted in a modest 5.7% increase in L-Homoserine production (from 7.02 g/L to 7.42 g/L). Another study [11] integrated a Ptrc-driven *ppc* replica into the genome of *E. coli*, producing the HOM-7 strain, which resulted in a significant increase in L-Homoserine production to 2.9 g/L — a 61.1% improvement over the control strain. Additionally, some researchers have inserted the *ppc* gene from *Corynebacterium glutamicum*, which performs better in L-aspartic acid production [46], into the *E. coli* genome [21]. These findings underscore the importance of enhancing the metabolic flow through the OAA-ASP pathway to improve L-Homoserine synthesis.

Attenuation of L-Homoserine degradation and branch metabolism

In *E. coli*, the *thrABC* operon plays a crucial role in the biosynthesis and degradation of L-Homoserine. L-Homoserine degradation occurs through homoserine kinase (encoded by *thrB*), which prevents its accumulation. Deleting genes involved in competing metabolic pathways is a common strategy to block such catabolic pathways and increase product production. However, this approach can lead to nutrient limitations, potentially harming industrial-scale production.

The self-regulated promoter PfliC offers a simple and cost-effective solution [47] and has been successfully employed to dynamically regulate the expression of ThrB, thereby mitigating L-Homoserine degradation [11, 29]. By substituting the natural promoter of the *thrABC* operon with PfliC, the expression of *thrB* and *thrC* can be dynamically regulated [11], while simultaneously increasing the copy number of $thrA^{fbr}$ driven by Ptrc in the genome. This strategy led to the development of HOM-5 (*thrB* driven by PfliC), which produced 1.8 g/L of L-Homoserine. Three methods were used to regulate *thrB* expression in *E. coli*: (1) gene deletion of *thrB*, (2) mutation of the start codon from ATG to GTG, and (3) replacement of the local *thrB* promoter with PfliC. When Ptrc-driven $thrA^{fbr}$ copies were increased, the strain lacking the *thrB* gene exhibited a significant decrease in biomass when supplemented with additional L-threonine. Conversely, the strain with PfliC-driven *thrB* grew efficiently without the need for supplementary L-threonine. These results indicate that dynamic regulation of metabolic flux offers a promising strategy for metabolic engineering.

Balancing redox pathway for L-Homoserine production

In the biosynthesis of L-Homoserine in *E. coli*, the conversion of one molecule of L-aspartate to L-Homoserine requires two molecules of NADPH, with key reactions catalyzed by aspartate-semialdehyde dehydrogenase and homoserine dehydrogenase being NADPH-dependent

(Fig. 1). A limited supply of NADPH thus represents a significant bottleneck in L-Homoserine production.

Previous research has demonstrated that overexpression of *pntAB*, which encodes NAD(P) transhydrogenase, can promote NADPH regeneration, balance intracellular cofactors, and enhance L-Homoserine production [21, 29, 37, 48]. Overexpressing of *pntAB* has been shown to convert NADH to NADPH, thereby increasing NADPH availability. In one study, the SHL5 strain overexpressing *pntAB* produced 1.2 g/L of L-Homoserine, a six-fold increase in yield compared to the control [49]. Integration of Ptrc-driven *pntAB* into the genome of *E. coli* resulted in the HOM-11 strain, which produced 10.7 g/L of L-Homoserine, a 21.6% increase compared to control [11]. Further overexpression of Ptrc-*pntAB* in strain H24 resulted in a production of 27.83 g/L L-Homoserine, a 21.7% improvement over the control. Additional copies of Ptrc-*pntAB* further increased the L-Homoserine titer to 33.77 g/L [29]. These results indicate that augmenting the NADPH pool through *pntAB* overexpression enhances both L-Homoserine production and bacterial growth.

An alternative strategy involves introducing NADH-dependent dehydrogenases to reduce NADPH consumption [50]. Aspartate dehydrogenase from *Pseudomonas aeruginosa* (encoded by *aspB-Pa*) and L-aspartate-4-semialdehyde dehydrogenase from *Titrella mobile* (encoded by *asd-Tm*) both utilize NADH in place of NADPH [51]. To minimize NADPH consumption and promote NADH generation, the genes *aspB_Pa* and *asd_Tm* were inserted into various expression constructs, including Ptrc-*asd_Tm*, Ptrc-*aspB_Pa*, Ptrc-*asd_Tm*-Ptrc-*aspB_Pa*, and Ptrc-*aspB_Pa*-Ptrc-*asd_Tm*, which were then integrated into plasmid pEC-thrA^{S345F}_Ec and introduced into strain Cg13-1. This led to the generation of strains Cg13-16, Cg13-17, Cg13-18, and Cg13-19, respectively. Notably, strain Cg13-19 produced 13.3 g/L of L-Homoserine, an 18% increase over the control [50].

Modification of the transport system

High intracellular concentrations of L-Homoserine are toxic to *E. coli* [52], and the accumulation of this product can induce toxic stress, which impedes both cell growth and L-Homoserine production [53, 54]. Consequently, enhancing the capacity of the L-Homoserine transport system and converting other toxic intermediates have become critical priorities for improving production.

The replacement of the local promoter with the Ptrc promoter to generate the HS3 strain (Ptrc-*rhtA*) resulted in a 30.9% increase in L-Homoserine production, reaching 2.63 g/L. In the HS4 strain (Ptrc-*eamA*), where the local promoter of the *eamA* gene was replaced by the Ptrc promoter, L-Homoserine production was 2.17 g/L. Further, overexpression of two copies of the *rhtA* and *eamA* genes under the Ptrc promoter in the chromosome, leading to the creation of strain HS5 (Δ*lacI*:Trc-*rhtA* Trc-*eamA*), achieved a 54.2% increase in L-Homoserine production, with a final titer of 3.14 g/L, compared to 2.04 g/L in the control strain HS2 [10]. Several overexpression strategies for the RhtA gene have been employed to increase the efflux capacity of recombinant strains for L-Homoserine [8]. These include: (1) overexpression controlled by the natural *PrhtA* promoter in the plasmid pBRmetL-*rhtA*, (2) the use of a mutant PrhtA23 promoter (with an A-for-G substitution at position −1 relative to the ATG start codon) in the plasmid pBRmetL-*rhtA23* to enhance *rhtA* expression, and (3) the use of the constitutive promoter pN25 in plasmid pBR*metL*-pN*rhtA* to drive *rhtA* overexpression. The final L-Homoserine titers for these three strains were 1.42 g/L, 1.81 g/L, and 2.12 g/L, respectively, compared to 1.04 g/L produced by the plasmid pBR*metL* in the control strain. Another study evaluated the effects of overexpressing *rhtA*, *eamA*, and the heterologous *brnFE* genes [29] in strains H17, H18, and H19 under Ptrc control. The H17 strain produced approximately 15 g/L of L-Homoserine, outperforming strains H18 and H19, which produced 13.28 g/L. Strain H20, which overexpressed *rhtA* under the stronger Plpp promoter, produced 22.86 g/L of L-Homoserine. However, efforts to further enhance L-Homoserine production in H20 by combining strategies such as *tdcC* and *sstT* deletions did not significantly improve the production.

Large-scale target recognition to increase L-Homoserine production

In recent years, gene expression at the transcriptional level has been efficiently regulated using CRISPR interference (CRISPRi) [55] and small regulatory RNAs (sRNAs) [56]. Both CRISPRi and sRNAs provide a versatile and rapid means of inhibiting target gene expression, allowing for tunable regulation through different levels of *dCas9* or sRNA expression. This approach significantly shortens the modification cycle compared to traditional gene knockouts and enables chromosomal gene regulation for large-scale screening of genes, optimizing metabolic pathways without the need for permanent genetic modifications. Table 1 summarizes the various metabolic engineering strategies employed to enhance L-Homoserine production in *E. coli*.

The L-Homoserine metabolic pathway can be divided into three distinct modules to identify potential target genes for metabolic engineering aimed at enhancing L-Homoserine production. Genes involved in glycolysis, by-product production, and amino acid biosynthesis within these modules are considered candidate targets. The CRISPRi system is then used to downregulate these genes, further boosting L-Homoserine production [10].

Table 1 Metabolic engineering strategies and manipulations for L-Homoserine production in *E. Coli*

Categories	Strategies	Manipulations
a	Restoring glucose uptake by modifying non-PTS sugar transporters	Knockout of the *ptsG and galR* genes, overexpression of *glK* gene
b	Increasing L-Homoserine by overexpression of key relevant genes	Overexpression of $thrA^{fbr}$,*asd*, *metL*, *ppc*, *aspA* and *aspC* genes
c	Attenuation L-Homoserine degradation and branch metabolism	Knockout of the *thrBC*, *metA*, *lysA*, *ldhA*, *poxB*, *pflB* genes
d	Export control	Knockout of the *tdcC and sstT* genes, overexpression of *rhtA*, *rhtB*, *eamA* and *brnFE* genes
e	Transcriptional regulation	Knockout of the *lacI* and *iclR* genes
f	Large-scale target recognition to increase L-Homoserine production	These metabolic nodes are involved in the glycolysis pathway, by-product production and amino acid biosynthesis
g	Synergistic use of NADPH and NADH to enhance L-Homoserine production	Overexpression of the *pntAB* gene

Table 2 Production of L-Homoserine by *E. Coli*

Strains	Strategies	Titer (g/L)	Yield (g/g)	Productivity (g/L/h)	Cultivation Mode	References
E. coli HM5 *(pBRmetL-pNrhtA)*	*b*+*c*+*d*+*e*	39.5	0.29	0.9	Fed-batch	[8]
E. coli LJL12	b+c+e	35.8	0.35	0.82	Fed-batch	[9]
E. coli HS33	a+b+c+d+e+f	37.6	0.31	0.35	Fed-batch	[10]
E. coli HOM-14	b+c+d+g	60.1	0.42	1.25	Fed-batch	[11]
E. coli HS15	b+c+d+e+g	84.1	0.5	1.96	Fed-batch	[15]
E. coli W-H18/pM2/pR1	a+b+c+d+e	110.8	0.64	1.82	Fed-batch	[30]
E. coli SHL17	b+c+d+g	44.4	0.21	0.93	Fed-batch	[17]
E. coli H28	b+c+d+e+g	85.3	0.43	1.78	Fed-batch	[21]

Studies have demonstrated that strains with sgRNA-directed downregulation of genes such as *ptsH*, *ptsI*, *crr*, *ptsG*, *tktA*, *rpe*, *talB*, *argA*, *argG*, *proB*, *gadA*, *zwf*, *pta*, and *poxB* in the three modules resulted in L-Homoserine production increases of over 50-100% [10]. These findings underscore the potential of sRNAs for large-scale target gene recognition. A library of 122 synthetic sRNAs was constructed to silence the expression of genes involved in the production or regulation of cadaverine. Notably, sRNAs targeting *ackA* (encoding acetate kinase) and *pdhR* (encoding pyruvate dehydrogenase complex regulator), which are not part of the main cadaverine biosynthesis pathway, enhanced cadaverine titers by approximately 30–40% [56]. Since cadaverine is a lysine derivative and both the cadaverine and L-Homoserine pathways stem from aspartic acid, these results suggest that sRNA-based strategies can effectively identify non-obvious target genes, making sRNA a promising tool for optimizing L-Homoserine production.

Downregulating the TCA cycle is considered a crucial strategy for boosting the biosynthesis of L-aspartate family amino acids [57, 58]. However, inhibiting the TCA cycle in *E. coli* often leads to reduced growth rates, limiting the production of important metabolic intermediates. Dynamic sRNA-mediated inhibition of TCA cycle genes provides a more controlled approach to balancing growth and production. By reducing TCA cycle flux, the availability of OAA for L-Homoserine synthesis can be increased. Consequently, critical enzymes involved in the glyoxylate shunt, such as the transcriptional regulator IclR, and enzymes of the TCA cycle, such as citrate synthase, can be disrupted to redirect carbon flux toward L-Homoserine production [9]. Additionally, since β-alanine and L-Homoserine both derive from aspartic acid, strategies for optimizing the TCA cycle for β-alanine production can also be applied to enhance L-Homoserine synthesis. For example, the deletion of *fumABC* (fumarate hydratase) has been shown to increase fumarate production, thereby enhancing the metabolic flux toward β-alanine [59]. Similarly, the deletion of malate dehydrogenase (encoded by *mdh*) increases the supply of OAA, while the overexpression of isocitrate dehydrogenase (encoded by *icd*) and downregulation of α-ketoglutarate dehydrogenase (encoded by *odhA*) can further optimize the supply of L-glutamate [60, 61]. These metabolic engineering strategies hold significant promise for improving the synthesis of L-Homoserine and other compounds. Table 2 provides a summary of engineered strains developed using various metabolic engineering strategies, along with the resulting L-Homoserine yields and related fermentation parameters.

The specific details of strategies *a*, *b*, *c*, *d*, *e*, *f*, and *g* mentioned in the strategy column are described in Table 1.

Development of L-Homoserine-producing strains for the production of other valuable chemicals

L-Homoserine-producing strains can also be leveraged for the biosynthesis of other high-value chemicals. 1,3-propanediol (1,3-PDO), a versatile compound with applications in adhesives, antifreeze, and coatings, has emerged as a promising industrial product [62, 63]. While natural 1,3-PDO producers utilize glycerol as the sole substrate, a recombinant *E. coli* strain has been engineered by integrating the glycerol biosynthesis pathway from *Saccharomyces cerevisiae* with the 1,3-PDO synthesis pathway from *Klebsiella pneumoniae* to enable the conversion of glucose into 1,3-PDO [62, 64]. In this system, the enzymes aspartate transaminase (encoded by *aspC*) from *E. coli*, pyruvate decarboxylase (encoded by *pdc*) from *Zymomonas mobilis*, and alcohol dehydrogenase (encoded by *yqhD*) from *E. coli* were co-optimized [65]. This engineered strain effectively converts L-Homoserine to 1,3-PDO, achieving a production of 0.32 g/L 1,3-PDO from glucose without the need for the costly addition of vitamin B12, thereby substantially reducing fermentation expenses.

O-Acetyl-L-homocysteine (OAH), a valuable intermediate for the production of L-methionine and other compounds, has seen rising market demand [66, 67]. A strain capable of accumulating 1.68 g/L of OAH was engineered by disrupting competing degradation pathways. Further optimization involved the development of the F147L-M182I-M240A mutant of MetX, which reduced by-product formation, enhanced OAA supply, and facilitated L-Homoserine biosynthesis [68]. Expression of this MetX mutant led to a 57.14% increase in O-Acetyl-L-homocysteine production.

Discussion

Microbial synthesis of L-homoserine plays a pivotal role in biotechnology, where the overexpression of pathway enzymes and metabolic engineering are critical to optimizing production. However, the overexpression of these enzymes can potentially disrupt the physiological state of the host microorganism [12, 69]. This disruption may interfere with intracellular metabolic processes, causing imbalances that increase the risk of substrate depletion and by-product accumulation, which can negatively affect both microbial growth and overall metabolic activity [69].

To mitigate these challenges, a comprehensive investigation into the functions of pathway enzymes and their impact on host microbial physiology is essential, alongside the development of effective regulatory strategies. Several approaches can be employed to address the physiological stresses resulting from enzyme overexpression. One promising strategy involves balancing cell growth with product biosynthesis by redirecting metabolic flux. It has been suggested that the supply of OAA constitutes a bottleneck in the synthesis of L-aspartic acid family amino acids [9, 10, 33]; at suboptimal gene expression levels, the biosynthetic pathway may experience metabolic imbalance, leading to intermediate accumulation or protein overload [70]. These challenges can be tackled using a strategy known as multivariable modular metabolic engineering (MMME). Xu et al. employed the multivariate modular metabolic engineering to engineer the β-alanine biosynthesis pathway and keep the balance of metabolic flux among the whole metabolic network, rationally and systematically. Ultimately, 37.9 g/L β-alanine was generated in fed-batch fermentation [60]. With the advancement of synthetic biology tools, such as promoter libraries and ribosome binding site (RBS) variants, more comprehensive and global optimization of strains can be achieved [71].

In addition to traditional static regulation methods, dynamic regulation has emerged as a powerful strategy for precision pathway engineering [72, 73]. The fliC promoter, which regulates flagellum construction, has been identified as a self-regulating promoter with significant potential for dynamic control [29]. In particular, the expression of the PfliC promoter is reduced in the stationary phase [47], which has been exploited to reduce L-Homoserine degradation by substituting the native promoter controlling the *thrB* gene, thus preventing the strain from becoming auxotrophic [11, 29]. Furthermore, metabolite-based biosensors offer a promising means of fine-tuning pathway flux in response to shifts in intracellular metabolite pools, thereby reducing the accumulation of toxic intermediates and alleviating the physiological burden on host cells, ultimately enhancing product synthesis. The development of specific L-Homoserine biosensors remains a key area for future research.

The toxicity of L-Homoserine to the host cell represents a major bottleneck in improving production yields. Adaptive laboratory evolution (ALE) has emerged as an effective technique for generating microbial strains with enhanced tolerance to toxic compounds, although it is time-consuming and labor-intensive [74, 75]. ALE can be employed to develop chassis cells with inherent desensitization to L-Homoserine toxicity, which can then be combined with L-Homoserine biosensors to screen for high-yielding strains with improved performance. The integration of modern biotechnologies such as whole-genome sequencing, transcriptomics, metabolomics, and the application of bioinformatics and computational modeling, will be crucial for advancing L-Homoserine production. By adopting a systems biology approach, involving global metabolic network analysis and flux reconstruction, further optimization of L-Homoserine biosynthesis can be achieved. The collective application of these methodologies holds the potential to

significantly enhance microbial L-Homoserine synthesis, paving the way for broader industrial applications and advancements in this field.

Abbreviations

E. coli	Escherichia coli
C. glutamicum	Corynebacterium glutamicum
EMP	Embden-Meyerhof pathway
TCA cycle	Tricarboxylic acid cycle
NADPH/$NADP^+$	Nicotinamide adenine dinucleotide phosphate
PTS	Phosphoenolpyruvate-dependent carbohydrate phosphotransferase system
PEP	Phosphoenolpyruvate
PYR	Pyruvate
Acetyl-CoA	Acetyl coenzyme A
CIT	Citrate
ICI	Isocitrate
α-KG	α-ketoglutarate
Suc-CoA	Succinyl coenzyme A
FUM	Fumarate
MAL	Malate
OAA	Oxaloacetate
L-Hom	L-Homoserine
L-Glu	L-glutamate
L-Asp	L-aspartate
Asp-P	Aspartyl-4-phosphate
Asp-SA	Aspartyl-4-semialdehyde
L-Lys	L-lysine
L-Thr	L-threonine
L-Met	L-methionine
TFBSs	Transcription factor binding sites
CRISPRi	Clustered regularly interspaced short palindromic repeats interference
AKI	Aspartate kinase I
AKII	Aspartate kinase II
AKIII	Aspartate kinase III
ALE	Adaptation to laboratory evolution
AFAAs	L-aspartic acid family amino acids
Fbr	Feedback resistance
RBS	Ribosome binding site
sRNA	Small regulatory RNA
1,3-propanediol	1,3-PDO
OAH	O-Acetyl-L-homocysteine
MMME	Multivariable modular metabolic engineering
ALE	Adaptive laboratory evolution

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Author contributions
Xin Jin proposed and conceived the conception, designed the study; cited, acquired, analyzed, and interpreted the data presented in the table and figure; filled in, compiled, and arranged the tables as well as drew the figure; drafted and revised the whole manuscript; Sumeng Wang, Yanbing Wang, and Qingsheng Qi participated in study design, cited, acquired and analyzed the data in the tables and figures, drafted parts of the manuscript; Quanfeng Liang proposed and conceived the conception, designed and coordinated the study, analyzed and interpreted the data presented in the tables and figures, drafted and revised the whole manuscript, supervised and administrated the scheme. All the authors read and approved the version before submission.

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No datasets were generated or analysed during the current study.

Declarations

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The authors declare no competing interests.

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