Improving the Plasmid Stability by a *Hok/Sok* System for L-Homoserine Production in *Escherichia Coli*

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Abstract

Background: The production of bioactive compounds using microbial hosts is considered a safe, cost competitive and scalable approach. However, the efficient engineering of cell factories with well stability, such as for the production of L-aspartate family amino acids and derivatives, remains an outstanding challenge.

Results: In the work, the toxin/antitoxin system and genome modification strategy were used to construct a stable Escherichia coli strain for L-homoserine production. The metabolic engineering strategies were focused on the enhancement of precursors for L-homoserine synthesis, reinforcement of the NADPH generation and efflux transporters using CRISPR-Cas9 system at the genome level. To improve the plasmid stability, two strategies were explored, including construction of the aspartate-auxotrophic and hok/sok systems. Constructing the auxotrophic complementation system to maintain plasmid stability was failed herein. The plasmid stability was improved by introducing the hok/sok system, resulting in 6.1 g/L (shake flask) and 44.4 g/L (5 L fermenter) L-homoserine production of the final engineered strain SHL19 without antibiotics addition. Moreover, the hok/sok system was also used to improve the plasmid stability for ectoine production, resulting in 36.7% and 46.5% higher titer of ectoine at shake flask and 5L fermenter without antibiotics addition, respectively.

Conclusion: This work provides valuable strategies to improve plasmid stability for producing L-aspartate family amino acids and derivatives and eliminate environmental concerns associated with the application of antibiotics.

1. Introduction

In the recent decades, public awareness toward green and sustainable biosynthesis is rapidly increasing worldwide due to non-renewable petroleum resources and environmental issues. [1-4] Many kinds of products have been achieved using microbial production, such as chemicals and natural products. [5, 6] For industrial bioproduction, one important issue is the stability of the engineered strain, which can maintain excellent performance and maximize the productivity during fermentation. Nevertheless, the efficient engineering of cell factories with well stability for industrial-scale applications has been still a challenge.

Several strategies have been used for the efficient engineering of cell factories with well stability, including modification at genome-level [3, 7] and maintaining the plasmid stability[8, 9]. Genome modification can be achieved using genome editing tools, such as the CRISPR-Cas9 system.[7] Generally, due to the overexpression of key genes, antibiotics and inducers need to be added during the process of fermentation to avoid plasmid losing and induce gene expression by recombinant plasmids.[3, 10] It has been observed that existence of expression plasmids in recombinant cells entails a metabolic burden of cells by replication, transcription and protein production, which can cause the loss of plasmid during fermentation process.[1] Additionally, the demand for antibiotics supplementation could increase the
costs and result in environmental contamination. \[11\] Since maintaining the plasmid stability is an important concern for enzyme expression and bioproduction using microbes, constructing an efficient system to maintain plasmid stability is necessary for the competitiveness of the plasmid-containing cells. \[10\] A number of approaches have been applied for maintaining the plasmid stability with the plasmid addition systems, including the toxin/antitoxin \[12, 13\] and auxotrophic complementation systems\[10, 11\]. The auxotrophic complementation system has been used in the production of viridiflorol and amorphadiene in *Escherichia coli*.\[10\] Park *et al.* introduced the toxin/antitoxin system to maintain the plasmid stability for high-level production of astaxanthin in *Escherichia coli*.\[12\]

The L-aspartate amino acids (AFAAs) are constituted of L-aspartate, L-lysine, L-methionine, L-threonine, L-isoleucine, and L-homoserine, which possess wide applications in food, animal feed, pharmaceutical and cosmetics industries.\[14\] Moreover, the AFAAs derivatives have also been reported with important commercial values, such as ectoine, the bioactive ingredients of skin protection and nasal spray due to its protective, moisturizing and anti-inflammatory qualities.\[15, 16\] To date, a number of amino acids, including AFAAs and its derivatives have been industrially produced by microbial fermentation, and the annual production of amino acids has dramatically increased worldwide.\[15, 17\] Nevertheless, to our limited knowledge, the metabolic engineering strategies focused on the enhancement of plasmid stability in the fermentation are still be restricted for the biosynthesis of AFAAs and their derivatives. Therefore, exploration of the effective way to construct genetic stable system is crucial for the efficient production of target products, such as AFAAs.

L-homoserine, as one of the AFAAs, is an attractive platform precursor for the biosynthesis of many important compounds, such as L-threonine, L-methionine, 1,4-butanediol, L-phosohinotheicin and butyrolactone.\[3, 18, 19\] Thus, L-homoserine has been attracted great interests and is widely used in food, agriculture, pharmaceutical, cosmetic product ingredient, and animal-feed additive.\[2, 3, 14\] Constructing an efficient and sustainable L-homoserine producing cell factory with high titer and stability is imperative for the fairly increasing market capacity demand and application of L-homoserine.\[19\] Due to the clear genetic background and ease of genetic manipulation, *Escherichia coli* is widely used as a host for producing amino acids. Biobased chemicals can be produced by microorganisms that are genetically modified on the basis of metabolic engineering and synthetic biology frameworks.\[4\] To obtain a cell factory with perfect performance, plenty of synthetic biology tools and applicable metabolic engineering strategies have been developed to remove the bottlenecks and fine-tune the metabolic flux in the chassis cell for the target chemical production.\[2\] In recent years, significant efforts have been made toward enhancing the production of L-homoserine in *E. coli* through metabolic engineering strategies. Zakataeva *et al.* have overexpressed the *thrB* and *rhtC* genes with plasmid pNPZ42 in mutant *E. coli* NZ10, which can accumulate 10.6 g/L of L-homoserine in 48 h \[20\]. Hua Li *et al.* conducted rational metabolic engineering strategies to improve the L-homoserine titer from 0 to 39.54 g/L in fed-batch fermentation by blocking the competing and degradation pathways and enhancing the carbon flux to L-homoserine. \[1\] Our group focused on the central metabolic pathway by enhancing oxaloacetate and phosphoenolpyruvate (PEP) metabolism in *E. coli* to obtain the engineered strain that generated 35.8 g/L L-homoserine with 0.82 g/L/h productivity.\[2\] Additionally, the L-homoserine production was
significantly increased by dynamically regulating \( \text{thrB} \) expression to attenuate L-homoserine degradation, and the engineered strain generated 60.1 g/L L-homoserine in a 5 L bioreactor.\[3\] Mu et al. have proposed a novel balanced redox metabolic network strategy for the production of L-homoserine, which achieved the highest yield ever reported (84.1 g/L).\[18\]

In the work, to construct genetic stable system for AFAAs, the metabolic engineering strategies were investigated using L-homoserine as an example. To achieve the accumulation of L-homoserine in \( E. \ coli \), the competing and degradative pathways of L-homoserine were blocked in our previous work \[2\]. The metabolic engineering strategies were focused on the enhancement of precursors for L-homoserine synthesis, reinforcement of the NADPH generation and efflux transporters through CRISPR-Cas9 genome editing system. To construct a genetic stable industrial strain for L-homoserine production, two strategies were explored, including construction of the aspartate-auxotrophic system and the toxin/antitoxin system to improve the plasmid stability. In addition, the established strategies for maintaining plasmid stability were also explored for the production of ectoine, which provides important guidance for industrial fermentation of AFAAs and their derivates with improved microbial productivity.

2. Materials And Methods

2.1. Strains, media and growth conditions

All strains and plasmids used in this work are listed in Table 1. \( E. \ coli \ W3110 \) was used as a parent strain and the LJL3 strain was selected as the base strain for L-homoserine producing \[2\]. For plasmid construction and propagation, \( E. \ coli \) DH5\( \alpha \) cells were routinely grown in Luria-Bertani (LB) broth (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) or on LB agar plates (1.5%, w/v, agar) supplemented with appropriate antibiotics as necessary (ampicillin (100 \( \mu \)g/mL), kanamycin (50 \( \mu \)g/mL), and spectinomycin (50 \( \mu \)g/mL)). Polymerase chain reaction (PCR) was performed using the PrimeSTAR MAX DNA polymerase (Takara Bio, Beijing, China). All restriction and modifying enzymes were purchased from Thermo Fisher (Thermo Fisher Scientific, USA). The primers used in this work are provided in supplemental Table S1.

The shake flask medium contained 20 g/L glucose, 5 g/L yeast extract, 5 g/L tryptone, 1 g/L MgSO\(_4\), 4 g/L KH\(_2\)PO\(_4\), 1 g/L (NH\(_4\))\(_2\)SO\(_4\), 2 g/L L-methionine, 2 g/L L-lysine, 2 g/L L-threonine, and 5 mL trace metal solution. The trace metal solution contains 10 g/L FeSO\(_4\)·7H\(_2\)O, 2.25 g/L ZnSO\(_4\)·7H\(_2\)O, 1.35 g/L CaCl\(_2\), 1 g/L CuSO\(_4\)·5H\(_2\)O, 0.5 g/L MnSO\(_4\)·4H\(_2\)O, 0.106 g/L (NH\(_4\))\(_6\)Mo\(_7\)O\(_{24}\)·4H\(_2\)O, 0.23 g/L Na\(_2\)B\(_4\)O\(_7\)·10H\(_2\)O, and 10 mL 35% HCl.

2.2. Plasmid and donor construction

The encoding gene \( \text{metL} \) was overexpressed with a medium copy plasmid pKK-miniPtac controlled by a constitutive \( \text{tac} \) promoter to obtain pKK-\( \text{metL} \), and the plasmid pKK-miniPtac was constructed as described previously \[2, 21\]. The primers pKK-\( \text{metL}-\text{F} \) and pKK-\( \text{metL}-\text{R} \) were used to carry out PCR (Polymerase Chain Reaction) using \( E. \ coli \ W3110 \) as a template, obtaining the \( \text{metL} \) gene. Then, the
plasmid pKK-miniPtac was simultaneously digested with the restriction enzymes *Kpn*I and *EcoRI*, and the ligation reaction was conducted using the Hieff clone Plus One Step Cloning kit, which was purchased from YEASEN (YEASEN Biotechnology Co., Shanghai, China), generating the plasmid pKK-*metL* for *metL* overexpression.

The *hok/sok* system was cloned from the genome of *E. coli* XL1-Blue[22], and then was inserted into two different sites of plasmid pKK-*metL* using the corresponding primers hok/sok-1-F and hok/sok-1-R to obtain plasmid pKK-*metL*-hok-1 and hok/sok-2-F and hok/sok-2-R to obtain plasmid pKK-*metL*-hok-2, respectively.

### 2.3. Genome manipulation by CRISPR-Cas9 system

To overexpress the *ppc* (encoding the PEP carboxylase), *thrA* (encoding the pyruvate kinase II), *asd* (encoding the pyruvate kinase I), *pntAB* (encoding the NAD(P) transhydrogenase) and *rhtA* (encoding a L-homoserine export protein) genes, the genome manipulation of *E. coli* strain was conducted using CRISPR-Cas9 system for replacing native promoter into constitutive *trc* promoter.

To construct the pTarget plasmid for gene editing, the plasmid pTarget-*ppc* for overexpressing *ppc* gene was taken as an example. First, a high-scoring 20 bp sgRNA was selected using the online tools (http://crispr.mit.edu/), which was designed into the forward primer pTargetF-*ppc* (*trc*). The pTarget-*ppc* plasmid was constructed using primers pTargetR-*ppc* (*trc*) and pTargetF-*ppc* (*trc*) and the original pTarget plasmid as a template. Similar methods were carried out to construct pTarget plasmids for other genes, including pTarget-*thrA* (using primers pTargetR-*thrA* (*trc*) and pTargetF-*thrA* (*trc*)), pTarget-*asd* (using primers pTargetR-*asd* (*trc*) and pTargetF-*asd* (*trc*)), pTarget-*pntAB* (using primers pTargetR-*pntAB* (*trc*) and pTargetF-*pntAB*(*trc*)), pTarget-*rhtA* (using primers pTargetR-*rhtA*(*trc*) and pTargetF-*rhtA*(*trc*)).

The upstream and downstream homologous arms were amplified using primers *ppc*-1/ *ppc*-2 and *ppc*-3/*ppc*-4, respectively. The *ppc* donor was generated by assembling the two fragments via overlap PCR. The donor fragment was approximately 800-1000 bp.

The *E. coli* strain containing pCas plasmid (the temperature-regulated plasmid) was inoculated into the 5 mL LB test-tube, and cultured in a shaker at 30 °C overnight. The culture was then transferred to 50 mL LB shake flask next day, with kanamycin (50 μg/mL) and L-arabinose (20 mmol/L) added, and was cultured in a shaker at 30 °C for 2-4 h until OD<sub>600</sub> 0.5~0.6 to prepare the electroporation-competent cells. Total 200 ng pTarget plasmid and 800 ng donor fragment were transferred into the electroporation-competent cells. After electroporating, the recombinant cells were cultured at the solid media with Kan and Spe added at 30 °C for 12-16 h.

Based on the strain LJL3, the *ppc*, *thrA*, *asd*, *pntAB*, and *rhtA* genes for L-homoserine synthesis were enhanced by replacing the native promoter with a strong *trc* promoter, finally generating strain SHL11. Considering the important role of *metL* in the L-homoserine synthesis pathway, the plasmid...
pKK-\textit{metL} was transformed into strain SHL11, generating the strain SHL12. Moreover, plasmid pKK-\textit{metL}-hok1 and pKK-\textit{metL}-hok2 were transformed into strain SHL11, resulting in strains SHL18 and SHL19, respectively. All of the validation primers are listed in supplemental Table S1.

### 2.4. Plasmid stability test

Plasmid stability was examined by subculturing the recombinant cells on antibiotic-containing medium versus antibiotic-free medium to determine the plasmid presence ratio, respectively. SHL12 (pKK-\textit{metL}), SHL18 (pKK-\textit{metL}-hok1), and SHL19 (pKK-\textit{metL}-hok2) strains were first grown in test tubes containing 5 mL LB medium at 37 °C without antibiotics. After cultivating for 12 h, 100 μL cultured broths at different dilution rates: 10×, 25× and 50× were transferred into solid LB medium with Amp or without Amp, respectively. The survival cell count assay was adopted to evaluate the efficiency of \textit{hok/sok} system for plasmid stability.

### 2.4. Batch and fed-batch cultivation condition

For batch fermentation, the single clone of \textit{E. coli} was transferred to 5 mL LB medium and was cultivated at 37°C overnight. Next day, the seeds were transferred to 500 mL baffled flask carrying 70 mL shake flake medium at an initial optical density measured at 600 nm (OD$_{600}$) of ~0.1 and were incubated at 37°C for 48 h with 200 rpm agitation. Bioreactor cultivations for L-homoserine was performed in a 5-L jar fermenter (Bailun bio, China Shanghai). The initial batch fermentation was started using modified fed-batch fermentation medium as follows: 20 g/L glucose, 5 g/L yeast extract, 5 g/L tryptone, 1 g/L MgSO$_4$, 4 g/L KH$_2$PO$_4$, 1 g/L (NH$_4$)$_2$SO$_4$, 2 g/L L-methionine, 2 g/L L-lysine, 2 g/L L-threonine, and 5 mL trace metal solution. The trace metal solution contained 10 g/L FeSO$_4$·7H$_2$O, 2.25 g/L ZnSO$_4$·7H$_2$O, 1.35 g/L CaCl$_2$, 1 g/L CuSO$_4$·5H$_2$O, 0.5 g/L MnSO$_4$·4H$_2$O, 0.106 g/L (NH$_4$)$_6$Mo$_7$O$_{24}$·4H$_2$O, 0.23 g/L Na$_2$B$_4$O$_7$·10H$_2$O, and 10 mL 35% HCl (pH 7.0). The glucose concentration was maintained at 5-10 g/L by supplementing 600 g/L of glucose. The temperature was controlled at 37°C, and the initial agitation was set to be 200 rpm and increased to maximally 800 rpm depending on the dissolved oxygen level, which was maintained above 30% in the fermentation medium by controlling the aeration and agitation speed. The pH of the fermentation medium was maintained at 6.9-7.1 by automatic feeding of NH$_4$OH (25%, v/v).

### 2.5. Analytical methods for L-homoserine, glucose, cell growth and ectoine

The production of L-homoserine and ectoine were quantified by high-performance liquid chromatography (HPLC, Agilent Technologies 1260 Infinity, U.S.A.). After fermentation, the culture broth samples were centrifuged to separate the supernatant from the fermentation cultures at 12,000 g for 10 min and then filtered using a 0.22 μm aqueous membrane. The supernatant containing product and by-product was diluted appropriately for subsequent analysis. Precolumn O-phthalaldehyde (OPA) derivatization was used to determine the concentration of L-homoserine as described previously [23]. Before the HPLC detection, total 800 μL OPA derivatization reagent was mixed with the broth supernatant for one minute. HPLC detection was conducted at a column temperature of 40°C and the detection
wavelength of 338 nm with a Phenomenex Luna C18 SB-Aq column (ODS 5 μm, 20 × 150 mm, Agilent, U.S.A.). 50 mM potassium dihydrogen phosphate/acetonitrile (81:19, v/v) was used as the mobile phase for L-homoserine detection at a flow rate of 1.0 mL/min, and the detection time was 15 min. The OD value of each sample was determined at 600 nm in triplicate using a Hitachi U-1100 spectrophotometer. The concentration of glucose in the broth was determined at 505 nm using an enzyme-coupled glucose assay kit (Rsbio, China). All values were average of three independent experiments.

3. Results And Discussion

3.1 Modification of the key genes for L-homoserine accumulation

Several system metabolic engineering strategies have been explored for the biosynthesis of AFAAs in *E. coli*, such as improving the supply of precursors and cofactors and facilitating the efflux efficiency.[2, 18] Improving the supply of precursors is regarded as an important approach for the biosynthesis of L-homoserine, *i.e.* oxaloacetate (OAA), L-aspartate (L-ASP), Aspartyl-β-phosphate (Asp-P), and aspartyl-β-semialdehyde (Asp-SA).[14] The OAA supply was considered as the bottleneck for the synthesis of AFAAs, and thus it was speculated that enhancing the OAA pool could facilitate L-homoserine production.[3]

Another strategy for increasing L-homoserine production is to overexpress the key enzymes to enhance the metabolic flux from OAA precursor toward the product of L-homoserine, especially these three enzymes: aspartokinase (AKI encoded by *thrA*, AKII encoded by *metL*, and AKIII encoded by *lysC*), aspartyl semialdehyde dehydrogenase (encoded by *asd*), and L-homoserine dehydrogenase (encoded by *metL* and *thrA*), as shown in Fig. 1. The ThrA and MetL are bifunctional enzymes with both aspartokinase and L-homoserine dehydrogenase activity [1, 19], which play essential roles in the synthesis pathway. The generation of NADPH as reducing equivalent is considered as a limiting step for optimizing the production of AFAAs. It can be found that two NADPH-dependent enzymatic reactions are involved in L-homoserine biosynthesis and two molecules of NADPH are needed to generate one molecule of L-homoserine (Fig. 1). Therefore, NADPH supply is a critical factor for L-homoserine production, which could be promoted by overexpressing the *pntAB* (encoding NAD(P) transhydrogenase) as described previously.[3, 18] Moreover, engineering the export system could facilitate the efflux of L-homoserine across the cell envelope to increase the extracellular product accumulation and alleviate the cellular metabolic burden.

Usually, L-homoserine cannot be accumulated in the wild type *E. coli* W3110 cells. In our previous work, LJL3 (ΔlysAΔmetAΔthrBC) was constructed by blocking the degradative and competing pathways to accumulate 0.2 g/L L-homoserine, which was chosen as the starting strain in the present work.[2] The L-homoserine production was still very low due to the limited availability of precursors. In *E. coli*, the anaplerotic pathway catalyzed by phosphoenolpyruvate carboxylase (encoded by *ppc*) is considered to be an important source of OAA.[3, 24] Therefore, the plasmid pKK-ppc was constructed to overexpress the *ppc* gene, which was then transferred into LJL3, obtaining strain SHL1. The L-homoserine titer of strain SHL1 reached 1.0 g/L in shake flask medium at 44 h, which was 5.0-fold higher than that of strain LJL3. It was indicated that *ppc* overexpression resulted in an increased carbon flux from glucose to OAA.
and enhanced the OAA supply for L-homoserine production. In addition, the *thrA*, *metL*, and *asd* genes were introduced into pKK-miniPtac, resulting in plasmids of pKK-*thrA*, pKK-*metL*, and pKK-*asd*, and were then transferred into LJL3 to obtain strains of SHL2, SHL3, and SHL4, respectively. As shown in Fig. 2A, the strains SHL2, SHL3, and SHL4 produced 0.8 g/L, 3.3 g/L and 0.6 g/L of L-homoserine in flask cultures, respectively. The results indicated that overexpressing *metL* could significantly improve the accumulation of L-homoserine. Another target gene *pntAB* (encoding a NAD(P) transhydrogenase) was overexpressed using plasmid pKK-*pntAB* to improve the NADPH generation, obtaining strain SHL5. SHL5 harboring pKK-*pntAB* produced 1.2 g/L L-homoserine, which showed a good capacity of the NADPH supply for L-homoserine biosynthesis.

Previous reports showed that high level of L-homoserine could cause the toxicity stress, which inhibited the cell growth, the activity of NADP\(^+\)-glutamate dehydrogenase, and L-homoserine production. [25, 26] Notably, the biomass of strains SHL2, SHL3 and SHL4 was significantly decreased due to the toxicity caused by increased accumulation of L-homoserine (OD\(_{600}\) from 9.9 to 7.5-8.3) (Fig. 2B). Efficient efflux of L-homoserine is an effective strategy to decrease the intracellular L-homoserine level, that can relieve the inhibition on cell growth. [20] RhtA (encoded by *rhtA*) has been identified as the potential exporter of L-homoserine. [3] To boost the efflux of L-homoserine, *rhtA* was overexpressed to obtain the strain SHL6 harboring pKK-*rhtA*, achieving a L-homoserine titer of 1.6 g/L, as shown in Fig. 2A. It was beneficial for relieving the toxicity stress arising from the L-homoserine accumulation.

Essentially, the increase in metabolic flux causes a higher demand for precursor substrates. Our results indicated that strengthening the intracellular key enzyme expression level was necessary for enhanced production of L-homoserine. Liu *et al*. have strengthened *thrA* and *metL* expression level, and Mu *et al*. have overexpressed *thrA* and *asd* to improve the production of L-homoserine. [2, 18] Meanwhile, Mu *et al*. have revealed the redox balance is important for increasing the NADPH supply and is beneficial for improving bioproducts, such as fatty acids and succinate. [18, 27] This strategy can also be applied to L-homoserine production as regeneration of NADPH from NADH by overexpressing *pntAB* gene. In short, overexpression of genes in enhancing the precursor flux (*i.e.* the OAA and L-ASP supply), the NADPH generation and the efflux of L-homoserine was essential for L-homoserine production.

### 3.2. Strengthening the genes involved in L-homoserine biosynthesis at the genome

To improve the stability of engineered strains, overexpressing the key genes by moderating the *E. coli* LJL3 genome is feasible. [2] Nowadays, CRISPR-mediated gene editing technology is beneficial for genome modification of microorganisms, owing to the high efficiency and seamless gene editing characteristics. [7] To further improve the production of L-homoserine and constructing a stable cell factory, five genes (*ppc, thrA, metL*, and *asd*, and *rhtA*) were individually up-regulated by replacing the native promoter with a strong *trc* promoter in LJL3 genome using the CRISPR-Cas9 system. First, the *ppc* gene in LJL3 was chromosomally enhanced by replacing its native promoter with the *trc* promoter, resulting in SHL7 to increase the carbon flux into L-homoserine accumulation. After 48 h of fermentation, L-homoserine production by the SHL7 strain was increased by 75% compared with that of LJL3, reaching
0.35 g/L (Fig. 2C). Moreover, to convert L-aspartate to L-homoserine, the thrA gene was subsequently upregulated by replacing the native promoter with the trc promoter in the genome of SHL7, obtaining strain SHL8. The L-homoserine titer of SHL8 was 0.7 g/L, which was 2.0-fold higher than that of SHL7. Next, the native promoter of asd gene was in situ replaced withPtrc to improve L-homoserine production, generating SHL9. As a result, the production of L-homoserine from flask cultivation of SHL9 strain was further increased to 0.9 g/L. To increase the NADPH pool, the promoter of pntAB driven by was also replaced withPtrc, generating strain SHL10. After 48 h of cultivation, strain SHL10 achieved 1.2 g/L L-homoserine, which was 33.3% higher than that of SHL10 (Fig. 2C). To boost the efflux of L-homoserine, rhtA was overexpressed by replacing native rhtA promoter withPtrc promoter of SHL10 genome, obtaining strain SHL11. L-homoserine production was significantly increased to 2.1 g/L in SHL11, 1.8-fold higher than that of SHL10 (Fig. 2C), which might be due to the higher transcription level of rhtA driven by the strong trc promoter. Moreover, the biomass (OD_{600}) was also improved from 10.4 to 12.3, 18.3% higher than that of SHL10, which was consistent with the production performance (Fig. 2D). The results showed that enhancing the efflux of L-homoserine facilitated both L-homoserine production and cell growth. Herein, the CRISPR-Cas9 mediated in situ promoter replacement was successfully applied in enhancing the expression of ppc, thrA, asd, pntAB and rhtA at the genome level, which was beneficial for the L-homoserine production. Nevertheless, the overall production of L-homoserine in the engineered strain was still low, and the increment was not significant as the overexpression at the plasmid level.

Considering the essential role of the bifunctional enzyme of MetL, the corresponding metL gene was overexpressed in SHL11 with the plasmid pKK-metL, generating the strain SHL12. As a result, the titer of 6.2 g/L was achieved for L-homoserine in SHL12, which was 3.0-fold higher than that of SHL11. It was proved that metL was the crucial gene to channel more carbon flux from glucose to L-homoserine, and the increased ASP-P pool was beneficial for improving the production of L-homoserine. However, enhancing the plasmid stability of pKK-metL was still an important issue due to the potential toxicity of L-homoserine. SHL12 cells begin to lose the plasmids after several rounds of division without any selection pressure (Fig. S1). When antibiotics are added, cells could harbor plasmids to obtain the resistance to the antibiotic. The plasmids can be unevenly distributed due to degradation or the shortage of selection pressure.[28] Thus, exploring strategies to maintain stability of pKK-metL plasmid without antibiotics addition was important for L-homoserine production.

### 3.3 Constructing auxotrophic complementation and hok/sok systems to improve the plasmid stability

Losing plasmid represents a major problem in large-scale cultivation of bacteria carrying plasmids, which are commonly used for overexpression of cloned genes. Several approaches have been conducted to enhance plasmid stability.[9, 11, 28] Herein, auxotrophic complementation and hok/sok systems were constructed to maintain the stability of pKK-metL plasmid. The auxotroph biosynthetic system, combined with the complementation of essential genes, could enable the stable maintenance of plasmid without the addition of antibiotics, because the essential genes should be expressed by plasmid instead of chromosome to ensure the cell growth. Moreover, choosing the proper gene directly related to cell viability could tightly control the plasmid copy number depending on its expression level and thus minimize cell-
to-cell variations during antibiotic-free cultivation.[9, 29] L-aspartate, the precursor of L-homoserine, is one of the most important 4-carbon amino acid platform compounds.[30, 31] As shown in Fig. 3A, the L-aspartate-amino-acid synthesis genes *aspA* and *aspC* encode aspartate aminotransferase, which could transfer fumarate and oxaloacetate to the key precursor of L-aspartate for L-homoserine, respectively. An auxotrophic complementation system was developed by deleting the chromosomal copies of these aspartate-amino-acid synthesis genes in the SHL11 genome to obtain SHL14 (Fig. 3B). The *aspA* and *aspC* genes were subsequently introduced into pKK-*metL* plasmid using a strong *trc* promoter and the native promoter to construct pKK-*metL-aspA-aspC*-1 and pKK-*metL-aspA-aspC*-2, respectively (Fig. 3B). The engineered strain SHL16 and SHL17 containing pKK-*metL-aspA-aspC*-1 and pKK-*metL-aspA-aspC*-2 were constructed, compared with the control strain SHL15 containing pKK-*metL* (Fig. 3B), respectively. The daughter cells were considered to be forced to maintain the plasmids as there was no L-ASP feeding in fermentation medium, making the cells totally dependent on the maintenance of plasmid. Unexpectedly, the titer of SHL16 was dramatically decreased to 2.2 g/L, while the titer of SHL17 was slightly higher than SHL16 (2.5 g/L), both lower than that of the control stain SHL15 (Fig. 3C). Thus, constructing the auxotrophic complementation system to maintain plasmid stability was failed herein. It was speculated that the overexpression of *aspA* amd *aspC* genes might affect the expression resources for *metL* gene, thus decreased the expression level of MetL and the L-homoserine production.

Plasmid instability is mainly caused by the factors leading to an uneven distribution of plasmids to the daughter cells during cell division. To improve the plasmid stability, the *hok/sok* system, one of the toxin/antitoxin systems, was employed to maintain the stability of plasmid pKK-*metL* herein. It was assumed that the plasmid-lacking daughter cells could be killed via a system of stable toxins and unstable antitoxins, both expressed from the plasmid (Fig. 4A).[12] The *hok/sok* gene cassette was cloned into two different locus of pKK-*metL* to investigate the effect of locations on gene expression and plasmid stability (Fig. 4B). One locus was next to the gene *metL* (generating plasmid pKK-*metL-hok1*), and the other locus was beside the antibiotic gene *amp* (generating plasmid pKK-*metL-hok2*). Then, the control strain of SHL12 (containing pKK-*metL*), two *hok/sok* constructed strains SHL18 (containing pKK-*metL-hok1*) and SHL19 (containing pKK-*metL-hok2*) were cultured in shake flasks without Amp (50 mg/L), respectively. Among these three strains, only SHL19 could retain the L-homoserine production regardless of the antibiotics added or not. As shown in Fig. 5A, the plasmid losing rate of strain SHL12 cultured without antibiotics was 94.0%. The plasmid losing rate of SHL19 (pKK-*metL-hok2*) cultured without antibiotics was 5.2%, which was much lower than that of SHL12 (Fig. 5A). The L-homoserine production of SHL19 was 6.1 g/L, which was similar with strain SHL12 (6.2 g/L). Single colony of SHL19 was inoculated into 3 bottles flask fermentation medium in parallel by 10 fermentation batches, respectively. As a result, all fermentation batches of SHL19 (10/10) could maintain the titer of 6 g/L (Fig. 5B).

The *hok/sok* system is a type I toxin/antitoxin system, which could enhance plasmid stability by the antisense RNA post-segregationally killing.[32] The half-life of *hok* mRNA is longer than that of the *sok* RNA. Thus, the daughter cells losing the plasmid would die during division since the *hok* mRNA translated to the toxin protein (Fig. 4A). [33] On the other hand, the daughter cells with plasmid will survive if the *sok*
antisense RNA binding with \textit{hok} mRNA to generate a complex that can be decayed by RNase III (Fig. 4A). [32-34] Introducing the \textit{hok/sok} system into plasmid pKK-\textit{metL} might make the cell growth dependent on the maintenance of plasmid, resulting in the improved plasmid stability without the selection pressure of antibiotics. The results indicated that the \textit{hok/sok} system was beneficial for maintaining plasmids for L-homoserine production in \textit{E. coli}.

3.4. Fed-batch fermentation for L-homoserine in a 5 L fermenter

To evaluate the productivity of strain SHL12 (harboring pKK-\textit{metL}) and SHL19 (harboring pKK-\textit{metL-hok2}) for L-homoserine production, the fed-batch fermentation was implemented in a 5 L fermenter with 2.5 L working volume without antibiotics addition. As shown in Fig. 6, the cell growth steadily increased, and the maximum OD$_{600}$ of strain SHL19 reached 68 at 48 h, while the OD$_{600}$ of SHL12 merely reached 48. The growth of strains SHL12 and SHL19 showed different trend: the OD$_{600}$ of SHL12 reached the maximum at 23.5 h and decreased at 35 h, while the OD$_{600}$ of SHL19 continuously increased. The accumulation of L-homoserine in strain SHL19 was initially detected at 4 h, and finally reached the maximum titer of 44.4 g/L at 48 h, which was 19.7% higher than that of SHL12 (35.1 g/L). It was indicated that the introduction of \textit{hok/sok} system into strain SHL19 was beneficial for the maintenance of plasmid and L-homoserine production during the fed-batch fermentation. The productivity of L-homoserine in strain SHL19 was 0.93 g/L/h, which was 1.26-fold higher than that of SHL12 (0.73 g/L/h). Compared with the L-homoserine producing strain LJL (0.82 g/L/h) reported previously[2], SHL19 showed higher productivity in fed-batch fermentation. The titer and productivity of L-homoserine obtained herein were lower than the two works reported in this year.[3, 18]

Nevertheless, there was no antibiotics added in this work, which could be beneficial for the environment. The production of L-homoserine could be further improved combing with other strategies, such as a dynamical controlling degradation system[3] and a balancing redox strategy[18]. Moreover, low OD$_{600}$ value (up to 68) was obtained in the fed-batch fermentation. Additional modifications of fermentation feeding strategy was also needed to optimize fermentation condition for higher production of L-homoserine.

3.5. Ectoine biosynthesis facilitated with the \textit{hok/sok} system

Ectoine is widely used in skin protection, pharmaceutics and cosmetics, which is an important derivative from AFAAs synthesis pathway using the same precursor ASP-SA with L-homoserine. Ning et al. constructed the engineered \textit{E. coli} strain with an ectoine titer of 25.1 g/L by introducing the \textit{ectABC} gene cluster from \textit{Ectothiorhodospira halochloris}, weakening the competitive pathway, releasing feedback resistant and improving the oxaloacetate pool.[35] To test the application of \textit{hok/sok} system for AFAAs synthesis, this system was applied for the ectoine production herein. First, the ectoine biosynthesis strain was constructed by introducing the \textit{ectABC} cluster into \textit{E. coli} MG1655, generating strain S00. The resultant S00 strain synthesized 1.8 g/L ectoine in the flask fermentation. Second, several metabolic engineering strategies were used by modifying the \textit{E. coli} MG1655 genome to obtain metabolic
engineering strain: improving the precursor OAA level by replacing the phosphoenolpyruvate carboxylase (ppc) promoter with a trc promoter to getting strain S01; enhancing the oxaloacetate supply by deleting the transcriptional repressor (encoded by iclR) to promote glyoxylate shunt to obtain strain S02; and concentrating the carbon flux from L-aspartate-β-semialdehyde to ectoine by deleting the bifunctional aspartokinase/homoserine dehydrogenase (encoding by thrA) to generating strain S03 (Fig. 7A). As shown in Fig. 7B, the ectoine titer of S03 containing plasmid ptrc99a-ectABC was increased to 3.0 g/L, which was 1.7-fold higher than that of the parent strain S00. Then, the hok/sok system was introduced into the plasmid ptrc99a-ectABC of strain S03, obtaining strain S04. Without antibiotics addition, the ectoine titer of S04 harboring ptrc99a-ectABC-hok reached 4.1 g/L, which was 36.7% higher than that of S03 (Fig. 7B). In order to evaluate the stability and ectoine production of the strains S04 and S03, the fed-batch fermentation was carried out in a 5 L fermenter. Finally, the ectoine titer of strain S04 reached 23.3 g/L, which was 1.5-fold higher than S03 (15.9 g/L). The growth of strains S03 and S04 showed different trend: the OD_{600} of S03 reached the maximum at 28 h and decreased at 36 h, so the accumulation rate of ectoine production of S03 slowed down at 28h; while the ectoine production of S04 continuously increased with growing biomass of S04. The productivity of S04 was 0.53 g/L/h, which was 1.2-fold higher than that of S03 (0.45 g/L/h) (Fig. 7C, 7D). It was indicated that the hok/sok system was also beneficial for improving plasmid stability and ectoine production, which also could eliminate the environmental concerns associated with the usage of antibiotics.

Conclusions

In the present work, we reported the application of hok/sok system and genome modification strategy for constructing stable E. coli strains to produce AFAAs and the derivatives (L-homoserine and ectoine) without adding antibiotics. The metabolic engineering strategy was focused on enhancing the carbon flux to L-homoserine synthesis, improving NADPH generation and boosting the L-homoserine efflux. The plasmid stability was improved by introducing the hok/sok system, and resulting in 44.4 g/L L-homoserine production of the final engineered strain SHL19 without antibiotics addition. Moreover, the hok/sok system was also beneficial for maintaining the plasmid stability and ectoine production. This work provides valuable strategies for producing AFAAs and the derivatives by improving plasmid stability and the elimination of environmental concerns associated with the application of antibiotics, which could be also used for producing other kinds of bioproducts in E. coli.

Abbreviations

AFAAs: L-aspartate amino acids; ppc: encoding the PEP carboxylase gene; thrA: encoding the pyruvate kinase II gene; asd: encoding the pyruvate kinase I gene; pntAB: encoding the NAD(P) transhydrogenase gene; rhtA: encoding a L-homoserine export protein gene; OAA: oxaloacetate; L-ASP: L-aspartate; Asp-P: Aspartyl-β-phosphate; Asp-SA: aspartyl-β-semialdehyde; aspA: encoding aspartate ammonia-lyase; aspC: encoding aspartate transamination gene.
Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during the study were included in this paper and its Additional file 1.

Competing interests

The authors declare no competing interests.

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Author contributions

BY S, HL S, and M L designed and carried out experiments, performed a complete analysis, and prepared a draft of the manuscript. BY S and HL S performed the fermentation experiments. XY T, B G, FQ W, QH L, YS M and J Z contributed reagents and materials. BY S, XY T, M L, and DZ W coordinated and supervised the study, and finalized the manuscript. All authors contributed to the manuscript, and read and approved the final version.

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**Tables**

Table 1 Strains and plasmids used in the work
| Strains/plasmids | Source | Relevant characteristic |
|------------------|--------|-------------------------|
| **Plasmids**     |        |                         |
| pKK-miniPtac     | Our laboratory | Plasmid for overexpression, $\text{Amp}^R$, miniPtac promoter |
| pKK-metL         | This study | Plasmid for overexpressing metL, $\text{Amp}^R$ |
| pKK-metL-hok-1   | This study | Plasmid for overexpressing metL, hok/sok system $\text{Amp}^R$ |
| pKK-metL-hok-2   | This study | Plasmid for overexpressing metL, hok/sok system, $\text{Amp}^R$ |
| pKK-ppc          | This study | Plasmid for overexpressing ppc, $\text{Amp}^R$ |
| pKK-asd          | This study | Plasmid for overexpressing asd, $\text{Amp}^R$ |
| pKK-thrA         | This study | Plasmid for overexpressing thrA, $\text{Amp}^R$ |
| pKK-pntAB        | This study | Plasmid for overexpressing pntAB, $\text{Amp}^R$ |
| pKK-rhtA         | This study | Plasmid for overexpressing rhtA, $\text{Amp}^R$ |
| pKK-metL-aspA-aspC-1 | This study | Plasmid for overexpressing metL, along with aspA, aspC, controlled by trc promoter, $\text{Amp}^R$ |
| pKK-metL-aspA-aspC-2 | This study | Plasmid for overexpressing metL, controlled by trc promoter; coupled with aspA, aspC, controlled by native promoter, $\text{Amp}^R$ |
| pTrc99a          | Our laboratory | Plasmid for overexpression, $\text{Amp}^R$ |
| pTrc99a-ectABC   | This study | Plasmid for overexpressing ectABC, $\text{Amp}^R$ |
| pTrc99a-ectABC-hok | This study | Plasmid for overexpressing ectABC, hok/sok system, $\text{Amp}^R$ |
| pTarget          | Our laboratory | $\text{pMB1 aadA sgRNA, Spe}^R$ |
| pTarget-ppc(trc) | This study | Plasmid for knockout gene ppc, $\text{Spe}^R$ |
| pTarget-asd(trc) | This study | Plasmid for knockout gene asd, $\text{Spe}^R$ |
| pTarget-thrA(trc) | This study | Plasmid for knockout gene rhtA, $\text{Spe}^R$ |
| pTarget-pntAB(trc) | This study | Plasmid for knockout gene \( pntAB, Spe^R \) |
|---------------------|-------------|---------------------------------|
| pTarget-rhtA(trc) | This study | Plasmid for knockout gene \( rhtA, Spe^R \) |
| pCas               | Our laboratory | \( \text{repA101(Ts)Kna}^R\text{Pcas-Cas9ParaB-Red lacIq P} \text{trc-sgRNA-pMB1} \) |

**Strains**

- **E. coli W3110**
  - Our laboratory
  - Host strain
- **E. coli MG1655**
  - Our laboratory
  - Host strain
- **LJL3**
  - Precious work
  - \( \text{W3110}^{\Delta lysA\Delta metA\Delta thrBC} \)
- **SHL1**
  - This study
  - LJL3 strain containing pKK-ppc
- **SHL2**
  - This study
  - LJL3 strain containing pKK-thra
- **SHL3**
  - This study
  - LJL3 strain containing pKK-metL
- **SHL4**
  - This study
  - LJL3 strain containing pKK-asd
- **SHL5**
  - This study
  - LJL3 strain containing pKK-pntAB
- **SHL6**
  - This study
  - LJL3 strain containing pKK-rhtA
- **SHL7**
  - This study
  - \( \text{W3110}^{\Delta lysA\Delta metA\Delta thrBC-ppc(trc)} \)
- **SHL8**
  - This study
  - \( \text{W3110}^{\Delta lysA\Delta metA\Delta thrBC-ppc(trc)-thrA(trc)} \)
- **SHL9**
  - This study
  - \( \text{W3110}^{\Delta lysA\Delta metA\Delta thrBC-ppc(trc)-thrA(trc)-asd(trc)} \)
- **SHL10**
  - This study
  - \( \text{W3110}^{\Delta lysA\Delta metA\Delta thrBC-ppc(trc)-thrA(trc)-asd(trc)-pntAB(trc)} \)
- **SHL11**
  - This study
  - \( \text{W3110}^{\Delta lysA\Delta metA\Delta thrBC-ppc(trc)-thrA(trc)-asd(trc)-pntAB(trc)-rhtA(trc)} \)
- **SHL12**
  - This study
  - SHL11 strain containing pKK-metL
- **SHL13**
  - This study
  - \( \text{W3110}^{\Delta lysA\Delta metA\Delta thrBC\Delta aspA-ppc(trc)-thrA(trc)-asd(trc)-pntAB(trc)-rhtA(trc)} \)
| SHL14 | This study | W3110ΔlysAΔmetAΔthrBCΔaspAΔaspC-ppc(trc)-thrA(trc)-asd(trc)-pntAB(trc)-rhtA(trc) |
|-------|------------|----------------------------------------------------------------------------------|
| SHL15 | This study | SHL14 strain containing pKK-metL                                                     |
| SHL16 | This study | SHL14 strain containing pKK-metL-aspA-aspC-1                                      |
| SHL17 | This study | SHL14 strain containing pKK-metL-aspA-aspC-2                                      |
| SHL18 | This study | SHL11 strain containing pKK-metL-hok-1                                            |
| SHL19 | This study | SHL11 strain containing pKK-metL-hok-2                                            |
| S00   | This study | MG1655 containing pTrc99a-ectAB                                                   |
| S01   | This study | MG1655-ppc(trc) containing pTrc99a-ectAB                                           |
| S02   | This study | MG1655ΔiclR-ppc(trc) containing pTrc99a-ectAB                                      |
| S03   | This study | MG1655ΔthrAΔiclR-ppc(trc) containing pTrc99a-ectAB                                 |
| S04   | This study | MG1655ΔthrAΔiclR-ppc(trc) containing pTrc99a-ectABC-hok                             |

**Figures**
Figure 1

The L-homoserine biosynthetic pathway from glucose in Escherichia coli. Red bold arrow denotes the enhanced pathway. Green bold cross denotes the deletion of genes. Abbreviations: PEP, phosphoenolpyruvate; Pyr, pyruvate; AcCoA, acetylcoenzyme A; CIT, citrate; OAA, oxaloacetate; FUM, fumarate; Asp, L-aspartate; Asp-P, Aspartyl-β-phosphate; Asp-SA, aspartyl-β-semialdehyde; L-Thr, L-threonine; L-Lys, L-lysine; L-Ile, L-isoleucine; L-HOM, L-homoserine; aspA, aspartate ammonia-lyase; aspC, aspartate transamination; pntAB, NAD(P) transhydrogenase; thrA, Aspartate kinase I; metL, Aspartate kinase II; ppc, PEP carboxylase; rhtA, L-homoserine export protein.
Figure 2

(A) Characterization of the recombinant strains of SHL1, SHL2, SHL3, SHL4, SHL5 and SHL6. (B) The biomass of these recombinant strains. The L-homoserine production and the biomass of the recombinant strains of SHL7, SHL8, SHL9, SHL10, SHL11 and SHL12 were shown in (C) and (D). The error bars represent standard deviation from triplicate experiments. Statistical analysis was performed using the Student’s t test (one-tailed; two-sample unequal variance; *p < 0.05, **p < 0.01, ***p < 0.001). All data represent the mean of n = 3 biologically independent samples and error bars show the standard deviation.
Figure 3

(A) The metabolic pathway for biosynthesis of the L-aspartate. (B) Schematic model of aspA/aspC-based auxotrophic complementation expression system. (C) The titer and biomass of SHL12, SHL16 and SHL17.
Figure 4

(A) Schematic model of the hok/sok system. (B) Two hok/sok plasmids harboring hok/sok system at different locations. Bent arrow indicates a promoter and T-shape bar indicates a transcription terminator. (C) Flask culture results of SHL12, SHL18 and SHL19 strains grown without antibiotics. Error bars represent standard deviations (n = 3).
Figure 5

(A) The fermentation broths of SHL12 and SHL19 cultured without adding antibiotics were diluted by 10, 25 and 50 times and cultured on solid plate with or without antibiotics, respectively. (B) L-homoserine titers of SHL19 in different fermentation batch.

Figure 6

Fed-batch fermentation profiles of recombination strains SHL12 (A) and SHL18 (B) in a 5L fermenter. Profiles of L-homoserine production, cell density, and glucose consumption of strain SHL12 (A) and SHL19 (B). The 5 L bioreactor used for fed-batch fermentation of SHL19 strain (C). The error bars represent standard deviation from triplicate experiments.
Figure 7

(A) The biosynthesis pathways for the production of ectoine. The enhanced pathway is shown in red lines and the deletion of gene is shown in green bold cross. (B) The ectoine and cell growth of engineered strains S00, S01, S02, S03 and S04. The ectoine production, glucose consuming and biomass of the engineered strain S03 (C) and S04 (D) in fed-batch fermentation were shown.

Supplementary Files

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