Application of *Corynebacterium glutamicum* in L- threonine biosynthesis

Jianhang Liu[a], Deqiang Zhu[b]

Key Laboratory of Microbiological Engineering of Shandong Province, Qilu University of Technology (Shandong Academy of Sciences), Jinan, China

[a]Liujx@tib.cas.cn; [b]zdq0819@qlu.edu.cn

Abstract. L-threonine is an essential amino acid for human and animal nutrition. L-threonine is the second limiting amino acid in pig feed and the third limiting amino acid in poultry feed, primarily used as a feed additive. Currently, industrial production of L-threonine is achieved through fermentation using *Escherichia coli*. *Corynebacterium glutamicum*, a bacterium commonly used as an industrial production chassis for amino acids such as glutamic acid and L-lysine, possesses advantages such as biosafety and strong environmental adaptability. It is considered a potential strain for the efficient production of L-threonine. However, current production of L-threonine by *C. glutamicum* often leads to the accumulation of significant amounts of other amino acid by-products, limiting the level of L-threonine production. Previous studies that aimed to block or weaken the pathways for by-product synthesis resulted in strains with nutritional deficiencies or only partial reduction in by-product accumulation.

Keywords: L-threonine; Corynebacterium glutamicum; metabolic engineering; biosynthesis.

1. Introduction

Amino acids are the fundamental building blocks of proteins in living organisms and are crucial for the nutritional health of humans and animals. With the development of technology, the variety of amino acids and their derivatives has expanded from over 50 in the 1960s to over 1000 now, widely used in fields such as feed, food, medicine, health products, cosmetics, pesticides, fertilizers, and leather making. Among them, the feed amino acid market has the largest scale, accounting for 50%-60% of the market share, followed by food amino acids accounting for about 30% of the market share [1].

Microorganisms can generally synthesize all 20 types of protein amino acids, so modifying and breeding microorganisms by fermenting sugars and other raw materials is currently the main production method for most amino acids. In the initial stage, due to a lack of understanding of microbial amino acid metabolism mechanisms and genetic modification techniques, more mutation screening methods were used for the selection of amino acid producing strains. The integration of the development of Synthetic biology and industrial biotechnology has hastened the birth of industrial Synthetic biology for the creation of industrial strains, reshaped the development and upgrading model of amino acid production strains, significantly improved the production level of various amino acids, and the synthesis conversion rate was close to the theoretical value. Some special amino acids have also been biosynthesized through the development of artificial strains and enzyme catalysts [2]

L-threonine is an essential amino acid in human and animal nutrition, which is widely used in food, medicine, feed and other fields, with a global market scale of more than 700000 tons/year. *C. glutamicum*, as a chassis cell for industrial production of amino acids, has the advantages of biological safety and strong environmental adaptability. It is a potential strain for efficient production of L-threonine. This paper systematic review the function and application of L-threonine, the pathway of *C. glutamicum* to synthesize L-threonine, and the application of metabolic engineering technology to improve the production of L-threonine.

2. Function and Application of L-Threonine

L-threonine is one of the eight essential amino acids for the human body, which plays an important role in the growth and development of organisms. In addition, L-threonine can also delay aging, improve the body's immunity and resistance, and has a good protective effect on brain and physical diseases. Lack of L-threonine will lead to damage to immune function. In addition to being an essential amino acid for the human body, L-threonine is also widely used in feed, food, medicine and other industries.

In the feed industry, L-threonine is the second limiting amino acid after L-lysine in pig feed, and the third limiting amino acid in poultry feed after L-lysine and L-methnine. In recent years, L-threonine has become one of the main bottlenecks in animal production performance. It is an essential amino acid for growth, promotes the production of mucin in the animal intestine, and plays an important role in food digestion. For the growth and development of animals, a good balance of each essential amino acid is very important, however, the amino acid content of most feed materials such as corn or wheat is not balanced, by adding L-threonine, the ratio of various amino acids in the feed can be increased Coordination, improve the quality of feed, and can effectively reduce unnecessary waste of resources[3].

In the food industry, as a food additive, L-threonine is widely used as a nutritional supplement, which can be used to enhance protein nutrition, improve the taste and quality of food, and formula food for special groups, such as infant formula milk powder, low-protein food wait. Moreover, L-threonine itself is a strong oxidant. By adding it to food, it has an antioxidant effect on food and can maintain the freshness of food. In addition, L-threonine can enhance the taste of food, making the mouthfeel more unique and delicious[4].

In the pharmaceutical industry, the status of L-threonine is also very important. As an essential amino acid in the human body, L-threonine plays an important role in the metabolism of the human body, which can improve the nutritional status, promote the growth and development of the body, and improve the body's immunity. For patients with oral injury or surgery, sometimes unable to take in enough nutrition, the amino acid injection with L-threonine can be added to obtain the necessary nutrients, and at the same time enhance the vitality of the body, which is also very helpful for the recovery of the wound[5].

3. Application of C glutamicum in amino acid production

C. glutamicum is a spore-free, non-pathogenic, Gram-positive bacterium that can grow at 30°C-40°C, with an optimum pH of 7.0-8.0[6, 7]. The cell shape is short rod rod, grows rapidly, is easy to cultivate, and the bacteria are smooth. Corynebacterium glutamicum meets food safety standards, is a safe strain[1], and does not produce endotoxin; it has strong environmental adaptability, can cope with rough industrial culture conditions, and can use a variety of carbon sources for growth and production of other compounds [8], so it is generally considered as an ideal strain for amino acid fermentation production.

The application of *C. glutamicum* in glutamic acid fermentation in 1957 was the beginning of amino acid fermentation. This discovery is not only a major breakthrough in the production of glutamic acid, but also indicates the feasibility of microbial fermentation to produce amino acids [9], making Corynebacterium glutamicum applied to L-lysine, L-glutamic acid and L- Production of various amino acids such as proline [10-12], thus meeting the growing market demand. Different amino acids are different in nutrition, flavor and physical and chemical properties, so they are widely used as raw materials in our daily life, such as cosmetics, food, chemical reagents, feed and medicine. At present, amino acid fermentation has become the pillar industry of my country's fermentation industry, and the market for amino acids is also increasing year by year, showing a steady growth trend. *C. glutamicum* has also gradually become an important strain for the production of amino acids by microbial fermentation in industry. In addition, it is also used in the production of alcohols [13], terpenoids [14] and organic acids [15] superior.

The traditional amino acid industrial production strains are usually obtained by mutation breeding, but these methods have certain disadvantages, such as unclear mechanism of random mutation, influence on strain growth and so on. With more and more studies on *C. glutamicum*, people have a clear understanding of its metabolic network and regulatory mechanism. Especially since Kalinowski et al. first sequenced the whole genome of *C. glutamicum* ATCC 13032 in 2003[16], as well as the continuous maturity of gene manipulation technology, it is convenient to use biological editing tools to carry out metabolic engineering of target genes, and then achieve Elimination of feedback inhibition of key enzymes in *C. glutamicum*, knockout of metabolic branches in the amino acid synthesis pathway, high-level expression of key genes, and knock-in of foreign genes, so as to finally obtain efficient amino acid production strains[17].

4. Modification of L-threonine metabolic pathway in *C. glutamicum*

Early amino acid production strains were mainly obtained by random mutation screening, but the strains obtained by this method tend to have problems such as unclear genetic background, unstable mutation effect, and strain safety in the industrial fermentation process [18]. At present, the application of metabolic engineering breeding in the breeding of L-threonine-producing strains is becoming more and more common, and the desired high-yielding strains of L-threonine are obtained by directional changes of target genes by means of molecular manipulation. With the continuous development of genetic metabolic engineering in recent years, a series of L-threonine-producing strains of *C. glutamicum* have also been obtained [19]. At present, the transformation strategy of L-threonine metabolic pathway mainly focuses on the following aspects: (1) strengthening the synthesis pathway of L-threonine; (2) enhancing the ability of L-threonine efflux; (3) reducing the capacity of L-threonine intracellular metabolism of threonine; (4) weakening the carbon flux of the L-threonine synthesis competition pathway.

5. Overexpression of key enzymes in biosynthetic pathways

Homoserine dehydrogenase (Hom) and homoserine kinase (ThrB) in the threonine production pathway of *C. glutamicum* are regulated by strict feedback inhibition: Hom is regulated by L-threonine in an allosteric noncompetitive manner Feedback inhibition; while ThrB is feedback-regulated by L-threonine through a competitive inhibition mechanism, which greatly limits the production level of threonine. To address this limitation to provide higher levels of L-threonine production, the following metabolic engineering strategies have been implemented in some studies: including overexpression of the gene *hom-thrB*, redirecting carbon flux from the L-lysine branch to L -Threonine branch [20], for example, the integration of three copies of *hom*^{r}*-thrB* in the chromosome of *C. glutamicum* MH20-22B makes the production of L-threonine reach 7.7 g/L, and the production of L-lysine from 30.4 g /L was significantly reduced to 8.5 g/L [21]. Further increasing the expression of *thrB* in the metabolic pathway to a higher level than the gene hom may effectively avoid the accumulation of the intermediate product L-homoserine.

Apart from this, lifting the feedback inhibition of the two enzymes is the most critical strategy to address this limitation. In terms of research on releasing the feedback inhibition of L-threonine on Hom, as early as 1991, Eikmann et al. used glutamic acid to replace glycine (G378E) to relieve the inhibition of threonine on the activity of the enzyme [22]; Archer et al. found that when When Hom has a C-terminal frameshift mutation, it can relieve the feedback inhibition of threonine. Recently, some studies have also screened some mutants that relieve Hom feedback inhibition by chemical mutagens: *hom*^{T285I}, *hom*^{R398Q} and *hom*^{G378W}, etc. can increase the production of threonine. On the other hand, since L-threonine competes with the substrate L-homoserine for ThrB binding, mutations that block the binding of L-threonine at the active site will also inhibit the binding of L-homoserine, which may Reduce the catalytic activity of the enzyme, so it is difficult to release the feedback inhibition of ThrB. In some studies, by rationally designing the binding site between ThrB and the

substrate, the resulting mutant has a higher selectivity for L-homoserine than L-threonine, thereby releasing the feedback inhibition and improving the catalytic activity of the enzyme [23].

6. Reduced intracellular consumption of L-threonine

The strategy of reducing the consumption of L-threonine to L-isoleucine and glycine in cells has been applied in *Ecoli* and *C. glutamicum*, and it has been proved that this strategy is effective in increasing the production of L-threonine and reducing By-product formation is effective [24]. When the $ilvA^{C290T}$ gene mutation was introduced into the chromosome in Escherichia coli and the tdh gene was knocked out, L-threonine consumption was reduced. In *C. glutamicum*, by down-regulating the promoter of the gene glyA, the *C. glutamicum* could be attenuated [25]. The conversion of threonine to glycine in DM368-2 increased the yield of L-threonine to 1.3 g/L, and the yield of glycine decreased accordingly; the reduction of threonine in *C. glutamicum* DM1800-T was achieved by down-regulating the promoter strength of *ilvA* conversion of acid to isoleucine, thereby increasing threonine production from 2.5 g/L to 4 g/L [26]

7. Increase the extracellular excretion of L-threonine

When threonine accumulates to a certain level in the cell, the capacity for extracellular secretion becomes the limiting amino acid production. High concentrations of threonine in cells will down-regulate key enzymes in biosynthesis in a feedback manner, and even inhibit cell growth. In *Ecoli*, when *rhtBEc*, rhtCEc and *thrECg* were overexpressed in MG422 strain, the production of threonine increased by 140, 200 and 290%, respectively. In another study, the transcription of *rhtA* in this strain was enhanced by introducing a point mutation (G → A) one base upstream of the start codon of the *rhtA* gene on the chromosome, resulting in an increase in L-threonine production from 18.4 g /L increased to 36.3 g/L [27].

In *C. glutamicum*, the introduction of a *thrECg*-containing recombinant plasmid into strain MH20-22B-(*hom*^{r}*-thrB*) 3 increased the L-threonine production from 5.8 g/L to 8.1 g/L while decreasing The accumulation of L-lysine, L-glycine and L-isoleucine was suppressed [25]. When heterologously expressing *rhtAEc*, *rhtCEc* and *yeaSEc* in Corynebacterium glutamicum, it is also beneficial to increase the production of threonine, for example, when the rhtCEc gene is overexpressed in *C. glutamicum* DM368-3 (AEC^r, AHV^r), the production of L-threonine increased from 0.9 g/L to 3.7 g/L. At the same time, when the gene was overexpressed in *C. glutamicum* DM1800 (pET-T18*hom*^{r}*-thrB-thrE*), the threonine production increased by 2.4 g/L, and there was no accumulation of L-homoserine [26].

8. Systemic Metabolic Engineering

There are relatively few reports on the production of threonine by metabolic engineering of *C. glutamicum*. Even with a similar genetic manipulation strategy in *C. glutamicum*, the production of threonine is still low: for example, studies have shown that in *C glutamicum* T11 (methionine and isoleucine deficiencies and release of hom feedback The *lysC-asdA* tandem expression cassette containing a strong promoter tac was inserted into the suppressor strain), although it was 27.8% higher than that of the starting strain, but the final L-threonine production in shake flask fermentation only reached 7.18 g/L. In recent studies The PLMC method was used to construct promoter libraries with different strengths for each gene in the threonine synthesis pathway, and the expression strength of each gene was adjusted by combination to make the final threonine yield reach 12.8 g/L, which was 6.1 times higher than that of the starting strain. In *C. glutamicum*, the catalytic activities of AKIII, HD and HK encoded by *lysC, hom* and *thrB* regulated by feedback inhibition may be the bottleneck limit of threonine production by *C. glutamicum*, and the feedback inhibition can be relieved to the

greatest extent It may be the best strategy to break through the limit and increase the production of threonine [28].

In addition, some studies have modified and combined a variety of related genes in glucose uptake, glycolysis, TCA cycle and pentose phosphate pathway in the threonine cell factory to increase the production of L-threonine. When knocking out the gene crr related to glucose uptake in *E. coli* MG1655 (activating other systems of glucose uptake GalP and MglABC) and inactivating the transcription factor *iclR* (inactivating the repressive transcription factor of glutamate dehydrogenase encoded by *gdhA* provided Nitrogen source), the threonine yield can reach 17.98 g/L [29]. Betaine is an osmoprotective compatible solute. Betaine supplementation can up-regulate the expression of intracellular zwf-encoded glucose-6-phosphate dehydrogenase, leading to an increase in NADPH, which is beneficial to the production of L-threonine. Betaine increases threonine production by 13.3% in *E. coli* THRD [30-32].

9. Summary and Outlook

At present, during the production of L-threonine by *C. glutamicum*, it is easy to accumulate other by-product amino acids, which affects the production of L-threonine. Therefore, it is particularly important to propose a new solution to the problem of by-product amino acid accumulation, and solving the problem of by-product accumulation is of great significance for improving the production of L-threonine. In this study, the by-products L-lysine and L-isoleucine increases the production of L-threonine, and successfully constructs a chassis strain for producing L-threonine. In this study, it was found that the existing L-threonine efflux proteins are not very specific. In the future work, excavating more specific L-threonine efflux proteins will be helpful for improving the L-threonine efflux protein. Acid production plays an important role. During the synthesis of L-threonine, the problem of L-homoserine accumulation has not been completely resolved. By expressing the homoserine kinase gene *thrB*, the accumulation of L-homoserine is further reduced, and it is not completely converted into L-threonine. Therefore, in future work, the problem of L-homoserine accumulation needs to be further solved, and homoserine kinases with higher enzyme activity levels can be excavated to promote the conversion of L-homoserine to L-threonine.

References

[1] Bampidis V, Azimonti G, Bastos ML, Christensen H, Dusemund B, Kouba M, Kos Durjava M, Lopez-Alonso M, Lopez Puente S, Marcon F, et al: Safety and efficacy of l-threonine produced by

[2] Usuda Y, Hara Y, Kojima H: Toward Sustainable Amino Acid Production. Amino Acid Fermentation 2017, 159:289-304.

[3] Jiang T, Li C, Teng Y, Zhang R, Yan Y: Recent advances in improving metabolic robustness of microbial cell factories. Curr Opin Biotechnol 2020, 66:69-77.

[4] Angelaccio S, Chiaraluce R, Consalvi V, Buchenau B, Giangiacomo L, Bossa F, Contestabile R: Catalytic and thermodynamic properties of tetrahydromethanopterin-dependent serine hydroxymethyltransferase from Methanococcus jannaschii. J Biol Chem 2003, 278:41789-41797.

[5] Mukherjee M, Sievers SA, Brown MT, Johnson PJ: Identification and biochemical characterization of serine hydroxymethyl transferase in the hydrogenosome of Trichomonas vaginalis. Eukaryot Cell 2006, 5:2072-2078.

[6] Hermann T: Industrial production of amino acids by coryneform bacteria. Journal of Biotechnology 2003, 104:155-172.

[7] Shiio I, Ozaki H: Regulation of Nicotinamide Adenine Dinucleotide Phosphate-specific Glutamate Dehydrogenase from Brevibacterium flavum, a Glutamate-producing Bacterium. The Journal of Biochemistry 1970, 68:633-647.

[8] Sasaki M, Jojima T, Inui M, Yukawa H: Simultaneous utilization of D-cellobiose, D-glucose, and D-xylose by recombinant Corynebacterium glutamicum under oxygen-deprived conditions. Appl Microbiol Biotechnol 2008, 81:691-699.

[9] Debabov VG: The threonine story. Advances in Biochemical Engineering Biotechnology 2003, 79:113-136.

[10] Wang XC, Liu J, Zhao J, Ni XM, Zheng P, Guo X, Sun CM, Sun JB, Ma YH: Efficient production of trans-4-hydroxy-l-proline from glucose using a new trans-proline 4-hydroxylase in Escherichia coli. J Biosci Bioeng 2018, 126:470-477.

[11] Xu JZ, Ruan HZ, Yu HB, Liu LM, Zhang W: Metabolic engineering of carbohydrate metabolism systems in Corynebacterium glutamicum for improving the efficiency of L-lysine production from mixed sugar. Microb Cell Fact 2020, 19:39.

[12] Liu J, Liu M, Shi T, Sun G, Gao N, Zhao X, Guo X, Ni X, Yuan Q, Feng J, et al: CRISPR-assisted rational flux-tuning and arrayed CRISPRi screening of an L-proline exporter for L-proline hyperproduction. Nat Commun 2022, 13:891.

[13] Jojima T, Noburyu R, Sasaki M, Tajima T, Suda M, Yukawa H, Inui M: Metabolic engineering for improved production of ethanol by Corynebacterium glutamicum. Appl Microbiol Biotechnol 2015, 99:1165-1172.

[14] Heider SAE, Peters-Wendisch P, Wendisch VF: Carotenoid biosynthesis and overproduction in Corynebacterium glutamicum. Bmc Microbiology 2012, 12.

[15] Wendisch VF, Bott M, Eikmanns BJ: Metabolic engineering of Escherichia coli and Corynebacterium glutamicum for biotechnological production of organic acids and amino acids. Current Opinion in Microbiology 2006, 9:268-274.

[16] Kalinowski J, Bathe B, Bartels D, Bischoff N, Bott M, Burkovski A, Dusch N, Eggeling L, Eikmanns BJ, Gaigalat L, et al: The complete Corynebacterium glutamicum ATCC 13032 genome sequence and its impact on the production of L-aspartate-derived amino acids and vitamins. Journal of Biotechnology 2003, 104:5-25.

[17] Wendisch VF: Metabolic engineering advances and prospects for amino acid production. Metab Eng 2020, 58:17-34.

[18] Ma Q, Xia L, Tan M, Sun Q, Yang M, Zhang Y, Chen N: Advances and prospects in metabolic engineering for the production of amino acids. Sheng Wu Gong Cheng Xue Bao 2021, 37:1677-1696.

[19] Kruse D, Kramer R, Eggeling L, Rieping M, Pfefferle W, Tchieu JH, Chung YJ, Jr Saier MH, Burkovski A: Influence of threonine exporters on threonine production in Escherichia coli. Appl Microbiol Biotechnol 2002, 59:205-210.

[20] Wei L, Xu N, Wang Y, Zhou W, Han G, Ma Y, Liu J: Promoter library-based module combination (PLMC) technology for optimization of threonine biosynthesis in Corynebacterium glutamicum. Appl Microbiol Biotechnol 2018, 102:4117-4130.

[21] REINSCHEID DJ: Stable Expression of hom-i-thrB in Corynebacterium glutamicum and Its Effect on the Carbon Flux to Threonine and Related Amino Acids. 1994.

[22] Eikmanns BJ, Metzger M, Reinscheid D, Kircher M, Sahm H: Amplification of three threonine biosynthesis genes in Corynebacterium glutamicum and its influence on carbon flux in different strains. Appl Microbiol Biotechnol 1991, 34:617-622.

[23] Petit C, Kim Y, Lee SK, Brown J, Larsen E, Ronning DR, Suh JW, Kang CM: Reduction of Feedback Inhibition in Homoserine Kinase (ThrB) of Corynebacterium glutamicum Enhances l-Threonine Biosynthesis. ACS Omega 2018, 3:1178-1186.

[24] Lee KH, Park JH, Kim TY, Kim HU, Lee SY: Systems metabolic engineering of Escherichia coli for L-threonine production. Mol Syst Biol 2007, 3:149.

[25] Simic P, Willuhn J, Sahm H, Eggeling L: Identification of glyA (encoding serine hydroxymethyltransferase) and its use together with the exporter ThrE to increase L-threonine accumulation by Corynebacterium glutamicum. Appl Environ Microbiol 2002, 68:3321-3327.

[26] Diesveld R, Tietze N, Furst O, Reth A, Bathe B, Sahm H, Eggeling L: Activity of exporters of Escherichia coli in Corynebacterium glutamicum, and their use to increase L-threonine production. J Mol Microbiol Biotechnol 2009, 16:198-207.

[27] Livshits VA, Zakataeva NP, Aleshin VV, Vitushkina MV: Identification and characterization of the new gene rhtA involved in threonine and homoserine efflux in Escherichia coli. Research in Microbiology 2003, 154:123-135.

[28] Li Y, Wei H, Wang T, Xu Q, Zhang C, Fan X, Ma Q, Chen N, Xie X: Current status on metabolic engineering for the production of l-aspartate family amino acids and derivatives. Bioresour Technol 2017, 245:1588-1602.

[29] Zhu L, Fang Y, Ding Z, Zhang S, Wang X: Developing an l-threonine-producing strain from wild-type Escherichia coli by modifying the glucose uptake, glyoxylate shunt, and l-threonine biosynthetic pathway. Biotechnol Appl Biochem 2019, 66:962-976.

[30] Su Y, Guo QQ, Wang S, Zhang X, Wang J: Effects of betaine supplementation on L-threonine fed-batch fermentation by Escherichia coli. Bioprocess Biosyst Eng 2018, 41:1509-1518.

[31] Wang S, Fang Y, Wang Z, Zhang S, Wang L, Guo Y, Wang X: Improving L-threonine production in Escherichia coli by elimination of transporters ProP and ProVWX. Microb Cell Fact 2021, 20:58.

[32] Li Y, Zhang D, Cai N, Han C, Mao Q, Wang T, Zhou Q, Chen N, Xie X: Betaine supplementation improved l-threonine fermentation of Escherichia coli THRD by upregulating zwf (glucose-6-phosphate dehydrogenase) expression. Electronic Journal of Biotechnology 2019, 39:67-73.