The Application of Corynebacterium glutamicum in L-Threonine Biosynthesis

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Abstract: L-threonine is an essential amino acid in human and animal nutrition. It is widely used in food, medicine, feed, and other fields. The global market scale exceeds 700,000 tons per year. Corynebacterium glutamicum, as a chassis cell for industrial amino acid production, has the advantages of biological safety and strong environmental adaptability, and is a potential strain for the efficient production of L-threonine. This article systematically reviewed the function and application of L-threonine, the pathway of C. glutamicum to synthesize L-threonine, and the use of metabolic engineering technology to improve the production of L-threonine.

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

1. Introduction

Amino acids, the basic building blocks of proteins, are essential for the nutritional health of humans and animals. Since the 1960s, owing to technological developments, the types of amino acids and their derivatives have increased from approximately 50 to more than 1000. Among them, L-threonine, one of the three major amino acids and an essential amino acid for living organisms, plays a pivotal role in the medicine, food, and feed industries, among others. Its high demand has driven the search for production methods based on simple raw materials, simple processes, low costs, and environmental friendliness. Microbial fermentation has stood out in this context. As microorganisms can synthesize their own desired amino acids, the large-scale production of target amino acids through the metabolic engineering of microorganisms using gene engineering techniques has attracted a great deal of attention from researchers [1].

Corynebacterium glutamicum is a nonpathogenic Gram-positive bacterium that meets food safety standards and does not produce spores. It is generally recognized as an ideal strain to produce amino acids via fermentation and has been applied in the industrial production of L-lysine and L-glutamic acid [2]. This article provides a systematic review of the function and application of L-threonine, the application of C. glutamicum in amino acid production, the metabolic pathways of L-threonine production using C. glutamicum, and the research progress on the use of metabolic engineering tools to promote L-threonine synthesis.

2. Functions and Applications of L-Threonine

Threonine is chemically known as α-amino-beta-hydroxybutyric acid. It was isolated from fibrin hydrolysate and was characterized by W.C. Rose in 1935. In 1936, Meger studied its spatial structure and named it threonine because of its structural similarity to threose. Threonine is usually present in the L-configuration and is naturally occurring and exerts physiological effect on the body. Therefore, this article mainly reviews the biosynthesis of L-threonine. L-threonine has a molecular formula of C₄H₉NO₃, a relative molecular
mass of 119.12, and a melting point of 255–256 °C, and it is a white crystal or crystalline powder that is slightly sweet and insoluble in organic solvents, such as ethanol, ether, and chloroform, among others.

L-threonine is an essential amino acid and plays an important role in slowing aging, improving immunity and resistance, and preventing diseases of the brain and body. Deficiencies in L-threonine can trigger various diseases such as impairments in immune function, and L-threonine is important for the growth and development of organisms. In addition to being an essential amino acid for humans, it is widely used in the feed, food, and pharmaceutical industries.

In the feed industry, threonine is listed as one of the four major feed additives, along with lysine, methionine, and tryptophan, and is fed mainly to immature piglets and poultry, among others. L-threonine serves as the second limiting amino acid in swine feed and the third in poultry feed, following L-lysine and L-methionine. It is also an essential amino acid for growth and promotes mucin production in the intestine for better food digestion [3]. However, in recent years, L-threonine has become a major bottleneck in animal performance due to the fact that a balance of each essential amino acid is pivotal for the growth and development of animals and that the amino acid contents of the raw materials for most feeds, such as maize or wheat, are not well balanced. Therefore, the addition of L-threonine can help ensure a balanced ratio of various amino acids in feed, enhance the biological value of feed protein, promote protein deposition, reduce the excretion of ammonia in animals, and alleviate environmental pollution, thereby minimizing resource waste. In addition, when appropriately added to feed, L-threonine can enhance amino acid absorption, elevate the nutritional value of the feed, accelerate livestock growth, lower feed raw material production costs, and ultimately improve economic income. Therefore, L-threonine as a livestock feed additive is of significant economic importance [4].

Currently, in the food industry, L-threonine holds a critical position, especially in the food processing industry, owing to its role in enhancing shelf life and ensuring ease of preservation. Its widespread availability worldwide makes it readily accessible for people to purchase and utilize. However, it can be easily destroyed in this process, which leads to the requirement for supplementation. Amino-acid-based fortifying agents are important in the production of food fortifying agents. L-Threonine can be used as a nutritional supplement to enhance protein nutrition and ensure the appropriate nutritional composition of food. In addition to its essential role in daily human nutrition, threonine finds valuable applications in special formulations catering to specific populations, like infant formula and low-protein foods. The recommended daily threonine intake stands at 7 mg/kg for adults, 35 mg/kg for children, and 87 mg/kg for infants. This versatile amino acid plays a crucial part in meeting the dietary needs of different age groups and specialized dietary requirements. Furthermore, because of its reducing properties, L-threonine is generally used in combination with other amino acids to fortify grains. When added as a food additive to foods such as confectionery and milk, the antioxidant properties of threonine can provide the food with a unique taste and freshness [5]. Moreover, heating threonine and glucose together produces a char flavor, which can enhance the aroma of food.

L-threonine has also been widely used in the pharmaceutical industry, where it plays an important role. Specifically, it can improve nutritional status, promote growth and development of the body, and improve immunity. Moreover, the ferric salt of L-threonine can be used as an anti-anemia agent. Additionally, L-threonine itself is an intermediate in the manufacture of carumonam, a highly effective antibiotic, and can be used in biochemical research. In clinical practice, L-threonine can promote the development of T-lymphocyte precursors in the bone marrow and their differentiation into mature T-lymphocytes. Moreover, L-threonine is one of the main components of many infusion solutions with amino-terminals, which are commonly used in adjuvant therapy for pre- and post-operative care, traumatic injuries, burns, bone fracture, malnutrition, and chronic wasting diseases, making it an amino acid with high clinical value. For patients with oral injuries or those undergoing surgery who are unable to obtain adequate nutrients, L-threonine injections can help patients obtain the necessary
nutrients to enhance physical vitality while also promoting wound healing [6]. Furthermore, L-threonine is the second limiting amino acid in gluten and contains hydroxyl groups, giving it a moisturizing effect on human skin.

3. Application of *C. glutamicum* in Amino Acid Production

The main strains used in L-threonine production via microbial fermentation include *C. glutamicum*, *Brevibacterium flavum*, *Brevibacterium lactofermentum*, *Corynebacterium crenatum*, and *E. coli*. Among them, most studies have used *C. glutamicum* and *E. coli* as original strains. *C. glutamicum* is an environmentally friendly Gram-positive bacterium that does not produce endospores and is non-pathogenic. The optimal pH for *C. glutamicum* is 7.0–8.0, and the optimal growth temperature is 30 °C under aerobic conditions. Moreover, although *C. glutamicum* stops growing under anaerobic conditions, its central metabolic pathway remains active, producing lactic, succinic, and acetic acids. The cells appear as short rods and are stick-like, with rapid growth rates and a smooth surface. *C. glutamicum* exhibits remarkable versatility in utilizing renewable carbon sources, particularly crop glycogen like sucrose, beet molasses, and starchy molasses from corn and cassava. However, given the unpredictable market prices of carbon sources, the variability of feedstocks becomes a crucial economic consideration. In pursuit of enhancing its substrate spectrum, researchers are actively engineering *C. glutamicum* to efficiently utilize diverse alternative feedstocks, including starch, hemicellulose, and glycerin. This promising endeavor aims to bolster its potential as a sustainable and cost-effective resource for various applications.

*C. glutamicum* has the following advantages over other industrial microbes. First, to prevent genetic instability in transgenic strains, large segments of phage-related elements have been deleted in *C. glutamicum*, making it resistant to phage infections. Second, *C. glutamicum* contains few isoenzymes, which significantly simplifies the metabolic engineering design and biotechnology applications. *C. glutamicum* does not produce endotoxins, and its safety is widely recognized, making it a safe bacterium that meets food safety standards [7]. Furthermore, acetic acid, which is detrimental, does not accumulate in *C. glutamicum*, and the limited accumulation of the target amino acid can be promoted by decreasing the activity of the enzymes that degrade endogenous amino acids. Moreover, *C. glutamicum* can utilize a variety of carbon sources to produce various compounds and is highly adaptable to the environment [8], as it can adapt to harsh industrial cultivation conditions. Therefore, it is widely recognized as an ideal strain for the fermentative production of amino acids.

For traditional strains used for the industrial production of amino acids, the classical mutation breeding approach has generally been applied to improve the overall performance of the strain. Research on the selection of *C. glutamicum* strains using different breeding strategies has rapidly increased its application potential in bioengineering, as it is easily manipulated. However, there are certain drawbacks associated with these methods, such as unclear mechanisms underlying random mutations and their impact on the growth of the bacteria. With increasing research on *C. glutamicum* and the rapid development of molecular biology and synthetic biology, double crossover homologous recombination and the clustered regularly interspaced short palindromic repeats system have been widely applied, and these technologies have rapidly become the most popular gene-editing tools. Scientists now have a clear understanding of the metabolic networks and regulatory mechanisms of *C. glutamicum*. In particular, following the determination of the first whole-genome sequence of *C. glutamicum* ATCC 13032 by Kalinowski et al. in 2003 [9], the *C. glutamicum* genome has been annotated, and the biosynthesis pathways and regulatory mechanisms of branched-chain amino acids have been elucidated. The development of genetic engineering techniques has facilitated the use of gene-editing tools for the metabolic engineering of target genes to conveniently alleviate the feedback inhibition of key enzymes in *C. glutamicum*, knock down the metabolic branches in the synthesis pathway of amino acids, express key genes with high efficiency, and knock in exogenous genes, thereby obtaining highly efficient amino acid-producing strains [10] that can be used for the production of a variety of compounds with commercial value.
4. Metabolic Pathways of l-Threonine Production by *C. glutamicum*

4.1. l-Threonine Synthesis Pathway

To better understand the process of l-threonine accumulation in production strains, the related biosynthetic pathways and corresponding regulatory mechanisms should be investigated and analyzed. In *C. glutamicum*, the l-threonine synthesis pathway can be broadly categorized into the following three parts (Figure 1): the glycolytic pathway, the tricarboxylic acid (TCA) cycle, and the aspartic acid family amino acid metabolic pathway. Glucose taken up by *C. glutamicum* is first subjected to the glycolytic pathway and is decomposed into a series of metabolites of phosphoenolpyruvic acid and pyruvic acid. Subsequently, the metabolites enter the TCA cycle and are converted to the intermediate metabolite oxaloacetic acid via the following two pathways: (i) phosphoenolpyruvate carboxykinase (encoded by the *ppc* gene) catalyzes the conversion of phosphoenolpyruvic acid to oxaloacetate; and (ii) pyruvate carboxylase (encoded by the *pyc* gene) catalyzes the conversion of pyruvic acid to oxaloacetate [11–13]. Thereafter, oxaloacetate enters the metabolic pathway for the aspartic acid family of amino acids to produce l-threonine [12,13].

First, aspartate transaminase (AT; encoded by the *aspC* gene) catalyzes the production of L-aspartate from oxaloacetate. Subsequently, aspartate kinase (AK; encoded by the *lysC* gene) catalyzes the production of L-aspartic acid phosphoric acid from L-aspartate, which is then utilized by aspartate semialdehyde dehydrogenase (ASD; encoded by the *asd* gene) to catalyze the production of L-aspartate semialdehyde. L-Aspartate semialdehyde is then utilized to produce phosphohomoserine through the sequential action of homoserine dehydrogenase (HD; encoded by the *hom* gene) and homoserine kinase (HK, encoded by the *thrB* gene) [14,15]. Finally, threonine synthase (TS; encoded by the *thrC* gene), catalyzes the synthesis of L-threonine from phosphohomoserine.

![Figure 1. l-threonine biosynthesis pathway and metabolic regulation in *C. glutamicum*. Notes: The green dashed line represents feedback deterrence, and the red dashed line represents feedback inhibition. PEPC, phosphoenolpyruvate carboxykinase; PC, pyruvate carboxylase; AT, aspartate transaminase; AK, aspartate kinase; ASD, aspartate semialdehyde dehydrogenase; HD, homoserine dehydrogenase; HK, homoserine kinase; TS, threonine synthase.](image-url)
As seen in Figure 1, L-threonine belongs to the aspartic acid family of amino acids. The L-threonine synthesis pathway begins with the production of L-aspartate from oxaloacetate catalyzed by AT. In total, five enzymes, including AK, ASD, HD, HK, and TS, are involved in the biosynthesis of L-threonine.

4.2. Regulatory Mechanisms

In terms of enzyme activity, AK is the first key enzyme in the L-threonine biosynthesis pathway, catalyzing the production of aspartyl phosphate from aspartate. AK is inhibited by feedback from the end products L-threonine and L-lysine. Such regulation of enzyme activity is a control point for the biosynthesis of amino acids of the aspartate family [14]. Among them, aspartyl phosphate is then converted to aspartate semialdehyde by ASD, which has also been shown to be subjected to feedback inhibition via the accumulation of lysine and threonine, owing to the close proximity of the gene sequences of asdA, the gene encoding ASD, and lysC, the gene encoding the enzyme AK, as the two genes are only separated by 12 bases or fewer. The co-existence of lysine and threonine would strongly inhibit the activity of ASD, with a loss of 90% of activity at a concentration of 10.0 mM [15]. Ohnishi et al. found that a mutation in the amino acid at position 311 of AK, from threonine to isoleucine, alleviates the feedback inhibition [16]. Furthermore, two other key enzymes in the L-threonine synthesis pathway, namely HD and HK, are also subjected to feedback inhibition mediated by L-threonine. HD is extremely sensitive to the allosteric inhibitory effect of L-threonine, and intracellular concentrations of L-threonine up to 2.0 mmol/L would completely inhibit the activity of HD [17]. HK is subjected to competitive inhibition mediated by L-threonine. However, this inhibitory effect is only manifested at high concentrations (60% inhibition with 30.0 mmol/L of threonine) [18]. Reinscheid et al. found that a mutation in the amino acid at position 378 of HD, from glycine to glutamic acid, alleviates the feedback inhibition on HD mediated by L-threonine; the enzyme activity of HD is unaffected by L-threonine concentrations up to 25.0 mmol/L, and the half maximal inhibitory concentration of L-threonine reaches 100.0 mmol/L [19]. At the transcriptional level, the lysC and asd genes, encoding AK and ASD, respectively, are located on the same operon, which is subjected to feedback deterrence mediated by L-threonine and L-isoleucine [20]. Similarly, the hom and thrB genes, encoding HD and HK, respectively, are located on the same operon, which is subjected to feedback deterrence at high intracellular concentrations of L-methionine [21].

4.3. Competitive Metabolic and Degradation Pathways

In C. glutamicum, the biosynthesis of L-lysine and L-methionine competes for the consumption of intermediate metabolites during the biosynthesis of L-threonine, which would reduce L-threonine production. Among them, aspartic semialdehyde is a common intermediate metabolite in the L-lysine and L-threonine synthesis pathways. Therefore, both pathways compete for the substrate aspartic semialdehyde. Moreover, dihydricipicolinate synthase (encoded by the dapA gene) is responsible for directing the carbon flux to the L-lysine synthesis pathway [22]. Similarly, the L-methionine and L-threonine synthetic pathways also compete for the common intermediate metabolite L-homoserine, whereas the presence of acetyltransferase (encoded by the metX gene) directs the carbon flux towards the L-methionine synthesis pathway [23].

Furthermore, the biosynthesis of L-isoleucine and glycine depletes threonine, which would reduce L-threonine production. For example, in the synthesis pathway of the byproduct isoleucine, the presence of threonine deaminase (encoded by the ilvA gene) would result in the degradation of L-threonine produced by the cells, thereby leading to the production of L-isoleucine. Moreover, serine hydroxymethyltransferase (encoded by the glyA gene) can use L-threonine as a substrate to produce the byproduct glycine, which serves as a one-carbon unit for cell growth [24].
4.4. Mode of L-Threonine Transmembrane Transport

L-threonine synthesized by cells is transported to the extracellular compartment mostly through active transport, a process mainly mediated by transport proteins [25]. Reinscheid et al. suggested that the low level of L-threonine exporter has severely limited threonine production. However, there were no reports on L-threonine efflux proteins [26] until 2001, when Simic et al. isolated the efflux protein ThrE (encoded by the thrE gene) [27] from *C. glutamicum* for the first time. However, it does not specifically export L-threonine [24,28]. Furthermore, in recent years, Zhang et al. identified SerE (encoded by the serE gene), another novel exporter for L-threonine, which can transport L-threonine from intracellular to extracellular compartments. The overexpression of SerE can be induced to increase L-threonine production [29].

5. Metabolic Engineering Tools to Increase L-Threonine Production

Earlier, high-yield strains for amino acid production were mainly obtained by screening random mutations. However, the strains obtained using such methods have unclear genetic backgrounds, unstable mutational effects, and safety issues [30]. In the current stage, metabolic engineering has been increasingly applied to the selection of L-threonine-producing strains (i.e., the targeted alteration of target genes through molecular approaches to obtain high-yield strains for L-threonine). Current strategies for the modification of the L-threonine metabolic pathways have focused on (1) enhancing the L-threonine synthesis pathway, (2) enhancing L-threonine transport engineering, (3) reducing the intracellular metabolism of L-threonine, (4) weakening carbon flux pathways competing with L-threonine synthesis, and (5) systems metabolic engineering. In recent years, a series of *C. glutamicum* strains with high L-threonine yields have been obtained [11].

5.1. Enhancing the L-Threonine Synthesis Pathway

L-threonine production can be increased by strengthening the expression of the target genes encoding the key enzymes involved in the L-threonine synthesis pathway and increasing the supply of precursors. The overexpression of key genes in the L-threonine synthesis pathway, such as *lysC*, *asd*, *hom*, and *thrB*, in the genome or plasmid can usually increase L-threonine production. Furthermore, L-threonine production could be further enhanced by alleviating the feedback inhibition through modifications to the genes encoding key enzymes to facilitate higher carbon flow into the L-threonine synthesis pathway. Wei et al. used a promoter library-based module combination (PLMC) to overexpress five genes for L-threonine synthesis, namely *lysC-asd*, *hom-thrB*, and *thrC*, to redirect the carbon flux from the L-lysine branch to the L-threonine branch, which resulted in an L-threonine yield of 12.8 g/L. Further, the expression levels of *thrB* in the metabolic pathway were further increased to make them greater than those of *hom* to avoid the accumulation of the intermediate product L-homoserine [31]. Huang et al. performed targeted mutation of the key rate-limiting enzyme-encoding gene *lysC* and obtained an A297T mutant. The genes of *lysC-asd* were tandemly overexpressed, and L-threonine production reached 7.2 g/L after shake-flask fermentation, which was 27.8% higher than that with the original strain [32].

5.2. Enhancing Threonine Transport Engineering

Excessive intracellular L-threonine concentrations in *C. glutamicum* would not only affect bacterial growth but also enhance the effects of feedback inhibition on key enzymes. Thus, enhancing the extracellular secretion of L-threonine could not only increase L-threonine production but also reduce L-threonine degradation. Simic et al. used *C. glutamicum* DR-17 as chassis cells and overexpressed the plasmids expressing the exporter ThrE to promote the transport of intracellularly produced L-threonine to the extracellular compartment, which resulted in an increase in L-threonine production from 5.8 g/L to 8.0 g/L [24]. Diesveld et al. overexpressed the exporters from *E. coli*, namely RhtA, RhtB, RhtC, and YeaS, in *C. glutamicum*. Except for RhtB, all of these proteins increased threonine production...
production, with the most significant increase observed with RhtC. The overexpression of RhtC in *C. glutamicum* DM368-3 (AEC\(^r\), AHV\(^r\)) increased \(L\)-threonine production from 0.9 g/L to 3.7 g/L [33].

5.3. Reducing the Intracellular Metabolism of \(L\)-Threonine

\(L\)-threonine is utilized as a substrate by cells to produce glycine or \(L\)-isoleucine via enzymatic catalysis. Among such enzymes, serine hydroxymethyltransferase (encoded by the *glyA* gene) utilizes \(L\)-threonine to enter the glycine synthesis pathway. Simic et al. used the inducible promoter *P\(_{lac}\)* in plasmids to reduce expression of the *glyA* gene, which increased \(L\)-threonine production from 0.9 g/L to 1.3 g/L [24]. Furthermore, \(L\)-threonine can be degraded by threonine deaminase (encoded by the *ilvA* gene) to produce \(\alpha\)-ketobutyric acid, which then enters the \(L\)-isoleucine synthesis pathway. Diesveld et al. generated an inactivating mutation (G96D) in the *ilvA* gene on the chromosome of *C. glutamicum* DM1800-\(T\), which reduced threonine deaminase activity and increased \(L\)-threonine production from 2.5 to 4.0 g/L [33]. Masaaki et al. produced \(L\)-threonine using *C. glutamicum* with \(L\)-isoleucine auxotrophy, and the production of \(L\)-threonine reached 57.7 g/L in fed-batch culture for 100 h [34].

5.4. Weakening Carbon Flux in the \(L\)-Threonine Synthesis Competitive Pathways

There are two pathways that compete with the \(L\)-threonine synthesis pathway, namely the \(L\)-lysine synthesis pathway and the \(L\)-methionine synthesis pathway. Weakening or shutting down these two competing pathways could help increase the supply of precursors for threonine production and reduce or eliminate the feedback effects of products of metabolic branches on key enzymes of the \(L\)-threonine synthesis pathway. Lv et al. knocked out the *dapA* and *metX* genes of *C. glutamicum* R102 and obtained an \(L\)-threonine yield of 3.0 g/L for *C. glutamicum* R102\(\Delta\)dapA\(\Delta\)metX, which was 1.7-fold higher than that obtained when using the original strain R102 [35]. Dong et al. increased \(L\)-threonine production by 28\% by knocking out *ddh*, a key gene in the \(L\)-lysine synthesis pathway, and *lysE*, the gene encoding the \(L\)-lysine efflux protein, from the genome of *C. glutamicum* ATCC 13869 [36].

5.5. Systems Metabolic Engineering

Few studies have been conducted on the metabolic engineering of *C. glutamicum* for high \(L\)-threonine production, and \(L\)-threonine yields have remained low [37]. In a recent study, Wei et al. performed PLMC to construct promoter libraries with different intensities and used them to regulate the expression of key enzymes in the \(L\)-threonine synthesis pathway. The final threonine yield reached 12.8 g/L after combining and adjusting the expression of the genes, which represented a 6.1-fold increase compared to that with the original strain [31]. In *C. glutamicum*, the catalytic activities of AK, HD, and HK (encoded by *lysC, hom*, and *thrB*, respectively), which are subjected to feedback inhibition, may represent the bottleneck limiting threonine production. Maximizing the alleviation of feedback inhibition might thus be the optimal strategy to overcome this limitation and increase threonine production.

6. Future Prospects

In recent years, *C. glutamicum* has remained an essential bacterium in the biomanufacturing of amino acids. Currently, the rapid development of novel mutagenesis, genome-editing, gene expression regulation, adaptive evolution, biosensors, and other metabolic engineering and synthetic biology methods and the rapid development of high-throughput screening techniques and gene-editing technologies have accelerated the construction and optimization of *C. glutamicum* as a microbial cell factory. The \(L\)-threonine industry is developing rapidly, and the international competition has intensified. Many focal points for future growth remain with respect to the threonine market, with great potential for development.
Currently, there have been limited breakthroughs in the synthesis capacity of L-threonine using C. glutamicum over the past 20 years, and the efficiency of these bacteria in producing L-threonine from raw materials should be further improved. Therefore, there is still a need to comprehensively analyze the functional genes of C. glutamicum using systematic biological techniques and to explore their metabolic potential using metabolic engineering approaches to ensure that the cell factories can produce L-threonine with high yields, at a high rate, and with high purity. Furthermore, pre-treated raw materials contain various inhibitors, which could affect the growth of the bacterial strain at higher concentrations. The co-utilization of a variety of carbon sources by the bacterial strain should be further improved to enhance its tolerance of high concentrations of products, substrates, or inhibitors, among others, to make it more suitable for industrialized production. In summary, this article reviews the current research on L-threonine production by C. glutamicum and provides ideas for the construction of high-yield strains, which is crucial for a better understanding of cellular metabolism and strain development in the future.

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