

Review

Expanding the Application of Threonine: Industrial Biomanufacturing of Threonine and Its Derivatives

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Abstract

Currently, the production methods for L-threonine (L-Thr) mainly include chemical synthesis, protein hydrolysis, and microbial fermentation. Among these, microbial fermentation has become an important method for the industrial production of L-Thr, owing to its advantages of abundant raw material sources, environmental friendliness, and high product purity. In recent years, gene editing, synthetic biology, and artificial intelligence have been integrated to significantly improve the synthesis efficiency and production stability of L-Thr and its derivatives through the rational design of metabolic networks, dynamic regulation of fermentation processes, and intelligent optimization of strain performance. This review systematically summarizes the progress of research on the biosynthesis of L-Thr and its derivatives, with emphasis on elucidating synthetic pathway regulation methods based on genetic engineering and metabolic engineering strategies, and summarizes the latest research developments in the synthesis of its derivatives, aiming to provide systematic references for efficient biomanufacturing in this field.

Keywords: L-Thr; metabolic engineering; fermentation; derivatives

1. Introduction

L-threonine (L-Thr), also known as α -amino- β -hydroxybutyric acid, is a multifunctional compound characterized by a molecular formula of $C_4H_9NO_3$ and a molecular weight of 119.12 [1]. It has found widespread applications in pharmaceuticals [2], food [3], and aquaculture [4] sectors, demonstrating significant market potential. In the pharmaceutical field, L-Thr is used as an enhanced nutritional supplement, where it can compensate for the deficiency of amino acids in the body and serve as an adjuvant therapeutic drug for digestive system ulcers [5]. It promotes organismic growth and development, alleviates physical fatigue [6], and enhances the differentiation and proliferation of bone marrow T lymphocytes, thereby strengthening the human immune system and exerting important physiological functions [7]. In the food sector, L-Thr is commonly co-heated with glucose to generate burnt and chocolate aromas for flavor enhancement. It is also employed in the formulation of amino acid infusions and comprehensive amino acid preparations, where its polarity and aqueous solubility enable hydrophobic, hydrophilic, and dipole–dipole interactions with solvent and solute molecules. These interactions stabilize macromolecular



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conformations, prevent protein denaturation and aggregation, and thereby preserve nutritional integrity [8]. In the aquaculture and livestock industries, L-Thr serves as an important feed nutritional fortifier. It is added to the feed of immature piglets [9] and poultry [10] to promote their growth and improve meat quality. It can also effectively improve the immune function and fat metabolism of the organism, thereby improving the nutritional value of feed [11,12] (Figure 1). The important functions and extensive applications of L-Thr have driven market demand, with global annual production reported to reach 700,000 tons [13]. With the continuous increase in market demand, the optimization of the L-Thr industry requires further enhancement of yield and reduction in production costs. Therefore, the construction of high-yield L-Thr production systems holds significant importance for social development and the national economy.

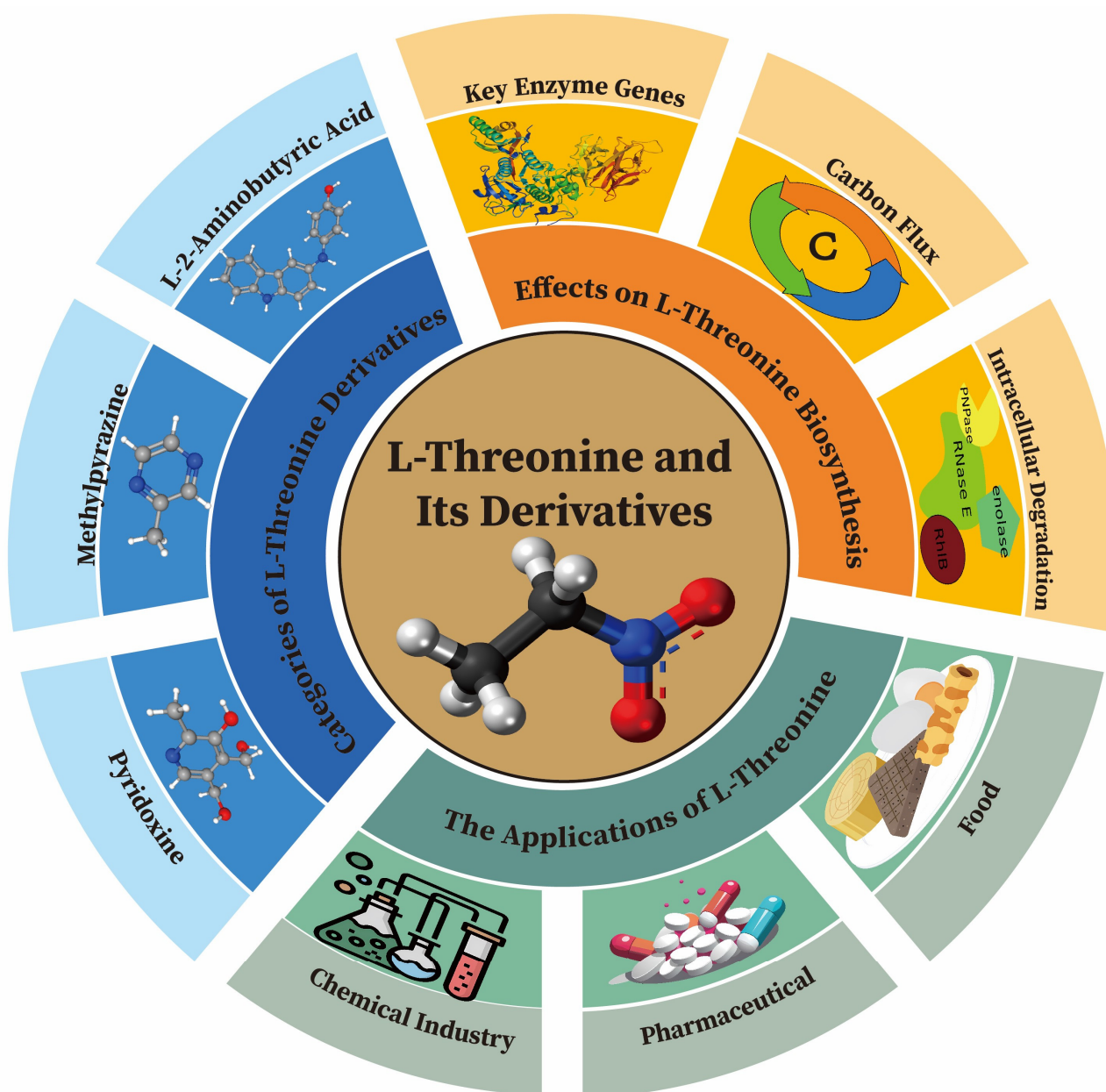


Figure 1. Applications of L-Thr and its derivatives, and metabolic engineering strategies.

L-Thr synthesis is mainly realized through protein hydrolysis, chemical synthesis and microbial fermentation. However, traditional chemical synthesis processes usually have cumbersome steps and easily lead to significant environmental pollution; and for

protein hydrolysis it is difficult to expand production scale due to raw material sourcing limitations. In this case, microbial fermentation has become the dominant technology in industrial production because of its economic efficiency, mild process and good environmental compatibility. Currently, microbial strains employed for L-Thr production primarily include *Escherichia coli* (*E. coli*) [14], *Corynebacterium glutamicum* (*C. glutamicum*) [15] and *Serratia marcescens* (*S. marcescens*) [16]. Among them, *E. coli* is the most widely used host for industrial-scale production of L-Thr. Its advantages lie in its clear genetic background, perfect genome annotation, short gene operation cycle, mature system, and rapid fermentation process, thus being widely adopted in research and industrial production [17]. In recent years, metabolic engineering modification of microorganisms has significantly improved the yield of L-Thr, but its fermentation level is still far lower than that of amino acids such as L-lysine and L-glutamic acid, the latter of which can reach above 181.5 g/L [18]. To break through this bottleneck, the application of synthetic biology strategies for precise design and optimization of microbial metabolic pathways to construct high-performance L-Thr production strains has become the core direction for promoting its industrialization process. The development of this direction will vigorously promote the industrialization process of L-Thr and have a positive impact on the expansion of related markets.

The synthesis methods of L-Thr derivatives can be divided into three categories: chemical synthesis, enzymatic or cell transformation methods and microbial fermentation [19]. Due to the serious environmental pollution problems and chiral separation difficulties of chemical synthesis, this method is being gradually eliminated. With the significant progress of fermentation technology for L-Thr production, the latter two methods are currently regarded as better alternatives for derivative research and development. Currently, the biosynthesis of these derivatives mainly relies on L-Thr produced by microbial fermentation as a precursor, which is realized through green processes such as enzymatic catalysis or whole-cell biotransformation, thereby significantly expanding the application scope and market potential of threonine.

To deeply explore the synthesis and regulation mechanism of L-Thr, this study first expounds the biosynthetic pathway of L-Thr. Then it focuses on metabolic engineering strategies to enhance L-Thr synthesis, and reviews and compares the effects of various strategies on improving L-Thr yield. Finally, the latest progress in biosynthesis of L-Thr derivatives is summarized, aiming to provide a more comprehensive understanding for the research on efficient synthesis of threonine.

2. Biosynthetic Pathway of L-Thr

2.1. Production of L-Thr

To elucidate the biosynthesis and metabolic regulation of L-Thr, the synthetic pathway can be dissected into two functionally distinct yet interconnected modules: the central metabolic module and L-Thr synthesis module (Figure 2). The central metabolic module utilizes glucose as the carbon source, primarily involving the glycolytic pathway (EMP), tricarboxylic acid cycle (TCA), and pentose phosphate pathway (PPP) [19]. The L-Thr synthesis module uses oxaloacetate (OAA), an intermediate metabolite of the TCA, as a precursor, and generates L-aspartate catalyzed by aspartate transaminase. L-Thr belongs to the aspartate family of amino acids, and its biosynthesis uses L-aspartate as a precursor substance; this synthesis pathway requires the synergistic catalysis of multiple key enzymes. Starting from L-aspartate, the L-Thr pathway comprises five enzymatic reactions: (1) aspartate kinase (AK) catalyzes the γ -carboxyl phosphorylation of L-aspartate to form aspartyl phosphate; (2) aspartate semialdehyde dehydrogenase (ASD) catalyzes the reduction of aspartyl phosphate to aspartate semialdehyde; (3) homoserine dehydrogenase (HD) utilizes Nicotinamide adenine dinucleotide phosphate (NADPH) as a reducing equiv-

alent to catalyze the decarboxylation of aspartate semialdehyde to generate L-homoserine (L-Hse); (4) homoserine kinase (HK) catalyzes the phosphorylation of L-Hse to produce phosphohomoserine; (5) threonine synthase (TS) catalyzes the dephosphorylation of phosphohomoserine to produce L-Thr.

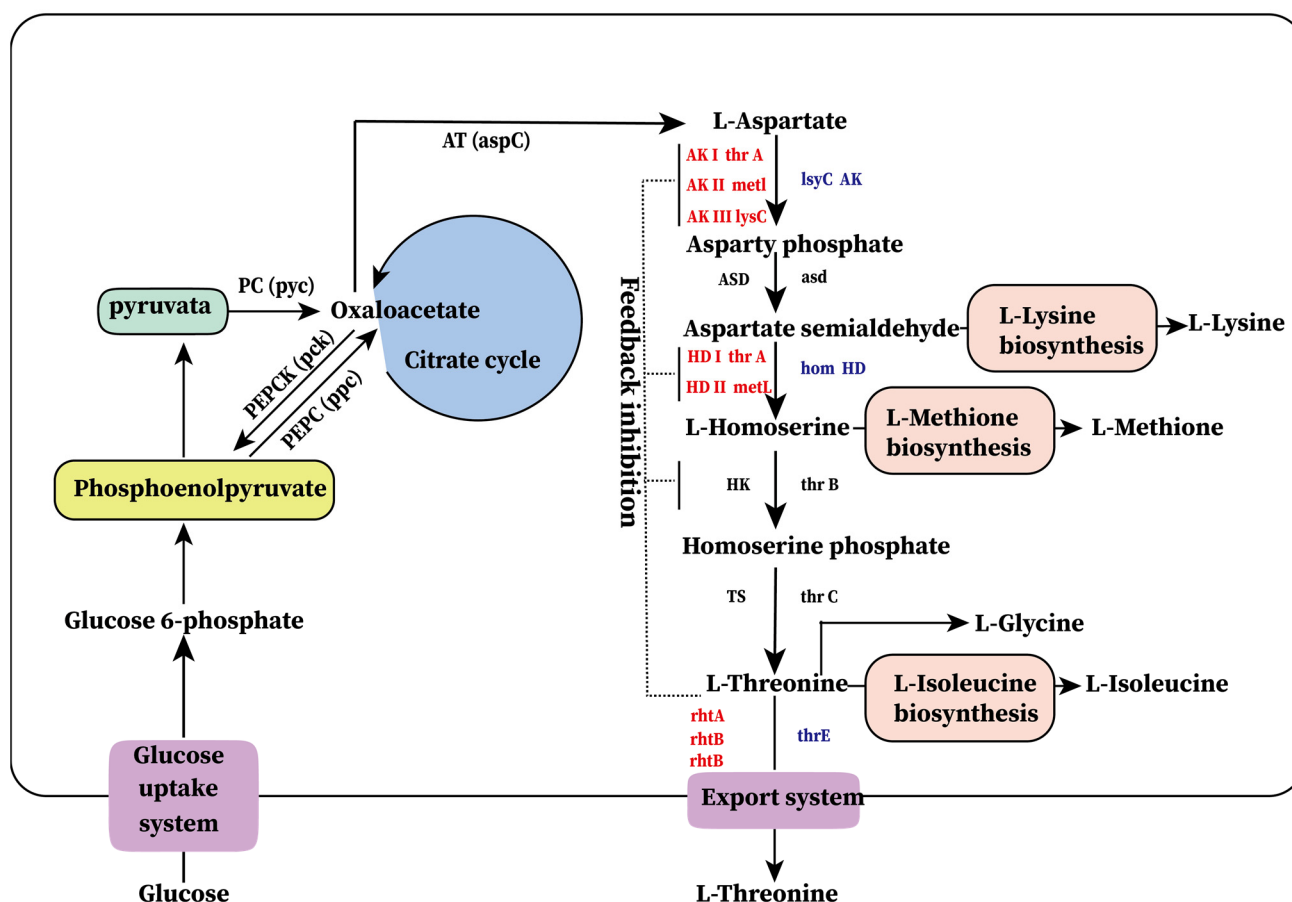


Figure 2. Engineering of L-threonine-producing strains in *E. coli* and *C. glutamicum*. AK: Aspartate kinase; ASD: Aspartate semialdehyde dehydrogenase; HD: Homoserine dehydrogenase; HK: Homoserine kinase; TS: Threonine synthase.

In *E. coli* and *C. glutamicum*, the key enzyme systems involved in threonine biosynthesis exhibit significant differences in organizational form. Specifically, *E. coli* AK exists as three isoenzymes (AKI, AKII, and AKIII) [20], whereas HD has two isoenzymes (HDI and HDII). Among these, AKI and HDI form a bifunctional enzyme encoded by *thrA*; similarly, AKII and HDII also constitute a bifunctional enzyme encoded by *metL* [21]. In contrast, AKIII is a monofunctional enzyme encoded by *lysC* [22]. Among the three AK isoenzymes, AKI exhibits the highest protein expression level, while AKII shows the lowest expression level. As for ASD, HK, and TS, they all exist as single enzymes in *E. coli*, encoded by *asd*, *thrB*, and *thrC*, respectively [23]. In contrast, in *C. glutamicum*, the genetic organization of the above five enzymes is more streamlined, with no isoenzymes identified: AK is encoded by *lysC*, ASD by *asd*, HD by *hom*, HK by *thrB*, and TS by *thrC* [24,25]. In microbial production of L-Thr, the transport of product from intracellular to extracellular space is generally regarded as a critical rate-limiting step. For this step, three functional proteins responsible for L-Thr efflux have been identified in *E. coli*, namely RhtA, RhtB, and RhtC, which are encoded by *rhtA*, *rhtB*, and *rhtC*, respectively [26]. In *C. glutamicum*, however, only one L-Thr efflux protein, ThrE, has been discovered, encoded by *thrE* [27].

2.2. Regulation Mechanism of L-Thr Biosynthesis Pathway

In *E. coli*, the transcription of genes involved in the L-Thr biosynthetic pathway and their corresponding enzymatic activities are subject to exquisite regulation by terminal amino acids. The *thrA*, *thrB*, and *thrC* genes, located within the *thrABC* operon [28], are transcriptionally repressed by L-Thr and L-isoleucine through attenuation mediated by the upstream *thrL* leader sequence; introduction of mutations at specific positions within this sequence can alleviate this regulatory control. Meanwhile, transcription of the *metL* gene is repressed by L-methionine, whereas the *lysC* gene is regulated by L-lysine through a translational attenuation mechanism [29]. Additionally, transcription of the *asd* gene is subject to multivalent repression by L-lysine, L-Thr, and L-methionine [30]. At the enzymatic level, the bifunctional enzyme AKI and HDI is subject to incomplete feedback inhibition by L-Thr, wherein AKI activity is modulated by allosteric regulation and substrate competition, while HDI activity exhibits non-competitive inhibition [31]. AKIII activity can be completely inhibited by L-lysine through conformational change, and specific point mutations can partially relieve this inhibition [27,31]. The feedback inhibition mechanism of HK is rather complex, being subject to competitive inhibition by L-Thr and non-competitive inhibition by L-lysine, as well as modulation by high concentrations of the substrate L-homoserine and ATP [31].

In *C. glutamicum*, transcriptional regulation of the L-Thr biosynthetic pathway is relatively limited, primarily targeting the operon composed of *hom* and *thrB*. This operon is arranged in the 5'-*hom*-*thrB*-3' orientation, with a long inverted repeat sequence upstream, suggesting that feedback repression of these two genes by L-methionine may be mediated through an attenuator mechanism [32]. At the enzymatic activity level, three key enzymes are subject to feedback inhibition by terminal amino acids. Among them, CgAK (encoded by *lysC*) is an allosteric enzyme, whose activity is synergistically inhibited by feedback from L-Thr and L-lysine; this inhibition can be completely abolished by introducing specific mutations [33]. CgHD (encoded by *hom*) activity is feedback-inhibited by L-Thr, an effect mediated by its C-terminal domain [34]; site-directed mutagenesis (e.g., G378E) can generate mutants completely insensitive to high concentrations of L-Thr [35]. CgHK (encoded by *thrB*) is subject to competitive feedback inhibition by L-Thr, with the degree of inhibition decreasing as substrate L-homoserine concentration increases; consequently, this inhibition can only be alleviated by elevating substrate concentration rather than by altering the enzyme protein structure [36].

3. Metabolic Engineering Strategies for Enhancing L-Thr Synthesis

Currently, industrial fermentation of L-Thr in *E. coli* has made progress; however, achieving high yields in *C. glutamicum* remains challenging. Metabolic engineering enhances production through systematic modification of strain metabolic networks, with its core lying in the directed regulation of carbon flux distribution and enzyme activity to maximize target product synthesis and minimize metabolic losses. In the metabolic engineering and synthetic pathway construction of the aspartate family of amino acids, multi-strategy combination approaches are commonly employed. Currently, the fermentation yield of L-Thr has reached 160.3 g/L in laboratory settings through systematic metabolic engineering modifications [37]. With the technology becoming increasingly mature, systematic metabolic engineering has become an ideal method for modifying strains to improve amino acid yields. Recent metabolic engineering strategies and yield levels for high-yield L-Thr strains are shown in Table 1. Based on established metabolic engineering principles, this section systematically elaborates four key strategies: flux amplification to strengthen precursor supply and key enzyme expression, elimination of competing pathways to reduce by-product formation, cofactor engineering to optimize reducing power balance, and

transport engineering to enhance product efflux and alleviate feedback inhibition. The integrated application of these methods is crucial for improving L-Thr synthesis efficiency.

Table 1. Engineering strategies and recent breakthroughs for L-Thr.

Strain Name	Microbial Sources	Engineered Strategy	Titer (g/L)	Refs.
WMZ016/pFW01-thrA*BC-rhtC	<i>E. coli</i>	Deletion of crr and iclR; replacement of native gltA promoter with Ptrc	17.98	[38]
TWF018	<i>E. coli</i>	Deletion of arcA, iclR, and tdcC	26.00	[39]
THRN7	<i>E. coli</i>	Obtained effective thrA mutants via in vivo directed evolution using the MutAT7 system, Deleted mic and introduced heterologous glvAC gen	121.26	[40]
TWF113/pFT24rpa1	<i>E. coli</i>	Derived from TWF106. Harbors the thermal switch vector pFT24rp, which overexpresses rhtC (threonine exporter) and pycmt (codon-optimized pyruvate carboxylase) under dynamic thermal control.	25.85	[41]
TDHR3-42-p226	<i>Halomonas bluephagenesis</i>	Replace inducible promoter for rhtC-lysC* with a strong constitutive promoter in TDHR3-42.	33	[42]
JLTHR	<i>E. coli</i>	Supplement 2 g/L betaine hydrochloride in glucose feed as the optimal osmoprotectant.	127.3	[43]
THPE5	<i>E. coli</i>	additional expression of membrane-bound pyridine nucleotide transhydrogenase (PNT, pntAB)	70.8	[44]
MH20-22B-(homA'-thrB)(pEC-T18mob2-thrE)	<i>C. glutamicum</i>	Introduction of a thrE-containing plasmid into the chromosomal integration strain to enhance export	38.1	[15]

* Mutant.

3.1. Flux Amplification

Flux amplification aims to increase carbon flux toward L-Thr biosynthesis by enhancing the expression of rate-limiting enzymes and bolstering precursor metabolite supply. The L-Thr synthesis module utilizes OAA as a direct precursor, which is replenished through anaplerotic reactions of the TCA. Notably, the mechanism of anaplerotic OAA formation differs between *E. coli* and *C. glutamicum*: in *E. coli*, phosphoenolpyruvate carboxylase (PEPC; encoded by ppc) catalyzes the carboxylation of phosphoenolpyruvate to OAA, whereas in *C. glutamicum*, pyruvate carboxylase (PYC; encoded by pyc) mediates the ATP-dependent carboxylation of pyruvate to OAA [45]. Consequently, targeted overexpression of these species-specific anaplerotic genes constitutes a fundamental strategy for enhancing precursor availability. Zhu et al. [38] investigated the effects of glucose uptake, the glyoxylate shunt, and threonine biosynthesis on L-Thr accumulation in *E. coli* through chromosomal editing and plasmid-based expression. Key modifications included overexpression of gltA (enhancing TCA flux) to increase OAA supply, enabling the engineered strain to produce 17.98 g/L L-Thr in shake-flask fermentation, compared to 0.68 g/L from the control. Zhao et al. [46] constructed a genetic circuit based on thrL and cysteine promoters (P-cys) to dynamically regulate the expression of seven key genes (iclR, aspC, arcA, cpxR, gadE, pykF, and fadR), increasing the L-Thr yield of the engineered strain TWF083 to 116.62 g/L, demonstrating the importance of optimizing central metabolism for precursor supply.

Beyond precursor supply, direct enhancements of the L-Thr biosynthesis pathway enzymes are critical, particularly given the feedback inhibition exerted by terminal amino acids on key enzymes such as AK and HDH. To overcome these constraints, introduction of feedback-resistant mutants has proven effective. Site-directed mutagenesis can generate mutants completely insensitive to high concentrations of L-Thr. Jin et al. [47] modified the *E. coli* threonine synthesis system using modular pathway optimization, dynamic transport regulation, and global transcription factor engineering strategies. After obtaining the Fur mutant through directed evolution, the engineered strain THR-48 was constructed, achieving a threonine yield of 154.2 g/L using glucose as the substrate. Based on THR-48, the THR-50 strain was constructed by integrating the sucrose utilization operon, which could directly produce 92.46 g/L threonine using untreated cane sugar as the carbon source, reducing production costs by 48%. Optimization of gene expression ratios also plays a key role in flux amplification. Hao et al. [48] systematically investigated the effect of varying the thrAB-to-thrC expression ratio on *E. coli* growth and L-Thr production, rather than adjusting strain ratios. This approach exemplifies single-strain metabolic engineering that circumvents the complexities of co-culture systems. The study found that when the ratio of thrAB to thrC was 3:5, the L-Thr yield was the highest, reaching 40.06 g/L after 60 h of fermentation, which was 96.85% higher than that of the initial control strain TH. Su et al. [49] constructed a dual-responsive biosensor based on the L-Thr inducible effect and riboswitch for ribosome binding site library metabolic pathway optimization, which increased the L-Thr yield from 0.34 g/L to 1.36 g/L and drove site-directed saturation mutagenesis of the ACT domain of thrA enzyme, obtaining mutant strain WA1 with a 7-fold increase in yield.

3.2. Elimination of Competing

Elimination of competing pathways directs metabolic flux toward threonine by knocking out genes involved in branch pathways and degradation routes, thereby minimizing carbon loss and reducing by-product accumulation. For L-Thr biosynthesis, carbon flux wastage primarily occurs through four pathways: (1) the lysine branch pathway initiated by dihydrodipicolinate synthase (DDPS) encoded by dapA; (2) the L-methionine branch pathway; (3) intracellular degradation to glycine after synthesis; (4) utilization for L-isoleucine synthesis [50]. The lysine branch represents the most significant competitive sink. In both *C. glutamicum* and *E. coli*, DDPS encoded by dapA catalyzes the first committed step toward lysine synthesis. Inhibition of DDPS activity has been shown to effectively redirect carbon flux toward threonine. Zhao et al. [51] performed a series of genetic engineering modifications on *C. glutamicum* WM001. By regulating the activities of PEPC, PYC, pyruvate kinase (PK), phosphoenolpyruvate carboxykinase, and oxaloacetate decarboxylase to finely adjust carbon flux, and inhibiting DDPS to reduce by-products, they achieved efficient synthesis of L-Thr. The final engineered strain TWZ024/pXTuf-thrE produced 78.3 g/L L-Thr in a 7 L bioreactor. The isoleucine pathway also competes for threonine as a substrate. Threonine dehydratase (TD), encoded by ilvA, catalyzes the conversion of L-Thr to 2-oxobutyrates, the first step in isoleucine biosynthesis. Gardner et al. [52] performed site-directed mutagenesis of the ilvA gene on the chromosome in *C. glutamicum* DM1800-T to inactivate the encoded TD, increasing L-Thr production from 2.5 g/L to 4 g/L. Yan et al. [53] increased L-Thr titer from 5.55 g/L to 8.65 g/L after knocking out the ilvA gene, and further improved L-Thr titer to 13.6 g/L by additionally knocking out the metA gene. Wu et al. [54] utilized the molecular dynamics-based allosteric prediction method and molecular mechanics-generalized born surface area energy decomposition to investigate the isoleucine allosteric inhibition mechanism of TD. They found that isoleucine concentrations above 0.1 mM caused wild-type TD activity loss exceeding 95%, confirming that intracellular accumulation severely

inhibits enzyme function. Through computer-aided screening, the mutant P441L was obtained, which maintained 80% activity at 2.5 mM isoleucine and increased leucine yield by over 400% in *E. coli* fermentation. Intracellular degradation of L-Thr represents another significant carbon loss. The main degradation products are glycine and acetyl-CoA, catalyzed by threonine dehydrogenase (TDH, encoded by *tdh*) and serine hydroxymethyltransferase (SHMT, encoded by *glyA*). Lee et al. [55] employed systematic metabolic engineering strategies to construct an engineered *E. coli* with a clear genetic background by deleting *tdh* in the L-Thr intracellular degradation pathway and mutating *ilvA* to decrease L-Thr intracellular consumption, achieving an L-Thr yield of 82.4 g/L and a productivity of 0.393 g/g glucose in fed-batch culture. Acetate formation represents an additional carbon loss in *E. coli*. Wang et al. [56] achieved L-Thr production of 96.4 g/L in a 3 L fermenter and 133.5 g/L in a 10 L fermenter with strain TWF001/pFW01-phaCAB through upregulation of key genes in the L-Thr biosynthesis pathway, downregulation of genes related to acetate formation, and upregulation of the *acs* gene encoding the enzyme that converts acetate to acetyl-coenzyme A. Xie et al. [57] systematically knocked out phosphofructokinase genes *pfkA* and *pfkB* and PK genes *pykF* and *pykA* in the *E. coli* L-Thr producer THRD to weaken glycolytic flux and redirect it toward the PPP, thereby significantly reducing acetate accumulation and improving L-Thr yield. The double knockout strain THRD Δ pfkB Δ pykF achieved an L-Thr yield of 111.37 g/L.

3.3. Cofactor Engineering

Cofactor engineering focuses on optimizing the supply of reducing equivalents, particularly NADPH, which is essential for several steps in L-Thr biosynthesis. HD utilizes NADPH as a reducing equivalent to catalyze the decarboxylation of aspartate semialdehyde to generate L-homoserine. Therefore, adequate NADPH supply is critical for maintaining high flux through the L-Thr synthesis pathway. Several strategies have been employed to enhance NADPH availability. Jin et al. [58] developed a dual-responsive biosensor for NADPH and L-Thr, and combined it with fluorescence-activated cell sorting (FACS) to obtain a high-yield (0.65 g/g) L-Thr-producing strain, demonstrating the importance of NADPH optimization. Overexpression of membrane-bound pyridine nucleotide transhydrogenase (PNT, encoded by *pntAB*) represents a direct approach to increase NADPH supply by converting NADH to NADPH. The engineered strain THPE5, with additional expression of *pntAB*, achieved an L-Thr yield of 70.8 g/L [44]. Vo et al. [59] adopted a modular metabolic engineering strategy to systematically optimize the acetate assimilation, TCA, and coenzyme A metabolic pathways of *E. coli* W3110, constructing the high-efficiency threonine-producing strain *E. coli* W-H31/pM2/pR1P, which achieved maximum titers of 44.1 g/L L-Hse and 45.8 g/L L-Thr in fed-batch fermentation, demonstrating the importance of cofactor balance. In the context of L-Thr derivative production, NADH oxidase has been employed to modulate redox balance. Liu et al. [60] engineered the L-Thr metabolic pathway in *E. coli* by overexpressing L-Thr dehydrogenase and NADH oxidase, constructing the engineered strain BL21(DE3)/pACYCDuet-1-Ectdh-Efnx, which successfully converted L-Thr to 2,5-dimethylpyrazine with a yield of 2009.31 mg/L. This approach demonstrates how cofactor engineering can be applied not only to enhance L-Thr production but also to improve the efficiency of downstream conversion processes. A compelling example of integrating cofactor engineering with complementary metabolic strategies is the work by Hanke et al. [61]. Employing combinatorial cloning coupled with a hybrid deep learning model, the authors iteratively engineered the L-Thr biosynthetic pathway in *E. coli* across three rounds of optimization. To augment NADPH availability, they overexpressed *pntAB* (encoding membrane-bound transhydrogenase). Concurrently, they eliminated competing metabolic branches to redirect carbon flux toward L-Thr by deleting

tdh (encoding threonine dehydrogenase, which catalyzes threonine degradation), dapA (encoding dihydrodipicolinate synthase, committing carbon to the lysine branch), dhaM (encoding a phosphotransferase system component influencing carbon partitioning), and metL (encoding a bifunctional aspartate kinase/homoserine dehydrogenase directing flux toward methionine). Furthermore, they enhanced precursor supply by overexpressing aspC (encoding aspartate aminotransferase, increasing aspartate availability) and ppc (encoding PEPC, replenishing the oxaloacetate pool). This multi-pronged, machine learning-guided optimization progressively elevated L-Thr titers from 2.7 g/L to 8.4 g/L. Although this study integrated multiple engineering strategies, the inclusion of pntAB overexpression specifically underscores the pivotal contribution of cofactor engineering to enhanced L-Thr production and exemplifies how synergistic implementation of diverse approaches can achieve substantial yield improvements.

3.4. Transport Engineering

Transport engineering addresses the critical bottleneck of product accumulation within the cell, which leads to feedback inhibition of key enzymes and limits further synthesis. Reducing excessive intracellular product accumulation is achieved by promoting extracellular secretion to decrease intracellular threonine concentration, thereby alleviating feedback inhibition on key enzymes in the synthesis pathway and enabling sustained high-rate synthesis. In microbial production of L-Thr, the transport of product from intracellular to extracellular space is generally regarded as a critical rate-limiting step. Kruse et al. [62] overexpressed endogenous *rhtB* and *rhtC* genes and the *thrE* gene from *C. glutamicum* in *E. coli*, confirming that *RhtB* and *RhtC* are stereoselective threonine exporters. The study found that intracellular threonine concentration in the high-producing strain BKIIM B-3996 pVIC40 remained consistently higher than extracellular concentration, reaching up to ten-fold, indicating that efflux capacity constitutes a production bottleneck. Overexpression of *rhtB*, *rhtC*, and heterologous *thrE* increased the yield of strain MG442 by 290%, 200%, and 250% to 290%, respectively. Zhao et al. [63] similarly overexpressed *ThrE* in the HK mutant R212Q strain, obtaining 86.4 g/L L-Thr. Dynamic control of transporter expression has emerged as an advanced strategy to balance growth and production. Song et al. [64] designed an artificial quorum sensing system that, through the combination of a switch module and a production module, enhances the L-Thr titer to 118.2 g/L by auto-inducing the expression of *PYC* and threonine extracellular transporter after cells reach a certain growth level, with a yield of 0.57 g/g glucose and a productivity of 2.46 g/(L·h). Fang et al. [41] developed a thermal switch vector pFT24rp that overexpresses *rhtC* under dynamic thermal control in strain TWF113/pFT24rpa1, achieving 25.85 g/L. Du et al. [42] engineered *Halomonas bluephagenesis* using CRISPR/Cas9 and modular regulation. They introduced the exporter *rhtC* to secrete threonine, deleted *ssT* (importer) and *tdh* (degradation enzyme) to prevent intracellular loss, and integrated feedback-resistant *thrABC* and *lysC* to enhance synthesis. The final strain produced 7.5 g/L in shake flasks and 33 g/L in a 7 L fermentor within 24 h, demonstrating the potential of this host for low-cost amino acid production.

4. Biosynthesis and Applications of L-Thr Derivatives

As an important terminal product, L-Thr is also an ideal precursor for the synthesis of various high-value-added chemicals. In recent years, research on converting L-Thr into functional derivatives through chemical synthesis, enzymatic or cellular transformation, and microbial fermentation has been continuously advancing, significantly expanding its application scope and enhancing its economic value. This conversion strategy primarily focuses on enzyme engineering, pathway design, and systematic metabolic modification,

gradually achieving precise carbon flux direction and efficiency optimization of the derivatization process. Microbial cell fermentation for L-Thr derivatization demonstrates significant advantages. By integrating the L-Thr synthesis module with the derivatization module in *E. coli* or *C. glutamicum*, one-step fermentation from glucose to target derivatives can be realized. Furthermore, through dynamic regulation strategies, accumulating L-Thr during the cell growth phase and initiating the expression of derivatization enzymes in the stationary phase can effectively alleviate product inhibition and improve final yield. This section reviews the following compounds (Table 2): pyridoxine (PN), 2,3,5-trimethylpyrazine (TMP) and 2,5-dimethylpyrazine (2,5-DMP), L-ABA, PA, 2-OBA, etc.

Table 2. Engineering strategies and recent breakthroughs for L-Thr derivatives.

Products	Strategies	Substrate	Titer (g/L)	Productivity (%)	Reference
Pyridoxine	Pathway engineering, RBS optimization, medium optimization, fed-batch fermentation	Glucose	174.6	NR	[65]
2,3,5-Trimethylpyrazine	Overexpression of BITDH (N157A), fermentation condition optimization (substrate ratio 1:2, IPTG 1.0 mM, fermentation for 4 d)	Glucose, L-Thr	44.52	NR	[66]
2,5-Dimethylpyrazine	Knockout of the kbl gene	L-Thr	2.82	17	[67]
L-2-Aminobutyric acid	Whole-cell catalysis using BL21/pET28a-R3ilvA-Esldh72Δ-fdh	L-Thr	121	95	[68]
Propionic Acid	Sequential fermentation using MG1655— <i>Pseudomonas putida</i>	L-Thr	62	>98	[69]
2-Oxobutyric Acid	Whole-cell catalysis using <i>Pseudomonas stutzeri</i> SDM	L-Thr	25.6	99.6	[70]

NR: Not Reported.

4.1. Pyridoxine

PN, also known as vitamin B6, is a water-soluble vitamin involved in more than 100 enzymatic reactions, particularly indispensable in amino acid metabolism, neurotransmitter synthesis, and hemoglobin production. PN is essential for maintaining normal immune function, cognitive function, and cardiovascular health. Clinically, it is used for the prevention and treatment of various diseases caused by vitamin B6 deficiency, including skin inflammation, anemia, and neurological disorders [71]. Additionally, PN is particularly important during pregnancy as it contributes to healthy fetal development. In medical applications, it can be used for screening neonatal pyridoxine-dependent epilepsy [72], diagnosing gyrate atrophy [73] and in the early treatment of autism [74]. In the food industry, PN is commonly used as a nutritional fortifier in cereal products and certain beverages. However, significant losses occur during the processing of cereals and milk powder, resulting in decreased actual content in the final products. Therefore, PN is typically additionally fortified in infant foods to ensure adequate intake by infants and young children [75]. With the deepening understanding of the physiological functions of PN, its application scope in the food industry is continuously expanding. Commichau et al. [76] heterologously constructed the DXP-dependent vitamin B6 synthesis pathway in *Bacillus subtilis* (*B. subtilis*), and enhanced the conversion efficiency from L-Thr metabolic flux to

PN by supplementing 4-hydroxy-L-threonine (4-HO-Thr) and deoxyxylulose, increasing PN production from 14 mg/L to 54 mg/L. Subsequently, Commichau et al. [77] utilized adaptive evolution and heterologous gene expression optimization (pdxA-Ec/pdxJ-Sm), combined with screening of deoxyxylulose utilization mutants (DXcsp) and metabolic regulation, to increase the titer of 4-HO-Thr to PN in *B. subtilis* from 12 mg/L to 70 mg/L.

4.2. 2,3,5-Trimethylpyrazine and 2,5-Dimethylpyrazine

Alkylpyrazines are a class of nitrogen-containing heterocyclic compounds with alkyl groups on the side chain. These compounds typically possess pleasant nutty, roasted, and toasted flavor characteristics at low odor thresholds [78]. Therefore, alkylpyrazines are considered important aroma compounds contributing to the distinctive flavors of raw, thermally processed, or fermented foods. In addition to their aroma contributions, alkylpyrazines also play important roles in the pharmaceutical industry. Pyrazine-based antibacterial agents represented by quinolones can effectively treat infections by inhibiting bacterial DNA gyrase [79]. In agriculture, triazine herbicides are widely used due to their efficient weed control capabilities, significantly enhancing crop yields. In the food industry, Ma et al. [80] investigated the interactions between bovine serum albumin (BSA) and pyrazine homologs (including MP, 2,5-DMP, TMP, and TTMP). The results indicated that the distribution of alkyl groups in the pyrazine ring affects flavor release in BSA solution by inducing conformational and polarity changes in BSA. Meanwhile, pyrazine compounds also play important roles in materials science. Due to their extensive biological activities and application value, pyrazine compounds have become a hotspot in scientific research and industrial development. Zhang et al. [67] conducted whole-cell catalysis of *B. subtilis* using L-Thr as the substrate and TDH as the enzyme catalyst, producing 0.27 mM 2,5-DMP after 48 h of incubation. Xu et al. [81] rewired the 2,5-DMP biosynthesis pathway and substrate transmembrane transport, with the final strain T6-47-7 producing 1.43 g/L 2,5-DMP in shake flask fermentation. Yang et al. [82] by overexpressing *tdh* and *soaao* genes, and blocked the competitive branch carbon flux metabolic pathway by knocking out *kbl*. After 24 h, they converted 9.21 g/L threonine to 1682 mg/L 2,5-DMP. Beyond overexpression, enzyme engineering strategies such as semi-rational design have been proven effective in enhancing catalytic efficiency. Structure-guided iterative saturation mutagenesis of 4-hydroxyphenylacetate 3-monooxygenase based on AlphaFold2 prediction and molecular docking identified key residues, and the resulting double mutant S210G/Y117A increased caffeic acid production by 1.68-fold in shake flasks and reached 2335.48 mg/L in a 5 L fermenter [83]. This approach provides a robust strategy for engineering TDH to advance 2,5-DMP biosynthesis.

4.3. L-2-Aminobutyric Acid

L-ABA is a non-proteinogenic amino acid that serves as an important precursor for the synthesis of various chiral drugs, such as the anti-tuberculosis drug ethambutol, and the anti-epileptic drugs levetiracetam and brivaracetam. This molecule is synthesized from L-Thr and L-aspartate through α -transamination reactions, and has been scaled up to 2000 L production [84]. In the pharmaceutical field, L-ABA is a key intermediate for various high-value-added drugs. It has regulatory effects on nervous system function and can be used to treat anxiety, epilepsy, and certain neurodegenerative diseases, particularly occupying an important position in the development of anticancer and antiviral drugs [85]. In the field of nutrition, L-ABA, as a supplement, is considered to enhance muscle growth and improve athletic performance. In veterinary medicine, L-ABA is used to improve growth performance and feed conversion efficiency in livestock. Additionally, L-ABA is also used as a flavor enhancer and preservative in the food industry. With the development

of synthetic biology and metabolic engineering, the production efficiency and application scope of L-ABA are expected to be further expanded, and its potential in biopharmaceuticals, functional foods, and agricultural technology is gradually being explored. Fu et al. [86] constructed an in vitro cascade enzymatic reaction by co-expressing TD, Leucine dehydrogenase (LDH), and Formate dehydrogenase (FDH) in a single-strain *E. coli* 3FT+L at a ratio of 1:1:0.2, producing 68.5 g/L of L-ABA. Li et al. [87] generated an optimized BtLDH triple mutant (BtLDHM3) by optimizing plasmids with different copy numbers to regulate enzyme expression and utilizing mechanism-based protein engineering, ultimately achieving one-pot conversion of L-Thr to L-ABA. Liao et al. [88] used 30 g/L glucose as the substrate and the high-yield L-Thr recombinant *E. coli* ATCC98082 for fermentation production of L-ABA, achieving a product concentration of 5.5 g/L.

4.4. Propionic Acid

PA is a naturally occurring colorless organic acid with certain corrosiveness. Its odor is pungent with slightly rancid characteristics, and its taste is predominantly sour with a faint cheese-like flavor [89]. PA is primarily utilized for its antimicrobial properties, with major applications as a food preservative or herbicide [90], widely used in bread and cheese products [91]. It also contains anti-inflammatory substances and possesses analgesic and antipyretic properties [92]. PA is widely present in metabolic processes of various organisms ranging from bacteria to humans, usually generated as a by-product rather than a primary fermentation product. During threonine production, PA is not the target product but rather an end product of its degradation metabolism. Its accumulation indicates that carbon flux is not completely directed toward threonine synthesis; instead, a portion is diverted to degradation pathways, which often leads to reduced conversion efficiency of the target product and consequently affects overall production yield [69]. Ma et al. [93] constructed the final engineered strain through chromosomal integration of heterologous L-Thr deaminase, permease, and acetyl-coenzyme A thioesterase, deletion of branch pathways, and overproduction of endogenous branched-chain α -keto acid dehydrogenase complex. Under optimized conditions, the PA titer reached 50.3 g/L after 48 h, with a productivity of 0.6 g/L/h. Mu et al. [69] designed a new coenzyme A-independent synthesis pathway from L-Thr to PA, optimized heterologous expression of 2-ketoisovalerate decarboxylase from *Lactococcus*, constructed an engineered *Pseudomonas* system, developed a sequential fermentation-biotransformation integrated process without L-Thr separation, and achieved efficient PA biosynthesis. In this, PA yield reached 43 g/L in batch conversion, and was further improved to 62 g/L in a fed-batch process, with a productivity of 1.07 g/L/h.

4.5. 2-Oxobutyrate

2-OBA, as an important biochemical material, is generated by the deamination of L-Thr catalyzed by TD. It exhibits extensive applications in the synthesis of pharmaceuticals, pesticides, and fragrances, demonstrating substantial value. This has motivated continuous exploration of more efficient solutions by industry, academia, and research institutions to support its deeper empowerment of related industrial development [94]. 2-OBA can be used for the synthesis of vitamins B1 and B6, serves as a critical step in synthesizing certain antibiotics and antifungal drugs, and plays a key role in preparing specific fragrance compounds. Zhang et al. [70] utilized whole cells of *Pseudomonas stutzeri* SDM as biocatalysts and optimized reaction conditions including pH, temperature, and substrate concentration to achieve efficient biotransformation of L-Thr to 2-OBA, obtaining a 2-OBA yield of 25.6 g/L with a molar conversion rate of 99.6% within 6 h. Zhang et al. [95] constructed an engineered strain by overexpressing threonine dehydratase (*ilvA*) and knocking out *ilvIH* and *rhtA* genes in *E. coli*, combined with a two-stage fermenta-

tion strategy involving temperature induction (shifting from 35 °C to 40 °C), successfully increasing 2-OBA production to 40.8 g/L and significantly improving its fermentation production efficiency.

4.6. Other Derivatives

In addition to common L-Thr derivatives such as PM, TMP, L-ABA, PA, and 2-OBA, other L-Thr derivatives include Chloramphenicol (CM), Norepinephrine (NE), and Thiourea (TU). CM is a broad-spectrum antibiotic widely used to treat severe infections caused by Gram-negative and Gram-positive bacteria, including meningitis [96], pneumonia, typhoid fever, and dysentery. Due to its efficacy against anaerobic bacteria, it is commonly used for the treatment of anaerobic infections. Additionally, chloramphenicol can be used for certain ocular and skin infections. Xi et al. [97] adopted a dual strategy involving directed evolution of L-Thr transaldolase and construction of an efficient acetaldehyde elimination system, achieving efficient production of the CM intermediate through a “one-pot” reaction, with the CM intermediate yield reaching 201.5 mM. NE is widely used in the treatment of cardiogenic and septic diseases due to its ability to stabilize blood pressure. It has been the only vasoactive drug recommended for cardiac arrest since 1974. It increases coronary perfusion pressure during cardiopulmonary resuscitation, thereby increasing the chance of return of spontaneous circulation [98]. Xu et al. [99] employed a biocatalytic cascade reaction, engineering PsLTTA to enhance multi-enzyme activity in single cells and fine-tune expression patterns, obtaining a robust whole-cell biocatalyst ES07 that achieved the highest space-time yield (3.38 g/L/h). TU has extensive applications in industry and agriculture, and holds significant potential in cancer treatment, diabetes management, viral inhibition, seizure control, alleviation of fungal infections, and eradication of bacterial pathogens [100]. Zheng et al. [101] introduced a chiral L-Thr skeleton modified through silyl etherification, performed fine-tuned activity and stereoselectivity alterations of substituents in the chiral scaffold derived from L-Thr, and synthesized bifunctional TU with tunable threonine skeletons.

5. Conclusions

This review summarizes the biosynthesis, research status, and applications of L-Thr and its derivatives in pharmaceuticals, food, and agriculture. L-Thr is mainly produced through microbial fermentation, and its synthesis pathway involves the TCA intermediate oxaloacetate. We summarize the improvement of threonine-producing strains through genetic engineering and metabolic engineering strategies to increase threonine yield, as well as the enhancement of threonine production through the modification of synthetic pathways and enzyme genes, making significant contributions to the research on constructing high-yield L-Thr strains. Additionally, the study discusses the modification and application of threonine derivatives, emphasizing the importance of bioengineering technology in improving the production of threonine and its derivatives. Currently, the sugar-to-acid conversion rate of microbial fermentation for L-Thr production generally ranges between 40% and 50%, which is far below the theoretical maximum conversion rate of 81%, indicating significant room for improvement. Compared with traditional breeding methods, using metabolic engineering to construct production strains has obvious advantages: on the one hand, it can greatly shorten the strain breeding cycle; on the other hand, due to the clear genetic background of the constructed strains, it helps simplify the optimization process of later fermentation. Therefore, using metabolic engineering technology to modify metabolic pathways and optimize fermentation processes to improve L-Thr synthesis capability is of great significance for promoting the development of the L-Thr fermentation industry. With the rapid development of the integration of artificial intelligence and synthetic biology, the

further development of biotechnology is promoted through gene design, rational design of enzyme, regulation of metabolic networks, model construction, and de novo design. Meanwhile, in the food industry, the integration of artificial intelligence and synthetic biology will further promote the development of functional factor mining, precise flavor regulation, and intelligent preservation, reshaping the technological path of the food industry [102].

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