Production of L-tryptophan by Microbial Fermentation

Xiaojing Wen, Ning Liu, Zhihao Jia

College of Bioengineering, Dalian Medical University, Liaoning, China

ABSTRACT

L-tryptophan is one of the eight amino acids necessary for human and animal life activities. It cannot be naturally synthesized in the human body and must be taken from food. It exists in the living body in a free state or in a living state. It plays a very important role in the physiological activities of animal growth, metabolism and so on. It is called the second essential amino acid and is widely used in food, feed and medical industry. L-tryptophan production methods are chemical synthesis, conversion and microbial fermentation. In recent years, with the successful use of metabolic engineering in the breeding of tryptophan bacteria, microbial fermentation has gradually become the main method of production of tryptophan. The metabolic engineering strategies involved in the production of tryptophan by microbial fermentation were reviewed, including the mechanism of metabolic regulation of biotinylated tryptophan and the measures and effects of pathway modification. In addition, the future development of L-tryptophan was also discussed.

KEYWORDS: L-tryptophan; metabolic engineering; microbial fermentation

1. Physical and chemical properties of L-tryptophan

L-tryptophan, chemical name B-indole-alanine and L-B-3-indolylalanine, also known as L-pancreatic protein amino acid. It has a molecular formula of C11H12O2N2 with relative molecular weight of 204.23 [1-3].

L-tryptophan belongs to neutral aromatic amino acids. It is white or slightly yellow crystalline or crystalline powder, odorless with slightly bitter taste. L-tryptophan slightly soluble in water, insoluble in ethanol, very insoluble in chloroform, soluble in formic acid, sodium hydroxide solution or dilute hydrochloric acid, and more stable in other acid and alkaline. But in the presence of other amino acids or saccharides L-tryptophan are easily decomposed. L-tryptophan has three kinds of optical isomers, easy to change color under long illumination. L-tryptophan is heated in water to produce a small amount of indole, produces a large amount of indole when heated with sodium hydroxide or copper sulfate.

Figure 1-1 Molecular structure of L-Trp

2. L-tryptophan

L-tryptophan cannot be natural synthesized in the body and need to be taken from food, it is an essential amino acid for animal and some fungi. L-tryptophan content in the protein is very low, the average content of about 1% or less [4]. L-tryptophan can regulate the synthesis of protein, regulate immunity and digestive function [5], increase the
metabolism of serotonin and enhance cognitive ability [6] and so on, and therefore plays an important role in human and
growth and development. These nutritional and medicinal values of L-tryptophan make it widely
used in pharmaceutical, feed and food industries.

3. L-tryptophan synthesis method

L-tryptophan production methods are chemical synthesis, conversion and microbial fermentation. Chemical synthesis
method has been gradually eliminated due to the existence of complex processes, complex product composition and
other reasons. While the conversion method (enzyme conversion method and microbial conversion method) has
been achieved in industrialization, but there are issues such as expensive raw materials and low conversion rate. The
production of tryptophan microbiological fermentation using cheap material such as glucose, is the earliest development
of tryptophan production, but this method cannot be used in industrial scale for a long period of time. The reason is
found mainly in the early studies, the researchers rely solely on the traditional chemical or physical mutagenesis of
tryptophan production bacteria. There is an extremely complex regulatory mechanism of the biosynthetic pathway
of tryptophan, and it is impossible to eradicate all its metabolic regulation by mutagenesis, so in this case, the
researchers cannot obtain excellent strains for production of L-tryptophan. In recent years, with the rapid development
of DNA recombination technology, especially the rise of metabolic engineering breeding methods, the researchers
gradually breed a number of high-yield tryptophan production strains, contributing to a substantial increase in microbial
fermentation production of tryptophan efficiency, making it the main production method of tryptophan in the industry [7].
This paper systematically reviews the metabolic engineering strategies involved in the production of L-tryptophan by
microbial fermentation and discusses its future development and trends.

4. L-tryptophan microbial synthesis mechanism

4.1. Microbial synthesis of L-tryptophan metabolic pathway

Currently used for the production of L-Trp microbial species are E. coli, Corynebacterium glutamicum,
Brevibacterium flavum, Bacillus subtilis, yeast and other bacteria. The L-Trp synthesis of various microorganisms is
slightly different. In Escherichia coli, L-Trp synthesis includes three parts: central metabolic pathway, aromatic amino
acid common pathway and L-Trp branch pathway [8]. The central metabolic pathway refers to both the erythritol-4-phosphate (E4P) and the phosphoenolpyruvate (PEP) in the glycolysis (EMP) pathway of glucose pentose (HMP)
(DHHP). The common route refers to the process from the beginning of the DAHP, shikimic acid (SHIK) to the
branched acid (CHA). The remaining pathway from CHA To the L-Trp moiety is referred to as the L-Trp branch
pathway (Figure 1-2). At present, most of the metabolic engineering studies on L-Trp focus on the common pathway
and the transformation of L-Trp branch pathway.

Glucose

![Figure 1-2 The synthesis pathway of L-Trp in Escherichia coli and its related regulation](image)
4.2. Microbial synthesis of L-tryptophan regulatory mechanism

Tryptophan microbial synthesis and metabolism of complex regulatory mechanisms, which strictly control the synthesis of tryptophan.

Feedback inhibition of rate-limiting enzyme

There are two significant rate limiting enzymes in the synthesis of tryptophan: DAHP synthase and anthranilate (ANTA) synthase (Figure 1-2). DAHP synthetase catalyzes the central metabolic pathway of PEP and E4P to produce DAHP, which strictly controls the carbon flow from the central metabolic pathway into the aromatic amino acid common pathway. The DAHP synthase of Escherichia coli is composed of three isozymes encoded by gene aroF, aroG and aroH, and the enzymatic activities of these three isozymes are inhibited by tyrosine, phenylalanine and tryptophan respectively. In Corynebacterium glutamicum, there are only two DAHP synthetases, encoded by aro and aroII, respectively. ANTA synthetase is responsible for catalyzing the common pathway of the terminal product CHA to generate ANTA, thereby entering the tryptophan branch pathway. ANTA synthetase is encoded by the gene trpED, and the enzyme activity is inhibited by terminal amino acid tryptophan feedback [9].

Transcriptional regulation of key enzymes

In addition to the inhibitory effect of enzyme activity, the transcriptional expression of some key enzymes is also regulated by terminal amino acids. In E. coli, the synthesis from the branched acid to tryptophan is accomplished by the tryptophan operon. A trpR gene is present upstream of the tryptophan operon, which encodes a trpR repressor protein involved in the transcriptional regulation of the tryptophan operon. When the concentration of intracellular tryptophan is higher than a certain threshold, the transcription of the operon is repressed by the action of trpR repressor. In addition, the transcription of the tryptophan operon is also regulated by the attenuator sequence. In the tryptophan operon sequence, there is a leader sequence consisting of 162 nucleotides before the structural gene. During the transcription process, when the intracellular tryptophan reaches a certain concentration, the secondary structure of the nucleotide sequence changes, thus preventing the continued transcription of the operon [9].

Transmutation control

Tryptophan in the culture medium is transported by microorganisms to the cells for absorption and utilization, controlled by a specific transport system. In E. coli, the three tryptophan transport genes mtr, tnaB and aroP regulate the extracellular to intracellular transport of tryptophan. (Figure 1-2). Among them, the mtr and tnaB genes respectively encode the high and low affinity tryptophan permease, all of which specifically regulate the intracellular absorption of tryptophan from, while the aroP gene encodes a transmembrane two-way transport of aromatic amino acid permease. In addition to regulating the absorption of tryptophan, the permease also involved in the regulation of phenylalanine and tyrosine absorption [10]. But in Corynebacterium glutamicum, the absorption of tryptophan is regulated only by the gene aroP protein [11].

Other regulatory mechanisms

In addition to the above regulatory mechanisms, there are some other important regulatory mechanisms for tryptophan anabolism. For example, microbes also regulate the synthesis of tryptophan by tryptophan degradation reactions and numerous competing pathways. Escherichia coli tnaA gene encodes tryptophanase, although the enzyme can catalyze pyruvate, indole and ammonia synthesis of tryptophan efficiently under high concentrations of pyruvate and ammonia, under normal circumstances it only plays the role of degradation of tryptophan to produce pyruvate, indole and ammonia, blocking the accumulation of tryptophan. Thus, inactivation of the tnaA gene facilitates the accumulation of more tryptophan in E. coli [12]. In addition, there are numerous competing pathways in the key nodes of the tryptophan synthesis pathway, further weakening the flow of carbon to the tryptophan synthesis pathway. For example, the terminal product CHA in the common pathway, in addition to the synthesis of tryptophan, is a common substrate for the other two aromatic amino acids phenylalanine and tyrosine, and it is also involved in folic acid, menadione, ubiquinone and other biosynthetic pathways.

5. Metabolic regulation strategy in enhancing L-tryptophan synthesis

5.1. Enzyme selection of anti-feedback inhibition

Anti-feedback inhibition of mutant enzymes is a prerequisite for microbial accumulation of L-Trp. In the process of inorganic culture of wild-type Escherichia coli, the enzyme activity of AroG and AroF isozyme accounts for more than 99% of the total activity of DAHP synthase, and AroH isoenzyme is less than 1% of total enzyme activity. Therefore,
most of the researchers studied the aroG and aroF genes (aroGfbr and aroFfbr) by screening anti-feedback mutations in the synthesis of aromatic amino acids to relieve the feedback inhibition of terminal amino acids to DAHP synthase. So far, many anti-feedback regulatory DAHP synthase genes have been reported. Among them, the anti-feedback inhibition of AroG isoenzyme mutations are: amino acid residue 148 proline mutations to leucine Pro148Leu, 152nd amino acid residue glutamine to iso-leucine Gln152Ile, and N Terminal 8 amino acid residues asparagine mutated to lysine Asn8Lys. Anti-feedback inhibition of AroG isoenzyme mutations were: 150th amino acid residues Proline mutations to leucine Pro150Leu; 146th amino acid residues aspartic acid mutations to asparagine Asp146Asn. In the aspect of anti-feedback regulation of ANTA synthase, according to the research results of Maureen.G et al., Dr Li Jianxin from Institute of Radiation and Radiation Medicine, Chinese Academy of Military Medical Sciences obtained a series of anti-feedback ANTA synthase genes from Salmonella typhimurium by chemical mutagenesis and sequence analysis. According to Maureen.G and other research results, the anti-L-Trp feedback was obtained by tailoring the mutant Escherichia coli ANTA synthase TrpE subunit 40th amino acid residue Ser for Phe, successfully suppressing ANTA synthase.

5.2. Common pathway and transformation of tryptophan branch pathway

Aromatic amino acid common pathway and L-Trp branch pathway transformation mainly involves anti-feedback regulation action enzyme (DHHIP synthase, ANTA synthase), other key enzyme genes (trp operon, serA, etc.), genomic TrpR repressor gene trpR and tryptophanase gene tnaA inactivation.

In 1982, Aiba et al. transformed the constitutive plasmid Psc101 trp.115 (TrpE, TrpD) with trp operon into trpR and tnaA double gene inactivated host bacteria, and fermented to the culture medium by adding ANTA for 27 hours. The strain can accumulate 6.2 g / L of L-Trp. On the basis of this, the L-Trp yield of the strain was increased to 30 g / L after the multi-round chemical mutagenesis. The final L-Trp yield was increased to 54.5 g / L by adding the appropriate amount of surfactant L61 in the middle stage of fermentation. This is the highest reported current production of Escherichia coli L-Trp. In 2002, Dodge et al. transformed the recombinant plasmid pBE7 containing trp operon (TrpE release feedback), aroG (release feedback inhibition) and serA gene into tNAA and serA gene inactivated anti-ANTA mutant host bacteria. By optimizing the fermentation process Glucose flow rate, the L-Trp yield of 42 g / L. This is the highest yield of L-Trp produced by E. coli in the absence of any precursors. In order to carry out research on L-Trp metabolic engineering breeding in China, Li Jianxin introduced the aroG and trpED genes in the plasmid pBV220, and transformed into the Escherichia coli K-12, which knocked out the trpR and tnaA genes. After fermentation, L-Trp of 0.168 g / L was produced. In the L-Trp strain selection study of Corynebacterium glutamicum, an L-Tyr and L-Phe deficient strain KY9456 was obtained in 1975 by Hagino and Nakayama. After screening by multiple mutagenesis, only 12 × 10 -3g / L of L-Trp is produced. On the basis of this, in 1993, Katsumata and Ikeda introduced plasmid pKW99 in co-expression, and the expression of L-Trp was significantly increased to 43 g / L, with accumulation of indole. In 1994, Ikeda et al. further amplified in the plasmid pKW99 key enzyme gene of the serine synthesis and metabolism serA, eliminating the indole accumulation. The L-Trp production increased further to 50 g / L. In this study, a strain of Corynebacterium glutamicum JXQ3-28 and 0.8 g / L of L-Trp were isolated from the soil in the year of 2007, and L-Tyr and L-Phe were obtained by multiple mutagenesis. Defective strain SG-116, fermented for 90 h, were producing 10.2 g / L of L-Trp [15].

5.3. Transformation of the central metabolic pathway

After the common pathway and L-Trp branch pathway transformation, optimizing the central metabolic pathway to increase the supply of L-Trp precursor PEP and E4P and promoting the biosynthesis of intermediate key product DAHP has become the focus of further research. As shown in Figure 1-3, the consumption of PEP in the central metabolic pathway is mainly derived from the same amount of PEP consumed by the cells using the phosphorl transferase (PTS) system to transfer glucose to the cells; and PEP is purified by PYR kinase (pykA and pykF) Into PYR, into the TCA cycle or through the PEP carboxylase (ppc) catalytic synthesis of oxaloacetate (OAA), which directly involved in TCA cycle. The accumulation of PEP mainly re-converts PYR to PEP by PEP synthase (pps). In addition, the study found that inactivation of carbon storage regulator (Csr) also contributes to the accumulation of PEP. Compared with PEP, E4P has no competitive pathway shunt, transgonal (tal) and transketolase (tkt) can catalyze the synthesis of E4P. In Escherichia coli, transketolase has two isozymes, encoded by tktA and tktB, respectively, in which TktA isoenzyme plays a major role in the synthesis of E4P.

In the literature on the optimization of metabolic pathways, the investigators identified the effect of pathway modification by analyzing the changes in DAHP synthesis in the cells. Flores et al. (1996) passed mutant inactivated E. coli PTS system (PTS-) and managed to make E. coli use galactose permease to normally absorb glucose, resulting in a 2.9-fold increase in DAHP synthesis. Gosset et al. investigated the effects of PYR kinase on PEP accumulation. It was found that neither of the two pyruvate kinase isofoms (pykA, pykF) alone did not affect the accumulation of PEP, but the metabolic flux of PEP was significantly enhanced when the when with both pykA and pykF gene was knocked out, and the yield of DAHP was 3 times higher than that before knockout. In order to enhance the expression of key enzymes, Liao et al. increased the intracellular DAHP concentration to 1.9 times by overexpressing the PEP synthase.
gene pps, while Patnaik and Liao were overexpressing aroG (deregulated inhibition), pps And tktA gene, so that the amount of DAHP production close to the theoretical maximum yield.

Ikeda et al. inserted the transketolase gene tktA into the low-copy plasmid pSW9911 containing the other L-Trp key enzyme gene in 1999, and transformed it into Corynebacterium glutamicum KY9218. The L-Trp yield was increased by 15%, reaching 58 g/L. In China, 2008, Wang Jing relieved feedback regulation of the aroG and trpED genes in sequence to the vector pZE12, while the gene ppsA and tktA in series to the vector pZA31, the two vectors simultaneously converted to knocked out trpR and tnaA gene in E. coli K-12. The yield of L-Trp was 1.3 g/L [14].

![Fig. 1-3 Schematic diagram of the central metabolic pathway of L-Trp in Escherichia coli](image)

### 5.4. Transformation of the transport route

Studies have shown that the higher the L-Trp concentration in the micro-organisms, the stronger the regulatory effect of the synthesis of L-Trp [17]. Therefore, the L-Trp synthesized in vivo by transporting the L-Trp in vitro is transported outside or extracellular accumulation of L-Trp is transferred to the cell, it will contribute to the biosynthesis of L-Trp. It has been shown that there is indeed a regulatory system for transporting L-Trp to the extracellular system in microbes, but its specific genes have not yet been fully defined [17]. While genes that control L-Trp uptake in E. coli and Corynebacterium glutamicum have been identified separately. In Escherichia coli, Yanofsky in 1991 indirectly investigated the effects of inactivation of L-Trp on the absorption of L-Trp by the inactivation of three transport genes, mtr, tnaB and aroP, which were controlled by L-Trp. The results showed that the inactivation of L-Trp was the most significant, and the inactivation of aroP had no effect on the uptake of L-Trp by cultivating Escherichia coli in acid-hydrolyzed casein. However, when Escherichia coli was cultured in medium lacking L-Phe and L-Tyr, the inactivation of aroP had an important effect on the absorption of L-Trp. In the case of Corynebacterium glutamicum, Ikeda and
6. Tryptophan Precursors

In the biosynthetic pathway of tryptophan, there are a series of intermediate products with important industrial value such as 3 - dehydro shikimic acid (DHS), SHIK and ANTA. With the metabolic engineering in the microbial synthesis of tryptophan in-depth study of these intermediates metabolic engineering research has gradually been taken seriously. The biosynthesis of DHS and SHIK is achieved primarily through the central metabolic pathway remodeling to increase the supply of precursor PEP and E4P, as well as to block the reaction of the target product after the aromatic amino acid consensus pathway (Figure 1). For example, Li et al. overexpressing tacA and aroFfbr in aroE inactivated Escherichia coli KL3, allowing the strain to produce 69 g / L of DHS. And Martínez et al. [18] overexpress tktA and aroGfbr genes in Escherichia coli PB12 deficient in PTS system but can normally absorb glucose, and the results showed that the strain could obtain high DHS and SHIK yield and yield in the medium with glucose and glycerol as mixed carbon source. Chandran et al. [18] co-expressed the aroFfbr and tktA genes derived from aroL and aroK inactivated and PTS system deficient Escherichia coli K-12 and Z. mobilis-derived glf and glk genes. The yield of shikimic acid were up to 87 g / L after fermentation. In addition, Ahn et al. [20] and Escalante et al. [21] performed similar gene operations in the study, in which Escalante et al. investigated effects of pykF or pykA inactivation on biosynthesis of SHIK and other aromatic compounds. O-aminobenzoic acid is another concern for the tryptophan intermediate. Baltas et al. [22] overexpresses aroGfbr, tktA, glk and galP 4 genes by Escherichia coli W3110 trpD9923 PTS- in the trpD gene. The yield of ANTA was 14 g / L.

7. Conclusions

The application of metabolic engineering in the research of tryptophan bacteria breeding has provided the necessary conditions and abundant means for rational transformation of tryptophan biosynthetic pathway, which greatly enhanced the production efficiency of tryptophan in microbial fermentation. There is a certain degree of limitations. First, the role of metabolic engineering, mainly based on the known tryptophan synthesis pathway and its regulatory mechanism of the relevant information, rational and effective anabolic transformation of tryptophan. However, previous studies have shown that, in view of the complexity of tryptophan biosynthesis, there are still many important enzymes of the tryptophan synthesis pathway and its regulatory mechanism is not fully elucidated, thus affecting the metabolic engineering pathway transformation effect. Secondly, the content and means of metabolic engineering in the breeding of tryptophan bacteria are still relatively simple. In the breeding of most tryptophan-producing strains, the researchers focused on metabolic engineering studies focused on the screening of aromatic amino acid common pathways and tryptophan branching pathways in a number of key enzyme anti-feedback regulatory mutants and their co-expression. It is worth noting that the synthesis pathway of tryptophan involves numerous branching pathways that are cloned only for several genes in a particular branch pathway and often do not significantly alter the flux of the entire metabolic pathway, and may even cause some accumulation of intermediate products, thus damaging the cells.

In recent years, leaping developments in DNA recombination technology and transcriptomics, metabolomics and other cellular analysis of the microbial synthesis of tryptophan metabolic engineering research has brought unprecedented opportunities. In the case where the genome sequence of the major microorganisms producing tryptophan has been published, the continuous progress of the DNA recombination technique allows the investigator to focus on the optimization of the entire tryptophan anabolism, thereby directly completing on the genome level such as a series of genetic modification of the anti-feedback regulatory mutant enzyme , which will greatly enhance the efficiency of metabolic engineering in the breeding of tryptophan producing bacteria. On the other hand, high-throughput bioinformatics analysis is one of the key factors in metabolic engineering. With the gradual maturation of various cellular analysis techniques, researchers can use the high-throughput bioinformatics analysis to comprehensively understand the metabolic regulation mechanism of tryptophan from the point of view of cell metabolism, and to accurately locate the effects of limiting factors in tryptophan production, and then perform more effective molecular transformation, recycling, and ultimately get more excellent tryptophan producing strains.

The application of metabolic engineering in the research of tryptophan bacteria is still in the initial stage. In addition to the use of rational molecular transformation in the breeding process, the existing tryptophan-producing strains have adopted a large number of irrational pathways of random mutagenesis, which inevitably leads to some uncertain and negative mutations. In recent years, in the microbial synthesis of threonine, alanine and other amino acid metabolic engineering research, by means of transcriptomics and other analytical techniques to locate the limiting factor of amino acid synthesis, and then completely rational molecular transformation, is the way to build a genetic background for determination of amino acid producing strains [22-23]. In short, although the current metabolic engineering technology in the application of tryptophan bacteria have many shortcomings, but the metabolic engineering is indeed providing a
great impetus to high fermentation production of tryptophan. The deepening of study will make a greater contribution to the development of the entire tryptophan industry.

References