Proceedings of the 6th International Conference on Biological Engineering and Medical Science
DOI: 10.54254/2753-8818/2026.38066

Metabolic Engineering of Microbial Cell Factories for L-Tryptophan and Its Derivatives

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Abstract. L-Tryptophan (L-Trp) is used in nutrition, animal feed, pharmaceuticals, and biomaterial-related applications, and many of its derivatives are also commercially valuable. Microbial fermentation offers a practical route to L-Trp production, but wild-type strains do not naturally direct enough carbon to this product because precursor supply, feedback control, transcriptional regulation, and competing branches all restrict pathway output. This review examines how these limitations have been addressed in Escherichia coli. The strategies discussed include mutagenesis with high-throughput screening, targeted modification of known metabolic nodes, and modular approaches for balancing pathway flux. We also summarize work in which L-Trp-producing strains were used as starting points for the biosynthesis of 5-hydroxytryptophan, serotonin, melatonin, and violacein. Remaining issues relevant to larger-scale production include host tolerance, cofactor and transport limitations, and the narrow choice of production hosts. Opportunities in computer-assisted pathway design and alternative chassis, particularly Saccharomyces cerevisiae, are considered in this context.

Keywords: Microbial cell factories, L-tryptophan, shikimate pathway, serotonin, violacein

1. Introduction

L-Tryptophan (L-Trp) is an essential aromatic amino acid. Besides its direct role in protein synthesis, it is the precursor of several biologically active metabolites, including serotonin, melatonin, and kynurenine. These compounds participate in nervous-system regulation, gastrointestinal physiology, and immune responses [1]. This combination of nutritional and biological functions explains why L-Trp and its derivatives are used in nutritional supplements, animal feed, pharmaceutical synthesis, and biomaterial development.

Demand for L-Trp and related products has encouraged the search for production methods that are both economical and sustainable. Microbial fermentation is attractive in this regard, but producing L-Trp from glucose is metabolically demanding. Carbon must pass through central metabolism and the shikimate pathway before entering the tryptophan-specific branch, while precursor competition, feedback inhibition, and transcriptional control restrict accumulation in wild-type strains [2]. Different engineering strategies address different parts of this problem. When the responsible genetic targets are not obvious, random mutagenesis coupled with biosensor-assisted high-throughput screening (HTS) can identify strains with improved production or tolerance [3].

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DOI: 10.54254/2753-8818/2026.38066

When bottlenecks are known, rational engineering can increase precursor availability and relieve regulatory constraints in the shikimate and tryptophan branches [2, 4]. Modular metabolic engineering is useful when several nodes need to be tuned together rather than independently [5]. Once a productive L-Trp chassis has been established, additional heterologous reactions can extend the pathway to high-value derivatives [6, 7]. Accordingly, this review first examines strategies for improving L-Trp production and then discusses representative derivative pathways, current limitations, and future directions for strain and process design.

2. Fundamentals of tryptophan

2.1. Introduction to tryptophan

Structurally, tryptophan contains an indole side chain attached to the amino acid backbone, and the naturally occurring form is the L-enantiomer. The indole group contributes to several reported biological properties of L-Trp and its derivatives, including anti-inflammatory and antioxidant activities [8]. Its hydrophobicity also affects protein structure by contributing to nonpolar interactions within proteins. Compared with many other proteinogenic amino acids, L-Trp is relatively scarce and may become limiting for protein synthesis under some conditions [9].

2.2. Tryptophan metabolic pathways

L-Trp is not only an end product of amino acid metabolism; it also supplies carbon and nitrogen to a variety of downstream compounds, including 5-hydroxytryptophan (5-HTP), serotonin (5-HT), melatonin, violacein, and non-natural tryptophan derivatives. In mammalian and microbial systems, three routes are commonly discussed: the kynurenine, serotonin, and indole pathways [10]. The kynurenine route accounts for about 95% of free L-Trp metabolism [11]. Indoleamine 2,3-dioxygenase (IDO) participates in the rate-limiting step and has been linked to regulation of T-cell differentiation [12]; quinolinic acid is one of the downstream metabolites associated with neurological homeostasis. Only a small fraction of L-Trp enters the serotonin pathway. Tryptophan hydroxylase (TPH) first converts L-Trp to 5-HTP, which is then decarboxylated to 5-HT [13]. Gut microorganisms provide another set of reactions, producing indole-3-lactic acid (ILA), indole-3-propionic acid (IPA), indoleacetic acid (IAA), indole-3-aldehyde (IAld), and related metabolites. These microbial products have been linked to processes such as intestinal-barrier maintenance and tumor progression [14].

3. Methods for tryptophan synthesis

3.1. Conventional tryptophan production methods and their drawbacks

Protein hydrolysis, chemical synthesis, and enzymatic conversion have all been used to produce tryptophan. Each route has practical limitations. Chemical or hydrolytic methods may generate difficult-to-separate products or damage the desired molecule, and some processes are costly [15]. Fermentation avoids several of these problems and is therefore widely considered for large-scale L-Trp production, although its performance depends strongly on strain design and process control.

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3.2. Microbial pathways for tryptophan synthesis

Escherichia coli, *Corynebacterium glutamicum*, and *Saccharomyces cerevisiae* are among the microbial hosts used for tryptophan production [16, 17]. *E. coli* has been studied particularly extensively because its physiology is well characterized and a broad genetic-engineering toolkit is available [18]. For discussion of pathway engineering, L-Trp formation in *E. coli* can be viewed in three connected segments. Central carbon metabolism first supplies phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P) through the Embden-Meyerhof-Parnas (EMP) and pentose phosphate (PPP) pathways. PEP and E4P then condense to form 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP), initiating the shikimate pathway. Chorismic acid (CHA) lies at the branch point where carbon can be directed toward tryptophan, phenylalanine, or tyrosine. The final segment is the tryptophan-specific branch encoded by the tryptophan operon, which converts pathway intermediates to L-Trp.

4. Construction and optimization of L-Trp cell factories

4.1. Strain library construction and high-throughput screening

A useful production strain can be obtained either by targeted engineering or by screening a diverse strain library. The latter is especially valuable when the genetic basis of a desirable phenotype is not yet clear. Random mutagenesis followed by HTS can reveal variants with improved L-Trp production or greater tolerance to the product. Biosensors make this workflow much faster because they translate intracellular L-Trp into a measurable output. For example, a riboswitch-based RNA sensor links L-Trp binding to green fluorescent protein (GFP) expression [2], whereas a *tnaC*-based sensor also converts changes in intracellular L-Trp into fluorescence [3]. After atmospheric and room-temperature plasma (ARTP) mutagenesis, fluorescence-activated cell sorting can enrich high-signal cells from a large population. Fermentation testing is still required afterward, because fluorescence is a screening proxy rather than a direct measure of final process performance. Repeating the mutagenesis-screening cycle can further enrich stable high-producing variants.

4.2. Precursor supply bottlenecks and strategies

PEP and E4P are the two substrates used to form DAHP, so their availability strongly influences how much carbon enters the shikimate pathway. Neither precursor is dedicated to L-Trp production. PEP is consumed during phosphotransferase system (PTS)-dependent glucose uptake and by other reactions in central metabolism, whereas E4P availability depends on PPP flux and cellular redox state [19, 20]. The practical aim is therefore not simply to enlarge the intracellular pools of PEP and E4P, but to increase the fraction that can be committed to aromatic amino acid synthesis. Several approaches have been used to raise E4P availability. Overexpression of the transketolase gene *tktA* can push more carbon through relevant PPP reactions. Manipulating glucose-6-phosphate dehydrogenase is less straightforward because the same intervention changes both carbon distribution and NADPH balance; regulation of *zwf1* can therefore improve or impair product formation depending on the extent of the change [19]. Another option is to introduce phosphoketolase activity. Heterologous *xfpk* expression creates an alternative route to E4P from sugar-phosphate intermediates and has also been explored in hosts other than *E. coli* [21].

PEP can be preserved in several ways. One is to improve PEP regeneration; another is to reduce the amount spent on glucose uptake or diverted to pyruvate and other products. Replacing part of

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the native PTS-dependent uptake with alternatives such as GalP-Glk, or introducing the *glf/glk* module from *Zymomonas mobilis*, lowers the PEP cost of glucose transport [19, 20]. Attenuating the pyruvate kinases encoded by *pykF* and *pykA* likewise reduces conversion of PEP to pyruvate, leaving more available for DAHP formation. Other studies have proposed routes that use carbon sources such as pyruvate together with E4P rather than relying only on conventional PEP supply [19].

More precursor is not automatically better. If PEP and E4P are increased to different extents, one can become excessive while the other remains limiting. This is why precursor balancing has become a separate engineering objective. By combining promoters of different strengths for *ppsA*, *tktA*, and *aroG*, investigators tuned the relative supply of the two precursors; an E4P-to-PEP ratio of about 0.75-0.78 was reported under the tested conditions together with improved L-Trp production [21, 22]. The value should therefore be interpreted as an experimentally optimized ratio for that system rather than a universal constant.

4.3. Feedback inhibition, transcriptional repression, and strategies

Precursor supply is only one level of control. The first step of the shikimate pathway is also regulated by three DAHP synthase isoenzymes in *E. coli*: AroF, AroG, and AroH. AroF and AroG contribute roughly 20% and 80% of total DAHP synthase activity, respectively [23]. Their activities are sensitive to end-product feedback: AroF responds to L-Tyr, AroG to L-Phe, and AroH to L-Trp. This allosteric control lowers catalytic activity without requiring a change in enzyme abundance. Transcription adds a second layer. TyrR, for example, responds to intracellular L-Tyr or L-Phe and binds regulatory regions associated with *aroF*, *aroG*, and other aromatic-amino-acid genes. Depending on the promoter context, TyrR can either activate or repress transcription [23]. The combination of enzyme-level feedback and transcriptional control limits the amount of carbon that proceeds through the shikimate pathway toward L-Trp [24].

Regulation continues after carbon enters the tryptophan-specific branch. When intracellular L-Trp rises, TrpR represses the *trpEDCBA* operon and other targets, including *aroH* [25]. TrpE, the anthranilate synthase component that catalyzes the first committed reaction of this branch, is also inhibited by L-Trp. As a result, high product levels feed back on both gene expression and enzyme activity. Engineering this section of the pathway therefore focuses on weakening these two forms of negative control rather than simply increasing precursor supply.

Studies have targeted both control levels. Tang et al. introduced the S180F feedback-resistant mutation into *aroG* in strain Trp04 and observed a 13.2% increase in L-Trp production [2]. Zhao et al. modified *E. coli* W3110 by deleting Ile11 from AroF and replacing Ser40 with Phe in TrpE. The resulting feedback-resistant variants increased L-Trp production by about 17-fold; deletion of *trpR* on this background produced a further approximately 3.2-fold increase [26]. Du et al. used indigo formation as an indirect readout of intracellular L-Trp. Disrupting *trpR*increased indigo production by 12.5%, and additional expression of $aroG^{FBR}$and $trpE^{FBR}$ raised it by about 1.47-fold [4]. Because indigo is derived from intracellular L-Trp, these data are consistent with increased precursor availability, although indigo production is not itself a direct measurement of L-Trp titer. Together, the studies show why feedback-resistant enzymes and transcriptional derepression are often combined rather than used as isolated interventions.

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4.4. Attenuation of competing pathways

Chorismate does not feed only the tryptophan branch; it is also the common precursor for L-Phe and L-Tyr. Carbon directed to *pheA*- and *tyrA*-dependent reactions is therefore unavailable for L-Trp synthesis. Complete removal of these branches is usually undesirable because L-Phe and L-Tyr are also needed for cell growth, and strong attenuation of *pheA* or *tyrA* can reduce biomass [2, 27]. Supplementing the missing amino acids is one solution, but dynamic control offers a way to retain endogenous synthesis while limiting unnecessary carbon loss. Tang et al. replaced the native *tyrA* and *pheA* promoters in strain Trp17 with the self-regulated P_{Flic} promoter. The resulting Trp28 strain produced 28.05% more L-Trp while maintaining biomass close to that of its parent [2]. This example illustrates the central trade-off in branch-pathway engineering: carbon must be redirected toward product formation without creating new growth limitations.

4.5. Multi-module engineering for coordinated pathway regulation

An intervention that improves one reaction does not necessarily improve the whole pathway. Once several connected nodes are changed, an upstream increase can exceed downstream capacity, or a heavily expressed module can impose a new burden on the host. Modular engineering was developed to deal with this coupling. Instead of optimizing every reaction independently, the pathway is divided into functional units whose expression or carbon demand can be adjusted relative to one another. In co-culture designs, the same idea can be extended across different strains by assigning different parts of the pathway to different hosts [5].

Two commonly discussed formats are multivariate modular metabolic engineering (MMME) and modular co-culture engineering (MCE). MMME keeps the modules in one host and tunes their relative expression, whereas MCE separates functions between strains or species so that pathway labor is shared [5]. Juminaga et al. used the first strategy for L-Tyr, optimizing pathway modules separately before combining them in *E. coli* [28]. Although the product was L-Tyr rather than L-Trp, the study is relevant because both routes draw on the aromatic amino acid network. Fu et al. used the second strategy, pairing *E. coli* with *Saccharomyces cerevisiae* in a co-culture for L-Trp and indole-derived production [6]. These examples emphasize a different design question from single-gene engineering: whether the supply and consumption capacities of whole pathway sections are matched. Better matching can reduce intermediate accumulation and unnecessary metabolic load, but the optimal arrangement depends on the host and pathway rather than on a fixed module design.

Modular designs also create new practical problems. Expressing several modules in one cell can increase energetic and metabolic burden, whereas a co-culture must maintain a workable population balance between its members. At larger scale, pathway complexity, genetic stability, process control, and production cost become equally important. Computer-assisted pathway design may help narrow the number of module combinations that need to be tested, and synthetic consortia provide additional ways to distribute pathway tasks [5]. Their value, however, ultimately depends on whether the resulting system remains stable and controllable during fermentation.

5. Microbial synthesis of L-tryptophan derivatives

Once a strain accumulates L-Trp efficiently, the same host can be repurposed as a starting platform for downstream products. The required reactions differ from one derivative to another, but the engineering principle is similar: add the relevant heterologous enzyme system and then check whether downstream conversion keeps pace with L-Trp supply. In this sense, L-Trp functions as a

DOI: 10.54254/2753-8818/2026.38066

platform precursor rather than only as the final fermentation product. The usefulness of the chassis therefore depends on the balance between precursor generation and the capacity of the newly introduced pathway.

5.1. Microbial synthesis of 5-HTP

5-Hydroxytryptophan (5-HTP) is formed by hydroxylation of L-Trp catalyzed by tryptophan hydroxylase (TPH), with tetrahydrobiopterin (BH4) serving as a required cofactor. Wild-type *E. coli* provides neither TPH nor a complete BH4 biosynthetic system. A 5-HTP-producing strain must therefore supply both the hydroxylase and a way to synthesize and regenerate BH4. In previous work, genes for BH4 synthesis and recycling were introduced together with a human TPH gene, allowing engineered *E. coli* to convert carbon sources to 5-HTP [7, 29]. This example shows that installing a new catalytic step may also require reconstruction of its cofactor cycle.

5.2. Microbial synthesis of 5-HT

Serotonin (5-hydroxytryptamine, 5-HT) lies one decarboxylation step downstream of 5-HTP. Tryptophan decarboxylase (TDC) can catalyze this reaction, but its higher affinity for L-Trp creates a competing route to tryptamine and reduces pathway selectivity [30, 31]. Dopa decarboxylase (DDC) has therefore been tested as an alternative enzyme for 5-HTP conversion. When DDC expression was added to a 5-HTP-producing *E. coli* system, sequential conversion from L-Trp to 5-HT was achieved, with a reported titer of 414.5 +/- 1.6 mg/L under the optimized conditions [31]. The case highlights enzyme substrate preference as a design variable: adding catalytic activity is useful only if it does not introduce a stronger competing reaction.

5.3. Microbial synthesis of melatonin

Melatonin synthesis extends the serotonin pathway by two reactions. AANAT/SNAT first acetylates 5-HT to N-acetylserotonin, after which ASMT catalyzes methylation to melatonin [32]. Byeon et al. expressed sheep SNAT together with rice COMT in *E. coli* and obtained 1.46 mg/L melatonin when 5-HT was supplied externally [33]. Wang et al. instead introduced human HsAANAT and HIOMT (ASMT) and reported 1.13 mg/L melatonin from L-Trp in a de novo system [34]. These titers should not be compared directly because the two studies started from different substrates and therefore placed different demands on the engineered pathway. Both nevertheless show that *E. coli* can support heterologous melatonin synthesis. Further improvement will depend on the activities and substrate preferences of the individual enzymes and on how well consecutive steps are matched in expression and flux.

5.4. Microbial synthesis of violacein

Violacein represents a different use of the L-Trp pool. It is an indole-derived natural product with reported antibacterial, antitumor, and antioxidant activities, but *E. coli* does not contain the native violacein biosynthetic cluster. Production therefore requires heterologous expression of *vio*genes. In an L-Trp-producing *E. coli* background, different configurations of *vioABCDE* were tested, and separating *vioABCD* from *vioE* gave the highest reported titer, 4.45 g/L [35]. Yang et al. further varied the composition of the *vio* cluster and directed the same chassis toward violacein, deoxyviolacein, proviolacein, and prodeoxyviolacein [36]. Here, pathway composition did more than change total output; it altered which products were formed. Future optimization can therefore

DOI: 10.54254/2753-8818/2026.38066

consider enzyme activity and relative gene expression together with the rate at which the upstream chassis supplies L-Trp.

The examples above make a common point. A high-L-Trp strain is useful as a derivative chassis only when the added pathway can consume the precursor at a compatible rate. The relevant bottleneck may be a missing cofactor cycle, poor substrate selectivity, uneven activity across consecutive steps, or an imbalanced multigene cluster. For this reason, derivative engineering should be treated as a new round of pathway balancing rather than as the simple addition of one or more heterologous enzymes. This view also leaves room to extend L-Trp-based cell factories to other natural and non-natural indole compounds.

6. Conclusion

Several lessons emerge from current L-Trp cell-factory studies. There is no single obligatory sequence of strain optimization. Random mutagenesis and HTS are useful when the relevant genetic targets are uncertain, whereas rational engineering is better suited to known limitations in precursor supply, feedback regulation, transcriptional control, or competing branches. When several interventions interact, modular engineering provides a way to tune pathway sections together rather than maximizing each one independently. In practice, these approaches are often complementary: screening can provide a productive starting strain, targeted modifications can remove identifiable bottlenecks, and module-level balancing can be applied when local improvements begin to create new constraints elsewhere in the network. The central objective is not simply to increase individual enzyme activities, but to move carbon through the pathway without creating a new growth or flux imbalance.

High-L-Trp strains can also be viewed as platform hosts for downstream products. By adding appropriate heterologous reactions, investigators have extended L-Trp metabolism to 5-HTP, 5-HT, melatonin, violacein, and related compounds. The examples reviewed here show that precursor supply and downstream catalytic capacity must be considered together; otherwise, improving the chassis alone may not increase derivative production.

Industrial translation introduces a different set of questions. High titer and yield must be maintained together with genetic stability, host tolerance, reproducible fermentation performance, and acceptable process cost. Cofactor engineering, transporter modification, and computer-assisted analysis may help identify bottlenecks that become important only after a strain has already reached relatively high production [37]. A second limitation is the heavy reliance on *E. coli*. *Saccharomyces cerevisiae* is already widely used in industrial biotechnology, yet the regulation of L-Trp metabolism in this host is less completely understood. Because L-Trp metabolism is connected with stress adaptation in *S. cerevisiae*, defining that regulatory relationship may reveal engineering targets for strains that remain productive under industrially relevant stress [38]. Future work will therefore need to combine pathway optimization with host selection and process-level validation rather than treating these as separate tasks.

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