



Review Article

Melatonin biosynthesis pathways in nature and its production in engineered microorganisms

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ABSTRACT

Melatonin is a biogenic amine that can be found in plants, animals and microorganism. The metabolic pathway of melatonin is different in various organisms, and biosynthetic endogenous melatonin acts as a molecular signal and antioxidant protection against external stress. Microbial synthesis pathways of melatonin are similar to those of animals but different from those of plants. At present, the method of using microorganism fermentation to produce melatonin is gradually prevailing, and exploring the biosynthetic pathway of melatonin to modify microorganism is becoming the mainstream, which has more advantages than traditional chemical synthesis. Here, we review recent advances in the synthesis, optimization of melatonin pathway. L-tryptophan is one of the two crucial precursors for the synthesis of melatonin, which can be produced through a four-step reaction. Enzymes involved in melatonin synthesis have low specificity and catalytic efficiency. Site-directed mutation, directed evolution or promotion of cofactor synthesis can enhance enzyme activity and increase the metabolic flow to promote microbial melatonin production. On the whole, the status and bottleneck of melatonin biosynthesis can be improved to a higher level, providing an effective reference for future microbial modification.

1. Introduction

Melatonin (*N*-acetyl-5-methoxytryptamine) is an important indoleamine derivative. In 1967, Sagan speculated that melatonin was synthesized by mitochondria and chloroplasts, which at the time were considered to have evolved from precursors similar to *Rhodospirillum rubrum* and cyanobacteria [1,2]. Melatonin has anti-inflammatory, antioxidant and autophagic properties [3,4]. The level of melatonin in plants affects their response to biotic and abiotic stress [5]. In animals, melatonin can regulate sleep and body temperature [6] and improve dairy production [7]. In humans, it was demonstrated to protect lymphocytes against DNA damage induced by ionizing radiation [8], regulate the circadian rhythm [9], protect the skin [10], and ameliorate diabetes [11]. Very recently, melatonin was also investigated as a potential adjuvant treatment for slowing down the effects of COVID-19

[12] (Fig. 1). Nowadays, it is estimated that 50 to 70 million Americans chronically suffer from a sleep or circadian disorder, and the global market of melatonin was estimated at USD 1 billion in 2020, and it is expected to reach 3.4 billion USD by 2026 (<https://marketresearchexpertz.com/report/global-melatonin-market-92220>). Recently, it was reported that more than 300 million people in China suffer from sleep disorders. However, sales of melatonin products in the global market have soared, and demand exceeds production, so that it is necessary to further improve industrial approaches for melatonin synthesis. Commercial melatonin is mainly produced by chemical synthesis, which is highly polluting. The raw material supply from animal and plant extraction is small, and the extraction rate is low. In recent years, the rapid development of microbial fermentation methods has offered hope for more economic and environmentally friendly production. It is a promising method to design, optimize and introduce melatonin

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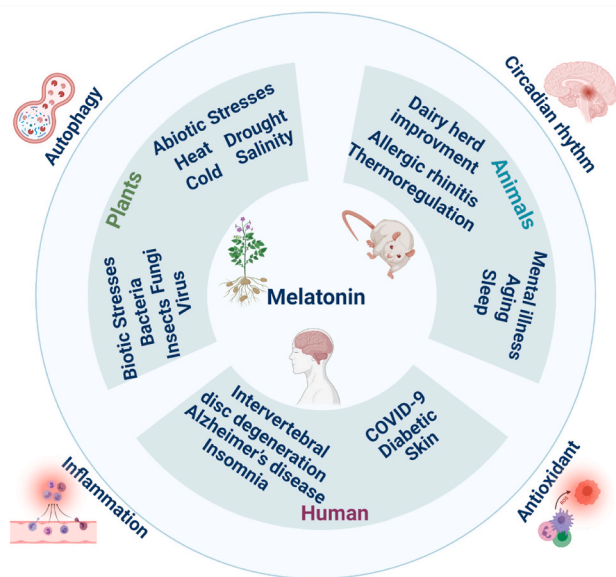


Fig. 1. The application value of melatonin.

synthesis pathways into microorganisms.

Due to the complex metabolic network and numerous secondary metabolites of microorganisms, the synthesis pathway of melatonin has not been investigated in many species. Only a few strains have been found to produce melatonin by themselves, including yeast and pseudomonads, but the synthesis efficiency is limited [13–16]. *Pseudomonas fluorescens* can synthesize melatonin by itself as a growth signal molecule or as a protective agent against ROS in the medium to promote early adaptation. However, the melatonin concentration reached a maximal value of only 1.32 $\mu\text{g/L}$ [16]. Back et al. speculated that the melatonin synthesis pathway of yeast is similar to that of cyanobacteria or plants [17]. Melatonin is a secondary metabolite of yeast which can interact with glycolytic proteins and has a positive effect on fermentative metabolism by increasing resistance to oxidative stress and UV radiation [18–20]. Yeast follows the Ehrlich pathway during early alcohol fermentation and will use amino acids such as L-tryptophan as the source of nitrogen [21]. The melatonin production of *Saccharomyces cerevisiae* QA23 and Levucell SC20 (from animal nutrition) reached 1.35 ± 1.69 and 93.14 ± 85.86 ng/mL during alcoholic fermentation, respectively [14,22].

Heterologous expression systems can be widely employed to screen various putative melatonin synthesis genes from animals and plants to overproduce melatonin in various microorganisms [23]. There are few reports on the ectopic overproduction of melatonin in *E. coli* because of either the low or insoluble expression of melatonin biosynthesis genes [23–25]. Zhang et al. combined the physostigmine biosynthetic genes from *Streptomyces albulus*, a gene encoding phenylalanine 4-hydroxylase from *Xanthomonas campestris* and caffeic acid 3-O-methyltransferase (COMT) from *Oryza sativa* in *E. coli*, which resulted in a melatonin yield of 136.17 ± 1.33 mg/L in a shake flask and 0.65 ± 0.11 g/L in a 2L-bioreactor [26]. When a melatonin-producing strain was assembled using the three plasmids ddc, aanat and asmt, encoding the cofactor pterin-4 α -carbinolamine dehydratase and using glucose as the sole carbon source for tryptophan supply, the final melatonin yield reached a record level of over 2 g/L [27].

However, the synthesis of melatonin in microorganisms is complex and the yield is generally low. The metabolic engineering approaches are not systematic and there is a lack of research on related pathways in the prior art. Therefore, we need to explore metabolic engineering strategies to facilitate the large-scale production of melatonin by microorganisms.

2. Natural melatonin synthesis pathways

2.1. Melatonin synthesis pathway in plants

Melatonin was first reported in plants in 2000 and was found to be distributed in various plant tissues [28]. Mitochondria and chloroplasts are the main sites of melatonin synthesis in plants. From an evolutionary point of view, the precursor of mitochondria probably was similar to a purple nonsulfur bacterium, and chloroplasts are probably the descendants of cyanobacteria [29,30]. Studies have shown that L-tryptophan can be converted into melatonin via two pathways in plants, one in healthy plants and the other under senescence or biological stress [28, 31].

Under normal plant growth, L-tryptophan is first carboxylated by L-tryptophan decarboxylase (TDC) into tryptamine in cytoplasm. The second committed enzyme tryptamine-5-hydroxylase (T5H), which catalyzes tryptamine into serotonin and requires acetyl-coenzyme A (Ac-CoA) on the endoplasmic reticulum. Usually in fresh plants, the third step is the acetylation of serotonin into N-acetylserotonin by serotonin N-acetyltransferase (SNAT) in the chloroplast. N-Acetylserotonin methyltransferase (ASMT) is believed to be the last enzyme, it catalyzes N-acetyl-serotonin into melatonin by methylation reaction in the cytoplasm. When plants under senescence and cadmium stress, the serotonin may be accumulated so that the melatonin synthesis would favor use ASMT catalyzes serotonin into 5-methoxytryptamine, and then synthesize melatonin by SNAT [32]. During the last step, the conversion of acetyl-5-hydroxytryptamine to melatonin is accompanied by the co-conversion of S-adenosyl-L-methionine (SAM) to S-adenosyl-L-homocysteine (SAH). Overall, when plants are subjected to biological stress and senescence, the same as the normal growth conditions of plants is that tryptophan is carboxylated and hydroxylated to produce serotonin. The difference is that serotonin is first methylated to produce 5-methyltryptamine, and then 5-methyltryptamine is acetylated to produce melatonin [17]. In plants, the existence of two melatonin metabolic pathways may be determined by the strict regulatory mechanisms of plants themselves (Fig. 2). However, the deeper regulation of enzymes that plants biosynthesize melatonin under different conditions needs to be explored.

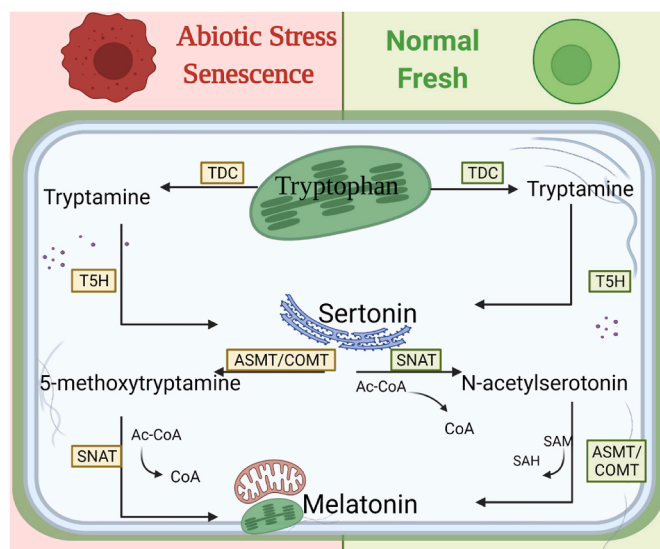


Fig. 2. Synthesis pathway of melatonin in plants
TDC, tryptophan decarboxylase; T5H, tryptamine-5-hydroxylase; ASMT, N-acetylserotonin methyltransferase; COMT, acetylserotonin O-methyltransferase; SNAT, serotonin N-acetyltransferase.

2.2. Melatonin synthesis pathway in animals

Animals have a single source of melatonin, mitochondria. Moreover, animals cannot synthesize L-tryptophan themselves, and it must be ingested externally. Therefore, animals have lower melatonin metabolism than plants. In animals, the synthesis of melatonin proceeds via a four-step pathway. First, tryptophan hydroxylase produces 5-hydroxytryptophan. This reaction requires the cofactor BH₄ and oxygen. Next, tryptophan carboxylase converts 5-hydroxytryptophan into serotonin, which is the main precursor of melatonin synthesis. This reaction is accompanied by the release of carbon dioxide. Subsequently, aralkylamine N-acetyltransferase produces N-acetyl serotonin at the expense of acetyl-CoA. Finally, N-acetyl-serotonin methyltransferase generates the final product melatonin accompanied by the conversion of the cofactor SAM into SAH [17,31] (Fig. 3).

3. Synthesis and modification pathways of melatonin in microorganisms

In addition to plants and animals, many microbes are also capable of melatonin synthesis [16,18,19,33]. The application of engineered microorganisms to synthesize melatonin is a potential industrial production method. The pathway through which microorganisms synthesize melatonin is similar to that of animals. It requires encompasses consecutive enzymatic reactions with the involvement of coenzymes or cofactors. However, there are few literature reports on the improvement of microbial melatonin production through metabolic engineering. According to the basic approaches of metabolic engineering, the production of melatonin can be improved by increasing the supply of precursors, optimizing the expression of pathway enzymes, or improving the utilization of cofactors and coenzymes.

3.1. L-tryptophan synthesis

L-tryptophan is a precursor for the synthesis of melatonin [34]. Normally, glucose is transported through the phosphotransferase system to produce glucose hexaphosphate, which then enters the glycolytic pathway and the pentose phosphate pathway to produce phosphoenolpyruvate pyruvic acid (PEP) and erythrose 4-phosphate (E4P), respectively. Then, 3-deoxy-D-arabinoheptulose (DAHP) is produced by the condensation of PEP and E4P, and passes through the shikimic acid pathway to generate chorismate. The biosynthetic pathway of L-tryptophan is summarized in Fig. 4. In *E. coli*, L-tryptophan is synthesized by a long pathway of enzymes encoded by the *trpEDCBA* operon [35]. The

main rate-limiting enzymes that have a major impact on L-tryptophan synthesis are anthranilate synthase encoded by *trpE* and DHAP synthase encoded by *aroG*. These enzymes are subject to feedback inhibition by tryptophan and phenylalanine, respectively. The transcriptional regulation of the synthesis and transport is mainly achieved by the repressor proteins encoded *trpR*. Weakening the TrpR repressor protein can relieve its inhibitory effect on the enzymes in the L-tryptophan synthesis pathway and increase the production of L-tryptophan [36,37]. Restraining the expression of *tnaA* (L-tryptophanase) was necessary to prevent the formation of serotonin and improve hydroxylation for increase the production of melatonin [38].

Both *E. coli* and yeast can synthesize L-tryptophan, which can be used to synthesize melatonin. At present, most strains with high tryptophan yield were constructed from *E. coli*, indicating that it may be a better chassis for the production of melatonin. Zhu et al. expressed heterologous glutamine synthetase in the engineered *E. coli* strain KW001, followed by the overexpression of *icd* and *gdhA*, as well as the expression of mutated *serA* and *thrA* variants. Finally, *sthA* and *pntAB*, encoding transhydrogenase, were overexpressed to maintain cofactor balance. The engineered strain could produce 1.71 g/L L-tryptophan in shake-flask fermentation, which was 2.76-times higher than the titer of the parental strain [39]. Zeng and Chen rationally engineered wild-type *E. coli* W3110 by knocking out tryptophanase (*tnaA*), 5-methyltetrahydrofolate-homocysteine methyltransferase (MTR), and 3-deoxy-7-phosphoheptanoic acid synthase (*aroFGH*), as well as overexpressing D-3-phosphoglycerate dehydrogenase (*serA*), which resulted in a final L-tryptophan titer of 30–34 g/L in a 1.5 L bioreactor [40]. Xiong et al. first introduced phosphoketolase from *Bifidobacterium adolescentis* to strengthen E4P formation, after which the phosphotransferase system was substituted with PEP-independent glucose transport, mediated by a glucose facilitator from *Zymomonas mobilis* and native glucokinase. Finally, they rewired the PEP-pyruvate-oxaloacetate node. Fed-batch fermentation in a 5-L bioreactor produced 41.7 g/L L-tryptophan, which is the highest yield reported to date [41].

3.2. Serotonin synthesis

Serotonin is formed by a two-step conversion of L-tryptophan. First, L-tryptophan is hydroxylated to 5-hydroxyserotonin by TPH, which in turn is carboxylated by TDC to yield serotonin. Serotonin is mainly known for its role as a major neurotransmitter and mood regulator, leading to the epithet ‘hormone of happiness’ based on the relationship between low serotonin levels and depression [34]. Serotonin is synthesized in the central nervous system and gastrointestinal tract of

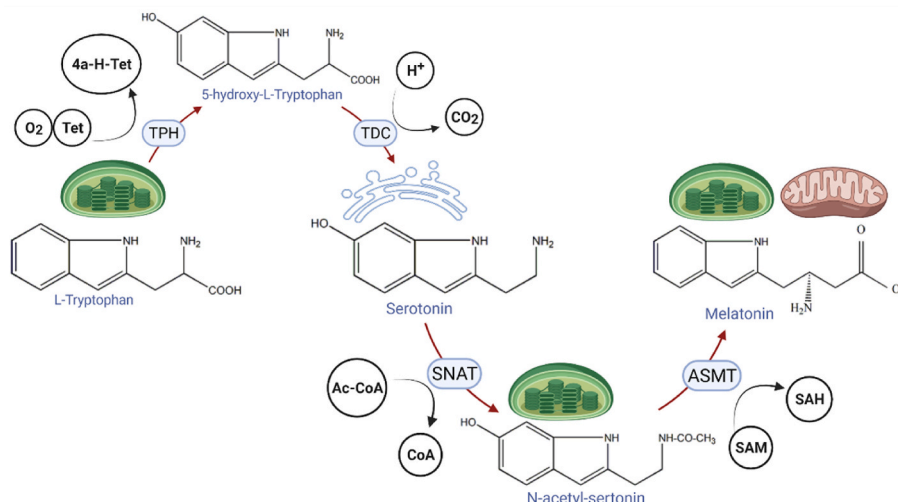


Fig. 3. The biosynthetic pathways of melatonin in all known living organisms.

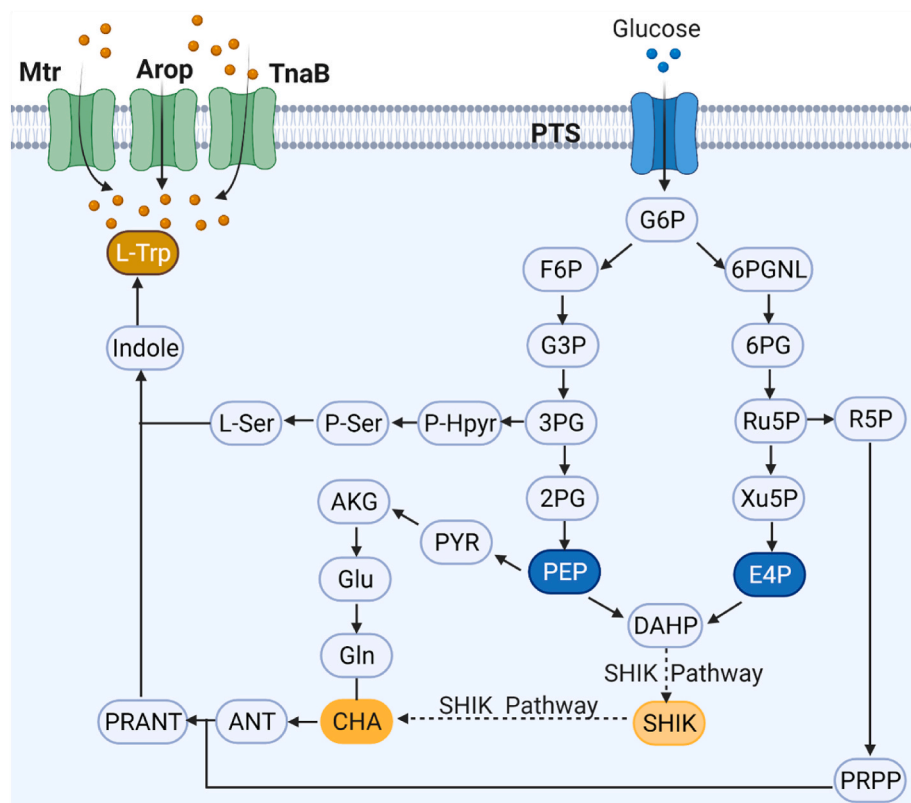


Fig. 4. The biosynthetic pathway of *L*-tryptophan. Abbreviations: G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; 6PGNL, 6-phosphoglucono-lactone; G3P, glyceraldehyde-3-phosphate; 3 PG, glyceralate-3-phosphate; 3-Hpyr, 3-phosphonoxypruvate; PEP, phosphoenolpyruvate; E4P, erythrose-4-phosphate; Ru5P, ribulose-5-phosphate; R5P, ribose-5-phosphate; PRPP, phosphoribosyl pyrophosphate; PYR, pyruvate; AKG, α -ketoglutarate; Glu, glutamate; Gln, glutamine; P-Ser, 3-phosphoserine; L-Ser, serine; DAHP, 3-deoxy-*D*-arabi-noheptulose-7-phosphate; SHIK, shikimate; CHA, chorismate; ANT, anthranilate; PRANT, *N*-(5'-phosphoribosyl)-anthranilate; Trp, tryptophan.

mammals [42]. The presence of serotonin is strictly related to the synthesis of melatonin in plants and animals. It is a key intermediate in the melatonin synthesis pathway. The biosynthetic pathway of serotonin from *L*-tryptophan is summarized in Fig. 5.

In microbial production, serotonin is mainly synthesized from externally added *L*-tryptophan. Germann et al. used truncated *H. sapiens* HsTPH146-460, lacking both the *N*- and *C*-terminal regulatory regions in order to increase heterologous expression and enhance protein stability, along with *H. sapiens* DDC, *Bos taurus* AANAT and *H. sapiens* ASMT to produce 2.4 mg/L of serotonin [18]. Tryptophan hydroxylase has been expressed in *E. coli* with low enzymatic activity, as the solubility and stability of the enzyme seem to be affected in this host. A serotonin-producing strain of *Saccharomyces cerevisiae* was constructed by introducing *L*-tryptophan hydroxylase from *Cupriavidus taiwanensis* together with a cofactor reconstitution pathway, and the yield of serotonin reached 154.3 ± 14.3 mg/L [43]. Therefore, introducing heterologous enzymes is helpful for the improvement of 5HT production. The production of serotonin can also be improved by regulating the expression levels of relevant enzymes. Directed evolution of enzymes can increase their affinity for the substrate and reduce side-reactions. Improving the supply of cofactors and oxygen are also effective ways to improve the titer of serotonin.

3.2.1. *L*-tryptophan hydroxylase

Tryptophan hydroxylase (TPH) is a mononuclear non-heme iron enzyme, which catalyzes the reaction between tryptophan, O₂, and tetrahydrobiopterin (BH₄) to produce 5-hydroxytryptophan and 4a-hydroxytetrahydrobiopterin [44]. The role of TPH is to replace the H at the C5 position on the benzene ring of tryptophan with an OH radical, which as the first step in the synthesis of serotonin. The hydroxylation reaction is the first and rate-limiting step in the synthesis of serotonin [45,46]. Therefore, it is crucial to find a suitable enzyme to increase serotonin production.

Directed evolution can improve the catalytic activity of enzymes.

Kino et al. found that L101F and W180F changes in the active center of *Pseudomonas aeruginosa* have an effect on substrate specificity and hydroxylase activity. The catalytic rate constant *K*_{cat} increased from 0.4 to 2.08 after mutation at the two sites [47,48]. Rosetta modeling is a useful tool for studying the effects of mutations on catalytic function in more detail. Generally, high-throughput screening based on biosensors can be used to identify effective mutations. Maxel et al. applied this selection platform to screen a NADPH-dependent-hydroxybenzoic acid hydroxylase that uses 3,4-dihydroxyphenyl acid as a substrate, and obtained variants with roughly 8-fold improved apparent catalytic efficiency (*K*_{cat}/*K*_m) for 3,4-DHBA, compared to the wild type [49,50].

Rational design of enzymes is becoming the dominant approach. Therefore, it is necessary to elucidate the structure of the enzyme and modify it to enhance the specificity of substrate binding by library construction and rational in silico design [51]. Windahl et al. solved the first crystal structure of an aromatic amino acid hydroxylase, TPH. The substrate specificity of the TPH is controlled through interactions with the side chain of tryptophan. The side chain of the tryptophan is bound in a hydrophobic pocket lined by residues Tyr236, Thr266, Pro267, Glu268, Pro269, His273, Phe314, Phe319, and Ile367 [44]. Human TPH can be expressed in *E. coli* and human embryonic kidney cells (HEK293). With *L*-tryptophan as the substrate, the *K*_m value of wild-type TPH is 41 μ M, while the *K*_m value of the TPH2 mutant R441H was 33 μ M [52]. (Table 1 and Table 2).

3.2.2. *L*-tryptophan decarboxylase

L-tryptophan decarboxylase (TDC) is a cytosolic type-II aromatic *L*-amino acid decarboxylase [53–57]. It catalyzes the final step in the microbial pathway of serotonin synthesis. TDC catalyzes two reactions, one of which produces tryptamine from *L*-tryptophan and the other serotonin from 5-hydroxytryptophan. Because TDC has an affinity for *L*-tryptophan, tryptamine is readily produced as a byproduct, which can affect the accumulation of serotonin. Therefore, to reduce the production of the by-product tryptamine, it is particularly important to select

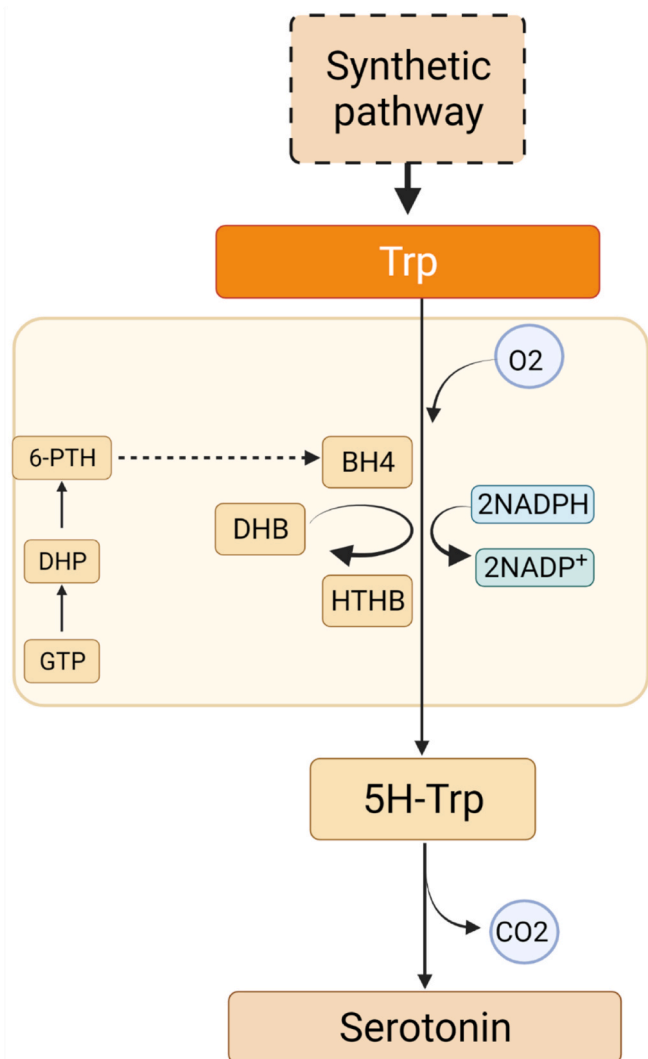


Fig. 5. Synthesis of serotonin from L-tryptophan.

appropriate enzymes.

TDC has different affinity for L-tryptophan and 5-hydroxytryptamine. The K_m value of TDC is generally higher in plants. Similar to the enzymes from rice and *Ophiorrhiza pumila*, *Withania somnifera* TDC is highly specific for tryptophan, and does not accept 5-hydroxytryptophan to produce serotonin [40]. The K_m values of TDC enzymes from different sources are summarized in Tables 1 and 2. TDC is a homodimer with strict specificity to the substrate tryptophan. The affinity between the enzyme and substrate is not only related to the size of the active site pocket, but is also affected by the hydrophobicity of the surrounding residues [58]. In the synthesis of serotonin, TDC can convert both tryptophan and 5-hydroxytryptophan, but exhibits different catalytic efficiency [58–61]. Zhou et al. used protein modeling to deduce that the bottom binding pocket and active cavity of TDC were mainly composed of hydrophobic residues of one subunit (W95, F103, F104 and L336) and adjacent subunits (V125, F127, L360 and V380) [58]. In contrast to 5-hydroxytryptophan, L-tryptophan lacks a hydroxyl group on the indole ring. Therefore, the potential reason why the TDC binding pocket can hold tryptophan is reduced steric hindrance and higher hydrophobicity. Thus, possible strategies to construct a TDC mutant that can use 5-hydroxytryptophan to initiate the synthesis of melatonin include increasing the size of the active site and promoting the formation of hydrogen bonds between the enzyme and the substrate. It was reported that H214 and Y359 are crucial for substrate recognition and influence

TDC activity [58,62,63]. The KOD-plus-neo PCR method can also be used for site-directed mutagenesis to change the size of the active pocket and achieve better binding of the substrate 5-hydroxytryptophan [58]. An optimization through promoter engineering and confirms the interaction model can be used which can also lift the rate-limiting bottleneck enzyme. Song Gao, using promoter regulation and directed evolution in the host *Saccharomyces cerevisiae*, established a high-throughput screening method to improve the catalytic efficiency of flavonoid 3'-hydroxylase, so that (2S)-eriodictyol reached the current highest titer of 3.3 g/L [64].

3.2.3. Cofactors

To increase product titers, it is necessary to not only overexpress pathway enzymes, but also boost the supply of cofactors such as BH4 and NADPH. The conversion of L-tryptophan into 5-hydroxytryptophan by TPH requires both oxygen and the cofactor BH4, which is produced from GTP [65]. Germann et al. constructed a strain of *S. cerevisiae* containing heterologous genes and increased the supply of cofactors to achieve a product titer of 14.5 mg/L [18,33]. However, *E. coli* cannot synthesize the cofactor BH4 by itself [47]. Directed metabolic pathway evolution was applied to achieve pterin-dependent hydroxylation of L-tryptophan in *E. coli*. Luo et al. used a minimum set of heterologous enzymes and a host *foIE* (T198I) mutation for achieving pterin-dependent L-tryptophan hydroxylation by directed metabolic pathway evolution. More important, a heterologously introduced BH4 biosynthetic pathway was deleted during adaptive laboratory evolution, the host strain repurposed one of its native cofactors, but it remained unclear exactly which *E. coli* pterin was repurposed as an alternative cofactor [66]. Heterologous expression of enzymes and the enzyme mutation *foIE* (T198I) in *E. coli* can be used to increase the production of GTP and BH4. A mutagenized TPH was expressed in *E. coli*, and 0.8 mM 5-HTP was synthesized after adding BH4 as a substrate. In addition, the BH4 regeneration pathway and glucose dehydrogenase of *Bacillus subtilis* were introduced to increase the utilization rate of BH4, and the production of 5-HTP was increased to 2.5 mM [38,47,66].

3.3. Melatonin synthesis

Melatonin produced by a two-step enzymatic conversion of serotonin. Serotonin is first converted by N-acetyltransferase, which replaces its hydrogen at the C3 position with N-acetyl-2-aminoethyl. Subsequently, it is methylated at hydroxy position [67]. In the pathway of melatonin synthesis from serotonin, ASMT is a rate-limiting enzyme. The biosynthetic pathway of melatonin from serotonin is summarized in Fig. 6.

3.3.1. Arylalkylamine N-acetyltransferase

Arylalkylamine N-acetyltransferase (AANAT/SNAT) is an enzyme of the GNAT (GCN-5 related N-acetyltransferase) superfamily, which is generally considered to be a crucial enzyme for melatonin synthesis in vertebrates [68]. It also converts serotonin to N-acetyl-serotonin during microbial synthesis of melatonin [69].

Increasing the yield of N-acetyl serotonin requires increasing the catalytic activity of the enzyme. Therefore, we need to understand its catalytic mechanism. AANAT has a catalytic funnel combined with reactive groups (amine and AcCoA). The catalytic histidines (His120, His122) at the bottom of the active site form a proton channel, which deprotonates the amino group to initiate the acetyl transfer reaction. The residue corresponding to Ile160 in AANAT of sea bream (*Sparus aurata*) is naturally mutated to methionine, which enhances acetylation and reduces the K_m value [70]. However, some studies reported that the effect of the mutation at position 160 is difficult to characterize because the enzyme has the same residue at that position. At the same time, mutation at position 114 will also cause differences in catalytic performance. Therefore, it was speculated that the catalytic reaction requires by the synergistic effect of the two mutations [71].

Table 1
Melatonin synthesis enzymes from plants and their Km values.

Enzymes	Organism	Km[mM]	Enzymes	Organism	Km[mM]
TDC	Catharanthus roseus	0.075 [84]	SNAT	Homo sapiens	0.06 [85]
	Vinca minor	1.3 [86]		Homo sapiens	0.096 [85]
	Withania coagulans	1.7 [87]		Homo sapiens	0.106 [85]
	Gelatoporia subvermispora	0.16 [88]		Homo sapiens	1.23 [85]
		0.16 [88]		Homo sapiens	1.35 [85]
		0.29 [88]		Ovis aries	0.125 [89]
		1.94 [88]		Ovis aries	0.64 [90]
		1.94 [88]		Rattus norvegicus	1.7 [90]
		0.3 [91]		Rattus norvegicus	2 [92]
		0.0147 [93]		Sparus aurata	0.05 [94]
T5H	Bacillus sp. (in: Bacteria)	0.015 [95]			2.05 [94]
	Homo sapiens	0.0217 [93]			0.16 [96]
		0.0249 [93]			0.23 [97]
		0.0265 [98]			0.42 [97]
		0.0289 [93]			0.232 [69]
		0.0351 [98]			0.371 [99]
		0.0423 [100]			0.467 [101]
		0.0518 [93]			13 [102]
		0.0684 [93]	ASMT	Oncorhynchus tshawytscha	0.005 [103]
		0.077 [104]		Ovibos moschatus	0.015 [105]
		0.315 [98]		Bos taurus	0.0291 [106]
		0.0142 [107]		Bos taurus	0.054 [106]
		0.031 [108]		Rattus norvegicus	0.04 [109]
		0.135 [107]		Rattus norvegicus	0.1 [110]
		0.294 [111]		Oryza sativa	0.243 [112]
		0.324 [113]		Oryza sativa	0.864 [25]
		0.0067 [114]		Arabidopsis thaliana	0.456 [115]
				Arabidopsis thaliana	0.456 [115]

In addition to complex enzyme modification, heterologous expression can also increase substrate conversion. During microbial melatonin synthesis, allogenic expression of enzymes from plants and animals (AANAT/SNAT) can increase the production of *N*-acetyl serotonin and melatonin. Ectopic expression of SNAT from Arabidopsis, alfalfa, tomato or apple leads to increased melatonin production in transgenic tomatoes and Arabidopsis [72,73]. When the heterologous AANAT from *Bos taurus* was introduced into *Saccharomyces cerevisiae*, the highest yield of *N*-acetyl-serotonin reached 9.1 mg/L in shake-flask cultures [18]. Overexpression of ovine AANAT in switchgrass increased the content of melatonin three times compared with the empty vector control [74]. The Km values of animal and plant enzymes are summarized in Tables 1 and 2

3.3.2. Acetylserotonin O-methyltransferase

N-acetyl-serotonin methyltransferase (ASMT/COMT) belongs to the O-methyltransferase family. The ASMT reaction seems to be the main bottleneck in the biosynthesis of melatonin [75]. The catalytic center of the enzyme exhibits different binding affinity for compounds with similar structures, resulting in variation of catalytic efficiency towards different substrates [76]. It can recognize both *N*-acetyl-serotonin and serotonin, and catalyzes the methylation reaction in the core pathway of O-melatonin biosynthesis. However, due to the presence of amide groups in the *N*-acetyl serotonin terminal chain, when the substrate enters the substrate binding pocket of *Arabidopsis thaliana* COMT, strong electrostatic repulsion will occur, preventing *N*-acetyl serotonin from binding to the catalytic pocket [77].

In order to improve the catalytic efficiency of ASMT with *N*-acetyl serotonin, we need to improve the substrate affinity of the enzyme. COMT has only three highly conserved amino acids (Met-230, Asp-233 and Met-386), among which the conserved hydrophobic residues Met-230 and Met-386 are isolated in the benzene ring, and the benzene ring faces the 5th position of the SAM aromatic ring provide the active hydroxyl. Based on notable differences in the terminal structure of caffeic acid and *N*-acetyl serotonin, mutants were designed to strengthen the interactions between the substrate binding pocket of the enzyme and the terminal structure of the unnatural substrate *N*-acetyl serotonin [77, 78]. Rational design not only eliminated electrostatic repulsion

(Gln310Leu), but also introduced an N single bond (Phe296), hydrogen- π interaction and hydrophobic interaction. These changes increased the catalytic efficiency in the conversion of *N*-acetyl serotonin to melatonin. The construction of COMT variant containing C303F and V321T mutations increased the production of melatonin 5-fold [26]. Molecular dynamics simulations and binding free energy analysis resulted in the construction of a triple mutant (C296F-Q310L-V314T) with a 9.5-fold increase of activity [77].

In addition to modifying enzymes, we can also express enzymes from different sources. ASMT/COMT orthologs from different plants and animals exhibit different activities. Compared with the enzymes from animals, ASMT orthologs from plants exhibit lower Km values (Tables 1 and 2) [77,78].

3.3.3. Coenzymes

In the synthesis process of serotonin to melatonin, the cofactors SAM and acetyl-CoA play a significant role. The conversion of serotonin to *N*-acetyl-serotonin requires the participation of acetyl-CoA [79]. In *Saccharomyces cerevisiae*, the supply of acetyl-CoA can be increased by overexpressing acetaldehyde dehydrogenase or by restricting oxygen [80]. However, anaerobic conditions will affect the upstream hydroxylation reactions. Kocharin et al. increased the acetyl-CoA supply by overexpressing acetaldehyde dehydrogenase, which enhanced the productivity of polyhydroxybutyrate approximately 16.5 times in bioreactor cultivations [81]. In addition, SAM is also an important cofactor for the last step of the pathway, in which ASMT methylates *N*-acetyl-serotonin to the final product melatonin. Thus, the conversion to melatonin is accompanied by the concomitant conversion of SAM to *S*-adenosyl-L-homocysteine (SAH). The SAM cycle is native and constitutively expressed in budding yeast [82]. Cofactor regulation also plays a vital role in constructing a highly efficient cell factory [39]. A comparison of the specific productivities of polyhydroxybutyrate producing strains revealed that employing the acetyl-CoA boost plasmid helps redirect carbon fluxes towards the polyhydroxybutyrate pathway [81, 83].

Table 2
Melatonin synthesis enzymes from animals and their Km values.

Enzymes	Organism	Km [mM]	Enzymes	Organism	Km[mM]
TPH	Homo sapiens	0.0075 [116]	TPH	Mus musculus	0.0075 [117]
		0.0078 [118]			0.00873 [119]
		0.0106 [120]			0.0166 [117]
		0.0131 [121]			0.0192 [117]
		0.0132 [122]			0.031–0.033 [111]
		0.015 [123]			0.0865 [111]
		0.017 [124]			0.119 [111]
		0.0185 [116]			0.119 [100]
		0.0186 [52]			0.002 [125]
		0.02 [126]			0.3 [125]
		0.0228 [120]			0.0021 [127]
		0.0228 [123]			0.0058 [107]
		0.023 [126]			0.032
		0.02426 [128]			0.0479 [129]
		0.0255 [52]			0.0077
		0.0282 [124]			0.016 [130]
		0.033 [52]			0.023 [131]
		0.0334 [52]			0.022 [131]
		0.0403 [121]			1.3 [132]
		0.0413 [52]			0.32 [88]
		0.0434 [120]			0.32 [88]
		0.0449 [100]			0.35 [88]
		0.0511 [52]			1.72 [88]
0.0545 [100]					
0.111 [120]					
			TDC	Catharanthus roseus	1.3 [132]
				Gelatoporia subvermispora	0.32 [88]
					0.32 [88]
					0.35 [88]
					1.72 [88]

4. Conclusions

In mammals, melatonin is released from the pineal gland into the third ventricle and from there into circulation. It is involved in regulating the body's sleep-wake cycle through its interactions with the suprachiasmatic nucleus of the hypothalamus and the retina, promoting sleep [133]. In today's stressful society, melatonin is a hot commodity. However, with the increase of annual demand, a green and efficient synthesis method is needed to meet the market demand, and the most promising approach is microbial fermentation. The synthesis pathways of melatonin differ between species. In animals and plants, the first two steps of melatonin synthesis are reversed and the Km of the involved enzymes are significantly different (Tables 1 and 2). Among microorganisms, the synthetic pathways of yeast and *Pseudomonas* are similar to those in animals. *E. coli* requires the expression of heterologous enzymes synthesize melatonin, such as TDC, SNAT, AANAT, ASMT and COMT. We summarized recent advances in the modification of microorganisms to produce melatonin. Therefore, recent and representative publications on the subject using different strategies were selected and

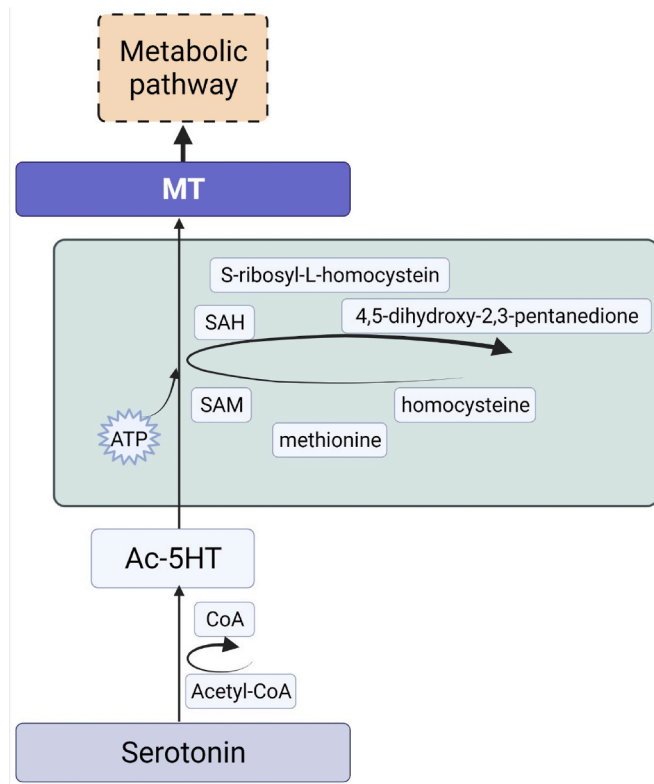


Fig. 6. Synthesis of melatonin from serotonin.

discussed, providing a good reference for future microbial metabolic engineering projects for the biosynthesis of melatonin.

Microbial metabolic networks are complex, and intermediate metabolites participate in many different metabolic modules. If the supply of the precursor L-tryptophan or serotonin is insufficient, there will not be enough impetus to produce melatonin [39]. Therefore, to improve L-tryptophan production, we can enhance the supply of precursors, such as glutamine, L-serine, shikimic acid and phosphoribose pyrophosphate. Feedback inhibition of key enzymes such as DAHP synthase (*aroGFH*) and anthranilate synthase (*trpED*) can be removed [35,134]. For the biosynthesis of melatonin in *E. coli*, TDC is the rate-limiting enzyme. However, TDC generates the by-product tryptamine, and TPH has a low affinity for tryptamine, which leads to the accumulation of a large amount of tryptamine, which affects cell growth [27]. Hence, to increase the precursor supply of serotonin, we can improve the activity of TPH and TDC through site-directed mutation guided by rational design or through directed evolution based on high-throughput screening [51]. To improve TDC, it is necessary to enhance the specific binding between the enzyme and substrate [58]. To improve the ASMT/COMT and SNAT/AANAT enzymes that synthesize melatonin, it is necessary to understand their structure, analyze their catalytic mechanism, identify the key sites and carry out site-directed mutagenesis.

With the rapid development of computer technology, there are endless ways to modify enzymes based on in silico analysis. Some scholars have combined the relevant theories of proteomics and bioinformatics to use protein design software to improve enzyme activity. For example, DeepMind has been developing the AlphaFold system, which system improves the accuracy of protein structure prediction by integrating novel neural network architectures and training programs based on evolutionary, physical, and geometric constraints of protein structure, which could be used as a computer-aided tool for enzyme modification in the future [135]. The development of metabolic engineering has brought greater appreciation of its feasibility for the industrial production of melatonin, and significant progress is expected in the

future.

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Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled “Melatonin biosynthesis pathways in nature and its production in engineered microorganisms”.

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