Combining protein and metabolic engineering strategies for biosynthesis of melatonin in *Escherichia coli*

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Abstract

**Background:** Melatonin has attracted substantial attention because of its excellent prospects for both medical applications and crop improvement. The microbial production of melatonin is a safer and more promising alternative to chemical synthesis approaches. Researchers have failed to produce high yields of melatonin in common heterologous hosts due to either the insolubility or low enzyme activity of proteins encoded by gene clusters related to melatonin biosynthesis.

**Results:** Here, a combinatorial gene pathway for melatonin production was successfully established in *Escherichia coli* by combining the physostigmine biosynthetic genes from *Streptomyces albulus* and gene encoding phenylalanine 4-hydroxylase (P4H) from *Xanthomonas campestris* and caffeic acid 3-O-methyltransferase (COMT) from *Oryza sativa*. A threefold improvement of melatonin production was achieved by balancing the expression of heterologous proteins and adding 3% glycerol. Further protein engineering and metabolic engineering were conducted to improve the conversion of N-acetylserotonin (NAS) to melatonin. Construction of COMT variant containing C303F and V321T mutations increased the production of melatonin by fivefold. Moreover, the deletion of speD gene increased the supply of S-adenosylmethionine (SAM), an indispensable cofactor of COMT, which doubled the yield of melatonin. In the final engineered strain EcMEL8, the production of NAS and melatonin reached 879.38 ± 71.42 mg/L and 136.17 ± 1.33 mg/L in a shake flask. Finally, in a 2-L bioreactor, EcMEL8 produced 1.06 ± 0.07 g/L NAS and 0.65 ± 0.11 g/L melatonin with tryptophan supplementation.

**Conclusions:** This study established a novel combinatorial pathway for melatonin biosynthesis in *E. coli* and provided alternative strategies for improvement of melatonin production.

**Keywords:** Melatonin, N-acetylserotonin, Metabolic engineering, *Streptomyces albulus*, S-adenosylmethionine

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Background

Melatonin is an ancient and ubiquitous molecule [1], widely distributed in almost all taxa of living organisms, including microorganisms, plants, and animals [2]. Melatonin is considered one of nature's most versatile biological signals, and its functions have diverged with organismal diversification [3]. Due to its multiple functions [4], melatonin has shown excellent prospects in both medical applications and crop improvement [5–7]. Melatonin has been extensively used as an over-the-counter drug and a dietary supplement for many years word wide. Currently, commercial melatonin relies on chemical synthesis, which is neither sustainable nor environmentally friendly [8–10]. The microbial production of melatonin is a safer and more promising alternative based on the understanding of the melatonin biosynthetic pathway [9].

The biosynthetic pathway of melatonin was first elucidated in animals [11]. In animals, melatonin is synthesized from tryptophan via the 5-hydroxytryptophan (5HTP), serotonin (5HT), and N-acetylserotonin (NAS) intermediates. Accordingly, four enzymes, including tryptophan-5-hydroxylase (TPH), tryptophan decarboxylase (TDC), serotonin N-acetyltransferase (SNAT), and N-acetylserotonin methyltransferase (ASMT), are involved in the catalytic process [11]. In plants, melatonin biosynthesis also begins with tryptophan and includes four enzymatic steps. However, the process of melatonin biosynthesis in plants differs from that in animals in several aspects [12]. First, the first enzymatic step is tryptophan decarboxylation rather than hydroxylation, as occurs in animals [13, 14]. Second, the subsequent step is the synthesis of 5-HT, which catalyzed by tryptamine 5-hydroxylase (T5H) [15]. Third, in addition to ASMT, caffeic acid O-methyltransferase (COMT), which is absent in animals, is another enzyme involved in the synthesis of melatonin. It is worth mentioning that COMT has a significantly higher catalytic efficiency than ASMT in the conversion of N-acetylserotonin to melatonin. The catalytic efficiency ($V_{max}/K_m$) for COMT activity was 709-fold higher than for ASMT in Arabidopsis thaliana, indicating a pivotal role of COMT in the synthesis of melatonin [16]. Therefore, in plants, a total of six enzymes, namely, TPH, SNAT, ASMT, TDC, T5H, and COMT, are involved in the biosynthetic pathway of melatonin, suggesting the complexity of melatonin biosynthesis [12]. In addition to the classic pathway, an alternate pathway, in which serotonin is first O-methylated and the resulting 5-MT is N-acetylated, was recently proposed, adding further complexity to the pathway of melatonin biosynthesis [12, 17]. Compared with the number of studies in animals and plants, there are few studies on the biosynthetic pathway in microorganisms, although melatonin is believed to have first appeared in bacteria as early as billions of years ago [3, 17]. The enzymes and corresponding genes involved in melatonin biosynthesis in microorganisms have remained almost unknown.

Therefore, almost all the genes necessary to establish the melatonin biosynthetic pathway in genetically engineered bacteria were cloned from animals and plants [18–20]. Germann et al. [19] established a de novo melatonin biosynthetic pathway in recombinant yeast. In that work, the genes encoding TPH were from either Homo sapiens or Schistosoma mansoni. Moreover, the genes encoding TDC and ASMT were cloned from H. sapiens, and the gene encoding SNAT was cloned from Bos taurus. Recently, Luo et al. established a recombinant melatonin biosynthetic pathway in E. coli. The genes encoding TPH and ASMT were cloned from H. sapiens and the genes encoding TDC and SNAT were cloned from Candidatus Koribacter versatilis and Streptomyces griseofuscus respectively [21]. In other attempts to produce melatonin in E. coli, genes encoding SNAT and ASMT were cloned from several animals and plants. When these enzymes derived from mammals and plants are heterologous expressed in E. coli, the expression level is extremely low or the expression product is inactive, which limits the high production of melatonin in prokaryotic cells [20, 22].

To date, the gene cluster involved in melatonin biosynthesis in microorganisms has not been identified. However, a gene cluster responsible for physostigmine biosynthesis in Streptomyces harbors the first three genes that encode enzymes to produce NAS, which is the immediate precursor of melatonin [23]. Physostigmine, a tryptophan-derived heterocyclic alkaloid is first discovered in the seeds of West African beans. As a potent acetylcholine inhibitor, physostigmine is widely used to treat glaucoma and Alzheimer’s disease but there are few studies regarding its in-situ function and biosynthesis pathway [24, 25]. After large scale-up screening, the researchers discovered that submerged cultivation of the actinomyces Streptomyces griseofuscus and Streptomyces pseudogriseolus can produce physostigmine [25]. In 2014, Liu et al. [23] identified the gene cluster psmA-H responsible for the biosynthesis of physostigmine in Streptomyces griseofuscus. Of these, PsmH and PsmF are required for the NAS biosynthesis starting from 5-HTP, which is shared with the melatonin pathway. Based on the physostigmine biosynthetic pathway, in this paper, a novel biosynthetic pathway for melatonin production was established in E. coli. In addition, metabolic engineering and enzyme engineering strategies were employed to optimize the biosynthetic pathway and the production of melatonin was effectively improved by 11-fold compared to the first generation strain.
Materials and methods

Bacterial strains and media

All bacterial strains used in this study are listed in Table 1. E. coli Trans1-T1 (TransGen, Beijing, China) was used as the host strain for plasmid construction and propagation. E. coli BW25113ΔtnaA was used for protein expression and in vivo hydroxylation of L-tryptophan to melatonin. For efficient biosynthesis of melatonin, expression and in vivo hydroxylation of L-tryptophan were used as the host strain for plasmid construction and propagation. (per liter) 10 g of glucose, 8 g of (NH4)2HPO4, 13.3 g of citrate dihydrate. The fed-batch medium contained 7H2O, 1 g of CuSO4·7H2O, 14.7 mg/L CaCl2·2H2O, 27.8 mg/L FeSO4·7H2O, and 2 g/L sodium citrate dihydrate. The fed-batch medium contained (per liter) 10 g of glucose, 8 g of (NH4)2HPO4, 13.3 g of KH2PO4, 1.2 g of MgSO4·7H2O, 1.7 g of citric acid, and 10 mL of a trace metal solution that contained (per liter of 5 M HCl) 10 g of FeSO4·7H2O, 2.25 g of ZnSO4·7H2O, 1 g of CuSO4·5H2O, 0.5 g of MnSO4·5H2O, 0.23 g of Na2B4O7·10H2O, 2 g of CaCl2·2H2O, and 0.1 g of (NH4)6Mo7O24. When necessary, the medium was supplemented with 50 μg/mL kanamycin, 100μg/mL ampicillin, and 17 μg/mL chloramphenicol.

DNA manipulation

All plasmids used in this study are listed in Table 1 and all primers used in this study for PCR are listed in Additional file 1: Table S1. XcP4H (NCBI Reference Sequence: WP_011035413.1), TIP4H (NCBI Reference Sequence: WP_028835852.1), HeP4H (NCBI Reference Sequence: WP_013332010.1), phhB (GenBank: RMS5283.1), folM (GenBank: EFM206776.1), SaPsmH (GenBank: A1A00687.1) and SaPsmF (NCBI Reference Sequence: WP_020929557.1) genes were cloned from the corresponding strains. SaP4H (W199F mutant) (NCBI Reference Sequence: WP_016572394.1), Ctp4H (W239F mutant) (NCBI Reference Sequence: WP_012354318.1), SaCOMT (NCBI Reference Sequence: WP_016577150.1) and OsCOMT (NCBI Reference Sequence: XP_015650053.1) genes were all codon optimized and synthesized by Generay (Shanghai, China). All plasmids were assembled by Gibson assembly method using the ClonExpress MultiS One Step Cloning Kit. To replace the promoters of the Mtn, luxS, metF, and metK genes, PCR was performed to obtain the upstream region, the kan resistance gene containing FRT at both ends, the promoter and RBS and the beginning of the gene’s ORF as the downstream region. Then, the PCR products described above were assembled to obtain the target genes. According to the λ-red recombination protocol, various E. coli BW25113ΔtnaA derivatives were constructed. Knockout of dcm and speD genes were performed by ligating the upstream region, the kan resistance gene containing FRT at both ends and the downstream region in order.

Production of melatonin in E. coli BW25113ΔtnaA

The melatonin-producing strains were cultured in 100 mL LB media (500-mL shaking flask) at 37 °C and 200 rpm until the OD600 reached 0.6. The enzymes were induced with 0.1% l-arabinose. After incubation at 30 °C and 200 rpm for 8 h, 200 OD cells were harvested by centrifugation at 4 °C and 5000×g for 10 min. This induced bacterial pellet was suspended into 20 mL M9Y media (50-mL flask) containing 2 g/L tryptophan, 4 g/L methionine (and 3% glycerin when necessary) and grown at 30 °C and 200 rpm. Samples were collected at 3 h, 6 h, 12 h, 24 h, 48 h, 72 h, and 96 h, and the concentrations of 5-HTP, NAS and melatonin were analyzed by HPLC.

Production of 5-HTP in E. coli BW25113ΔtnaA

E. coli BW25113ΔtnaA was transformed with the pBAD-Sa5HTP, pBAD-Xc5HTP, pBAD-Tf5HTP, pBAD-He5HTP and pBAD-Ct5HTP plasmids. The culture methods were the same as described above (production of melatonin), and 200 OD-induced cultures were harvested and suspended into 20 mL of M9Y medium containing 2 g/L tryptophan and grown at 30 °C and 200 rpm. Samples were collected at the time points described above and analyzed by HPLC.

Fed-batch fermentation and optimization

A single colony of Ec-MEL8 from a cell plate was inoculated into 5 mL LB media and cultured at 37 °C and 200 rpm overnight. Then, 1% (v/v) of the culture was transferred into 100 mL LB media (500-mL flask) and
| Strains and plasmids | Relevant characteristics | Source |
|---------------------|-------------------------|--------|
| **Strains**         |                         |        |
| E. coli Trans1-T1   | F-g801lacZDm15ΔlacX74hsdR (rk-, mk+) ΔrecA1398endA1tonA | Transgene company |
| E. coli BW25113     |                         |        |
| E. coli BW25113ΔtnaA | wild type ΔtnaA         | Laboratory storage |
| EcSc5HTP BW25113ΔtnaA | harboring pBAD-Sa5HTP  | This work |
| EcXc5HTP BW25113ΔtnaA | harboring pBAD-Xc5HTP  | This work |
| EcHe5HTP BW25113ΔtnaA | harboring pBAD-He5HTP  | This work |
| EcTs5HTP BW25113ΔtnaA | harboring pBAD-Ts5HTP  | This work |
| EcCsHTP BW25113ΔtnaA | harboring pBAD-CsHTP  | This work |
| EcSaCOMT BW25113ΔtnaA | harboring pBAD-SaCOMT | This work |
| EcOsCOMT BW25113ΔtnaA | harboring pBAD-OsCOMT | This work |
| EcMEL1 BW25113ΔtnaA | harboring pBAD-MEL1    | This work |
| EcMEL2 BW25113ΔtnaA | harboring pBAD-MEL2    | This work |
| EcMEL3 BW25113ΔtnaA | harboring pBAD-Xc5HTP and pZS-MEL1 | This work |
| EcMEL4 BW25113ΔtnaA | harboring pBAD-Xc5HTP and pZS-MEL2 | This work |
| EcMEL5 BW25113ΔtnaA | harboring pZS-MEL1    | This work |
| EcMEL5 BW25113ΔtnaA | harboring pBAD-SHTP7COMTsa and pZS-PsmHF | This work |
| EcMEL6 BW25113ΔtnaA | harboring pBAD-SHTP7COMTos and pZS-PsmHF | This work |
| EcMEL7 BW25113ΔtnaA | harboring pBAD-XcP4H-OsCOMT and pZS-SaPsmHF-phhBfolM | This work |
| EcMEL7-1 | EcMEL7 + over expression of metk | This work |
| EcMEL7-2 | EcMEL7 + over expression of mtn | This work |
| EcMEL7-3 | EcMEL7 + over expression of luxS | This work |
| EcMEL7-4 | EcMEL7 + over expression of mtn and luxS | This work |
| EcMEL7-5 | EcMEL7ΔspeD | This work |
| EcMEL7-6 | EcMEL7Δdcm | This work |
| EcMEL8 BW25113ΔtnaAΔspeD | harboring pBAD-XcP4H-OsCOMT2 and pZS-SaPsmHF-phhBfolM | This work |
| EcMELCXPM BW25113ΔtnaAΔspeD | harboring pBAD-OsCOMT2-XcP4H-PhhB-FolM and pZS-SaPsmHF | This work |
| EcMELCX BW25113ΔtnaAΔspeD | harboring pBAD-OsCOMT2-XcP4H and pZS-SaPsmHF-PhhB-FolM | This work |
| EcMELCS BW25113ΔtnaAΔspeD | harboring pBAD-OsCOMT2 and pZS-XcP4H-SaPsmHF-PhhB-FolM | This work |
| EcMELCXPM BW25113ΔtnaAΔspeD (DE3) | harboring pBAD-P7OsCOMT2 and pZS-XcP4H-SaPsmHF-PhhB-FolM | This work |
| EcMELCXPM | EcMELCXPM | This work |
| EcMELCXPM | EcMELCXPM | This work |
| EcMELCXPM | EcMELCXPM | This work |
| **Plasmids**        |                         |        |
| pKD46 | AmpR, λ-Red recombinase expression plasmid, ara-inducible expression, temperature sensitive replication | Laboratory storage |
| pKD13 | KanR, ori plasmid containing an FRT-aph-FRT cassette | Laboratory storage |
| pCP20 | AmpR, CmR, repA(Ts), pSC101 based vector expressing the yeast Flp recombinase | Laboratory storage |
| pBAD/HisA | AmpR, pBR322 origin, araBAD promoter, araC gene | Laboratory storage |
| pZS | CmR, p15A origin, pBAD based vector expressing recombinase | Laboratory storage |
| pBAD-Sa5HTP | pBAD containing SaP4H, phhB, FoM genes | This work |
| pBAD-Xc5HTP | pBAD containing XcP4H, phhB, FoM genes | This work |
| pBAD-He5HTP | pBAD containing HeP4H, phhB, FoM genes | This work |
| pBAD-Ts5HTP | pBAD containing TsP4H, phhB, FoM genes | This work |
| pBAD-CsHTP | pBAD containing CsP4H, phhB, FoM genes | This work |
| pBAD-SaCOMT | pBAD containing SaCOMT | This work |
| pBAD-OsCOMT | pBAD containing OsCOMT | This work |
| pBAD-MEL1 | pBAD containing SaP4H, phhB, FoM, SaPsmH, SaPsmF, SaCOMT | This work |
| pBAD-MEL2 | pBAD containing SaP4H, phhB, FoM, SaPsmH, SaPsmF, OsCOMT | This work |
| pZS-MEL1 | pZS containing SaPsmH, SaPsmF, SaCOMT | This work |
grown at 37 °C and 200 rpm for approximately 8–10 h. 5% (v/v) of the cultures were transferred to a 2-L bioreactor (BXBIO, Shanghai, China) with 1 L fed-batch media. The pH was controlled at 6.8 by automatic feeding of 30% (v/v) NH₄OH, and the temperature was set at 37 °C. The dissolved oxygen concentration was maintained above 20% air saturation by supplying air at 1 vvm (air volume/working volume/minute) and by automatically controlling the agitation speed up to 700 rpm. When the initial 10 g/L glucose was consumed, a feeding solution containing 500 g of glucose and 10 g of MgSO₄·7H₂O per liter was periodically added. When the OD₆₀₀ reached 20, the culture was induced by adding L-arabinose to a final concentration of 1 g/L, the temperature was set at 30 °C, and a solution of 4 g/L tryptophan, 6 g/L methionine and 3% (v/v) glycerol was added to the media. When the OD₆₀₀ reached 20, the culture was induced by adding L-arabinose to a final concentration of 1 g/L, the temperature was set at 30 °C, and a solution of 4 g/L tryptophan, 6 g/L methionine and 3% (v/v) glycerol was added to the media.

HPLC analysis and LC/MS

L-tryptophan from Sigma (St. Louis, USA), 5-HTP from Aladdin (Shanghai, China), NAS from Sigma and melatonin from Aladdin (Shanghai, China) were used as the standards. The standards and the supernatant concentration of tryptophan, 5-HTP and NAS were measured after dilution of the samples with methanol/water (15:85 v/v). For analysis of melatonin, standard and supernatant of cultures were diluted to a final concentration of methanol/water (40:60 v/v). The samples were filtered through a 0.22-μm nylon filter and then analyzed by HPLC (Agilent 1260 series, Hewlett-Packard) using an Agilent ZORBAX Eclipse Plus C18 column (4.6 × 100 mm, 3.5-Micron). Tryptophan, 5-HTP and NAS were quantified under 275 nm UV detection with methanol/water (15:85 v/v) as the mobile phase. Melatonin was quantified under 290 nm UV detection with methanol/water (40:60 v/v) as the mobile phase. NAS and melatonin were identified by QTRAP 6500 and AB SCIEX using Gemini 3 µm NX-C18 110 Å (50 × 2 mm) with 95% methanol (containing 0.1% formic acid) and 5% water (containing 0.1% formic acid) (v/v).

Results and discussion

Conceptual design of the melatonin biosynthetic pathway by virtue of the physostigmine pathway

When searching tryptophan derivatives in prokaryotes, we found three intermediates of physostigmine biosynthesis (5-HTP, 5-HT, and NAS) in Streptomyces albulus are shared by melatonin biosynthesis. Enlightened by the physostigmine biosynthetic pathway, a novel biosynthetic pathway for melatonin production was designed (Fig. 1). In the first catalytic step of physostigmine biosynthesis, the production of 5-HTP was proposed to be catalyzed by TPH. However, the gene encoding TPH was not included in the physostigmine biosynthesis gene cluster and was not specifically identified in the report. In animals, TPH and phenylalanine 4-hydroxylase (P4H) are two subgroups of aromatic amino acid hydroxylases (AAAHs) that share high sequence similarity. BLASTP analysis of the TPH homolog in the S. albulus genome revealed a putative AAAH, which has typical characteristics of P4H (SaP4H). It was reported that some bacterial P4Hs exhibit activity towards both phenylalanine and tryptophan. Moreover, the substitution of a small
number of residues (e.g., W179F substitution) causes the preferred substrate of P4H to change from phenylalanine to tryptophan [22]. Therefore, SaP4H was used to establish the 5-HTP biosynthetic pathway. Alternative P4Hs from other bacteria, including Xanthomonas campes -
tris [22], Halomonas elongata, Thermomonas fusca, and Cupriavidus taiwanensis [27], were also selected to catalyze the first step of melatonin biosynthesis. The genes encoding homologs of PsmH and PsmF were selected from S. albulus to establish the second and third catalytic steps of melatonin biosynthesis. For the final step of melatonin biosynthesis, two kinds of enzymes: ASMT and COMT have been identified to exhibit the catalytic activity. ASMT, as a methyltransferase initially identified in animals and plants, had low enzyme activities, indicating its role as a rate-limiting enzyme in melatonin synthesis [28]. Besides, ASMT was noncompetitively inhibited by NAS and melatonin and competitively inhibited by its product S-adenosylhomocysteine (SAH) [29, 30]. Compared with ASMT, COMT, which is a methyltransferase found in plants [31], also O-methylates NAS to melatonin with a much higher catalytic activity (100 times) than that of ASMT [20, 32]. Therefore, COMT from Oryza sativa (OsCOMT) was the preferred enzyme, and this enzyme exhibits the highest activity for converting NAS to melatonin as ever reported [12]. In S. albulus, BLASTP analysis revealed a putative gene encoding COMT, which shared the highest sequence identity with OsCOMT. The resulting SaCOMT and OsCOMT were then selected to establish the final catalytic step of melatonin biosynthesis.

Above all, we designed a new melatonin biosynthetic pathway in E. coli based on the physostigmine biosynthetic pathway. All four proteins from S. albulus, namely, SaP4H, SaCOMT, SaPsmF and SaPsmH, were expressed well as soluble recombinant proteins in E. coli. Among these genes, the genes encoding SaP4H and SaCOMT were optimized according to the E. coli codon preference, while the genes encoding SaPsmF and SaPsmH were not optimized. The 5-HTP biosynthetic module and COMT were involved in the first and final steps of melatonin biosynthesis. According to previous reports, these two steps were proposed to be the rate-limiting of melatonin production and were tested first [9, 20].

**Engineering the biosynthetic module to produce 5-HTP in E. coli**

The 5-HTP biosynthetic module was engineered in three ways (Fig. 2a). First, the W179F substitution was discovered to change the preferred substrate of P4H from phenylalanine to tryptophan and hence to enhance its TPH activity. Thus, the W179F substitution was introduced into various P4Hs (Fig. 2b), and the resulting genes encoding the W179F mutant were cloned into the pBAD vector under the control of the arabinose-inducible araBAD promoter. Second, a tetrahydromonapterin (MH4) recycling system was constructed to provide cofactor to P4Hs. The genes encoding PhhB from P. aeruginosa and FolM from E. coli were cloned and inserted downstream of the genes encoding the W179F mutants of P4Hs in the pBAD plasmid (Fig. 2a). The resulting plasmids were named pBAD-Sa5HTP, pBAD-Xc5HTP, pBAD-Tt5HTP, pBAD-He5HTP and pBAD-Ct5HTP. Third, the E. coli mutant strain (abbreviated EcΔtnaA), in which the tnaA gene encoding tryptophanase was deleted to block the degradation of tryptophan and 5-HTP, was generated (Fig. 2a). The pBAD-5HTP plasmids were transformed into the EcΔtnaA strain, and all the recombinant proteins were well expressed (Additional file 1: Fig. S1a). The whole-cell biocatalysis of tryptophan to 5-HTP in these strains at different intervals was analyzed and compared. As shown in Fig. 2c, all the strains could produce 5-HTP after the addition of tryptophan, confirming the successful establishment of the 5-HTP biosynthesis module. However, the strain harboring the putative SaP4H from S. albulus displayed the lowest level of 5-HTP production. It seemed that the putative P4H-W199F of S. albulus might exhibit lower catalytic activity toward tryptophan. It is also possible that other unknown tryptophan hydroxylases were involved in the conversion...
of tryptophan to 5-HP during physostigmine biosynthesis. The XcP4H-W179F exhibited superior catalytic activity toward tryptophan (Fig. 2c), which is in accordance with a previous report. It produced ~500 mg/L 5-HTP in the shake flask with tryptophan supplied. Thus, XcP4H-W179F was used as the 5-HTP biosynthetic module in subsequent pathway engineering.

**COMT overexpression enabled melatonin production from NAS**

Two genes encoding COMT from *S. albulus* and *O. sativa* were singly cloned into the pBAD vector under the control of the araBAD promoter. The resulting pBAD-SaCOMT and pBAD-OsCOMT plasmids were transformed into the strain EcΔtnaA, resulting in EcSaCOMT and EcOsCOMT, respectively, and the soluble proteins were obtained (Additional file 1: Fig. S1b). By adding NAS and methionine, the capabilities of the two COMTs in the biocatalysis of NAS to melatonin were tested. As shown in Fig. 3, both COMTs facilitated the conversion of NAS to melatonin, suggesting that both COMTs could be used in engineering a pathway for melatonin production. The strain harboring OsCOMT produced significantly higher levels of melatonin than the strain harboring SaCOMT, and SDS-PAGE analysis showed that the expression of OsCOMT was much higher than that of SaCOMT. Two COMT genes were coupled with other intermediate product synthesis genes to be constructed on the co-expression plasmid to verify their ability to increase the production of melatonin. As far as we know, this was the first reported COMT protein identified in microorganisms, which has expanded the COMT candidate gene pool involved in melatonin biosynthesis. Combined with SaP4H, SaPsmH and SaPsmF, a putative pathway of melatonin synthesis in *S. albulus* was identified.

**Melatonin production pathway engineering**

Based on the 5-HTP biosynthetic module and the COMT from *S. albulus* or *O. sativa*, a complete melatonin production pathway was constructed (Additional file 1: Fig. S2). With all the genes responsible for melatonin biosynthesis expressed from a single pBAD plasmid in the EcΔtnaA strain, the recombinant strains EcMEL1 and EcMEL2 (with either SaCOMT or OsCOMT) were
assembled. Besides, another two strains, namely EcMEL3 and EcMEL4, were made by placing the genes encoding SaPsmH, SaPsmF and COMT on another plasmid (pZS), with the 5-HTP biosynthetic module on the pBAD plasmid. Unexpectedly, melatonin was not produced in all these four strains, while significant NAS accumulation was observed (Fig. 4a). Results of SDS-PAGE (Additional file 1: Fig. S3) showed that the proteins necessary for the synthesis of melatonin were well expressed, except for the last enzyme, COMT, which catalyzed NAS to melatonin. These results indicated that the weak or absent expression of COMT might restricted the production of melatonin. To improve the expression of COMT in the recombinant strains, a series of plasmid modifications were carried out (Additional file 1: Fig S2C–G). When we moved COMT from pZS plasmid to pBAD plasmid, a clear OsCOMT band was observed and a total of 12.29 ± 0.13 mg/L melatonin was produced by Ec-MEL6 in flasks, while no obvious melatonin or SaCOMT was produced by EcMEL5 (Fig. 4a, Additional file 1: Fig S4a). Thus, OsCOMT is more superior for the bioconversion of NAS to melatonin than SaCOMT in this system. Whereas the synthesis of melatonin was still blocked at the step catalyzed by COMT. During the whole-cell biocatalytic, the production of NAS in EcMEL6 was nearly 200 mg/L, which was about 15-fold of melatonin. This indicated that COMT expression is of vital importance for melatonin production.

In order to enhance the expression of OsCOMT and achieved higher melatonin yields, the next melatonin-producing strain, EcMEL7, was made by placing the gene encoding OsCOMT immediately downstream of the XcP4H. Results showed that the production of melatonin by the EcMEL7 strain was increased to 18.64 ± 0.46 mg/L (Fig. 4a). SDS-PAGE analysis confirmed that the expression of OsCOMT was enhanced in the EcMEL7 cells (Additional file 1: Fig S4b). To further increase the expression of OsCOMT, we also shifted OsCOMT to the front of XP4H or put OsCOMT singly on the pBAD plasmid (Additional file 1: Fig S2J). However, when OsCOMT was shifted to the upstream of XP4H, the recombinant strains did not show an increased in COMT expression but had a significantly decreased soluble expression of XP4H (Additional file 1: Figure S4c), which caused the

![Fig. 4](image-url)
synthesis of melatonin blocked in the first step. When putting OsCOMT on the pBAD plasmid alone, and even replacing Para with a stronger promoter such as P_{T7} or P_{prot}, the expression of COMT and the production of melatonin were not increased compared to that of EcMEL7 (Additional file 1: Figure S5a, b). It seemed that the expression of COMT has reached its limit in the current six enzymes co-expression system. Even when the gene elements involved in the COMT expression were enhanced, a stronger expression would not be achieved due to the limitation of resources related to transcription and translation.

Improved production of melatonin by Cofactor engineering and protein engineering

After the establishment of the complete melatonin biosynthetic pathway, cofactor engineering and protein engineering were conducted to further enhance melatonin production. First, cofactor engineering was conducted to increase the supply of acetyl-CoA and SAM, resulting in a significant increase in melatonin production. Acetyl-CoA is an important cofactor of PsmF that is involved in the biosynthesis of NAS from 5-HT. HPLC analysis of the supernatant of EcMEL7 showed a large amount of 5-HT accumulation (data not shown). The low conversion efficiency of 5-HT to NAS might indicate the lack of acetyl-CoA. Glucose, acetate and glycerol were reported to be able to provide energy and the acetate group for acetyltransferase in _E. coli_ [33, 34]. 10 g/L glucose, 50 mM acetate or 5% glycerol was added to M9 medium and the whole-cell biocatalytic efficiency of EcMEL7 was evaluated. Results showed that glycerol effectively increased the yield of NAS compared with that of glucose and acetate (Additional file 1: Fig S6). As shown in Additional file 1: Fig S7, the level of Acetyl Coenzyme A in the supernatant was increased by 55% after adding glycerol, which confirmed that glycerol augmented the PsmF activity by facilitating the availability of Acetyl Coenzyme A cofactor. Therefore, different concentrations of glycerol were tested in the whole-cell biocatalysis of melatonin from tryptophan. The highest production of NAS was 569.82 ± 16.88 mg/L in the shake flask (Fig. 4b). Moreover, the addition of glycerol also significantly increased the production of melatonin (Fig. 4b). The increase in the production of melatonin was glycerol concentration-dependent. The highest level of melatonin was achieved: 35.13 ± 0.66 mg/L (Fig. 4b) when 3% glycerol was added into the whole-cell catalysis system. Thus, subsequent optimization experiments all added 3% glycerol.

Radical S-adenosylmethionine (SAM), the other important cofactor, plays a key role in the process of COMT catalysis. SAM is a common methyl donor in vivo that participates in the rate-limiting steps of the biosynthesis of multiple compounds and undergoes many genome modifications to remove inhibition [35]. Research on the enzyme activity of COMT in plants showed that it was positively correlated with the SAM/SAH ratio [35–37]. To test whether increased SAM levels and decreased SAH accumulation could improve the potential of COMT and hence enhance the production of melatonin in _E. coli_, a series of genes related to SAM recycling were deleted or overexpressed (Fig. 5a). Among the deleted and overexpressed genes, deletion of the _speD_ gene increased the production of melatonin by two-fold (Fig. 5c). The SpeD catalyzed decarboxylation of SAM to produce dSAM: one of the precursors involving spermidine synthesis. Deletion of _speD_ gene reduced the consumption of SAM and provided more methyl donors to enhance melatonin synthesis. In addition, _speD_ knockout has no significant effect on the growth of BW25113ΔtnaA (Additional file 1: Fig S8). The modification of _mtn_, _luxS_, and _dcm_ increased the production of NAS, but does not caused significant differences (p > 0.05) in melatonin production (Fig. 5c).

Wang et al. reported that amino acid substitutions near the NAS-binding pocket (C296F, Q310L, and V314T) significantly enhanced the catalytic activity of COMT from _Arabidopsis thaliana_ (AtCOMT) [38]. The corresponding residues of C296 and V314 are conserved in OsCOMT, while Q310 is not (Fig. 5b). Thus, the corresponding substitutions of C296F and V314T were introduced to OsCOMT, yielding OsCOMT2. OsCOMT2 increased melatonin production to 122.83 ± 4.44 mg/L, which was fivefold higher than the melatonin production with OsCOMT (Fig. 5c). By combining the mutation of OsCOMT and the deletion of _speD_, the EcMEL8 strain was obtained. EcMEL8 produced 136.17 ± 1.33 mg/L melatonin and 879 ± 71.42 mg/L NAS in a shake flask (Fig. 5c). Figure 5d showed the HPLC analysis of the EcMEL8 fermentation supernatant, and the target products were confirmed by LC–MS (Fig. 5e). The results showed that almost all the consumed tryptophan (4.94 mM) was converted into NAS and melatonin (4.12 mM + 0.56 mM), and other tryptophan derivatives were negligible. As reported by Luo et al., the engineered strain produced high levels of byproduct AcTRPM when achieved high yields of melatonin in the fed-batch fermentation process with tryptophan as substrate, due to the TRPM-forming activity of the TDC (tryptophan decarboxylase) [21]. However, the PsmH and PsmF proteins from the physostigmine pathway were very specific to their substrates [23], and the EcMEL8 strain did not produce obvious byproducts. Compared to the EcMEL7 strain, the yield of melatonin produced by EcMEL8 was effectively improved and the ratio of NAS/melatonin
Fig. 5  Cofactor engineering and protein engineering for high-level production of melatonin. a Metabolic engineering for cofactor SAM of COMT. The red arrow indicates the enhanced expression of the gene, and the blue cross indicates the knockout of the gene. b Alignment of the OsCOMT and AtCOMT protein sequences. c Conversion of tryptophan to NAS and melatonin by recombinant E. coli strains. d HPLC analysis of the standard and bioconversion products of the representative EcMEL8 strain. e LC–ESI–MS analysis of symbols from panel d: exact mass of compound 1[M+H]+[m/z](219.1), compound 2[M+H]+[m/z](233.0). Data are the means ± standard deviations of triplicate experiments.
production was reduced, suggested that the bioconversion of NAS to melatonin was successfully enhanced. Nevertheless, the fivefold higher level of NAS than melatonin in EcMET8 indicated that COMT was still the rate-limiting step for the production of melatonin in this study.

**High-level melatonin production in a fermenter**

Whole-cell bioconversion of tryptophan to melatonin in EcMET8 was also performed in a 2-L fermenter with a higher cell density ($OD_{600} = 38$). Melatonin yield reached its highest level in the medium of $0.32 \pm 0.03$ g/L at 71 h with the synthesis efficiency of melatonin produced via per g of dry cell weight (DCW) at 35 mg/g DCW. NAS production continued to increase even after 83 h and reached $3.0 \pm 0.26$ g/L (Fig. 6a). Thus, both melatonin and NAS production were significantly higher in this system than in shake flasks. The cell transformation efficiency was reduced compared with that of the shake flask. This may be due to the limitations of the fermentation process. After the optimization of fermentation conditions, Fig. 6b showed that the accumulation of NAS decreased to $1.06 \pm 0.07$ g/L, and the melatonin production finally reached $0.65 \pm 0.11$ g/L at 180 h. The higher OD value, as expected, brought a higher production of melatonin. Under the optimized fermentation conditions, the melatonin synthesis efficiency of EcMEL8 reached 59 mg/g DCW, which was even higher than the strain reported by Luo et al. However, the limitation of the final step of melatonin synthesis in EcMEL8 was not completely eliminated: high levels of NAS accumulated in the fermenter. Subsequent further work should focus on removing the problem of NAS accumulation to improve melatonin production.

**Conclusions**

In this study, the melatonin synthesis pathway in *Streptomyces albicans* was identified, and a new melatonin biosynthesis pathway in *Escherichia coli* was successfully constructed on this basis. By balancing heterologous proteins expression elements, optimizing cofactors supplementation, and modifying the rate-limiting enzyme COMT, the production of melatonin was increased by 11-fold. In this study, the gene resource library of the melatonin synthesis pathway was extended to prokaryotic genes, which provided a fast and excellent synthesis pathway for the production of tryptophan derivatives in genetically engineered strains.

**Supplementary Information**

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Additional file 1. Supplementary Fig. 1. (a), SDS-PAGE analysis of pBAD-5HTPs. The red arrow indicates the expression of P4Hs. (b), SDS-PAGE analysis of EcSaCOMT and EcOsCOMT. Supplementary Fig. 2. Construction of plasmids for expression of melatonin-related proteins. Supplementary Fig 3. SDS-PAGE analysis of EcMEL1, EcMEL2, EcMEL3, and EcMEL4. Supplementary Fig 4. SDS-PAGE analysis of EcMEL5, EcMEL6, EcMEL7, EcMELCX and EcMELCXPM. Supplementary Fig. 5. Comparison of the melatonin production by EcMEL7, EcMELCS, EcMEL-CtacS and EcMEL-CT7S. Supplementary Fig. 6. Comparison of adding glucose, acetate and glycerol in M9Y medium for biosynthesis of NAS and melatonin. Supplementary Fig. 7. The concentration of Acetyl Coenzyme A in EcMEL7 and EcMEL7 +5% glycerol during the whole-cell biocatalysis. Supplementary Fig 8. The grow curve of BW25113 ΔtnaA and BW25113 ΔtnaA ΔspeD. Table S1. Primers used in DNA manipulation. Table S2. The OD600 of Melatonin Producing Strains. Table S3. The OD600 of Melatonin Producing Strains after adding glucose, glycerol, and acetate.
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Authors’ contributions
YFZ and SZ conceived the project and designed the experiments. YFZ, YZH, NZ and JJI performed the experiments. SZ, YFZ and YZH analyzed the data and wrote the manuscript. ZYD provided resources and revised the manuscript. All authors read and approved the final manuscript.

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Declarations
Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
This work has been included in patent applications by the Institute of Microbiology, Chinese Academy of Sciences.

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