Commentary

Biomanufacturing of melatonin is now possible

Lei Yang[1*], Bernhard O Palsson[1,2*]

[1]Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, Kemitorvet, Building 220, 2800 Kgs. Lyngby, Denmark
[2]Department of Bioengineering, University of California, San Diego, La Jolla, CA 92093, USA
*Correspondence: leiya@biosustain.dtu.dk or palsson@ucsd.edu, Tel: +45 3196 4881

Running title: Biomanufactured melatonin

Received: January 5, 2023; Accepted: February 11, 2023

ABSTRACT

Currently, the majority of the melatonin manufactured is chemically synthesized replacing the previous method that extracted it from biological materials. Due to environmental concerns, biomanufacturing of chemical-related products is now gaining favor over chemical synthesis. Melatonin is synthesized from tryptophan in a four-step pathway that includes a hydroxylase and methyl transferase, both representing major challenges for metabolic engineering. Using novel technologies, including genome editing and laboratory evolution, the metabolic pathway of melatonin synthesis has been reconstructed in a microbial host which has the capacity to produce melatonin in gram-level quantities.

Key words: Melatonin, 5-hydroxytryptophan, serotonin, biomanufacture, biosynthesis, metabolic pathway, fermentation, scale-up.

1. INTRODUCTION

Melatonin is an indoleamine naturally produced by the pineal gland in vertebrates including humans to regulate the circadian rhythm. It is best known as a sleep aid, but its applications have been expanded to other areas including beverages (1, 2), cosmetics (3), horticulture (4), AgBios (5), and antioxidant and anti-aging (6, 7). Since melatonin exhibits antiviral activity which can be used for COVID-19 treatment, this drove up its sale in the US market to reach $1.26 billion in 2021 (8). This tendency will likely continue, judging from the increased awareness of its health benefits (9–12). The rapidly increasing melatonin demand requires an environmentally-friendly process to replace the chemically synthesized melatonin which inevitably generates some environmental biohazards (4). Therefore, the biomanufacturing of melatonin using microbial cell factories and renewable feedstocks will eventually become the dominant process to synthesize melatonin.

2. MELATONIN PATHWAY ENGINEERING

Melatonin is biosynthesized from tryptophan by four consecutive steps: 1) hydroxylation of tryptophan to form 5-hydroxytryptophan (5-HTP), 2) decarboxylation to produce 5-hydroxytryptamine (5-HT, or serotonin), 3) acetylation to form acetyl-5-hydroxytryptamine (AcHT), and 4) methylation to generate melatonin (Figure 1). Achieving functional expression of these enzymes and maintaining a supply of the substrates in microbial hosts are among the current challenges.

Microbial production of the intermediates of the melatonin pathway has been reported, including 5-HTP and serotonin (13–15). Production of melatonin using microbial cell factories was first demonstrated in *Saccharomyces cerevisiae*, reporting a titer of 14 mg/L use glucose as the initial substrate (16). Later, an *Escherichia coli* melatonin production host was constructed with the melatonin yield up to 1g/L in fed batch fermentation co-feed with glucose and tryptophan (17). In this study, Hao *et al.* reconstituted the melatonin synthetic pathway in *E. coli* using heterologous enzymes selected from various origins. The Ddc and Aanat are promiscuous enzymes which can result in adverse effects on other metabolic processes and generate unwanted toxic metabolic products. It is thus necessary to fine-tune their expression levels to limit the accumulation of unwanted byproducts while maintaining high metabolic fluxes through the targeted reactions (Figure 1).

Further engineering of a host genome, such as deleting *tnaA* gene encoding tryptophanase that catalyzes the degradation of both tryptophan and 5-HTP, is also necessary to enable the melatonin synthetic pathway to function at high generating rate. Knocking out the *trpR* (tryptophan transcription repressor) gene also enhanced melatonin production by the derepression of genes involved in tryptophan synthesis and uptake. Numerous other adjustments are needed to fine-tune the gene expression of melatonin production hosts (17).

Among the four steps of melatonin biosynthesis, hydroxylation of tryptophan to form 5-HTP and methylation of acetyl-serotonin to form melatonin are difficult to manipulate. These two enzymes do not function well in *E. coli* and require metabolic cofactors (see Figure 1) that have their own complex synthesis and regeneration machinery. Here we briefly describe the novel strategies used to overcome these bottlenecks (18, 19).

2.1. Tryptophan hydroxylation.

The first step of melatonin synthesis is the production of 5-HTP through hydroxylation of tryptophan, using tetrahydrobiopterin (BH_4), Fe^{2+} and O_2 as cofactors. There are two major engineering challenges with this reaction: the efficiency of hydroxylase and cofactor tetrahydrobiopterin (BH_4) regeneration.

Hao *et. al* have expressed human TpH in *E. coli* with an N-terminal truncation, which is necessary for the solubility of this enzyme (19). Lin *et. al*. have alternatively used prokaryotic phenylalanine 4-hydroxylases, which is better expressed in *E. coli*, but has a preference of phenylalanine as the substrate. They altered its substrate preference to better utilize tryptophan through protein engineering guided by rational design (13).

BH_4 is the cofactor for TpH to synthesize 5-HTP. It is derived from guanosine triphosphate (GTP). Wang *et. al.* have reconstituted the 5-step human BH_4 synthesis and regeneration system in *E. coli*, which enabled production of high level (1.2g/L) 5-HTP in fed batch fermentation (14). This was the highest 5-HTP titer reported using microbial cell factories. Hao *et. al* have designed

a novel strategy using growth-coupling and adaptive laboratory evolution (ALE) to repurpose *E. coli* native pterin as an efficient cofactor for TpH. They also obtained beneficial mutations that enhanced TpH abundance, allowing for considerably higher production of 5-HTP (19).

2.2. SAM-dependent methylation.

Another bottleneck in the melatonin biosynthesis pathway is the methylation of acetyl-serotonin to form melatonin. This reaction is catalyzed by methyltransferase (Mtase) using S-adenosylmethionine (SAM) as a cofactor, one of the most abundant cofactors used in metabolism across different kingdoms. SAM donates a methyl group to the substrate and gets regenerated in the SAM cycle from methionine. Both SAM availability and Mtase efficiency are critical but prove difficult to metabolically engineer.

In a study examining the production of a different methylated product, vanillin, Kunjapur *et al.* have improved SAM synthesis and regeneration by deleting *metJ*, a gene encoding a repressor of methionine synthetic genes as well as overexpressing the feedback resistant variants of two key enzymes in SAM biosynthesis (20). Another strategy they employed was to overexpress two enzymes, *luxS* and *mtn,* which are for SAM regeneration. This strategy also results in enhanced SAM availability and vanillin titer. All these approaches are potentially beneficial for melatonin production. It is worth noting that GTP is involved in both SAM and pterin biosynthesis and transformation (Figure 1), thus, optimizing pterin-dependent hydroxylation and SAM-dependent methylation in the melatonin biosynthesis pathway can be much more challenging due to the potential limitation of GTP or other related reactions.

In order to enhance methyltransferase enzyme activities, Luo *et al.* designed a growth-coupling system by rewiring the SAM cycle to cysteine synthesis (18). First, they deleted the *cysE* gene to make *E. coli* a cysteine auxotroph. Second, they added two genes from yeast to enable the synthesis of cysteine from homocysteine, which is an intermediate of the SAM cycle. The resulting strain requires a high flux of SAM-dependent methylation to provide sufficient cysteine to grow. Improved variants of the Mtases or upstream enzymes can be obtained from ALE of the growth-coupled strains. This method provided a fast and efficient workflow for improving the biosynthesis of melatonin and other methylated products.

2.3. Transporter engineering.

Membrane transporters play an important role in achieving a high-performance production strain. Enrichment of metabolic precursor supply is favorable for the downstream reactions, while high intracellular concentration of the product may inhibit enzymes or cellular growth. It is beneficial to identify and engineer membrane transporters to prevent the leakage of precursors or intermediates, as well as increase the efflux of the product.

In a process where tryptophan is fed as the precursor, deleting the known tryptophan transporter gene *yddG* increases melatonin titer significantly (Figure 1) (17). YddG plays a major role in export of aromatics amino acids including tryptophan. Deleting it can help increase the intracellular tryptophan concentration and improve melatonin production. Melatonin inhibits the growth of *E. coli* (21), so it is beneficial to increase strain tolerance to melatonin. However, it is generally difficult to identify transporters for xenobiotic chemicals in microbial hosts. Several studies have succeeded in addressing this difficulty, using methods like adaptive evolution or screening metagenomics libraries (22–25).

Yang *et al.* have developed a high throughput workflow in which they screened an *E. coli* single gene knockout library containing more than 400 predicted membrane transporters in the presence of a high concentration of melatonin (26). In total, five knockout strains showed growth defects in melatonin, indicating that these five transporters play a role in exporting melatonin. One of the transporters, YhjV, was annotated as a putative transporter. Its function was previously uncharacterized. They further over-expressed genes encoding the five transporters and found that *yhjV* overexpression increased melatonin titer by 27% in *E. coli*. Enhancing production strain tolerance to melatonin is important for developing an efficient process to commercial-relevant scales.

A practical metabolic production strain was generated after meeting all these metabolic engineering requirements that included over 10 genomic edits (26).

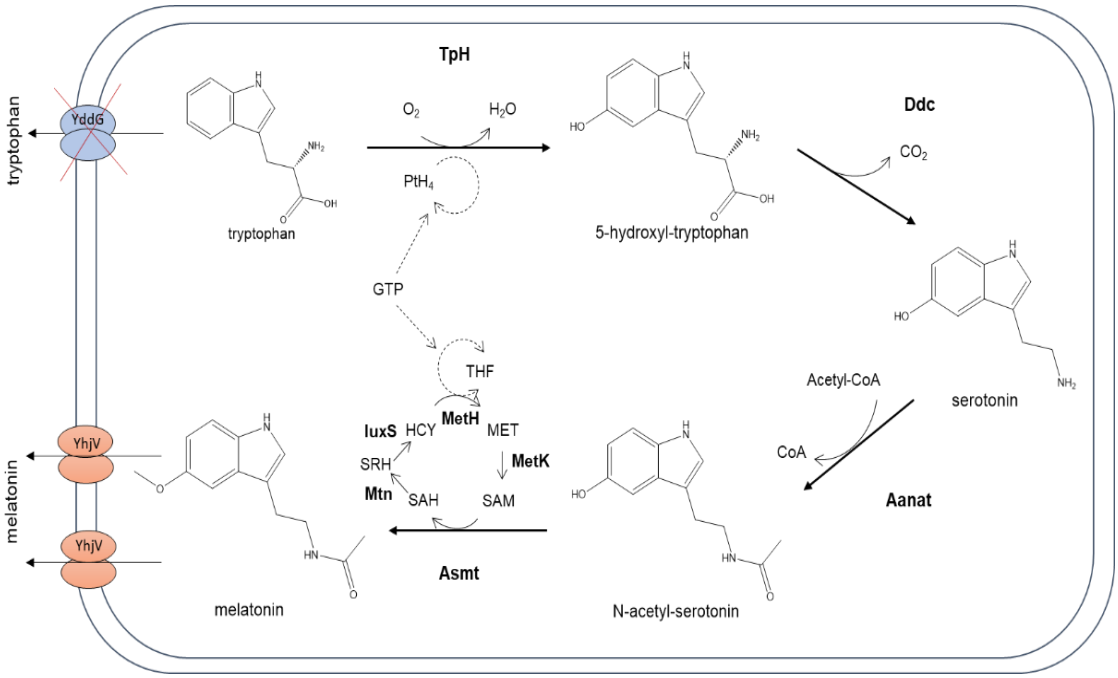

Fig. 1. Melatonin biosynthetic pathway.

Melatonin can be synthesized from tryptophan in four steps by four enzymes: hydroxylation by tryptophan hydroxylase (TpH), decarboxylation by decarboxylase (Ddc), acetylation by aralkylamine acetyltransferease (Aanat), and methylation by N-Acetylserotonin O-methyltransferase (Asmt). Hydroxylation requires either tetrahydrobiopterin or tetrahydromonopterin (both being GTP derivatives, indicated as PtH_4) as a cofactor. The last reaction methylation is dependent on S-adenosylmethionine (SAM) as a cofactor, which is mostly regenerated in the SAM cycle. Intermediates of the SAM cycle include S-adenosyl-L-homocysteine (SAH), S-ribosyl-L-homosystein (SRH), homocysteine (HCY), and methionine (MET). The enzymes involved in the SAM cycle include SAH nucleosidase (mtn), SAH lyase (luxS), methionine synthase (metE or metH) and methionine adenosyltransferase (metK). The SAM cycle is also linked to GTP through tetrhydrofolate synthesis and transformation. Transporter engineering is also beneficial for melatonin production, e.g., knockout yddG and overexpression yhjV, which encode transporters responsible for efflux of tryptophan and melatonin, respectively.

3. CHALLENGES IN SCALING-UP MELATONIN PRODUCTION

To biomanufacture melatonin at commercial scales, a robust production strain with high titer, yield, or productivity is needed. One critical challenge during scale-up is product inhibition. On one hand, melatonin is an antioxidant agent playing an important role in the evolution of aerobic metabolism (27), which might be beneficial in fermentation in oxygen-rich conditions (28). On the other hand, melatonin exerts a toxic effect on *E. coli*, especially at high concentrations. Interestingly, it appears to repel *E. coli*, as it upregulates the chemotaxis machinery, causing *E. coli* to swim away from high melatonin concentrations (21).

It is thus essential to understand the mechanisms of melatonin inhibition in order to develop robust production strains that can perform well in large-scale fermentation. Gulcin *et. al.* have reported that melatonin can effectively chelate metal ions including zinc, copper, and iron as a way of reducing lipid peroxidation in human cells (29). However, the ion chelating effect can be detrimental for the production strain of microbes, causing poor fermentation performance as melatonin accumulates in the fermenter. Moreover, melatonin was reported to inhibit citrate synthase in the TCA cycle in gram negative bacteria, which could also lead to growth arrest of the production strain (30). Finally, melatonin has a low solubility (2g/L) in water (Scientific committee on consumer safety, opinion on melatonin, 23 March 2010). A commercially feasible titer should exceed 2g/L. In that case, melatonin will most likely precipitate and form insoluble particles in the fermentation broth, which might also decrease strain performance in the reactors as melatonin accumulates (31). Improving strain tolerance towards these stresses by genome engineering can help increase fermentation performance. Alternatively, removing melatonin during fermentation using methods like liquid extraction is another option to reduce product inhibition (32).

In conclusion, the challenges of making melatonin through a bioprocess are great. The introduction of four heterologous enzymes and multiple genome edits were needed to get the pathway to function in a microbial host. Thus, the practical biomanufacturing of melatonin is now possible. Although the current production strain can go into a biomanufacturing process, the deployment of additional advanced biological tools such as transcriptomics, metabolomics, and integrated multi-omics data analysis are likely to continuously improve the productive strain and the fermentation process. Biomanufactured melatonin is thus on its way into myriad products that are anticipated to appear in the coming years.

ACKNOWLEDGMENTS

The authors would like to thank the Novo Nordisk Foundation for financial support (grants NNF20CC0035580 and NNF10CC1016517). We would also like to thank Dr. Andreas Worberg and Pre-Pilot Plant team, including Dr. Ann Dorrit Enevoldsen, Dr. Milica Randelovic, Dr. Minkle Jain and Dr. Gossa Garedew Wordofa for their efforts and discussion on the scale-up and downstream process development. We also appreciate the comments from Dr. Emre Özdemir and Dr. Se Hyeuk Kim on this manuscript.

AUTHORSHIP

LY and BOP both wrote the paper.

CONFLICT OF INTERESTS

L. Yang is an inventor of the following patent application: WO/2020/187739. 2020. B.O. Palsson sits on the Board of Directors of Conarium BioWorks.

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Please cite this paper as:
Yang, L. and Palsson, B. 2023. Biomanufacturing of melatonin is now possible. Melatonin Research. 6, 1 (Feb. 2023), 72-78. DOI:https://doi.org/https://doi.org/10.32794/mr112500142.