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Glucose-based microbial production of the hormone melatonin in yeast *Saccharomyces cerevisiae*


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Melatonin is a natural mammalian hormone that plays an important role in regulating the circadian cycle in humans. It is a clinically effective drug exhibiting positive effects as a sleep aid and a powerful antioxidant used as a dietary supplement. Commercial melatonin production is predominantly performed by complex chemical synthesis. In this study, we demonstrate microbial production of melatonin and related compounds, such as serotonin and N-acetylserotonin. We generated *Saccharomyces cerevisiae* strains that comprise heterologous genes encoding one or more variants of L-tryptophan hydroxylase, 5-hydroxy-L-tryptophan decarboxylase, serotonin acetyltransferase, acetylserotonin O-methyltransferase, and means for providing the cofactor tetrahydrobiopterin via heterologous biosynthesis and recycling pathways. We thereby achieved de novo melatonin biosynthesis from glucose. We furthermore accomplished increased product titers by altering expression levels of selected pathway enzymes and boosting co-factor supply. The final yeast strain produced melatonin at a titer of 14.50 ± 0.57 mg L⁻¹ in a 76 h fermentation using simulated fed-batch medium with glucose as sole carbon source. Our study lays the basis for further developing a yeast cell factory for biological production of melatonin.

Keywords: EasyClone vectors · Melatonin · Microbial production · *Saccharomyces cerevisiae* · Serotonin

See accompanying commentary by Tao Sun, Lei Chen and Weiwen Zhang DOI 10.1002/biot.201500604

1 Introduction

Melatonin is a natural hormone produced by the pineal gland in the brain [1, 2]. In humans, it regulates the body’s circadian sleep-wake cycle [3] and is used as a sleep-aid [4, 5], anti-oxidant [6], and over-the-counter dietary supplements based on melatonin have been available for many years in the US. Moreover studies suggest that the melatonin hormone is also an effective cancer inhibitor [7, 8], can be beneficial in treating neurodegenerative diseases [9], and is useful to fight depression and anxiety [10]. The first commercially available melatonin was produced via extraction of pineal glands of animals [1]. However, one of the biggest drawbacks of the extraction process is the danger of viral contamination of the animal tissue. Therefore, melatonin is typically chemically synthesized,
requiring toxic solvents and catalysts [11, 12], albeit that all of these solvents and chemical intermediates are abundantly available.

This has created a compelling case for a biological production process, which could potentially be the dominant production process for melatonin in the future. In mammals, melatonin is primarily synthesized in the pineal gland in a four-step pathway using L-tryptophan as a precursor, which is a downstream metabolite of the intrinsic shikimate pathway [13, 14]. For recombinant melatonin production, we chose budding yeast *Saccharomyces cerevisiae* as the host, since the product is intended for food supplement and medical applications. To the best of our knowledge, this is the first report on heterologous production of melatonin from glucose.

2 Materials and methods

2.1 Strains and chemicals

*Escherichia coli* DH5α was used for cloning, *S. cerevisiae* CEN.PK strains were obtained from Peter Kötter (Johan Wolfgang Goethe-University Frankfurt, Germany). All oligos are listed in Supporting information, Table S1. DNA BioBricks are listed in Supporting information, Table S2. plasmids are listed in Supporting information, Table S3, and yeast strains are listed in Supporting information, Table S4. EasyClone plasmids used in this study are described in Jensen et al. [15]. All chemicals were from Sigma-Aldrich, except nourseothricin (Werner BioAgents).

2.2 Media

*E. coli* was cultivated at 37°C in LB medium. *S. cerevisiae* was cultivated at 30°C on synthetic complete medium or drop-out media prepared with pre-mixed drop-out powders from Sigma-Aldrich. Mineral medium was prepared as described previously [15]. Synthetic fed-batch (FITT) medium M-Sc.syn-1000 was prepared according to manufacturer’s instructions (M2P labs GmbH). FITT medium was supplemented with the supplied vitamins solution (final concentration 1% v/v) and the enzyme mix (final concentration 0.5% v/v) immediately prior to use.

2.3 Cultivation and analysis

For cultivating yeast strains, single colonies originating from independent transformants were inoculated from a pre-culture grown in selective minimal medium into 500 μL FIT medium or mineral medium supplemented with 20 g L⁻¹ glucose in a 96-deep well microtiter plate with air-penetrable lid (EnzyScreen, NL). The microtiter plates were incubated at 30°C with 300 rpm agitation at 5 cm orbit cast, cells were allowed to grow for 72 h. For some experiments, the mineral medium was supplemented with 500 mg L⁻¹ methionine or 500 mg L⁻¹ tryptophan. For harvesting, an equal amount of 96% ethanol was added to the supernatant or the total cell suspension, mixed thoroughly, the ethanol-extracted culture filtered (0.22 μm), and subjected to LC-ESI-MS analysis (details of the LC-ESI-MS method are found in the Supporting information).

2.4 Statistics

For statistical evaluations of the changes in production levels, data were analyzed for significant differences by unpaired two-tailed t-tests using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, California, USA).

3 Results and discussion

3.1 Pathway engineering for sugar-based recombinant biosynthesis of melatonin in yeast

In this study, a de novo pathway for melatonin production was established in *S. cerevisiae* by the extension of the shikimate synthesis pathway. The biosynthesis pathway is described in Fig. 1, the connections to the intrinsic yeast metabolism are depicted in Supporting information, Fig. S1. The amino acid L-tryptophan is the intrinsic precursor, which is converted to 5-hydroxy-L-tryptophan (5-HTP) by tryptophan hydroxylase (TPH) [16]. TPH requires both oxygen and the cofactor tetrahydrobiopterin (THB, also known as BH4). 5-HTP is decarboxylated to serotonin by 5-hydroxy-L-tryptophan decarboxylase (DDC) [17], and serotonin is acetylated to N-acetylseryotonin by serotonin N-acetyltransferase (AANAT), requiring acetyl-coenzyme A (acetyl-CoA) [18]. Finally, N-acetylseryotonin O-methyltransferase (ASMT) methylates N-acetylseryotonin to the final product melatonin [19]. During this last step, the conversion to melatonin is accomplished by the co-conversion of S-adenosyl-L-methionine (SAM) to S-adenosyl-L-homocysteine (SAH). SAH can then be recycled back to SAM via the S-adenosyl-L-methionine cycle (SAM cycle) (Fig. 1). The SAM cycle is native and constitutively expressed in budding yeast [20].

In order to establish a functional heterologous pathway that produces melatonin de novo, we ventured to construct a melatonin producing strain that utilizes endogenous L-tryptophan generated from glucose. Since we had concerns about the stability of 5-HTP observed in initial experiments (data not shown), we directly constructed production strains that contained the entire pathway from L-tryptophan to melatonin. Additionally, we introduced the BH4 biosynthesis and regeneration pathways in order to avoid the need to add chemically synthesized BH4, which is the cofactor for TPH. The pathway for BH4 bio-

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synthesis comprises a GTP cyclohydrolase I (GCH1, FOL2 in *S. cerevisiae*), a 6-pyruvoyl-tetrahydropterin synthase (PTS) and a sepiapterin reductase (SPR), and converts GTP into BH4 [21, 22] (Fig. 1). Conveniently, the enzyme GCH1/Fol2 is natively present in wild-type *S. cerevisiae*, whereas heterologous PTS and SPR were introduced to enable BH4 production in budding yeast. The enzymes 4a-hydroxytetrahydrobiopterin dehydratase (PCBD1) and 6-pyruvoyl-tetrahydropterin synthase (DHPR) complete the BH4 regeneration pathway.

For all pathway genes, a variety of homologues were chosen based on functional conservation to the mammalian melatonin pathway enzymes, and the most promising candidates from initial functionality analysis for single substrate conversion in *E. coli* (data not shown) were chosen for strain engineering in yeast. We generated recombinant strains overexpressing *Rattus norvegicus* RnPTS, *R. norvegicus* RnSPR, one of two homologues of PCBD1 (*Lactobacillus ruminis* LrPCBD1 or *Pseudomonas aeruginosa* PaPCBD1), and 6-pyruvoyl-tetrahydropterin synthase (DHPR) complete the BH4 regeneration pathway.

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The derived strains were analyzed after small-scale cultivation in mineral medium with glucose as sole carbon source. The cells produced substantially 0.3–0.8 mg L⁻¹ of melatonin, 0.0–2.4 mg L⁻¹ of serotonin, and 4.0–9.1 mg L⁻¹ of N-acetylserotonin (Fig. 2B; Supporting information, Table S5). None of these three compounds were detected in the reference non-engineered yeast strain. Having confirmed the functionality of the de novo biosynthetic pathway, we ventured to improve the metabolic flux towards melatonin by optimizing gene expression.

### 3.2 Improved production of melatonin by higher expression of two flux controlling enzymes

Next we investigated whether improved overexpression of TPH is beneficial in order to boost the flux towards melatonin. To analyze this, we used an integrative vector that causes multi-copy integration into Ty retrotransposon sites. Integration of the rate-limiting enzyme into yeast Ty sites has previously been shown to be beneficial for production of 3-hydroxypropionic acid [26]. Yeast retrotransposons are a family of transposable elements that are dispersed throughout the eukaryotic genome in high numbers [27, 28], and therefore represent attractive target sites for multi-copy integration. Accordingly, we introduced the genes coding *Schistosoma mansoni* SmTPH [24] into the conversion of L-tryptophan to 5-HTP. Here, truncated SmTPH lacking both the N- and the C-terminus regulatory regions was used in order to increase heterologous expression and enhance protein stability [25]. Subsequently, the downstream pathway genes for producing melatonin from 5-HTP were integrated, i.e. *H. sapiens* HsDDC, *Bos taurus* BtAANAT, and *H. sapiens* HsASMT (Fig. 2A). The derived strains were analyzed after small-scale cultivation in mineral medium with glucose as sole carbon source. The cells produced substantially 0.3–0.8 mg L⁻¹ of melatonin, 0.0–2.4 mg L⁻¹ of serotonin, and 4.0–9.1 mg L⁻¹ of N-acetylserotonin (Fig. 2B; Supporting information, Table S5). None of these three compounds were detected in the reference non-engineered yeast strain. Having confirmed the functionality of the de novo biosynthetic pathway, we ventured to improve the metabolic flux towards melatonin by optimizing gene expression.
most importantly that expression levels are substantially increased compared to single integration (Supporting information, Fig. S3, Table S6). The derived strains were tested for melatonin production after small-scale cultivation in mineral medium. The cells with HsTPH2146-460 overexpressed from Ty2 sites did not show any significant improvement, we measured melatonin at 0.4–0.9 mg L⁻¹, and N-acetylserotonin at 4.9–7.2 mg L⁻¹ (Fig. 2B; Supporting information, Table S5). Interestingly, we observed a clear effect of choice of BH4 recycling pathway on production performance. Strains carrying PaPCBD1/RnDHPR produced five-fold more melatonin than those carrying the LrPCBD1/HsDHPR homologues. Importantly, we achieved significantly higher titer of melatonin at 1.9 mg L⁻¹, and highly increased titer of N-acetylserotonin at 16.7 mg L⁻¹ when overexpressing SmTPH from Ty2 sites (Ty2::SmTPH) in the presence of PaPCBD1/RnDHPR (Fig. 2B–D; and Supporting information, Table S5), and could obtain high clonal reproducibility with regards to metabolite production (Supporting information, Fig. S2). This represents an approximate two-fold increase in melatonin production in comparison to HsTPH2 146-460, indicating that S. mansoni TPH clearly outperforms H. sapiens TPH. Regarding the expression of SmTPH from Ty2 sites, we tested three independent clones and observed variable copy numbers of 2, 3 and 11. However, RT-OPCR revealed that mRNA expression levels were increased to similar high levels of 13.6 ± 1.6 relative to S. cerevisiae ScACT1 (Supporting information, Fig. S3, Table S6). Interestingly, the choice of BH4 recy-
The clamping pathway had an even more pronounced impact on melatonin production with SmTPH overexpressed from Ty2 sites, as we observed an approximate 64-fold increase in melatonin production when PaPCBD1/RnDHPR were integrated compared to LrPCBD1/HsDHPR (Fig. 2B; Supporting information, Table S5). We can therefore conclude that the BH4 recycling pathway with PaPCBD1 and RnDHPR is beneficial for melatonin production. In order to verify that the BH4 recycling pathway is essential for melatonin production, we constructed strains that contain the complete pathway including the TPH variants in single or multi-copy, but lack PTS, SPR, PCBD1 and DHPR. Indeed, we did not observe any production in these strains (Fig. 3C). For future improvement, the investigation of other variants of BH4 cofactor regeneration enzymes, optimizing protein expression, or different promoter choice might be of interest.

Interestingly, we consistently observed about 10-fold elevated levels of N-acetylserotonin compared to melatonin in all melatonin-producing strains (Fig. 2B; Sup-
higher titers of melatonin at 13.2 ± 2.1 mg L\(^{-1}\) in the parent at 1.4 ± 0.3 mg L\(^{-1}\) (Fig. 3B; Supporting information, Table S5). We therefore analyzed whether we could enhance conversion by increasing the expression of HsASMT. To test this hypothesis, we introduced an episomal high-copy (2µ) plasmid bearing HsASMT into two independent clones of the melatonin production strain that overexpress SmTPH from Ty2 sites. We observed a 1.5-fold decrease of serotonin and a 1.2-fold decrease of N-acetylserotonin. However, both were not significantly different to the control. Importantly, we found a significant 1.8-fold increase of melatonin when HsASMT was overexpressed from the high-copy plasmid (p = 0.0202) (Fig. 3A), supporting the notion that the pathway flux is pushed towards product formation. Future research might focus on balancing other enzymatic steps in this heterologous pathway. In addition, we investigated whether we could improve conversion of N-acetylserotonin by enriching S-adenosyl-L-methionine (SAM) in the cell in order to shift the ASMT reaction equilibrium towards the product. We cultured biological triplicates of all eight melatonin-producing strains in media supplemented with 500 mg L\(^{-1}\) methionine, the precursor of SAM. However, we did not observe any significant improvement of production (data not shown). This might be due to the presence of feedback inhibiting or other regulatory processes in the cell. As an alternative, we conducted genomic engineering approaches in order to increase SAM supply. In S. cerevisiae, there are two isogenes, SAM1 and SAM2, encoding SAM synthetase. ScSAM2 was chosen for overexpression since it has been shown that the expression of the ScSAM2 gene increases during cell growth, and overrides the repressive effect of SAM [29]. We also overexpressed the S. cerevisiae ethionine resistance conferring gene ScERC1, since it was reported that overexpressing ScERC1 improves the accumulation of SAM in yeast [30]. We introduced these genes into the best producing strain SCE-iL3-HM-43 (carrying RnPTS, RnSPR, PaPCBD1, RnDHPR, HsDDC, BtAANAT, HsASMT and Ty2::SmTPH), cultivated independent clones in mineral or FIT medium, and performed ethanol extraction of the total cell suspension, as opposed to the extraction of supernatant in previous experiments. This different extraction method of the parent strain cultured in mineral medium resulted in increased titer of the 5-ACs in the intermediate metabolites serotonin at 65.9 ± 1.0 mg L\(^{-1}\), and N-acetylserotonin at 22.1 ± 0.2 mg L\(^{-1}\). However, serotonin production was not increased and remained at 1.4 ± 0.3 mg L\(^{-1}\) (Fig. 3B; Supporting information, Table S7). Strikingly, we achieved highly decreased titers of serotonin at 2.0 ± 0.0 mg L\(^{-1}\), strongly increased titers of N-acetylserotonin at 43.3 ± 7.1 mg L\(^{-1}\), and significantly higher titers of melanin at 13.2 ± 2.1 mg L\(^{-1}\) in the parent strain when cultured in FIT medium, which represents a strong shift toward product formation (Fig. 3B; Supporting information, Table S7). Meanwhile, overexpression of ScSAM2 or ScERC1 was not beneficial for melanin production, as we observed no significant changes compared to parent strain (Fig. 3C; Supporting information, Table S8). Supplementing these strains with methionine decreased metabolite production even more (data not shown). We conclude that improving melatonin titers by metabolic engineering of the SAM cofactor supply is not as straightforward as anticipated. This is however very worthwhile investigating in the future, since the metabolite profile in FIT medium shows high accumulation of N-acetylserotonin, and therefore the ASMT reaction seems to be the major bottleneck.

### 3.3 Further strategies for improving production titers of melatonin

Furthermore, we investigated whether increased precursor L-tryptophan availability is beneficial for melatonin production. There are a number of tryptophan overproducers available in E. coli [31] or cereal crops [32]. One might consider investigating these platform strains as alternative chassis for melatonin production in future research. In any case, amino acid pools are very tightly regulated in yeast S. cerevisiae, and to the best of our knowledge, there is no yeast tryptophan overproducer available yet. We therefore analyzed melatonin production in media supplemented with tryptophan. However, this approach did not lead to any measurable improvement (data not shown). We then ventured to analyze whether the deletion of the S. cerevisiae ScARO9 gene might be beneficial for melatonin production, since we observed reduced degradation of 5-HTP in initial single enzymatic conversion studies [33]. Aro9 is an aminotransferase II and catalyzes the first step of tryptophan, phenylalanine, and tyrosine catabolism [34]. We deleted ScARO9 in the best producing strain SCE-iL3-HM-43, cultivated the clones in FIT or mineral medium, and measured production. We did not observe any significant change in melatonin levels (Fig. 3C; Supporting information, Table S8). Possibly, the DDC reaction is efficient enough to compete with the 5-HTP degradation pathways and redirects the flux towards serotonin production. Therefore, deleting ScARO9 has no effect on overall production.

Alternatively, increasing acetyl-CoA levels might improve titers of melatonin, since it is consumed during the acetylation of serotonin by AANAT [18]. Therefore, a higher acetyl-CoA/CoA ratio could favor product generation. Feeding carbon sources such as glucose improves acetyl-CoA availability in the cell, but there are many competing metabolic pathways consuming acetyl-CoA. Limiting oxygen can inhibit a subset of these competing pathways and thereby increase acetyl-CoA supply [35]. However, we did not further investigate this strategy, since the upstream conversion of L-tryptophan to 5-HTP requires oxygen, and is probably not functional under low oxygen limiting conditions. Therefore, we ventured to improve the supply of acetyl-CoA, by overexpressing
the cytosolic acetaldehyde dehydrogenase ScALD6 of S. cerevisiae or acetyl-CoA synthase SeACS of Salmonella enterica [36]. We introduced these modifications into the best producer SCE-iL3-HM-43, cultivated the clones in FIT or mineral medium, and observed a minor increase in melatonin production by 5% (SeACS) or 10% (ScALD6) (Fig. 3C; Supporting information, Table S8). However, these changes are not significant. Nevertheless, we were confident to have obtained an improved best producing strain overexpressing ScALD6 (SCE-iL3-HM-60), since it produces melatonin with very low clonal variation at 14.5 ± 0.6 mg L⁻¹. Lastly, increasing NADH and NADPH pools might be of interest in future investigations for improving titers of melatonin, since they are consumed during the acetylation step by AANAT [18] or during the BH4 biosynthesis and regeneration pathways, respectively.

4 Concluding remarks

In contrast to biological tissue extraction and chemical synthesis, production of active biological ingredients using metabolically engineered microorganisms fermenting renewable feedstocks is an attractive process. In this study, we have assembled a recombinant melatonin pathway in S. cerevisiae and demonstrated de novo production of melatonin and its related compounds from glucose for the first time. We have identified the ASMT reaction as the major bottleneck, and gene expression optimization was proven to be beneficial. Overall, our initial exploration is providing a promising basis for future improvements and investigations.

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The authors declare competing interests. The results of this publication are the basis of a European patent application [33].

5 References


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