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

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Rapid Communication

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 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 (FTT) medium M-Sc.syn-1000 was prepared according to manufacturer’s instructions (M2P labs GmbH). FTT 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 FTT 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 tetrahydropterin (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-acetylserotonin by serotonin N-acetyltransferase (AANAT), requiring acetyl-coenzyme A (acetyl-CoA) [18]. Finally, N-acetylserotonin O-methyltransferase (ASMT) methylates N-acetylserotonin to the final product melatonin [19]. During this last step, the conversion to melatonin is accompanied 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-
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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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 SmTPH and HsTPH2 146-460 under the control of the strong P_{PGK1} promoter in multi-copy via expression vectors targeting yeast retrotransposon Ty2 sites. We investigated TPH copy number and mRNA expression levels by QPCR and RT-QPCR, and could confirm that there are more copies integrated, and...
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 HsTPH2-146-460 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 titers 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 recycler...
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 \( \pm \) 2.1 mg L\(^{-1}\) in the parent melatonin production was not increased and remained tured in mineral medium resulted in increased titers of the This different extraction method of the parent strain cul-
tered clones in mineral or FIT medium, and performed alternative chassis for melatonin production in future research. In any case, aromatic amino acid pools are very tightly regulated in yeast \( S. \) cerevisiae, and to the best of our knowledge, there is no yeast tryptophan overpro-
ducer 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 \( \text{ScARO9} \) gene might be beneficial for melatonin production, since we observed reduced degradation of 5-HTP in initial single enzymatic conversion studies [33]. \( \text{Aro9} \) is an aromatic aminotransferase II and catalyzes the first step of tryptophan, phenylalanine, and tyrosine catabolism [34]. We deleted \( \text{ScARO9} \) in the best producing strain \( \text{SCE-IL3-HM-43} \), 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 degrada-
tion pathways and redirects the flux towards serotonin production. Therefore, deleting \( \text{ScARO9} \) has no effect on overall production.

3.3 Further strategies for improving production titers of melatonin

Furthermore, we investigated whether increased precur-
lor L-tryptophan availability is beneficial for melatonin production. There are a number of tryptophan overpro-
ducers 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, aromatic amino acid pools are very tightly regulated in yeast \( S. \) cerevisiae, and to the best of our knowledge, there is no yeast tryptophan overpro-
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Alternatively, increasing acetyl-CoA levels might improve titers of melatonin, since it is consumed during the acetylation of serotonin by \( \text{AANAT} \) [18]. Therefore, a higher acetyl-CoA/CoA ratio could favor product genera-
tion. 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 oxygen limiting conditions. Therefore, we ventured to improve the supply of acetyl-CoA, by overexpressing
the cytosolic acetaldehyde dehydrogenase ScALD6 of *Saccharomyces 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].

### References


Cover illustration
This regular issue of BTJ includes articles on biocatalysis, biomaterials, and industrial biotechnology. The cover picture shows several aspects of bacteriophage therapy, including phages adsorbing to bacterial cell surfaces, commercial preparations Pypo- and Intesi-phages that are currently produced and used in Georgia, and administration of phages by medical staff to a patient. Image is provided by Aleksandre Ujmajuridze, Jon Caplin, Nino Chanishvili, and Aidan Coffey authors of “Silk route to the acceptance and re-implementation of bacteriophage therapy” (http://dx.doi.org/10.1002/biot.201600023).


Forum
Silk route to the acceptance and re-implementation of bacteriophage therapy
Wilbert Sybesma, Jean-Paul Pirnay
http://dx.doi.org/10.1002/biot.201600023

Commentary
Microbial production of mammalian melatonin – a promising solution to melatonin industry
Tao Sun, Lei Chen and Weiwen Zhang
http://dx.doi.org/10.1002/biot.201500604

Research Article
Extracellular vesicles concentration is a promising and important parameter for industrial bioprocess monitoring
Apolonija Bedina-Zavec, Veronika Kralj-Iglic, Matjaž Brinc, Tanja Ficko-Trček, Drago Kuzman, Ana Schweiger and Gregor Anderluh
http://dx.doi.org/10.1002/biot.201500049

Research Article
Dynamics of immature mAb glycoform secretion during CHO cell culture: An integrated modelling framework
Ioscani Jimenez del Val, Yuzhou Fan and Dietmar Weilguny
http://dx.doi.org/10.1002/biot.201400663

Research Article
An artificial self-sufficient cytochrome P450 directly nitrates fluorinated tryptophan analogs with a different regio-selectivity
Ran Zuo, Yi Zhang, Jose C. Huquet-Tapia, Mishal Mehta, Evelina Dedic, Steven D. Bruner, Rosemary Loria and Yousong Ding
http://dx.doi.org/10.1002/biot.201500416

Research Article
A novel regulatory element (E77) isolated from CHO-K1 genomic DNA enhances stable gene expression in Chinese hamster ovary cells
Shin-Young Kang, Yeon-Gu Kim, Seunghee Kang, Hong W. Lee and Eun G. Lee
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Research Article
Exploring mild enzymatic sustainable routes for the synthesis of bio-degradable aromatic-aliphatic oligoesters
Alessandro Pellis, Alice Guarneri, Martin Brandauer, Enrique Herrero Acero, Henricus Peerlings, Lucia Gardossi and Georg M. Guebitz
http://dx.doi.org/10.1002/biot.201500544

Research Article
MapMaker and PathTracer for tracking carbon in genome-scale metabolic models
Christopher J. Tervo and Jennifer L. Reed
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Research Article
Human pluripotent stem cell culture density modulates YAP signaling
Cheston Hsiao, Michael Lampe, Songkhun Niillasithanukroh, Wenqing Han, Xiaojun Lian and Sean P. Palecek
http://dx.doi.org/10.1002/biot.201500374

Research Article
High-throughput cell quantification assays for use in cell purification development – enabling technologies for cell production
Sarah Zimmermann, Sarah Gretzinger, Christian Scheeder, Marie-Luise Schwab, Stefan A. Oelmeier, Anna Osbergerhaus, Eric Gottwald and Jürgen Hubbuch
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Research Article
Functionalized silk assembled from a recombinant spider silk fusion protein (Z-4RepCT) produced in the methylotrophic yeast *Pichia pastoris*
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Research Article
Rapid ester biosynthesis screening reveals a high activity alcohol-O-acyltransferase (AATase) from tomato fruit
Jyun-Liang Lin, Jie Zhu and Ian Wheeldon
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Research Article
An integrated practical implementation of continuous aqueous two-phase systems for the recovery of human IgG: From the microfluidic device to a multistage bench-scale mixer-settler device
Edith Espitia-Saloma, Patricia Vázquez-Villegas, Marco Rito-Palomares and Oscar Aguilar
http://dx.doi.org/10.1002/biot.201400565

Rapid Communication
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