



## Engineered cells for production of indole-derivatives

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(54) Title: ENGINEERED CELLS FOR PRODUCTION OF INDOLE-DERIVATIVES

(57) Abstract: Integral membrane proteins capable of transporting melatonin and other indole-derivatives across biological membranes, and uses thereof.



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## ENGINEERED CELLS FOR PRODUCTION OF INDOLE-DERIVATIVES

## FIELD OF THE INVENTION

The present invention relates to integral membrane proteins capable of transporting melatonin and other indole-derivatives across biological membranes, and well as the use of such integral membrane proteins in cell factories for production of melatonin and other indole-derivatives.

## BACKGROUND OF THE INVENTION

Melatonin is an antioxidant hormone produced in mammals that can be used for treating sleep disorders. One of the intermediates in the biological pathway for production of melatonin ; 5-hydroxy-L-tryptophan (5HTP), has also been used as a sleep aid as well as an antidepressant. Commercial production of melatonin and 5HTP has so far predominantly relied on chemical synthesis, or, in the case of 5HTP, extraction from plants. Producing 'natural' melatonin or 5HTP biologically in so-called cell factories, i.e., cells engineered to produce these compounds, would be highly desirable. Such recombinant production of melatonin, 5HTP and other indole-derivatives from tryptophan is described in, e.g., WO 2013/127915 A1, WO 2015/032911 A1, WO 2017/167866 A1, WO 2017/202897 A1 and WO 2018/037098 A1 (Danmarks Tekniske Universitet).

Transporters are responsible for cellular exchange of both small molecules and macromolecules (Kell, 2018; WO13093737 A1 (BASF)). Transporters can be engineered to decrease intracellular product concentration, improve substrate uptake, reduce feedback inhibition or improve cellular growth. Transporters can therefore play an important role in bio-based production for improving product titers in fermentation as well as facilitating downstream purification process.

EP2267145 A1 (Evonik Degussa GmbH) relates to a process for the production of L-amino acids, in particular L-threonine, in recombinant microorganisms of the family *Enterobacteriaceae*, in which at the open reading frame (ORF) of *yhaO* is deleted, optionally together with a deletion or downregulation of, e.g., *yhjV*. UniProtKB database entry P37660 (YHJV\_ECOLI) describes *E. coli* protein YhjV as an inner membrane transport protein, based on phylogenetic grounds. Sargentini *et al.* (2018) reports a screen for genes involved in radiation survival of *E. coli*, mentioning *yhjV* among the genes identified.

Despite these and other progresses in the art, however, there is a need for recombinant cells and their use in methods providing for cost-effective production of melatonin, 5HTP and other desirable bioproducts. So, one object of the invention is to identify transporters useful for engineering cells towards improved export of melatonin, 5HTP and other indole-derivatives.

## 5 SUMMARY OF THE INVENTION

In a first aspect, the present invention relates to a recombinant cell comprising a biosynthetic pathway for producing an indole-derivative selected from melatonin, N-acetylserotonin, serotonin and 5-hydroxytryptophan (5HTP), wherein the recombinant cell is genetically modified to overexpress a gene encoding an integral membrane protein selected from YhjV (SEQ ID NO:2), GarP (SEQ ID NO:4), ArgO (SEQ ID NO:6), AcrAB (SEQ ID NOS:10 and 12) and LysP (SEQ ID NO:8), a functionally active variant and/or fragment of any thereof, or a combination of any two or more thereof, and wherein the amino acid sequence of the variant has a sequence identity of at least about 80 % to the amino acid sequence of the integral membrane protein and the fragment comprises at least about 80 % of the amino acid sequence of the integral membrane protein.

In some embodiments, the recombinant cell overexpresses a gene encoding YhjV, GarP, ArgO or AcrAB, or a combination of any two or more thereof.

In some embodiments, the recombinant cell overexpresses a gene encoding YhjV or a functionally active variant thereof.

In some embodiments, the biosynthetic pathway comprises (a) an L-tryptophan hydroxylase; (b) an L-tryptophan hydroxylase and a 5HTP decarboxylase; (c) an L-tryptophan hydroxylase, a 5HTP decarboxylase, and a serotonin acetyltransferase; (d) an L-tryptophan hydroxylase, a 5HTP decarboxylase, a serotonin acetyltransferase and an acetylserotonin O-methyltransferase; (e) a tryptophan decarboxylase and a tryptamine 5-hydroxylase; (f) a tryptophan decarboxylase; a tryptamine 5-hydroxylase, and a serotonin acetyltransferase; or (g) a tryptophan decarboxylase; a tryptamine 5-hydroxylase, a serotonin acetyltransferase and an acetylserotonin O-methyltransferase.

In some embodiments, the overexpression of the gene encoding the integral membrane protein provides for an increased production of the indole-derivative, an increased tolerance to the indole-derivative, or both, by the recombinant cell as compared to a non-modified control cell.

In a second aspect, the invention relates to a recombinant cell capable of producing a second indole-derivative from a first indole-derivative via a biosynthetic pathway, wherein the recombinant cell is genetically modified to overexpress a gene encoding YhjV (SEQ ID NO:2),  
5 or a functionally active variant and/or fragment thereof, wherein the amino acid sequence of the variant has a sequence identity of at least about 80% to SEQ ID NO:2 and the fragment comprises at least about 80% of SEQ ID NO:2.

In some embodiments, the recombinant cell overexpresses a gene encoding YhjV or a functionally active variant thereof. In some embodiments, the first and second indole-  
10 derivatives are: (a) tryptophan and melatonin, respectively; (b) tryptophan and N-acetylserotonin, respectively; (c) tryptophan and serotonin, respectively; and (d) tryptophan and 5-hydroxytryptophan, respectively.

In some embodiments, the overexpression of the gene encoding YhjV or the functionally active variant and/or fragment thereof provides for an increased production of the second  
15 indole-derivative, an increased tolerance to the second indole-derivative, or both, by the recombinant cell as compared to a non-modified control cell.

In some embodiments, the recombinant cell according to any aspect or embodiment herein, the integral membrane protein is expressed from a transgene or from an upregulated endogenous gene. In some embodiments, at least one of the enzymes of the biosynthetic  
20 pathway is expressed from a transgene, optionally a heterologous transgene. In some embodiments, the recombinant cell is a bacterial cell, a yeast cell, a filamentous fungal cell, an algal cell or a mammalian cell. In some embodiments, the recombinant cell is a bacterial cell, optionally of the family *Enterobacteriaceae*, such as an *Escherichia coli* cell.

In a third aspect, the invention relates to a method for producing an indole-derivative  
25 selected from melatonin, N-acetylserotonin, serotonin and 5HTP, comprising the step of culturing the recombinant cell according to any aspect or embodiment herein in a culture medium comprising a carbon source, and optionally, isolating the indole-derivative. In some embodiments, the medium further comprises tryptophan.

In a fourth aspect, the invention relates to a method for producing a second indole-derivative  
30 from a first indole-derivative, comprising the step of culturing the recombinant cell of any aspects or embodiment herein in a culture medium comprising a carbon source and the first indole-derivative, and optionally, isolating the second indole-derivative. In some

embodiments, the first and second indole-derivatives are tryptophan and melatonin, respectively.

In a fifth aspect, the invention provides for functionally active variants or fragments of the integral membrane proteins described herein. For example, in one embodiment, the invention provides for a functionally active variant or fragment of YhjV (SEQ ID NO:2) having a sequence identity of at least about 70%, such as at least about 80%, such as at least 84%, such as at least 85%, such as at least 87%, such as at least about 90%, such as at least about 93%, such as at least about 95%, such as at least about 96%, such as at least about 97%, such as at least about 98%, such as at least about 99% to SEQ ID NO:2 or a portion thereof and comprising one or more mutations, optionally an amino acid substitution in one or more residues selected from V176, G108, I151, I182, F187, A260, C78, A260, P385, I155, N186, S268, S75 and K402. In a particular embodiment, the variant comprises a V176M, G108W, A260V, F187L, I182T, I151F, C78S, A260T, P385T, I55F, N186K, S268N, S75R or K402T amino acid substitution, such as a V176M, G108W, A260V, F187L, I182T or I151F amino acid substitution, or a combination of two or more such amino acid substitutions. In one embodiment, the invention provides for a fragment of GarP (SEQ ID NO:4), the fragment comprising or consisting of amino acid residues 1-134 of SEQ ID NO:4.

These and other aspects and embodiments are described in more detail below.

#### FIGURE LEGENDS

Figure 1. Metabolic pathways for the production of melatonin, serotonin, N-acetylserotonin, and/or 5FITP. TRP = L-tryptophan; Met = methionine; SAM = S-adenosyl methionine; SAH = S-adenosyl homocysteine.

Figure 2. High-concentration of melatonin decreased cell growth of *E. coli*. Melatonin stock-solution was made by dissolving melatonin into 70% ethanol (ETOFI). The growth medium was M9 supplemented with 0.2% glucose and 2 to 7 g/L melatonin. Note that the control condition contains 4% ETOFI to be comparable with growth in melatonin. The ordinate scale reflects optical density.

Figure 3. Workflow for screening a transporter knockout library to identify melatonin transporters. See Example 1 for details.

Figure 4. Growth profiles of *E. coli* BW25113 and knockout strains in the presence of 4g/L of melatonin. (A) *acrB*; (B) *yhjV*; (C) *garP*; (D) *lysP*; (E) *argO*. Note that the control condition contains 4% ETOH to be comparable with growth in melatonin.

5 Figure 5. Workflow for transporter overexpression ("o.e." means "overexpressed"; "diff." means "different").

Figure 6. Melatonin production in small scale assay with glucose and tryptophan feeding. Black bar: control strains without transporter overexpression.

10 Figure 7. Melatonin production in fed-batch fermentation with glucose and tryptophan feeding. Strains HMP3337 (*YhjV* overexpression) and HMP2818 (control) (**A**) as well as strains HMP3355 (*YhjV* overexpression) and HMP2820 (control) (**B**) were tested with glucose and tryptophan feeding. A and B represent two different strain backgrounds. Strains HMP3428 (*YhjV*+) and HMP3427 (control) (**C**) were tested with glucose feeding only.

Figure 8. 5HTP production in a small-scale assay with glucose and tryptophan feeding.

15 Figure 9. Complementation of *yhjV* gene restored growth defect of *yhjV* knockout strain in melatonin.

Figure 10. Knockout of *yhjV* gene negatively affects *E. coli* growth in tryptophan.

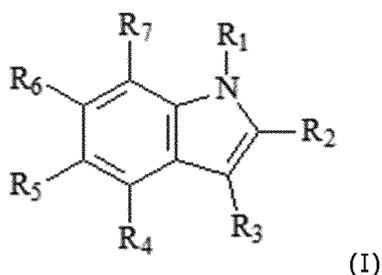
20 Figure 11. Selected *YhjV* mutants that improved melatonin tolerance. Strain containing mutants gave rise to growth (gained biomass after 48h of cultivation) benefit in melatonin (A). One of them also increased melatonin titer in a small-scale production assay (B). pHM635 is the control plasmid that contains the wild-type *YhjV* protein sequence. Error bars indicate standard deviation of 3 replicates.

## DETAILED DISCLOSURE OF THE INVENTION

### Definitions

25 Unless otherwise indicated or contradicted by context, an "indole-derivative" as used herein is a compound which comprises, as part of its chemical structure, an indole group according to Formula I, wherein each of **R<sub>i</sub>** to **R<sub>7</sub>** designates hydrogen (H) or a substituent at the indicated position, e.g., independently selected from hydrogen, an alkyl group, an alkylaryl

group, a substituted alkyl group, a substituted alkylaryl group, a hydroxyl group, an amino group, a carboxyl group, a carboxylic acid group, or an ester group. In some embodiments,  $R_5$  is not H. In some embodiments,  $R_3$  is not H. In some embodiments,  $R_3$  and  $R_5$  are not H. In some preferred embodiments,  $R_1$ ,  $R_2$ ,  $R_4$ ,  $R_6$ , and  $R_7$  are H. Non-limiting examples of indole-derivatives include melatonin, N-acetylserotonin, serotonin, 5HTP, L- tryptophan, indoxyl, indican, indigo, indole-3-acetic acid (IAA), 5,6-dihydroxyindole-2-carboxylate (DHICA), tryptamine, Indometacin (2-[1-(4-chlorobenzoyl)-5-methoxy-2-methylindol-3-yl]acetic acid), Almotriptan (N,N-dimethyl-2-[5-(pyrrolidin-1-ylsulfonylmethyl)-1H-indol-3-yl]ethanamine), Bopindolol ([1-(tert-butylamino)-3- [(2-methyl- 1H-indol-4-yl)oxy]propan-2-yl] benzoate), Frovatriptan ((6R)-6-(methylamino)-6,7,8,9-tetrahydro-5H-carbazole-3-carboxamide) and Zolmitriptan ((4S)-4-[[3-[2-(dimethylamino)ethyl]-1H-indol-5-yl]methyl]-1,3-oxazolidin-2-one). In one preferred embodiment, an indole-derivative is a compound having the general chemical structure of Formula I, wherein  $R_5$  is -OFI or -OCF<sub>3</sub> and  $R_3$  is CH<sub>2</sub>CH<sub>2</sub>N(H)C(=O)CH<sub>3</sub> or CH<sub>2</sub>CH<sub>2</sub>NH<sub>2</sub> or CH<sub>2</sub>CH<sub>2</sub>(NH<sub>2</sub>)COOH. Particularly preferred indole-derivatives are melatonin, N-acetylserotonin, serotonin, 5HTP and tryptamine.



A "recombinant cell" or "a recombinant host cell" as used herein refers to a cell which has been genetically modified, e.g., to introduce one or more transgenes, typically via transformation of a host cell with a vector, or to upregulate or downregulate one or more endogenous genes.

As used herein, a "biosynthetic pathway" for a compound of interest refers to an enzymatic pathway resulting in the production of the compound in a host cell. In some embodiments, at least one of the enzymes is expressed from a transgene, *i.e.*, a gene added to the host cell genome by transformation or other means. In such cases, the biosynthetic pathway may be referred to as a "recombinant biosynthetic pathway" or a "heterologous biosynthetic pathway." In some cases, the recombinant biosynthetic pathway also comprises a deletion of one or more native genes in the host cell. The compound of interest is typically an indole-derivative, and may be the actual end product or a precursor or intermediate in the production of another end product.

The term "substrate" or "precursor", as used herein in relation to a specific enzyme, refers to a molecule upon which the enzyme acts to form a product. When used in relation to a biosynthetic pathway, the term "substrate" or "precursor" refers to the molecule(s) upon which the first enzyme of the referenced pathway acts. When referring to an enzyme-catalyzed reaction in a cell, an "endogenous" substrate or precursor is a molecule which is native to or biosynthesized by the microbial cell, whereas an "exogenous" substrate or precursor is a molecule which is added to the microbial cell, via a medium or the like.

The term "gene" refers to a nucleic acid sequence that encodes a cellular function, such as a protein, optionally including regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence. An "endogenous gene" refers to a native gene in its natural location in the genome of an organism. A "transgene" is a gene, native or heterologous, that has been introduced into a cell, either by natural uptake or by a genetic engineering technique, e.g., a transformation, transduction, or transduction procedure. Gene names are herein set forth in italicised text with a lower-case first letter (e.g., *yhjV*) whereas protein names are set forth in normal text with a capital first letter (e.g., YhjV).

The term "heterologous", when used to characterize a gene or protein with respect to a host cell or species, refers to a gene or protein which has a nucleic acid or amino acid sequence not normally found in the host cell or species.

As used herein, the terms "native" or "endogenous", when used to characterize a gene or protein with respect to a host cell or species, refer to a gene or protein normally found in the host cell or microbial species in question.

As used herein the term "transformation" refers to the transfer of a nucleic acid fragment, such as a gene, into a host cell. Host cells containing a gene introduced by transformation or a "transgene" are referred to as "transgenic" or "recombinant" or "transformed" cells.

The term "expression", as used herein, refers to the process in which a gene is transcribed into mRNA, and may optionally include the subsequent translation of the mRNA into an amino acid sequence, *i.e.*, a protein or polypeptide.

As used herein, "reduced expression" or "downregulation" of an endogenous gene in a host cell means that the levels of the mRNA, protein and/or protein activity encoded by the gene are significantly reduced in the host cell, typically by at least 25%, such as at least 50%, such as at least 75%, such as at least 90%, such as at least 95%, as compared to a control. Typically, when the reduced expression is obtained by a genetic modification in the host cell,

the control is the unmodified host cell. The knocking-out of a gene typically results in the native mRNA and functional protein encoded by the gene being completely absent from the host cell.

"Increased expression", "upregulation", "overexpressing" or the like, when used in the context of a gene, means increasing the level of protein encoded by said gene within a cell, typically by genetic modification of the cell. This can be determined by, e.g., comparing the level of the protein, level of mRNA encoding the protein, or the activity provided by the protein, in the genetically modified cell to that in a non-modified control (or parent) cell. Overexpression can, for example, result in an increase of the protein activity or protein or mRNA level by at least about 5%, such as at least about 10%, such as at least about 20%, such as at least about 30%, such as at least about 50%, such as at least about 100% or more. Overexpression of a gene can, for example, be achieved by placing the gene under the control of a promoter, optionally tuning the (over)expression by use of degenerate sequences in ribosome binding sites (RBSs) or other expression control sequences to obtain a diversity of expression levels and then testing for the desired protein activity or protein/mRNA level. Non-limiting examples of strong promoters suitable for, e.g., *E. coli* cells are Ptrc, Plac, PlacUV5, PT7, and PTrp. Non-limiting examples of strong promoters suitable for, e.g., yeast cells are TEF1, PGK1, HXT7 and TDH3.

As used herein, an "integral membrane protein" is a protein that is integrated in or permanently attached to a biological membrane, such as a cell membrane, which typically is a phospholipid bilayer. An integral protein may be a "transmembrane protein", spanning the entire biological membrane. Single-pass membrane proteins cross the membrane only once, while the sequence of multi-pass membrane proteins weave in and out, crossing the membrane several times

As used herein, a "transporter" is an integral membrane protein assisting in, or facilitating, the passage of a molecule across a biological membrane, such as a cell membrane. An "efflux transporter" or "exporter" for a molecule of interest is a transporter which primarily assists or facilitates the passage of the molecule across a cell membrane from an intracellular compartment (typically the cytoplasm) to an extracellular compartment. An "influx transporter" or "importer" for a molecule of interest is a transporter which primarily assists or facilitates the passage of the molecule across a cell membrane from an extracellular compartment to an intracellular compartment (typically the cytoplasm). A "symporter" is an importer or exporter which can transport two or more molecules at the same time and in the same direction across a biological membrane, e.g., functioning as a cotransporter. An "antiport transporter" or "antiporter" is a transporter which can transport two molecules at the same time in opposite directions across a biological membrane. See, e.g., Kell, 2018.

Standard recombinant DNA and molecular cloning techniques used here are well known in the art and are described by Sambrook, J., Fritsch, E. F. and Maniatis, T. *Molecular Cloning: A Laboratory Manual*, 4<sup>th</sup> ed.; Cold Spring Harbor Laboratory: Cold Spring Harbor, New York, 2012; and by Silhavy, T. J., Bannan, M. L. and Enquist, L. W. *Experiments with Gene Fusions*; Cold Spring Harbor Laboratory: Cold Spring Harbor, New York, 1984; and by Ausubel, F. M. *et al.*, In *Current Protocols in Molecular Biology*, published by John Wiley & Sons (1995); and by Datsenko and Wanner, 2000; and by Baba *et al.*, 2006; and by Thomason *et al.*, 2007.

A "variant" of a parent or reference protein comprises one or more mutations, such as amino acid substitutions, insertions and deletions, as compared to the parent or reference protein. Typically, the variant has a high sequence identity to the amino acid sequence of the parent or reference protein, e.g., at least about 70%, such as at least about 80%, such as at least 84%, such as at least 85%, such as at least 87%, such as at least about 90%, such as at least about 93%, such as at least about 95%, such as at least about 96%, such as at least about 97%, such as at least about 98%, such as at least about 99%, over at least the functionally or catalytically active portion, optionally over the full length.

Unless otherwise stated, the term "sequence identity" for amino acid sequences as used herein refers to the sequence identity calculated as  $(n_{ref} - n_{diff}) \cdot 100 / n_{ref}$  wherein  $n_{diff}$  is the total number of non-identical residues in the two sequences when aligned and wherein  $n_{ref}$  is the number of residues in one of the sequences. Hence, the amino acid sequence GSTDYTQNWA will have a sequence identity of 80% with the sequence GSTGYTQAWA ( $n_{diff}=2$  and  $n_{ref}=10$ ). The sequence identity can be determined by conventional methods, e.g., Smith and Waterman (*Adv. Appl. Math.* 1981;2:482), by the 'search for similarity' method of Pearson & Lipman (*Proc. Natl. Acad. Sci. USA* 1988;85:2444), using the CLUSTAL W algorithm of Thompson *et al.* (*Nucleic Acids Res* 1994; 22:467380), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group), or the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *J. Mol. Biol.* 48: 443- 453) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice *et al.*, 2000, *Trends Genet.* 16: 276- 277), e.g., as provided at the European Bioinformatics Institute website ([www.ebi.ac.uk](http://www.ebi.ac.uk)). The BLAST algorithm (Altschul *et al.*, (1990), *Mol. Biol.* 215:403-10) for which software may be obtained through the National Center for Biotechnology Information ([www.ncbi.nlm.nih.gov/](http://www.ncbi.nlm.nih.gov/)) may also be used. When using any of the aforementioned algorithms, the default parameters for "Window" length, gap penalty, etc., may be used.

A residue in one amino acid sequence which "corresponds to" a specific reference residue in a reference amino acid sequence is the residue which aligns with the reference residue, e.g., as determined by use of sequence alignment software described in the preceding paragraph.

5 A "conservative" amino acid substitution in a protein is one that does not negatively influence protein activity. Typically, a conservative substitution can be made within groups of amino acids sharing physicochemical properties, such as, e.g., basic amino acids (arginine, lysine and histidine), acidic amino acids (glutamic acid and aspartic acid), polar amino acids (glutamine and asparagines), hydrophobic amino acids (leucine, isoleucine, valine and methionine), aromatic amino acids (phenylalanine, tryptophan and tyrosine), and small  
10 amino acids (glycine, alanine, serine, and threonine). Most commonly, substitutions can be made between Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, Asp/Gly.

A "fragment" of a protein comprises at least the part of the protein which is responsible for its function of interest, that is, the functionally active portion (e.g., in the case of an enzyme, its catalytically active portion). Typically, a "fragment" comprises a segment corresponding to  
15 at least about 30%, such as at least about 50%, such as at least about 60%, such as at least about 70%, such as at least about 80%, such as at least about 90%, such as at least about 95%, of the full length protein.

A "functionally active variant" or "functionally active fragment" of a protein comprises  
20 mutations and/or truncations, respectively, which do not substantially affect the function of the variant or fragment as compared to the parent or reference protein, and can substitute at least partially for the parent or reference protein in terms of the function of interest. In the case of an enzyme having a specific catalytic activity, this can also be referred to as a "catalytically active" variant or fragment. Typically, a functionally active variant and/or  
25 fragment retains, as determined by a suitable activity assay, at least 50%, such as at least 80%, such as at least 90%, such as about 100% or more, e.g., 50-150%, such as 80-120%, such as 90%-110%, such as 95%-105% or more, of the activity of a parent or reference protein which does not comprise the mutations and/or truncations in question. Suitable activity assays for comparing the transport activity of variants or fragments of transporter  
30 proteins can be found in the present Examples. For example, to test the transport activity of a variant or fragment of a transporter, the growth of a recombinant cell (e.g., an *E. coli* strain) expressing a variant or fragment of the transporter can be compared with that of the parent transporter (typically the native transporter) in 4% of ethanol and 4-5 g/L of melatonin (see Examples 1 and 5). As an alternative, the transport activity of a fragment or  
35 variant of a transporter can be evaluated in a small-scale melatonin production assay as described in Examples 1, 2 and 5, preparing a recombinant cell (e.g., an *E. coli* strain)

expressing the proteins and enzymes expressed by plasmid pHM345 (Table 5) as well as the fragment or variant of the transporter, and comparing the melatonin production of that recombinant cell with a corresponding recombinant cell expressing instead of the parent transporter (typically the native transporter).

- 5 Standard recombinant DNA and molecular cloning techniques useful for construction of appropriate expression vectors and other recombinant or genetic modification techniques for practising the invention, are well known in the art and are described by, e.g., Green and Sambrook, *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory Press (Cold Spring Harbor, N.Y.) (2012); by Silhavy, T. J., Bennis, M. L. and Enquist, L. W. Experiments with Gene Fusions; Cold Spring Harbor Laboratory: Cold Spring Harbor, New York, 1984; by Ausubel *et al.*, *Short Protocols in Molecular Biology, Current Protocols*, John Wiley and Sons (New Jersey) (2002), and references cited herein. Appropriate microbial cells and vectors are available commercially through, for example, the American Type Culture Collection (ATCC), Rockville, Md.
- 10
- 15 Enzymes referred to herein can be classified on the basis of the handbook *Enzyme Nomenclature* from NC-IUBMB, 1992), see also the ENZYME site at the internet: <http://www.expasy.ch/enzyme/>. This is a repository of information relative to the nomenclature of enzymes, and is primarily based on the recommendations of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUB-MB). It describes each type of characterized enzyme for which an EC (Enzyme Commission) number has been provided (Bairoch A., *The ENZYME database*, 2000, *Nucleic Acids Res* 28:304-305). The IUBMB Enzyme nomenclature is based on the substrate specificity and occasionally on their molecular mechanism.
- 20

#### *Specific embodiments of the invention*

- 25 The present inventors found that melatonin is toxic to *E. coli* cell growth in high concentrations (Figure 2). So, they identified a need to reduce the intracellular concentration of melatonin in order to support high melatonin fermentation titers and yields. They subsequently identified proteins and polypeptides capable of transporting melatonin, 5HTP, and other indole-derivatives across a biological membrane, e.g., a cell membrane.
- 30 For example, YhjV, GarP, ArgO, AcrB, and LysP were identified as transporters providing for melatonin export (Example 1). YhjV was further identified as providing for 5HTP export (Example 2) and for L-tryptophan import (Example 3). Without being limited to theory, YhjV may thus assist or facilitate both the import of tryptophan or other indole-derivatives into a

cell and the export of other indole-derivatives such as melatonin and 5HTP out of the cell, optionally functioning as an antiport transporter.

The novel transporters identified can thus be beneficial for improving the tolerance of *E. coli* and other cells to higher concentrations of melatonin and other indole-derivatives. Further, the novel transporters identified may also allow for the development of cell factories for more efficient production of melatonin and other indole-derivatives via biosynthetic pathways, e.g., from a tryptophan substrate or precursor.

In particular, overexpression of the gene encoding the integral membrane protein may provide for an increased production of the indole-derivative, an increased tolerance to the indole-derivative, or both, by the recombinant cell as compared to a non-modified control cell. This can be tested according to the assays described in the present Examples.

So, in some aspects, the invention relates to cells that are genetically modified to overexpress one or more of the genes encoding the transporters indicated in Table 1, or a functionally active variant or derivative thereof.

Table 1. *E. coli* transporters of indole-derivatives

Name (gene)	Description and UniProtKB reference	Sequences*
YhjV ( <i>yhjV</i> )	Putative amino acid transporter; UniProtKB - P37660 (YHJV_ECOLI)	Gene: 1272 bp (SEQ ID NO:1) Protein: 432 aa (SEQ ID NO:2)
GarP ( <i>garP</i> )	Galactarate /glucarate/glycerate transporter; UniProtKB - B1LFM8 (B1LFM8_ECOSM)	Gene: 1335 bp (SEQ ID NO:3) Protein: 444 aa (SEQ ID NO:4)
ArgO ( <i>argO</i> )	L-arginine efflux transporter; UniProtKB - P11667 (ARGO_ECOLI)	Gene: 636 bp (SEQ ID NO:5) Protein: 211 aa (SEQ ID NO:6)
LysP ( <i>lysP</i> )	lysine:H <sup>+</sup> symporter; UniProtKB - P25737 (LYSP_ECOLI)	Gene: 1470 bp (SEQ ID NO:7) Protein: 489 aa (SEQ ID NO:8)
AcrA ( <i>acrA</i> )	Multidrug efflux pump subunit; UniProtKB - P0AE06 (ACRA_ECOLI)	Gene: 1194 bp (SEQ ID NO:9) Protein: 397 aa (SEQ ID NO:10)
AcrB ( <i>acrB</i> )	Multidrug efflux pump subunit; UniProtKB - P31224 (ACRB_ECOLI)	Gene: 3150 bp (SEQ ID NO:11) Protein: 1049 aa (SEQ ID NO:12)

\* bp= base pairs; aa = amino acids

In some embodiments, the recombinant cell is genetically modified to overexpress a gene encoding an integral membrane protein selected from YhjV (SEQ ID NO:2), GarP (SEQ ID NO:4), ArgO (SEQ ID NO:6), AcrAB (SEQ ID NOS: 10 and 12) and LysP (SEQ ID NO:8), a functionally active variant and/or fragment of any thereof, or a combination of any two or more thereof.

Functionally active fragments and variants of YhjV (SEQ ID NO:2), GarP (SEQ ID NO:4), ArgO (SEQ ID NO:6), AcrAB (SEQ ID NOS: 10 and 12) and LysP (SEQ ID NO:8) can be identified by a person of skill in the art.

For example, the full-length sequence can be truncated from the N- and/or C-terminal to remove portions not necessary for its transport activity while preserving the portion of the protein responsible for its transport activity with respect to melatonin, 5HTP, L-tryptophan or other indole-derivative. For example, such a fragment may comprise at least about 30%, such as at least about 50%, such as at least about 60%, such as at least about 70%, such as at least about 80%, such as at least about 90%, such as at least about 95%, of the full length of the protein, counting by its amino acid residues in the native, full-length sequence. Alternatively, such a fragment can be obtained by removing, e.g., 1, 2, 3, 4, 5, 10, 20 or more amino acid residues from the C-terminal, N-terminal, or both.

Variants of the transporter comprising one or more amino acid substitutions, deletions or insertions can also be prepared and identified by screening or testing them for their transport activity with respect to melatonin, 5HTP, L-tryptophan or other indole-derivative, selecting variants that are functionally active. For example, such variants may have a sequence identity of at least about 70%, such as at least about 80%, such as at least 84%, such as at least 85%, such as at least 87%, such as at least about 90%, such as at least about 93%, such as at least about 95%, such as at least about 96%, such as at least about 97%, such as at least about 98%, such as at least about 99% to the native, full-length sequence or a portion thereof. Alternatively, such a variant may comprise up to 10 mutations, such as 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 mutations. In one embodiment, the variant differs from the native sequence only by conservative amino acid substitutions.

In some embodiments, the C- or N-terminal of the integral membrane protein, or functionally active variant or fragment thereof, is fused or conjugated to a second polypeptide, such as a protein tag. Suitable protein tags for facilitating detection of a protein or for improving stability, solubility or other properties of a protein are well-known in the art. Non-limiting proteins tags for facilitating protein expression include Green Fluorescent protein (GFP) and Red Fluorescent Protein (RFP).

Suitable assays for testing the transport activity of such fragments and variants are provided in Examples 1 to 3. The transport activity of the native, full-length transporter (see Table 1) can advantageously serve as a control to identify functionally active fragment and variants of the transporter in question, which typically retain at least 50%, such as at least 80%, such as 5 100% or more, e.g., 50-150%, such as 80-120%, such as 90%-110%, such as 95%-105%, of the activity of the native, full-length transporter.

### YhjV

In some embodiments, the integral membrane protein is YhjV (SEQ ID NO:2) or a functionally active variant and/or fragment thereof. YhjV is an uncharacterised protein which 10 has earlier been assigned, based on phylogenetic grounds, to the Hydroxy/Aromatic Amino Acid Permease (HAAAP) Family within the Amino Acid-Polyamine-Organocation (APC) Superfamily. Based on its amino acid sequence, the protein has been predicted to comprise 11 transmembrane helices and a C-terminus located in the periplasm. In one preferred embodiment, the integral membrane protein is YhjV (SEQ ID NO:2).

15 Contemplated fragments and variants of YhjV (SEQ ID NO:2) include those exemplified above, including fragments which are N- and/or C-terminally truncated forms and variants comprising one or more amino acid substitutions, any of which may further be fused or conjugated to a second polypeptide.

In one embodiment, the variant is a functionally active fragment or variant which has a 20 sequence identity of at least about 70%, such as at least about 80%, such as at least 84%, such as at least 85%, such as at least 87%, such as at least about 90%, such as at least about 93%, such as at least about 95%, such as at least about 96%, such as at least about 97%, such as at least about 98%, such as at least about 99% to SEQ ID NO:2 or a portion thereof and further comprises a mutation in one or more residues.

25 In one embodiment, at least one mutation is in a residue selected from V176, G108, 1151, 1182, F187, A260, C78, A260, P385, 155, N186, S268, S75 and K402. In a particular embodiment, the variant comprises a V176M, G108W, A260V, F187L, I182T, I151F, G108W, A260V, F187L, V176M, I151F, C78S, A260T, P385T, I55F, N186K, S268N, S75R or K402T amino acid substitution, or a combination of two or more such amino acid substitutions. In 30 one embodiment, at least one mutation is in a residue selected from V176, G108, 1151, 1182, F187, and A260, such as in V176. In a particular embodiment, the variant comprises a V176M, G108W, A260V, F187L, I182T or I151F amino acid substitution, or a combination of two or more such amino acid substitutions. In one embodiment, at least one mutation is in a

residue selected from C78, A260, P385, 155, N186, S268, S75 and K402. In a particular embodiment, the variant comprises a C78S, A260T, P385T, I55F, N186K, S268N, S75R or K402T amino acid substitution, or a combination of two or more such amino acid substitutions. In a specific embodiment, the variant is a functionally active fragment or variant which has a sequence identity of at least about 70%, such as at least about 80%, such as at least 84%, such as at least 85%, such as at least 87%, such as at least about 90%, such as at least about 93%, such as at least about 95%, such as at least about 96%, such as at least about 97%, such as at least about 98%, such as at least about 99% to SEQ ID NO:2 or a portion thereof and comprises a mutation in residue V176, such as a V176M amino acid substitution.

Further contemplated are variants which are homologs or orthologs of YhjV, examples of which are shown in Table 2 below.

Table 2. Homologs or orthologs identified by protein BLAST (BLASTP) of *E. coli* K-12 MG1655 proteins against protein databases from selected reference organisms. "SID" refers to sequence identify to YhjV (SEQ ID NO:2). "Sequence" refers to UniProtKB, RefSeq, or Genbank identifier.

Protein (organism)	SID	Description	Sequence
YhaO ( <i>E. coli</i> )	45%	Serine transporter (443aa)	P42628
TdcC ( <i>E. coli</i> )	23.9%	Threonine/Serine transporter (443aa)	P0AAD8
SdaC ( <i>E. coli</i> )	25.3%	Serine importer (429aa)	P0AAD6
YqeG ( <i>E. coli</i> )	21.5%	Amino acid transporter (409aa)	P63340
( <i>Rodentibacter pneumotropicus</i> )	47.9%	Uncharacterized Protein	WP_077664799.1
( <i>Methanoculleus sp. SDB</i> )	21%	Sodium:calcium symporter	KQC04638.1
( <i>Citrobacter koseri</i> )	88.5%	Transporter	WP_104868054.1
( <i>Shigella sonnei</i> Ss046)	100%	Putative transporter	AAZ90392.1

GarP

In some embodiments, the integral membrane protein is GarP (SEQ ID NO:4) or a functionally active variant and/or fragment thereof. GarP has been earlier assigned as a member of the major facilitator superfamily (MFS) of transporters, and as a galactarate  
5 /glucarate/glycerate transporter. In one preferred embodiment, the integral membrane protein is GarP (SEQ ID NO:4).

Contemplated fragments and variants of GarP (SEQ ID NO:4) include those exemplified above, including fragments which are N- and/or C-terminally truncated forms and variants comprising one or more amino acid substitutions, any of which may further be fused or  
10 conjugated to a second polypeptide.

In one embodiment, the integral membrane protein is a fragment of GarP comprising at least amino acid residues 1-130 SEQ ID NO:4, such as at least amino acid residues 1-134 of SEQ ID NO:4. In a particular embodiment, the fragment is residues 1-134 of SEQ ID NO:4, which has been shown in the Examples to be functionally active (where it is referred to as  
15 "garP(R135\*"). Variants of GarP which are fragments comprising residues 1-130, such as residues 1-134, of SEQ ID NO:4 and further comprises one or more mutations as compared to the native amino acid sequence are also specifically contemplated.

ArgO

In some embodiments, the integral membrane protein is ArgO (SEQ ID NO:6) or a  
20 functionally active variant and/or fragment thereof. ArgO has been earlier assigned as a member of the LysE family of lysine efflux transporters and as a L-arginine efflux transporter, with 5 predicted transmembrane regions. Experimental topology analysis suggests that it adopts an NimCout conformation. In one preferred embodiment, the integral membrane protein is ArgO (SEQ ID NO:6).

25 Contemplated fragments and variants of ArgO (SEQ ID NO:6) include those exemplified above, including fragments which are N- and/or C-terminally truncated forms and variants comprising one or more amino acid substitutions, any of which may further be fused or conjugated to a second polypeptide.

AcrAB

In some embodiments, the integral membrane protein is AcrAB, with the AcrA and AcrB subunits having the amino acid sequences of SEQ ID NO: 10 and 12, respectively, or a functionally active variant and/or fragment of AcrAB, comprising a variant and/or fragment of  
5 AcrA, AcrB or both. AcrB, the RND (resistance-nodulation-division) family protein, is known as the inner membrane component of the tripartite, proton dependent, drug efflux pump AcrAB-TolC. AcrB contains 12 potential transmembrane regions and two large hydrophilic domains. AcrA is the periplasmic lipoprotein component of the AcrAB-TolC and AcrAD-TolC multidrug efflux pumps. In one preferred embodiment, the integral membrane protein is  
10 AcrAB, with the AcrA and AcrB subunits having the amino acid sequences of SEQ ID NO: 10 and 12, respectively.

Contemplated fragments and variants of AcrAB include those exemplified above, including fragments which comprise N- and/or C-terminally truncated forms and variants of AcrA (SEQ ID NO: 10), AcrB (SEQ ID NO: 12) or both and variants comprising one or more amino acid  
15 substitutions in AcrA, AcrB or both, any of which may be fused or conjugated to a second polypeptide. In a particular embodiment, fragments and variants of AcrAB include fragments which comprise N- and/or C-terminally truncated forms or variants of AcrB (SEQ ID NO: 12) and variants comprising one or more amino acid substitutions, any of which may be fused or conjugated to a second polypeptide.

20 LvsP

In some embodiments, the integral membrane protein is LysP (SEQ ID NO:8) or a functionally active variant and/or fragment thereof. LysP has been earlier assigned as a lysine-specific permease, with 12 transmembrane segments. In one preferred embodiment, the integral membrane protein is LysP (SEQ ID NO:8).

25 Contemplated fragments and variants of LysP (SEQ ID NO:8) include those exemplified above, including fragments which are N- and/or C-terminally truncated forms and variants comprising one or more amino acid substitutions, any of which may further be fused or conjugated to a second polypeptide.

Biosynthetic pathways

Preferably, the recombinant cell comprises a biosynthetic pathway for producing one or more indole-derivatives, most preferably melatonin, N-acetylserotonin, serotonin and/or 5HTP, and typically from an L-tryptophan precursor or substrate.

- 5 For production of 5HTP, the biosynthetic pathway may comprise an L-tryptophan hydroxylase (TPH), catalyzing the conversion of L-tryptophan into 5HTP. For production of serotonin, the biosynthetic pathway may further comprise a 5HTP decarboxylase (DDC), catalyzing the conversion of 5HTP to serotonin. For production of N-acetylserotonin, the biosynthetic pathway may further comprise a serotonin acetyltransferase (AANAT), catalyzing
- 10 the conversion of serotonin to N-acetylserotonin. For production of melatonin, the biosynthetic pathway may further comprise an acetylserotonin O-methyltransferase (ASMT), catalyzing the conversion of N-acetylserotonin to melatonin.

- The enzymes of the biosynthetic pathway may be endogenous to the cell. For example, at least in vertebrates, melatonin is biosynthesized via the Shikimate pathway from the native precursor L-tryptophan (Figure 1). Accordingly, in embodiments where the recombinant cell is a vertebrate cell, e.g., a mammalian cell, such as a *Homo sapiens* cell, the endogenous enzymes can provide for the desired biosynthetic pathway, optionally upregulating the endogenous genes or overexpressing genes encoding one or more of the native enzymes from recombinantly introduced transgenes. In embodiments where the desired end-product is
- 15 N-acetylserotonin, serotonin or 5HTP, endogenous genes encoding enzymes catalyzing the subsequent reaction steps in the Shikimate pathway can optionally be downregulated or knocked-out, e.g., using CRISPR-Cas9 technology or other methods known in the art.
- 20

- Alternatively, one or more of the enzymes of the biosynthetic pathway may be heterologous to the recombinant host cell, introduced by recombinant techniques known in the art and described, e.g., in WO 2013/127914 AI, WO 2013/127915 AI, WO 2015/032911 AI, WO 2017/167866 AI, WO 2017/202897 AI, WO 2018/037098 AI and WO 2018/108966 AI (all Danmarks Tekniske Universitet) and in US 2014/134689 AA (University of California), all of which hereby incorporated by reference in their entireties. Suitable, non-limiting, sources of heterologous enzymes for introducing a biosynthetic pathway for melatonin, N-
- 25 acetylserotonin, serotonin, 5HTP or other indole-derivatives are shown in Table 3 and Table 4. Typically, the host cell is transformed with transgenes encoding the heterologous enzymes under the control of a promoter suitable for the selected host cell and/or linked to elements that promote integration of the nucleic acid sequence into the host cell genome.
- 30

Table 3. Examples of enzyme sources for biosynthetic pathways

<b>Name (EC #)</b>	<b>Species</b>	<b>Genbank, RefSeq or UniProtKB accession No.</b>
L-tryptophan hydroxylase (EC 1.14.16.4) (TPH)	<i>Oryctolagus cuniculus</i> TPH1 <i>Homo sapiens</i> TPH1 <i>Homo sapiens</i> TPH2 <i>Gallus gallus</i> <i>Mus musculus</i> <i>Equus caballus</i> <i>Schistosoma mansoni</i>	P17290-1, v2 NP_004170.1 NP_775489.2 NP_990287.1 NP_033440.1 NP_001075252.1 AAD01923.1
5HTP decarboxylase (EC 4.1.1.28) (DDC)	<i>Acidobacterium capsulatum</i> <i>Rattus norvegicus</i> <i>Sus scrofa</i> <i>Homo sapiens</i> <i>Capsicum annuum</i> <i>Drosophila caribiana</i> <i>Maricaulis maris</i> (strain MCS10) <i>Oryza sativa</i> subsp. Japonica <i>Pseudomonas putida</i> S16 <i>Catharanthus roseus</i> <i>Candidatus Koribacter versatilis</i> Ellin345 <i>Draconibacterium orientale</i> <i>Verrucosipora maris</i>	WP_015898075.1 XP_006251536.1 NP_999019.1 P20711-1, v2 NP_001312016.1 AAM80956.1 ABI65701.1 XP_015648768.1 WP_013972057.1 P17770-1, v1 ABF41161.1 AHW60462.1 WP_013735011.1
serotonin acetyltransferase (EC 2.3.1.87 or 2.3.1.5) (AANAT)	<i>Streptomyces griseofuscus</i> <i>Chlamydomonas reinhardtii</i> <i>Bos Taurus</i> , optionally with A55P mutation <i>Gallus gallus</i> <i>Homo sapiens</i> <i>Mus musculus</i> <i>Oryctolagus cuniculus</i> <i>Ovis aries</i>	AHL44344.1 / W8QGX9 BAH10512.1 DAA18183.1 NP_990489.1 NP_001079.1 XP_011246971.1 XP_008249128.1 NP_001009461.1
acetylserotonin O-methyltransferase (EC 2.1.1.4) (ASMT)	<i>Homo sapiens</i> <i>Ocimum basilicum</i> <i>Bos taurus</i> <i>Takifugu rubripes</i> <i>Macaca mulatta</i> <i>Elephantulus edwardii</i> <i>Oryza sativa</i> <i>Rattus norvegicus</i> <i>Gallus gallus</i> <i>Chromobacterium violaceum</i> <i>Desulfotomaculum kuznetsovii</i> DSM 6115 <i>Xenopus (Silurana) tropicalis</i> <i>Pseudomonas fluorescens</i> <i>Candidatus Solibacter usitatus</i> <i>Fenneropenaeus chinensis</i> <i>Arabidopsis thaliana</i>	P46597-1, v1 Q9XGV9-1, v1 P10950-1, v2 XP_011609423.1 NP_001028112.1 XP_006902482.1 XP_015610997.1 NP_653360.2 NP_990674.1 WP_011135808.1 YP_004515712.1 NP_001011409.1 WP_019095725.1 WP_011682595.1 AAZ66373.1 NP_200227.1
pterin-4-alpha-carbinolamine dehydratase (EC 4.2.1.96) (PCBD1)	<i>Pseudomonas aeruginosa</i> <i>Bacillus cereus</i> var. <i>anthracis</i> <i>Corynebacterium glutamicum</i> ATCC 14067 <i>Lactobacillus ruminis</i> ATCC 25644 <i>Rhodobacteraceae bacterium</i> HTCC2083 <i>Homo sapiens</i> <i>Chromobacterium violaceum</i>	WP_003085898.1 WP_000979542.1 WP_003860504.1 WP_003692157.1 WP_009831434.1 NP_000272.1 WP_011135913

Other biosynthetic pathways for the production of, e.g., serotonin or melatonin can also be used. For example, in one aspect, the recombinant cell additionally or alternatively comprises a biosynthetic pathway for producing serotonin from L-tryptophan via a tryptamine intermediate. Park et al. (Appl Microbiol Biotechnol 201 1;89(5) :1387-1394) describes the

5 production of serotonin in *E. coli* by dual expression of a tryptophan decarboxylase (TDC) and tryptamine 5-hydroxylase (T5H), with TDC decarboxylating tryptophan into tryptamine, after which T5H hydroxylates tryptamine into serotonin. Adding an AANAT and an ASMT to the biosynthetic may then provide for the production of N-acetylserotonin and melatonin.

10 Suitable TDCs and T5Hs include those endogenous to plants, such as tomato or *Oryza sativa* subsp. *japonica* (Rice), UniProtKB - Q6ZJK7 (TDCI\_ORYSJ) and UniProtKB - Q2QUC5 (C71PI\_ORYSJ), respectively.

Preferably, the biosynthetic pathway comprises one or more enzymes shown in Table 4. In some embodiments, the biosynthetic pathway is for producing 5HTP, and comprises a TPH

15 enzyme described in WO 2017/167866 A1, preferably SEQ ID NO: 13 of WO 2017/167866 A1 with E2K, N97I and P99C mutations introduced. In some embodiments, the biosynthetic pathway is for producing serotonin, and further comprises a DDC, e.g. *Candidatus Koribacter versatilis* *EHin345* (ABF41161.1). In some embodiments, the biosynthetic pathway is for producing N-acetylserotonin, and further comprises an AANAT described in WO 2018/108966

20 A1, preferably *Streptomyces griseus* AANAT (WP\_011135913) with a D63G mutation. In some embodiments, the biosynthetic pathway is for producing melatonin, and further comprises an ASMT described in WO 2017/202897 A1, e.g., *Homo sapiens* ASMT (P46597.1) comprising an A258E, G260D or T272A mutation, such as an A258E mutation and, optionally, a V305A mutation.

25 As described herein, YhjV can provide for import and export of indole-derivatives via antiport transporter activity. For example, YhjV provides for export of melatonin and for import of L-tryptophan, and for export of 5HTP and for import of L-tryptophan. This can be particularly advantageous in embodiments where a first indole-derivative is the substrate for a biosynthetic pathway where a second indole-derivative is the product of interest. In such

30 cases, each molecule of imported substrate may be coupled with the export of one molecule of exported product, thereby avoiding undue intracellular accumulation of either substrate or product. So, in a particular aspect, the recombinant cell is genetically modified to overexpress a gene encoding YhjV (SEQ ID NO:2), or a functionally active variant and/or fragment thereof, and is capable of producing a second indole-derivative from a first indole-

35 derivative via the biosynthetic pathway. The first and second indole-derivatives can be chosen from any substrate/product indole-derivatives pair for which a biosynthetic pathway is

known in the art. In the Shikimate pathway, any compound can serve as the first or the second indole-derivative. In some embodiments, the first indole-derivative is L-tryptophan. In some embodiments, the first indole-derivative is tryptamine. In some embodiments, the first indole-derivative is serotonin. In some embodiments, the first indole-derivative is N-acetylserotonin. In some embodiments, the first indole-derivative is melatonin. In some  
5  
embodiments, the second indole-derivative is selected from melatonin, N-acetylserotonin, serotonin, 5HTP and tryptamine. In a particular embodiment, the second indole-derivative is melatonin. In specific embodiments, the first and second indole-derivatives are (a) tryptophan and melatonin, respectively; (b) tryptophan and N-acetylserotonin, respectively;  
10 (c) tryptophan and serotonin, respectively; and (d) tryptophan and 5-hydroxytryptophan, respectively. In other specific embodiments, the second and first indole-derivatives are (a) tryptophan and melatonin, respectively; (b) tryptophan and N-acetylserotonin, respectively; (c) tryptophan and serotonin, respectively; and (d) tryptophan and 5-hydroxytryptophan, respectively.

15 Other indole-derivatives of interest, typically as a second indole-derivative, include Almotriptan, Alosetron, Bopindolol, Bromocriptine, Cabergoline, Carprofen, Carvedilol, Ceruletide, Delavirdine, Deserpidine, Dihydroergotamine, Dihydroergotamine, Dolasetron, Eletriptan, Ergoloid mesylate, Ergonovine, Ergotamine, Etodolac, Fluvastatin, Frovatriptan, Gonadorelin, Goserelin, Indomethacin, Lisuride, Methylergonovine, Methysergide, Nafarelin,  
20 Naratriptan, Nicergoline, Octreotide, Ondansetron, Pentagastrin, Pergolide, Pindolol, Rescinnamine, Reserpine, Rizatriptan, Sertindole, Sumatriptan, Tadalafil, Vapreotide, Vilazodone, Vinblastine, Vincristine, Vindesine, Vinorelbine, Voacamine, Yohimbine, Zafirlukast, Zolmitriptan, (5-hydroxyindol-3-yl)acetaldehyde, 5,6-dihydroxyindole-2-carboxylate, 5-Flydroxyindoleacetate, 5-Methoxyindoleacetate, 6-Flydroxymelatonin, indol-3-ylacetaldehyde, indole-3-acetate, N-Methylserotonin and tryptaminium.  
25

#### Flost cell

The recombinant cell (host cell) is preferably tryptophan autotrophic (/i.e., capable of endogenous biosynthesis of L-tryptophan), grows on synthetic medium with suitable carbon sources, and expresses a suitable RNA polymerase (such as, *e.g.*, T7 polymerase). In all  
30 known microorganisms, tryptophan production takes place via a single metabolic pathway (Somerville, R. L., Flerrmann, R. M., 1983, Amino acids, Biosynthesis and Genetic Regulation, Addison-Wesley Publishing Company, U.S.A. : 301-322 and 351-378; Aida *et al.*, 1986, Bio-technology of amino acid production, progress in industrial microbiology, Vol. 24, Elsevier Science Publishers, Amsterdam: 188-206). Tryptophan precursor can also be provided  
35 exogenously to the host cell, *e.g.*, by adding L-tryptophan to the medium of a recombinant

host cell. In some embodiments, particularly suitable for host cells of the family *Enterobacteriaceae*, such as *E. coli*, the host cell expresses *E. coli* TrpE (Anthranilate synthase component 1; Uniprot P00895) or a S40F mutant thereof (Caligiuri et al., Science 1991;252(5014): 1845-8; Caligiuri et al., J Biol Chem 1991; 266(13):8328-35), known to promote the first step of the subpathway that synthesizes L-tryptophan from chorismate.

The recombinant host cell is also typically capable of biosynthesizing and/or regenerating the cofactors used by the enzymes in the biosynthetic pathway. In particular, the recombinant host cell is preferably capable of biosynthesizing, regenerating, or bio-synthesizing and regenerating, one or more cofactors for TPH, AANAT and ASMT (Figure 1).

10 To provide cofactor for TPH-catalyzed hydroxylation of tryptophan, the recombinant host cell is preferably capable of biosynthesizing one or both of THB and MH4 via endogenous or heterologous (introduced) pathways. For example, endogenous pathways for THB biosynthesis are present in mammalian cells. Microbial cells generally do not biosynthesize THB endogenously, but it has been reported that the endogenous compound MH4 may substitute for or replace THB as cofactor for TPH in such cells (US 2014/134689 AA; University of California). GTP cyclohydrolase I (such as, e.g. FolE) -catalyzed pterin biosynthesis resulting in MH4 takes place in many organisms including both prokaryotes and eukaryotes (see, e.g. Figure 9 of US 2014/134689 AA). So, for example, in one embodiment, the microbial host cell is an *E. coli* cell, and comprises the endogenous enzymes FolE, FolX, P-ase, and FolM (Uniprot P0AFS3), optionally upregulated or expressed from a transgene on one or more introduced vectors. Preferably, at least FolM is overexpressed. Further, useful FolE variants such as, e.g., FolE (T198I) are described in, e.g., WO 2017/167866. Orthologs of these enzymes in other microbial host cells, e.g., of the family *Enterobacteriaceae* can also be identified and upregulated or overexpressed.

25 Alternatively, enzymes of biosynthetic pathways for producing and/or regenerating THB can be introduced recombinantly, as described in, e.g. WO 2013/127914 AI, WO 2013/127915 AI and WO 2015/032911 AI (Danmarks Tekniske Universitet) and in US 2014/134689 AA (University of California). Briefly, in one embodiment, the recombinant cell comprises an exogenous pathway producing THB from GTP and herein referred to as "first THB pathway", comprising a GTP cyclohydrolase I (GCH1), a 6-pyruvoyl-tetrahydropterin synthase (PTPS), and a sepiapterin reductase (SPR). The addition of such a pathway to microbial cells such as *E. coli* (JM 101 strain), *S. cerevisiae* (KA31 strain) and *Bacillus subtilis* (1A1 strain (TrpC2)) has also been described in, e.g., U.S. 7,807,421. In one embodiment, the recombinant cell comprises a pathway producing THB by regenerating THB from HTHB, herein referred to as "second THB pathway", comprising a 4a-hydroxytetrahydrobiopterin dehydratase (PCBD1) and, optionally, a 6-pyruvoyl-tetrahydropterin synthase (DHPR). The second THB pathway

can convert the HTHB formed by the L-tryptophan hydroxylase-catalyzed hydroxylation of L-tryptophan back to THB, thus allowing for a more cost-efficient 5HTP synthesis. Preferably, at least microbial host cells, such as host cells of the family *Enterobacteriaceae*, e.g., *E. coli*, may be transformed with a heterologous gene expressing a PCBD1, such as a PCBD1 shown in Table 3, e.g., *Chromobacterium violaceum* PCBD1 (WP\_011135913).

Most types of host cells (e.g., mammalian host cells, yeast host cells such as *S. cerevisiae*, bacteria such as *E. coli*, etc.) are capable of producing and regenerating acetyl-CoA (AcCoA) and SAM; the cofactors for AANAT and ASMT, respectively. AcCoA serves as a metabolic cofactor in the AANAT reaction, but is also part of other, endogenous pathways in, e.g., microbial cells. SAM is a principal methyl donor in various intracellular transmethylation reactions. It is synthesized in the cell through SAM synthetase from methionine and ATP, and natively generated through the SAM cycle, which consists of a methyl transferase, an S-adenosyl-L-homocysteine hydrolase, a folate transferase, and an S-adenosyl-methionine synthetase (Lee *et al.*, Korean J. Chem. Eng. 2010, 27, 587-589). Accordingly, in the ASMT-catalyzed, last reaction in the production of melatonin from L-tryptophan, N-acetylserotonin and SAM are converted to melatonin and SAH. SAH can then be recycled back to SAM via the SAM-cycle in microbial cells where the S-adenosyl-L-methionine cycle is native (or exogenously added) and constitutively expressed, such as, e.g., in *E. coli*. The enzymes of such native pathways can also, in needed, be upregulated or expressed from an exogenously introduced vector, using well-known recombinant techniques known in text books referenced elsewhere herein. Non-limiting and exemplary nucleic acids encoding enzymes of the SAM cycle for use in aspects and embodiments of the present invention include those shown in Table 1 of WO 2015/032911 A1, which is hereby specifically incorporated by reference.

#### Process

In one aspect, the invention also provides for a process of preparing the recombinant host cell. Typically, the process comprises introducing into the host cell, typically via transformation, one or more vectors comprising transgenes encoding the desired integral membrane protein and, optionally, enzymes providing for the biosynthetic pathway, using standard methods known in the art and cited elsewhere herein. The introduction of a vector into a bacterial host cell may, for instance, be effected by protoplast transformation (see, e.g., Chang and Cohen, 1979, Molecular General Genetics 168: 111-115), using competent cells (see, e.g., Young and Spizizen, 1961, Journal of Bacteriology 81 : 823-829, or Dubnau and Davidoff-Abelson, 1971, Journal of Molecular Biology 56: 209-221), electroporation (see, e.g., Shigekawa and Dower, 1988, Biotechniques 6: 742-751), or conjugation (see, e.g., Koehler and Thome, 1987, Journal of Bacteriology 169: 5771-5278). As described

above, the vector, once introduced, may be maintained as a chromosomal integrant or as a self-replicating extra-chromosomal vector.

Preferably, for transformation of an *E. coli* or other bacterial host cell, the vectors are designed as follows: A *P<sub>trc</sub>* promoter is used to control the expressions of a gene or an artificial operon containing up to three genes connected with a linker sequence, in order to  
5 express the genes at a suitable level so that the introduction of heterologous genes/pathways do not overdraw substrates or energy in the host cell. In one particular embodiment, the recombinant microbial cell, preferably derived from a bacterial cell, is transformed according to a strategy outlined in the Examples.

10 Preferably, for transformation of a yeast host cell such as *S. cerevisiae*, the heterologous genes are integrated onto chromosome using a homologous recombination based method (Mikkelsen et al., 2012). As compared with gene expression based on plasmids, the chromosomally integrated genes can be expressed with higher fidelity and resulted in better protein translation, in particular for multiple gene co-expression systems.

15 The transformation can be confirmed using methods well known in the art. Such methods include, for example, nucleic acid analysis such as Northern blots or polymerase chain reaction (PCR) amplification of mRNA, or immunoblotting for expression of gene products, or other suitable analytical methods to test the expression of an introduced nucleic acid  
20 sequence or its corresponding gene product, including those referred to above and relating to measurement of 5HTP production. Expression levels can further be optimized to obtain sufficient expression using methods well known in the art and as disclosed herein.

In a preferred embodiment, the host cell is a microbial cell. The microbial host cell for use in the present invention is typically unicellular and can be, for example, a bacterial cell, a yeast host cell, a filamentous fungal cell, an algal cell, or a mammalian cell.

25 In one embodiment, the host cell is bacterial cell, e.g., of the family *Enterobacteriaceae*, a *Bacillus* cell such as a *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus brevis*, *Bacillus circulans*, *Bacillus clausii*, *Bacillus coagulans*, *Bacillus lautus*, *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus stearothermophilus*, *Bacillus subtilis*, or a *Bacillus thuringiensis* cell; or a *Streptomyces* cell such as a *Streptomyces lividans* or *Streptomyces*  
30 *murinus* or *Streptomyces coelicolor* cell. In a particular embodiment, the recombinant microbial cell is derived from cell of the *Escherichia* genus, such as an *Escherichia coli* cell. In another particular embodiment, the host cell is of an *E. coli* strain selected from the group consisting of K12.DH1 (Proc. Natl. Acad. Sci. USA, volume 60, 160 (1968)), JM101, JM103

(Nucleic Acids Research (1981), 9, 309), JA221 (J. Mol. Biol. (1978), 120, 517), HB101 (J. Mol. Biol. (1969), 41, 459) and C600 (Genetics, (1954), 39, 440) and BL21.

In one embodiment, the host cell is a fungal cell, such as, e.g., a yeast cell. Exemplary yeast cells include *Candida*, *Hansenula*, *Kluyveromyces*, *Pichia*, *Saccharomyces*,

5 *Schizosaccharomyces* and *Yarrowia* cells. In a particular embodiment, the host cell is an *S. cerevisiae* cell. In another particular embodiment, the host cell is of an *S. cerevisiae* strain selected from the group consisting of *S. cerevisiae* KA31, AH22, AH22R-, NA87-11A, DKD-5D and 20B-12, *S. pombe* NCYC1913 and NCYC2036 and *Pichia pastoris* KM71.

10 In one embodiment, the host cell is a mammalian cell. Examples of mammalian cells include, but are not limited to, CHO, CHO-S, HEK, HEK293, HEK-293F, Expi293F, PER.C6, NS0 cells, Sp2/0 cells and lymphocytic cells.

For example, for overexpression of YhjV, or a fragment or variant thereof, the following strategies are contemplated: In an *E. coli* host cell, the native YhjV expression level may be enhanced by, for example, exchanging the promoter or 5'-UTR sequences. Alternatively, in 15 an *E. coli* host cell, additional YhjV gene copies, or transgenes encoding a fragment or variant of YhjV, may be inserted in the genome or introduced via plasmids, e.g., low-copy plasmids, and the additional gene expression driven by a promoter. In other microbial cells, such as yeast cells, or mammalian cells, a transgene encoding YhjV or the fragment or derivative thereof may be introduced via a plasmid and/or into the genome.

20 In another aspect, the invention provides for compositions comprising a recombinant cell according to any aspect or embodiment described herein in a medium comprising at least 2 g/L melatonin, such as at least 2.5 g/L melatonin, such as at least 3 g/L, such as at least 4 g/L, such as at least 5 g/L, such as at least 10 g/L. In a particular embodiment, the recombinant cell is of the family *Enterobacteriaceae*, such as, e.g., an *E. coli* cell.

25 The preparation of recombinant cells capable of producing indole-derivatives such as, e.g., melatonin has been extensively described elsewhere, see, e.g., WO 2013/127915 A1, WO 2015/032911 A1, WO 2017/167866 A1, WO 2017/202897 A1 and WO 2018/037098 A1 (Danmarks Tekniske Universitet). Suitable methods and plasmids for transformation of bacterial cells, such as e.g. *E. coli* cells, with enzymes of a melatonin-production pathway are 30 described in Example 1 and Table 5, respectively. Based on the information provided, a person of skill in the art would be able to prepare other types of host cells, e.g., yeast or mammalian cells, comprising the relevant biosynthetic pathway.

## Methods

In some aspects, the invention provides for a method for producing an indole-derivative using the recombinant cells of any aspect or embodiment described herein. Preferably, the indole-derivative is selected from melatonin, N-acetylserotonin, serotonin and 5HTP.

- 5 Typically, the method comprises culturing the recombinant cell in a culture medium comprising a carbon source. Suitable fermentation methods include batch (batch culture), fed batch (feed), repeated fed batch (repetitive feed) and continuous process. In one embodiment, the method is a fed-batch fermentation process, optionally according to the one described in Example 1. In some embodiments, the medium further comprises tryptophan.
- 10 The desired compound can then optionally be isolated or retrieved from the medium, and optionally further purified. Also provided is a method of preparing a composition comprising such an indole-derivative, further comprising adding one or more suitable excipients to obtain the composition.

- 15 Suitable carbon sources include carbohydrates such as monosaccharides, oligosaccharides and polysaccharides, such as, e.g., glucose, fructose, sucrose, xylose, mannose, galactose, rhamnose, arabinose, fatty acids, glycerine, glycerol, acetate, pyruvate, gluconate, starch, glycogen, amylopectin, amylose, cellulose, cellulose acetate, cellulose nitrate, hemicellulose, xylan, glucuronoxylan, arabinoxylan, glucomannan, xyloglucan, lignin, and lignocellulose. Other polymers or monomers may also be used.

- 20 The culture conditions are adapted to the recombinant cell, and can be optimized to maximize production of the indole-derivative by varying culture conditions and media components as is well-known in the art. In one embodiment, no L-tryptophan is added to the medium, instead relying on L-tryptophan precursor biosynthesized by the recombinant cell. In one embodiment, L-tryptophan is added to the medium.

- 25 For a recombinant *Escherichia coli* cell, exemplary media include LB medium and M9 medium (Miller, Journal of Experiments in Molecular Genetics, 431-433, Cold Spring Harbor Laboratory, New York, 1972), optionally supplemented with one or more amino acids. When an inducible promoter is used, the inductor can also be added to the medium. Examples include the lac promoter, which can be activated by adding isopropyl-beta-thiogalactopyranoside (IPTG) and the GAL/BAD promoter, in which case galactose/arabinose can be
- 30 added. The culturing can be carried out a temperature of about 10 to 40 °C for about 3 to 72 hours, if desired, with aeration or stirring.

For a recombinant yeast cell, Burkholder minimum medium (Bostian, K. L., *et al.* Proc. Natl. Acad. Sci. USA, volume 77, 4505 (1980)), SD medium containing 0.5% of Casamino acid

(Bitter, G. A., *et al.*, Proc. Natl. Acad. Sci. USA, volume 81, 5330 (1984), and Delft medium (Verduyn *et al.*, Yeast 1992, 8, 501-517) can be used. The pH is preferably adjusted to about 5-8.

5 Isolation of the desired product from the cell culture can be achieved, *e.g.*, by separating the compound from the cells using a membrane, using, for example, centrifugation or filtration methods. The product-containing supernatant is then collected. Further purification of the desired compound can then be carried out using known methods, such as, *e.g.*, salting out and solvent precipitation; molecular-weight-based separation methods such as dialysis, ultrafiltration, and gel filtration; charge-based separation methods such as ion-exchange chromatography; and methods based on differences in hydrophobicity, such as reversed-phase HPLC; and the like. In one embodiment, ion-exchange chromatography is used for purification of serotonin. In one embodiment, reverse-phase chromatography is used for separation and/or purification of melatonin. An exemplary method for purification of these indolamines using reversed-phase chromatography is described in Harumi *et al.*, (1996) (J Chromatogr B 675:152-156).

Once a sufficiently pure preparation has been achieved, suitable excipients, stabilizers can optionally be added and the resulting preparation incorporated in a composition for use in preparing a product such as, *e.g.*, a dietary supplement, a pharmaceutical, a cosmeceutical, or a nutraceutical. For a dietary supplement comprising melatonin, each serving can contain, *e.g.*, from about 0.01 mg to about 100 mg melatonin, such as from about 0.1 mg to about 10 mg, or about 1-5 mg, such as 2-3 mg. Emulsifiers may be added for stability of the final product. Examples of suitable emulsifiers include, but are not limited to, lecithin (*e.g.*, from egg or soy), and/or mono- and di-glycerides. Other emulsifiers are readily apparent to the skilled artisan and selection of suitable emulsifier(s) will depend, in part, upon the formulation and final product. Preservatives may also be added to the nutritional supplement to extend product shelf life. Preferably, preservatives such as potassium sorbate, sodium sorbate, potassium benzoate, sodium benzoate or calcium disodium EDTA are used.

The invention is illustrated by the following Examples, which are not to be construed as limiting.

#### 30 EXAMPLE 1

This Example describes testing *E. coli* cells for melatonin tolerance, the identification of melatonin transporters in *E. coli*, as well as the effects of overexpressing the transporters on melatonin production.

## Materials and methods

### Screening using Growth Profiler

The *E.coli* Keio strains (single gene deleted strains) were inoculated into 400  $\mu$ l of M9+0.2% glucose media supplemented with kanamycin (50 pg/ml) in 96 deep well-plate picking the single colonies in LB-agar plates. Then, the plate was incubated at 30 °C with 300 rpm overnight (incubated for 18-24 h). The wild type strain *E.coli* BW25113 was used as a control. The following day, the saturated pre-cultures were diluted 100 fold into 300  $\mu$ l of M9+0.2% glucose media (and antibiotics as required) supplemented with 4.4% of ethanol or 4.4% of ethanol and 4.4 g/L of melatonin so that the resulting final concentration would be 4% of ethanol and 4 g/L of melatonin in 96 well MTP plate. In case of the RBS library of *yhjV*, screening was performed at 5 g/L of melatonin instead of 4 g/L. Then, the culture plates were incubated in Growth Profiler (EnzyScreen, Heemstede, Netherland) at 30 °C and 250 RPM for 48 h with constant monitoring of the growth by taking picture in every 20 minutes interval. Using the GP software (GP960Viewer version 1.0.0.4, EnzyScreen, Heemstede, Netherland), the pictures data was converted into digital OD600nm values and the growth curves were compared.

### Preparation of strains

Melatonin production strains were derived from BW25113. Heterologous genes introducing 5HTP decarboxylase (DDC); aralkylamine N-acetyltransferase (AANAT); tryptophan hydroxylase (TPH); a pterin-4-alpha-carbinolamine dehydratase (PCBD1), and acetylserotonin O-methyltransferase (ASMT) enzymatic activities were overexpressed using constitutive promoters. In some strains, *E. coli* FolM (P0AFS3) and a mutant of *E. coli* TrpE (S40F; Caligiuri et al., Science 1991;252(5014): 1845-8; Caligiuri et al., J Biol Chem 1991; 266(13):8328-35) were also overexpressed. The details of the enzymes used to construct the melatonin biosynthetic pathway are described in Table 4. The details of the strains used in this Example are listed in Table 5. The sequences of promoters used for expressing the enzymes are listed Table 6.

Table 4. Enzymes

Activity	Source (Genbank or UniProtKB accession No.)	Reference
TPH	Residues M1 and E147 to T460 of <i>Homo sapiens</i> TPH2 (NP_775489.2); E2K, N97I and P99C substitutions	WO 2017/167866 A1
DDC	<i>Candidatus Koribacter versatilis</i> Ellin345 (ABF41161.1)	
PCBD1	<i>Chromobacterium violaceum</i> PCBD1 (WP_011135913)	
AANAT	<i>Streptomyces griseus</i> AANAT (WP_011135913), D63G	WO 2018/108966 A1
ASMT	<i>Homo sapiens</i> ASMT (P46597.1), A258E, V305A	WO 2017/202897 A1

Table 5. Melatonin production strain list

Strains	Overexpression	Other information*
HMP2360	Ptrc_ddc, Ptrc_aanat J23107_tph_pcbd1_asmt	$\Delta$ TnaA $\Delta$ TrpR FoIE(T198I) $\Delta$ PfoIE::PJ23100
HMP2993	P2_ddc; J23101_aanat	$\Delta$ TnaA $\Delta$ TrpR FoIE(T198I) $\Delta$ PfoIE::PJ23100 $\Delta$ FhuA
HMP2818	P2_ddc; J23101_aanat J23107_tph_pcbd1_asmt	$\Delta$ TnaA $\Delta$ TrpR FoIE(T198I) $\Delta$ PfoIE::PJ23100 $\Delta$ FhuA
HMP3337	P2_ddc; J23101_aanat J23107_tph_pcbd1_asmt J23107_yhjV	$\Delta$ TnaA $\Delta$ TrpR FoIE(T198I) $\Delta$ PfoIE::PJ23100 $\Delta$ FhuA
HMP3333	P2_ddc; J23101_aanat J23107_tph_pcbd1_asmt J23107_garP(R135*)	$\Delta$ TnaA $\Delta$ TrpR FoIE(T198I) $\Delta$ PfoIE::PJ23100 $\Delta$ FhuA
HMP3335	P2_ddc; J23101_aanat J23107_tph_pcbd1_asmt J23107_acrAB	$\Delta$ TnaA $\Delta$ TrpR FoIE(T198I) $\Delta$ PfoIE::PJ23100 $\Delta$ FhuA

HMP3339	P2_ddc; J23101_aanat J23107_tph_pcbd1_asmt J23107_argO	$\Delta$ TnaA $\Delta$ TrpR FoIE(T198I) $\Delta$ PfoIE::PJ23100 $\Delta$ FhuA
HMP2820	P2_ddc; J23101_aanat J23107_tph_pcbd1_asmt	$\Delta$ TnaA $\Delta$ TrpR FoIE(T198I) $\Delta$ PfoIE::PJ23100 $\Delta$ FhuA PJ23107-foIM
HMP3355	P2_ddc; J23101_aanat J23107_tph_pcbd1_asmt J23107_yhjV	$\Delta$ TnaA $\Delta$ TrpR FoIE(T198I) $\Delta$ PfoIE::PJ23100 $\Delta$ FhuA PJ23107-foIM
HMP3427	P2_ddc; J23101_aanat J23107_tph_pcbd1_asmt	$\Delta$ TnaA $\Delta$ TrpR FoIE(T198I) $\Delta$ PfoIE::PJ23100 $\Delta$ FhuA TrpE(S40F)
HMP3428	P2_ddc; J23101_aanat J23107_tph_pcbd1_asmt J23107_yhjV	$\Delta$ TnaA $\Delta$ TrpR FoIE(T198I) $\Delta$ PfoIE::PJ23100 $\Delta$ FhuA TrpE(S40F)
HMP3403	J23107_tph_pcbd1	$\Delta$ TnaA $\Delta$ TrpR FoIE(T198I) PfoIE::PJ23100 $\Delta$ gstA $\Delta$ FhuA
HMP3404	J23107_tph_pcbd1 J23107_yhjV	$\Delta$ TnaA $\Delta$ TrpR FoIE(T198I) PfoIE::PJ23100 $\Delta$ gstA $\Delta$ FhuA
pHM345 (plasmid)	J23107_tph_pcbd1_asmt	
pHM635 (plasmid)	J23107_yhjV	

\*"PfoIE::PJ23100" indicates that the native promoter of the foIE gene has been replaced by J23100.

Table 6. Promoter sequences used

Promoter	Sequence	SEQ ID NO:
P <sub>trc</sub>	AACTGTTAATTAGTAGGCCGAGCATATTAC	13
P <sub>2</sub>	AAAAAGAGTATTGACTTCGCATCTTTTTGTACCTATAATGTGTGGA	14
J23101	TTTACAGCTAGCTCAGTCCTAGGTATTATGCTAGC	15
J23107	TTTACGGCTAGCTCAGCCCTAGGTATTATGCTAGC	16

J23100	TTGACGGCTAGCTCAGTCCTAGGTACAGTGCTAGC	17
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#### Small-scale production assay

To measure the production of melatonin, bacterial cells were cultivated in deep-well plates in glucose slow release (GSR) medium mimicking fed-batch fermentation at 30 °C. 1 L of GSR medium contains 15 g of Maltodextrin (dextrose equivalent 4.0-7.0, Sigma Aldrich 419672), 200U of Amyloglucosidase (Sigma Aldrich 10115, ~70 U/mg) for glucose release, 40 g of MES monohydrate (Sigma Aldrich), 1.2 g of K<sub>2</sub>HPO<sub>4</sub>, 7 g of Ammonium sulfate, 120 mg of Sodium citrate, 8 mg of ZnCl<sub>2</sub>, 12 mg of FeSO<sub>4</sub>·7H<sub>2</sub>O, 9 µM of CaCl<sub>2</sub>, 12.5 mM of MgSO<sub>4</sub>·7H<sub>2</sub>O, as well as trace elements and vitamins. The media pH was adjusted to 6.4. The medium was supplemented with 500 mg/L tryptophan, 50 mg/L kanamycin and 100 mg/L ampicillin. The bacterial cells were cultivated at 30°C in M9 medium for 24 hours. The precultures were diluted 100 times into GSR medium and cultured at 30°C. After 24 hours, the supernatant was collected by filtering the broth with 0.2µm filters (Pall, New York, USA). The concentration of melatonin in the supernatant was determined by HPLC.

#### Fed-batch fermentation

Fed-batch fermentation was carried out using ambr250 reactors (Sartorius, Goettingen, Germany). The cryo stocks of strains were transferred into 50 ml of M9 media with 0.2% glucose and required antibiotics. The flasks were incubated at 250 RPM and 37 °C overnight. Two millilitres of saturated overnight cultures were inoculated into 100 ml of M9 media with 0.2% glucose and required antibiotics in 250 ml single-use bioreactors (ambr250, Sartorius, Germany).

The cultivations were performed at 30 °C using the cascade of stirring and aeration to control the levels of dissolved oxygen at 40%. The level of stirring in cascade was kept between 1000 rpm and 4000 rpm, while airflow levels were kept between 100 ml/min and 250 ml/min. The pH of the cultures was maintained at 6.0, using 7% ammonium hydroxide.

After the batch phase, 36% w/v glucose solution with other M9 media components and antibiotics was fed in the reactors with strains HMP3427 and HMP3428 using the exponential feeding with specific growth rate of 0.05 h<sup>-1</sup>. For the strains HMP3337, HMP2818, HMP3355 and HMP2820, the feed medium contained, in addition to 36% w/v glucose solution and M9 media components, 15 g/L of tryptophan and it was added using the exponential feeding profile with specific growth rate of 0.05 h<sup>-1</sup>.

The duration of the feeding phase was up to 72h, depending on the strain used in the specific cultivation. The culture samples of 2 ml were withdrawn every 7h to track the progress of cultivation. The biomass concentration was determined using optical density measurement at the wavelength 600nm. The HPLC analysis was used for determination of residual medium components and metabolites concentrations. For determination of extracellular metabolites and residual sugars, 1 ml of the culture broth was centrifuged at 4000 rpm at 4 C° for 10 min and the supernatant filtered and injected into HPLC. For determination of total metabolite concentration, 500 pi of culture broth was mixed with 500 pi of acetonitrile: isopropanol solvent mixture (ratio 1:1) and vigorously vortexed for 30 seconds and frozen at -80 C°. The frozen samples were thawed and extracted at room temperature by shaking at 2000 rpm for 30 min. Subsequently, the samples were centrifuged at 4000 rpm and 4 C° for 10min. The supernatant was filtered and injected into HPLC.

#### HPLC analytics

The same HPLC protocol was used to separate and detect melatonin pathway intermediates and possible by-products, including melatonin, tryptophan, 5-hydroxytryptophan (5-HTP), serotonin, N-acetylserotonin, and N-acetyl-tryptamine.

N-acetyl-tryptamine was purchased from Santa Cruz Biotechnology (Dallas, Texas, US). All other compounds were purchased from Sigma Aldrich (St. Louis, Missouri, USA).

Water for all solutions was purified in a Milli-Q system (Millipore, Milford, MA, US). LC-MS grade acetic acid (Sigma Aldrich, St. Louis, Missouri, USA) and Acetonitrile (Merck KGaA, Darmstadt, Germany) was use for solutions and eluents.

A Dionex 3000 HPLC (Thermo Fisher Scientific, Waltham, Massachusetts, US) was equipped with a Zorbax Eclipse Plus C18 (4.6x100mm, 3.5µm) column from Agilent Technologies and a precolumn filter from Phenomenex (Torrance, CA, US). The column temperature was set at 30°C. The mobile phase consisted of a A: 0.05% (v/v) acetic acid in MilliQ water and B: acetonitrile in the following gradient program: 0 min 95%A decreasing to 38.7%A in 9.4 min. and holding for 0.6 min Then returning to initial composition after 11 min and holding for 1 min. The total run time was 12 min. The flow rate was constant at 1 ml/min and injection volume was 1µl. Detection was done by UV at wavelength 210 nm, 240 nm, 280 nm and 300 nm and 3D UV scan.

Calibration standards were prepared by diluting stock solutions of the investigated compounds. Matrix-matched standards were used to prevent matrix effects, and were

prepared by mixing standards with spent media. The concentration of the calibration standards were: 0.5, 1, 2.5, 5, 10, 25, 50, 100, 250, 500, 750, 1000, 2500, 5000 mg/L. All solutions were kept in a freezer (-18°C) when not in use. Calibration standards were prepared freshly every week.

- 5 Data were processed by Chromeleon 7.1.3 software (Thermo Fisher Scientific, Waltham, Massachusetts, US) and the concentration of melatonin and melatonin pathway intermediates were calculated by bracketing calibration from standard curves.

## Results

### Growth of *E. coli* in the presence of melatonin

- 10 In order to elucidate whether melatonin production at high titers is toxic to *E. coli*, we tested the growth of *E. coli* strain in the presence of different concentrations of melatonin. Melatonin was externally added into M9 medium supplemented with 0.2% glucose and the growth curves were monitored. As shown in Figure 2, high concentrations of melatonin inhibited *E. coli* growth. Compared to the control, the biomass yield decreased ~30% at 2 g/L melatonin,  
15 and further decreased about 30% at 7 g/l melatonin.

### Identification of melatonin transporters

- To identify any melatonin-specific native transporters in *E. coli*, an *E. coli* knockout library of 430 strains was screened, testing cell growth in the presence of 4 g/L (or 5 g/L) melatonin using the Growth Profiler. In such a screen, improved growth parameters (especially higher  
20 growth rate and/or lower lag time) compared to wild-type strain can be expected in case an importer is knocked-out, whereas decreased growth parameters can be expected in case an exporter is knocked-out (Figure 3).

- From the results of the first screening round, 30 candidate transporter targets could be identified. In a second round, the growth test was repeated in triplicates. From the triplicates  
25 runs, 5 transporters responsible for melatonin export were identified - YhjV, GarP, ArgO, AcrB and LysP (Table 1).

No importers were identified. Without being limited to theory, it is possible that there were too many importers for the loss of one to be noticeable in this assay.

Result of Growth Profiler screening for 5 target transporter candidates:

The growth curves of the five selected transporter candidate deletion strains (target candidate knocked-out Keio strains) were accessed after running growth profiler experiments as mentioned above. With the exception of the *garP* deletion strain, deletion of the  
5 transporter candidate gene did not have any effect on the growth rates when the strains were grown in M9+0.2% glucose media supplemented with 4% ethanol. However, when the strains were grown in the presence of 4 g/L of melatonin in media, the growth rate was decreased for each of the 5 strains, with the most prominent effects resulting from the *acrB* (no growth at all), *yjhV* (longer lag time and reduced growth rate) and *garP* (longer lag time  
10 and reduced growth rate) deletions. The *lysP* and *argO* deletion strains also showed reduced growth rates but not much difference in lag time as compared to the BW25113 control strain (Figure 4).

These results show that knocking out these genes can increase the toxicity of externally supplied melatonin to the host cells, presumably because the knockouts are less able to  
15 export melatonin taken up by the cells.

Overexpression of melatonin transporters to improve melatonin production

In order to increase the tolerance of *E. coli* to melatonin, each transporter was overexpressed in a low copy number plasmid. In order to tune the expression level of each transporter, degenerate sequences in ribosome binding sites (RBS) were introduced to obtain a diversity  
20 of expression levels and subjected for selection in the presence of melatonin (Figure 5). The 5' untranslated region (5'UTR) containing RBS sequence was tcttaatcatgcnngannnttaacttt (SEQ ID NO: 18) and it was cloned upstream of the coding sequence of each gene. The plasmid libraries were transformed into strain HMP2993 (Table 5) together with pHM345 containing some pathway genes (Table 5) and cultured in the presence of 5 g/L melatonin.

25 Nine to 16 isolates were obtained for each transporter and their growth profiles and melatonin production tested. The growth profiles of strains with overexpression of the transporter genes are illustrated in Figure 6, with YhjV as an example. Small-scale production assay (400 pi cultures) was performed to test melatonin production of the selected isolates (Figure 6). Strains carrying GarP, AcrAB, YhjV and ArgO showed improved melatonin titers,  
30 whereas LysP did not show significant improvement with any selected RBS sequences.

Four strains containing GarP, AcrAB, YhjV, ArgO overexpression plasmids were further tested in fed-batch fermentation (250 ml culture). For each transporter gene, the RBS sequence giving rise to the highest biomass melatonin yield in Figure 6 was selected (Table 7).

5 The final titers of fed batch fermentation of each strain as well as the control are listed in Table 8. One of the transporters, YhjV, increased melatonin titers from 3.9 to 5 g/L. We further tested this transporter in 2 other background strains by re-transforming the plasmid pHM635 containing the *yhjV* gene. In both cases, we observed 15%-40% increases of melatonin titers. The details of melatonin production during fed-batch fermentation of YhjV overexpression in 3 different background strains are shown in Figure 7.

10 *Table 7. RBS sequences used*

Transporter	5'UTR sequence	SEQ ID NO:
GarP	tcttaatcatgcgggggagtgtaacttt	19
AcrAB	tcttaatcatgcggttgaggattaacttt	20
YhjV	tcttaatcatgccggggacggtaacttt	21
ArgO	tcttaatcatgctggggagggttaacttt	22

*Table 8. Melatonin titers in fed-batch fermentation with novel transporters implemented*

Strain	Transporter	Final titer (g/L)
HMP2818	control	3.9
HMP3333	GarP	3.6
HMP3335	AcrAB	3.9
HMP3337	YhjV	5.0
HMP3339	ArgO	2.7

## EXAMPLE 2

This Example shows that overexpression of YhjV transporter improved 5HTP production.

An *E. coli* 5FITP-production strain was generated, and the effect of the YhjV transporter on 5HTP production tested. Briefly, the plasmid providing YhjV overexpression (pHM635) was transformed into a 5FITP production strain (FIMP3403), resulting in strain FIMP3404. In a small-scale production assay (GSR medium supplemented with 500 mg/L tryptophan and appropriate antibiotics as described materials and methods in Example 1), the new strain overexpressing YhjV improved 5FITP titers by 15% compared to the control strain (Figure 8).

#### EXAMPLE 3

This Example shows that complementation of YhjV restored melatonin-tolerance of *yhjV* knockout strain. Briefly, we complemented *yhjV* knockout strain with plasmid pHM635 expressing YhjV constitutively. The growth was tested in growth profiler in M9 medium supplemented with 0.2% glucose. As shown in Figure 9, pHM635 restored the growth of *yhjV* knockout strain to the same level as the wild type in the presence of 4g/L melatonin. This confirms that YhjV is responsible for melatonin export.

#### EXAMPLE 4

This Example describes testing the influence of YhjV on the uptake of amino acids. The growth test was performed using M9 medium supplemented with 0.2% glucose and 1 mM tryptophan, as well as appropriate antibiotics (100mg/L ampicillin or 50mg/L kanamycin). The growth curves were monitored with Growth Profiler (EnzyScreen, Heemstede, Netherland) at 30 °C and 250 RPM for 48 h.

We first tested how YhjV transporter affects the growth of *E. coli* tryptophan. We followed the growth curves of *E. coli* wild type BW25113 (HMP0\_wt), YhjV knockout (HMP3406\_YhjV KO) and YhjV overexpression (HMP3405\_YhjV O. E., made by transforming pHM635 into HMP0) in M9 medium containing 0.2% glucose supplemented with 1 mM tryptophan.

We found that when growing in the presence of tryptophan, YhjV knockout led to a growth defect, resulting in a much lower biomass yield compared to the wild type (Figure 10). These results support that YhjV is also responsible for tryptophan uptake.

#### EXAMPLE 5

This example describes using random mutagenesis and screening to identify YhjV mutants resulting higher melatonin tolerance.

## Materials and Methods

### Random mutagenesis of YhiV

To generate error-prone PCR libraries of YhjV gene, we used pHM635 as the template, and primers HP-1647 (ggcagcagcctaggttaatttta) and HP-1652 (ccggggacgggtaactttatg) for  
5 amplification of the YhjV coding sequence. GeneMorph II Random Mutagenesis Kit (Agilent, Santa Clara, California, United States) was used for error-prone PCR. In order to generate the plasmid libraries containing random mutagenesis library of YhjV, we performed a circular PCR using the error-prone PCR as a mega-primer and plasmid pHM635 as a template. Phusion high fidelity DNA polymerase (Thermo Fisher Scientific, Waltham, Massachusetts,  
10 US) was used for the circular PCR. The resulting PCR/plasmid library was treated with DpnI restriction enzyme (Thermo Fisher Scientific) to remove the template plasmid. Finally, the DpnI treated PCR library was diluted 5 times with water and transformed into OneShot Top10 cells (Thermo Fisher Scientific) by electroporation. To check the quality of the library, 1 pi of transformants was plated on LB+amp agar plates to estimate the library size. We also  
15 sequenced 8 random colonies and 5 of them contain 1-3 mutations. The rest of the transformants were cultivated in 50ml liquid LB medium supplemented with ampicillin (100 pg/ml) overnight. The plasmids were prepared from this cell culture using QIAprep spin miniprep kit (Qiagen, Hilden, Germany). The plasmids were stored as the random mutagenesis library of YhjV.

### 20 Screening of beneficial mutants

To screen for beneficial mutants of yhiV from the random mutagenesis library, we transformed the random mutagenesis library into a background strain HMP2993 (Table 5). The transformants were selected by growing in M9+0.2% glucose medium supplemented with 4g/L or 5g/L melatonin, 0.2% glucose and 100pg/ml ampicillin at 30 °C for 24h to  
25 enrich cell population containing YhjV mutants that improve melatonin tolerance. The culture was spread on LB+ampicillin agar plates to obtain single colonies. We picked 89 colonies randomly and tested their growth again in the presence of melatonin. All 89 colonies as well as the control strain (pHM635 transformed in HMP2993) was inoculated into 400 pi of M9+0.2% glucose media supplemented with ampicillin (100 pg/ml) in 96 deep-well plate.  
30 Then, the plate was incubated at 30 °C with 300 rpm overnight (incubated for 24 h). The following day, the saturated pre-cultures were diluted 100 fold into 300 pi of M9+0.2% glucose media (and ampicillin 100 pg/ml) supplemented with 4.5g/L melatonin in 96 well MTP plate. Note that melatonin stock solution was prepared by dissolving in 75% ethanol to 170g/L. The culture plates were incubated in Growth Profiler (EnzyScreen, Heemstede,

Netherland) at 30 °C and 250 RPM for 48 h. Using the GP software (GP960Viewer version 1.0.0.4, EnzyScreen, Fleemstede, Netherland), the pictures data was converted into digital OD600nm values.

### Results

- 5 The random mutagenesis library of YhjV was generated and screened as described in materials and methods. Seven out of selected 89 colonies showed improved tolerance toward melatonin and carry mutations in *yhjV* gene as shown in Table 9. The growth presented by OD600 difference between 48h and 0h in the presence of 4.5 g/L melatonin was shown in Figure 11A. The 7 strains carry YhjV mutations showed better growth compared to the
- 10 control which is HMP2993 transformed with pHM635 containing wild-type YhjV. In order to test the melatonin production of these mutants, we transformed another plasmid pHM345 (containing pathway genes, Table 5) and performed a small-scale production assay (see Example 1 materials and methods for details). The V176M mutant (pHM679) showed increased melatonin titer after 24h of cultivation (Figure 11B). We conclude that the
- 15 efficiency of YhjV can be further improved by mutagenesis to benefit the tolerance or production of targeted chemicals.

*Table 9. YhjV mutations accumulated in high melatonin screening*

Plasmid ID	Mutation in YhjV protein
pHM670	G108W
pHM671	A260V
pHM672	F187L
pHM676	I182T
pHM679	V176M
pHM680	I151F

## LIST OF REFERENCES

UniProtKB - P37660 (YHJV\_ECOLI)

Kell, "Control of metabolite efflux in microbial cell factories: current advances and future prospects". Available at: [www.osf.io/7t8gm/online](http://www.osf.io/7t8gm/online) [Accessed 1 Oct 2018]

- 5 Sargentini et al., Mutation Research 793-794 (2016) 1-14.  
EP2267145 AI (Evonik Degussa GmbH)  
WO 2013/093737 AI (BASF)  
WO 2013/127915 AI (Danmarks Tekniske Universitet)  
WO 2015/032911 AI (Danmarks Tekniske Universitet)  
10 WO 2017/167866 AI (Danmarks Tekniske Universitet)  
WO 2017/202897 AI (Danmarks Tekniske Universitet)  
WO 2018/037098 AI (Danmarks Tekniske Universitet)  
WO 2018/108966 AI (Danmarks Tekniske Universitet)

## CLAIMS

1. A recombinant cell comprising a biosynthetic pathway for producing an indole-derivative, optionally selected from melatonin, N-acetylserotonin, serotonin and 5-hydroxytryptophan (5HTP),
  - 5 wherein the recombinant cell is genetically modified to overexpress a gene encoding an integral membrane protein selected from YhjV (SEQ ID NO:2), GarP (SEQ ID NO:4), ArgO (SEQ ID NO:6), AcrAB (SEQ ID NOS: 10 and 12) and LysP (SEQ ID NO:8), a functionally active variant and/or fragment of any thereof, or a combination of any two or more thereof, and
  - 10 wherein the amino acid sequence of the variant has a sequence identity of at least about 80 % to the amino acid sequence of the integral membrane protein and the fragment comprises at least about 80 % of the amino acid sequence of the integral membrane protein.
2. The recombinant cell according to claim 1, overexpressing a gene encoding YhjV, GarP, ArgO or AcrAB, or a combination of any two or more thereof.
- 15 3. The recombinant cell according to any one of the preceding claims, overexpressing a gene encoding YhjV or a functionally active variant thereof.
4. The recombinant cell according to any one of the preceding claims, wherein the biosynthetic pathway comprises
  - (a) an L-tryptophan hydroxylase;
  - 20 (b) an L-tryptophan hydroxylase and a 5HTP decarboxylase;
  - (c) an L-tryptophan hydroxylase, a 5HTP decarboxylase, and a serotonin acetyltransferase;
  - (d) an L-tryptophan hydroxylase, a 5HTP decarboxylase, a serotonin acetyltransferase and an acetylserotonin O-methyltransferase;
  - 25 (e) a tryptophan decarboxylase and a tryptamine 5-hydroxylase;
  - (f) a tryptophan decarboxylase; a tryptamine 5-hydroxylase, and a serotonin acetyltransferase; or

(g) a tryptophan decarboxylase; a tryptamine 5-hydroxylase, a serotonin acetyltransferase and an acetylserotonin O-methyltransferase.

5. A recombinant cell capable of producing a second indole-derivative from a first indole-derivative via a biosynthetic pathway, wherein the recombinant cell is genetically modified to  
5 overexpress a gene encoding YhjV (SEQ ID NO:2), or a functionally active variant and/or  
fragment thereof,

wherein the amino acid sequence of the variant has a sequence identity of at least about 80% to SEQ ID NO:2 and the fragment comprises at least about 80% of SEQ ID NO:2.

6. The recombinant cell according to claim 5, overexpressing a gene encoding YhjV or a  
10 functionally active variant thereof.

7. The recombinant cell according to claim 5 or 6, wherein the first and second indole-derivatives are:

(a) tryptophan and melatonin, respectively;

(b) tryptophan and N-acetylserotonin, respectively;

15 (c) tryptophan and serotonin, respectively; and

(d) tryptophan and 5-hydroxytryptophan, respectively.

8. The recombinant cell according to

(a) any one of claims 1 to 4, wherein the overexpression of the gene encoding  
20 the integral membrane protein provides for an increased production of the  
indole-derivative, an increased tolerance to the indole-derivative, or both,  
by the recombinant cell as compared to a non-modified control cell;

(b) any one of claims 5 to 7, wherein the overexpression of the gene encoding  
YhjV or the functionally active variant and/or fragment thereof provides for  
an increased production of the second indole-derivative, an increased  
25 tolerance to the second indole-derivative, or both, by the recombinant cell  
as compared to a non-modified control cell.

9. The recombinant cell according to any one of the preceding claims, wherein the integral membrane protein is expressed from a transgene or from an upregulated endogenous gene.

5 10. The recombinant cell according to any one of the preceding claims, wherein at least one of the enzymes of the biosynthetic pathway is expressed from a transgene, optionally a heterologous transgene.

11. The recombinant cell according to any one of the preceding claims, which is a bacterial cell, a yeast cell, a filamentous fungal cell, an algal cell or a mammalian cell.

10 12. The recombinant cell according to claim 11, which is a bacterial cell, optionally of the family *Enterobacteriaceae*, such as an *Escherichia coli* cell.

13. A method for producing an indole-derivative selected from melatonin, N-acetylserotonin, serotonin and 5HTP, comprising the step of culturing the recombinant cell according to any one of the preceding claims in a culture medium comprising a carbon source, and optionally, isolating the indole-derivative.

15 14. The method of claim 13, wherein the medium further comprises tryptophan.

15. A method for producing a second indole-derivative from a first indole-derivative, comprising the step of culturing the recombinant cell of any one of claims 5 to 12 in a culture medium comprising a carbon source and the first indole-derivative, and optionally, isolating the indole-derivative.

20 16. A functionally active variant and/or fragment of YhjV (SEQ ID NO:2) having a sequence identity of at least about 80% to SEQ ID NO:2 and comprising a mutation in one or more amino acid residues selected from V176, G108, I151, F187, I182, C78, A260, P385, I155, N186, S268, S75 and K402.

25 17. The functionally active variant and/or fragment of YhjV according to claim 16, comprising a V176M, G108W, A260V, F187L, I182T or I151F amino acid substitution, or a combination of two or more such amino acid substitutions.

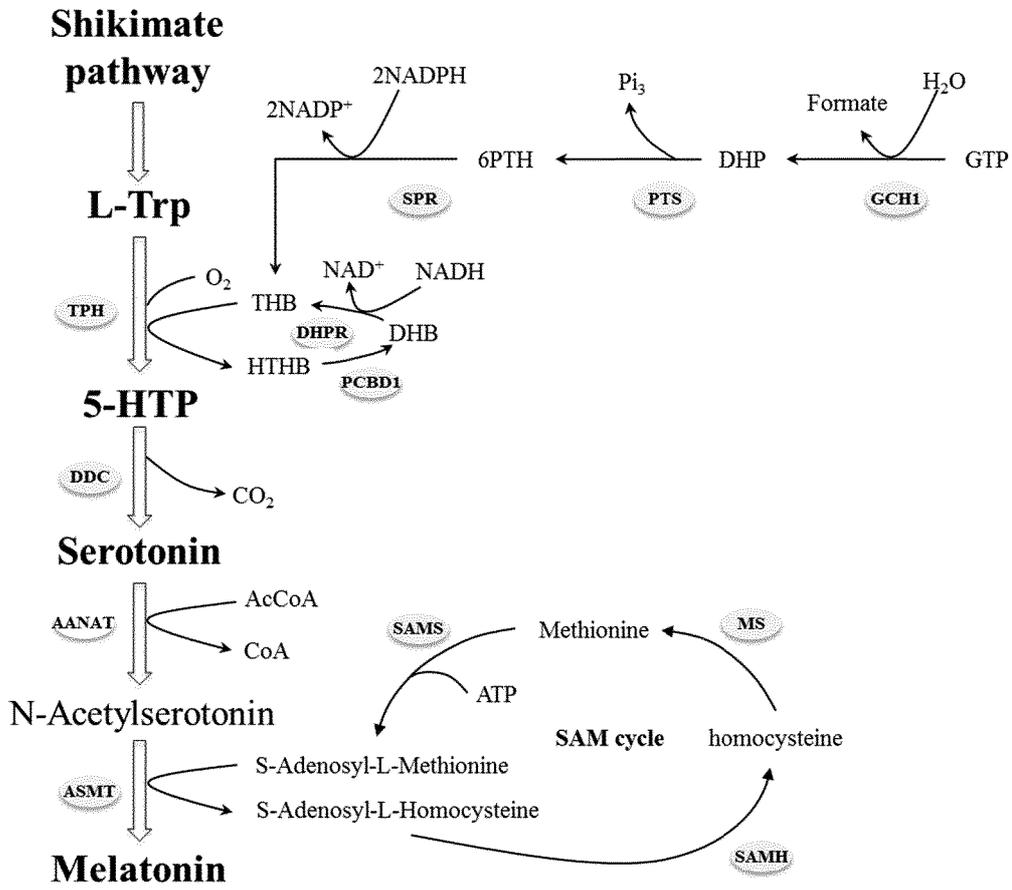


Fig. 1

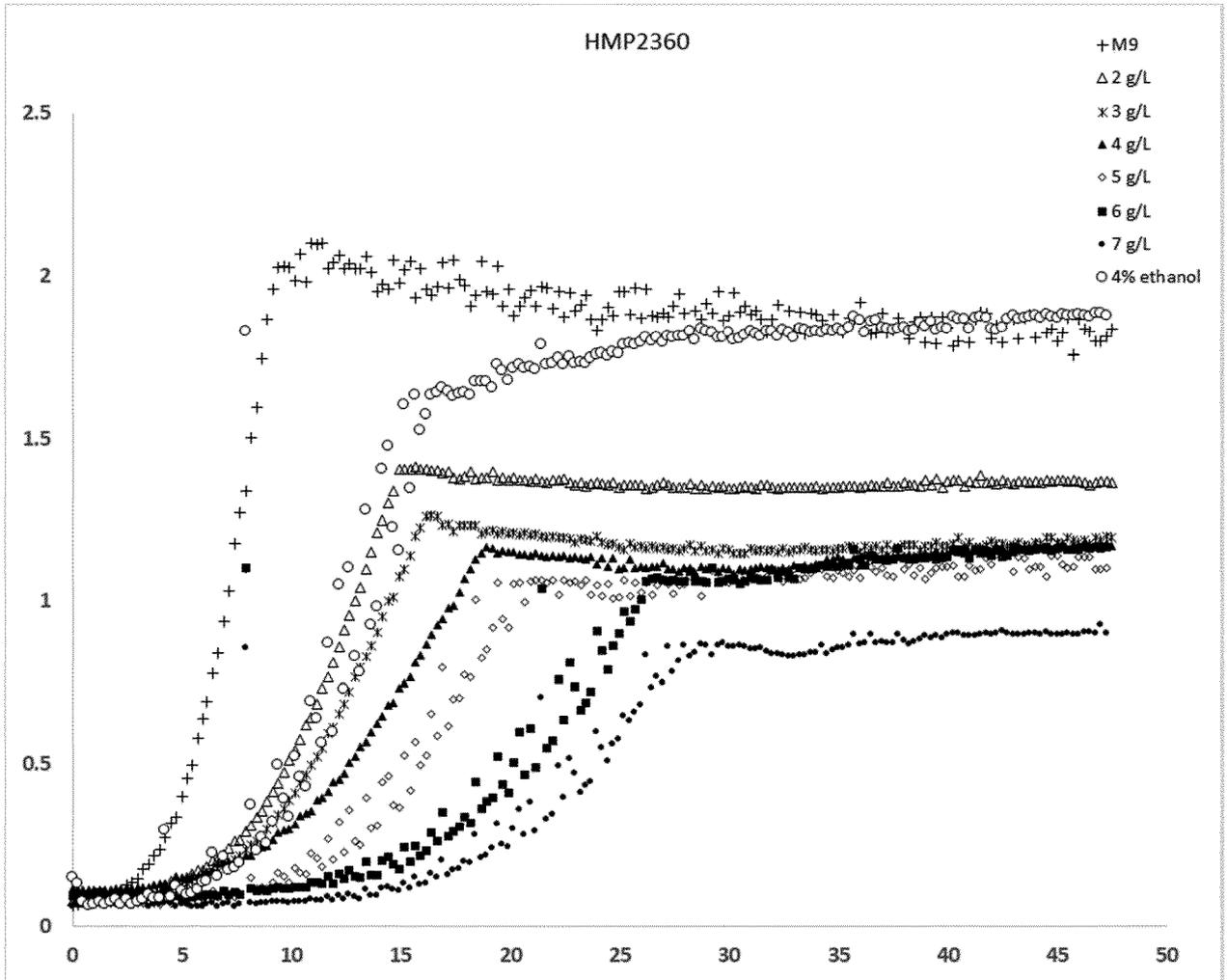


Fig. 2

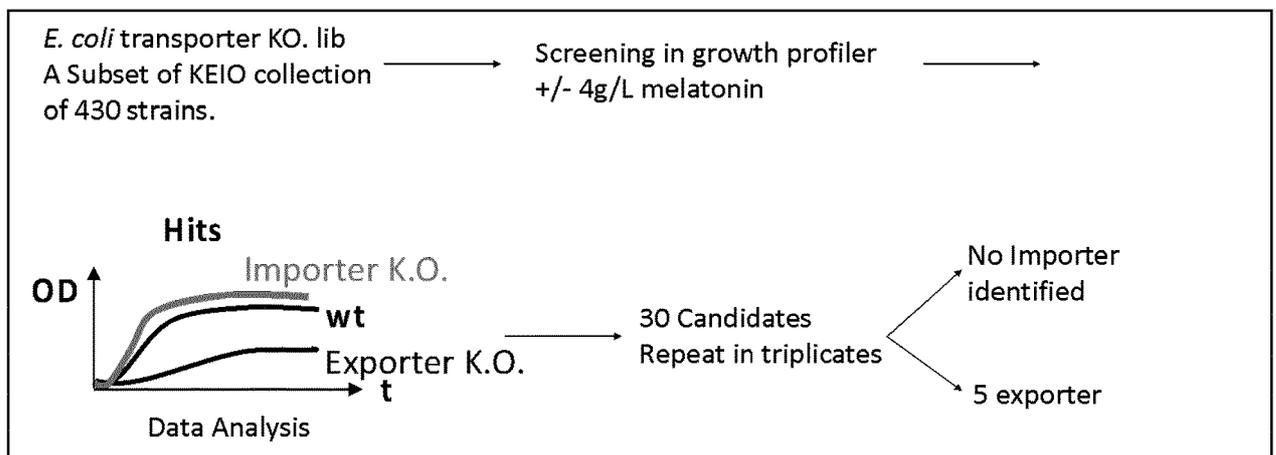


Fig. 3

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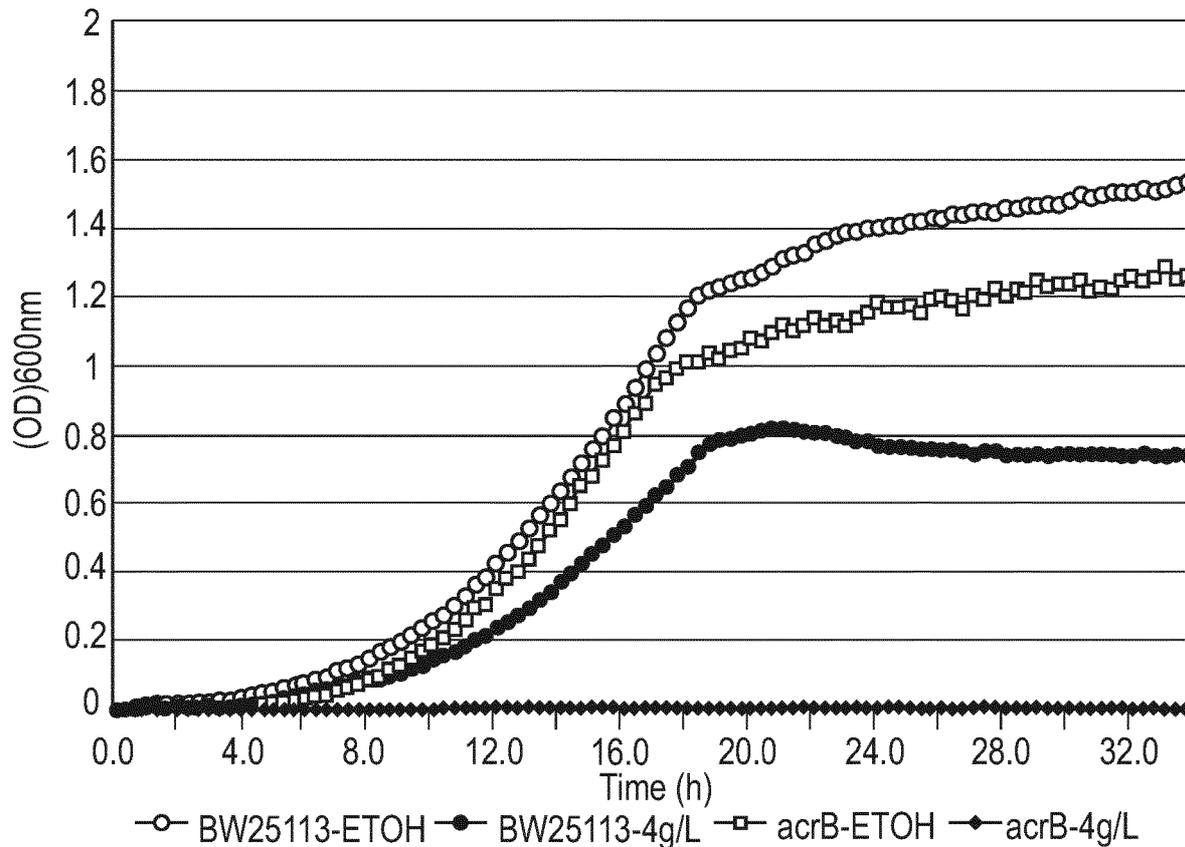


Fig. 4A

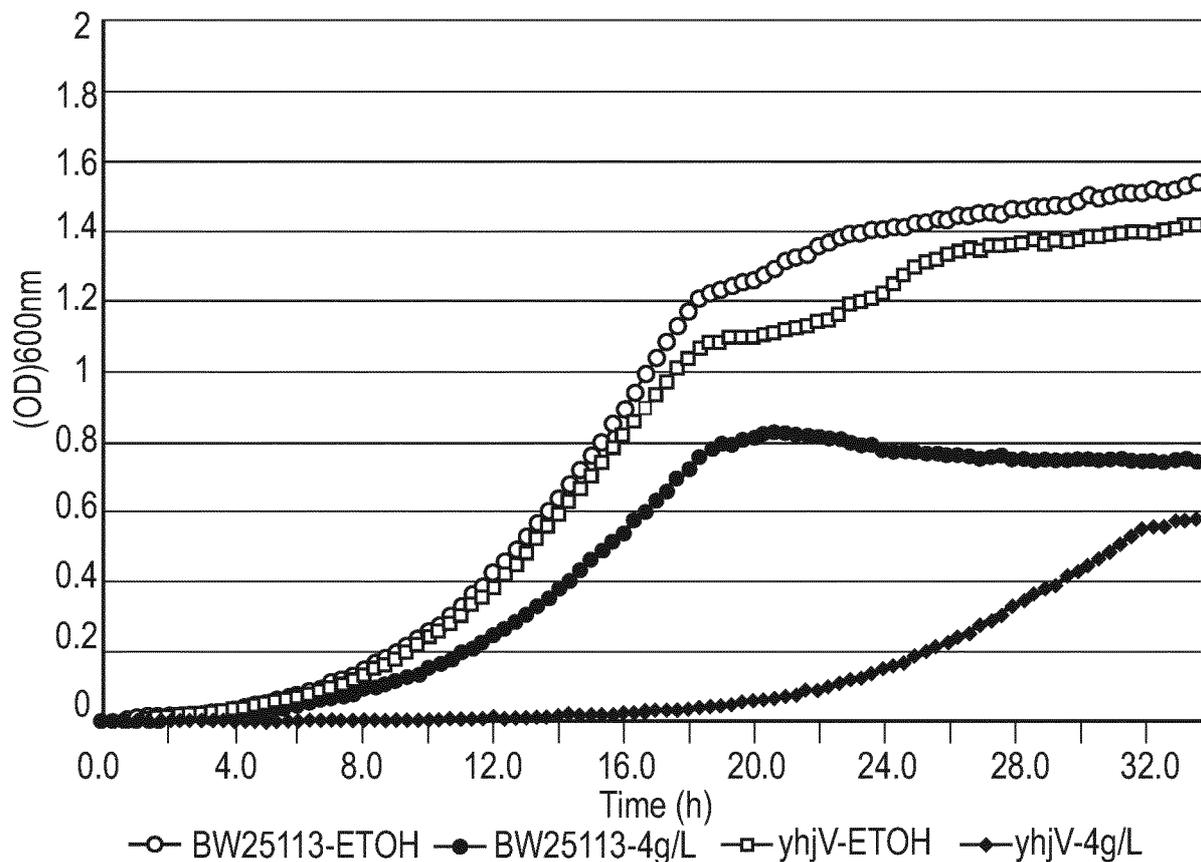


Fig. 4B

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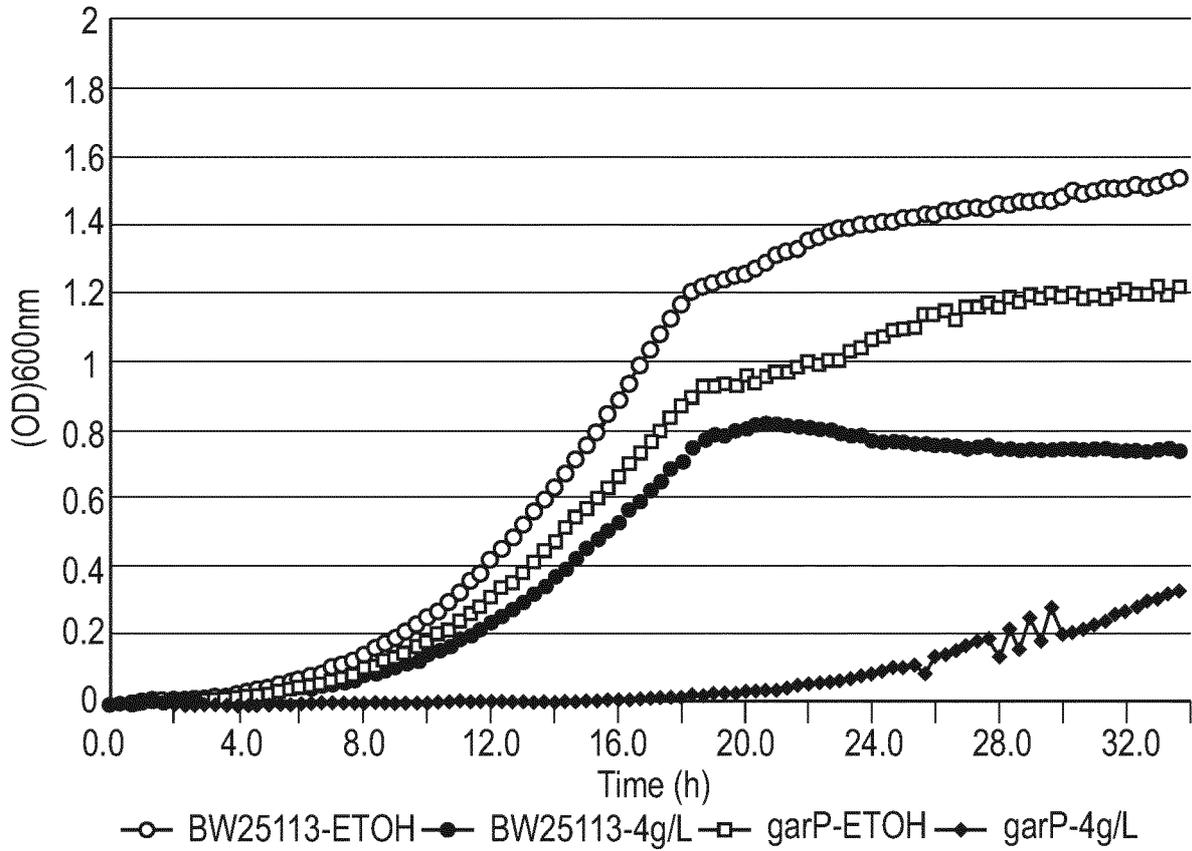


Fig. 4C

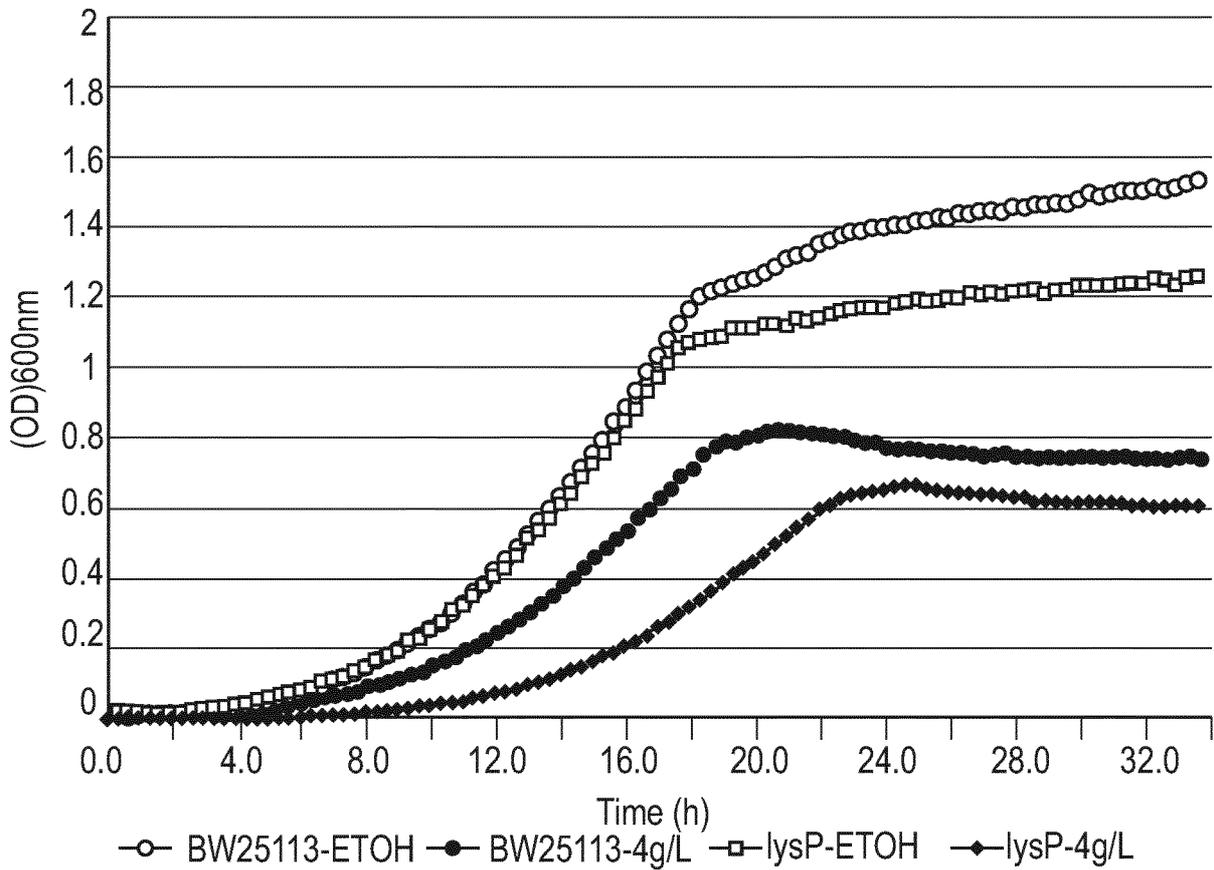


Fig. 4D

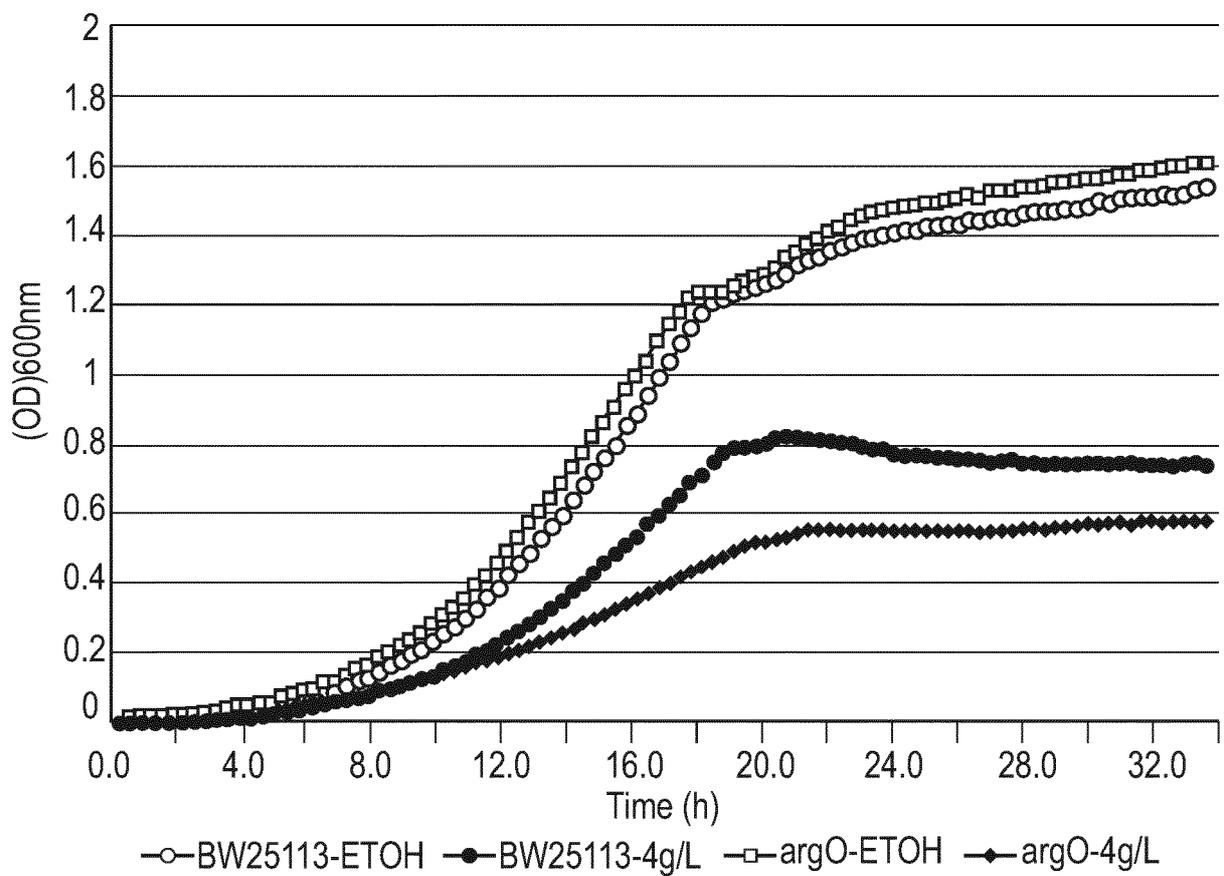
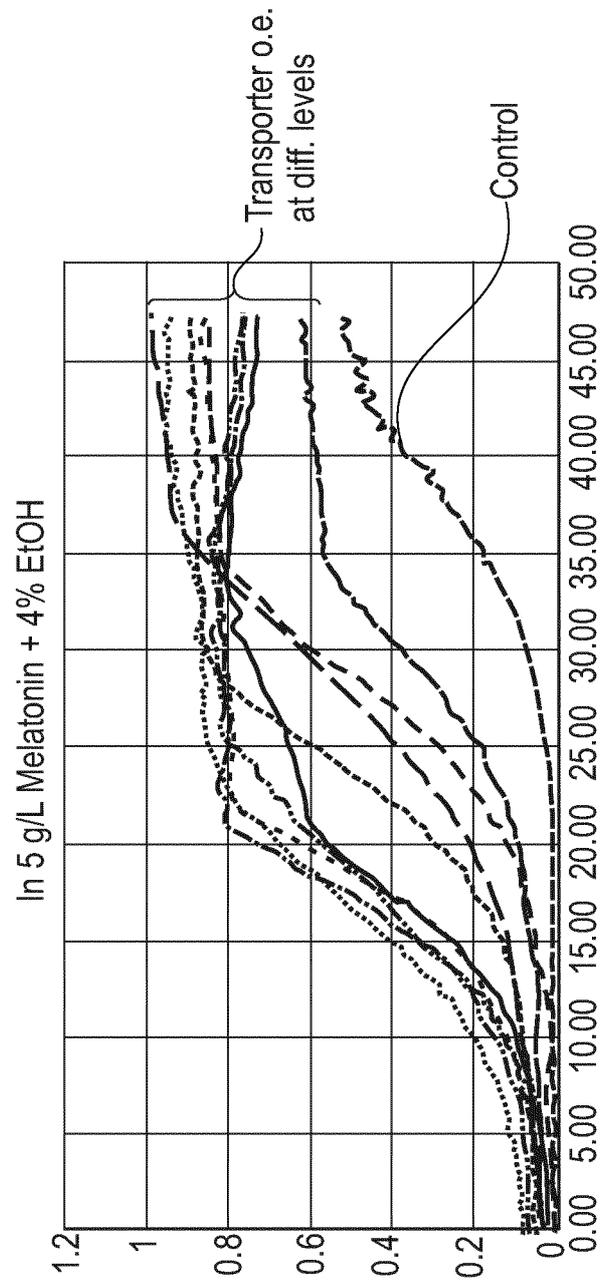
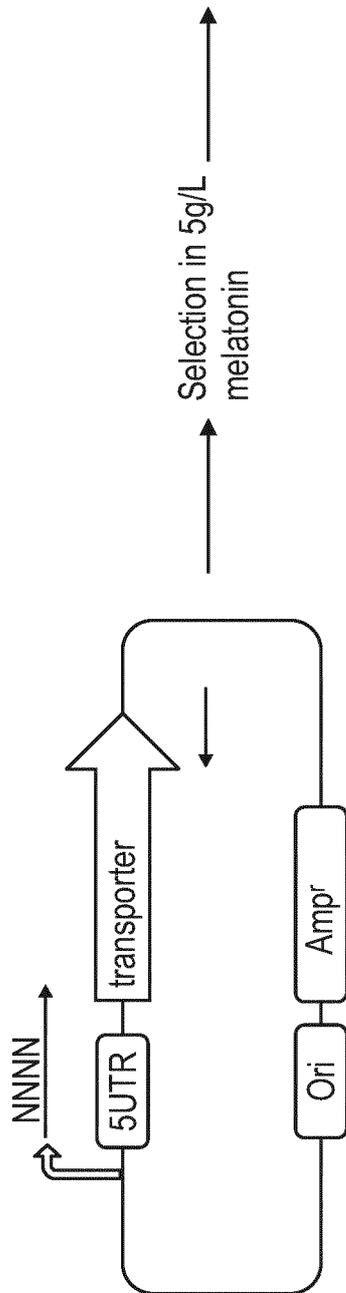


Fig. 4E



Overexpression of transporters improved strain tolerance to melatonin

Fig. 5

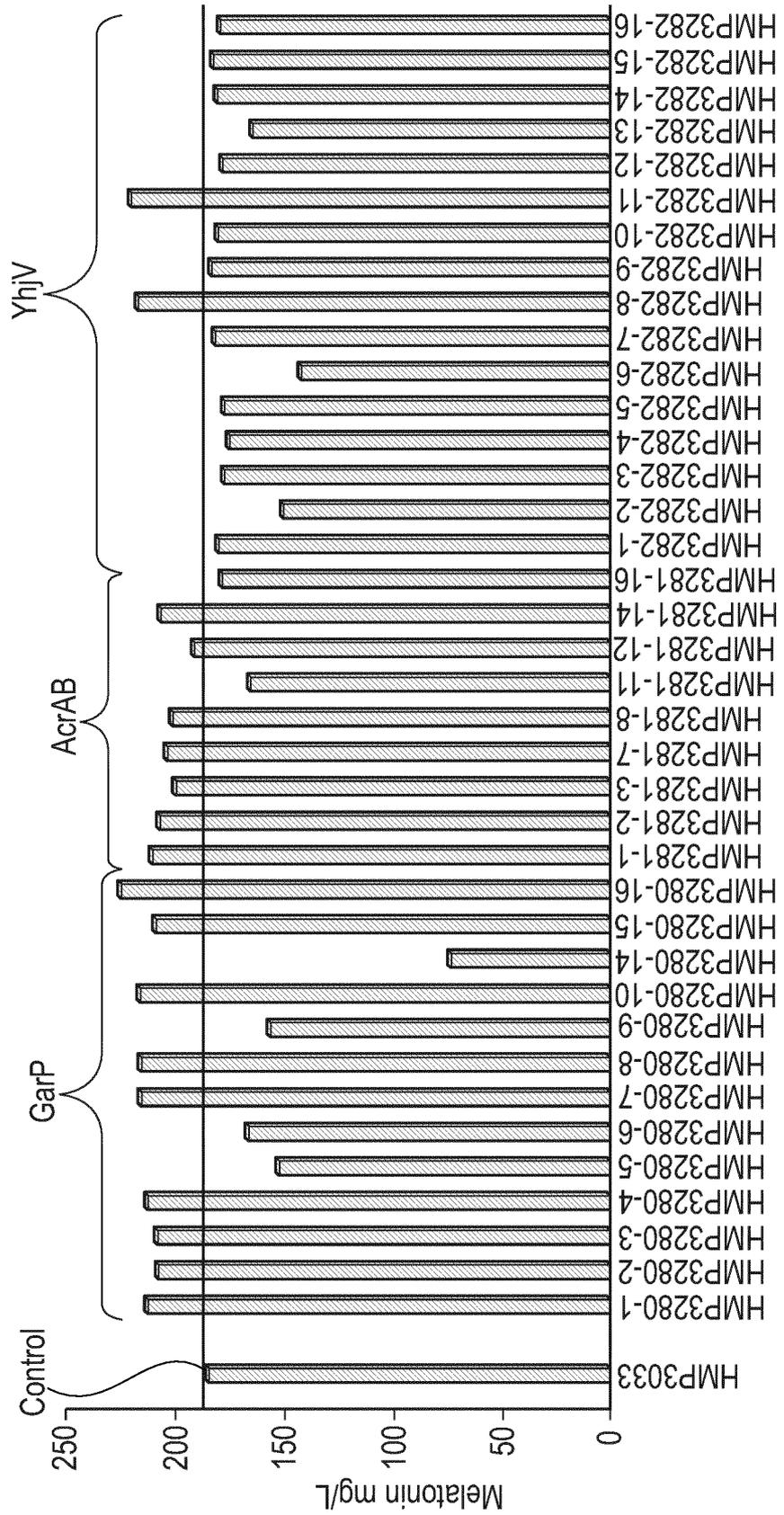


Fig. 6

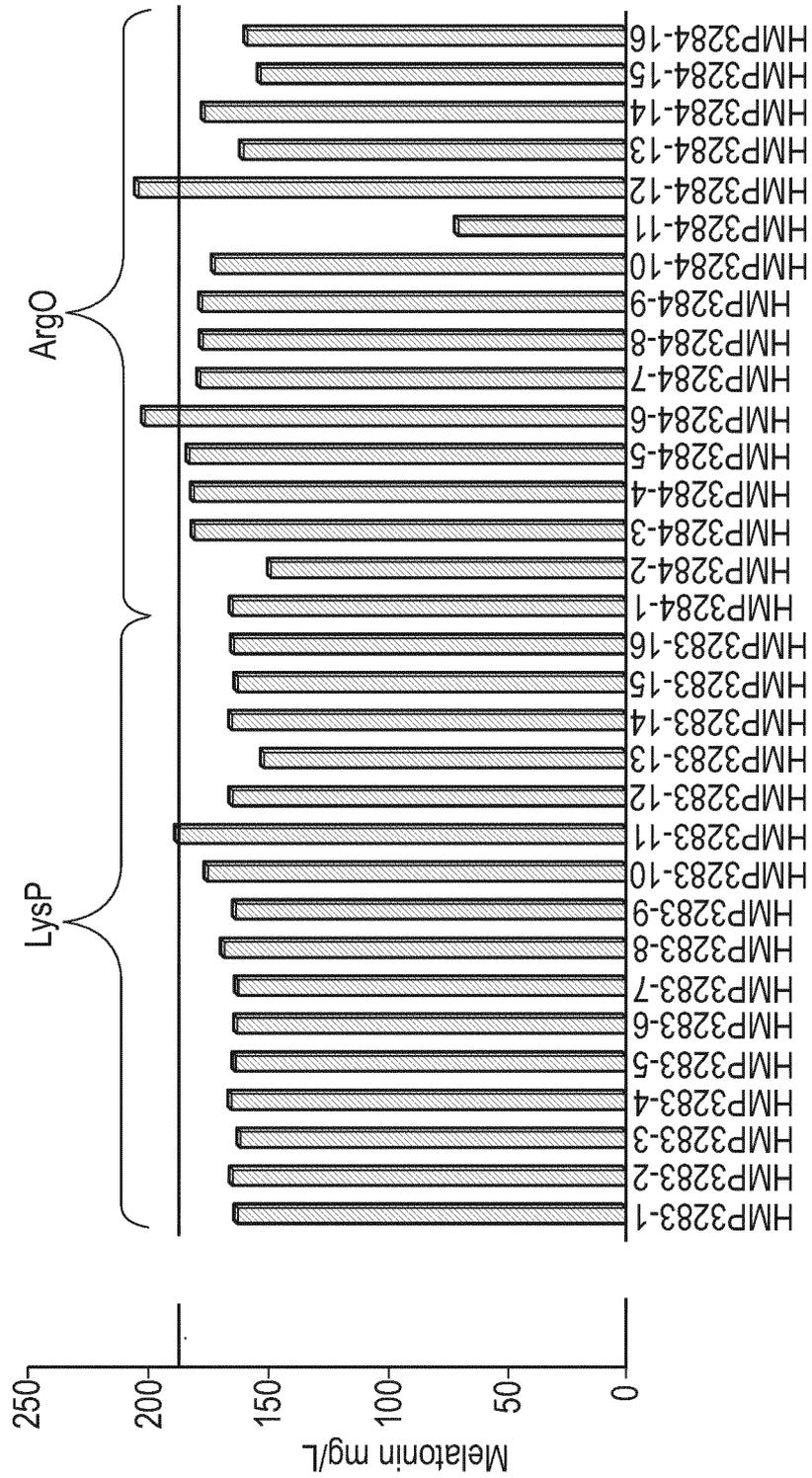
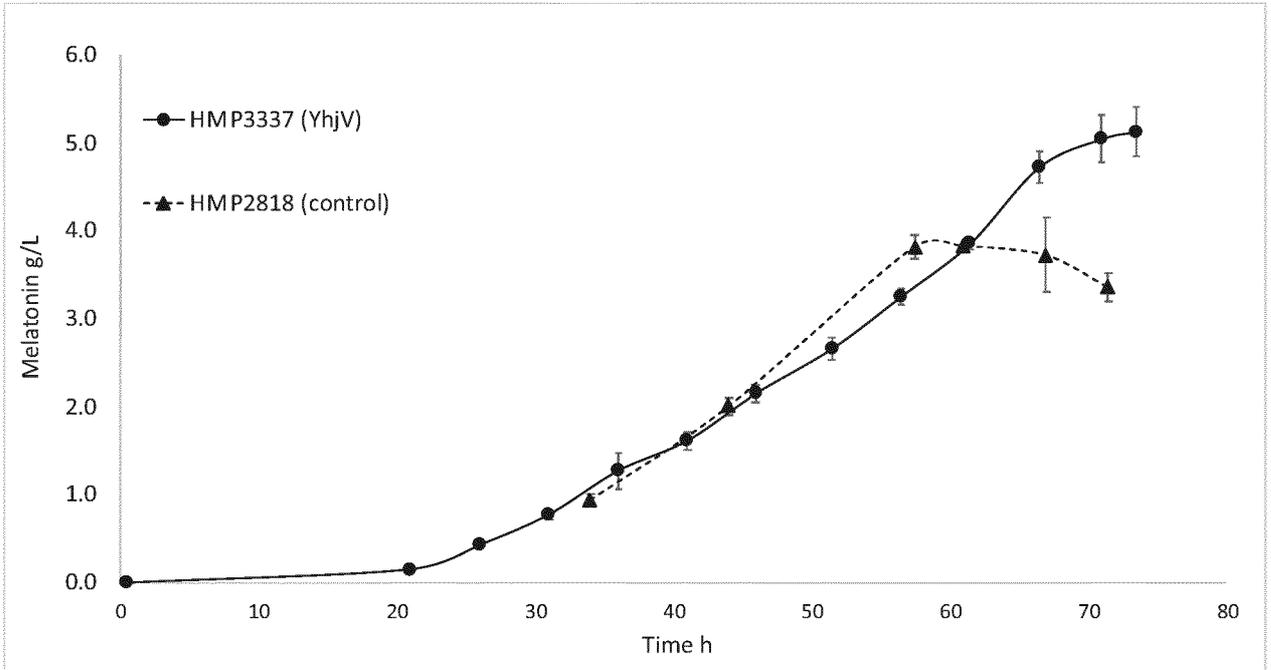


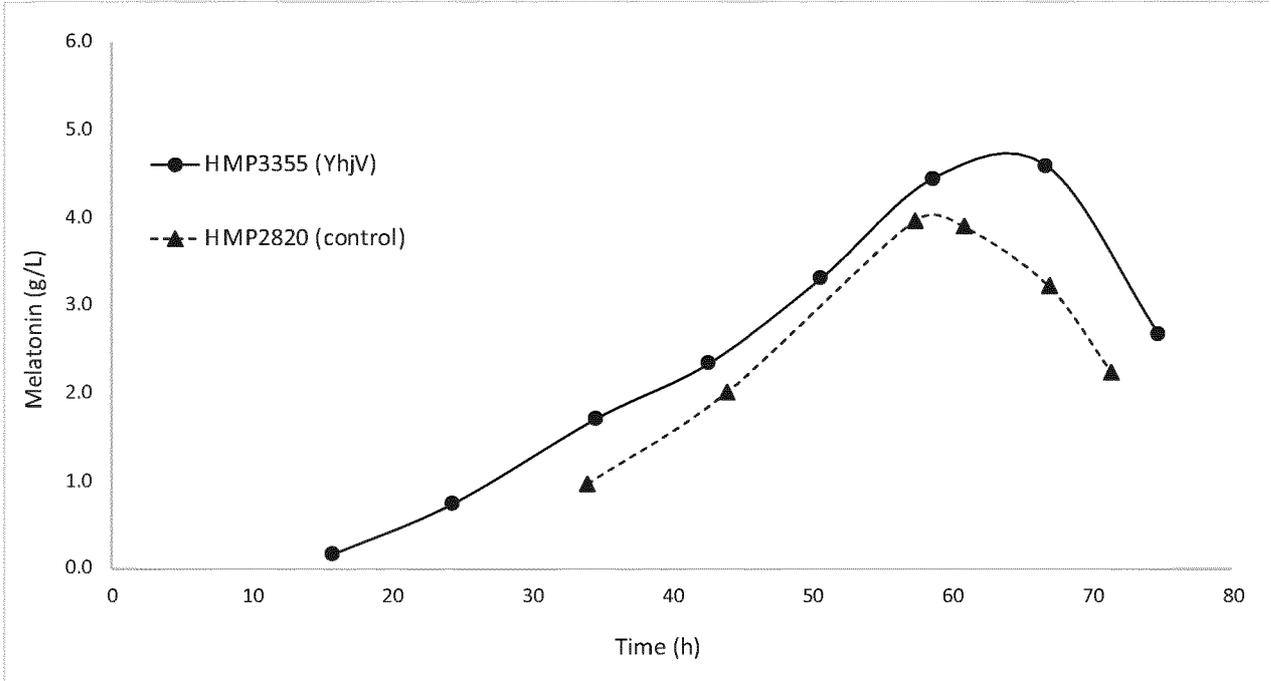
Fig. 6 (continued)

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**A**



**B**



**Fig. 7**

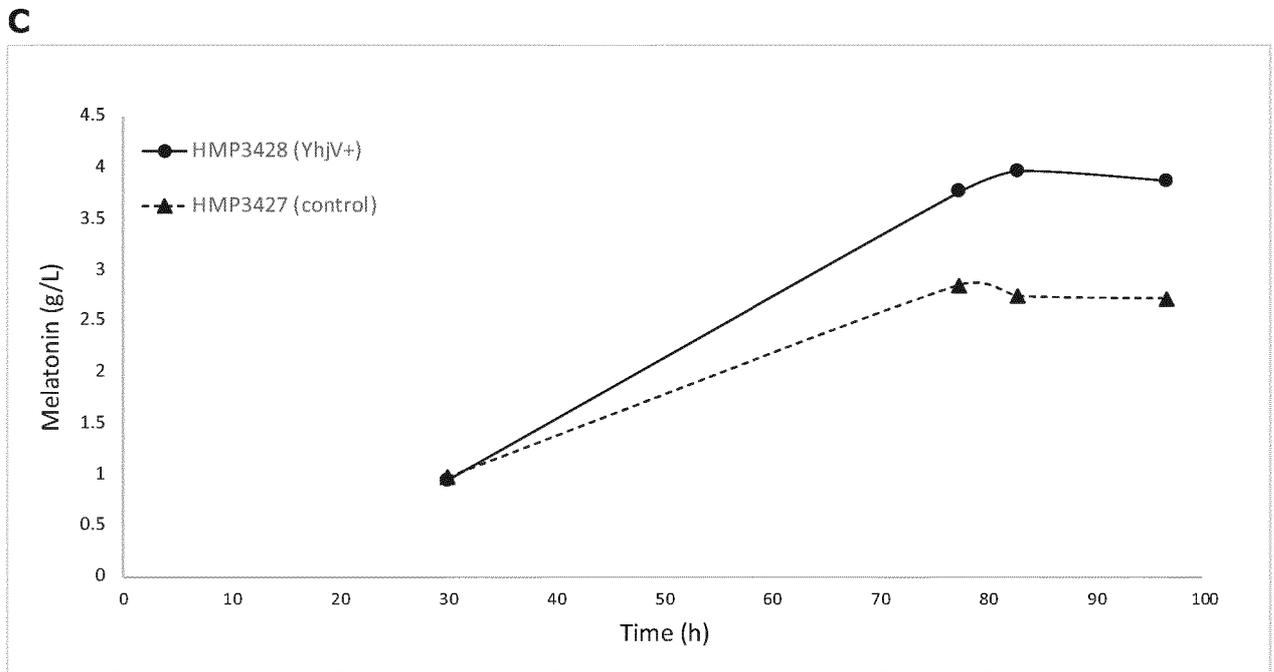


Fig. 7 (continued)

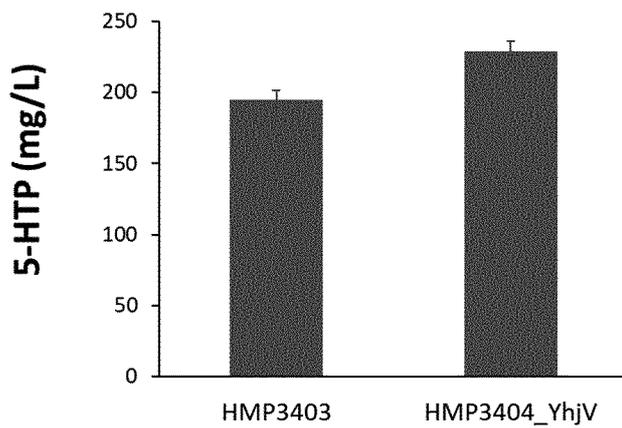


Fig. 8

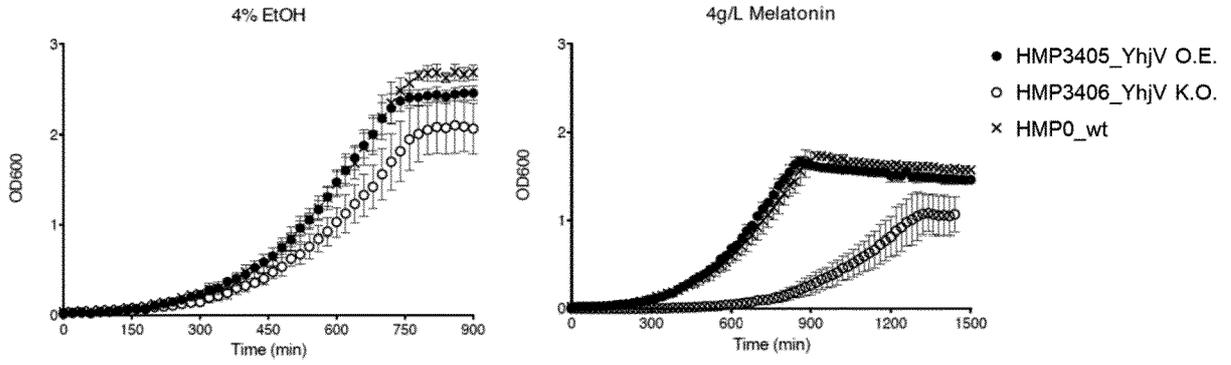


Fig. 9

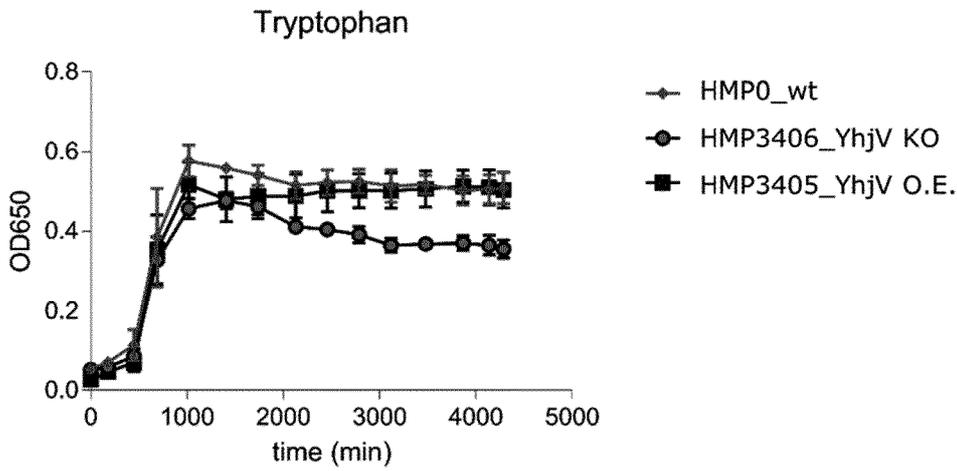


Fig. 10

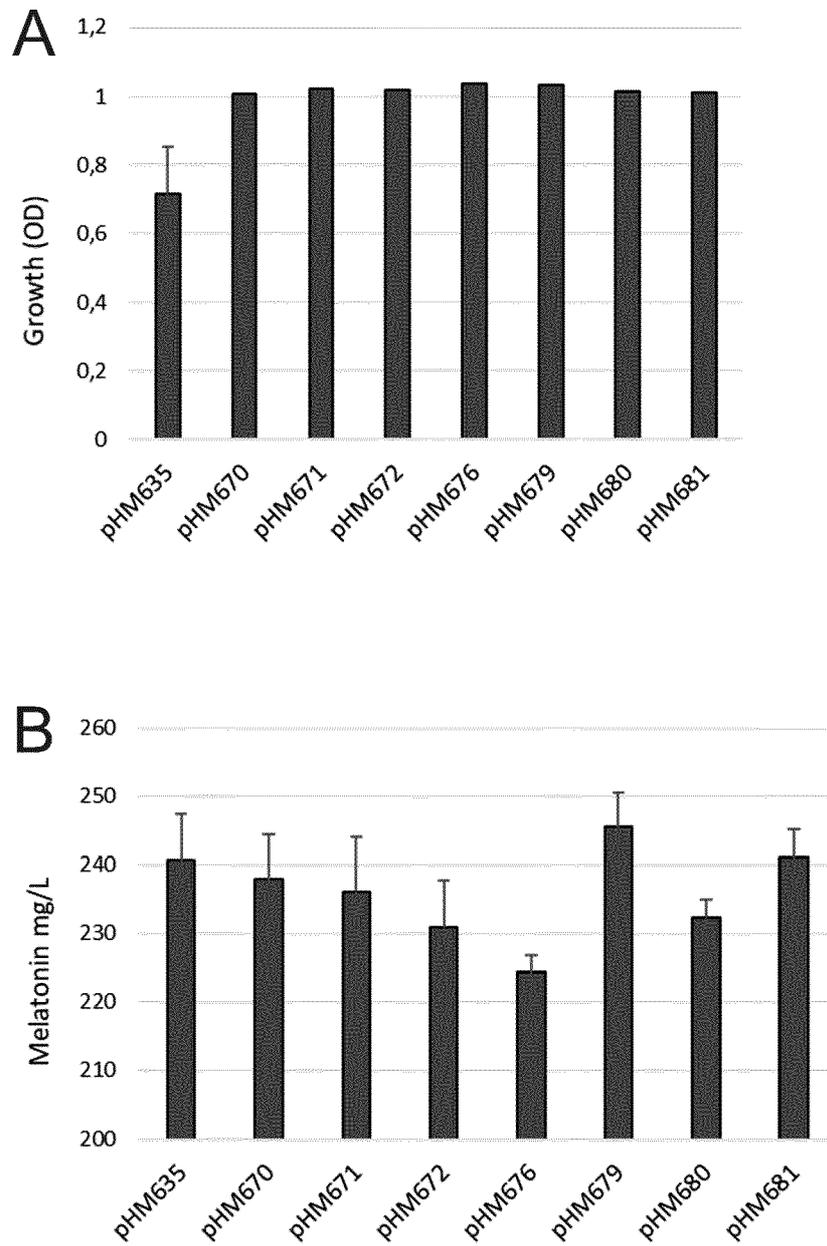


Fig. 11

INTERNATIONAL SEARCH REPORT

International application No  
PCT/EP2020/056828

A. CLASSIFICATION OF SUBJECT MATTER  
INV. C07K14/715  
ADD.  
  
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED  
Minimum documentation searched (classification system followed by classification symbols)  
C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
EPO-Internal , Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2013/127915 A1 (UNIV DANMARKS TEKNISKE [DK]) 6 September 2013 (2013-09-06) claims 1-19; figures 1-6,8-10 -----	1-15
A	WO 2006/113897 A2 (CARGILL INC [US]; LAPLAZA JOSE [US] ET AL.) 26 October 2006 (2006-10-26) claims 1-31 ----- -/--	1-15

Further documents are listed in the continuation of Box C.  See patent family annex.

\* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E" earlier application or patent but published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search  27 April 2020	Date of mailing of the international search report  15/05/2020
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Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer  Strobel, Andreas
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## INTERNATIONAL SEARCH REPORT

International application No  
PCT/EP2020/056828

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>JOSÉ-ANÍBAL MORA-VILLALOBOS ET AL: "Synthetic pathways and processes for effective production of 5-hydroxytryptophan and serotonin from glucose in Escherichia coli", JOURNAL OF BIOLOGICAL ENGINEERING, vol. 12, no. 1, 15 March 2018 (2018-03-15), XP055594649, DOI: 10.1186/s13036-018-0094-7 abstract; figures 1,3; table 2</p> <p>-----</p>	1-15
A	<p>WEN-JING YANG ET AL: "Overexpression of TaCOMT Improves Melatonin Production and Enhances Drought Tolerance in Transgenic Arabidopsis", INTERNATIONAL JOURNAL OF MOLECULAR SCIENCES, vol. 20, no. 3, 2 February 2019 (2019-02-02), page 652, XP055594657, DOI: 10.3390/ijms20030652 abstract; figure 2</p> <p>-----</p>	1-15
A	<p>DAMLA HUC CETOGULLARI ET AL: "Metabolic engineering of microorganisms for production of aromatic compounds", MICROBIAL CELL FACTORIES, vol. 18, no. 1, 1 February 2019 (2019-02-01), page 41, XP055594648, DOI: 10.1186/s12934-019-1090-4 page 21, right-hand column, paragraph 2 - page 24, left-hand column, paragraph 2; table 4</p> <p>-----</p>	1-14
X	<p>DATABASE REFSeq [Online] NCBI; 10 April 2020 (2020-04-10), "Transporter Escherichia coli", XP002798836, Database accession no. WP_033554172 abstract</p> <p>-----</p>	16,17

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2020/056828

Patent document cited in search report	Publication date	Patent family member(s)	Publication date	
WO 2013127915	A1	06-09-2013	EP 2820121 A1	07-01-2015
			US 2015024440 A1	22-01-2015
			WO 2013127915 A1	06-09-2013
-----				
WO 2006113897	A2	26-10-2006	CN 101223444 A	16-07-2008
			EP 1883817 A2	06-02-2008
			JP 5059746 B2	31-10-2012
			JP 2008536523 A	11-09-2008
			US 2007105938 A1	10-05-2007
			WO 2006113897 A2	26-10-2006
-----				