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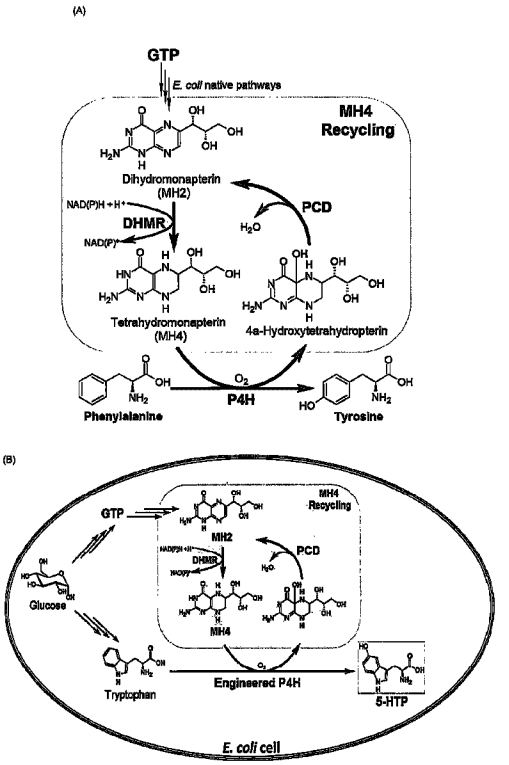


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5-hydroxytryptophan (5-HTP), a precursor of serotonin, is produced in a microbial host cell. A modified bacterial phenylalanine 4-hydroxylase (P4H) catalyzes the tryptophan 5 -hydroxylation reaction. Optionally the host cell includes a cofactor regeneration mechanism, allowing continuous production of 5-HTP without supplementation of exogenous cofactors.

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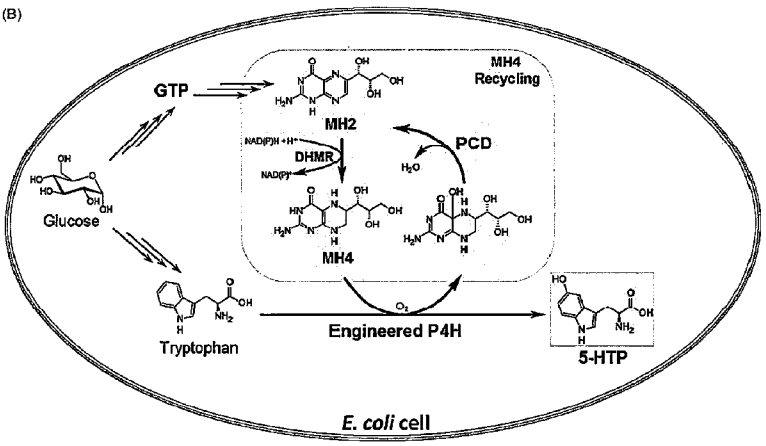
*Fig. 2*




(57) **Abstract**: 5-hydroxytryptophan (5-HTP), a precursor of serotonin, is produced in a microbial host cell. A modified bacterial phenylalanine 4-hydroxylase (P4H) catalyzes the tryptophan 5 -hydroxylation reaction. Optionally the host cell includes a cofactor regeneration mechanism, allowing continuous production of 5-HTP without supplementation of exogenous cofactors.

# MICROBIAL APPROACH FOR THE PRODUCTION OF 5-HYDROXYTRYPTOPHAN

This application claims the benefit of U.S. Provisional Application Serial No. 61/994,413, filed May 16, 2014.

## BACKGROUND

Depression is a common mental disorder that threatens millions of people over the world. A deficit of the neurotransmitter serotonin in the central nervous system (CNS) is thought to be an important physiological factor for depression. 5-Hydroxytryptophan (5-HTP) is the direct biosynthetic precursor to serotonin in humans and animals. It has been shown to be clinically effective in treating depression with relatively few side effects. In most European countries, 5-HTP is a commonly prescribed drug for multiple treatment purposes; while in North America market it is sold as an "over-the-counter" dietary supplement.

In addition to depression, 5-HTP has been shown to be effective in treating insomnia, fibromyalgia, obesity, cerebellar ataxia, chronic headaches, etc. (Birdsall, Altern. Med. Rev. 1998; 3(4):271-280), as evidenced by studies conducted in the past two decades indicating that 5-HTP may have positive effects in the treatment of disorders such as fibromyalgia (Caruso et al., J Int Med Res. 1990;18:201-209; Puttini et al., J Int Med Res. 1992; 20:182-189), insomnia (Attele et al., Altern Med Rev. 2000; 5(3):249-259), migraines and headaches (Ribeiro, 2000 Jun; 40(6):451-6), eating disorders such as polyphagia (Cangiano et al., Int J Obes Relat Metab Disord. 1998; 22:648-654; Ceci et al., J Neural Transm.1989; 76:109-117) as an aid in the management of obesity and diabetes, and hot flashes (Curcio et al., Altern Med Rev. 2005; 10(3):216-21; Freedman, Maturitas. 2010 Apr; 65(4):383-5).

5-HTP is also considered as a potential feed additive for farm animals, as either a preventative of, or therapeutic agent against, mastitis in cattle (Jury et al., PLoS One. 2015 Feb 17;10(2):e0117339; Pai et al., Biomed Res Int. 2015;2015:364746; Horseman et al., Annu Rev Anim Biosci. 2014 Feb 2:353-74), and in the regulation of lactation function in mammals (Marshall



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et al., J Mammary Gland Biol Neoplasia. 2014 Mar; 19(1):139-46; Collier et al., Domest Anim Endocrinol. 2012 Aug; 43(2):161-70; Hernandez et al., J Endocrinol. 2009 Oct; 203(1):123-31), thus having great potential to increase milk production by said animals. In certain avians (e.g., chickens) 5-HTP appears to be associated with the regulation of circadian cycle (Thomas et al., Cell. Mol. Neurobiol.1991, 11(5):511-527) and other positive health effects (Moneva et al., Bulgarian J. Ag Sci. 2008 14(4):424-431) with positive impact in the animal growth and productivity.

Currently, 5-HTP is produced through extraction from the seeds of *Griffonia simplicifolia*, a woody climbing shrub grown in Africa. The season- and region-dependent supply of the raw materials has limited its cost-effective production and broad clinical applications. In addition, *Griffonia* derived 5-HTP has been contaminated with a compound called Peak X, leading the USDA to briefly remove the supplement from shelves in the US.

The current bulk wholesale price for 5-HTP ranges from 400 to 1000 USD/kg. Despite the current high production cost and limited supply, the global market of 5-HTP is still about 120,000 kg (bulk value 50-100 million dollars) with an annual growth rate of about 7%. The 5-HTP end products are even more expensive, and the retail market values of the end products are much higher.

In 2012, North America represented about 48% of the market followed by Europe and Asia Pacific with the major market segment being human nutritional supplement. Emerging market segments (e.g., animal nutrition, human therapeutic co-adjuvant) are likely to grow at rates much higher than those predicted for the over-the-counter human nutritional supplement.

## SUMMARY OF THE INVENTION

The present invention provides compounds, compositions, and methods useful for microbial production of 5-hydroxytryptophan (5-HTP), a precursor of serotonin (5-hydroxytryptamine). A host cell is metabolically engineered to express a non-naturally occurring enzyme that catalyzes the conversion of tryptophan to 5-HTP via 5-hydroxylation of tryptophan. A preferred host cell is one that overproduces that starting material, tryptophan. Optionally, the host cell is further engineered to include a cofactor regeneration mechanism, allowing continuous production of 5-HTP without supplementation with exogenous cofactors. The present invention can result in improved titers of 5-HTP and permits low-cost, large scale production without the need for supplementation with precursors or coenzymes.

In one aspect, the invention includes a genetically engineered microbial cell, such as a bacterial or yeast cell, which includes a bacterial phenylalanine-4-hydroxylase enzyme (P4H) that has been modified to show increased affinity for tryptophan compared to the corresponding wild-type P4H. The modified bacterial enzyme can be based on or derived from, for example, a P4H from *Pseudomonas*, *Chromobacterium*, *Ralstonia*, or *Xanthomonas*. In one embodiment, the modified bacterial P4H contains an amino acid mutation at any one, any two, or all three of amino acid positions 98, 179, and 231 of *Xanthomonas campestris* P4H, or at any one, any two, or all three corresponding amino acid positions in a bacterial P4H enzyme from another species, preferably a bacterial species (see, e.g., Fig 5F). Examples of amino acid mutations include any one, any two, or all three mutations selected from the group consisting of L98Y, W179F and Y231C (numbered according to the *X. campestris* P4H amino acid sequence). An exemplary modified phenylalanine 4-hydroxylase (P4H) is an *X. campestris* phenylalanine 4-hydroxylase that incorporates at least the mutation W179F. Optionally, the modified bacterial P4H further includes at least one additional mutation, which additional mutation is at a position corresponding to any one, any two, or all three of amino acid positions 85, 223 and 282 of *X. campestris* P4H, or any one, any two, or all three corresponding amino acid positions in a bacterial P4H enzyme from another species, preferably a bacterial species.

Optionally, the genetically engineered bacterial or yeast cell is a cell that has been genetically engineered to overproduce or accumulate tryptophan.

Optionally, the genetically engineered bacterial or yeast cell includes a cofactor recycling system that includes at least one of a pterin-4α-carbinolamine dehydratase enzyme (PCD) and a dihydromonapterin reductase enzyme (DHMR). Preferably, the cell includes both a pterin-4α-carbinolamine dehydratase enzyme (PCD) and a dihydromonapterin reductase enzyme (DHMR). In one embodiment, the dihydromonapterin reductase (DHMR) is encoded by the *E. coli* gene *folM*.

The genetically engineered bacterial or yeast cell can contain one or more plasmids encoding any or all of the modified P4H, PCD and/or DHMR enzymes. The enzymes may be encoded by separate plasmids, or two or more enzymes may be encoded by the same plasmid. In some embodiments, the genetically engineered bacterial or yeast cell contains a first plasmid that includes a polynucleotide operably encoding the modified bacterial P4H, and one or both of a pterin-4α-carbinolamine dehydratase (PCD) and a dihydromonapterin reductase (DHMR). The first plasmid can be a low, medium or high copy number plasmid, and is preferably a medium copy number plasmid. Optionally, the genetically engineered cell contains a second plasmid that

includes a polynucleotide operably encoding all or a portion of a *trp* operon, so as to cause the overproduction and/or accumulation of the substrate tryptophan. The second plasmid can be a low, medium, or high copy number plasmid, and is preferably low copy number plasmid. In one embodiment, the *trp* operon or portion thereof can include a mutation S40F in the TrpE gene.

In some embodiments, the genetically engineered bacterial cell does not contain a tetrahydrobiopterin (BH4) cofactor. In other embodiments, the genetically engineered bacterial cell contains an endogenous tetrahydrobiopterin (BH4). Additionally or alternatively, the genetically engineered bacterial cell can contain an endogenous tetrahydromonapterin (MH4) cofactor.

Exemplary genetically engineered bacterial cells include a genetically engineered *Escherichia coli* cell, a genetically engineered *Bacillus subtilis* cell, or a genetically engineered *Corynebacterium glutamicum* cell.

Methods of making and using the genetically engineered cells are also included in the invention.

In another aspect, the invention includes a method of making 5-hydroxytryptophan (5-HTP). The method involves culturing a genetically engineered microbial cell of the invention, preferably a genetically engineered bacterial cell, under conditions and for a time sufficient to produce 5-HTP. Optionally, the 5-HTP can be isolated and/or purified. The method can further include incorporating the 5-HTP into a food product. The food product can be fit for human consumption and/or it can be an animal feed or a beverage. The method can include packaging the 5-HTP or food product for sale and optionally providing instructions for use of the 5-HTP or food product as a food additive, a food supplement, or a nutraceutical. In one embodiment, the food additive, food supplement, or nutraceutical is packaged for use as an animal feed or beverage.

In an embodiment, there is provided a genetically engineered bacterial cell comprising a modified bacterial phenylalanine-4-hydroxylase (P4H) that catalyzes the 5-hydroxylation of tryptophan in the presence of a tetrahydromonapterin (MH4) cofactor; and in the absence of a tetrahydrobiopterin (BH4) cofactor or 6,7-dimethyl-5,6,7,8-tetrahydropterine hydrochloride, wherein the modified bacterial P4H comprises an amino acid mutation at any one, any two, or all three of amino acid positions 98, 179, and 231 of *Xanthomonas campestris* P4H (SEQ ID NO:1), or at any one, any two, or all three corresponding amino acid positions in a bacterial P4H enzyme from another species.



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In an embodiment, there is provided a method of making 5-hydroxytryptophan (5-HTP) comprising: culturing the genetically engineered bacterial cell of any of the preceding claims under conditions and for a time sufficient to produce 5-HTP; and isolating the 5-HTP.

The words "preferred" and "preferably" refer to embodiments of the invention that may afford certain benefits, under certain circumstances. However, other embodiments may also be preferred, under the same or other circumstances. Furthermore, the recitation of one or more preferred embodiments does not imply that other embodiments are not useful, and is not intended to exclude other embodiments from the scope of the invention.

The terms "comprises" and variations thereof do not have a limiting meaning where these terms appear in the description and claims.



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Unless otherwise specified, "a," "an," "the," and "at least one" are used interchangeably and mean one or more than one.

Also herein, the recitations of numerical ranges by endpoints include all numbers subsumed within that range (e.g., 1 to 5 includes 1, 1.5, 2, 2.75, 3, 3.80, 4, 5, etc.).

For any method disclosed herein that includes discrete steps, the steps may be conducted in any feasible order. And, as appropriate, any combination of two or more steps may be conducted simultaneously.

The above summary of the present invention is not intended to describe each disclosed embodiment or every implementation of the present invention. The description that follows more particularly exemplifies illustrative embodiments. In several places throughout the application, guidance is provided through lists of examples, which examples can be used in various combinations. In each instance, the recited list serves only as a representative group and should not be interpreted as an exclusive list.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the phylogenetic relationship of prokaryotic phenylalanine 4-hydroxylases (P4Hs) and animal aromatic amino acid hydroxylases (AAAHs); the protein sequence alignment was performed using ClustalX 2.1; the phylogenetic tree was constructed with MEGA 5.02 by using the neighbor-joining method; the bootstrapping method was used for phylogeny test (1000 replications); the numbers associated with the branches refer to the bootstrap values representing the substitution frequencies per amino acid residue.

Figure 2 shows (A) reconstitution of prokaryotic P4H activity in *E. coli;* wherein the black- and grey-colored arrows indicate the *E. coli* native pathways and heterologous reactions, respectively; bold arrows refer to the over-expressed steps; the introduced MH4 recycling system is indicated by the grey-colored box, and abbreviations are as follows: GTP, guanosine-5'-triphosphate; PCD, pterin-4α-carbinolamine dehydratase; DHMR, dihydromonapterin reductase; P4H, phenylalanine 4-hydroxylase; and (B) introduction of modified bacterial P4H activity into the *E. coli* cell of (A), further allowing for the *de novo* production of 5-hydroxytryptophan (5-HTP) from simple carbon sources, such as sugars.

Figure 3 shows modification of XcP4H via protein engineering; (A), comparative illustration of the positions of the 3 critical residues (L98, W179 and Y231) in the structures of

XcP4H, the P4H from human (HumP4H; PDB entry 1MMK) and the T5H1 from human (HumT5H; PDB entry 3HF6); (B), *in vivo* activities of wild type XcP4H and its mutants, wherein grey- and black-colored bars indicate the activities towards tryptophan and phenylalanine, respectively; (C), whole-cell bioconversion of tryptophan into 5-HTP, wherein solid and dotted lines indicate the time courses of 5-HTP production and cell density, respectively, and black- and grey-colored lines indicate the profiles at 30 and 37°C, respectively, and wherein all data are reported as mean ± s.d. from three independent experiments and error bars are defined as s.d.

Figure 4 shows *de novo* production of 5-HTP from glucose; (A), schematic presentation of the complete 5-HTP biosynthetic pathway wherein the black- and grey-colored arrows indicate the *E. coli* native pathways and heterologous reactions, respectively; (B), production of tryptophan from glucose at 30 and 37°C, wherein data are reported as mean ± s.d. from two independent experiments; (C), profiles of cell growth and 5-HTP production from glucose of two host strains BW*ΔtnaA* (grey) and QH4*ΔtnaA* (black), wherein all data are reported as mean ± s.d. from three independent experiments and error bars are defined as s.d.

Figure 5 shows (A), the amino acid sequence of P4H from *Pseudomonas aeruginosa* (SEQ ID NO:2) (B), the amino acid sequence of P4H from *Pseudomonas fluorescens* (SEQ ID NO:3); (C), the amino acid sequence of P4H from *Pseudomonas putida* (SEQ ID NO:4); (D), the amino acid sequence of P4H from *Ralstonia eutropha* (SEQ ID NO:5); (E), the amino acid sequence of P4H from *Xanthomonas campestris* (SEQ ID NO:1), wherein for (A) through (E), * indicates termination of a protein sequence; (F), an amino acid sequence alignment of P4H enzymes from *P. aeruginosa* (SEQ ID NO:2), *P. fluorescens* (SEQ ID NO:3), *P. putida* (SEQ ID NO:4), *R. eutropha* (SEQ ID NO:5) and *X. campestris* (SEQ ID NO:1), wherein the brackets [ ] indicate the number of amino acids omitted from the graphical depiction of the sequence in order to facilitate alignment, including the termination designator * at the end of the sequence, and wherein candidate mutation sites are indicated at L98, W179, and Y231 (boxed residues), numbered according to the *X. campestris* P4H sequence; and (G), an amino acid sequence alignment of animal AAAHs *Homo sapiens* T5H2 (SEQ ID NO:6), *Mus musculus* T5H2 (SEQ ID NO:7), *Gallus gallus* T5H2 (SEQ ID NO:8), *Bos taurus* T5H1 (SEQ ID NO:9), *Homo sapiens* T5H1 (SEQ ID NO:10), *Mus musculus* T5H1 (SEQ ID NO:11), *Gallus gallus* T5H1 (SEQ ID NO:12), *Homo sapiens* P4H (SEQ ID NO:13), *Oryctolagus cuniculus* P4H (SEQ ID NO:14), *Mus musculus* P4H (SEQ ID NO:15), *Bos taurus* P4H (SEQ ID NO:16),*Gallus gallus* P4H (SEQ ID NO:17); and XcP4H (SEQ ID NO:1); the circled arrows indicate candidate mutation sites at L98, W179, and Y231 as in (F); the uncircled

arrows indicate additional candidate mutation sites at amino acid positions corresponding to *X. campestris* positions Q85, L223, and L282.

## DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

The present invention provides compounds, compositions, and methods useful for microbial production of 5-hydroxytryptophan (5-HTP). The invention involves metabolically engineering a microbial host cell to express a non-naturally occurring hydroxylase enzyme, preferably a hydroxylase that is of microbial origin, that catalyzes the conversion of tryptophan to 5-HTP via 5-hydroxylation of tryptophan. Genetically engineered cells are referred to herein as "metabolically engineered" cells when the genetic engineering is directed to disruption or alteration of a metabolic pathway so as to cause a change in the cell's metabolism.

A preferred microbial host cell is one that overproduces that starting material, tryptophan. Optionally, the microbial host cell is further engineered to include a cofactor regeneration mechanism, allowing continuous production of 5-HTP without supplementation of exogenous cofactors.

Microbial enzymes that catalyze the conversion of tryptophan to 5-HTP via 5-hydroxylation of tryptophan (referred to as tryptophan hydroxylase, “TPH” or “T5H” enzymes) are not known. Some animals possess a tryptophan hydroxylase capable of catalyzing this reaction (utilizing a pterin cofactor, typically tetrahydrobiopterin, also known as “BH4”); however, due to factors such as low solubility, low stability, and inefficient or absent post-translational modification mechanisms, expression of a eukaryotic 5-hydroxylase in microbial systems has proven problematic. Some microbes possess a 4-hydroxylase that catalyzes the conversion of phenylalanine to tyrosine (“P4H” enzymes), but this enzyme possesses little or no 5-hydroxolase activity for the substrate tryptophan.

This unique biotechnological production process of the present invention combines protein engineering and metabolic engineering to achieve microbial production of 5-HTP. Protein engineering, for example site-directed mutagenesis, is employed to produce an effective, non-naturally occurring hydroxylase enzyme that effectively catalyzes the conversion of tryptophan to 5-HTP, using site-directed mutation of a naturally occurring microbial enzyme, such as a phenylalanine-4-hydroxylase (P4H). The mutated naturally occurring microbial enzyme is referred to herein as a “modified” enzyme, for example, a modified bacterial phenylalanine-4-hydroxylase

(P4H). A modified enzyme that is "derived from" a particular organism means a modified enzyme that has been created by mutating the amino acid sequence of the wild-type enzyme produced by that organism, for example by way of one or more site mutations at specified amino acid positions. This engineered or "modified" microbial-origin enzyme is better suited for use in a microbial host cell than its eukaryotic counterparts. Metabolic engineering is used to alter one or more metabolic pathway involved in the production or accumulation of tryptophan within the cell, and/or to incorporate a heterologous cofactor regeneration system into the host cell. Bacterial cofactors that can be recycled by way of a heterologous cofactor regeneration system include tetrahydropterins such as tetrahydromonapterin (MH4) and tetrahydrobiopterin (BH4). Incorporation of a tetrahydromonapterin (MH4) cofactor regeneration system into an *E. coli* cell, for example, allows the use of the endogenous *E. coli* MH4 as an efficient hydrogen donor.

The present invention provides a cost-effective microbial process for the efficient production of 5-HTP from inexpensive carbon sources such as tryptophan and glucose. This technology is expected to facilitate creation of a biocatalytic platform to convert cheap feedstock to the high purity product 5-HTP, which will dramatically lower its production cost compared with the conventional extraction approach, greatly improve its availability to the less well-treated patients, and further expand its market.

*Host Cell*

The invention makes possible the production of 5-HTP in a host cell, preferably a microbial host cell such as a bacterial or yeast cell. Exemplary bacterial host cells include *Escherichia coli*, *Bacillus spp.*, such as *B. subtilis, Lactobacillus spp.*, such as *L. acidophilus*, *Bifidobacteria* and *Corynebacterium* spp., such as *C. glutamicum*. Exemplary yeast cells include yeasts from the genus *Saccharomyces* (e.g., *S. cerevisiae*) and the genus *Pichia*. Yeast cells may contain an endogenous cofactor, BH4; in some embodiments, a BH4 cofactor recycling system is introduced into the host yeast cell, in addition to or instead of the MH4 cofactor recycling system described herein.

In a preferred embodiment, the invention provides for the introduction of bacterial P4H activity into a bacterial cell, for example *E. coli* or *B. subtilis* (which have not been shown to contain a native P4H enzyme) via the introduction of a modified bacterial P4H from a different microbe (for example, and without limitation, *Pseudomonas*, *Chromobacterium*, *Ralstonia*, or *Xanthomonas*), which modified enzyme advantageously and surprisingly is able to utilize an endogenous *E. coli* cofactor, MH4 (typically, the pterin cofactor BH4 is associated with AAAH

enzymes). In another preferred embodiment, the *E. coli* cell is further engineered to contain a recycling system to recycle MH4 as described herein.

Optionally, the host cell is genetically engineered to increase the amount of tryptophan in the cell, i.e., to overproduce and/or accumulate tryptophan, the starting material for the biosynthesis of 5-HTP. Overproduction or accumulation of tryptophan can be achieved through a variety of methods. In one embodiment, overproduction or accumulation of tryptophan can be achieved by knocking out one or more enzymes involved in tryptophan degradation. For example, the amount of tryptophan in a cell can be increased by knocking out the gene for tryptophanase (*tnaA*), a protein reported to catalyze the degradation of tryptophan and 5-HTP. Alternatively or additionally, *pheA, pheLA,* and/or *tyrA* can be downregulated or knocked out to increase the amount of tryptophan in a cell.

In another embodiment, tryptophan overproduction or accumulation can be achieved by increasing the biosynthesis of tryptophan. For example, the host cell can be engineered to overexpress the *trp* operon or portions thereof. A gene product, such as an enzyme or regulatory protein, is "overexpressed" in a metabolically engineered cell when the gene product is expressed in the metabolically engineered cell at a level higher than the level at which it is expressed in a comparable wild-type cell. In cells that do not endogenously express a particular gene product, any level of expression of that gene product in the cell is deemed an "overexpression" of that gene product for purposes of the present invention. Typically, as a means to overexpress one or more gene products from the *trp* operon, additional copies of the *trp* operon are introduced into the cell on a plasmid, cosmid or other DNA or RNA vector. In one embodiment, the host cell is engineered to overexpress the complete *trp* operon, including *trpEDCBA*. In another embodiment, the host cell is engineered to overexpress only portion of the *trp* operon, for example *trp ED* or *trpE*.

Optionally, the *trp* operon is engineered to reduce or eliminate feedback inhibition. For example, a mutation at amino acid position S40, such as the mutation S40F, can incorporated into TrpE to reduce or eliminate feedback inhibition.

In another embodiment, tryptophan overproduction or accumulation can be achieved altering the host cell's endogenous tryptophan expression. For example, tryptophan overproduction or accumulation can be achieved by altering expression of, downregulating or inactivating the tryptophan transcriptional repressor protein (TrpR).

In another embodiment, tryptophan overproduction or accumulation can be achieved by overproducing a precursor to tryptophan biosynthesis, such as chorismate. A host cell can be

engineered to express or overexpress one or more enzymes that increase chorismate availability. Several rate limiting steps in the production of chorismate are known and can be manipulated to result in a microbe that produces more chorismate. Enzymes that increase carbon flow toward chorismate include, for example, shikimate kinase, phosphoenolpyruvate synthase, transketolase, and 3-deoxy-*D*-arabino-heptulosonate-7-phosphate synthase, preferably feedback-inhibition-resistant 3-deoxy-*D*-arabino-heptulosonate-7-phosphate synthase, 3-dehydroquinate synthase, 3-dehydroquinate dehydratase, shikimate dehydrogenase, 3-phosphoshikimate-1-carboxyvinyltransferase, and chorismate synthase. Examples of enzymes include, but are not limited to, AroL, AroF, AroH, AroG, PpsA, and TktA. In some embodiments, one or more of the AroL, AroF, AroH, AroG enzymes may be in the form of feedback-inhibition-resistant (fbr) enzymes. Exemplary polynucleotides which encode preferred enzymes are *aroL, ppsA, tktA, and* $aroG^{fbr}$, respectively. In one embodiment, an *E. coli* host cell can express *aroL*, *ppsA*, *tktA,* and $aroG^{fbr}$ to increase chorismate availability. Exemplary enzymes are also described in Lin et al., *Metab Eng*. 2014, 23:62-9; Lin et al., *Nat. Commun*. 2013, 4:2603; and US Patent Application Publication No. 2014/0370557, published December 18, 2014.

In another embodiment, tryptophan overproduction or accumulation can be achieved by inactivation of the tryptophan transcriptional repressor protein (TrpR). In yet another embodiment, tryptophan overproduction or accumulation can be achieved by inactivation of the transcriptional regulator protein TyrR.

Any known method for overproduction or accumulation of tryptophan can be used, including, for example, the methods described in U.S Patent No. 5,756,345 and Chinese Patent CN 102453691 B; see also Zhao et al., *J. Ind. Microbiol. Biotechnol*., 2011, 39:1921-1929.

Optionally, one or more overproduction or accumulation techniques can be combined. For example, the tryptophan-degradation pathway can be knocked out, and the host cell can overexpress the entire *trp* operon. As another example, the host cell can overexpress the entire *trp* operon as well as one or more enzymes to increase the production of the tryptophan precursor chorismate. When the host cell is engineered to express or overexpress a plurality of enzymes, a single exogenous construct, such as a plasmid, can be utilized, or multiple exogenous constructs, such as plasmids, can be utilized, as described in more detail herein. Alternatively or additionally, genetic engineering can be accomplished using chromosomal additions, deletions, or mutations.



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*Engineered hydroxylase*

In the present invention, a modified bacterial hydroxylase, rather than a eukaryotic hydroxylase (see, e.g., WO2013127914A1), is advantageously utilized to catalyze the conversion of tryptophan to 5-HTP. The use of a modified bacterial hydroxylase in a bacterial host cell permits the use of endogenous bacterial cofactors, which may include MH4 and/or BH4, and can avoid limitations associated with the use of eukaryotic enzymes including low solubility, low stability, and inefficient or absent post-translational modification mechanisms and/or cofactors (see, e.g., McKinney et al. (2004), Protein Expr. Purif. 33(2):185-194; Martinez et al. (2001) Curr. Med. Chem. 8(9):1077-1091). In contrast to animal 5-hydroxylases that have been truncated to improve solubility (see, e.g., WO2013/127914 A1), the modified bacterial 4-hydroxylases of the present invention demonstrate increased catalytic efficiency.

Naturally occurring bacterial phenylalanine 4-hydroxylases (P4H) have little activity towards tryptophan. The invention involves the expression in the host cell of a bacterial hydroxylase, preferably a 4-hydroxylase, more preferably a phenylalanine 4-hydroxylase, that has been modified to increase its 5-hydroxylase activity toward the substrate tryptophan. Preferably, the modification is achieved through site-directed mutagenesis. Not all bacteria contain P4H enzymes, but any bacterial P4H enzyme can be modified according the invention to increase its activity toward tryptophan. Suitable bacterial P4H enzymes that can be modified according to the invention to increase their activity toward tryptophan include, without limitation, P4H enzymes from *Pseudomonas*, *Chromobacterium*, *Ralstonia*, *Burkolderia*, *Xanthomonas*, *Bacillus, Stenotrophomonas, Arenimonas, Lysobacter, Dyella, Rhodanobacter, Cupriavidus, Sphingobium, Polaromonas, Micavibrio, Caulobacter,* and the like. Exemplary species include *C. violaceum, P. putida*, *P. protegens*, *P. aeruginosa*, *R. eutropha*, *B. pseudomallei*, *X. campestris*, and *B. cereus*. Preferred bacterial P4H enzymes include those from *Pseudomonas aeruginosa* (PaP4H), *Pseudomonas putida* (PpP4H), *Pseudomonas fluorescens* (PfP4H), *Ralstonia eutropha* (ReP4H), and *Xanthomonas campestris* (XcP4H). A preferred bacterial P4H enzyme is XcP4H. Bacterial P4H may be encoded by the gene *phhA*.

The bacterial P4H enzyme is mutated at one more residues to produce a modified P4H enzyme with greater activity toward tryptophan. As an example, XcP4H can be mutated at residues W179, L98, Y231, Q85, L223, and/or L282. In one embodiment, XcP4H is mutated at residues W179, L98, and Y231. In other embodiments, XcP4H is mutated only at a single residue, for example only at residue W179, only at residue L98, or only at residue Y231. In other embodiments,

XcP4H is mutated at two or more residues, for example, residues W179 and L98; or at W179 and Y231; or at L98 and Y231. In a preferred embodiment, W179 is replaced with the amino acid phenylalanine (F). In another embodiment, L98 is replaced with tyrosine (Y). In yet another embodiment, Y231 is replaced with cysteine (C). In one embodiment, XcP4H includes mutations at each of the following residues: W179F, L98Y, and Y231C. In other embodiments, XcP4H contains the following mutations: W179F and L98Y; or W179F and Y231C; or L98Y and Y231C. One or more corresponding residues in bacterial P4H enzymes PaP4H, PpP4H, PfP4H, and ReP4H (see, e.g., Fig. 5F) or any other microbial or animal P4H enzyme (see, e.g., Fig. 5G) could also be mutated at one or more corresponding amino acid positions to produce the modified P4H enzyme with enhanced preference for tryptophan. Preferably, a bacterial P4H enzyme (exemplified by the sequences in Fig. 5F) is modified to produce the modified P4H enzyme. However, animal enzymes can also be used. Fig. 5G shows exemplary animal P4H and T5H amino acid sequences aligned with bacterial P4H from *Xanthomonas campestris*; amino acid positions corresponding to XcP4H positions W179, L98Y, and Y231C are indicated with circled arrows, and amino acid positions corresponding to XcP4H positions Q85, L223, and L282 are indicated with uncircled arrows. Any one, some or all of these positions are candidates for mutation to produce a modified P4H enzyme of the invention.

*Cofactor Recycling*

Optionally, the invention further provides for the increased production of and/or recycling of a cofactor for the modified P4H enzyme. The cofactor can be a pterin, such as a tetrahydropterin exemplified by tetrahydrobiopterin (BH4) or tetrahydomonapterin (MH4). Preferably, the cofactor used by the modified P4H enzyme is endogenous to the host cell. In a preferred embodiment, the cofactor is a bacterial MH4, for example *E. coli* MH4.

Advantageously, a cofactor regeneration mechanism can be introduced into the host cell to facilitate recycling of the endogenous pterin cofactor (e.g.MH4 or BH4) and thereby promote increased overproduction or accumulation of 5-HTP. To this end, the host cell is preferably engineered to express a pterin 4a-carbinolamine dehydratase (PCD), a dihydromonapterin reductase (DHMR), or both, so as to allow recycling of the MH4 or BH4 pterin cofactor used by the P4H enzyme. The PCD and/or DHMR enzymes can be native to the host cell (in which case the cell is engineered to overexpress the enzyme) or they can be heterologous (foreign) to the host cell. In some embodiments, one of the enzymes is native, and the other is foreign. Preferably the enzymes

are bacterial enzymes, although they may be eukaryotic. A preferred PCD is from *P. aeruginosa*, but a suitable PCD can be obtained from any convenient prokaryote or eukaryote, preferably a bacterium or yeast having an endogenous P4H enzyme. A preferred DHMR is form *E. coli*, but a suitable DHMR can be obtained from any prokaryote or eukaryote.

*Plasmids*

The modified bacterial P4H, the optional enzymes involved in recycling its cofactor, and the optional *trp* operon, or portions of the *trp* operon, as well as any other enzymes of interest, can be expressed or overexpressed in the host cell through the introduction of one or more plasmids containing polynucleotide sequences that operably encoding the enzyme(s). Suitable exemplary plasmids are described in Example I and include but are not limited to pZE12-luc, pCS27, and pSA74. A person having skill in the art will appreciate that additional plasmids with different promoters, antibiotic resistance, and origins of replication (ori) can also be used.

Additionally, it should be noted that any or all of the nucleotide sequences that operably encode the enzymes described herein can instead be genomically integrated into the bacterial genome, if desired, using well-known genetic engineering techniques and protocols.

The plasmid can be a high-copy number, a medium copy-number, or a low-copy number plasmid. While the boundaries associated with the art-recognized designations "high," "medium" and "low" copy number are indistinct and may in practice overlap, in general a high copy number plasmid is characterized by copy numbers within a cell of, for example, greater than 50 or 60 copies, a medium copy number plasmid is characterized by copy numbers within a cell of, for example, between 10 and 60 copies (e.g., 15-20 copies), and a low copy number plasmid is characterized by copy numbers within a cell of, for example, fewer than 10 or 15 copies (e.g., 3-8 copies). Plasmids pZE12-luc, pCS27, pSA74 exemplify high-, medium- and low-copy number plasmids, respectively. In some embodiments, pZE12-luc has a copy number of about 60-70, pCS27 has a copy number of about 15-20, and pSA74 has a copy number of about 5-10.

Expression of P4H, preferably a modified bacterial P4H, can be achieved by introducing into the host cell a plasmid containing *phhA* encoding the modified P4H, preferably *phhA* that has been mutated to encode a modified P4H with greater activity toward the substrate tryptophan, as described herein. The *phhA* that encodes the modified P4H can be obtained from any suitable organism, such as, for example and without limitation, *P. aeruginosa* (Fig. 5A, SEQ ID NO:2), *P.*

*fluorescens* (Fig. 5B, SEQ ID NO:3), *P. putida* (Fig. 5C, SEQ ID NO:4), *R. eutropha* (Fig. 5D, SEQ ID NO:5) and *X. campestris* (Fig. 5E, SEQ ID NO:1), as described herein.

Expression of one or more enzymes involved in cofactor recycling, such as PCD and/or DHMR, can be achieved by introducing into the host cell a plasmid containing, for example, *phhB* (encoding PCD from, for example, *P. aeuroginosa*) and/or *folM* (encoding DHMR from, for example, *E. coli*). Polynucleotide sequences encoding PCD and DHMR can be obtained from any suitable organism; for example, they can be obtained from a microorganism that endogeneously expresses P4H.

In one embodiment, *phhA* (mutated to encode a modified P4H with greater activity toward tryptophan), *phhB*, and *folM* are cloned into two or more separate plasmids. In a preferred embodiment, *phhA* (mutated as described), *phhB*, and *folM* are cloned into the same plasmid. Preferably, the genes are cloned into a medium-copy number plasmid, for example, pCS27, although high copy number and low copy number plasmids can be used.

The *trp* operon, or portions of the *trp* operon, can also be expressed or overexpressed in the host cell by means of a plasmid. The *trp* operon can include mutations, including but not limited to, mutation S40F in TrpE. In an exemplary embodiment, the *trp* operon including mutation S40F is cloned into a low copy number plasmid, such as pSA74 to make pSA-TrpEDCBA, although medium copy number and high copy number plasmids may be used.

*Method of Making 5-HTP*

Microbial host cells expressing the modified bacterial P4H and other features as described herein, such as a cofactor recycling system and/or tryptophan overproduction or accumulation, can advantageously be grown in a medium containing a simple carbon source such as glucose or glycerol. Other suitable carbon sources include xylose, arabinose, and other renewable sugars, such as sugars present in a plant or lignocellulosic hydrolysate. Optionally, the medium is supplemented with tryptophan. In order to optimize cell growth and production efficiency, the host cells can be incubated at a temperature, including but not limited to 21°C, 22°C, 23°C, 24°C, 25°C, 26°C, 27°C, 28°C, 29°C, 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C, 37°C, 38°C, 39°C, 40°C, 41°C, 42°C, 43°C, 44°C, 45°C, 46°C. In one embodiment, the host cells are incubated at 30°C. In an alternative embodiment, the host cells are incubated at 37°C. Glucose is preferably used as carbon source for cell growth and maintenance (e.g., 0.1-100g/L); optionally, citrate can be added to promote cell

growth increase the titer (e.g., 0.1-100g/L); optionally, other growth supplements such as tryptone can be added.

Optionally, 5-HTP is isolated from the host cell. The isolated 5-HTP may be purified. The purified 5-HTP may be used as the starting material for other chemical or enzymatic reactions to produce other biochemicals of interest, such as melatonin.

Optionally, the isolated 5-HTP is incorporated into a food product as a food additive, food supplement, or nutraceutical. For example, the isolated 5-HTP can be incorporated into an animal feed, such as feed for domestic or farm animals. Supplementation with 5-HTP can have beneficial effects relating to calcium metabolism and is especially suitable for pregnant and lactating farm animals such as cows. The method of making 5-HTP therefore optionally includes packaging and/or marketing the 5-HTP as an animal food supplement, additive or nutraceutical, as well as incorporating the 5-HTP into a food product such as an animal feed or beverage.

Advantageously, in host cells that have been engineered to contain the cofactor recycling system as described herein, there is no need to supplement with expensive pterin coenzymes or precursors; the cells can utilize simple renewable carbon sources. The method thus holds great potential for scale-up production of 5-HTP.

The present invention is illustrated by the following examples. It is to be understood that the particular examples, materials, amounts, and procedures are to be interpreted broadly in accordance with the scope and spirit of the invention as set forth herein.

## EXAMPLES

### Example I. Engineering Bacterial Phenylalanine 4-Hydroxylase for Microbial Synthesis of Human Neurotransmitter Precursor 5-Hydroxytryptophan

5-Hydroxytryptophan (5-HTP) is a clinically effective drug against depression, insomnia, obesity, cerebellar ataxia, chronic headaches, etc. It is only commercially produced by the extraction from the seeds of *Griffonia simplicifolia* due to lack of synthetic methods. Here, we report the efficient microbial production of 5-HTP via combinatorial protein and metabolic engineering approaches. Instead of using the less tractable animal tryptophan 5-hydoxylase, we attempted to modify a bacterial phenylalanine 4-hydroxylase (P4H) to alter its substrate specificity.

First we reconstituted and screened prokaryotic phenylalanine 4-hydroxylase (P4H) activity in *Escherichia coli*. Then, we used sequence and structure-based protein engineering to dramatically shift its substrate preference, allowing for efficient conversion of tryptophan into 5-HTP. Importantly, *E. coli* endogenous tetrahydromonapterin (MH4) was able to be utilized as the coenzyme, when a foreign MH4 recycling mechanism was introduced. Whole-cell bioconversion enabled the high-level production of 5-HTP (1.1-1.2 g $l^{-1}$) from tryptophan in shake flasks. Metabolic engineering efforts were further made to achieve the *de novo* 5-HTP biosynthesis from glucose. See Lin et al., ACS Synth. Biol., 2014, 3(7), pp 497–505. This process does not require the supplementation of expensive pterin coenzymes or precursors, and is able to utilize renewable carbon sources. This work not only holds great scale-up potential but also demonstrates a strategy to expand native metabolism of microorganisms.

**Introduction**

The World Health Organization (WHO) reported that depression is a common mental disorder affecting more than 350 million people globally. It results in around one million suicides per year. Unfortunately, over 50% of sufferers over the world (over 90% in some regions) have never received medical treatment[1]. Alterations in serotonin (5-hydroxytryptamine) metabolism were thought to be an important physiological factor for depression[2]. Dysfunction of the serotonergic mechanism in the central nervous system (CNS) has been implicated in the etiology of depression[3]. However, supply of serotonin via oral administration is not clinically effective against depression because it cannot pass through the brain-blood barrier. Unlike the conventional antidepressants (e.g. selective serotonin re-uptake inhibitors) acting on minimizing serotonin loss, 5-hydroxytryptophan (5-HTP) functions as the direct precursor to increase serotonin supply. Orally administered 5-HTP can easily pass through the blood-brain barrier without requiring transport molecules. Then it can be efficiently converted into serotonin in the CNS by endogenous decarboxylase[3]. Detailed clinical trials have demonstrated its efficacy in alleviating depression symptoms. Meanwhile, the therapeutic administration of 5-HTP has been shown to be effective in treating insomnia, fibromyalgia, obesity, cerebellar ataxia, and chronic headaches[4]. Importantly, relatively few adverse effects are associated with its use in treatment[2]. In most European countries, 5-HTP is a commonly prescribed drug for multiple treatment purposes; while in North America it is sold as an "over-the-counter" dietary supplement.

Due to the difficulty in regio-selective hydroxylation of tryptophan via chemical approaches, the commercial production of 5-HTP relies only on the isolation from the seeds of *Griffonia simplicifolia*, a woody climbing shrub grown in West and Central Africa[3, 4]. The season- and region-dependent supply of the raw materials has been largely limiting its cost-effective production and broad clinical applications. The recent development of metabolic engineering and protein engineering in combination with fundamental genetics, biochemistry and bioinformatics is providing new strategies to synthesize natural and non-natural molecules using microbial systems. On the basis of accumulated knowledge on natural biosynthetic mechanisms of target products, especially the genetic and biochemical information of the involved enzymes, heterologous enzymatic reactions can be reconstituted, modified and optimized in genetically superior microbial hosts to achieve efficient production of pharmaceutically important compounds that are scarce in nature[5-11].

5-HTP is natively produced in humans and animals from *L*-tryptophan by the action of tryptophan 5-hydroxylase (T5H) and then converted to the neurotransmitter serotonin under normal physiological conditions[4]. T5Hs belong to the class of pterin-dependent aromatic amino acid hydroxylases (AAAHs) that also include two other subgroups: phenylalanine 4-hydroxylases (P4Hs) and tyrosine 3-hydroxylases (T3Hs). AAAHs were broadly identified and extensively studied in animals due to their close relationship with human diseases such as phenylketonuria, Parkinson's disease, and neuropsychiatric disorders[12]. These enzymes consist of three domains that are the N-terminal regulatory domain, the central catalytic domain, and the C-terminal domain involved in tetramer formation, and usually utilize tetrahydrobiopterin (BH4) as the coenzyme (or co-substrate)[13]. Animal T5Hs were proved to be unstable and hard to be functionally expressed in a microbial host[14, 15]. A very recent patent reported the use of truncated T5H1 from *Oryctolagus cuniculus*, which produced up to 0.9 mM (equivalent to 198 mg $l^{-1}$) of 5-HTP from tryptophan in *Escherichia coli*. To supply the pterin coenzyme, the animal BH4 biosynthetic pathway coupled with a regeneration system including a total of five enzymes was required to be co-expressed in *E. coli*[16]. However, the production efficiency is still not satisfying for scale-up production.

A few AAAHs were also found in bacteria such as *Pseudomonas* and *Chromobacterium* species[17, 18]. So far, all of them were identified as P4Hs with little activity for tryptophan hydroxylation; but such activity was reported to be improved *in vitro* when mutations were introduced into the P4H from *Chromobacterium violaceum*[19]. Prokaryotic P4Hs consist of only one domain corresponding to the catalytic domain of animal AAAHs[13]. Recent experimental evidence

indicated that bacterial P4Hs may utilize tetrahydromonapterin (MH4) instead of BH4 as the native pterin coenzyme[20], since BH4 does not naturally occur in most bacteria. Interestingly, MH4 is the major form of pterin in *E. coli,* although its function is still unknown. In this work, we report the reconstitution of bacterialP4H activity in *E. coli* through utilization and recycling of its endogenous MH4. Combined bioprospecting and protein engineering approaches enabled the development of the P4H mutants that are highly active on converting tryptophan to 5-HTP, which allowed the establishment of an efficient 5-HTP production platform via further metabolic engineering efforts. This *de novo* process does not require supplementation of expensive pterin co-factors or precursors but only utilizes renewable simple carbon sources, which holds great potential for scale-up production of 5-HTP in microorganisms.

## RESULTS AND DISCUSSION

**Phylogenetic analysis of aromatic amino acid hydroxylases (AAAHs).** Compared with animal AAAHs that include three sub-groups, their prokaryotic counterparts were all identified or annotated as P4H only. Previous biochemical and structural studies revealed that in addition to the central catalytic domain, animal AAAHs usually consist of two additional domains that are the N-terminal regulatory domain and the C-terminal domain involved in tetramer formation; while prokaryotic AAAHs (e.g. the P4H from *C. violaceum*) are monomers with only one single domain that shares moderate sequence similarity (about 30%) with the catalytic domains of animal AAAHs[21]. To explore the evolutionary relationship among AAAHs, 25 amino acid sequences from both prokaryotes and animals were randomly selected and a phylogenetic tree was constructed using MEGA 5.02 based on the neighbor-joining method (Fig. 1)[22]. The tree reflects a considerable evolutionary separation between prokaryotic and animal AAAHs. The three subfamilies (P4Hs, T5Hs, and T3Hs) of animal AAAHs are distinctly separated as well, among which P4Hs show closer phylogenetic relationship with T5Hs than with T3Hs. These results are consistent with a previous phylogenetic study on AAAHs[23].

Considering the phylogenetic evidence in combination with the development of functional diversity, we inferred that the animal AAAHs were evolved from prokaryotic P4Hs through duplication and divergence. Therefore, we hypothesized that even after a long-term evolution process, prokaryotic and animal P4Hs may still share some conserved amino acid residues that determine their substrate preference towards phenylalanine. Meanwhile, animal P4Hs and T5Hs

share high sequence similarity, suggesting that the interchange of substrate preference from phenylalanine to tryptophan may only involve the substitution of a small number of residues. Based on these hypotheses, we speculated that by performing a comprehensive alignment analysis of the sequences of animal AAAHs and prokaryotic P4Hs, we may be able to identify the substrate-determining residues from the latter group and artificially evolve them into T5Hs.

**Bio-prospecting and reconstitution of prokaryotic P4Hs in *E. coli*.** Before exploring the substrate-determining amino acid residues, we picked five P4Hs from different microorganisms (*P. aeruginosa*, *Pseudomonas putida*, *Pseudomonas fluorescence*, *Ralstonia eutropha* and *Xanthomonas campestris*) to verify and compare their activities and substrate preferences, since most of the prokaryotic P4Hs are still putative enzymes without experimental confirmation of their function. The P4H from *P. aeruginosa* (PaP4H) was previously identified *in vitro* and its crystal structure has been resolved[24]. Some genetic and biochemical evidence suggested that PaP4H utilizes MH4 instead of BH4 as the native pterin coenzyme[20]. Thus, we first selected it as a prototype to establish its *in vivo* activity in *E. coli*, since MH4 is the major pterin produced by *E. coli* (Fig. 2). To achieve the expression of PaP4H, its gene *phhA* was amplified from the genomic DNA of *P. aeruginosa* and cloned into a high-copy number plasmid under the control of an IPTG-inducible promoter $P_L$*lacO1*. The resulting expression vector pZE-PaphhA was introduced into *E. coli* strain BW25113*ΔtnaA* (abbreviated as BW*ΔtnaA*). Since tryptophanase encoded by *tnaA* was reported to catalyze the degradation of tryptophan and 5-HTP[25], the gene was knocked out from all the strains used in this study. We observed that the cell growth of BW*ΔtnaA* carrying pZE-PaphhA was significantly retarded. Its $OD_{600}$ values only reached 0.8-1.0 after 8-hour cultivation, dramatically lower than those of the control strain (BW*ΔtnaA* carrying an empty vector) with $OD_{600}$ values at 5.5-6.0. A similar effect was also observed in a previous study[26]. When the cells were incubated with phenylalanine (500 mg $l^{-1}$), almost no hydroxylated product (tyrosine) was detected. Indeed, *P. aeruginosa* possesses a pterin 4a-carbinolamine dehydratase (PCD, encoded by *phhB*) responsible for the regeneration of dihydromonapterin (MH2) which can be further reduced to MH4. But *E. coli* does not have such a mechanism natively. To establish an artificial MH4 recycling system (Fig. 2), *phhB* from *P. aeruginosa* and *folM* from *E. coli* (encoding dihydromonapterin reductase, DHMR) were co-expressed along with the *phhA* using the vector pZE-PaABM. Interestingly, the *E. coli* strain harboring this vector dramatically improved cell viability which was comparable with the control strain. Its $OD_{600}$ values reached 4.5-5.5 after cultivation for 8 hours. When these cells were collected and incubated with phenylalanine, a large amount of tyrosine was produced at a rate of

83.50 μM/h/$OD_{600}$, as was shown in the *in vivo* assays (Table 1). These results indicated that introduction of the MH4 recycling system not only restored the cell growth but also enabled the *E. coli* strain to convert phenylalanine to tyrosine. Moreover, this strain was also capable of converting tryptophan into 5-HTP (Table 1), although the production rate (0.19 μM/h/$OD_{600}$) was much lower, only equivalent to 0.23% of that towards phenylalanine.

**Table 1** *In vivo* activities of P4Hs from different microorganisms

| Source of P4H | *In vivo* activity[a] (μM/h/$OD_{600}$) | | Preference (Phe:Trp) |
|---|---|---|---|
| | Phenylalanine | Tryptophan | |
| *Pseudomonas aeruginosa* | 83.50 ± 16.00 | 0.19 ± 0.02 | 439.5 |
| *Pseudomonas putida* | 76.32 ± 10.02 | 0.12 ± 0.03 | 636.0 |
| *Pseudomonas fluorescence* | 82.47 ± 12.05 | 0.20 ± 0.05 | 412.4 |
| *Ralstonia eutropha H16* | 73.33 ± 4.63 | 1.22 ± 0.04 | 60.1 |
| *Xanthomonas campestris* | 97.40 ± 4.42 | 2.91 ± 0.21 | 33.5 |

[a] All data are reported as mean ± s.d. from three independent experiments.

On this basis, another four P4Hs were also tested by replacing the PaP4H gene on pZE-PaABM with their respective genes. As shown in Table 1, all the identified P4Hs showed high activity and strong substrate preference towards phenylalanine in *E. coli*. Among them, the P4H from *X. campestris* (XcP4H) exhibited the highest activity towards both phenylalanine and tryptophan. The three from *pseudomonas* species showed the most similar catalytic properties, which is consistent with their close phylogenetic relationship. Therefore, we confirmed that all the tested P4Hs can function well by utilizing the *E. coli* endogenous pterin coenzyme MH4 in the presence of a recycling system.

**Modification of the XcP4H substrate preference through protein engineering.** XcP4H was selected for protein engineering due to its superior catalytic potential. To investigate the substrate-determining amino acid residues, its sequence was aligned with animal P4Hs and T5Hs. Comparison of the sequences of only animal P4Hs and T5Hs led to the identification of a number of residues that are conserved within each group but varied between groups. However, when these residues were aligned with the XcP4H sequence, only six of them were found to be conserved in P4Hs and probably critical to the substrate selectivity, which are Q85, L98, W179, L223, Y231, and L282, numbered according to the numbering system for *X. campestris* (Fig. 5G, six arrows). To further investigate their locations in the enzyme structure, a homology model was built using the crystal structure of the P4H from *C. violaceum* (PDB code 3TK2) as a template. The conserved residues were well aligned with those in the crystal structures of the human P4H (PDB code

1MMK) and T5H1 (PDB code 3HF6), indicating the reliability of the model. In this structure, W179 is located inside the catalytic pocket just at the predicted phenylalanine binding site, while L98 and Y231 are near the entrance to the pocket, which are closer to the coenzyme MH4 binding site (Fig. 3A). However, Q85, L223 and L282 are not located near the catalytic pocket, suggesting these residues are less relevant to the enzyme's substrate selection. Therefore, we selected W179, L98 and Y231 as the targets for further mutation analysis. We hypothesized that if these residues were replaced with their respective residues in T5Hs that are F, Y and C, respectively, the mutants might exhibit stronger preference towards tryptophan. As a result, the W179F mutant of XcP4H exhibited a 17.4-fold increase in tryptophan hydroxylation activity compared with the wild-type (WT) enzyme; meanwhile its activity towards phenylalanine decreased by about 20%. The substrate preference towards phenylalanine over tryptophan was shifted from 33.5 to 1.5 (Table 2). When the mutations L98Y or Y231C were combined with W179F, the substrate preference further shifted towards tryptophan, although their activities towards tryptophan were not as high as that of W179F alone. The triple mutant showed almost the same preference towards the two substrates (Fig. 3B). As mentioned, L98 and Y231 are closer to the MH4 binding site, suggesting that these two residues might not contribute to the aromatic amino acid substrate selection.

**Table 2** *In vivo* activities and substrate preferences of XcP4H mutants

| XcP4H mutants | Substrate | | | | Preference (Phe:Trp) |
|---|---|---|---|---|---|
| | Phenylalanine | | Tryptophan | | |
| | *In vivo* activity[b] ($\mu M/h/OD_{600}$) | R.A.[a] (%) | *In vivo* activity ($\mu M/h/OD_{600}$) | R.A. (%) | |
| WT | 97.40 ± 4.42 | 100 | 2.91 ± 0.21 | 100 | 33.5 |
| W179F | 78.05 ± 4.34 | 80 | 50.60 ± 4.72 | 1739 | 1.5 |
| W179F/L98Y | 44.49 ± 4.95 | 46 | 35.13 ± 1.67 | 1207 | 1.3 |
| W179F/Y231C | 50.92 ± 4.36 | 52 | 27.71 ± 2.99 | 952 | 1.8 |
| W179F/L98Y/Y231C | 16.56 ± 1.86 | 17 | 16.58 ± 2.59 | 570 | 1.0 |

[a] R.A., relative activity, setting the R.A. of WT XcP4H as 100%
[b] All data are reported as mean ± s.d. from three independent experiments.

To further explore the potential of XcP4H mutant W179F for whole-cell biocatalysis, feeding experiments were conducted by incubating pre-cultured *E. coli* cells harboring pZE-XcABMW179F (initial $OD_{600}$ = 12-13) with 2.0 g $l^{-1}$ of tryptophan. As shown in Fig. 3C, the initial conversion rates were similar at 30 and 37 °C, although the cells grew slightly faster at 37°C. However, the production efficiency at 30°C became obviously higher after 5 hours. By the end of 16 hours, the cultures at 30 and 37°C accumulated 1114.8 and 758.3 mg $l^{-1}$ of 5-HTP at the expense of 1503.2 and 1417.1 mg $l^{-1}$ tryptophan, respectively. Meanwhile, we observed that the color of the

cultures gradually turned dark after 5 hours, especially at 37°C, probably due to the oxidation of 5-HTP and tryptophan under aerobic conditions.

***De novo* microbial synthesis of 5-HTP via metabolic engineering.** After achieving the efficient bioconversion of tryptophan to 5-HTP, we proceeded with the construction of a 5-HTP producing strain which allows the utilization of endogenous tryptophan generated from simple carbon sources (Fig. 4A). Our first attempt was focused on the construction of a tryptophan overproducer. In *E. coli*, tryptophan biosynthesis is branched from the shikimate pathway at chorismate by the action of the *trp* regulon (Fig. 4A) and negatively regulated by tryptophan transcriptional repressor (TrpR) in response to intracellular tryptophan levels. To circumvent the intrinsic regulation at the transcription level, the complete *trp* operon including *trpEDCBA* was cloned into a low-copy plasmid under the control of an IPTG inducible promoter. Meanwhile, to eliminate the feed-back inhibition effect, a mutation S40F was incorporated into TrpE according to a previous study[27], resulting in plasmid pSA-TrpEDCBA. When the plasmid was introduced into *E. coli* BW*ΔtnaA,* the resulting strain produced 292.2 mg $l^{-1}$ of tryptophan at 37 °C after 24-hour cultivation; however, the titers dramatically decreased after 48 h (74.4 mg $l^{-1}$) probably due to oxidative degradation[28]. This problem was solved when the growth temperature was changed to 30 °C (Fig. 4B). In addition to BW*ΔtnaA*, we also attempted to use QH4*ΔtnaA* as the host for boosting carbon flux through the shikimate pathway, because QH4 is a derivative of the well-developed phenylalanine overproducer ATCC31884 with *pheLA* and *tyrA* disrupted and has been successfully engineered for the enhanced production of caffeic acid, salicylic acid and muconic acid in our previous studies[29, 30]. However, in this study, QH4*ΔtnaA* harboring pSA-TrpEDCBA did not significantly improve the production of tryptophan but showed slightly improved titers at 30°C compared with the BW*ΔtnaA* host. By the end of 48 h, up to 304.4 mg $l^{-1}$ of tryptophan was produced (Fig. 4B). The control strain QH4*ΔtnaA* without the over-expression of the *trp* operon did not accumulate tryptophan at either temperature.

As the tryptophan production and the bioconversion of tryptophan to 5-HTP were achieved and 30°C worked better for both cases, our further efforts were directed to the establishment of *de novo* biosynthesis of 5-HTP at this temperature by integrating the two modules. When pZE-XcABMW179F was co-transferred together with pSA-TrpEDCBA into *E. coli* BW*ΔtnaA* and QH4*ΔtnaA*, the generated strains only produced 19.9 and 11.5 mg $l^{-1}$ of 5-HTP, respectively, without accumulating tryptophan in the cultures. Apparently, the introduction of the 5-hydroxylation reaction using a high-copy number plasmid exerted negative influence on the carbon

flow through tryptophan compared with their parent strains. We speculated that the excessive expression of the XcP4H mutant with the MH4 recycling system might have resulted in metabolic imbalance and disturbed carbon flux towards tryptophan. To test this hypothesis, we cloned the coding sequences of XcP4H mutant W179F, PCD and DHMR into a medium-copy-number plasmid instead of the high-copy-number one, yielding plasmid pCS-XcABMW179F. Interestingly, we observed dramatic improvement on 5-HTP production for both BW*ΔtnaA* and QH4*ΔtnaA* harboring pCS-XcABMW179F; by the end of 48 hours, the two strains produced 128.6 and 152.9 mg $l^{-1}$ of 5-HTP, respectively (Fig. 4C), at the expense of 8.5 and 9.7 g $l^{-1}$ of glucose consumption, respectively. Meanwhile, we detected the accumulation of tryptophan at the concentrations of 166.3 and 339.7 mg $l^{-1}$ for the two strains, indicating that the carbon flux towards tryptophan was fully recovered. Other by-products were also detected as depicted in Table 3. The 5-HTP producing strains followed growth-dependent production patterns (Fig. 4C).

**Table 3** Intermediates and by-products produced by 5-HTP producing strains

| Host strain[a] | Biomass ($OD_{600}$) | Intermediates and by-products[b] | | | |
|---|---|---|---|---|---|
| | | Tyrosine (mg/L) | Tryptophan (mg/L) | Acetate (g/L) | Pyruvate (g/L) |
| BW*ΔtnaA* | 4.44 ± 0.02 | 52.30 ± 0.36 | 166.29 ± 1.52 | 2.72 ± 0.00 | 1.41 ± 0.06 |
| QH4*ΔtnaA* | 3.19 ± 0.05 | 34.85 ± 3.34 | 339.68 ± 18.15 | 1.55 ± 0.22 | 2.11 ± 0.27 |

[a] Both host strains containing plasmid pSA-TrpEDCBA and pCS-XcABMW179F.
[b] All data are reported as mean ± s.d. from 3 independent experiments. Error bars are defined as s.d.

**Discussion.** The high price of medication has become a major burden of families over the world. High price indeed deprives the low-income population of access to the use of some drugs that are hard to obtain and expensive. The causes of this issue are either the low efficiency in isolating these pharmaceuticals from natural sources or the high cost for their chemical synthesis. Microbial biosynthesis and biocatalysis provide a facile and eco-friendly way for the production of pharmaceutically valuable compounds. The development of metabolic engineering and synthetic biology tools enables tailored assembly of heterologous and artificial pathways in desirable host strains for the biosynthesis of target products[31].

Lack of suitable enzymes is one of the most frequently encountered problems in pathway engineering in microbes. Functional expression of eukaryotic enzymes is often problematic due to their low solubility, low stability and/or requirements for post-translational modification. For example, the tryptophan 5-hydroxylation reaction has been understood for a long time and many

T5Hs has been identified and characterized from human and animals. However, animal AAAHs were hard to be expressed in *E. coli* in a soluble and stable form[14, 32]. In addition, their activities are usually regulated by phosphorylation as well as their products[33]. Although the use of truncated or fusion proteins can help obtain soluble and active enzymes[32], the catalytic efficiency seems still low in the production of 5-HTP in *E. coli* using truncated animal T5Hs[16]. In recent years, protein engineering has become a potent tool for enzyme modification in order to obtain desired catalytic properties[34]. In our study, a highly active P4H that utilizes MH4 was successfully engineered to catalyze the tryptophan 5-hydroxylation reaction. Another group recently reported 5-HTP production in *E. coli* with a mutant P4H from *C. violaceum*[35]. However, its function had to completely rely on the supplementation of exogenous pterin coenzyme 6,7-dimethyl-5,6,7,8-tetrahydropterine hydrochloride[35], which is disadvantageous in terms of economic viability.

Self-supply or regeneration of cofactors (including co-enzymes and co-substrates) is one of the greatest advantages for whole-cell biosynthesis and biocatalysis. In *E. coli* host, many of such molecules can be natively generated along with cell growth, such as FMN/$FMNH_2$, FAD/$FADH_2$, $NAD(P)^+$/NAD(P)H, coenzyme A, acetyl-CoA, malonyl-CoA, MH4, etc. Although it is more convenient and economical to utilize these endogenous cofactors, sometimes heterologous enzymes require the cofactor(s) not produced by the host strain. To solve such a problem, one approach is to supplement exogenous cofactors into the culture medium. But it should be noted that most of the cofactors such as tetrahydropterine are so expensive that the supplementation of them is not economically viable for commercial production. Another approach is to introduce the cofactor biosynthetic and/or regeneration mechanism(s) into the host strain. As in the study using animal T5H to produce 5-HTP, a BH4 biosynthetic pathway starting from GTP was introduced into *E. coli*[16]. Meanwhile, a BH4 regeneration system was necessary to achieve continuous production. Recently, an interesting study reported that the mouse T3H can also utilize *E. coli* MH4 in the presence of a BH4 regeneration system, although the efficiency was proved to be low[36]. In this work, with minimal modifications of the host strains' metabolism, the prokaryotic P4Hs and the mutants were able to utilize and recycle *E. coli* endogenous MH4 and NAD(P)H (Figs. 2A and B).

**Conclusion.** This work simultaneously solved two problems in the biological production of 5-HTP, which are related to enzyme compatibility and cofactor self-supply. To our knowledge, the titer of 5-HTP (1.1-1.2 g $l^{-1}$) generated from tryptophan in this work is significantly higher than those in previous studies, showing great scale-up potential. Moreover, this work also demonstrates the *de novo* production of 5-HTP without needing to supplement precursors and coenzymes. Since

the high-level production of tryptophan (up to 48.7 g $l^{-1}$) has already been achieved in *E. coli*[37], introduction and optimization of the 5-hydroxylation reaction into tryptophan overproducers is expected to result in efficient and low-cost production of 5-HTP.

## MATERIALS AND METHODS

**Experimental materials.** *E. coli* XL1-Blue was employed as the host strain for cloning and plasmid propagation; *E. coli* BW25113*ΔtnaA* was used as the host strain for *in vivo* enzyme assays, feeding experiments, and *de novo* production of tryptophan and 5-HTP. QH4 was previously constructed with the disruption of *pheLA* and *tyrA* from a phenylalanine producer *E. coli* ATCC31884[29]. Luria-Bertani (LB) medium containing 10 g $l^{-1}$ tryptone, 5 g $l^{-1}$ yeast extract, and 10 g $l^{-1}$ NaCl was used for cell cultivation and enzyme expression. M9 minimal medium containing 5 g $l^{-1}$ glycerol, 6 g $l^{-1}$ $Na_2HPO_4$, 0.5 g $l^{-1}$ NaCl, 3 g $l^{-1}$ $KH_2PO_4$, 1 g $l^{-1}$ $NH_4Cl$, 246.5 mg $l^{-1}$ $MgSO_4 \cdot 7H_2O$, 14.7 mg $l^{-1}$ $CaCl_2$ and 27.8 mg $l^{-1}$ $FeSO_4 \cdot 7H_2O$, was used for *in vivo* assays of P4Hs. Modified M9 (M9Y) medium was used for *de novo* production of tryptophan and 5-HTP. M9Y medium contains 10 g $l^{-1}$ glucose, 6 g $l^{-1}$ Na2HPO4, 0.5 g $l^{-1}$ NaCl, 3 g $l^{-1}$ $KH_2PO_4$, 1 g $l^{-1}$ $NH_4Cl$, 246.5 mg $l^{-1}$ $MgSO_4 \cdot 7H_2O$, 14.7 mg $l^{-1}$ $CaCl_2 \cdot 2H_2O$, 27.8 mg $l^{-1}$ $FeSO_4 \cdot 7H_2O$, 2 g $l^{-1}$ yeast extract and 2 g $l^{-1}$ sodium citrate dihydrate. When necessary, kanamycin, ampicillin and/or chloramphenicol were supplemented to the media at the final concentration of 50, 100 and 34 $mgl^{-1}$, respectively. pZE12-luc, pCS27, pSA74 are high-, medium- and low-copy number plasmids[30], respectively, which were used for expressing enzymes in *E. coli*. Details of the strains and plasmids used in this study are depicted in Table 4.

**Table 4** Strains and plasmids used in this study

| Strain | Genotype | Source |
|---|---|---|
| XL1-Blue | *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*$^q$*ZΔM15 Tn10* (Tet$^r$)] | Stratagene |
| JW3686-7 | F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), *λ-*, *rph-1*, *ΔtnaA739::kan*, *Δ(rhaD-rhaB)568*, *hsdR514* | CGSC |
| QH4 | *E. coli* ATCC31884 with *pheLA* and *tyrA* disrupted | (28) |
| BW25113*ΔtnaA* | JW3686-7 with *kan* deleted | This study |
| QH4 | QH4 with *tnaA* deleted | This study |
| **Plasmids** | **Description** | **Reference** |
| pZE12-luc | $P_L$lacO1, *colE* ori, *luc*, Amp$^r$ | (29) |
| pCS27 | $P_L$lacO1, P15A ori, Kan$^r$ | (29) |
| pSA74 | $P_L$lacO1, pSC101 ori, Cm$^r$ | (29) |
| pZE-PaphhA | pZE12-luc containing *phhA* from *P. aeruginosa* PAO1 | This study |
| pZE-PaABM | pZE12-luc containing *phhA and phhB* from *P. aeruginosa* PAO1, and *folM* from *E. coli* MG1655 | This study |
| pZE-PfABM | pZE12-luc containing *phhA* from *P. fluorescens* Migula, *phhB* from *P. aeruginosa* PAO1, and *folM* from *E. coli* MG1655 | This study |
| pZE-PpABM | pZE12-luc containing *phhA* from *P. putida* KT2440, *phhB* from *P. aeruginosa* PAO1, and *folM* from *E. coli* MG1655 | This study |
| pZE-ReABM | pZE12-luc containing *phhA* from *R. eutropha* H16, *phhB* from *P. aeruginosa* PAO1, and *folM* from *E. coli* MG1655 | This study |
| pZE-XcABM | pZE12-luc containing *phhA* from *X. campestris* ATCC 33913, *phhB* from *P. aeruginosa* PAO1, and *folM* from *E. coli* MG1655 | This study |
| pZE-XcABMW179F | pZE-XcABM with mutation W179F on the P4H | This study |
| pSA-XcABM2Ma | pZE-XcABM with mutations W179F and L98Y on the P4H | This study |
| pZE-XcABM2Mb | pZE-XcABM with mutations W179F and Y231C on the P4H | This study |
| pZE-XcABM3M | pZE-XcABM with mutations W179F, L98Y and Y231C on the P4H | This study |
| pCS-XcABMW179F | pCS27 containing *phhA* (W179F) from *X. campestris* ATCC 33913, *phhB* from *P. aeruginosa* PAO1, and *folM* from *E. coli* MG1655 | This study |
| pSA-TrpEDCBA | pSA74 containing *trpEDCBA* with S40F on TrpE | This study |

**DNA manipulation.** *E. coli strain* BW25113 *ΔtnaA::kan* (JW3686-7) was purchased from Coli Genetic Stock Center (CGSC). The kanamycin resistant marker was deleted according to the

reported protocol[38]. Deletion of the *tnaA* gene from QH4 was performed using the reported Red disruption method[38]. Plasmid pZE-PaphhA was constructed by inserting the amplified *phhA* gene from *Pseudomonas aeruginosa* into pZE12-luc using restriction sites Acc65I and XbaI. pZE-PaABM was constructed by inserting *phhA* and *phhB* from *P. aeruginosa* and *folM* from *E. coli* into pZE12-luc via multi-piece ligation using Acc65I/NdeI, NdeI/HindIII and HindIII/XbaI. pZE-PpABM, pZE-PfABM, pZE-ReABM and pZE-XcABM were constructed using the same approach with the respective *phhA* genes in place of the *phhA* gene from *P. aeruginosa*. pSA-trpEDCBA was constructed by inserting the DNA fragment of *trpEDCBA* from *E. coli* into pSA74 using Acc65I and BamHI. Site-directed mutagenesis was conducted by overlap PCR. Plasmids pZE-XcABMW179F, pZE-XcABM2Ma, pZE-XcABM2Mb, pZE-XcABM3M were constructed by replacing the wild type *phhA* gene from *X. campestris* with the respective mutant genes (Table 4)

**Construction of phylogenetic tree and homology modeling.** The AAAH sequences were randomly selected from GenBank using "phenylalanine 4-hydroxylase", "tyrosine 3-hydroxylase" and "tryptophan 5-hydroxylase" as the searching keywords. The alignment of the AAAH amino acid sequences was conducted by using ClustalX 2.1. The phylogenetic tree was constructed by Molecular Evolutionary Genetics Analysis (MEGA) version 5.02 using the neighbor-joining method[22]. Bootstrapping test was performed to evaluate the reliability (1000 replicates). All other used parameters were the default of the software. The homology model of XcP4H was built with the SWISS-MODEL online server by using the crystal structure of the P4H from *C. violaceum* (PDB code 3TK2) as a template.

***In vivo* assays of wild-type and mutant P4Hs.** *E. coli* BW25113*ΔtnaA* carrying pZE-PaABM was inoculated in 50 ml of LB liquid medium containing 0.5 mM of IPTG and 100 $\mu g ml^{-1}$ of ampicillin, and grown aerobically at 37°C for about 8 h till $OD_{600}$ reached 4.5-5.5. Then the cells were harvested, and re-suspended in the M9 minimal medium ($OD_{600}$ = 4.5-5.5). After adaption for 20 min, phenylalanine or tryptophan was added into the cell suspension to a final concentration of 500 mg $l^{-1}$. At the same time, 1 mM of ascorbic acid was added to avoid the product oxidation. The flasks were incubated with shaking (300 rpm) at 37 °C for 1 h. Subsequently, samples were taken by removing cell pellets and the products (tyrosine and tryptophan) were quantitatively measured with HPLC. The same method was used to measure the *in vivo* activities of other P4Hs and XcP4H mutants. The *in vivo* activities of P4Hs were expressed as $\mu M/min/OD_{600}$.

**Bioconversion of tryptophan to 5-HTP.** *E. coli* strain BW25113*ΔtnaA* was transformed with plasmid pZE-XcABMW179F. Single colonies were inoculated into 50 ml LB medium

containing 0. 5 mM of IPTG and grown aerobically at 37°C for about 8 h till $OD_{600}$ reached around 5.0. Then cells were harvested, re-suspended in 20 ml of M9Y medium (at $OD_{600}$ =12-13) containing 2g $l^{-1}$ of tryptophan and left to grow at 30 and 37°C. Samples were taken at 2 h, 5 h, 10 h and 16 h. The concentrations of produced 5-HTP were analyzed by HPLC.

***De novo* production of tryptophan and 5-HTP**. Overnight LB cultures of the producing strains were inoculated at 2% into the M9Y medium containing appropriated antibiotics and cultivated at 30 and 37°C with shaking at 300 rpm. IPTG was added to the cultures to a final concentration of 0.5 mM at 0 h. Samples were taken every 12 hours. The $OD_{600}$ values were measured and the concentrations of the products, intermediates and by-products were analyzed by HPLC.

**HPLC analysis.** *L*-tyrosine (from SIGMA ALDRICH), *L*-tryptophan (from SIGMA ALDRICH) and 5-HTP (from Acros Organics) were used as standards. Both the standards and samples were quantified by HPLC (Dionex Ultimate 3000 installed with an Ultimate 3000 Photodiode Array Detector and a reverse phase ZORBAX SB-C18 column). A gradient elution method was used according to our previous study[39]. Quantification of tryptophan, tyrosine and 5-HTP was based on the peak areas at specific wavelength (276 nm). Glucose, acetate and pyruvate were quantified using a previously described method[40].

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40. Shen, X., Lin, Y., Jain, R., Yuan, Q., and Yan, Y. (2012) Inhibition of acetate accumulation leads to enhanced production of (R,R)-2,3-butanediol from glycerol in *Escherichia coli*, *J. Ind. Microbiol. Biotechnol.39*, 1725-1729.

## Example II. 5-Hydroxytryptophan as an Animal Feed Supplement

Milk fever (also called periparturient hypocalcemia) is a metabolic disorder frequently occurring among farm animals around calving period. About 0-10% of dairy cows experience milk fever (clinical hypocalcaemia), while this number can increase to 25% in calving cows (DeGaris et al. 2008, Vet. J. 176, 58-69). Moreover, a majority of dairy cows exhibit different degrees of subclinical hypocalcaemia. Milk fever results in substantial economic losses to dairy farmers, including cow deaths (about 5% of affected cows), reduction in the productive lifespan (3.4 years for each affected cow), decrease in milk production, deterioration in reproductive performance, and costs on prevention and treatment of this disease. On average, the total loss related to milk fever is estimated to be $334 per incidence (Goff et al., 2003, Acta Vet. Scand. Suppl. 97, 145-147). The Food and Agriculture Organization of the United Nations (FAO) has reported that in 2011there



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were 260 million dairy cows in the world (World Dairy Cow Number, 2014). Accordingly, the estimated loss caused by milk fever is about $4.34 billion globally (assuming the incidence of severe milk fever is 5%). In contrast, the cows that went through the transition period free of disease usually show a much higher chance to have productive lactation periods, as well as better reproductive performance (Hernandez, 2011, Calsium Homeostasis in Transition Dairy Cattle.

Extensive studies have shown that elevation of serotonin level during the dry period can increase calcium levels and prevent milk fever. Current practices mainly focus on the manipulation of dietary cation-anion difference in dry cow diets. However, these diets are unpalatable to the animals and also very costly (Id.). Therefore, it is desirable to develop new strategies to control milk fever that are both palatable to the animals and inexpensive for large-scale practice. A recent USDA-funded study entitled "Calcium Homeostasis in Transition Dairy Cattle" revealed that supplementation of 5-HTP resulted in increased calcium mobilization in animals transitioning from pregnancy to lactation, a mechanism that can prevent milk fever effectively (Id.). Horseman et al. claim that 5-HTP supplementation is efficient for the prevention and/or treatment of periparturient hypocalcemia in lactating female mammals (WO2013112873 A2). It should be noted that milk fever occurs among not only cows but also many other farm mammals. However, the high cost and limited supply of the 5-HTP produced by current botanical extraction prevent its use in farm animal feed. Our new technology will reduce the production cost by up to 90%, which would make it economically viable to use 5-HTP as an animal feed additive or supplement in order to prevent milk fever.

The foregoing detailed description and examples have been given for clarity of understanding only. No unnecessary limitations are to be understood therefrom. The invention is not limited to the exact details shown and described, for variations obvious to one skilled in the art will be included within the invention defined by the claims.

Unless otherwise indicated, all numbers expressing quantities of components, molecular weights, and so forth used in the specification and claims are to be understood as being modified in all instances by the term "about." Accordingly, unless otherwise indicated to the contrary, the numerical parameters set forth in the specification and claims are approximations that may vary depending upon the desired properties sought to be obtained by the present invention. At the very least, and not as



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an attempt to limit the doctrine of equivalents to the scope of the claims, each numerical parameter should at least be construed in light of the number of reported significant digits and by applying ordinary rounding techniques.

Notwithstanding that the numerical ranges and parameters setting forth the broad scope of the invention are approximations, the numerical values set forth in the specific examples are reported as precisely as possible. All numerical values, however, inherently contain a range necessarily resulting from the standard deviation found in their respective testing measurements.

In the event that any inconsistency exists between the disclosure of the present application and the disclosure(s) of any document cited herein, the disclosure of the present application shall govern.

All headings are for the convenience of the reader and should not be used to limit the meaning of the text that follows the heading, unless so specified.



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CLAIMS:

1. A genetically engineered bacterial cell comprising a modified bacterial phenylalanine-4-hydroxylase (P4H) that catalyzes the 5-hydroxylation of tryptophan in the presence of a tetrahydromonapterin (MH4) cofactor; and in the absence of a tetrahydrobiopterin (BH4) cofactor or 6,7-dimethyl-5,6,7,8-tetrahydropterine hydrochloride, wherein the modified bacterial P4H comprises an amino acid mutation at any one, any two, or all three of amino acid positions 98, 179, and 231 of *Xanthomonas campestris* P4H (SEQ ID NO:1), or at any one, any two, or all three corresponding amino acid positions in a bacterial P4H enzyme from another species.

2. The genetically engineered bacterial cell of claim 1, wherein the modified bacterial phenylalanine-4-hydroxylase has increased affinity for tryptophan compared to the corresponding wild-type phenylalanine-4-hydroxylase.

3. The genetically engineered bacterial cell of claim 1 or 2, which is genetically engineered to overproduce or accumulate tryptophan.

4. The genetically engineered bacterial cell of any one of claims 1 to 3, wherein the genetically engineered bacterial cell comprises an endogenous or genetically engineered tetrahydromonapterin (MH4) or tetrahydrobiopterin (BH4) cofactor biosynthetic mechanism.

5. The genetically engineered bacterial cell of any one of claims 1 to 4, wherein the genetically engineered bacterial cell comprises an endogenous or genetically engineered tetrahydromonapterin (MH4) or tetrahydrobiopterin (BH4) cofactor regeneration mechanism.

6. The genetically engineered bacterial cell of claim 5 wherein the cofactor regeneration mechanism comprises at least one of a pterin-4α-carbinolamine dehydratase (PCD) and a dihydromonapterin reductase (DHMR).

7. The genetically engineered bacterial cell of claim 6 wherein the dihydromonapterin reductase is encoded by the *E. coli* gene *folM*.



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8. The genetically engineered bacterial cell of any one of claims 1 to 7 wherein the modified bacterial P4H is derived from *Pseudomonas*, *Chromobacterium*, *Ralstonia*, or *Xanthomonas*.

9. The genetically engineered bacterial cell of claim 1 wherein the amino acid mutation comprises any one, any two, or any three mutations selected from the group consisting of L98Y, W179F and Y231C of the *X. campestris* P4H, or corresponding amino acid positions in a bacterial P4H enzyme from another species.

10. The genetically engineered bacterial cell of claim 1 or 9 wherein the modified bacterial P4H further comprises at least one further mutation, said further mutation at any one, any two, or all three of amino acid positions 85, 223 and 282 of the *X. campestris* P4H, or at any one, any two, or all three corresponding amino acid positions in a bacterial P4H enzyme from another species.

11. The genetically engineered bacterial cell of any one of claims 1 to 10, the bacterial cell comprising a first plasmid comprising a polynucleotide operably encoding (a) the modified bacterial P4H and (b) at least one of a pterin-4α-carbinolamine dehydratase (PCD) and a dihydromonapterin reductase (DHMR).

12. The genetically engineered bacterial cell of claim 11 wherein the first plasmid is a medium copy number plasmid.

13. The genetically engineered bacterial cell of claim 11 or 12 further comprising a second plasmid comprising a polynucleotide operably encoding all or a portion of a *trp* operon.

14. The genetically engineered bacterial cell of claim 13 wherein the *trp* operon or portion thereof comprises a mutation S40F in the TrpE gene.

15. The genetically engineered bacterial cell of claim 13 or 14 wherein the second plasmid is a low copy number plasmid.



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16. The genetically engineered bacterial cell of any one of claims 1 to 15 which is an *Escherichia coli* cell, a *Bacillus spp.* cell, a *Lactobacillus spp.* cell, a *Bifidobacteria* spp. cell, or a *Corynebacterium spp. glutamicum* cell.

17. The genetically engineered bacterial cell of any one of claims 1 to 16 which does not comprise a tetrahydrobiopterin (BH4) cofactor.

18. The genetically engineered bacterial cell of any one of claims 1 to 16 which comprises an endogenous tetrahydrobiopterin (BH4) cofactor.

19. The genetically engineered bacterial cell of any one of claims 1 to 18 which comprises an endogenous tetrahydromonapterin (MH4) cofactor.

20. A method of making 5-hydroxytryptophan (5-HTP) comprising:

culturing the genetically engineered bacterial cell of any one of claims 1 to 19 under conditions and for a time sufficient to produce 5-HTP; and

isolating the 5-HTP.

21. The method of claim 20, wherein culturing the genetically engineered bacterial cell comprises supplying the cell with at least one carbon source selected from the group consisting of glucose, glycerol, xylose, and arabinose.

22. The method of claim 20 further comprising purifying the 5-HTP.

23. The method of any one of claims 20 to 22 further comprising incorporating the 5-HTP into a food product.

24. The method of claim 23 wherein the food product is an animal feed or beverage.

25. The method of claim 23 or 24 further comprising packaging the 5-HTP or food product for sale.



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26. The method of claim 25 further comprising providing instructions for use of the 5-HTP or food product as a food additive, a food supplement, or a nutraceutical.

27. The method of claim 26 wherein the food additive, food supplement, or nutraceutical is packaged for use as an animal feed or beverage.

28. The method of any one of claims 20 to 27 wherein the culture is not supplemented with a pterin cofactor.



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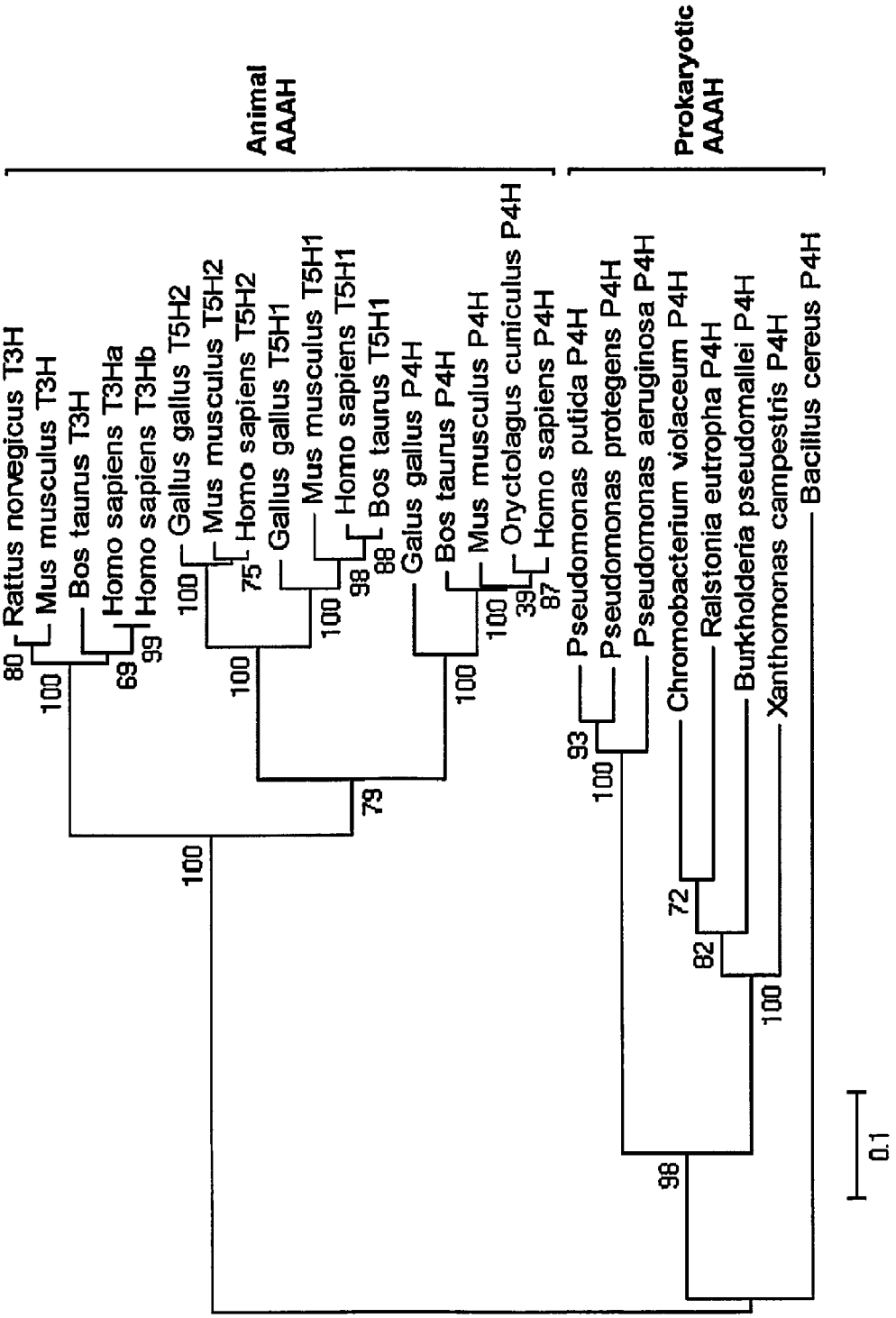
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Prokaryotic AAAH
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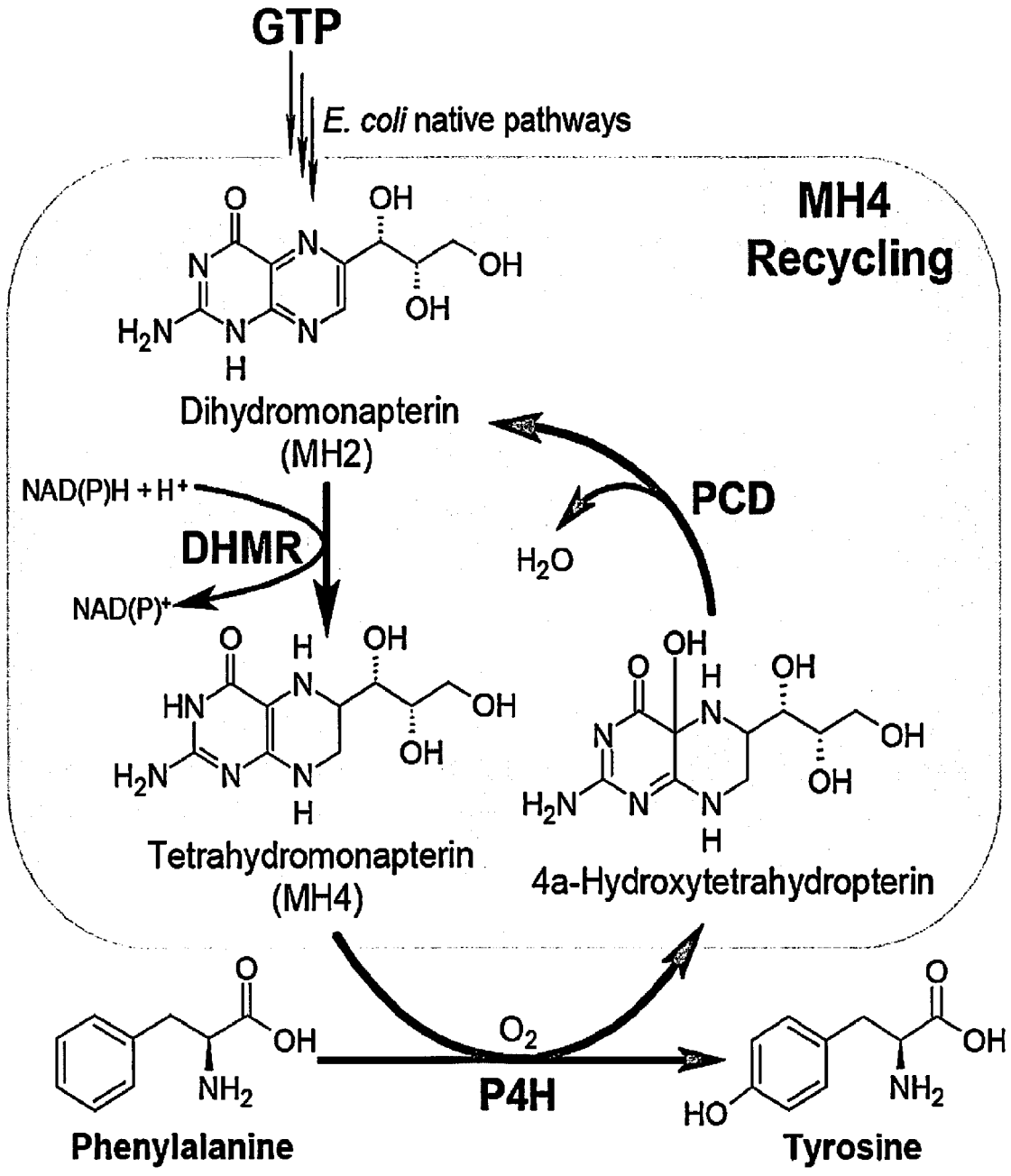
Mus musculus T3H
Bos taurus T3H
Homo sapiens T3Ha
Homo sapiens T3Hb
Gallus gallus T5H2
Mus musculus T5H2
Homo sapiens T5H2
Gallus gallus T5H1
Mus musculus T5H1
Homo sapiens T5H1
Bos taurus T5H1
Galus gallus P4H
Bos taurus P4H
Mus musculus P4H
Oryctolagus cuniculus P4H
Homo sapiens P4H
Pseudomonas putida P4H
Pseudomonas protegens P4H
Pseudomonas aeruginosa P4H
Chromobacterium violaceum P4H
Ralstonia eutropha P4H
Burkholderia pseudomallei P4H
Xanthomonas campestris P4H
Bacillus cereus P4H
0.1

*Fig. 1*

*Fig. 2*

(A)


GTP
E. coli native pathways
MH4
Recycling
Dihydromonapterin
(MH2)
NAD(P)H + H+
DHMR
NAD(P)+
PCD
H2O
Tetrahydromonapterin
(MH4)
4a-Hydroxytetrahydropterin
O2
P4H
Phenylalanine
Tyrosine

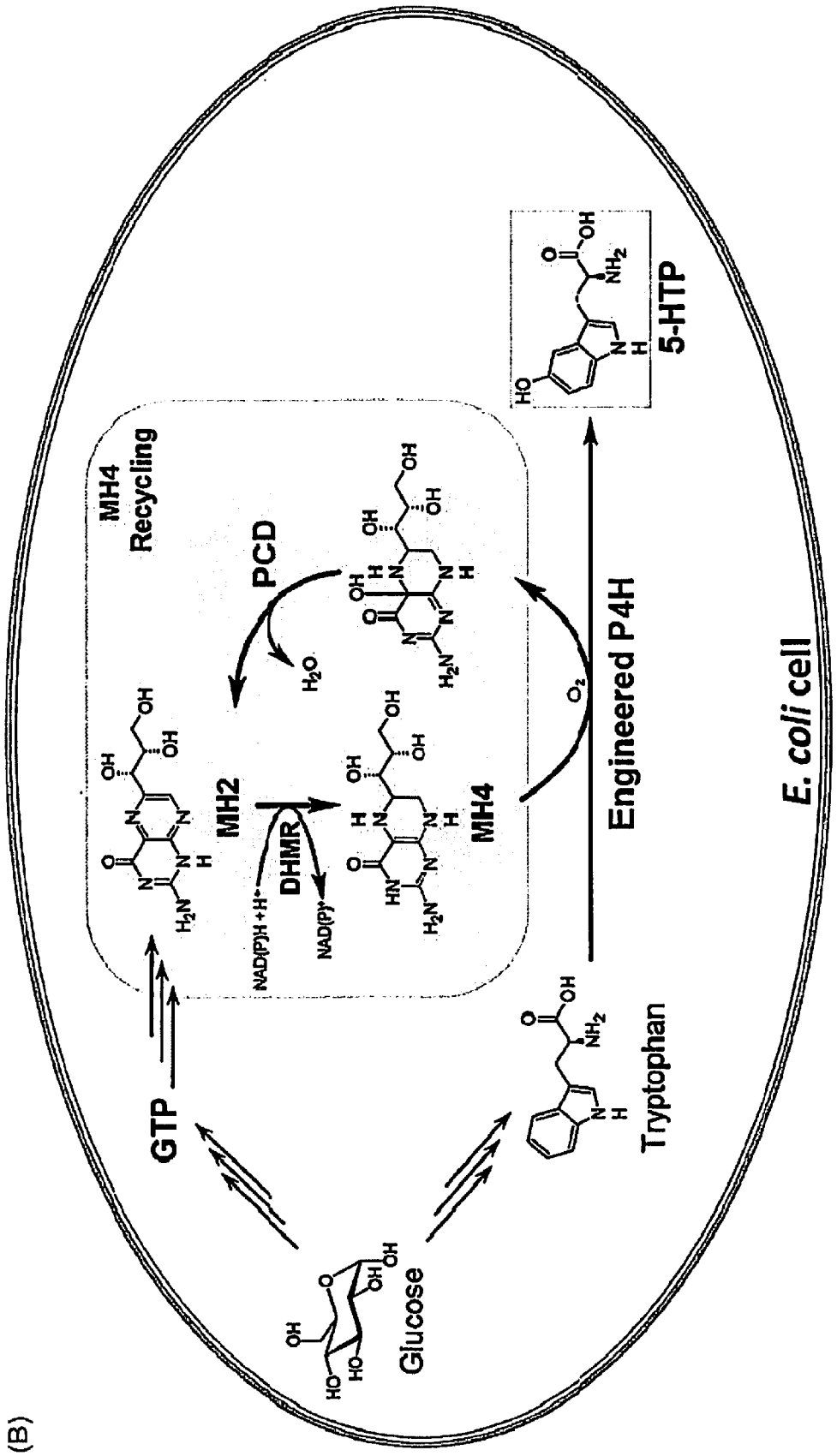

E. coli cell
Glucose
GTP
Tryptophan
MH4 Recycling
MH2
DHMR
NAD(P)H + H+
NAD(P)+
PCD
H2O
MH4
O2
Engineered P4H
5-HTP

Fig. 2

(B)

*Fig. 3*

(A)

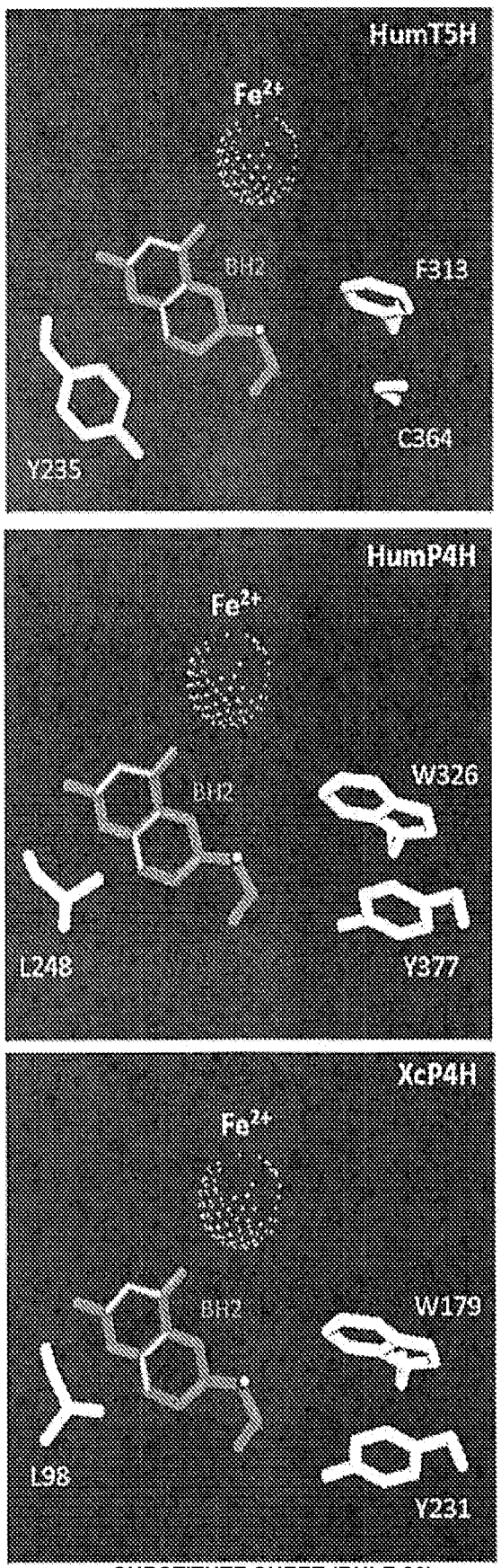
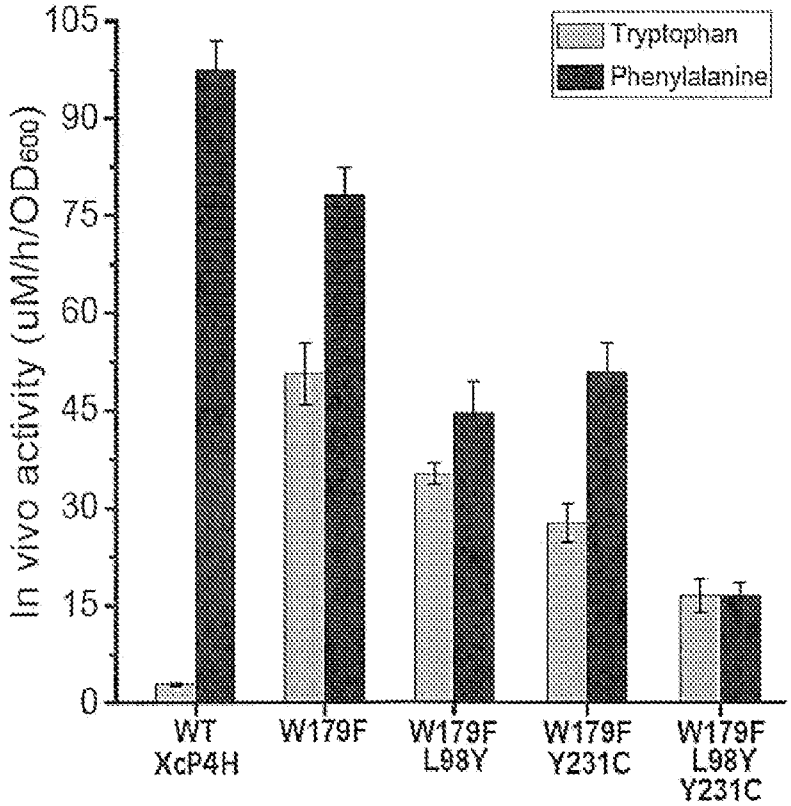
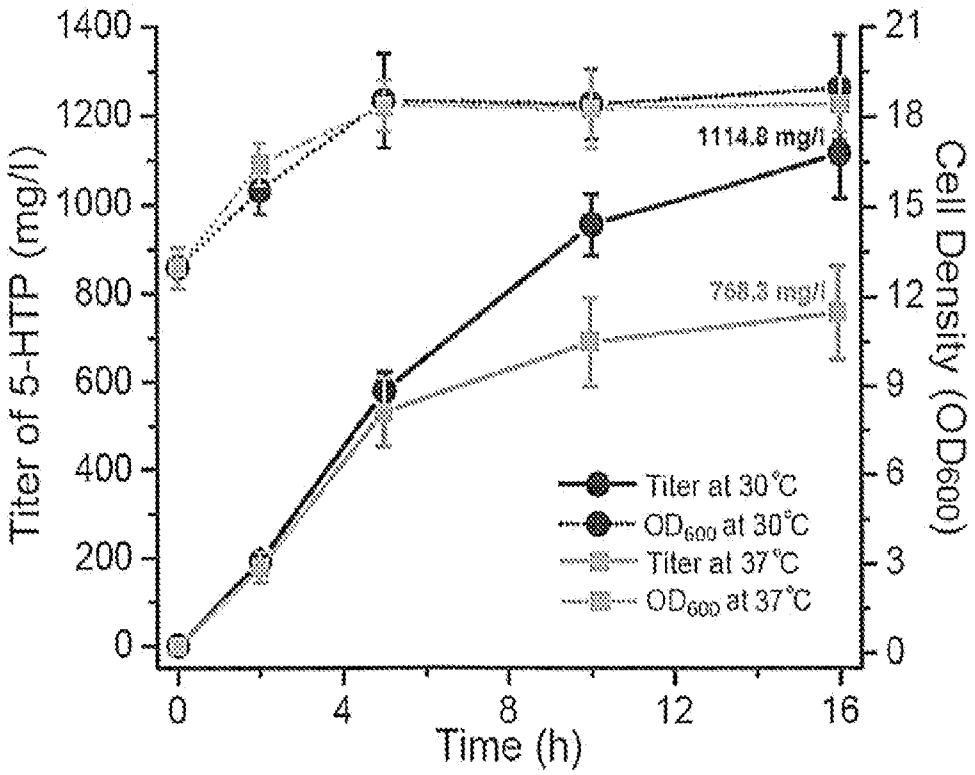

HumT5H
Fe2+
BH2
F313
C364
Y235
HumP4H
Fe2+
BH2
W326
L248
Y377
XcP4H
Fe2+
BH2
W179
L98
Y231

*Fig. 3*

(B)


105
90
75
60
45
30
15
0
In vivo activity (uM/h/OD600)
Tryptophan
Phenylalanine
WT XcP4H
W179F
W179F L98Y
W179F Y231C
W179F L98Y Y231C

(C)


1400
1200
1000
800
600
400
200
0
Titer of 5-HTP (mg/l)
21
18
15
12
9
6
3
0
Cell Density (OD600)
1114.8 mg/l
768.3 mg/l
Titer at 30°C
OD600 at 30°C
Titer at 37°C
OD600 at 37°C
0
4
8
12
16
Time (h)

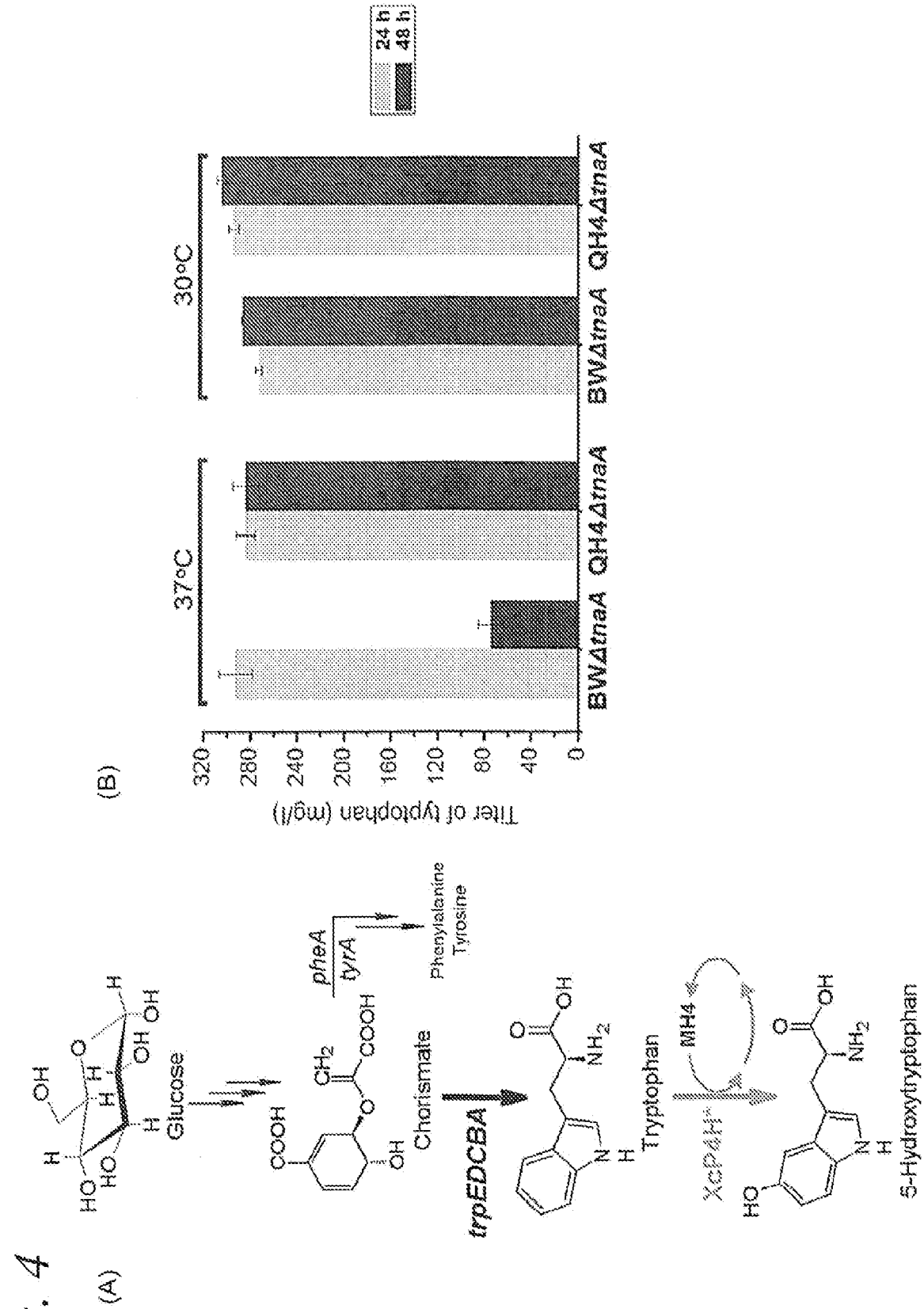

(A)
Glucose
Chorismate
pheA
tyrA
Phenylalanine
Tyrosine
trpEDCBA
Tryptophan
MH4
XcP4H
5-Hydroxytryptophan
(B)
Titer of tryptophan (mg/l)
320
280
240
200
160
120
80
40
0
37°C
30°C
24 h
48 h
BWΔtnaA
QH4ΔtnaA
BWΔtnaA
QH4ΔtnaA

Fig. 4

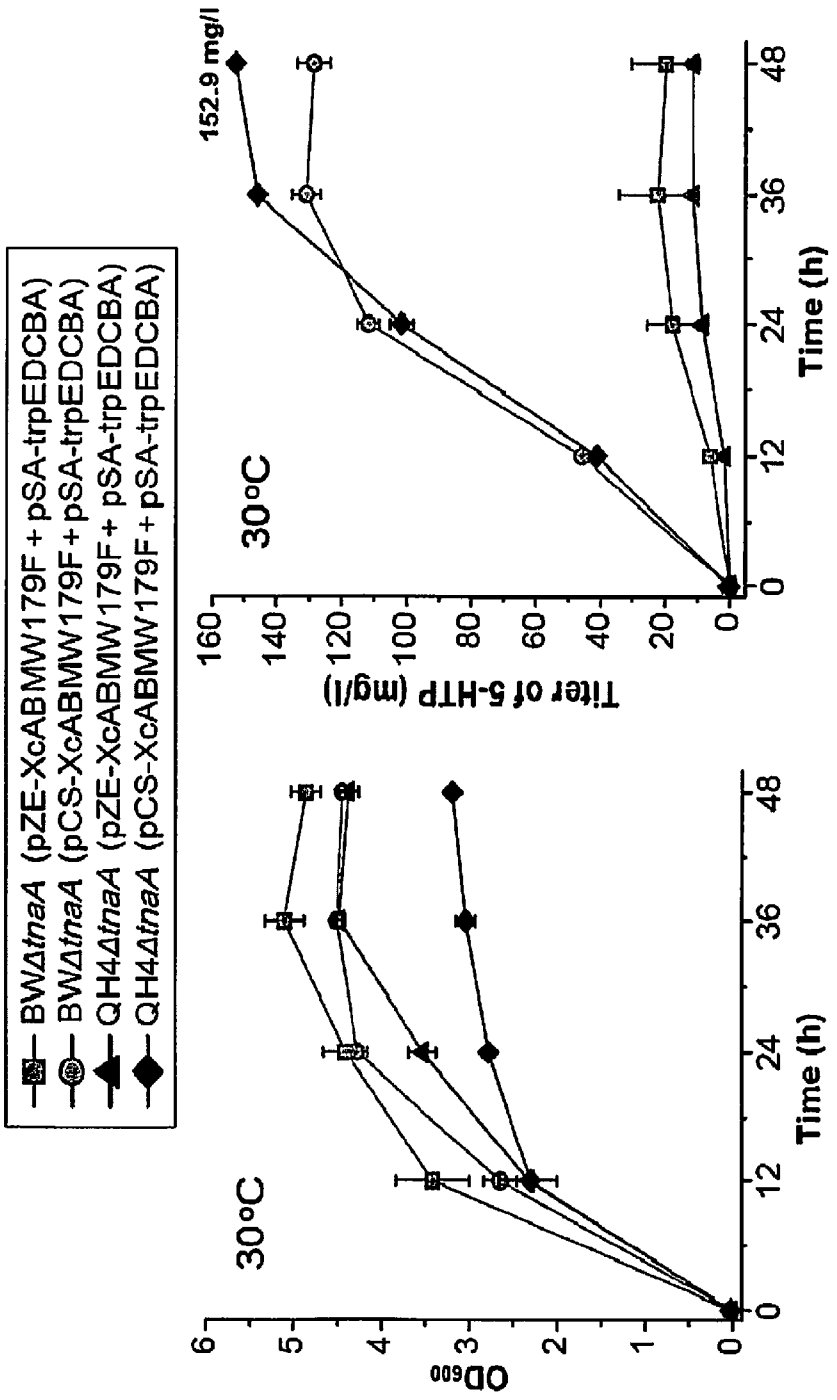

BWΔtnaA (pZE-XcABMW179F + pSA-trpEDCBA)
BWΔtnaA (pCS-XcABMW179F + pSA-trpEDCBA)
QH4ΔtnaA (pZE-XcABMW179F + pSA-trpEDCBA)
QH4ΔtnaA (pCS-XcABMW179F + pSA-trpEDCBA)
30°C
OD600
Time (h)
30°C
Titer of 5-HTP (mg/l)
152.9 mg/l
Time (h)

*Fig. 4*

(C)

*Fig. 5*

(A)

*Pseudomonas aeruginosa* (PaP4H) **Protein Sequence**

MKTTQYVARQPDDNGFIHYPETEHQVWNTLITRQLKVIEGRAC
QEYLDGIEQLGLPHERIPQLDEINRVLQATTGWRVARVPALIP
FQTFFELLASQQFPVATFIRTPEELDYLQEPDIFHEIFGHCPL
LTNPWFAEFTHTYGKLGLKASKEERVFLARLYWMTIEFGLVET
DQGKRIYGGGILSSPKETVYSLSDEPLHQAFNPLEAMRTPYRI
DILQPLYFVLPDLKRLFQLAQEDIMALVHEAMRLGLHAPLFPP
KQAA*

(B)

*Pseudomonas fluorescens* (PfP4H) **Protein Sequence**

MKQTQYVAREPDAQGFIDYPPEEHAVWNTLITRQLKVIEGRAC
QEYLDGIDKLGLPHDRIPQLGEINKVLGETTGWQVARVPALIP
FQTFFELLASKRFPVATFIRTREELDYLQEPDIFHEIFGHCPL
LTNPWFAEFTHTYGKLGLQATKEERVYLARLYWMTIEFGLVDT
PAGRRIYGGGILSSPKETVYSLSEEPEHQAFDPLEAMRTPYRI
DILQPIYFTLPNLKRLFDLAHEDIMALVHQGMQLGLHAPKFPP
KPKAA*

*Fig. 5*

(C)

*Pseudomonas putida* (PpP4H) **Protein Sequence**

MKQTQYVAREPDAHGFIDYPQQEHAVWNTLITRQLKVIEGRAC
QEYLDGIDQLKLPHDRIPQLGEINKVLGATTGWQVARVPALIP
FQTFFELLASKRFPVATFIRTPEELDYLQEPDIFHEIFGHCPL
LTNPWFAEFTHTYGKLGLAATKEQRVYLARLYWMTIEFGLMET
AQGRKIYGGGILSSPKETVYSLSDEPEHQAFDPIEAMRTPYRI
DILQPVYFVLPNMKRLFDLAHEDIMGMVHKAMQLGLHAPKFPP
KVAA*

(D)

*Ralstonia eutropha* (ReP4H) **Protein Sequence**

MSIATATEAPGAFQGTLTDKLKEQFDAGLLSGQELRPDFTIAQ
PVHRYTSTDHAIWRKLYERQAAMLQGRVSDEFLQGLATLGMDK
DRVPDFDQLNETLMRATGWQVVAVPGLVPDQVFFEHLANRRFP
ASWWMRKPEQLDYLQEPDCFHDVFGHVPLLINPVFADYMEAYG
KGGLKANGLGALDMLSRLYWYTVEFGLIRTAQGLRIYGAGILS
SQGESIYSLDSASPNRIGFDVRRIMRTRYRIDTFQKTYFVIDS
FEQLFDATRPDFAPLYEELRAQPTLGAGDVAPGDQVLNVGTRE
GWADTEDI*

*Fig. 5*

(E)

*Xanthomonas campeteris* (XcP4H) **Protein Sequence**

MNTAPRRVENQLTDKGYVPVYTTAVVEQPWDGYSADDHATWGT
LYRRQRALLVGRACDEFLQAQDAMGMDDTQIPRFDALNAVLQA
TTGWTLVGVEGLLPELDFFDHLANRRFPVTWWIRRPDQIDYIA
EPDLFHDLFGHVPLLMNPLFADFMQAYGRGGVKAHGIGPDALQ
NLTRLYWYTVEFGLIATPQGLRIYGAGIVSSKGESLHSLESAA
PNRVGFDLQRVMRTRYRIDSFQKTYFVIDSFTQLMDATAPDFT
PIYAALAQQPQVPAGEVLATDHVLQRGSGEGWSRDGDV*

*Fig. 5*

(F)

```
PaP4H   1   MKTTQ    YVARQ---PD   DNGFIHYPETEHQVWNTLITRQLKVIEGRACQEYLDGIEQLGLPHERIPQLDEINRVL  70
PfP4H   1   MKQTQ    YVARE---PD   AQGFIDYPPEEHAVWNTLITRQLKVIEGRACQEYLDGIDKLGLPHDRIPQLGEINKVL  70
PpP4H   1   MKQTQ    YVARE---PD   AHGFIDYPQQEHAVWNTLITRQLKVIEGRACQEYLDGIDQLKLPHDRIPQLGEINKVL  70
ReP4H   1   MSIAT[23]LLSGQelrPD[4]-QPVHRYTSTDHAIWRKLYERQAAMLQGRVSDEFLQGLATLGMDKDRVPDFDQLNETL  99
XcP4H   1   MNTA-[12]YV------PV[6]EQPWDGYSADDHATWGTLYRRQRALLVGRACDEFLQAQDAMGMDDTQIPRFDALNAVL  84

PaP4H   71  QATTGWRVARVPALIPFQTFFELLASQQFPVATFIRTPEELDYLQEPDIFHEIFGHCPLLTNPWFAEFTHTYGKLGLKAS  150
PfP4H   71  GETTGWQVARVPALIPFQTFFELLASKRFPVATFIRTREELDYLQEPDIFHEIFGHCPLLTNPWFAEFTHTYGKLGLQAT  150
PpP4H   71  GATTGWQVARVPALIPFQTFFELLASKRFPVATFIRTPEELDYLQEPDIFHEIFGHCPLLTNPWFAEFTHTYGKLGLAAT  150
ReP4H   100 MRATGWQVVAVPGLVPDQVFFEHLANRRFPASWWMRKPEQLDYLQEPDCFHDVFGHVPLLINPVFADYMEAYGKGGLKAN  179
XcP4H   85  QATTGWTLVGVEGLLPELDFFDHLANRRFPVTWWIRRPDQIDYIAEPDLFHDLFGHVPLLMNPLFADFMQAYGRGGVKAH  164

PaP4H   151 ---KEERVFLARLYWMTIEFGLVETDQGKRIYGGGILSSPKETVYSLSD-EPLHQAFNPLEAMRTFYRIDILQPLYFVLP  226
PfP4H   151 ---KEERVYLARLYWMTIEFGLVDTPAGRRIYGGGILSSPKETVYSLSE-EPEHQAFDPLEAMRTFYRIDILQPIYFTLP  226
PpP4H   151 ---KEQRVYLARLYWMTIEFGLMETAQGRKIYGGGILSSPKETVYSLSD-EPEHQAFDPIEAMRTFYRIDILQPVYFVLP  226
ReP4H   180 glg--ALDMLSRLYWYTVEFGLIRTAQGLRIYGAGILSSQGESIYSLDSaSPNRIGFDVRRIMRTRYRIDTFQKTYFVID  257
XcP4H   165 gigPDALQNLTRLYWYTVEFGLIATPQGLRIYGAGIVSSKGESLHSLESaAPNRVGFDLQRVMRTRYRIDSFQKTYFVID  244

PaP4H   227 DLKRLFQLAQEDIMALVHEAMRLGLHAPLFPPKQ-AA*       263
PfP4H   227 NLKRLFDLAHEDIMALVHQGMQLGLHAPKFPPKPKAA*       264
PpP4H   227 NMKRLFDLAHEDIMGMVHKAMQLGLHAPKFPPKV-AA*       263
ReP4H   258 SFEQLFDATRPDFAPLYEELRA----QPTLGAGDVAPG[19]   310
XcP4H   245 SFTQLMDATAPDFTPIYAALAQ----QPQVPAGEVLAT[19]   297
```

Fig. 5

(G)

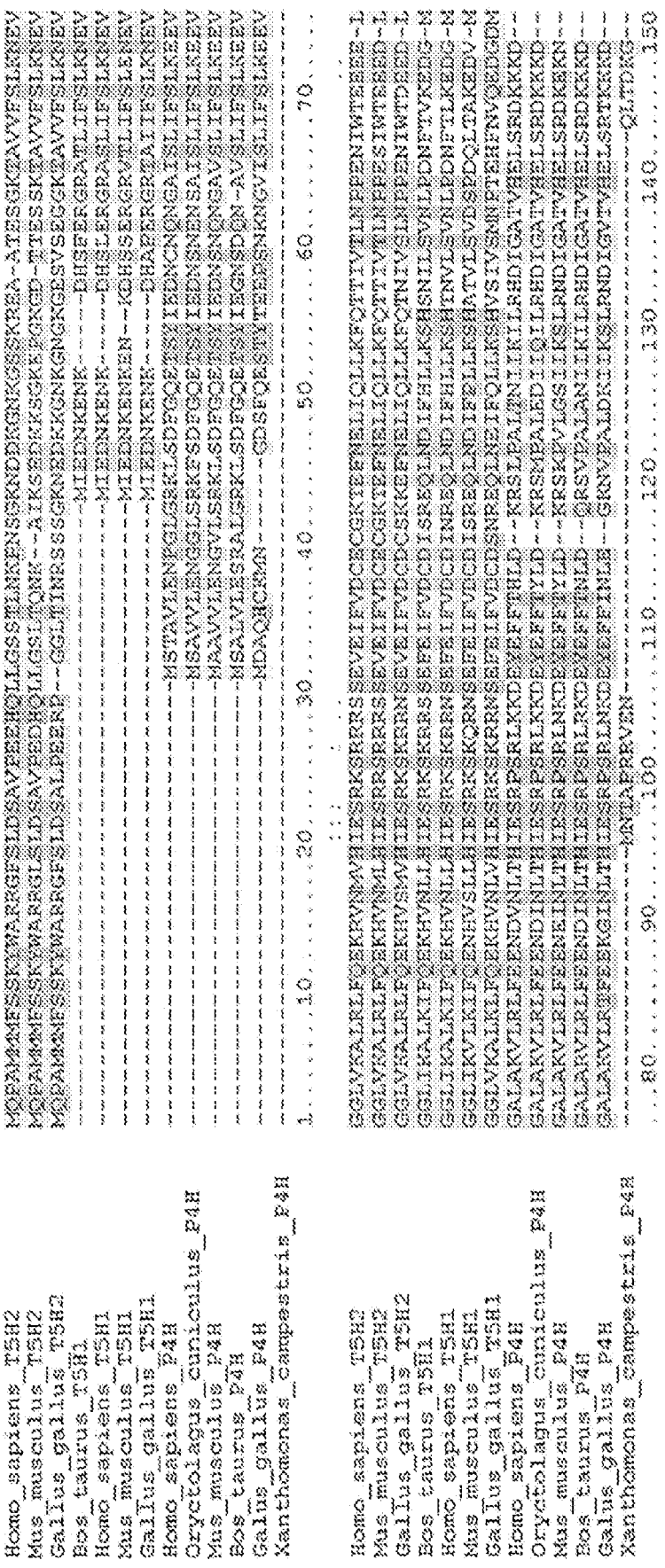


*Fig. 5*
(G)
(cont.)

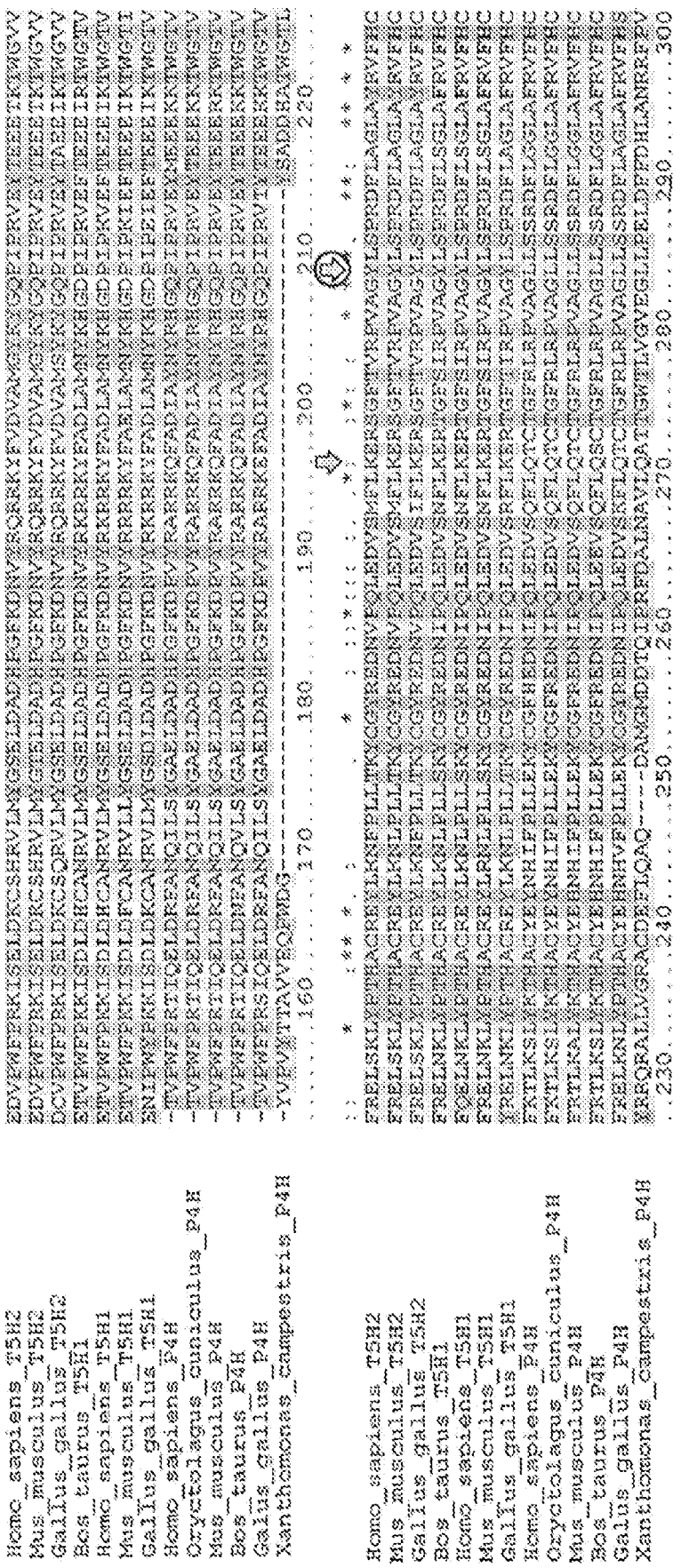
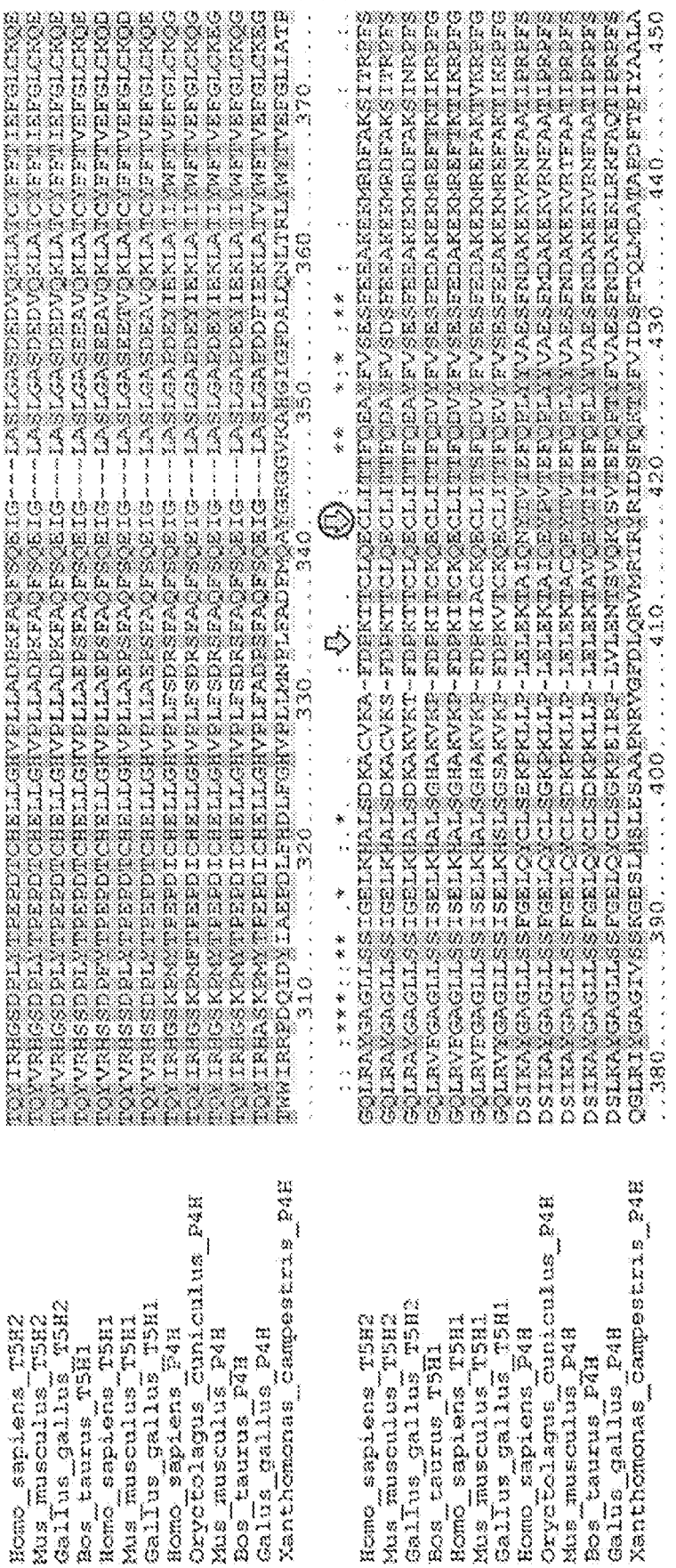


Fig. 5
(G)
(cont.)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Gallus_gallus_T5H2
Bos_taurus_T5H1
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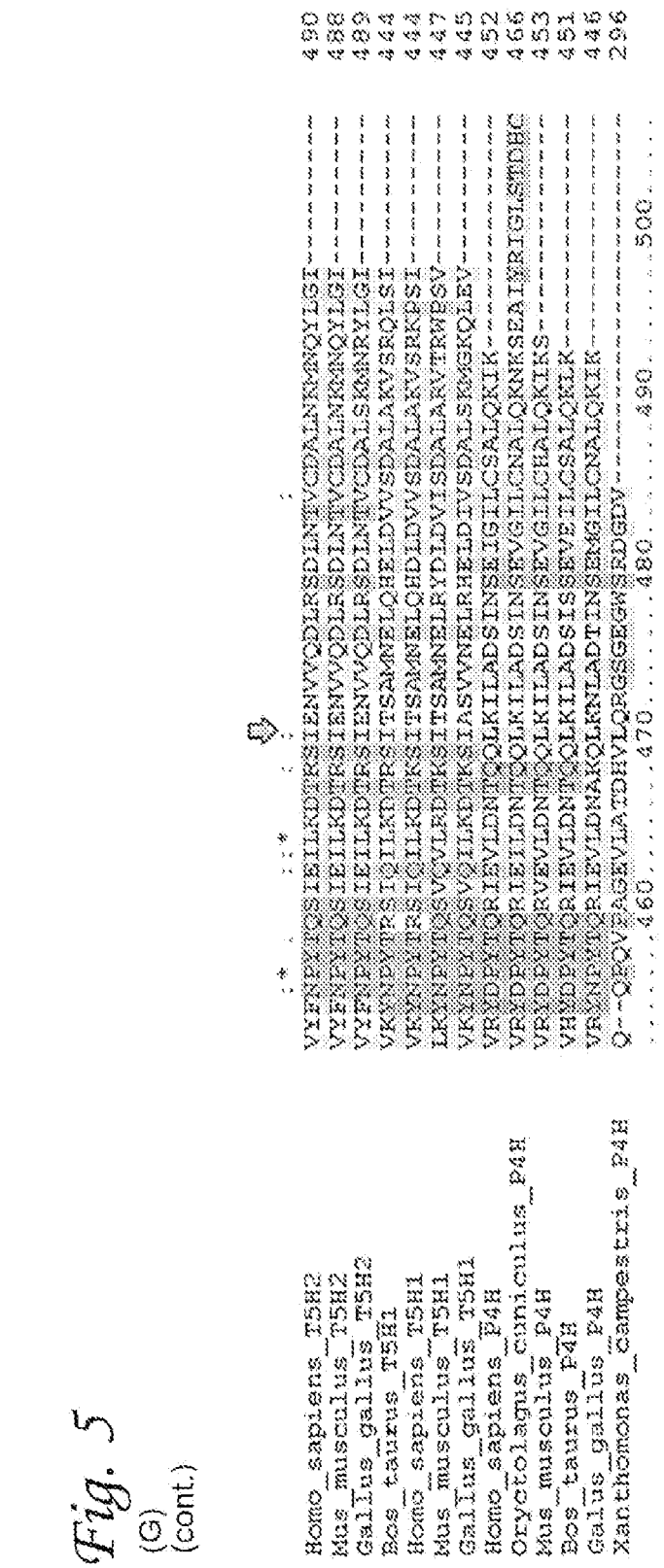
Gallus_gallus_T5H1
Homo_sapiens_P4H
Oryctolagus_Cuniculus_P4H
Mus_musculus_P4H
Bos_taurus_P4H
Gallus_gallus_P4H
Xanthomonas_campestris_P4H
310 320 330 340 350 360 370
Homo_sapiens_T5H2
Mus_musculus_T5H2
Gallus_gallus_T5H2
Bos_taurus_T5H1
Homo_sapiens_T5H1
Mus_musculus_T5H1
Gallus_gallus_T5H1
Homo_sapiens_P4H
Oryctolagus_Cuniculus_P4H
Mus_musculus_P4H
Bos_taurus_P4H
Gallus_gallus_P4H
Xanthomonas_campestris_P4H
380 390 400 410 420 430 440 450

Fig. 5
(G)
(cont.)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Mus_musculus_T5H2
Gallus_gallus_T5H2
Bos_taurus_T5H1
Homo_sapiens_T5H1
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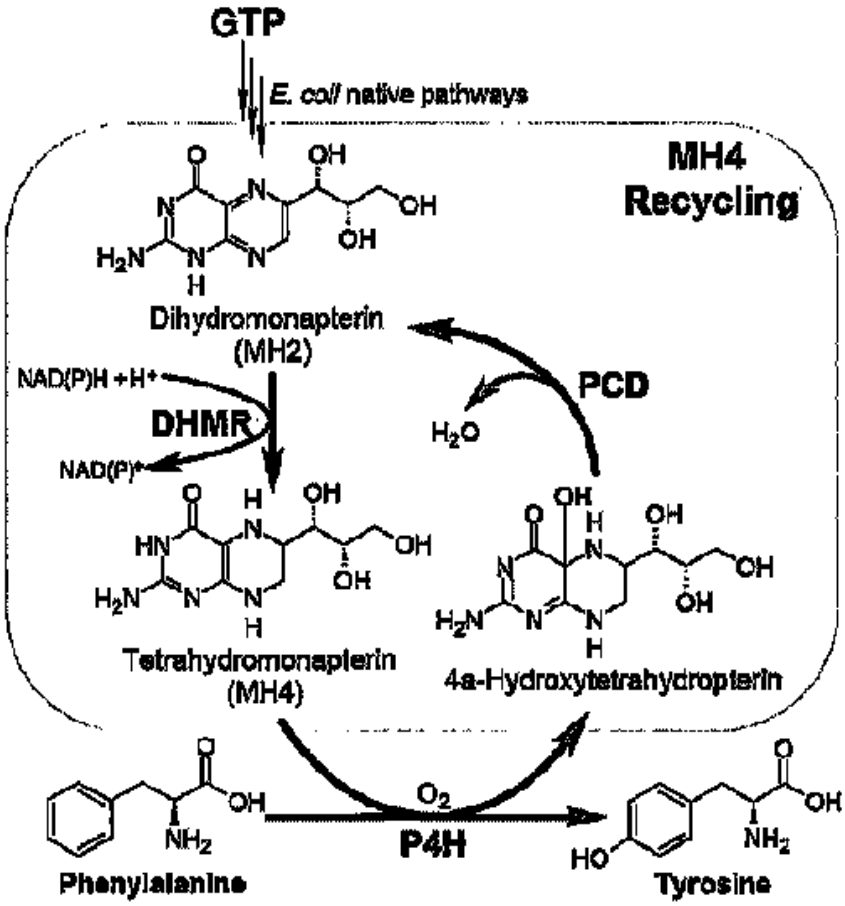
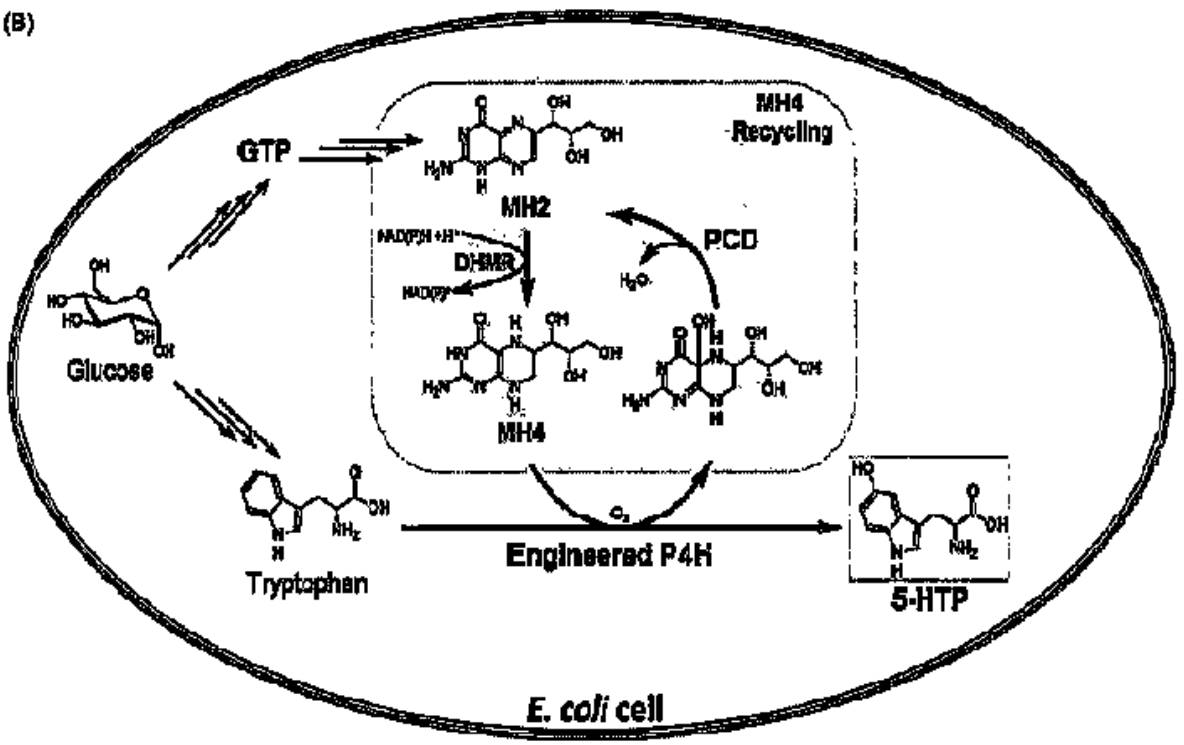
Gallus_gallus_T5H1
Homo_sapiens_P4H
Oryctolagus_cuniculus_P4H
Mus_musculus_P4H
Bos_taurus_P4H
Gallus_gallus_P4H
Xanthomonas_campestris_P4H
460
470
480
490
500
490
488
489
444
444
447
445
452
466
453
451
445
296

(A)


GTP
E. coli native pathways
MH4
Recycling
Dihydromonapterin
(MH2)
NAD(P)H +H+
DHMR
NAD(P)+
PCD
H2O
Tetrahydromonapterin
(MH4)
4a-Hydroxytetrahydropterin
O2
P4H
Phenylalanine
Tyrosine

(B)


GTP
MH4
Recycling
MH2
DHMR
PCD
H2O
MH4
Glucose
Tryptophan
Engineered P4H
5-HTP
E. coli cell