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(54) Title: ENGINEERED BIOSYNTHETIC PATHWAYS FOR PRODUCTION OF TYRAMINE BY FERMENTATION

(57) Abstract: The present disclosure describes the engineering of microbial cells for fermentative production of tyramine and provides novel engineered microbial cells and cultures, as well as related tyramine production methods.

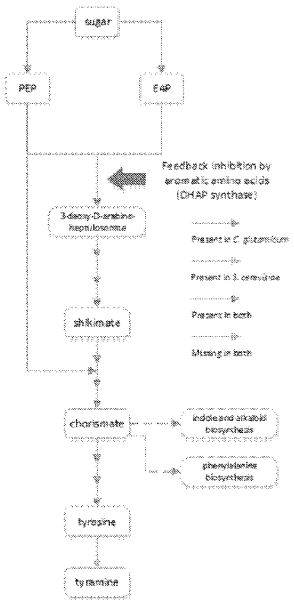

Fig. 1

WO 2018/203947 A2

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ENGINEERED BIOSYNTHETIC PATHWAYS FOR PRODUCTION OF TYRAMINE BY FERMENTATION

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. provisional application no. 62/455,428, filed February 6, 2017 which is hereby incorporated by reference in its entirety.

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

[0002] This invention was made with Government support under Agreement No. HR0011-15-9-0014, awarded by DARPA. The Government has certain rights in the invention.

INCORPORATION BY REFERENCE OF THE SEQUENCE LISTING

[0003] This application includes a sequence listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. This ASCII copy, created on February 6, 2018, is named 2018-02-05_ZMGNP001WO_seqlist.txt and is 331,364 bytes in size.

FIELD OF THE DISCLOSURE

[0004] The present disclosure relates generally to the area of engineering microbes for overproduction of tyramine by fermentation.

BACKGROUND

[0005] Tyramine is known to exist in nature as the decarboxylation product of tyrosine. Often tyramine is produced in environments or processes where protein-rich materials have rotted or decayed. Tyramine is present in foods produced from fermentation of protein-rich substances such as animal milk or legumes. These processes rely on an external source of proteins containing aromatic amino acids and microbes expressing tyrosine decarboxylases.

SUMMARY

[0006] Various embodiments conetemplated herein may include, but need not be limited to, one or more of the following:

[0007] Embodiment 1: An engineered microbial cell, wherein the engineered microbial cell expresses: (a) a heterologous tyrosine decarboxylase (TYDC); and (b) the engineered microbial cell includes increased activity of one or more upstream enzyme(s) in the tyramine biosynthesis pathway, said increased activity being increased relative to a control cell.

[0008] Embodiment 2: The engineered microbial cell of embodiment 1, wherein the one or more upstream enzyme(s) includes 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase.

[0009] Embodiment 3: An engineered microbial cell, wherein the engineered microbial cell expresses: (a) a heterologous tyrosine decarboxylase (TYDC); and (b) the engineered microbial cell includes increased activity of one or more enzyme(s) selected from the group consisting of a dehydroquinate synthase, a dehydroquinate dehydratase, a shikimate dehydrogenase, a shikimate kinase, EPSP synthase, aromatic pentafunctional enzyme, a chorismate synthase, a chorismate mutase, a prephenate dehydratase, a phenyalananine aminotransferase, a prephenate dehydrogenase, a prephenate aminotransferase, an arogenate dehydrogenase, a phenylalanine hydroxylase, and a tyrosine aminotransferase, said increased activity being increased relative to a control cell; wherein the engineered microbial cell produces tyramine.

[0010] Embodiment 4: The engineered microbial cell of embodiment 3, wherein the engineered microbial cell additionally expresses: (c) a feedback-disregulated 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase or a feedback-disregulated chorismate mutase.

[0011] Embodiment 5: An engineered microbial cell, wherein the engineered microbial cell includes: (a) means for expressing a heterologous tyrosine decarboxylase (TYDC); and (b) means for increasing the activity of one or more upstream enzyme(s) in the tyramine biosynthesis pathway.

[0012] Embodiment 6: The engineered microbial cell of embodiment 5, wherein the one or more upstream enzyme(s) includes 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase.

[0013] Embodiment 7: An engineered microbial cell, wherein the engineered microbial cell includes: (a) means for expressing a heterologous tyrosine decarboxylase (TYDC); and (b) means for increasing the activity of one or more enzyme(s) selected from the group consisting of a dehydroquinate synthase, a dehydroquinate dehydratase, a shikimate dehydrogenase, a shikimate kinase, EPSP synthase, aromatic pentafunctional enzyme, a chorismate synthase, a chorismate mutase, a prephenate dehydratase, a prephenate aminotransferase, an arogenate dehydrogenase, a phenylalanine hydroxylase, a phenyalananine aminotransferase, a prephenate dehydrogenase, and a tyrosine aminotransferase, said increased activity being increased relative to a control cell; wherein the engineered microbial cell produces tyramine.

[0014] Embodiment 8: The engineered microbial cell of embodiment 7, wherein the engineered microbial cell additionally expresses: (c) means for expressing a feedback-disregulated DAHP synthase or a feedback-disregulated chorismate mutase.

[0015] Embodiment 9: The engineered microbial cell of any one of embodiments 1-8, wherein the engineered microbial cell produces tyramine by fermentation of a substrate, wherein at least 50% of the substrate is not derived from protein or amino acid sources.

[0016] Embodiment 10: The engineered microbial cell of any one of embodiments 4, 8, or 9, wherein the engineered microbial cell includes: (a) a heterologous TYDC; and (b) a feedback-disregulated DAHP synthase.

[0017] Embodiment 11: The engineered microbial cell of any one of embodiments 3-10, wherein the engineered microbial cell includes a fungal cell.

[0018] Embodiment 12: The engineered microbial cell of any one of embodiments 3-11, wherein the engineered microbial cell includes a yeast cell.

[0019] Embodiment 13: The engineered microbial cell of embodiment 8, wherein the yeast cell includes a cell of the genus *Saccharomyces*.

[0020] Embodiment 14: The engineered microbial cell of embodiment 13, wherein the yeast cell is a cell of the species *cerevisiae*.

[0021] Embodiment 15: The engineered microbial cell of any one of embodiments 4, 8, or 9-14, wherein the DAHP synthase is a variant of a *S. cerevisiae* DAHP synthase.

[0022] Embodiment 16: The engineered microbial cell of any one of embodiments 3-15, wherein the heterologous TYDC includes a TYDC having at least 70% amino acid sequence identity with a TYDC from *Papaver somniferum*.

[0023] Embodiment 17: The engineered microbial cell of any one of embodiments 4, 8, or 9-16, wherein the: (a) heterologous TYDC includes a *P. somniferum* Tyrosine/DOPA decarboxylase 2; and the (b) feedback-disregulated DAHP synthase is a *S. cerevisiae* DAHP synthase encoded by the *Aro4* gene that additionally includes a K229L mutation.

[0024] Embodiment 18: The engineered microbial cell of any one of embodiments 3-17, wherein the engineered microbial cell includes increased activity of a prephenate dehydrogenase relative to the control cell.

[0025] Embodiment 19: The engineered microbial cell of embodiment 18, wherein the engineered microbial cell expresses an extra copy of a wild-type *S. cerevisiae* prephenate dehydrogenase gene.

[0026] Embodiment 20: The engineered microbial cell of embodiment 18, wherein the engineered microbial cell expresses an extra copy of a wild-type *S. cerevisiae* transaldolase gene.

[0027] Embodiment 21: The engineered microbial cell of embodiment 2 or embodiment 6, wherein the engineered microbial cell includes a yeast cell.

[0028] Embodiment 22: The engineered microbial cell of embodiment 21, wherein the yeast cell includes a cell of the genus *Yarrowia*.

[0029] Embodiment 23: The engineered microbial cell of embodiment 22, wherein the yeast cell is a cell of the species *lipolytica*.

[0030] Embodiment 24: The engineered microbial cell of embodiment 22 or embodiment 23, wherein the heterologous TYDC includes a TYDC having at least 70% amino acid sequence identity with a TYDC from *Enterococcus faecium*.

[0031] Embodiment 25: The engineered microbial cell of any one of embodiments 22-24, wherein the DAHP synthase includes a DAHP synthase having at least 70% amino acid sequence identity with a DAHP synthase from *S. cerevisiae*.

[0032] Embodiment 26: The engineered microbial cell of embodiment 25, wherein the: (a) heterologous TYDC includes a pyridoxal-dependent decarboxylase (TYDC) from

E. faecium Com15; and (b) DAHP synthase includes a phospho-2-dehydro-3-deoxyheptonate aldolase (DAHP synthase) from *S. cerevisiae* S288c.

[0033] Embodiment 27: An engineered microbial cell which is a yeast cell including a heterologous tyrosine decarboxylase (TYDC) having at least 70% amino acid sequence identity to a TYDC from *Papaver somniferum*, wherein the engineered yeast cell produces tyramine.

[0034] Embodiment 28: The engineered microbial cell of embodiment 27, wherein the engineered yeast cell is a cell of the genus *Saccharomyces*.

[0035] Embodiment 29: The engineered microbial cell of embodiment 28, wherein the engineered yeast cell is a cell of the species *cerevisiae*.

[0036] Embodiment 30: The engineered microbial cell of any one of embodiments 3-10, wherein the engineered microbial cell is a bacterial cell.

[0037] Embodiment 31: The engineered microbial cell of embodiment 30, wherein the bacterial cell is a cell of the genus *Corynebacteria*.

[0038] Embodiment 32: The engineered microbial cell of embodiment 31, wherein the bacterial cell is a cell of the species *glutamicum*.

[0039] Embodiment 33: The engineered microbial cell of any one of embodiments 30-32, wherein the bacterial cell includes a feedback-disregulated DAHP synthase that is a variant of an *S. cerevisiae* DAHP synthase.

[0040] Embodiment 34: The engineered microbial cell of any one of embodiments 30-33, wherein the heterologous TYDC includes a TYDC having at least 70% amino acid sequence identity with a TYDC from *Enterococcus faecium* or having at least 70% amino acid sequence identity with a TYDC from *Zygosaccharomyces bailii*.

[0041] Embodiment 35: The engineered microbial cell of embodiment 33 or embodiment 34, wherein the: (a) heterologous TYDC includes an *E. faecium* TYDC; and the (b) feedback-disregulated DAHP synthase is a *S. cerevisiae* DAHP synthase encoded by the *Aro4* gene that additionally includes a K229L mutation.

[0042] Embodiment 36: The engineered microbial cell of embodiment 33 or embodiment 34, wherein the: (a) heterologous TYDC includes an *Z. bailii* TYDC; and the (b) feedback-disregulated DAHP synthase is a *S. cerevisiae* DAHP synthase encoded by the *Aro4* gene that additionally includes a K229L mutation.

[0043] Embodiment 37: The engineered microbial cell of embodiment 34, additionally including increased activity of chorismate synthase or prephrenate dehydrogenase, relative to a control cell.

[0044] Embodiment 38: The engineered microbial cell of embodiment 37, wherein the engineered microbial cell includes increased activity of chorismate synthase and expresses a heterologous chorismate synthase.

[0045] Embodiment 39: The engineered microbial cell of embodiment 38, wherein the heterologous chorismate synthase includes a chorismate synthase having at least 70% amino acid sequence identity to a *S. cerevisiae* chorismate synthase.

[0046] Embodiment 40: The engineered microbial cell of embodiment 39, wherein the heterologous chorismate synthase includes a *S. cerevisiae* chorismate synthase.

[0047] Embodiment 41: The engineered microbial cell of embodiment 37, wherein the engineered microbial cell includes increased activity of prephenate dehydrogenase and expresses an additional copy of a prephenate dehydrogenase gene.

[0048] Embodiment 42: The engineered microbial cell of embodiment 41, wherein the additional copy of the prephenate dehydrogenase gene encodes a prephenate dehydrogenase having at least 70% amino acid sequence identity to a prephenate dehydrogenase from *S. cerevisiae*.

[0049] Embodiment 43: The engineered microbial cell of embodiment 42, wherein the additional copy of the prephenate dehydrogenase gene encodes a prephenate dehydrogenase from *S. cerevisiae*.

[0050] Embodiment 44: The engineered microbial cell of embodiment 2 or embodiment 6, wherein the engineered microbial cell includes a bacterial cell.

[0051] Embodiment 45: The engineered microbial cell of embodiment 44, wherein the bacterial cell includes a cell of the genus *Corynebacterium* or *Bacillus*.

[0052] Embodiment 46: The engineered microbial cell of embodiment 45, wherein the bacterial cell is a cell of the species *glutamicum* or *subtilis*, respectively.

[0053] Embodiment 47: The engineered microbial cell of embodiment 45 or embodiment 46, wherein the heterologous TYDC includes a TYDC having at least 70% amino acid sequence identity with a TYDC from *Enterococcus faecium*.

[0054] Embodiment 48: The engineered microbial cell of any one of embodiments 45-47, wherein the DAHP synthase includes a DAHP synthase having at least 70% amino acid sequence identity with a DAHP synthase from *S. cerevisiae*.

[0055] Embodiment 49: The engineered microbial cell of any one of embodiments 45-48, wherein the engineered microbial cell includes increased activity of a shikimate kinase relative to a control cell.

[0056] Embodiment 50: The engineered microbial cell of embodiment 49, wherein the shikimate kinase includes a shikimate kinase having at least 70% amino acid sequence identity with a shikimate kinase from *Escherichia coli*.

[0057] Embodiment 51: The engineered microbial cell of embodiment 50, wherein the: (a) heterologous TYDC includes a pyridoxal-dependent decarboxylase (TYDC) from *E. faecium* Com15; (b) DAHP synthase includes a phospho-2-dehydro-3-deoxyheptonate aldolase (DAHP synthase) from *S. cerevisiae* S288c; and (c) shikimate kinase includes a shikimate kinase from *E. coli* K12.

[0058] Embodiment 52: The engineered microbial cell of any one of embodiments 13-43, wherein, when cultured, the engineered microbial cell produces tyramine at a level greater than 100 mg/L of culture medium.

[0059] Embodiment 53: The engineered microbial cell of embodiment 52, wherein the engineered microbial cell produces tyramine at a level of at least 2.5 g/L of culture medium.

[0060] Embodiment 54: An engineered microbial cell which is a bacterial cell including a heterologous tyrosine decarboxylase (TYDC) having at least 70% amino acid sequence identity with a TYDC from *Enterococcus faecium*, wherein the engineered bacterial cell produces tyramine.

[0061] Embodiment 55: The engineered microbial cell of embodiment 54, wherein the bacterial cell is of the genus *Corynebacteria*.

[0062] Embodiment 56: The engineered microbial cell of embodiment 55, wherein the bacterial cell is of the species *glutamicum*.

[0063] Embodiment 57: An engineered microbial cell which is a bacterial cell including a heterologous tyrosine decarboxylase (TYDC) having at least 70% amino acid

sequence identity with a TYDC from *Zygosaccharomyces bailii*, wherein the engineered bacterial cell produces tyramine.

[0064] Embodiment 58: The engineered microbial cell of embodiment 57, wherein the bacterial cell is of the genus *Corynebacteria*.

[0065] Embodiment 59: The engineered microbial cell of embodiment 58, wherein the bacterial cell is of the species *glutamicum*.

[0066] Embodiment 60: A culture of engineered microbial cells according to any one of embodiments 13-59.

[0067] Embodiment 61: The culture of embodiment 60, wherein the tyramine is produced from fermentation of a substrate wherein at least 50% of the substrate is not derived from protein or amino acid sources.

[0068] Embodiment 62: The culture of embodiment 61, wherein the substrate includes a carbon source and a nitrogen source selected from the group consisting of urea, an ammonium salt, ammonia, and any combination thereof.

[0069] Embodiment 63: The culture of any one of embodiments 60-62, wherein the engineered microbial cells are present in a concentration such that the culture has an optical density at 600 nm of 10-500.

[0070] Embodiment 64: The culture of any one of embodiments 60-63, wherein the culture includes tyramine.

[0071] Embodiment 65: The culture of any one of embodiments 60-64, wherein the culture includes tyramine at a level greater than 100 mg/L of culture medium.

[0072] Embodiment 66: The culture of any one of embodiments 60-65, wherein the culture includes tyramine at a level of at least 2.5 g/L of culture medium.

[0073] Embodiment 67: A method of culturing engineered microbial cells according to any one of embodiments 1-59, the method including culturing the cells in the presence of a fermentation substrate including a non-protein carbon and a non-protein nitrogen source, wherein the engineered microbial cells produce tyramine.

[0074] Embodiment 68: The method of embodiment 67, wherein the method includes fed-batch culture, with an initial glucose level in the range of 1-100 g/L, followed controlled sugar feeding.

[0075] Embodiment 69: The method of embodiment 67 or embodiment 68, wherein the fermentation substrate includes glucose and a nitrogen source selected from the group consisting of urea, an ammonium salt, ammonia, and any combination thereof.

[0076] Embodiment 70: The method of any one of embodiments 67-69, wherein the culture is pH-controlled during culturing.

[0077] Embodiment 71: The method of any one of embodiments 67-70, wherein the culture is aerated during culturing.

[0078] Embodiment 72: The method of any one of embodiments 67-71, wherein the engineered microbial cells produce tyramine at a level greater than 100 mg/L of culture medium.

[0079] Embodiment 73: The method of any one of embodiments 67-72, wherein the engineered microbial cells produce tyramine at a level of at least 2.5 g/L of culture medium.

[0080] Embodiment 74: The method of any one of embodiments 67-73, wherein the method additionally includes recovering tyramine from the culture.

BRIEF DESCRIPTION OF THE DRAWINGS

[0081] Figure 1: Pathway for production of tyramine by fermentation.

[0082] Figure 2: A "split-marker, double-crossover" genomic integration strategy, which was developed to engineer *S. cerevisiae* strains. Two plasmids with complementary 5' and 3' homology arms and overlapping halves of a URA3 selectable marker (direct repeats shown by the hashed bars) were digested with meganucleases and transformed as linear fragments. A triple-crossover event integrated the desired heterologous genes into the targeted locus and re-constituted the full URA3 gene. Colonies derived from this integration event were assayed using two 3-primer reactions to confirm both the 5' and 3' junctions (UF/IF/wt-R and DR/IF/wt-F). See Example 1.

[0083] Figure 3: A "loop-in, single-crossover" genomic integration strategy, which was developed to engineer *C. glutamicum* strains. Loop-in only constructs (shown under the heading "Loop-in") contained a single 2-kb homology arm (denoted as "integration locus"), a positive selection marker (denoted as "Marker")), and gene(s) of interest (denoted as "promoter-gene-terminator"). A single crossover event integrated the plasmid into the *C. glutamicum* chromosome. Integration events are stably maintained in the genome by growth in the presence of antibiotic (e.g., 25μg/ml kanamycin). Correct genomic

integration in colonies derived from loop-in integration were confirmed by colony PCR with UF/IR and DR/IF PCR primers. Loop-in, loop-out constructs (shown under the heading "Loop-in, loop-out) contained two 2-kb homology arms (5' and 3' arms), gene(s) of interest (arrows), a positive selection marker (denoted "Marker"), and a counter-selection marker. Similar to "loop-in" only constructs, a single crossover event integrated the plasmid into the chromosome of *C. glutamicum*. Note: only one of two possible integrations is shown here. Correct genomic integration was confirmed by colony PCR and counter-selection was applied so that the plasmid backbone and counter-selection marker could be excised. This results in one of two possibilities: reversion to wild-type or the desired pathway integration. Again, correct genomic loop-out is confirmed by colony PCR. (Abbreviations: Primers: UF = upstream forward, DR = downstream reverse, IR = internal reverse, IF = internal forward.) See Example 1.

[0084] Figure 4A-4C: Fig. 4A shows a graph of biomass as a function of culture time under different process conditions for a culture of *S. cerevisiae* engineered to produce tyramine. The data come from a study described in Example 2. Fig. 4B shows the tyramine titer for the same culture. Fig. 4C shows the glucose concentration in this culture as a function of culture time.

[0085] Figure 5: Tyramine titers measured in extracellular broth following fermentation by the first-round engineered host *Yarrowia lipolytica*. Strain designs tested the expression of additional heterologous enzymes.

[0086] Figure 6: Tyramine titers measured in extracellular broth following fermentation by the first round engineered host *Bacillus subtilis*. Strain designs tested the expression of additional heterologous enzymes.

[0087] Figure 7: Tyramine titers measured in extracellular broth following fermentation by the host *S. cerevisiae* engineered to express the host evaluation designs for production of tyramine.

[0088] Figure 8: Tyramine titers measured in extracellular broth following fermentation by the host *C. glutamicum* engineered to express the host evaluation designs for production of tyramine.

[0089] Figure 9: Tyramine titers measured in extracellular broth following fermentation by the (third round) engineered host *S. cerevisiae*. Strain designs tested the expression of additional heterologous enzymes.

[0090] Figure 10: Structure of tyramine.

DETAILED DESCRIPTION

[0091] The present disclosure describes the engineering of microbial cells for fermentative production of tyramine and provides novel engineered microbial cells and cultures, as well as related tyramine production methods.

Definitions

[0092] Terms used in the claims and specification are defined as set forth below unless otherwise specified.

[0093] The term "fermentation" is used herein to refer to a process whereby a microbial cell converts one or more substrate(s) into a desired product (such as tyramine) by means of one or more biological conversion steps, without the need for any chemical conversion step.

[0094] The term "engineered" is used herein, with reference to a cell, to indicate that the cell contains at least one targeted genetic alteration introduced by man that distinguishes the engineered cell from the naturally occurring cell.

[0095] The term "endogenous" is used herein to refer to a cellular component, such as a polynucleotide or polypeptide, that is naturally present in a particular cell.

[0096] The term "heterologous" is used herein, with reference to a polynucleotide or polypeptide introduced into a host cell, to refer to a polynucleotide or polypeptide, respectively, derived from a different organism, species, or strain than that of the host cell. A heterologous polynucleotide or polypeptide has a sequence that is different from any sequence(s) found in the same host cell.

[0097] As used with reference to polypeptides, the term "wild-type" refers to any polypeptide having an amino acid sequence present in a polypeptide from a naturally occurring organism, regardless of the source of the molecule; i.e., the term "wild-type" refers to sequence characteristics, regardless of whether the molecule is purified from a natural source; expressed recombinantly, followed by purification; or synthesized. The term wild-type is also used to denote naturally occurring cells.

[0098] A "control cell" is a cell that is otherwise identical to an engineered cell being tested, including being of the same genus and species as the engineered cell, but lacks the specific genetic modification(s) being tested for in the engineered cell.

[0099] Enzymes are identified herein by the reactions they catalyze and, unless otherwise indicated, refer to any polypeptide capable of catalyzing the identified reaction. Unless otherwise indicated, enzymes may be derived from any organism and may have a naturally occurring or mutated amino acid sequence. As is well known, enzymes may have multiple functions and/or multiple names, sometimes depending on the source organism from which they derive. The enzyme names used herein encompass orthologs, including enzymes that may have one or more additional functions or a different name.

[0100] The term "feedback-disregulated" is used herein with reference to an enzyme that is normally negatively regulated by a downstream product of the enzymatic pathway (i.e., feedback-inhibition) in a particular cell. In this context, a "feedback-disregulated" enzyme is a form of the enzyme that is less sensitive to feedback-inhibition than the wild-type enzyme endogenous to the cell. A feedback-disregulated enzyme may be produced by introducing one or more mutations into a wild-type enzyme. Alternatively, a feedback-disregulated enzyme may simply be a heterologous, wild-type enzyme that, when introduced into a particular microbial cell, is not as sensitive to feedback-inhibition as the endogenous, wild-type enzyme. In some embodiments, the feedback-disregulated enzyme shows no feedback-inhibition in the microbial cell.

[0101] The term "tyramine" refers to 4-(2-aminoethyl)phenol (CAS#51-67-2).

[0102] The term "sequence identity," in the context of two or more amino acid or nucleotide sequences, refers to two or more sequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same, when compared and aligned for maximum correspondence, as measured using a sequence comparison algorithm or by visual inspection.

[0103] For sequence comparison to determine percent nucleotide or amino acid sequence identity, typically one sequence acts as a "reference sequence," to which a "test" sequence is compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence relative to the

reference sequence, based on the designated program parameters. Alignment of sequences for comparison can be conducted using BLAST set to default parameters.

[0104] The term "titer," as used herein, refers to the mass of a product (e.g., tyramine) produced by a culture of microbial cells divided by the culture volume.

[0105] As used herein with respect to recovering tyramine from a cell culture, "recovering" refers to separating the tyramine from at least one other component of the cell culture medium.

Engineering Microbes for Tyramine Production

Tyramine Biosynthesis Pathway

[0106] Tyramine is derived from the aromatic branch of amino acid biosynthesis, based on the precursors phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P). This pathway is illustrated in Fig. 1. The first step of the amino acid biosynthesis pathway, catalyzed by 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase, is subject to feedback inhibition by the aromatic amino acids tyrosine, tryptophan and phenylalanine. Many microbes lack the enzyme that catalyzes the final step in this pathway, namely tyrosine decarboxylase (TYDC). Production of tyramine in such microbial hosts requires the addition of at least one heterologous TYDC enzyme.

Engineering for Microbial Tyramine Production

[0107] Any TYDC that is active in the microbial cell being engineered may be introduced into the cell, typically by introducing and expressing the gene encoding the enzyme using standard genetic engineering techniques. Suitable TYDCs may be derived from any source, including plant, archaeal, fungal, gram-positive bacterial, and gram-negative bacterial sources. Exemplary sources include, but are not limited to: *Papaver somniferum, Petroselinum crispum, Oryza sativa, Methanosphaerula palustris Methanocaldococcus jannaschii, Zygosaccharomyces bailii, Penicillium marneffei, Talaromyces stipitatus, Trichophyton equinum, Propionibacterium sp. oral, Enterococcus faecium, Streptomyces hygroscopicus, Streptomyces sviceus, Modestobacter marinus, Pseudomonas putida, Sinorhizobium fredii.* Some sources, such as *P. somniferum*, may include more than one form of TYDC, and any of these can be used in the methods described herein.

[0108] One or more copies of a TYDC can be introduced into a selected microbial host cell. If more than one copy of a TYDC gene is introduced, the copies can be copies of the same or different TYDC gene. In some embodiments, the heterologous TYDC gene(s) is/ are expressed from a strong, constitutive promoter. In some embodiments, the heterologous TYDC gene(s) is/are expressed from inducible promoters. The heterologous genes can optionally be codon-optimized to enhance expression in the selected microbial host cell. Codon-optimization tables are available for common microbial host cells. The codon-optimization tables used in the Examples are as follows: *Bacillus subtilis* Kazusa codon table: www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=1423&aa=1&style=N; *Yarrowia lipolytica* Kazusa codon table: www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=4952&aa=1&style=N; *Corynebacteria glutamicum* Kazusa codon table: www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=340322&aa=1&style=N; *Saccharomyces cerevisiae* Kazusa codon table: http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=4932&aa=1&style=N. Also used, was a modified, combined codon usage scheme for *S. cereviae* and *C. glutamicum*, which is reproduced below.

Amino Acid	Codon	Fraction
A	GCG	0.22
A	GCA	0.29
A	GCT	0.24
A	GCC	0.25
C	TGT	0.36
C	TGC	0.64
D	GAT	0.56
D	GAC	0.44
E	GAG	0.44
E	GAA	0.56
F	TTT	0.37
F	TTC	0.63
G	GGG	0.08
G	GGA	0.19
G	GGT	0.3
G	GGC	0.43
H	CAT	0.32
H	CAC	0.68
I	ATA	0.03
I	ATT	0.38
I	ATC	0.59
K	AAG	0.6

Amino Acid	Codon	Fraction
K	AAA	0.4
L	TTG	0.29
L	TTA	0.05
L	CTG	0.29
L	CTA	0.06
L	CTT	0.17
L	CTC	0.14
M	ATG	1
N	AAT	0.33
N	AAC	0.67
P	CCG	0.22
P	CCA	0.35
P	CCT	0.23
P	CCC	0.2
Q	CAG	0.61
Q	CAA	0.39
R	AGG	0.11
R	AGA	0.12
R	CGG	0.09
R	CGA	0.17
R	CGT	0.34
R	CGC	0.18
S	AGT	0.08
S	AGC	0.16
S	TCG	0.12
S	TCA	0.13
S	TCT	0.17
S	TCC	0.34
T	ACG	0.14
T	ACA	0.12
T	ACT	0.2
T	ACC	0.53
V	GTG	0.36
V	GTA	0.1
V	GTT	0.26
V	GTC	0.28
W	TGG	1
Y	TAT	0.34
Y	TAC	0.66

[0109] In Example 1, *C. glutamicum* was engineered to express a TYDC from *E. faecium* (SEQ ID NO: 1), which yielded a tyramine titer of 80 µg/L.

Engineering for Increased Tyramine Production

Increasing the Activity of Endogenous Upstream Enzymes

[0110] One approach to increasing tyramine production in a microbial cell which expresses a heterologous TYDC is to increase the activity of one or more upstream enzymes in the tyramine biosynthesis pathway. Upstream pathway enzymes include all enzymes involved in the conversions from a feedstock all the way to tyrosine. In certain embodiments, the upstream pathway enzymes refer specifically to the enzymes involved in the conversion of key precursors (i.e., E4P and PEP) into the last native metabolite (i.e. tyrosine) in the pathway leading to tyramine. In some embodiments, the activity of one or more upstream pathway enzymes is increased by modulating the expression or activity of the endogenous enzyme(s). In some embodiments, the activity of one or more upstream pathway enzymes is supplemented by introducing one or more of the corresponding genes into the TYDC-expressing microbial host cell. Such genes include those encoding a dehydroquinate synthase, a dehydroquinate dehydratase, a shikimate dehydrogenase, a shikimate kinase, EPSP synthase, aromatic pentafunctional enzyme, a chorismate synthase, a chorismate mutase, a prephenate dehydratase, a phenyalanine aminotransferase, a prephenate dehydrogenase, a prephenate aminotransferase, an arogenate dehydrogenase, a phenylalanine hydroxylase, an aromatic amino acid transferase such as a tyrosine aminotransferase, a glyceraldehyde-3-phosphate dehydrogenase, a transaldolase, a transketolase, a DAHP synthase, a phosphoenolpyruvate synthase, a glutamate synthase. Suitable upstream pathway genes may be derived from any source, including, for example, those discussed above as sources for a heterologous TYDC gene.

[0111] Example 1 describes the successful engineering of a microbial host cell to express a heterologous TYDC, along with an introduced gene encoding an upstream gene; either a chorismate synthase or a prephenate dehydrogenase. In particular, *S. cerevisiae* was engineered to express a TYDC from *P. somniferum* (SEQ ID NO:2) and an additional copy of the *S. cerevisiae* gene encoding either chorismate synthase (SEQ ID NO:3) or prephenate dehydrogenase (SEQ ID NO:4). The results are provided in Example 1, below.

[0112] An introduced upstream pathway gene may be heterologous or may simply be an additional copy of an endogenous gene. In some embodiments, one or more such genes are introduced into the TYDC-expressing microbial host cell and expressed from a strong constitutive promoter and/or can optionally be codon-optimized to enhance

expression in the selected microbial host cell. A TYDC-expressing microbial cell can, for example, be engineered to express one or more copies of one or more upstream pathway genes.

[0113] In various embodiments, the engineering of a TYDC-expressing microbial cell to increase the activity of one or more upstream pathway enzymes increases the tyramine titer by at least 10, 20, 30, 40, 50, 60, 70, 80, or 90 percent or by at least 2-fold, 2.5-fold, 3-fold, 3.5-fold, 4-fold, 4.5-fold, 5-fold, 5.5-fold, 6-fold, 6.5-fold, 7-fold, 7.5-fold, 8-fold, 8.5-fold, 9-fold, 9.5-fold, 10-fold, 11-fold, 12-fold, 13-fold, 14-fold, 15-fold, 16-fold, 17-fold, 18-fold, 19-fold, 20-fold, 21-fold, 22-fold, 23-fold, 24-fold, 25-fold, 30-fold, 35-fold, 40-fold, 45-fold, 50-fold, 55-fold, 60-fold, 65-fold, 70-fold, 75-fold, 80-fold, 85-fold, 90-fold, 95-fold, or 100-fold. In various embodiments, the increase in tyramine titer is in the range of 10 percent to 100-fold, 2-fold to 50-fold, 5-fold to 40-fold, 10-fold to 30-fold, or any range bounded by any of the values listed above. (Ranges herein include their endpoints.) These increases are determined relative to the tyramine titer observed in a tyramine-producing microbial cell that lacks any increase in activity of upstream pathway enzymes. This reference cell may have one or more other genetic alterations aimed at increasing tyramine production, e.g., the cell may express a feedback-disregulated enzyme.

[0114] In various embodiments, the tyramine titers achieved by increasing the activity of one or more upstream pathway genes are at least 10, 50, 75, 100, 200, 300, 400, 500, 600, 700, 800, or 900 mg/L or at least 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, or 10 gm/L. In various embodiments, the titer is in the range of 10 mg/L to 10 gm/L, 100 mg/L to 5 gm/L, 200 mg/L to 4 gm/L, 300 mg/L to 3 gm/L, or any range bounded by any of the values listed above.

Introduction of Feedback-Disregulated Enzymes

[0115] Since aromatic amino acid biosynthesis is subject to feedback inhibition, another approach to increasing tyramine production in a microbial cell engineered to express a heterologous TYDC is to introduce feedback-disregulated forms of one or more enzymes that are normally subject to feedback inhibition in the TYDC-expressing microbial cell. Examples of such enzymes include DAHP synthase and chorismate mutase. A feedback-disregulated form can be a heterologous, wild-type enzyme that is less sensitive to feedback inhibition than the endogenous enzyme in the particular microbial host cell. Alternatively, a feedback-disregulated form can be a variant of an endogenous or

heterologous enzyme that has one or more mutations rendering it less sensitive to feedback inhibition than the corresponding wild-type enzyme. Examples of the latter include variant DAHP synthases (two from *S. cerevisiae*, one from *E. coli*) that have known point mutations rendering them resistant to feedback inhibition, e.g., *S. cerevisiae ARO4*Q166K (SEQ ID NO:5), *S. cerevisiae ARO4*K229L (SEQ ID NO:6), and *E. coli AroG*D146N (SEQ ID NO:7). The last 5 characters of these designations indicate amino acid substitutions, using the standard one-letter code for amino acids, with the first letter referring to the wild-type residue and the last letter referring to the replacement reside; the numbers indicate the position of the amino acid substitution in the translated protein.

[0116] Example 1 describes the successful engineering of a fungal and bacterial host cells to express a heterologous TYDC, along with an introduced gene encoding a feedback-disregulated DAHP synthase. In particular, *S. cerevisiae* was engineered to express a TYDC 2 from *P. somniferum* (SEQ ID NO:2) and *S. cerevisiae ARO4*K229L (SEQ ID NO:6), which gave a tyramine titer of 387 μg/L.

[0117] In various embodiments, the engineering of a TYDC-expressing microbial cell to express a feedback-disregulated enzymes increases the tyramine titer by at least 10, 20, 30, 40, 50, 60, 70, 80, or 90 percent or by at least 2-fold, 2.5-fold, 3-fold, 3.5-fold, 4-fold, 4.5-fold, 5-fold, 5.5-fold, 6-fold, 6.5-fold, 7-fold, 7.5-fold, 8-fold, 8.5-fold, 9-fold, 9.5-fold, 10-fold, 11-fold, 12-fold, 13-fold, 14-fold, 15-fold, 16-fold, 17-fold, 18-fold, 19-fold, 20-fold, 21-fold, 22-fold, 23-fold, 24-fold, 25-fold, 30-fold, 35-fold, 40-fold, 45-fold, 50-fold, 55-fold, 60-fold, 65-fold, 70-fold, 75-fold, 80-fold, 85-fold, 90-fold, 95-fold, or 100-fold. In various embodiments, the increase in tyramine titer is in the range of 10 percent to 100-fold, 2-fold to 50-fold, 5-fold to 40-fold, 10-fold to 30-fold, or any range bounded by any of the values listed above. These increases are determined relative to the tyramine titer observed in a tyramine-producing microbial cell that does not express a feedback-disregulated enzyme. This reference cell may (but need not) have other genetic alterations aimed at increasing tyramine production, i.e., the cell may have increased activity of an upstream pathway enzyme resulting from some means other than feedback-insensitivity.

[0118] In various embodiments, the tyramine titers achieved by using a feedback-disregulated enzyme to increase flux though the tyramine biosynthetic pathway are at least 50, 75, 100, 200, 300, 400, 500, 600, 700, 800, or 900 mg/L or at least 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, or 5 gm/L. In various embodiments, the titer is in the range of 100 mg/L to 5 gm/L,

200 mg/L to 4 gm/L, 300 mg/L to 3 gm/L, or any range bounded by any of the values listed above.

[0119] The approaches of supplementing the activity of one or more endogenous enzymes and/or introducing one or more feedback-disregulated enzymes can be combined in TYDC-expressing microbial cells to achieve even higher tyramine production levels.

Microbial Host Cells

[0120] Any microbe that can be used to express introduced genes can be engineered for fermentative production of tyramine as described above. In certain embodiments, the microbe is one that is naturally incapable of fermentative production of tyramine. In some embodiments, the microbe is one that is readily cultured, such as, for example, a microbe known to be useful as a host cell in fermentative production of compounds of interest. Bacteria cells, including gram positive or gram negative bacteria can be engineered as described above. Examples include, in addition to *C. glutamicum* cells, *P. citrea, B. subtilis, B. licheniformis, B. lentus, B. brevis, B. stearothermophilus, B. alkalophilus, B. amyloliquefaciens, B. clausii, B. halodurans, B. megaterium, B. coagulans, B. circulans, B. lautus, B. thuringiensis, S. albus, S. lividans, S. coelicolor, S. griseus, Pseudomonas sp., P. alcaligenes, Lactobacilis spp.* (such as *L. lactis*, *L. plantarum*), *L. grayi, E. coli, E. faecium, E. gallinarum, E. casseliflavus, and/or E. faecalis* cells.

[0121] There are numerous types of anaerobic cells that can be used as microbial host cells in the methods described herein. In some embodiments, the microbial cells are obligate anaerobic cells. Obligate anaerobes typically do not grow well, if at all, in conditions where oxygen is present. It is to be understood that a small amount of oxygen may be present, that is, there is some level of tolerance level that obligate anaerobes have for a low level of oxygen. Obligate anaerobes engineered as described above can be grown under substantially oxygen-free conditions, wherein the amount of oxygen present is not harmful to the growth, maintenance, and/or fermentation of the anaerobes.

[0122] Alternatively, the microbial host cells used in the methods described herein can be facultative anaerobic cells. Facultative anaerobes can generate cellular ATP by aerobic respiration (e.g., utilization of the TCA cycle) if oxygen is present. However, facultative anaerobes can also grow in the absence of oxygen. Facultative anaerobes engineered as described above can be grown under substantially oxygen-free conditions, wherein the amount of oxygen present is not harmful to the growth, maintenance, and/or

fermentation of the anaerobes, or can be alternatively grown in the presence of greater amounts of oxygen.

[0123] In some embodiments, the microbial host cells used in the methods described herein are filamentous fungal cells. (See, e.g., Berka & Barnett, Biotechnology Advances, (1989), 7(2):127-154). Examples include *Trichoderma longibrachiatum, T. viride, T. koningii, T. harzianum, Penicillium sp., Humicola insolens, H. lanuginose, H. grisea, Chrysosporium sp., C. lucknowense, Gliocladium sp., Aspergillus sp.* (such as *A. oryzae, A. niger, A. sojae, A. japonicus, A. nidulans, or A. awamori*), *Fusarium sp.* (such as *F. roseum, F. graminum F. cerealis, F. oxysporuim, or F. venenatum*), *Neurospora sp.* (such as *N. crassa or Hypocrea sp.*), *Mucor sp.* (such as *M. miehei*), *Rhizopus sp.*, and *Emericella sp.* cells. In particular embodiments, the fungal cell engineered as described above is *A. nidulans, A. awamori, A. oryzae, A. aculeatus, A. niger, A. japonicus, T. reesei, T. viride, F. oxysporum,* or *F. solani*. Illustrative plasmids or plasmid components for use with such hosts include those described in U.S. Patent Pub. No. 2011/0045563.

[0124] Yeasts can also be used as the microbial host cell in the methods described herein. Examples include: *Saccharomyces sp., Schizosaccharomyces sp., Pichia sp., Hansenula polymorpha, Pichia stipites, Kluyveromyces marxianus, Kluyveromyces spp., Yarrowia lipolytica* and *Candida sp.* In some embodiments, the *Saccharomyces sp.* is *S. cerevisiae* (See, e.g., Romanos et al., Yeast, (1992), 8(6):423-488). Illustrative plasmids or plasmid components for use with such hosts include those described in U.S. Pat. No. 7,659,097 and U.S. Patent Pub. No. 2011/0045563.

[0125] In some embodiments, the host cell can be an algal cell derived, e.g., from a green algae, red algae, a glaucophyte, a chlorarachniophyte, a euglenid, a chromista, or a dinoflagellate. (See, e.g., Saunders & Warmbrodt, "Gene Expression in Algae and Fungi, Including Yeast," (1993), National Agricultural Library, Beltsville, Md.). Illustrative plasmids or plasmid components for use in algal cells include those described in U.S. Patent Pub. No. 2011/0045563.

[0126] In other embodiments, the host cell is a cyanobacterium, such as cyanobacterium classified into any of the following groups based on morphology: *Chlorococcales, Pleurocapsales, Oscillatoriales, Nostocales, Synechosystic or Stigonematales* (See, e.g., Lindberg et al., Metab. Eng., (2010) 12(1):70-79). Illustrative plasmids or plasmid components for use in cyanobacterial cells include those described in

U.S. Patent Pub. Nos. 2010/0297749 and 2009/0282545 and in Intl. Pat. Pub. No. WO 2011/034863.

Genetic Engineering Methods

[0127] Microbial cells can be engineered for fermentative tyramine production using conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, and biochemistry, which are within the skill of the art. Such techniques are explained fully in the literature, see e.g., "Molecular Cloning: A Laboratory Manual," fourth edition (Sambrook et al., 2012); "Oligonucleotide Synthesis" (M. J. Gait, ed., 1984); "Culture of Animal Cells: A Manual of Basic Technique and Specialized Applications" (R. I. Freshney, ed., 6th Edition, 2010); "Methods in Enzymology" (Academic Press, Inc.); "Current Protocols in Molecular Biology" (F. M. Ausubel et al., eds., 1987, and periodic updates); "PCR: The Polymerase Chain Reaction," (Mullis et al., eds., 1994); Singleton et al., Dictionary of Microbiology and Molecular Biology 2nd ed., J. Wiley & Sons (New York, N.Y. 1994).

[0128] Vectors are polynucleotide vehicles used to introduce genetic material into a cell. Vectors useful in the methods described herein can be linear or circular. Vectors can integrate into a target genome of a host cell or replicate independently in a host cell. For many applications, integrating vectors that produced stable transformants are preferred. Vectors can include, for example, an origin of replication, a multiple cloning site (MCS), and/or a selectable marker. An expression vector typically includes an expression cassette containing regulatory elements that facilitate expression of a polynucleotide sequence (often a coding sequence) in a particular host cell. Vectors include, but are not limited to, integrating vectors, prokaryotic plasmids, episomes, viral vectors, cosmids, and artificial chromosomes.

[0129] Illustrative regulatory elements that may be used in expression cassettes include promoters, enhancers, internal ribosomal entry sites (IRES), and other expression control elements (e.g,. transcription termination signals, such as polyadenylation signals and poly-U sequences). Such regulatory elements are described, for example, in Goeddel, Gene Expression Technology: Methods In Enzymology 185, Academic Press, San Diego, Calif. (1990).

[0130] In some embodiments, vectors may be used to introduce systems that can carry out genome editing, such as CRISPR systems. See U.S. Patent Pub.

No. 2014/0068797, published 6 March 2014; *see also* Jinek M., *et al.*, "A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity," Science 337:816–21, 2012). In Type II CRISPR-Cas9 systems, Cas9 is a site-directed endonuclease, namely an enzyme that is, or can be, directed to cleave a polynucleotide at a particular target sequence using two distinct endonuclease domains (HNH and RuvC/RNase H-like domains). Cas9 can be engineered to cleave DNA at any desired site because Cas9 is directed to its cleavage site by RNA. Cas9 is therefore also described as an "RNA-guided nuclease." More specifically, Cas9 becomes associated with one or more RNA molecules, which guide Cas9 to a specific polynucleotide target based on hybridization of at least a portion of the RNA molecule(s) to a specific sequence in the target polynucleotide. Ran, F.A., *et al.*, ("*In vivo* genome editing using *Staphylococcus aureus* Cas9," Nature 520(7546):186-91, 2015, Apr 9], including all extended data) present the crRNA/tracrRNA sequences and secondary structures of eight Type II CRISPR-Cas9 systems. Cas9-like synthetic proteins are also known in the art (*see* U.S. Published Patent Application No. 2014-0315985, published 23 October 2014).

[0131] Example 1 describes two illustrative integration approaches for introducing polynucleotides into the genomes of *S. cerevisiae* and *C. glutamicum* cells.

[0132] Vectors or other polynucleotides can be introduced into microbial cells by any of a variety of standard methods, such as transformation, conjugation, electroporation, nuclear microinjection, transduction, transfection (e.g., lipofection mediated or DEAE-Dextrin mediated transfection or transfection using a recombinant phage virus), incubation with calcium phosphate DNA precipitate, high velocity bombardment with DNA-coated microprojectiles, and protoplast fusion. Transformants can be selected by any method known in the art. Suitable methods for selecting transformants are described in U.S. Patent Pub. Nos. 2009/0203102, 2010/0048964, and 2010/0003716, and International Publication Nos. WO 2009/076676, WO 2010/003007, and WO 2009/132220.

Engineered Microbial Cells

[0133] The above-described methods can be used to produce engineered microbial cells that produce, and in certain embodiments, overproduce, tyramine. Engineered microbial cells can have at least 1, 2, 3, 4, 5, 6 ,7, 8, 9, 10, or more genetic alterations, such as 30-40 alterations, as compared to a wild-type microbial cell, such as any of the microbial host cells described herein. Engineered microbial cells described in the Example below

have one, two, or three genetic alterations, but those of skill in the art can, following the guidance set forth herein, design microbial cells with additional alterations. In some embodiments, the engineered microbial cells have not more than 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, or 4 genetic alterations, as compared to a wild-type microbial cell. In various embodiments, microbial cells engineered for tyramine production can have a number of genetic alterations falling within the any of the following illustrative ranges: 1-10, 1-9, 1-8, 2-7, 2-6, 2-5, 2-4, 2-3, 3-7, 3-6, 3-5, 3-4, etc.

[0134] In some embodiments, an engineered microbial cell expresses at least one heterologous tyrosine decarboxylase (TYDC). This is necessary in the case of a microbial host cell that does not naturally produce tyramine. In various embodiments, the microbial cell can include and express, for example: (1) a single heterologous TYDC gene, (2) two or more heterologous TYDC genes, which can be the same or different (in other words, multiple copies of the same heterologous TYDC genes can be introduced or multiple, different heterologous TYDC genes can be introduced), (3) a single heterologous TYDC gene and one or more additional copies of an endogenous TYDC gene, or (4) two or more heterologous TYDC genes, which can be the same or different, and one or more additional copies of an endogenous TYDC gene.

[0135] This engineered host cell can include at least one additional genetic alteration that increases flux through the pathway leading to the production of tyrosine (the immediate precursor of tyramine). These "upstream" enzymes in the pathway include: dehydroquinate synthase, dehydroquinate dehydratase, shikimate dehydrogenase, shikimate kinase, EPSP synthase, aromatic pentafunctional enzyme, chorismate synthase, chorismate mutase, prephenate dehydratase, phenyalananine aminotransferase, prephenate dehydrogenase, prephenate aminotransferase, arogenate dehydrogenase, phenylalanine hydroxylase, and tyrosine aminotransferase, including any isoforms, paralogs, or orthologs having these enzymatic activities (which as those of skill in the art readily appreciate may be known by different names). The at least one additional alteration can increase the activity of the upstream pathway enzyme(s) by any available means, e.g., by: (1) modulating the expression or activity of the endogenous enzyme(s), (2) expressing one or more additional copies of the genes for the endogenous enzymes, or (3) expressing one or more copies of the genes for one or more heterologous enzymes.

[0136] In some embodiments, increased flux through the pathway can be achieved by expressing one or more genes encoding a feedback-disregulated enzyme, as discussed

above. For example, the engineered host cell can include and express: (1) one or more feedback-disregulated DAHP synthase genes, (2) one or more feedback-disregulated chorismate mutase genes, or (3) one or more feedback-disregulated DAHP synthase genes and one or more feedback-disregulated chorismate mutase genes. Thus, an engineered microbial cell having any of these genetic alterations can also include at least one heterologous TYDC and, optionally, one more genetic alterations that increase the activity of one or more upstream pathway enzymes.

[0137] The engineered microbial cells can contain introduced genes that have a wild-type nucleotide sequence or that differ from wild-type. For example, the wild-type nucleotide sequence can be codon-optimized for expression in a particular host cell. The amino acid sequences encoded by any of these introduced genes can be wild-type or can differ from wild-type. In various embodiments, the amino acid sequences have at least 0 percent, 70 percent, 75 percent, 80 percent, 85 percent, 90 percent, 95 percent or 100 percent amino acid sequence identity with a wild-type amino acid sequence.

[0138] The engineered microbial cells can, in various embodiments, be capable of producing tyramine at high titer, as described above. In some embodiments, the engineered microbial cell can produce tyramine by fermentation of a substrate, wherein at least 20 percent of the substrate is not derived from protein or amino acid sources. In various embodiments, at least 25 percent, 30 percent, 35 percent, 40 percent, 45 percent, 50 percent, 55 percent, 60 percent, 65 percent, 70 percent, 75 percent, 80 percent, 85 percent, 90 percent, 95 percent or 100 percent of the substrate is not derived from protein or amino acid sources. In some embodiments, the percentage of the fermentation substrate that is not derived from protein or amino acid sources falls within any of the following illustrative ranges: 40-100 percent, 40-90 percent, 40-80 percent, 50-100 percent, 50-90 percent, 50-80 percent, 60-100 percent, 60-90 percent, 60-80 percent, etc.

[0139] The approach described herein has been carried out in fungal cells, namely the yeast *S. cerevisiae* (a eukaryote), and in bacterial cells, namely *C. glutamicum* (a prokaryote). (See Example 1.)

Illustrative Engineered Fungal Cells

Illustrative Engineered *Saccharomyces cerevisiae* Cells

[0140] In certain embodiments the engineered yeast (e.g., *S. cerevisiae*) cell expresses a heterologous tyrosine decarboxylase (TYDC) having at least 70 percent, 75 percent, 80 percent, 85 percent, 90 percent, 95 percent or 100 percent amino acid sequence identity to a TYDC from *Papaver somniferum*. In various embodiments, the *P. somniferum* TYDC can include SEQ ID NO:20. This may be the only genetic alteration of the engineered yeast cell, or the yeast cell can include one or more additional genetic alterations, as discussed more generally above.

[0141] In particular embodiments, the engineered yeast (e.g., *S. cerevisiae*) cell additionally expresses a variant of a *S. cerevisiae* DAHP synthase, which typically has at least 70 percent, 75 percent, 80 percent, 85 percent, 90 percent, or 95 percent amino acid sequence identity to the wild-type *S. cerevisiae* DAHP synthase. In an illustrative embodiment, the engineered yeast (e.g., *S. cerevisiae*) cell expresses a *P. somniferum* Tyrosine/DOPA decarboxylase 2 (SEQ ID NO:2) and a feedback-disregulated *S. cerevisiae* DAHP synthase encoded by the *Aro4* gene that additionally comprises a K229L mutation (SEQ ID NO:6).

[0142] An illustrative yeast (e.g., *S. cerevisiae*) cell having a third genetic alteration can additionally have increased activity of an upstream pathway enzyme, such as prephenate dehydrogenase, relative to the control cell, e.g., produced by introducing an additional copy of a wild-type *S. cereviseae* prephenate dehydrogenase (SEQ ID NO:4) gene into the cell or a gene encoding a prephenate dehydrogenase having at least 70 percent, 75 percent, 80 percent, 85 percent, 90 percent, or 95 percent amino acid sequence identity to the wild-type *S. cereviseae* prephenate dehydrogenase.

[0143] An illustrative yeast (e.g., *S. cerevisiae*) cell having a fourth genetic alteration can additionally have increased activity of an upstream pathway enzyme, such as transaldolase, relative to the control cell, e.g., produced by introducing an additional copy of a wild-type *S. cereviseae* transaldolase (SEQ ID NO:8) gene into the cell or a gene encoding a transaldolase having at least 70 percent, 75 percent, 80 percent, 85 percent, 90 percent, or 95 percent amino acid sequence identity to the wild-type *S. cereviseae* transaldolase. In an illustrative embodiment, an engineered *S. cereviseae* cell expresses versions of these genes

that are codon-optimized using a using a modified combined codon table for *Corynebacterium glutamicum* and *S. cereviseae*.

Illustrative Engineered *Yarrowia lipolytica* Cells

[0144] In certain embodiments the engineered yeast (e.g., *Yarrowia lipolytica*) cell expresses a heterologous tyrosine decarboxylase (TYDC) having at least 70 percent, 75 percent, 80 percent, 85 percent, 90 percent, 95 percent or 100 percent amino acid sequence identity to a pyridoxal-dependent decarboxylase (TYDC) from *Enterococcus faecium* (e.g., Com15). In various embodiments, the *E. faecium* TYDC can include SEQ ID NO:1. This may be the only genetic alteration of the engineered yeast cell, or the yeast cell can include one or more additional genetic alterations, as discussed more generally above.

[0145] In particular embodiments, the engineered yeast (e.g., *Y. lipolytica*) cell additionally expresses a *S. cerevisiae* DAHP synthase, which typically has at least 70 percent, 75 percent, 80 percent, 85 percent, 90 percent, or 95 percent amino acid sequence identity to the wild-type *S. cerevisiae* (e.g., S288c) phospho-2-dehydro-3-deoxyheptonate aldolase (DAHP synthase)(SEQ ID NO: 9). In an illustrative embodiment, an engineered *Y. lipolytica* cell expresses versions of these genes that are codon-optimized for *Y. lipolytica*(SEQ ID NO: 10).

Illustrative Engineered Bacterial Cells

Illustrative Engineered *Corynebacterium glutamicum* Cells

[0146] In certain embodiments the engineered bacterial (e.g., *C. glutamicum*) cell expresses a heterologous tyrosine decarboxylase (TYDC) having at least 70 percent, 75 percent, 80 percent, 85 percent, 90 percent, 95 percent or 100 percent amino acid sequence identity to a TYDC from *Enterococcus faecium* (e.g., Com15) or from *Zygosaccharomyces bailii*. For example, the *E. faecium* TYDC can include SEQ ID NO:1, and the *Z. bailii* TYDC can include SEQ ID NO:11. Expression of a heterologous TYDC may be the only genetic alteration of the engineered bacterial cell, or the bacterial cell can include one or more additional genetic alterations, as discussed more generally above.

[0147] In particular embodiments, the engineered bacterial (e.g., *C. glutamicum*) cell additionally expresses a *S. cerevisiae* DAHP synthase (e.g., phospho-2-dehydro-3-deoxyheptonate aldolase from strain 288c) or a variant thereof, which typically has at least

70 percent, 75 percent, 80 percent, 85 percent, 90 percent, or 95 percent amino acid sequence identity to the wild-type *S. cerevisiae* DAHP synthase (SEQ ID NO:9). Both genes can be codon-optimized, for example, for *S. cerevisiae*. In illustrative embodiments, the engineered bacterial (e.g., *C. glutamicum*) cell expresses either (or both) an *E. faecium* TYDC (SEQ ID NO:1) or a *Z. bailii* TYDC (SEQ ID NO:11) in combination with a feedback-disregulated *S. cerevisiae* DAHP synthase encoded by the *Aro4* gene that additionally comprises a K229L mutation (SEQ ID NO:6).

[0148] Alternatively, or in addition to expressing a DAHP synthase variant, a TYDC-expressing bacterial (e.g., *C. glutamicum*) cell can have increased activity of an upstream pathway enzyme, such as chorismate synthase and/or prephrenate dehydrogenase relative to the control cell. In an illustrative embodiment, the engineered bacterial (e.g., *C. glutamicum*) cell expresses an *E. faecium* TYDC (SEQ ID NO:1) in combination with a copy of a wild-type *S. cereviseae* chorismate synthase (SEQ ID NO:12) gene or a gene encoding a chorismate synthase having at least 70 percent, 75 percent, 80 percent, 85 percent, 90 percent, or 95 percent amino acid sequence identity to the wild-type *S. cereviseae* chorismate synthase (SEQ ID NO: 3). In another illustrative embodiment, the engineered bacterial (e.g., *C. glutamicum*) cell expresses an *E. faecium* TYDC (SEQ ID NO:1) in combination with a copy of a wild-type *S. cereviseae* prephenate dehydrogenase (SEQ ID NO:13) gene or a gene encoding a prephenate dehydrogenase having at least 70 percent, 75 percent, 80 percent, 85 percent, 90 percent, or 95 percent amino acid sequence identity to the wild-type *S. cereviseae* prephenate dehydrogenase (SEQ ID NO: 4).

Illustrative Engineered *Bacillus subtilus* Cells

[0149] In certain embodiments the engineered bacterial (e.g., *Bacillus subtilus*) cell expresses a heterologous tyrosine decarboxylase (TYDC) having at least 70 percent, 75 percent, 80 percent, 85 percent, 90 percent, 95 percent or 100 percent amino acid sequence identity to a TYDC from *Enterococcus faecium* (e.g., Com15). For example, the *E. faecium* TYDC can include SEQ ID NO:1. Expression of a heterologous TYDC may be the only genetic alteration of the engineered bacterial cell, or the bacterial cell can include one or more additional genetic alterations, as discussed more generally above.

[0150] In particular embodiments, the engineered bacterial (e.g., *B. subtilus*) cell additionally expresses a variant of a *S. cerevisiae* DAHP synthase (e.g., phospho-2-dehydro-3-deoxyheptonate aldolase from strain 288c), which typically has at least 70 percent, 75

percent, 80 percent, 85 percent, 90 percent, or 95 percent amino acid sequence identity to the wild-type *S. cerevisiae* DAHP synthase (SEQ ID NO:9).

[0151] An illustrative bacterial (e.g., *B. subtilus*) cell having a third genetic alteration can additionally have increased activity of an upstream pathway enzyme, such as shikimate kinase, relative to the control cell, e.g., produced by introducing an additional copy of a wild-type *E. coli* (e.g., K12) shikimate kinase 2 (SEQ ID NO:14) gene into the cell or a gene encoding a shikimate kinase having at least 70 percent, 75 percent, 80 percent, 85 percent, 90 percent, or 95 percent amino acid sequence identity to the wild-type *E. coli* shikimate kinase 2 (SEQ ID NO: 15). In an illustrative embodiment, an engineered *B. subtilus* cell expresses versions of these genes that are codon-optimized for *S. cerevisiae*.

Culturing of Engineered Microbial Cells

[0152] Any of the microbial cells described herein can be cultured, e.g., for maintenance, growth, and/or tyramine production. Generally, tyramine is produced from fermentation of a substrate wherein at least 20% of the substrate is not derived from protein or amino acid sources. Accordingly, cultures of the engineered microbial cells described herein include a fermentation substrate, wherein at least 20 percent of the substrate is not derived from protein or amino acid sources. In various embodiments, at least 25 percent, 30 percent, 35 percent, 40 percent, 45 percent, 50 percent, 55 percent, 60 percent, 65 percent, 70 percent, 75 percent, 80 percent, 85 percent, 90 percent, 95 percent or 100 percent of the substrate is not derived from protein or amino acid sources. In some embodiments, the percentage of the fermentation substrate that is not derived from protein or amino acid sources falls within any of the following illustrative ranges: 40-100 percent, 40-90 percent, 40-80 percent, 50-100 percent, 50-90 percent, 50-80 percent, 60-100 percent, 60-90 percent, 60-80 percent, etc.

[0153] In some embodiments, the cultures are grown to an optical density at 600 nm of 10-500, such as an optical density of 50-150.

[0154] In various embodiments, the cultures include produced tyramine at titers of at least 10, 50, 75, 100, 200, 300, 400, 500, 600, 700, 800, or 900 mg/L or at least 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, or 10 gm/L. In various embodiments, the titer is in the range of 10 mg/L to 10 gm/L, 100 mg/L to 5 gm/L, 200 mg/L to 4 gm/L, 300 mg/L to 3 gm/L, or any range bounded by any of the values listed above.

Culture Media

[0155] Microbial cells can be cultured in any suitable medium including, but not limited to, a minimal medium, i.e., one containing the minimum nutrients possible for cell growth. Minimal medium typically contains: (1) a carbon source for microbial growth; (2) salts, which may depend on the particular microbial cell and growing conditions; and (3) water. Suitable media can also include any combination of the following: a nitrogen source for growth and product formation, a sulfur source for growth, a phosphate source for growth, metal salts for growth, vitamins for growth, and other cofactors for growth.

[0156] Any suitable carbon source can be used to cultivate the host cells. The term "carbon source" refers to one or more carbon-containing compounds capable of being metabolized by a microbial cell. In various embodiments, the carbon source is a carbohydrate (such as a monosaccharide, a disaccharide, an oligosaccharide, or a polysaccharide), or an invert sugar (e.g., enzymatically treated sucrose syrup). Illustrative monosaccharides include glucose (dextrose), fructose (levulose), and galactose; illustrative oligosaccharides include dextran or glucan, and illustrative polysaccharides include starch and cellulose. Suitable sugars include C6 sugars (e.g., fructose, mannose, galactose, or glucose) and C5 sugars (e.g., xylose or arabinose). Other, less expensive carbon sources include sugar cane juice, beet juice, sorghum juice, and the like, any of which may, but need not be, fully or partially deionized.

[0157] The salts in a culture medium generally provide essential elements, such as magnesium, nitrogen, phosphorus, and sulfur to allow the cells to synthesize proteins and nucleic acids.

[0158] Minimal medium can be supplemented with one or more selective agents, such as antibiotics.

[0159] To produce tyramine, the culture medium can include, and/or is supplemented during culture with, glucose and/or a nitrogen source such as urea, an ammonium salt, ammonia, or any combination thereof.

Culture Conditions

[0160] Materials and methods suitable for the maintenance and growth of microbial cells are well known in the art. See, for example, U.S. Pub. Nos. 2009/0203102, 2010/0003716, and 2010/0048964, and International Pub. Nos. WO 2004/033646, WO 2009/076676, WO 2009/132220, and WO 2010/003007, Manual of Methods for General

Bacteriology Gerhardt et al., eds), American Society for Microbiology, Washington, D.C. (1994) or Brock in Biotechnology: A Textbook of Industrial Microbiology, Second Edition (1989) Sinauer Associates, Inc., Sunderland, Mass.

[0161] In general, cells are grown and maintained at an appropriate temperature, gas mixture, and pH (such as about 20°C to about 37°C, about 6% to about 84% CO_2, and a pH between about 5 to about 9). In some aspects, cells are grown at 35°C. In certain embodiments, such as where thermophilic bacteria are used as the host cells, higher temperatures (e.g., 50°C -75°C) may be used. In some aspects, the pH ranges for fermentation are between about pH 5.0 to about pH 9.0 (such as about pH 6.0 to about pH 8.0 or about 6.5 to about 7.0). Cells can be grown under aerobic, anoxic, or anaerobic conditions based on the requirements of the particular cell.

[0162] Standard culture conditions and modes of fermentation, such as batch, fed-batch, or continuous fermentation that can be used are described in U.S. Publ. Nos. 2009/0203102, 2010/0003716, and 2010/0048964, and International Pub. Nos. WO 2009/076676, WO 2009/132220, and WO 2010/003007. Batch and Fed-Batch fermentations are common and well known in the art, and examples can be found in Brock, Biotechnology: A Textbook of Industrial Microbiology, Second Edition (1989) Sinauer Associates, Inc.

[0163] In some embodiments, the cells are cultured under limited sugar (e.g., glucose) conditions. In various embodiments, the amount of sugar that is added is less than or about 105% (such as about 100%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, or 10%) of the amount of sugar that can be consumed by the cells. In particular embodiments, the amount of sugar that is added to the culture medium is approximately the same as the amount of sugar that is consumed by the cells during a specific period of time. In some embodiments, the rate of cell growth is controlled by limiting the amount of added sugar such that the cells grow at the rate that can be supported by the amount of sugar in the cell medium. In some embodiments, sugar does not accumulate during the time the cells are cultured. In various embodiments, the cells are cultured under limited sugar conditions for times greater than or about 1, 2, 3, 5, 10, 15, 20, 25, 30, 35, 40, 50, 60, or 70 hours or even up to about 5-10 days. In various embodiments, the cells are cultured under limited sugar conditions for greater than or about 5, 10, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 95, or 100% of the total length of time the cells are cultured. While not intending to be bound by

any particular theory, it is believed that limited sugar conditions can allow more favorable regulation of the cells.

[0164] In some aspects, the cells are grown in batch culture. The cells can also be grown in fed-batch culture or in continuous culture. Additionally, the cells can be cultured in minimal medium, including, but not limited to, any of the minimal media described above. The minimal medium can be further supplemented with 1.0% (w/v) glucose (or any other six-carbon sugar) or less. Specifically, the minimal medium can be supplemented with 1% (w/v), 0.9% (w/v), 0.8% (w/v), 0.7% (w/v), 0.6% (w/v), 0.5% (w/v), 0.4% (w/v), 0.3% (w/v), 0.2% (w/v), or 0.1% (w/v) glucose. In some cultures, significantly higher levels of sugar (e.g., glucose) are used, e.g., at least 10% (w/v), 20% (w/v), 30% (w/v), 40 % (w/v), 50% (w/v), 60% (w/v), 70% (w/v), or up to the solubility limit for the sugar in the medium. In some embodiments, the sugar levels falls within a range of any two of the above values, e.g.: 0.1-10% (w/v), 1.0-20% (w/v), 10-70 % (w/v), 20-60 % (w/v), or 30-50 % (w/v). Furthermore, different sugar levels can be used for different phases of culturing. For fed-batch culture (e.g., of *S. cerevisiae* or *C. glutamicum)*, the sugar level can be about 100-200 g/L (10-20 % (w/v)) in the batch phase and then up to about 500-700 g/L (50-70 % in the feed).

[0165] Additionally, the minimal medium can be supplemented 0.1% (w/v) or less yeast extract. Specifically, the minimal medium can be supplemented with 0.1% (w/v), 0.09% (w/v), 0.08% (w/v), 0.07% (w/v), 0.06% (w/v), 0.05% (w/v), 0.04% (w/v), 0.03% (w/v), 0.02% (w/v), or 0.01% (w/v) yeast extract. Alternatively, the minimal medium can be supplemented with 1% (w/v), 0.9% (w/v), 0.8% (w/v), 0.7% (w/v), 0.6% (w/v), 0.5% (w/v), 0.4% (w/v), 0.3% (w/v), 0.2% (w/v), or 0.1% (w/v) glucose and with 0.1% (w/v), 0.09% (w/v), 0.08% (w/v), 0.07% (w/v), 0.06% (w/v), 0.05% (w/v), 0.04% (w/v), 0.03% (w/v), or 0.02% (w/v) yeast extract. In some cultures, significantly higher levels of yeast extract can be used, e.g., at least 1.5% (w/v), 2.0% (w/v), 2.5% (w/v), or 3 % (w/v). In some cultures (e.g., of *S. cerevisiae* or *C. glutamicum)*, the yeast extract level falls within a range of any two of the above values, e.g.: 0.5-3.0% (w/v), 1.0-2.5% (w/v), or 1.5-2.0% (w/v).

[0166] Illustrative materials and methods suitable for the maintenance and growth of the engineered microbial cells described herein can be found below in Example 1.

Tyramine Production and Recovery

[0167] Any of the methods described herein may further include a step of recovering tyramine. In some embodiments, the produced tyramine contained in a so-called harvest stream is recovered/harvested from the production vessel. The harvest stream may include, for instance, cell-free or cell-containing aqueous solution coming from the production vessel, which contains tyramine as a result of the conversion of production substrate by the resting cells in the production vessel. Cells still present in the harvest stream may be separated from the tyramine by any operations known in the art, such as for instance filtration, centrifugation, decantation, membrane crossflow ultrafiltration or microfiltration, tangential flow ultrafiltration or microfiltration or dead end filtration. After this cell separation operation, the harvest stream is essentially free of cells.

[0168] Further steps of separation and/or purification of the produced tyramine from other components contained in the harvest stream, i.e., so-called downstream processing steps may optionally be carried out. These steps may include any means known to a skilled person, such as, for instance, concentration, extraction, crystallization, precipitation, adsorption, ion exchange, chromatography, distillation, electrodialysis, bipolar membrane electrodialysis and/or reverse osmosis. Any of these procedures can be used alone or in combination to purify tyramine. Further purification steps can include one or more of, e.g., concentration, crystallization, precipitation, washing and drying, treatment with activated carbon, ion exchange and/or re-crystallization. The design of a suitable purification protocol may depend on the cells, the culture medium, the size of the culture, the production vessel, etc. and is within the level of skill in the art.

[0169] The following example is given for the purpose of illustrating various embodiments of the disclosure and is not meant to limit the present disclosure in any fashion. Changes therein and other uses which are encompassed within the spirit of the disclosure, as defined by the scope of the claims, will be identifiable to those skilled in the art.

EXAMPLE 1 – Construction and Selection of Strains of *Saccharomyces cerevisiae* and *Corynebacterium glutamicum* Engineered to Produce Tyramine

Plasmid/DNA Design

[0170] All strains tested for this work were transformed with plasmid DNA designed using proprietary software. Plasmid designs were specific to one of the two host organisms engineered in this work. The plasmid DNA was physically constructed by a standard DNA assembly method. This plasmid DNA was then used to integrate metabolic pathway inserts by one of two host-specific methods, each described below.

S. cerevisiae Pathway Integration

[0171] A "split-marker, double-crossover" genomic integration strategy has been developed to engineer S. cerevisiae strains. Fig. 2 illustrates genomic integration of complementary, split-marker plasmids and verification of correct genomic integration via colony PCR in *S. cerevisiae*. Two plasmids with complementary 5' and 3' homology arms and overlapping halves of a URA3 selectable marker (direct repeats shown by the hashed bars) were digested with meganucleases and transformed as linear fragments. A triple-crossover event integrated the desired heterologous genes into the targeted locus and re-constituted the full URA3 gene. Colonies derived from this integration event were assayed using two 3-primer reactions to confirm both the 5' and 3' junctions (UF/IF/wt-R and DR/IF/wt-F). For strains in which further engineering is desired, the strains can be plated on 5-FOA plates to select for the removal of URA3, leaving behind a small single copy of the original direct repeat. This genomic integration strategy can be used for gene knock-out, gene knock-in, and promoter titration in the same workflow.

C. glutamicum Pathway Integration

[0172] A "loop-in, single-crossover" genomic integration strategy has been developed to engineer *C. glutamicum* strains. Fig. 3 illustrates genomic integration of loop-in only and loop-in/loop-out constructs and verification of correct integration via colony PCR. Loop-in only constructs (shown under the heading "Loop-in") contained a single 2-kb homology arm (denoted as "integration locus"), a positive selection marker (denoted as "Marker")), and gene(s) of interest (denoted as "promoter-gene-terminator"). A single crossover event integrated the plasmid into the *C. glutamicum* chromosome. Integration events are stably maintained in the genome by growth in the presence of antibiotic (25µg/ml

kanamycin). Correct genomic integration in colonies derived from loop-in integration were confirmed by colony PCR with UF/IR and DR/IF PCR primers.

[0173] Loop-in, loop-out constructs (shown under the heading "Loop-in, loop-out) contained two 2-kb homology arms (5' and 3' arms), gene(s) of interest (arrows), a positive selection marker (denoted "Marker"), and a counter-selection marker. Similar to "loop-in" only constructs, a single crossover event integrated the plasmid into the chromosome of *C. glutamicum*. Note: only one of two possible integrations is shown here. Correct genomic integration was confirmed by colony PCR and counter-selection was applied so that the plasmid backbone and counter-selection marker could be excised. This results in one of two possibilities: reversion to wild-type (lower left box) or the desired pathway integration (lower right box). Again, correct genomic loop-out is confirmed by colony PCR. (Abbreviations: Primers: UF = upstream forward, DR = downstream reverse, IR = internal reverse, IF = internal forward.)

Cell Culture

[0174] Separate workflows were established for *C. glutamicum* and *S. cerevisiae* due to differences in media requirements and growth. Both processes involved a hit-picking step that consolidated successfully built strains using an automated workflow that randomized strains across the plate. For each strain that was successfully built, up to four replicates were tested from distinct colonies to test colony-to-colony variation and other process variation. If fewer than four colonies were obtained, the existing colonies were replicated so that at least four wells were tested from each desired genotype.

[0175] The colonies were consolidated into 96-well plates with selective medium (BHI for *C. glutamicum*, SD-ura for *S. cerevisiae*) and cultivated for two days until saturation and then frozen with 16.6% glycerol at -80°C for storage. The frozen glycerol stocks were then used to inoculate a seed stage in minimal media with a low level of amino acids to help with growth and recovery from freezing. The seed plates were grown at 30°C for 1-2 days. The seed plates were then used to inoculate a main cultivation plate with minimal medium and grown for 48-88 hours. Plates were removed at the desired time points and tested for cell density (OD600), viability and glucose, supernatant samples stored for LC-MS analysis for product of interest.

Cell Density

[0176] Cell density was measured using a spectrophotometric assay detecting absorbance of each well at 600nm. Robotics were used to transfer fixed amounts of culture from each cultivation plate into an assay plate, followed by mixing with 175mM sodium phosphate (pH 7.0) to generate a 10-fold dilution. The assay plates were measured using a Tecan M1000 spectrophotometer and assay data uploaded to a LIMS database. A non-inoculated control was used to subtract background absorbance. Cell growth was monitored by inoculating multiple plates at each stage, and then sacrificing an entire plate at each time point.

[0177] To minimize settling of cells while handling large number of plates (which could result in a non-representative sample during measurement) each plate was shaken for 10-15 seconds before each read. Wide variations in cell density within a plate may also lead to absorbance measurements outside of the linear range of detection, resulting in underestimate of higher OD cultures. In general, the tested strains so far have not varied significantly enough for this be a concern.

Cell Viability

[0178] Two methods were used to measure cell viability. The first assay utilized a single stain, propidium iodide, to assess cell viability. Propidium iodide binds to DNA and is permeable to cells with compromised cell membranes. Cells that take up the propidium iodide are considered non-viable. A dead cell control was used to normalize to total number of cells, by incubating a cell sample of control culture at 95°C for 10 minutes. These control samples and test samples were incubated with the propidium iodide stain for 5 minutes, washed twice with 175mM phosphate buffer, and fluorescence measured in black solid-bottom 96-well plates at 617nm.

Glucose

[0179] Glucose is measured using an enzymatic assay with 16U/mL glucose oxidase (Sigma) with 0.2 U/mL horseradish peroxidase (Sigma) and 0.2mM Amplex red in 175mM sodium phosphate buffer, pH 7. Oxidation of glucose generates hydrogen peroxide, which is then oxidized to reduce Amplex red, which changes absorbance at 560nm. The change is absorbance is correlated to the glucose concentration in the sample using standards of known concentration.

Liquid-Solid Separation

[0180] To harvest extracellular samples for analysis by LC-MS, liquid and solid phases were separated via centrifugation. Cultivation plates were centrifuged at 2000 rpm for 4 minutes, and the supernatant was transferred to destination plates using robotics. 75μL of supernatant was transferred to each plate, with one stored at 4°C, and the second stored at 80°C for long-term storage.

[0181] A first round of genetic engineering and screening was carried out using *C. glutamicum* and *S. cerevisiae* as host cells. A heterologous TYDC was expressed in the host cells, in some cases, along with a feedback-disregulated DAHP synthase. In some cases, the TYDC nucleotide sequence was codon-optimized for either *C. glutamicum* or *S. cerevisiae*. The strains were produced and cultured as described above, and the tyramine titer in the culture media was measured by LC-MS. The strains and results are shown in Table 1. The best-performing strain was an *S. cerevisiae* strain expressing a *P. somniferum* TYDC (SEQ ID NO: 2), along with an *S. cerevisiae* DAHP synthase with a K229L amino acid substitution (SEQ ID NO: 6), which gave a tyramine titer of almost 387 μg/L of culture medium. This strain was selected for a second round of genetic engineering and screening.

Table 1 – First-Round Results

Titer µg/L	E1 activity name	E1 source organism	E1 taxonomic region	E1 codon opt	E2 activity name	E2 source organism	E2 taxonomic region	E2 modifications
C. glutamicum								
80.20	TYDC*	*E. faecium (S. faecium)*	Bacteria	Cg				
54.6927	TYDC	*Z. bailii ISA1307*	Fungi	Cg				
14.3711	TYDC	*E. faecium (S. faecium)*	Bacteria	Cg	DAHP synthase**	*S. cerevisiae* ***	Fungi	Q166K, reduces pathway feedback inhibition
4.8087	TYDC	*M. palustris (strain ATCC BAA-1556 / DSM 19958 / E1-9c)*	Archaea	Cg				
2.2345	TYDC	*Propionibacterium sp. oral taxon 192 str. F0372*	Bacteria	Cg				
0.9617	TYDC	*P. crispum*	Viridi-plantae	Cg	DAHP synthase	*E. coli***	Bacteria	D146N, reduces pathway feedback inhibition
0.5946	TYDC	*T. equinum (strain ATCC MYA-4606 / CBS 127.97) (Horse ringworm fungus)*	Fungi	Cg	DAHP synthase	*E. coli*	Bacteria	D146N, reduces pathway feedback inhibition
0.1045	TYDC	*S. sviceus ATCC 29083*	Bacteria	Cg				
0.0825	TYDC	*P. putida (strain KT2440)*	Bacteria	Cg	DAHP synthase	*E. coli*	Bacteria	D146N, reduces pathway feedback inhibition
0.0269	TYDC	*M. marinus (strain BC501)*	Bacteria	Cg				
0.0024	TYDC	*P. somniferum*	Viridi-plantae	Cg	DAHP synthase	*S. cerevisiae*	Fungi	Q166K, reduces pathway feedback inhibition
0.0018	TYDC	*T. equinum (strain ATCC MYA-4606 / CBS 127.97) (Horse ringworm fungus)*	Fungi	Cg				
0.0008	TYDC	*S. fredii USDA 257*	Bacteria	Cg				
0	TYDC	*P. somniferum)*	Viridi-plantae	Sc	DAHP synthase	*S. cerevisiae*	Fungi	K229L, reduces pathway feedback

Titer µg/L	E1 activity name	E1 source organism	E1 taxonomic region	E1 codon opt	E2 activity name	E2 source organism	E2 taxonomic region	E2 modifications
								inhibition
S. cerevisiae								
386.7658	TYDC	*P. somniferum*	Viridi-plantae	Sc	DAHP synthase	*S. cerevisiae*	Fungi	K229L, reduces pathway feedback inhibition
162.2137	TYDC	*P. somniferum*	Viridi-plantae	Cg	DAHP synthase	*E. coli*	Bacteria	D146N,reduces pathway feedback inhibition
115.9018	TYDC	*P. somniferum*	Viridi-plantae	Sc				
4.7859	TYDC	*O. sativa subsp. Japonica*	Viridi-plantae	Cg	DAHP synthase	*E. coli*	Bacteria	D146N,reduces pathway feedback inhibition
3.2748	TYDC	*S. fredii USDA 257*	Bacteria	Sc				
1.1297	TYDC	*P. putida (strain KT2440)*	Bacteria	Sc				
1.0391	TYDC	*M. palustris (strain ATCC BAA-1556 / DSM 19958 / E1-9c)*	Archaea	Sc				
0.6421	TYDC	*M. jannaschii*	Archaea	Sc				
0.4238	TYDC	*O. sativa subsp. Japonica*	Viridi-plantae	Cg	DAHP synthase	*E. coli*	Bacteria	D146N,reduces pathway feedback inhibition
0.1481	TYDC	*M. marinus (strain BC501)*	Bacteria	Sc				

***TYDC GO ID: GO:0004837**

****DAHP Synthase: GO ID: GO:0003849**

*****S.cerevisiae DAHP synthase taxon ID: Sc; Uniprot ID: P32449**

****** E. coli DAHP synthase taxon ID: 83333; Uniprot ID: P0AB91**

Codon optimization was for *C. glutamicum* (Cg) or *S. cerevisiae* (Sc)

[0182] In the second round of engineering/screening, a third enzyme was expressed in the *S. cerevisiae* strain expressing a *P. somniferum* TYDC, along with an *S. cerevisiae* DAHP synthase with a K229L amino acid substitution from the first round. In some cases, the nucleotide sequence encoding the third enzymes was codon-optimized. Table 2 shows the third enzymes tested and the resultant tyramine titers. The higher titer was about 346 mg/L, an almost 1000-fold improvement, which was achieved by the strain expressing native *S. cerevisiae* prephenate dehydrogenase as the third enzyme.

Table 2 – Second-Round Results

Titer (µg/L)	Enzyme3 -activity name	Enzyme3 – source organism	E3 UniProt id	E3 codon opt
238453.9398	DAHP synthase (D146N)	*Escherichia coli*	P0AB91	Sc
288361.4515	DAHP synthase (Q166K)	*Saccharomyces cerevisiae*	P32449	Cg
228876.649	DAHP synthase (D146N)	*Escherichia coli*	P0AB91	Sc
285231.8609	DAHP synthase (Q166K)	*Saccharomyces cerevisiae*	P32449	Cg
283175.3481	DAHP synthase (wild type)	*Saccharomyces cerevisiae CEN.PK2*	N1P8J9	native
253015.743	phospho-2-dehydro-3-deoxyheptonate aldolase	*Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)*	P14843	native
243755.6441	phospho-2-dehydro-3-deoxyheptonate aldolase	*Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)*	P32449	native
249195.9423	chorismate synthase	*Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)*	P28777	native
345546.6876	prephenate dehydrogenase	*Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)*	P20049	native
300138.6771	aromatic/aminoadipate aminotransferase 1; 2-aminoadipate transaminase, L-phenylalanine:2-oxoglutarate aminotransferase	*Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)*	P53090	native
284884.7302	aromatic amino acid aminotransferase 2; 2-aminoadipate transaminase, L-phenylalanine:2-oxoglutarate aminotransferase, kynurenine-oxoglutarate transaminase	*Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)*	P38840	native
334432.6849	prephenate dehydrogenase	*Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)*	P20049	native
245114.4577	phospho-2-dehydro-3-deoxyheptonate aldolase	*Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)*	P14843	native
271440.4966	phospho-2-dehydro-3-deoxyheptonate aldolase	*Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)*	P32449	native

EXAMPLE 2A – Improvement-Round (Third-Round) Results in *S. cerevisiae* Constructions and Evaluation of Different Microbial Host Cells for Production of Tyramine

Summary

[0183] The best-performing strain of Example 1 was selected as the control strain to in which to test a third round of genetic engineering in an effort to improve tyramine in *Saccharomyces cerevisiae* ("Improvement Round"). The control strain expressed prephenate dehydrogenase from *S. cerevisiae* (UniProt ID P20049)(SEQ ID NO: 4), DAHP synthase from *S. cerevisiae* S288c (UniProt ID P32449)(SEQ ID NO: 6) harboring the amino acid substitution K229L and tyrosine/DOPA decarboxylase 2 from *Papaver somniferum* (UniProt ID P54769)(SEQ ID NO: 2). Tyramine production was improved in *S. cerevisiae* for eight strains relative to the control, and of these strains the strain giving the highest titer (300 mg/L vs. 266 mg/L for the control) expressed transaldolase from *S. cerevisiae* (UniProt ID P15019)(SEQ ID NO: 8).

Strain Designs Tested

[0184] Tyramine production was improved by expression of each of the following heterologous enzymes or combinations of heterologous enzymes relative to the control:

1) Aromatic amino acid aminotransferase (SEQ ID NO: 28)
2) Phosphoenolpyruvate synthase (UniProt ID P23538) from *Escherichia coli* K12 (SEQ ID NO: 29)
3) Transketolase (UniProt ID P23254) from *S. cerevisiae* (SEQ ID NO: 30)
4) Transaldolase (UniProt ID P15019) from *S. cerevisiae* (SEQ ID NO: 8)
5) Transketolase (UniProt ID P23254) from *S. cerevisiae* (SEQ ID NO: 30) AND transaldolase (UniProt ID P15019) from *S. cerevisiae* (SEQ ID NO: 8)
6) Transketolase (UniProt ID P23254) from *S. cerevisiae* (SEQ ID NO: 30) AND phosphoenolpyruvate synthase (UniProt ID P23538) from *E. coli* K12 (SEQ ID NO: 29).
7) Transaldolase (UniProt ID P15019) from *S. cerevisiae* (SEQ ID NO: 8) AND DAHP synthase (UniProt ID P32449) from *S. cerevisiae* harboring amino acid substitution K299L (SEQ ID NO: 6).
8) Glyceraldehyde-3-phosphate dehydrogenase (UniProt ID P30724) from *Gracilaria gracilis* (SEQ ID NO: 31)

Results

[0185] The results are shown in Table 7 (below) and Fig. 9.

[0186] It further designs, production of tyramine can be tested for improvement in strains containing the addition of the following heterologous enzymes or combinations of heterologous enzymes to the best-performing strain from this Example:

1) 2-dehydro-3-deoxyphosphoheptonate aldolase (SEQ ID NO: 32)
2) Chorismate mutase (SEQ ID NO: 33)
3) Chorismate mutase (SEQ ID NO: 33) and Prephenate dehydrogenase (SEQ ID NO:
4) Shikimate kinase1 (SEQ ID NO: 34)
5) shikimate dehydrogenase (SEQ ID NO: 35)
6) prephenate dehydrogenase (SEQ ID NO: 4)
7) Tyrosine aminotransferase (SEQ ID NO: 36)
8) Prephenate dehydrogenase (SEQ ID NO: 4)
9) Glutamate synthase (GOGAT) large subunit (SEQ ID NO: 37)
10) Glutamine synthetase (GS) (SEQ ID NO: 39)
11) Glutamate synthase small subunit (SEQ ID NO: 38)
12) Shikimate dehydrogenase (SEQ ID NO: 35)
13) Shikimate kinase2 (SEQ ID NO: 15)
14) Chorismate mutase (SEQ ID NO: 33) and Prephenate dehydrogenase (SEQ ID NO: 4)

EXAMPLE 2B – Evaluation of Different Microbial Host Cells for Production of Tyramine

Summary

[0187] Host evaluation designs were tested in *Yarrowia lipolytica, Bacillus subtilus*, *S. cerevisiae* and *Corynebacteria glutamicum*. Tyramine production was demonstrated in all strains tested. The best-performing *Y. lipolytica* strain on average produced 54.5 mg/L tyramine. The best-performing *B. subtilus* strain produced 19.9 mg/L tyramine. The best-performing *S. cerevisiae* strain from the host evaluation produced 189 mg/L tyramine. The best-performing *C. glutamicum* strain produced 467 mg/L tyramine.

Results

[0188] The best performing *Y. lipolytica* strain on average produced 54.5 mg/L tyramine and expressed the pyridoxal-dependent decarboxylase (TYDC) from *Enterococcus faecium* Com15 (UniProt ID C9ASN2) (SEQ ID NO: 1), phospho-2-dehydro-3-deoxyheptonate aldolase (DAHP synthase) from *S. cerevisiae* S288c (UniProt ID P32449) (SEQ ID NO: 9), where the DNA sequences for both enzymes were codon-optimized for *Y. lipolytica*. (Table 3, Fig. 5.) (SEQ ID Nos: 40 and 10, respectively).

[0189] The best-performing *B. subtilis* strain produced 19.9 mg/L tyramine and expressed the pyridoxal-dependent decarboxylase (TYDC) from Enterococcus faecium Com15 (UniProt ID C9ASN2) (SEQ ID NO: 1), phospho-2-dehydro-3-deoxyheptonate aldolase (DAHP synthase) from Saccharomyces cerevisiae S288c (UniProt ID P32449) (SEQ ID NO: 9), and shikimate kinase 2 from Escherichia coli K12 (UniProt ID P0A6E1) (SEQ ID NO: 15), where the DNA sequences for all three enzymes were codon-optimized for *S. cerevisiae*. (Table 4, Fig. 6.) (SEQ ID NOs: 43, 42, 41)

[0190] The best-performing *S. cerevisiae* strain from the host evaluation produced 189 mg/L tyramine and expressed pyridoxal-dependent decarboxylase (TYDC) from *E. faecium* Com15 (UniProt ID C9ASN2) (SEQ ID NO: 1), phospho-2-dehydro-3-deoxyheptonate aldolase (DAHP synthase) from *S. cerevisiae* S288c (UniProt ID P32449) harboring the amino acid substitution K229L (SEQ ID NO: 6), where the DNA sequences for both enzymes were codon-optimized using a modified combined codon table for *C. glutamicum* and *S. cerevisiae*. (Table 5, Fig. 7.) (SEQ ID NOs: 45, 44).

[0191] The best-performing *C. glutamicum* strain produced 467 mg/L tyramine and expressed pyridoxal-dependent decarboxylase (TYDC) from *E. faecium* Com15 (UniProt ID C9ASN2) (SEQ ID NO: 1), phospho-2-dehydro-3-deoxyheptonate aldolase from (DAHP synthase) *S. cerevisiae* S288c (UniProt ID P32449) (SEQ ID NO: 9), where the DNA sequences for both enzymes was codon-optimized for *S. cerevisiae*. (Table 6, Fig. 8.) (SEQ ID NOs: 43, 42).

[0192] To improve a platform *C. glutamicum* strain for production of stilbenes and (2S)-flavanones Kallscheuer et al. (see References below) deleted genes and operons that degrade aromatic rings including polypropanoid degradation operon (phdBCDE, cg0344-47); 4-hydroxybenzoate-3-hydrolase (pobA (cg1226); the gene cluster harboring cat, ben, pca (cg2625-40), which is essential for degradation of 4-hydroxybenzoate, catechol, benzoate, and protocatechuate; and qsuE (cg0502), which is part of an operon comprised of

essential genes of the anabolic shikimate pathway. Production of tyramine in *C. glutamicum* can also be tested for further improvement by deleting or lowering expression of these enzymes which degrade aromatics, since tyramine contains an aromatic ring.

Table 3 - Host Evaluation Results for *Yarrowia lipolytica* Strains Engineered to Produce Tyramine

Strain name	Titer (micro-gram/L)	E1 Uni-prot ID	E1 - activity name	E1 - source organism	E1 CO	E2 Uni-prot ID	E2 - activity name	E2 Modi-fica-tions	E2 - source organism	E2 CO	E3 Uni-prot ID	E3 - activity name	E3 - source organism	E3 CO
YITYR MN_ 01	22.7	P54 769	Tyrosine/ DOPA decarbox-ylase 2	Papaver somniferum	Bs	P32 449	Phospho-2-dehydro-3-deoxy-heptonate aldolase	K229L	S. cerevisiae S288c	Bs				
YITYR MN_ 02	6069.1	P54 769	Tyrosine/ DOPA decarbox-ylase 2	Papaver somniferum	Mod	P32 449	Phospho-2-dehydro-3-deoxy-heptonate aldolase	K229L	S. cerevisiae S288c	Mod				
YITYR MN_ 03	13.9	P54 769	Tyrosine/ DOPA decarbox-ylase 2	Papaver somniferum	Sc	P32 449	Phospho-2-dehydro-3-deoxy-heptonate aldolase	K229L	S. cerevisiae S288c	Sc				
YITYR MN_ 04		P54 769	Tyrosine/ DOPA decarbo-xylase 2	Papaver somniferum	Yl	P32 449	Phospho-2-dehydro-3-deoxy-heptonate aldolase	K229L	S. cerevisiae S288c	Yl				
YITYR MN_ 05	16.8	C9A SN2	Pyridoxal-dependent decarbox-ylase	Entero-coccus faecium Com15	Bs	P32 449	Phospho-2-dehydro-3-deoxy-heptonate aldolase		S. cerevisiae S288c	Bs				

Strain name	Titer (micro-gram/L)	E1 Uni-prot ID	E1 - activity name	E1 - source organism	E1 CO	E2 Uni-prot ID	E2 - activity name	E2 Modi-fica-tions	E2 - source organism	E2 CO	E3 Uni-prot ID	E3 - activity name	E3 - source organism	E3 CO
YITYRMN_06	12804.6	C9ASN2	Pyridoxal-dependent decarbox-ylase	Entero-coccus faecium Com15	Mod	P32449	Phospho-2-dehydro-3-deoxy-heptonate aldolase		S. cerevisiae S288c	Mod				
YITYRMN_07	7.5	C9ASN2	Pyridoxal-dependent decarbox-ylase	Entero-coccus faecium Com15	Sc	P32449	Phospho-2-dehydro-3-deoxy-heptonate aldolase		S. cerevisiae S288c	Sc				
YITYRMN_08	54503.8	C9ASN2	Pyridoxal-dependent decarbox-ylase	Entero-coccus faecium Com15	Yl	P32449	Phospho-2-dehydro-3-deoxy-heptonate aldolase		S. cerevisiae S288c	Yl				
YITYRMN_09	14.6	P54769	Tyrosine/ DOPA decarbox-ylase 2	Papaver somniferum	Bs	P32449	Phospho-2-dehydro-3-deoxy-heptonate aldolase		S. cerevisiae S288c	Bs	P0A6E1	Shikimate kinase 2	Escherichia coli K12	Bs
YITYRMN_10	15	P54769	Tyrosine/ DOPA decarbox-ylase 2	Papaver somniferum	Sc	P32449	Phospho-2-dehydro-3-deoxy-heptonate aldolase		S. cerevisiae S288c	Sc	P0A6E1	Shikimate kinase 2	Escherichia coli K12	Sc
YITYRMN_11	18461.8	P54769	Tyrosine/ DOPA decarbox-ylase 2	Papaver somniferum	Yl	P32449	Phospho-2-dehydro-3-deoxy-heptonate aldolase		S. cerevisiae S288c	Yl	P0A6E1	Shikimate kinase 2	Escherichia coli K12	Yl

CO = Codon Optimization; Bs = Bacillus subtilus; Mod = codon usage for Cg and Sc; Sc = Saccharomyces cerevisiae Yl = Yarrowia lipolytica.

Table 4 - Host Evaluation Results for *Bacillus subtilus* Strains Engineered to Produce Tyramine

Strain name	Titer (micro-gram/L)	E1 Uni-prot ID	E1 - activity name	E1 - source organism	E1 CO	E2 Uni-prot ID	Enzyme 2 - activity name	E2 Modi-fica-tions	E2 - source organism	E2 CO	E3 Uni-prot ID	Enzyme 3 - activity name	E3 - source organism	E3 CO
BsTY RMN_ 01		P54 769	Tyrosine/ DOPA decarbox-ylase 2	Papaver somniferum	Yl	P32 449	Phospho-2-dehydro-3-deoxy-heptonate aldolase	K229L	S. cerevisiae S288c	Yl				
BsTY RMN_ 02		C9A SN2	Pyridoxal-dependent decarbox-ylase	Entero-coccus faecium Com15	Yl	P32 449	Phospho-2-dehydro-3-deoxy-heptonate aldolase		S. cerevisiae S288c	Yl				
BsTY RMN_ 03		P54 769	Tyrosine/ DOPA decarbox-ylase 2	Papaver somniferum	Bs	P32 449	Phospho-2-dehydro-3-deoxy-heptonate aldolase	K229L	S. cerevisiae S288c	Bs				
BsTY RMN_ 04	564.7	P54 769	Tyrosine/ DOPA decarbox-ylase 2	Papaver somniferum	Mod	P32 449	Phospho-2-dehydro-3-deoxy-heptonate aldolase	K229L	S. cerevisiae S288c	Mod				
BsTY RMN_ 05		P54 769	Tyrosine/ DOPA decarbox-ylase 2	Papaver somniferum	Sc	P32 449	Phospho-2-dehydro-3-deoxy-heptonate aldolase	K229L	S. cerevisiae S288c	Sc				
BsTY		C9A	Pyridoxal-	Entero-	Bs	P32	Phospho-2-		S.	Bs				

Strain name	Titer (microgram/L)	E1 Uniprot ID	E1 - activity name	E1 - source organism	E1 CO	E2 Uniprot ID	Enzyme 2 - activity name	E2 Modifications	E2 - source organism	E2 CO	E3 Uniprot ID	Enzyme 3 - activity name	E3 - source organism	E3 CO
RMN_06		SN2	dependent decarboxylase	coccus faecium Com15		449	dehydro-3-deoxy-heptonate aldolase		cerevisiae S288c					
BsTY RMN_07		C9A SN2	Pyridoxal-dependent decarboxylase	Enterococcus faecium Com15	Mod	P32 449	Phospho-2-dehydro-3-deoxy-heptonate aldolase		S. cerevisiae S288c	Mod				
BsTY RMN_08		C9A SN2	Pyridoxal-dependent decarboxylase	Enterococcus faecium Com15	Sc	P32 449	Phospho-2-dehydro-3-deoxy-heptonate aldolase		S. cerevisiae S288c	Sc				
BsTY RMN_09		P54 769	Tyrosine/ DOPA decarboxylase 2	Papaver somniferum	Bs	P32 449	Phospho-2-dehydro-3-deoxy-heptonate aldolase		S. cerevisiae S288c	Bs	P0A 6E1	Shikimate kinase 2	Escherichia coli (strain K12)	Bs
BsTY RMN_10		P54 769	Tyrosine/ DOPA decarboxylase 2	Papaver somniferum	Mod	P32 449	Phospho-2-dehydro-3-deoxy-heptonate aldolase		S. cerevisiae S288c	Mod	P0A 6E1	Shikimate kinase 2	Escherichia coli (strain K12)	Mod
BsTY RMN_11	19873.2	P54 769	Tyrosine/ DOPA decarboxylase 2	Papaver somniferum	Sc	P32 449	Phospho-2-dehydro-3-deoxy-heptonate aldolase		S. cerevisiae S288c	Sc	P0A 6E1	Shikimate kinase 2	Escherichia coli (strain K12)	Sc
BsTY RMN_	124.7	P54 769	Tyrosine/ DOPA	Papaver somniferum	Yl	P32 449	Phospho-2-dehydro-3-		S. cerevisiae	Yl	P0A 6E1	Shikimate kinase 2	Escherichia coli (strain	Yl

Strain name	Titer (micro-gram/L)	E1 Uni-prot ID	E1 - activity name	E1 - source organism	E1 CO	E2 Uni-prot ID	Enzyme 2 - activity name	E2 Modi-fica-tions	E2 - source organism	E2 CO	E3 Uni-prot ID	Enzyme 3 - activity name	E3 - source organism	E3 CO
12			decarbox-ylase 2				deoxy-heptonate aldolase		S288c				K12)	
BsTY RMN_ 13		P54 769	Tyrosine/ DOPA decarbox-ylase 2	Papaver somniferum	Mod	P32 449	Phospho-2-dehydro-3-deoxy-heptonate aldolase		S. cerevisiae S288c	Mod	P20 049	Prephenate dehydro-genase	Saccharom yces cerevisiae S288c	Mod

CO = Codon Optimization; Bs = Bacillus subtilus; Mod = codon usage for Cg and Sc; Sc = Saccharomyces cerevisiae Yl = Yarrowia lipolytica.

Table 5 - Host Evaluation Results for *Saccharomyces cerevisiae* Strains Engineered to Produce Tyramine

Strain name	Titer (micro-gram/L)	E1 Uni-prot ID	E1 - activity name	E1 - source organism	E1 CO	E2 Uni-prot ID	E2 - activity name	E2 Modi-fica-tions	E2 - source organism	E2 CO	E3 Uni-prot ID	E3 - activity name	E3 - source organism	E3 CO
ScTY RMN_ 114	164602	P54 769	Tyrosine/ DOPA decarbox-ylase 2	Papaver somniferum	Bs	P32 449	Phospho-2-dehydro-3-deoxy-heptonate aldolase	K229L	S. cerevisiae S288c	Bs				
ScTY RMN_ 115	184762	P54 769	Tyrosine/ DOPA decarbox-ylase 2	Papaver somniferum	Mod	P32 449	Phospho-2-dehydro-3-deoxy-heptonate aldolase	K229L	S. cerevisiae S288c	Mod				
ScTY RMN_ 116	188	P54 769	Tyrosine/ DOPA decarbox-ylase 2	Papaver somniferum	Sc	P32 449	Phospho-2-dehydro-3-deoxy-heptonate aldolase	K229L	S. cerevisiae S288c	Sc				
ScTY RMN_ 117	171171	P54 769	Tyrosine/ DOPA decarbox-ylase 2	Papaver somniferum	Yl	P32 449	Phospho-2-dehydro-3-deoxy-heptonate aldolase	K229L	S. cerevisiae S288c	Yl				
ScTY RMN_ 118	66972	P54 769	Tyrosine/ DOPA decarbox-ylase 2	Papaver somniferum	Bs	P32 449	Phospho-2-dehydro-3-deoxy-heptonate aldolase		S. cerevisiae S288c	Bs	P0A 6E1	Shikimate kinase 2	Escherichia coli (strain K12)	Bs
ScTY RMN_	89422	P54 769	Tyrosine/ DOPA	Papaver somniferum	Sc	P32 449	Phospho-2-dehydro-3-		S. cerevisiae	Sc	P0A 6E1	Shikimate kinase 2	Escherichia coli (strain	Sc

Strain name	Titer (micro-gram/L)	E1 Uni-prot ID	E1 - activity name	E1 - source organism	E1 CO	E2 Uni-prot ID	E2 - activity name	E2 Modi-fica-tions	E2 - source organism	E2 CO	E3 Uni-prot ID	E3 - activity name	E3 - source organism	E3 CO
119			decarbox-ylase 2				deoxy-heptonate aldolase		S288c				K12)	
ScTY RMN_ 120	81170	P54 769	Tyrosine/ DOPA decarbox-ylase 2	Papaver somniferum	Yl	P32 449	Phospho-2-dehydro-3-deoxy-heptonate aldolase		S. cerevisiae S288c	Yl	P0A 6E1	Shikimate kinase 2	Escherichia coli (strain K12)	Yl

CO = Codon Optimization; Bs = Bacillus subtilus; Mod = codon usage for Cg and Sc; Sc = Saccharomyces cerevisiae Yl = Yarrowia lipolytica.

Table 6 - Host Evaluation Results for *Corynebacteria glutamicum* Strains Engineered to Produce Tyramine

Strain name	Titer (micro-gram/L)	E1 Uni-prot ID	E1 - activity name	E1 - source organism	E1 CO	E2 Uni-prot ID	E2 - activity name	E2 Modi-fica-tions	E2 - source organism	E2 CO	E3 Uni-prot ID	E3 - activity name	E3 - source organism	E3 CO
CgTY RMN_ 69		P54 769	Tyrosine/ DOPA decarbox-ylase 2	Papaver somniferum	Bs	P32 449	Phospho-2-dehydro-3-deoxy-heptonate aldolase	K229L	S. cerevisiae S288c	Bs				
CgTY RMN_ 70		P54 769	Tyrosine/ DOPA decarbox-ylase 2	Papaver somniferum	Mod	P32 449	Phospho-2-dehydro-3-deoxy-heptonate aldolase	K229L	S. cerevisiae S288c	Mod				
CgTY RMN_ 71		P54 769	Tyrosine/ DOPA decarbox-ylase 2	Papaver somniferum	Sc	P32 449	Phospho-2-dehydro-3-deoxy-heptonate aldolase	K229L	S. cerevisiae S288c	Sc				
CgTY RMN_ 72		P54 769	Tyrosine/ DOPA decarbox-ylase 2	Papaver somniferum	Yl	P32 449	Phospho-2-dehydro-3-deoxy-heptonate aldolase	K229L	S. cerevisiae S288c	Yl				
CgTY RMN_ 73		C9A SN2	Pyridoxal-dependent decarbox-ylase	Entero-coccus faecium Com15	Bs	P32 449	Phospho-2-dehydro-3-deoxy-heptonate aldolase		S. cerevisiae S288c	Bs				
CgTY RMN_	466779	C9A SN2	Pyridoxal-dependent	Entero-coccus	Sc	P32 449	Phospho-2-dehydro-3-		S. cerevisiae	Sc				

Strain name	Titer (micro-gram/L)	E1 Uni-prot ID	E1 - activity name	E1 - source organism	E1 CO	E2 Uni-prot ID	E2 - activity name	E2 Modi-fica-tions	E2 - source organism	E2 CO	E3 Uni-prot ID	E3 - activity name	E3 - source organism	E3 CO
74			decarbox-ylase	faceium Com15			deoxy-heptonate aldolase		S288c					
CgTY RMN_ 75		C9A SN2	Pyridoxal-dependent decarbox-ylase	Entero-coccus faecium Com15	Yl	P32 449	Phospho-2-dehydro-3-deoxy-heptonate aldolase		S. cerevisiae S288c	Yl				
CgTY RMN_ 76		P54 769	Tyrosine/ DOPA decarbox-ylase 2	Papaver somniferum	Bs	P32 449	Phospho-2-dehydro-3-deoxy-heptonate aldolase		S. cerevisiae S288c	Bs	P0A 6E1	Shikimate kinase 2	Escherichia coli (strain K12)	Bs
CgTY RMN_ 77	14	P54 769	Tyrosine/ DOPA decarbox-ylase 2	Papaver somniferum	Sc	P32 449	Phospho-2-dehydro-3-deoxy-heptonate aldolase		S. cerevisiae S288c	Sc	P0A 6E1	Shikimate kinase 2	Escherichia coli (strain K12)	Sc
CgTY RMN_ 78	49	P54 769	Tyrosine/ DOPA decarbox-ylase 2	Papaver somniferum	Yl	P32 449	Phospho-2-dehydro-3-deoxy-heptonate aldolase		S. cerevisiae S288c	Yl	P0A 6E1	Shikimate kinase 2	Escherichia coli (strain K12)	Yl

CO = Codon Optimization; Bs = Bacillus subtilus; Mod = codon usage for Cg and Sc; Sc = Saccharomyces cerevisiae Yl = Yarrowia lipolytica.

Table 7 – Improvement-Round Results for *Saccharomyces cerevisiae* Strains Engineered to Produce Tyramine

	Titer (micro-gram/L)	E1 Uni-prot ID	E1 - activity name	E1 Modi-ficat-ions	E1 - source organism	E1 CO	E2 Unii prot ID	E2 - activity name	E2 Modi-fica-tions	E2 - source organism	E2 CO	E3 Uni-prot ID	E3 - activity name	E3 Modi-fica-tions	E3 - source organism	E3 CO
ScTY RMN _100	263842	Q8U 0A9	2-dehydro-3-deoxy-phospho-heptonate aldolase		Pyrococcus furiosus ATCC 43587	Mod	P23 538	Phospho-enolpyruvate synthase		Escherichia coli (strain K12)	Mod					
ScTY RMN _101	233752	A5D B21	Chorismate mutase		Meyerozyma guilliermondii ATCC 6260	Mod	P22 259	Phospho-enolpyruvate carboxy-kinase		Escherichia coli (strain K12)	Mod					
ScTY RMN _102	200881	P070 23	Chorismate mutase and Prephenate dehydro-genase	M53I, A354V	Escherichia coli K12	Mod	P32 449	Phospho-2-dehydro-3-deoxy-heptonate aldolase	K229L	S. cerevisiae S288c	Mod					
ScTY RMN _103	155522	P0A6 E1	Shikimate kinase		Escherichia coli K12	Mod	P32 449	Phospho-2-dehydro-3-deoxy-heptonate aldolase	K229L	S. cerevisiae S288c	Mod					
ScTY RMN _104	193051	P0A6 D7	Shikimate kinase		Escherichia coli K12	Mod										
ScTY RMN _105	131046	P108 80	Shikimate kinase	C162S	Dickeya chrysanthemi	Mod										
ScTY	180373	P085	shikimate	Error	S. cerevisiae	Mod										

	Titer (micro-gram/L)	E1 Uni-prot ID	E1 - activity name	E1 Modi-fica-tions	E1 - source organism	E1 CO	E2 Unii prot ID	E2 - activity name	E2 Modi-fica-tions	E2 - source organism	E2 CO	E3 Uni-prot ID	E3 - activity name	E3 Modi-fica-tions	E3 - source organism	E3 CO
RMN_106		66	dehydro-genase		S288c											
ScTY RMN_107	154577	P0A6E1	Shikimate kinase		Escherichia coli K12	Mod										
ScTY RMN_108	248412	P0A6E1	Shikimate kinase		Escherichia coli K12	Mod										
ScTY RMN_109	226394	P20049	prephenate dehydro-genase		S. cerevisiae S288c	Mod	P09831	Glutamate synthase		Escherichia coli (strain K12)	Mod					
ScTY RMN_110	203759	P32449	Phospho-2-dehydro-3-deoxy-heptonate aldolase	K229L	S. cerevisiae S288c	Mod										
ScTY RMN_111	270601	P38840	Aromatic amino acid amino-transferase		S. cerevisiae S288c	Mod										
ScTY RMN_112	235307	P17735	Tyrosine amino-transferase		Homo sapiens	Mod										
ScTY RMN_54	265899	P20049	Prephenate dehydro-genase		S. cerevisiae S288c	Mod										
ScTY RMN_75	271983	P23538	Phospho-enolpyruvate synthase		Escherichia coli K12	Mod										
ScTY RMN	220715	P23538	Phospho-enolpyruvate		Escherichia coli K12	Mod										

	Titer (micro-gram/L)	E1 Uni-prot ID	E1 - activity name	E1 Modi-fica-tions	E1 - source organism	E1 CO	E2 Uniiprot ID	E2 - activity name	E2 Modi-fica-tions	E2 - source organism	E2 CO	E3 Uni-prot ID	E3 - activity name	E3 Modi-fica-tions	E3 - source organism	E3 CO
_76			synthase													
ScTY RMN _77	296242	P232 54	Transketolase		S. cerevisiae S288c	Mod										
ScTY RMN _78	299143	P150 19	Transaldolase		S. cerevisiae S288c	Mod										
ScTY RMN _79	296289	P232 54	Transketolase		S. cerevisiae S288c	Mod	P15 019	Transaldolase		S. cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)	Mod					
ScTY RMN _80	208329	P232 54	Transketolase		S. cerevisiae S288c	Mod	P08 566	shikimate dehydro-genase		S. cerevisiae S288c	Mod	P0A 6E1	Shikimate kinase		E. coli K12	Mod
ScTY RMN _81	244921	P232 54	Transketolase		S. cerevisiae S288c	Mod	P08 566	shikimate dehydro-genase		S. cerevisiae S288c	Mod	P0A 6E1	Shikimate kinase		E. coli K12	Mod
ScTY RMN _82	274335	P232 54	Transketolase		S. cerevisiae S288c	Mod	P23 538	Phospho-enolpyruvate synthase		Escherichia coli K12	Mod					
ScTY RMN _83	220523	P232 54	Transketolase		S. cerevisiae S288c	Mod										
ScTY RMN _84	252995	P232 54	Transketolase		S. cerevisiae S288c	Mod										

	Titer (micro-gram/L)	E1 Uni-prot ID	E1 - activity name	E1 Modi-fications	E1 - source organism	E1 CO	E2 Uniiprot ID	E2 - activity name	E2 Modi-fications	E2 - source organism	E2 CO	E3 Uni-prot ID	E3 - activity name	E3 Modi-fications	E3 - source organism	E3 CO
ScTY RMN_85	274284	P15019	Transaldolase		S. cerevisiae S288c	Mod	P32449	Phospho-2-dehydro-3-deoxy-heptonate aldolase, tyrosine-inhibited (EC 2.5.1.54) (3-deoxy-D-arabino-heptulosonate 7-phosphate synthase) (DAHP synthase) (Phospho-2-keto-3-deoxy-heptonate aldolase)	K229L	Fungi	Mod						
ScTY RMN_86	199	O52631	Glycer-aldehyde-3-phosphate dehydro-genase		Clostridium acetobutylicum ATCC 824	Mod	P08566	shikimate dehydro-genase		S. cerevisiae S288c	Mod					
ScTY RMN_87	215006	P25856	Glycer-aldehyde-3-phosphate dehydro-genase		Arabidopsis thaliana	Mod	P08566	shikimate dehydro-genase		S. cerevisiae S288c	Mod	P07023	Chorismate mutase and Prephenate dehydro-genase	M53I,A354V	E. coli K12	Mod
ScTY RMN	141184	P50362	Glycer-aldehyde-3-		Chlamy-domonas	Mod	P08566	shikimate dehydro-		S. cerevisiae	Mod	P07023	Chorismate mutase and	M53I,A354V	E. coli K12	Mod

	Titer (micro-gram/L)	E1 Uni-prot ID	E1 - activity name	E1 Modi-fica-tions	E1 - source organism	E1 CO	E2 Unii prot ID	E2 - activity name	E2 Modi-fica-tions	E2 - source organism	E2 CO	E3 Uni-prot ID	E3 - activity name	E3 Modi-fica-tions	E3 - source organism	E3 CO
_88			phosphate dehydro-genase		reinhardtii			genase		S288c			Prephenate dehydro-genase			
ScTY RMN _89	238075	Q5H QV4	Glycer-aldehyde-3-phosphate dehydro-genase		Staphylo-coccus epidermidis ATCC 35984	Mod	P08 566	shikimate dehydro-genase		S. cerevisiae S288c	Mod	P07 023	Chorismate mutase and Prephenate dehydro-genase	M53I,A 354V	E. coli K12	Mod
ScTY RMN _90	277029	P307 24	Glycer-aldehyde-3-phosphate dehydro-genase		Gracilaria gracilis (Red alga)	Mod										
ScTY RMN _91	263562	P098 32	Glutamate synthase		Escherichia coli K12	Mod										
ScTY RMN _92	245798	P322 88	Glutamine synthetase		S cerevisiae S288c	Mod										
ScTY RMN _93	231297	P324 49	Phospho-2-dehydro-3-deoxy-heptonate aldolase	Q166 K	S cerevisiae S288c	Mod										
ScTY RMN _94	264041	P324 49	Phospho-2-dehydro-3-deoxy-heptonate aldolase	K229L	S cerevisiae S288c	Mod										
ScTY RMN	245229	P0A B91	Phospho-2-dehydro-3-	D146 N	Escherichia coli K12	Mod										

	Titer (micro-gram/L)	E1 Uni-prot ID	E1 - activity name	E1 Modi-fica-tions	E1 - source organism	E1 CO	E2 Unii prot ID	E2 - activity name	E2 Modi-fica-tions	E2 - source organism	E2 CO	E3 Uni-prot ID	E3 - activity name	E3 Modi-fica-tions	E3 - source organism	E3 CO
_95			deoxy-heptonate aldolase													
ScTY RMN _96	64088	P0A B91	Phospho-2-dehydro-3-deoxy-heptonate aldolase	P150L	Escherichia coli K12	Mod										
ScTY RMN _97	236618	P008 88	Phospho-2-dehydro-3-deoxy-heptonate aldolase	N8K	Escherichia coli K12	Mod										
ScTY RMN _98	140	Q9Z MU5	Phospho-2-dehydro-3-deoxy-heptonate aldolase		Helicobacter pylori ATCC 700824	Mod										
ScTY RMN _99	244825	Q9Y EJ7	Phospho-2-dehydro-3-deoxy-heptonate aldolase		Aero-pyrum pernix ATCC 700893	Mod										

Table 8 – SEQ ID NO Cross-Reference Table

SEQ ID NO	SEQ ID NO from Provisional	Sequence Type with Modifications	Uniprot ID	Activity name	Source organism	Codon Optimization Abbrev.
1	2	AA seq for enzyme C9ASN2	C9ASN2	Pyridoxal-dependent decarboxylase	Enterococcus faecium Com15	
2	20	AA seq for enzyme P54769	P54769	Tyrosine/DOPA decarboxylase 2 [Includes: DOPA decarboxylase	Papaver somniferum (Opium poppy)	
3	45	AA seq	P28777	chorismate synthase	S cerevisiae	
4	47	AA seq for enzyme P20049	P20049	Prephenate dehydrogenase [NADP	Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)	
5	31	AA seq for enzyme P32449 with substitution Q166K	P32449	Phospho-2-dehydro-3-deoxyheptonate aldolase, tyrosine-inhibited (EC 2.5.1.54) (3-deoxy-D-arabino-heptulosonate 7-phosphate synthase) (DAHP synthase) (Phospho-2-keto-3-deoxyheptonate aldolase)	Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)	
6	37+39 (seqs were duplicates)	AA seq for enzyme P32449 with substitution K229L	P32449	Phospho-2-dehydro-3-deoxyheptonate aldolase, tyrosine-inhibited	Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)	
7	33+35 (seqs were duplicates)	AA seq for enzyme P0AB91 with substitution D146N	P0AB91	Phospho-2-dehydro-3-deoxyheptonate aldolase, Phe-sensitive (EC 2.5.1.54) (3-deoxy-D-arabino-heptulosonate 7-phosphate synthase) (DAHP synthase) (Phospho-2-keto-3-deoxyheptonate aldolase)	Escherichia coli (strain K12)	
8		AA seq for enzyme P15019	P15019	Transaldolase (EC 2.2.1.2)	Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)	
9	43	AA seq for enzyme P32449	P32449	Phospho-2-dehydro-3-deoxyheptonate aldolase, tyrosine-inhibited	Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)	
10		DNA seq4 for enzyme P32449	P32449	Phospho-2-dehydro-3-deoxyheptonate aldolase, tyrosine-inhibited	Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)	Yarrowia lipolytica
11	4	AA	W0VJZ5	TYDC	Zygosaccharomyces bailii	
12	44	DNA	P28777	chorismate synthase	S cerevisiae	
13	46	DNA seq3 for enzyme P20049	P20049	Prephenate dehydrogenase [NADP(+)]	Saccharomyces cerevisiae S288c	modified codon usage for Corynebacterium

SEQ ID NO	SEQ ID NO from Provisional	Sequence Type with Modifications	Uniprot ID	Activity name	Source organism	Codon Optimization Abbrev.
						glutamicum and Saccharomyces cerevisiae
14		DNA seq1 for enzyme P0A6E1	P0A6E1	Shikimate kinase 2	Escherichia coli (strain K12)	Bacillus subtillus
15		AA seq for enzyme P0A6E1	P0A6E1	Shikimate kinase 2	Escherichia coli (strain K12)	
16	6	AA	B8GDM7	TYDC	Methanosphaerula palustris (strain ATCC BAA-1556 / DSM 19958 / E1-9c)	
17	8	AA	S3X9X3	TYDC	Propionibacterium sp. oral taxon 192 str. F0372	
18	10	AA	Q06085	TYDC	Petroselinum crispum	
19	12+22 (seqs were duplicates)	AA	F2PNN9	TYDC	Trichophyton equinum (strain ATCC MYA-4606 CBS 127.97) (Horse ringworm fungus)	
20	14	AA	B5HRY3	TYDC	Streptomyces sviceus ATCC 29083	
21	16	AA	Q88JU5	TYDC	Pseudomonas putida (strain KT2440)	
22	18	AA	I4EZJ8	TYDC	Modestobacter marinus (strain BC501)	
23	24	AA	I3X3G3	TYDC	Sinorhizobium fredii USDA 257	
24	27	AA	Q7XHL3	TYDC	Oryza sativa subsp. Japonica	
25	29	AA	Q60358	TYDC	Methanocaldococcus jannaschii	
26	41	AA	P14843	Phospho-2-dehydro-3-deoxyheptonate aldolase, tyrosine-inhibited (EC 2.5.1.54) (3-deoxy-D-arabino-heptulosonate 7-phosphate synthase) (DAHP synthase) (Phospho-2-keto-3-deoxyheptonate aldolase)	Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)	
27	49	AA	P53090	aromatic amino acid aminotransferase 2_2-aminoadipate transaminase, L-phenylalanine:2-oxoglutarate aminotransferase, kynurenine-oxoglutarate transaminase	Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)	
28	51	AA seq for enzyme P38840	P38840	Aromatic amino acid aminotransferase 2 (EC 2.6.1.57) (Aromatic amino acid aminotransferase II)	Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)	

SEQ ID NO	SEQ ID NO from Provisional	Sequence Type with Modifications	Uniprot ID	Activity name	Source organism	Codon Optimization Abbrev.
				(Aromatic amino acid-requiring protein 9) (Kynurenine aminotransferase I) (KAT I) (EC 2.6.1.7)		
29		AA seq for enzyme P23538	P23538	Phosphoenolpyruvate synthase (PEP synthase) (EC 2.7.9.2) (Pyruvate, water dikinase)	Escherichia coli (strain K12)	
30		AA seq for enzyme P23254	P23254	Transketolase	Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)	
31		AA seq for enzyme P30724	P30724	Glyceraldehyde-3-phosphate dehydrogenase, chloroplastic (EC 1.2.1.13) (NADP-dependent glyceraldehydephosphate dehydrogenase)	Gracilaria gracilis (Red alga)	
32		AA seq for enzyme Q8U0A9	Q8U0A9	2-dehydro-3-deoxyphosphoheptonate aldolase (EC 2.5.1.54)	Pyrococcus furiosus (strain ATCC 43587 / DSM 3638 / JCM 8422 / Vc1)	
33		AA seq for enzyme A5DB21	A5DB21	Chorismate mutase (EC 5.4.99.5)	Meyerozyma guilliermondii (strain ATCC 6260 / CBS 566 / DSM 6381 / JCM 1539 / NBRC 10279 / NRRL Y-324) (Yeast) (Candida guilliermondii)	
34		AA seq for enzyme P0A6D7	P0A6D7	Shikimate kinase 1 (SK 1) (EC 2.7.1.71) (Shikimate kinase I) (SKI)	Escherichia coli (strain K12)	
35		AA seq for enzyme P08566	P08566	shikimate dehydrogenase	Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)	
36		AA seq for enzyme P17735	P17735	Tyrosine aminotransferase (TAT) (EC 2.6.1.5) (L-tyrosine:2-oxoglutarate aminotransferase)	Homo sapiens (Human)	
37		AA seq for enzyme P09831	P09831	Glutamate synthase [NADPH] large chain (EC 1.4.1.13) (Glutamate synthase subunit alpha) (GLTS alpha chain) (NADPH-GOGAT)	Escherichia coli (strain K12)	
38		AA seq for enzyme P09832	P09832	Glutamate synthase [NADPH] small chain (EC 1.4.1.13) (Glutamate synthase subunit beta) (GLTS beta chain) (NADPH-GOGAT)	Escherichia coli (strain K12)	
39		AA seq for enzyme P32288	P32288	Glutamine synthetase (GS) (EC 6.3.1.2) (Glutamate--ammonia ligase)	Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)	
40		DNA seq4 for enzyme C9ASN2	C9ASN2	Pyridoxal-dependent decarboxylase	Enterococcus faecium Com15	Yarrowia lipolytica

SEQ ID NO	SEQ ID NO from Provisional	Sequence Type with Modifications	Uniprot ID	Activity name	Source organism	Codon Optimization Abbrev.
41		DNA seq2 for enzyme P0A6E1	P0A6E1	Shikimate kinase 2	Escherichia coli (strain K12)	Saccharomyces cerevisiae
42		DNA seq3 for enzyme P32449	P32449	Phospho-2-dehydro-3-deoxyheptonate aldolase, tyrosine-inhibited	Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)	Saccharomyces cerevisiae
43		DNA seq3 for enzyme C9ASN2	C9ASN2	Pyridoxal-dependent decarboxylase	Enterococcus faecium Com15	Saccharomyces cerevisiae
44		DNA seq2 for enzyme P32449 with substitution K229L	P32449	Phospho-2-dehydro-3-deoxyheptonate aldolase, tyrosine-inhibited	Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)	modified codon usage for Corynebacterium glutamicum and Saccharomyces cerevisiae
45		DNA seq2 for enzyme C9ASN2	C9ASN2	Pyridoxal-dependent decarboxylase	Enterococcus faecium Com15	modified codon usage for Corynebacterium glutamicum and Saccharomyces cerevisiae
46		AA seq for enzyme P22259	P22259	Phosphoenolpyruvate carboxykinase (ATP) (PCK) (PEP carboxykinase) (PEPCK) (EC 4.1.1.49)	Escherichia coli (strain K12)	
47		AA seq for enzyme P07023 with substitution M53I,A354V	P07023	T-protein [Includes: Chorismate mutase (CM) (EC 5.4.99.5); Prephenate dehydrogenase (PDH) (EC 1.3.1.12)]	Escherichia coli (strain K12)	
48		AA seq for enzyme P10880 with substitution C162S	P10880	Shikimate kinase 2 (SK 2) (EC 2.7.1.71) (Shikimate kinase II) (SKII)	Dickeya chrysanthemi (Pectobacterium chrysanthemi) (Erwinia chrysanthemi)	
49		AA seq for enzyme O52631	O52631	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (EC 1.2.1.12) (NAD-dependent glyceraldehyde-3-phosphate dehydrogenase)	Clostridium acetobutylicum (strain ATCC 824 / DSM 792 / JCM 1419 / LMG 5710 / VKM B-1787)	
50		AA seq for enzyme P25856	P25856	Glyceraldehyde-3-phosphate dehydrogenase GAPA1, chloroplastic (EC 1.2.1.13) (NADP-dependent glyceraldehydephosphate dehydrogenase A subunit 1)	Arabidopsis thaliana (Mouse-ear cress)	
51		AA seq for enzyme P50362	P50362	Glyceraldehyde-3-phosphate dehydrogenase A,	Chlamydomonas reinhardtii	

SEQ ID NO	SEQ ID NO from Provisional	Sequence Type with Modifications	Uniprot ID	Activity name	Source organism	Codon Optimization Abbrev.
				chloroplastic (EC 1.2.1.13) (NADP-dependent glyceraldehydephosphate dehydrogenase subunit A)	(Chlamydomonas smithii)	
52		AA seq for enzyme Q5HQV4	Q5HQV4	Glyceraldehyde-3-phosphate dehydrogenase 1 (GAPDH 1) (EC 1.2.1.12) (NAD-dependent glyceraldehyde-3-phosphate dehydrogenase)	Staphylococcus epidermidis (strain ATCC 35984 / RP62A)	
53		AA seq for enzyme P00888 with substitution N8K	P00888	Phospho-2-dehydro-3-deoxyheptonate aldolase, Tyr-sensitive (EC 2.5.1.54) (3-deoxy-D-arabino-heptulosonate 7-phosphate synthase) (DAHP synthase) (Phospho-2-keto-3-deoxyheptonate aldolase)	Escherichia coli (strain K12)	
54		AA seq for enzyme Q9ZMU5	Q9ZMU5	Phospho-2-dehydro-3-deoxyheptonate aldolase (EC 2.5.1.54)	Helicobacter pylori (strain J99 / ATCC 700824) (Campylobacter pylori J99)	
55		AA seq for enzyme Q9YEJ7	Q9YEJ7	Phospho-2-dehydro-3-deoxyheptonate aldolase (EC 2.5.1.54)	Aeropyrum pernix (strain ATCC 700893 / DSM 11879 / JCM 9820 / NBRC 100138 / K1)	
56		AA seq for enzyme P0AB91 with substitution P150L	P0AB91	Phospho-2-dehydro-3-deoxyheptonate aldolase, Phe-sensitive (EC 2.5.1.54) (3-deoxy-D-arabino-heptulosonate 7-phosphate synthase) (DAHP synthase) (Phospho-2-keto-3-deoxyheptonate aldolase)	Escherichia coli (strain K12)	
57		DNA seq1 for enzyme P54769	P54769	Tyrosine/DOPA decarboxylase 2 [Includes: DOPA decarboxylase	Papaver somniferum (Opium poppy)	Bacillus subtillus
58		DNA seq1 for enzyme P32449 with substitution K229L	P32449	Phospho-2-dehydro-3-deoxyheptonate aldolase, tyrosine-inhibited	Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)	Bacillus subtillus
59		DNA seq2 for enzyme P54769	P54769	Tyrosine/DOPA decarboxylase 2 [Includes: DOPA decarboxylase	Papaver somniferum (Opium poppy)	modified codon usage for Corynebacterium glutamicum and Saccharomyces cerevisiae
60		DNA seq3 for enzyme P54769	P54769	Tyrosine/DOPA decarboxylase 2 [Includes: DOPA	Papaver somniferum (Opium poppy)	Saccharomyces

SEQ ID NO	SEQ ID NO from Provisional	Sequence Type with Modifications	Uniprot ID	Activity name	Source organism	Codon Optimization Abbrev.
				decarboxylase		cerevisiae
61		DNA seq3 for enzyme P32449 with substitution K229L	P32449	Phospho-2-dehydro-3-deoxyheptonate aldolase, tyrosine-inhibited	Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)	Saccharomyces cerevisiae
62		DNA seq4 for enzyme P54769	P54769	Tyrosine/DOPA decarboxylase 2 [Includes: DOPA decarboxylase	Papaver somniferum (Opium poppy)	Yarrowia lipolytica
63		DNA seq4 for enzyme P32449 with substitution K229L	P32449	Phospho-2-dehydro-3-deoxyheptonate aldolase, tyrosine-inhibited	Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)	Yarrowia lipolytica
64		DNA seq1 for enzyme C9ASN2	C9ASN2	Pyridoxal-dependent decarboxylase	Enterococcus faecium Com15	Bacillus subtillus
65		DNA seq1 for enzyme P32449	P32449	Phospho-2-dehydro-3-deoxyheptonate aldolase, tyrosine-inhibited	Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)	Bacillus subtillus
66		DNA seq2 for enzyme P32449	P32449	Phospho-2-dehydro-3-deoxyheptonate aldolase	Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)	modified codon usage for Corynebacterium glutamicum and Saccharomyces cerevisiae
67		DNA seq3 for enzyme P0A6E1	P0A6E1	Shikimate kinase 2	Escherichia coli (strain K12)	Yarrowia lipolytica
68		DNA seq4 for enzyme P0A6E1	P0A6E1	Shikimate kinase 2	Escherichia coli (strain K12)	modified codon usage for Corynebacterium glutamicum and Saccharomyces cerevisiae
69		DNA seq5 for enzyme P54769	P54769	Tyrosine/DOPA decarboxylase 2 [Includes: DOPA decarboxylase	Papaver somniferum (Opium poppy)	modified codon usage for Corynebacterium glutamicum and Saccharomyces cerevisiae
70	36	DNA seq5 for enzyme P32449 with substitution K229L	P32449	Phospho-2-dehydro-3-deoxyheptonate aldolase, tyrosine-inhibited	Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)	modified codon usage for Corynebacterium glutamicum and Saccharomyces

SEQ ID NO	SEQ ID NO from Provisional	Sequence Type with Modifications	Uniprot ID	Activity name	Source organism	Codon Optimization Abbrev.
						cerevisiae
71		DNA seq1 for enzyme P20049	P20049	Prephenate dehydrogenase [NADP	Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)	modified codon usage for Corynebacterium glutamicum and Saccharomyces cerevisiae
72		DNA seq1 for enzyme Q8U0A9	Q8U0A9	2-dehydro-3-deoxyphosphoheptonate aldolase (EC 2.5.1.54)	Pyrococcus furiosus (strain ATCC 43587 / DSM 3638 / JCM 8422 / Vc1)	modified codon usage for Corynebacterium glutamicum and Saccharomyces cerevisiae
73		DNA seq1 for enzyme P23538	P23538	Phosphoenolpyruvate synthase (PEP synthase) (EC 2.7.9.2) (Pyruvate, water dikinase)	Escherichia coli (strain K12)	modified codon usage for Corynebacterium glutamicum and Saccharomyces cerevisiae
74		DNA seq1 for enzyme A5DB21	A5DB21	Chorismate mutase (EC 5.4.99.5)	Meyerozyma guilliermondii (strain ATCC 6260 / CBS 566 / DSM 6381 / JCM 1539 / NBRC 10279 / NRRL Y-324) (Yeast) (Candida guilliermondii)	modified codon usage for Corynebacterium glutamicum and Saccharomyces cerevisiae
75		DNA seq1 for enzyme P22259	P22259	Phosphoenolpyruvate carboxykinase (ATP) (PCK) (PEP carboxykinase) (PEPCK) (EC 4.1.1.49)	Escherichia coli (strain K12)	modified codon usage for Corynebacterium glutamicum and Saccharomyces cerevisiae
76		DNA seq1 for enzyme P07023 with substitution M53I,A354V	P07023	T-protein [Includes: Chorismate mutase (CM) (EC 5.4.99.5); Prephenate dehydrogenase (PDH) (EC 1.3.1.12)]	Escherichia coli (strain K12)	modified codon usage for Corynebacterium glutamicum and Saccharomyces cerevisiae
77		DNA seq6 for enzyme P32449	P32449	Phospho-2-dehydro-3-deoxyheptonate aldolase,	Saccharomyces cerevisiae (strain	modified codon usage

SEQ ID NO	SEQ ID NO from Provisional	Sequence Type with Modifications	Uniprot ID	Activity name	Source organism	Codon Optimization Abbrev.
		with substitution K229L		tyrosine-inhibited (EC 2.5.1.54) (3-deoxy-D-arabino-heptulosonate 7-phosphate synthase) (DAHP synthase) (Phospho-2-keto-3-deoxyheptonate aldolase)	ATCC 204508 / S288c) (Baker's yeast)	for Corynebacterium glutamicum and Saccharomyces cerevisiae
78		DNA seq5 for enzyme P0A6E1	P0A6E1	Shikimate kinase 2 (SK 2) (EC 2.7.1.71) (Shikimate kinase II) (SKII)	Escherichia coli (strain K12)	modified codon usage for Corynebacterium glutamicum and Saccharomyces cerevisiae
79		DNA seq1 for enzyme P0A6D7	P0A6D7	Shikimate kinase 1 (SK 1) (EC 2.7.1.71) (Shikimate kinase I) (SKI)	Escherichia coli (strain K12)	modified codon usage for Corynebacterium glutamicum and Saccharomyces cerevisiae
80		DNA seq1 for enzyme P10880 with substitution C162S	P10880	Shikimate kinase 2 (SK 2) (EC 2.7.1.71) (Shikimate kinase II) (SKII)	Dickeya chrysanthemi (Pectobacterium chrysanthemi) (Erwinia chrysanthemi)	modified codon usage for Corynebacterium glutamicum and Saccharomyces cerevisiae
81		DNA seq1 for enzyme P08566	P08566	shikimate dehydrogenase	Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)	modified Corynebacterium glutamicum codon usage
82		DNA seq2 for enzyme P20049	P20049	prephenate dehydrogenase	Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)	modified codon usage for Corynebacterium glutamicum and Saccharomyces cerevisiae
83		DNA seq1 for enzyme P09831	P09831	Glutamate synthase [NADPH] large chain (EC 1.4.1.13) (Glutamate synthase subunit alpha) (GLTS alpha chain) (NADPH-GOGAT)	Escherichia coli (strain K12)	modified codon usage for Corynebacterium glutamicum and Saccharomyces

SEQ ID NO	SEQ ID NO from Provisional	Sequence Type with Modifications	Uniprot ID	Activity name	Source organism	Codon Optimization Abbrev.
						cerevisiae
84		DNA seq1 for enzyme P38840	P38840	Aromatic amino acid aminotransferase 2 (EC 2.6.1.57) (Aromatic amino acid aminotransferase II) (Aromatic amino acid-requiring protein 9) (Kynurenine aminotransferase I) (KAT I) (EC 2.6.1.7)	Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)	modified codon usage for Corynebacterium glutamicum and Saccharomyces cerevisiae
85		DNA seq1 for enzyme P17735	P17735	Tyrosine aminotransferase (TAT) (EC 2.6.1.5) (L-tyrosine:2-oxoglutarate aminotransferase)	Homo sapiens (Human)	modified codon usage for Corynebacterium glutamicum and Saccharomyces cerevisiae
86		DNA seq1 for enzyme P23254	P23254	Transketolase	Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)	modified codon usage for Corynebacterium glutamicum and Saccharomyces cerevisiae
87		DNA seq1 for enzyme P15019	P15019	Transaldolase (EC 2.2.1.2)	Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)	modified codon usage for Corynebacterium glutamicum and Saccharomyces cerevisiae
88		DNA seq1 for enzyme O52631	O52631	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (EC 1.2.1.12) (NAD-dependent glyceraldehyde-3-phosphate dehydrogenase)	Clostridium acetobutylicum (strain ATCC 824 / DSM 792 / JCM 1419 / LMG 5710 / VKM B-1787)	modified codon usage for Corynebacterium glutamicum and Saccharomyces cerevisiae
89		DNA seq1 for enzyme P25856	P25856	Glyceraldehyde-3-phosphate dehydrogenase GAPA1, chloroplastic (EC 1.2.1.13) (NADP-dependent glyceraldehydephosphate dehydrogenase A subunit 1)	Arabidopsis thaliana (Mouse-ear cress)	modified codon usage for Corynebacterium glutamicum and Saccharomyces cerevisiae
90		DNA seq1 for enzyme P50362	P50362	Glyceraldehyde-3-phosphate dehydrogenase A,	Chlamydomonas reinhardtii	modified codon usage

SEQ ID NO	SEQ ID NO from Provisional	Sequence Type with Modifications	Uniprot ID	Activity name	Source organism	Codon Optimization Abbrev.
				chloroplastic (EC 1.2.1.13) (NADP-dependent glyceraldehydephosphate dehydrogenase subunit A)	(Chlamydomonas smithii)	for Corynebacterium glutamicum and Saccharomyces cerevisiae
91		DNA seq1 for enzyme Q5HQV4	Q5HQV4	Glyceraldehyde-3-phosphate dehydrogenase 1 (GAPDH 1) (EC 1.2.1.12) (NAD-dependent glyceraldehyde-3-phosphate dehydrogenase)	Staphylococcus epidermidis (strain ATCC 35984 / RP62A)	modified codon usage for Corynebacterium glutamicum and Saccharomyces cerevisiae
92		DNA seq1 for enzyme P30724	P30724	Glyceraldehyde-3-phosphate dehydrogenase, chloroplastic (EC 1.2.1.13) (NADP-dependent glyceraldehydephosphate dehydrogenase)	Gracilaria gracilis (Red alga)	modified codon usage for Corynebacterium glutamicum and Saccharomyces cerevisiae
93		DNA seq1 for enzyme P09832	P09832	Glutamate synthase [NADPH] small chain (EC 1.4.1.13) (Glutamate synthase subunit beta) (GLTS beta chain) (NADPH-GOGAT)	Escherichia coli (strain K12)	modified codon usage for Corynebacterium glutamicum and Saccharomyces cerevisiae
94		DNA seq1 for enzyme P32288	P32288	Glutamine synthetase (GS) (EC 6.3.1.2) (Glutamate--ammonia ligase)	Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)	modified codon usage for Corynebacterium glutamicum and Saccharomyces cerevisiae
95		DNA seq1 for enzyme P32449 with substitution Q166K	P32449	Phospho-2-dehydro-3-deoxyheptonate aldolase, tyrosine-inhibited (EC 2.5.1.54) (3-deoxy-D-arabino-heptulosonate 7-phosphate synthase) (DAHP synthase) (Phospho-2-keto-3-deoxyheptonate aldolase)	Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)	modified codon usage for Corynebacterium glutamicum and Saccharomyces cerevisiae
96		DNA seq1 for enzyme P0AB91 with substitution D146N	P0AB91	Phospho-2-dehydro-3-deoxyheptonate aldolase, Phe-sensitive (EC 2.5.1.54) (3-deoxy-D-arabino-heptulosonate 7-phosphate synthase) (DAHP	Escherichia coli (strain K12)	modified codon usage for Corynebacterium glutamicum and

SEQ ID NO	SEQ ID NO from Provisional	Sequence Type with Modifications	Uniprot ID	Activity name	Source organism	Codon Optimization Abbrev.
				synthase) (Phospho-2-keto-3-deoxyheptonate aldolase)		Saccharomyces cerevisiae
97		DNA seq1 for enzyme P0AB91 with substitution P150L	P0AB91	Phospho-2-dehydro-3-deoxyheptonate aldolase, Phe-sensitive (EC 2.5.1.54) (3-deoxy-D-arabino-heptulosonate 7-phosphate synthase) (DAHP synthase) (Phospho-2-keto-3-deoxyheptonate aldolase)	Escherichia coli (strain K12)	modified codon usage for Corynebacterium glutamicum and Saccharomyces cerevisiae
98		DNA seq1 for enzyme P00888 with substitution N8K	P00888	Phospho-2-dehydro-3-deoxyheptonate aldolase, Tyr-sensitive (EC 2.5.1.54) (3-deoxy-D-arabino-heptulosonate 7-phosphate synthase) (DAHP synthase) (Phospho-2-keto-3-deoxyheptonate aldolase)	Escherichia coli (strain K12)	modified codon usage for Corynebacterium glutamicum and Saccharomyces cerevisiae
99		DNA seq1 for enzyme Q9ZMU5	Q9ZMU5	Phospho-2-dehydro-3-deoxyheptonate aldolase (EC 2.5.1.54)	Helicobacter pylori (strain J99 / ATCC 700824) (Campylobacter pylori J99)	modified codon usage for Corynebacterium glutamicum and Saccharomyces cerevisiae
100		DNA seq1 for enzyme Q9YEJ7	Q9YEJ7	Phospho-2-dehydro-3-deoxyheptonate aldolase (EC 2.5.1.54)	Aeropyrum pernix (strain ATCC 700893 / DSM 11879 / JCM 9820 / NBRC 100138 / K1)	modified codon usage for Corynebacterium glutamicum and Saccharomyces cerevisiae
101	1	DNA	C5IXK9	TYDC	Enterococcus faecium (Streptococcus faecium)	codon optimized for Cg
102	3	DNA	W0VJZ5	TYDC	Zygosaccharomyces bailii ISA1307	codon optimized for Cg
103	5	DNA	B8GDM7	TYDC	Methanosphaerula palustris (strain ATCC BAA-1556 / DSM 19958 / E1-9c)	codon optimized for Cg
104	7	DNA	S3X9X3	TYDC	Propionibacterium sp. oral taxon 192 str. F0372	codon optimized for Cg
105	9	DNA	Q06085	TYDC	Petroselinum crispum	codon optimized for Cg
106	11+21	DNA	F2PNN9	TYDC	Trichophyton equinum (strain ATCC	codon optimized for Cg

SEQ ID NO	SEQ ID NO from Provisional	Sequence Type with Modifications	Uniprot ID	Activity name	Source organism	Codon Optimization Abbrev.
	(seqs were duplicates)				MYA-4606 / CBS 127.97) (Horse ringworm fungus)	
107	13	DNA	B5HRY3	TYDC	Streptomyces sviceus ATCC 29083	codon optimized for Cg
108	15	DNA	Q88JU5	TYDC	Pseudomonas putida (strain KT2440)	codon optimized for Cg
109	17	DNA	I4EZJ8	TYDC	Modestobacter marinus (strain BC501)	codon optimized for Cg
110	19	DNA	P54769	TYDC	Papaver somniferum	codon optimized for Cg
111	23	DNA	I3X3G3	TYDC	Sinorhizobium fredii USDA 257	codon optimized for Cg
112	25	DNA	P54769	TYDC	Papaver somniferum	codon optimized for Sc
113	26	DNA	Q7XHL3	TYDC	Oryza sativa subsp. Japonica	codon optimized for Cg
114	28	DNA	Q60358	TYDC	Methanocaldococcus jannaschii	codon optimized for Sc
115	30	DNA with substitution Q166K	P32449	Phospho-2-dehydro-3-deoxyheptonate aldolase, tyrosine-inhibited (EC 2.5.1.54) (3-deoxy-D-arabino-heptulosonate 7-phosphate synthase) (DAHP synthase) (Phospho-2-keto-3-deoxyheptonate aldolase)	Saccharomyces cerevisiae	codon optimized for Cg
116	32	DNA with substitution D146N	P0AB91	Phospho-2-dehydro-3-deoxyheptonate aldolase, tyrosine-inhibited (EC 2.5.1.54) (3-deoxy-D-arabino-heptulosonate 7-phosphate synthase) (DAHP synthase) (Phospho-2-keto-3-deoxyheptonate aldolase)	Escherichia coli	codon optimized for Sc
117	34	DNA with substitution D146N	P0AB91	Phospho-2-dehydro-3-deoxyheptonate aldolase, tyrosine-inhibited (EC 2.5.1.54) (3-deoxy-D-arabino-heptulosonate 7-phosphate synthase) (DAHP synthase) (Phospho-2-keto-3-deoxyheptonate aldolase)	Escherichia coli	codon optimized for Cg
118	38	DNA	N1P8J9	Phospho-2-dehydro-3-deoxyheptonate aldolase, tyrosine-inhibited (EC 2.5.1.54) (3-deoxy-D-arabino-heptulosonate 7-phosphate synthase) (DAHP synthase) (Phospho-2-keto-3-deoxyheptonate aldolase)	Saccharomyces cerevisiae CEN.PK2	
119	40	DNA	P14843	phospho-2-dehydro-3-deoxyheptonate aldolase 02	Saccharomyces cerevisiae (strain	

SEQ ID NO	SEQ ID NO from Provisional	Sequence Type with Modifications	Uniprot ID	Activity name	Source organism	Codon Optimization Abbrev.
					ATCC 204508 / S288c) (Baker's yeast)	
120	42	DNA	P32449	phospho-2-dehydro-3-deoxyheptonate aldolase 03	Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)	
121	48	DNA	P53090	aromatic/aminoadipate aminotransferase 1_2-aminoadipate transaminase, L-phenylalanine:2-oxoglutarate aminotransferase	Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)	
122	50	DNA	P38840	aromatic amino acid aminotransferase 2_2-aminoadipate transaminase, L-phenylalanine:2-oxoglutarate aminotransferase, kynurenine-oxoglutarate transaminase	Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)	

REFERENCES

[0193] Noda, S. et al., PLoS One, DOI:10.1371/journal.pone.0125488 May 21, 2015.

[0194] Koma, D. et al., Appl Microbiol Biotechnol (2012) 93:815–829, DOI 10.1007/s00253-011-3735-z, November, 2011.

[0195] WO 2008/064835, filed November 27, 2007, priority date November 27, 2006, assigned to DSM IP ASSETS B.V.

[0196] Zhang, C. et al., FEMS Microbiol Lett 353 (2014) 11–18, 0.1111/1574-6968.12397, 2014.

[0197] Kallscheuer, N., et al., Construction of a Corynebacterium glutamicum platform strain for the production of stilbenes and (2S)-flavanones. Metab Eng, 2016. **38**: p. 47-55.

CLAIMS

What is claimed is:

1. An engineered microbial cell, wherein the engineered microbial cell expresses:

(a) a heterologous tyrosine decarboxylase (TYDC); and

(b) the engineered microbial cell comprises increased activity of one or more upstream enzyme(s) in the tyramine biosynthesis pathway, said increased activity being increased relative to a control cell.

2. The engineered microbial cell of claim 1, wherein the one or more upstream enzyme(s) comprises 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase.

3. An engineered microbial cell, wherein the engineered microbial cell expresses:

(a) a heterologous tyrosine decarboxylase (TYDC); and

(b) the engineered microbial cell comprises increased activity of one or more enzyme(s) selected from the group consisting of a dehydroquinate synthase, a dehydroquinate dehydratase, a shikimate dehydrogenase, a shikimate kinase, EPSP synthase, aromatic pentafunctional enzyme, a chorismate synthase, a chorismate mutase, a prephenate dehydratase, a phenyalananine aminotransferase, a prephenate dehydrogenase, a prephenate aminotransferase, an arogenate dehydrogenase, a phenylalanine hydroxylase, and a tyrosine aminotransferase, said increased activity being increased relative to a control cell;

wherein the engineered microbial cell produces tyramine.

4. The engineered microbial cell of claim 3, wherein the engineered microbial cell additionally expresses:

(c) a feedback-disregulated 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase or a feedback-disregulated chorismate mutase.

5. An engineered microbial cell, wherein the engineered microbial cell comprises:

(a) means for expressing a heterologous tyrosine decarboxylase (TYDC); and

(b) means for increasing the activity of one or more upstream enzyme(s) in the tyramine biosynthesis pathway.

6. The engineered microbial cell of claim 5, wherein the one or more upstream enzyme(s) comprises 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase.

7. An engineered microbial cell, wherein the engineered microbial cell comprises:

(a) means for expressing a heterologous tyrosine decarboxylase (TYDC); and

(b) means for increasing the activity of one or more enzyme(s) selected from the group consisting of a dehydroquinate synthase, a dehydroquinate dehydratase, a shikimate dehydrogenase, a shikimate kinase, EPSP synthase, aromatic pentafunctional enzyme, a chorismate synthase, a chorismate mutase, a prephenate dehydratase, a prephenate aminotransferase, an arogenate dehydrogenase, a phenylalanine hydroxylase, a phenyalananine aminotransferase, a prephenate dehydrogenase, and a tyrosine aminotransferase, said increased activity being increased relative to a control cell;

wherein the engineered microbial cell produces tyramine.

8. The engineered microbial cell of claim 7, wherein the engineered microbial cell additionally expresses:

(c) means for expressing a feedback-disregulated DAHP synthase or a feedback-disregulated chorismate mutase.

9. The engineered microbial cell of any one of claims 1-8, wherein the engineered microbial cell produces tyramine by fermentation of a substrate, wherein at least 50% of the substrate is not derived from protein or amino acid sources.

10. The engineered microbial cell of any one of claims 4, 8, or 9, wherein the engineered microbial cell comprises:

(a) a heterologous TYDC; and

(b) a feedback-disregulated DAHP synthase.

11. The engineered microbial cell of any one of claims 3-10, wherein the engineered microbial cell comprises a fungal cell.

12. The engineered microbial cell of any one of claims 3-11, wherein the engineered microbial cell comprises a yeast cell.

13. The engineered microbial cell of claim 8, wherein the yeast cell comprises a cell of the genus *Saccharomyces*.

14. The engineered microbial cell of claim 13, wherein the yeast cell is a cell of the species *cerevisiae*.

15. The engineered microbial cell of any one of claims 4, 8, or 9-14, wherein the DAHP synthase is a variant of a *S. cerevisiae* DAHP synthase.

16. The engineered microbial cell of any one of claims 3-15, wherein the heterologous TYDC comprises a TYDC having at least 70% amino acid sequence identity with a TYDC from *Papaver somniferum*.

17. The engineered microbial cell of any one of claims 4, 8, or 9-16, wherein the:

(a) heterologous TYDC comprises a *P. somniferum* Tyrosine/DOPA decarboxylase 2; and the

(b) feedback-disregulated DAHP synthase is a *S. cerevisiae* DAHP synthase encoded by the *Aro4* gene that additionally comprises a K229L mutation.

18. The engineered microbial cell of any one of claims 3-17, wherein the engineered microbial cell comprises increased activity of a prephenate dehydrogenase relative to the control cell.

19. The engineered microbial cell of claim 18, wherein the engineered microbial cell expresses an extra copy of a wild-type *S. cerevisiae* transaldolase gene.

20. The engineered microbial cell of claim 2 or claim 6, wherein the engineered microbial cell comprises a yeast cell.

21. The engineered microbial cell of claim 20, wherein the yeast cell comprises a cell of the genus *Yarrowia*.

22. The engineered microbial cell of claim 21, wherein the yeast cell is a cell of the species *lipolytica*.

23. The engineered microbial cell of claim 21 or claim 22, wherein the heterologous TYDC comprises a TYDC having at least 70% amino acid sequence identity with a TYDC from *Enterococcus faecium*.

24. The engineered microbial cell of any one of claims 21-23, wherein the DAHP synthase comprises a DAHP synthase having at least 70% amino acid sequence identity with a DAHP synthase from *S. cerevisiae*.

25. The engineered microbial cell of claim 24, wherein the:

(a) heterologous TYDC comprises a pyridoxal-dependent decarboxylase (TYDC) from *E. faecium* Com15; and

(b) DAHP synthase comprises a phospho-2-dehydro-3-deoxyheptonate aldolase (DAHP synthase) from *S. cerevisiae* S288c.

26. An engineered microbial cell which is a yeast cell comprising a heterologous tyrosine decarboxylase (TYDC) having at least 70% amino acid sequence identity to a TYDC from *Papaver somniferum*, wherein the engineered yeast cell produces tyramine.

27. The engineered microbial cell of claim 26, wherein the engineered yeast cell is a cell of the genus *Saccharomyces*.

28. The engineered microbial cell of claim 27, wherein the engineered yeast cell is a cell of the species *cerevisiae*.

29. The engineered microbial cell of any one of claims 3-10, wherein the engineered microbial cell is a bacterial cell.

30. The engineered microbial cell of claim 29, wherein the bacterial cell is a cell of the genus *Corynebacteria*.

31. The engineered microbial cell of claim 30, wherein the bacterial cell is a cell of the species *glutamicum*.

32. The engineered microbial cell of any one of claims 29-31, wherein the bacterial cell comprises a feedback-disregulated DAHP synthase that is a variant of an *S. cerevisiae* DAHP synthase.

33. The engineered microbial cell of any one of claims 29-32, wherein the heterologous TYDC comprises a TYDC having at least 70% amino acid sequence identity with a TYDC from *Enterococcus faecium* or having at least 70% amino acid sequence identity with a TYDC from *Zygosaccharomyces bailii*.

34. The engineered microbial cell of claim 32 or claim 33, wherein the:

(a) heterologous TYDC comprises an *E. faecium* TYDC; and the

(b) feedback-disregulated DAHP synthase is a *S. cerevisiae* DAHP synthase encoded by the *Aro4* gene that additionally comprises a K229L mutation.

35. The engineered microbial cell of claim 32 or claim 33, wherein the:

(a) heterologous TYDC comprises an *Z. bailii* TYDC; and the

(b) feedback-disregulated DAHP synthase is a *S. cerevisiae* DAHP synthase encoded by the *Aro4* gene that additionally comprises a K229L mutation.

36. The engineered microbial cell of claim 33, additionally comprising increased activity of chorismate synthase or prephrenate dehydrogenase, relative to a control cell.

37. The engineered microbial cell of claim 36, wherein the engineered microbial cell comprises increased activity of chorismate synthase and expresses a heterologous chorismate synthase.

38. The engineered microbial cell of claim 36, wherein the engineered microbial cell comprises increased activity of prephenate dehydrogenase and expresses an additional copy of a prephenate dehydrogenase gene.

39. The engineered microbial cell of claim 2 or claim 6, wherein the engineered microbial cell comprises a bacterial cell.

40. The engineered microbial cell of claim 39, wherein the bacterial cell comprises a cell of the genus *Corynebacterium* or *Bacillus*.

41. The engineered microbial cell of claim 40, wherein the bacterial cell is a cell of the species *glutamicum* or *subtilis*, respectively.

42. The engineered microbial cell of claim 40 or claim 41, wherein the heterologous TYDC comprises a TYDC having at least 70% amino acid sequence identity with a TYDC from *Enterococcus faecium*.

43. The engineered microbial cell of any one of claims 40-42, wherein the DAHP synthase comprises a DAHP synthase having at least 70% amino acid sequence identity with a DAHP synthase from *S. cerevisiae*.

44. The engineered microbial cell of any one of claims 40-43, wherein the engineered microbial cell comprises increased activity of a shikimate kinase relative to a control cell.

45. The engineered microbial cell of claim 44, wherein the shikimate kinase comprises a shikimate kinase having at least 70% amino acid sequence identity with a shikimate kinase from *Escherichia coli*.

46. The engineered microbial cell of claim 45, wherein the:

(a) heterologous TYDC comprises a pyridoxal-dependent decarboxylase (TYDC) from *E. faecium* Com15;

(b) DAHP synthase comprises a phospho-2-dehydro-3-deoxyheptonate aldolase (DAHP synthase) from *S. cerevisiae* S288c; and

(c) shikimate kinase comprises a shikimate kinase from *E. coli* K12.

47. The engineered microbial cell of any one of claims 13-46, wherein, when cultured, the engineered microbial cell produces tyramine at a level greater than 100 mg/L of culture medium.

48. The engineered microbial cell of claim 47, wherein the engineered microbial cell produces tyramine at a level of at least 2.5 g/L of culture medium.

49. An engineered microbial cell which is a bacterial cell comprising a heterologous tyrosine decarboxylase (TYDC) having at least 70% amino acid sequence identity with a TYDC from *Enterococcus faecium*, wherein the engineered bacterial cell produces tyramine.

50. The engineered microbial cell of claim 49, wherein the bacterial cell is of the genus *Corynebacteria.*

51. The engineered microbial cell of claim 50, wherein the bacterial cell is of the species *glutamicum*.

52. An engineered microbial cell which is a bacterial cell comprising a heterologous tyrosine decarboxylase (TYDC) having at least 70% amino acid sequence identity with a TYDC from *Zygosaccharomyces bailii*, wherein the engineered bacterial cell produces tyramine.

53. The engineered microbial cell of claim 52, wherein the bacterial cell is of the genus *Corynebacteria.*

54. The engineered microbial cell of claim 53, wherein the bacterial cell is of the species *glutamicum*.

55. A culture of engineered microbial cells according to any one of claims 13-54.

56. The culture of claim 55, wherein the tyramine is produced from fermentation of a substrate wherein at least 50% of the substrate is not derived from protein or amino acid sources.

57. The culture of claim 56, wherein the substrate comprises a carbon source and a nitrogen source selected from the group consisting of urea, an ammonium salt, ammonia, and any combination thereof.

58. The culture of any one of claims 55-57, wherein the engineered microbial cells are present in a concentration such that the culture has an optical density at 600 nm of 10-500.

59. The culture of any one of claims 55-58, wherein the culture comprises tyramine.

60. The culture of any one of claims 55-59, wherein the culture comprises tyramine at a level greater than 100 mg/L of culture medium.

61. The culture of any one of claims 55-60, wherein the culture comprises tyramine at a level of at least 2.5 g/L of culture medium.

62. A method of culturing engineered microbial cells according to any one of claims 1-54, the method comprising culturing the cells in the presence of a fermentation substrate comprising a non-protein carbon and a non-protein nitrogen source, wherein the engineered microbial cells produce tyramine.

63. The method of claim 62, wherein the method comprises fed-batch culture, with an initial glucose level in the range of 1-100 g/L, followed controlled sugar feeding.

64. The method of claim 62 or claim 63, wherein the fermentation substrate comprises glucose and a nitrogen source selected from the group consisting of urea, an ammonium salt, ammonia, and any combination thereof.

65. The method of any one of claims 62-64, wherein the culture is pH-controlled during culturing.

66. The method of any one of claims 62-65, wherein the culture is aerated during culturing.

67. The method of any one of claims 62-66, wherein the engineered microbial cells produce tyramine at a level greater than 100 mg/L of culture medium.

68. The method of any one of claims 62-67, wherein the engineered microbial cells produce tyramine at a level of at least 2.5 g/L of culture medium.

69. The method of any one of claims 62-68, wherein the method additionally comprises recovering tyramine from the culture.

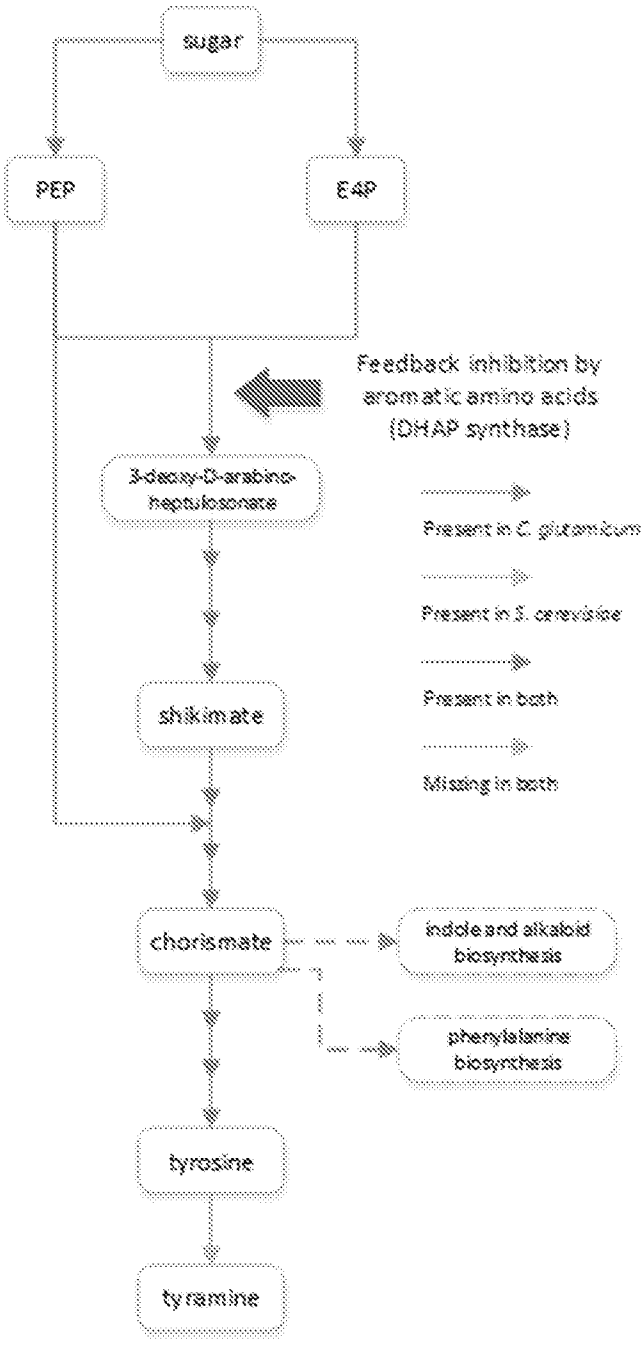
sugar
PEP
E4P
Feedback inhibition by aromatic amino acids (DHAP synthase)
3-deoxy-D-arabino-heptulosonate
shikimate
chorismate
indole and alkaloid biosynthesis
phenylalanine biosynthesis
tyrosine
tyramine
Present in C. glutamicum
Present in S. cerevisiae
Present in both
Missing in both

Fig. 1

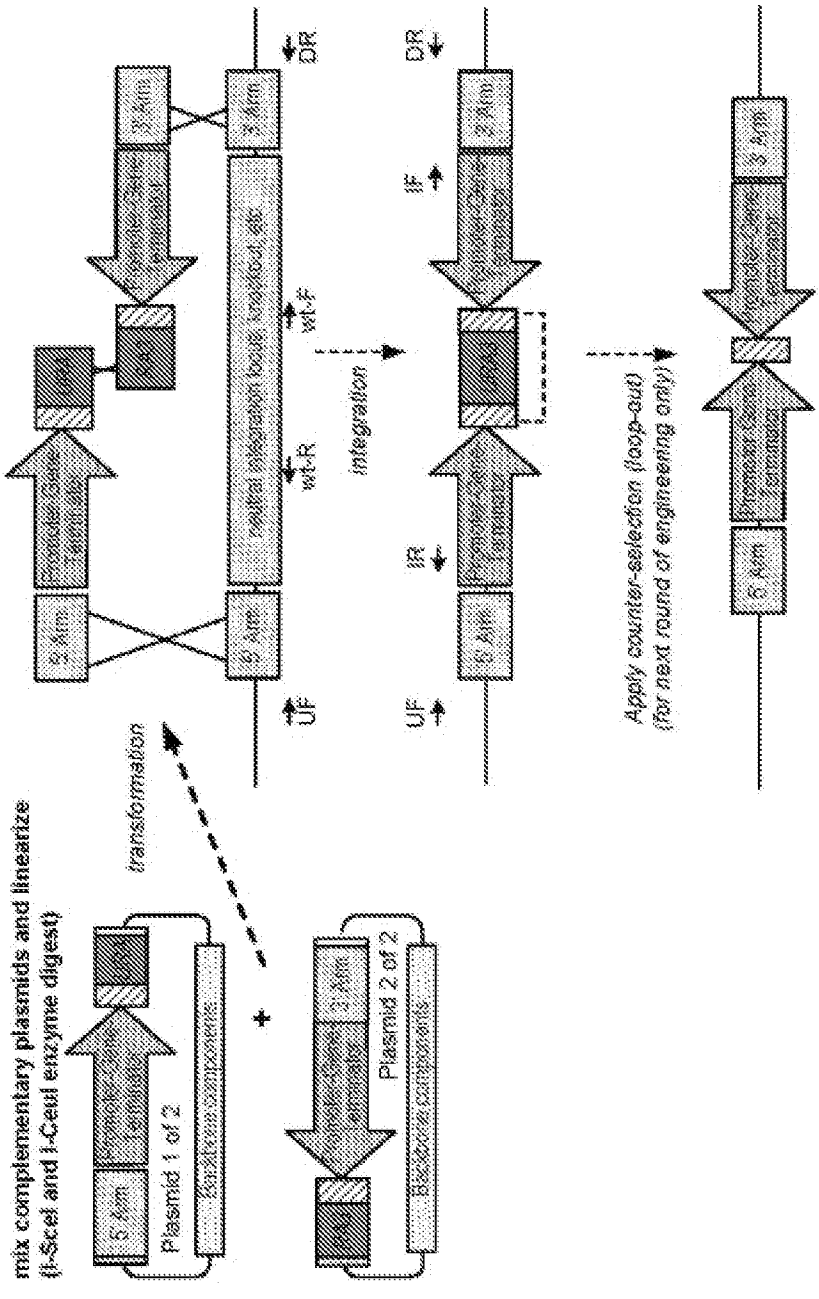
mix complementary plasmids and linearize
(I-SceI and I-CeuI enzyme digest)
transformation
Plasmid 1 of 2
Plasmid 2 of 2
integration
Apply counter-selection (loop-out)
(for next round of engineering only)
UF
DR
IF
IR
wt-F
wt-R

FIG. 2

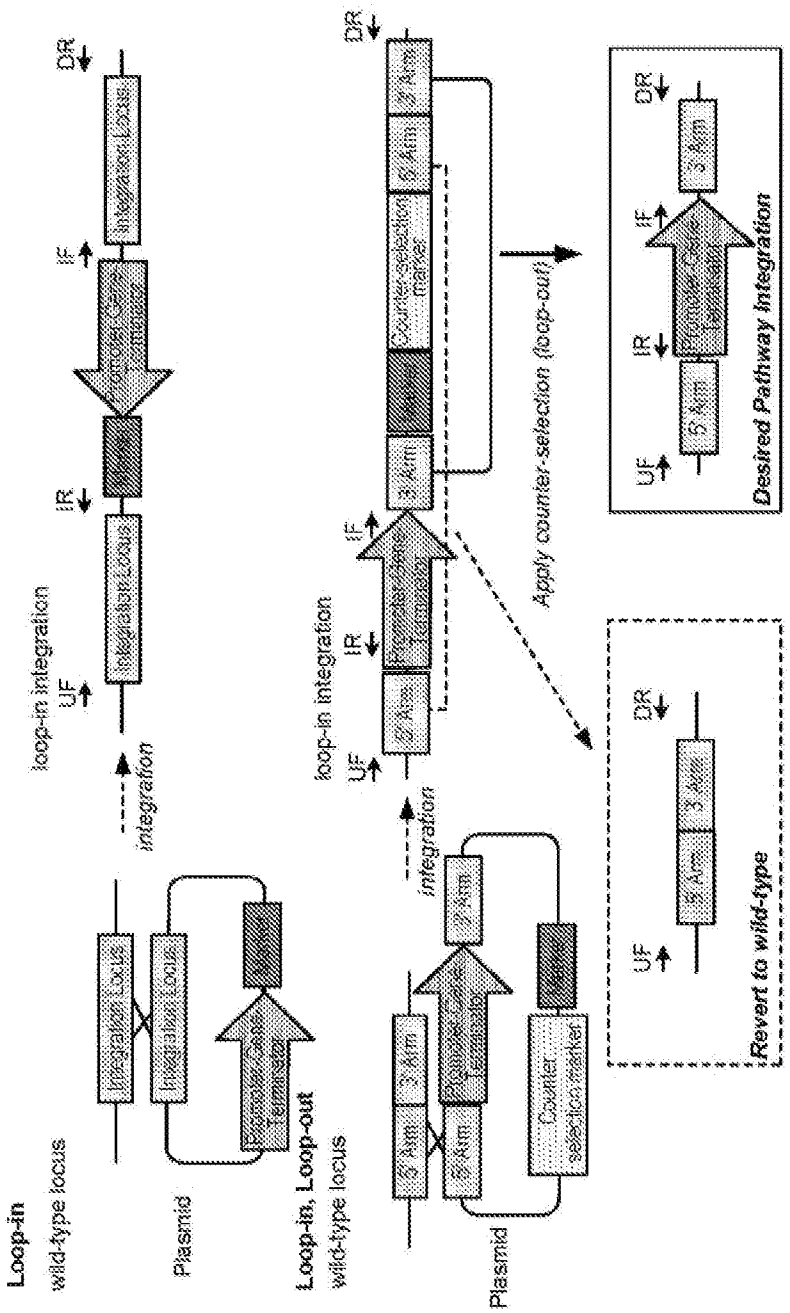
Loop-in
wild-type locus
Plasmid
Loop-in, Loop-out
wild-type locus
Plasmid
loop-in integration
integration
Apply counter-selection (loop-out)
Revert to wild-type
Desired Pathway Integration
DR
IF
IR
UF
5' Arm
3' Arm
Integration Locus
Counter-selection marker
Counter selection marker

FIG. 3

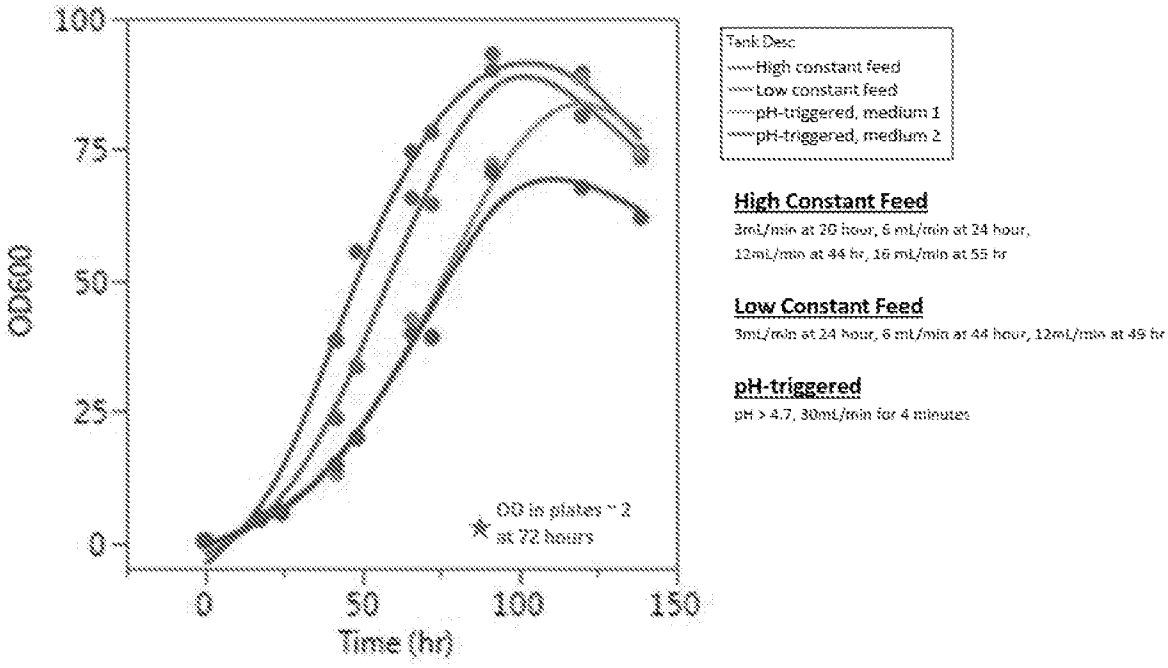
100
75
50
25
0
OD600
0
50
100
150
Time (hr)
OD in plates * 2
at 72 hours
Tank Desc
High constant feed
Low constant feed
pH-triggered, medium 1
pH-triggered, medium 2
High Constant Feed
3mL/min at 20 hour, 6 mL/min at 24 hour,
12mL/min at 44 hr, 18 mL/min at 55 hr
Low Constant Feed
3mL/min at 24 hour, 6 mL/min at 44 hour, 12mL/min at 49 hr
pH-triggered
pH > 4.7, 30mL/min for 4 minutes

Fig. 4A

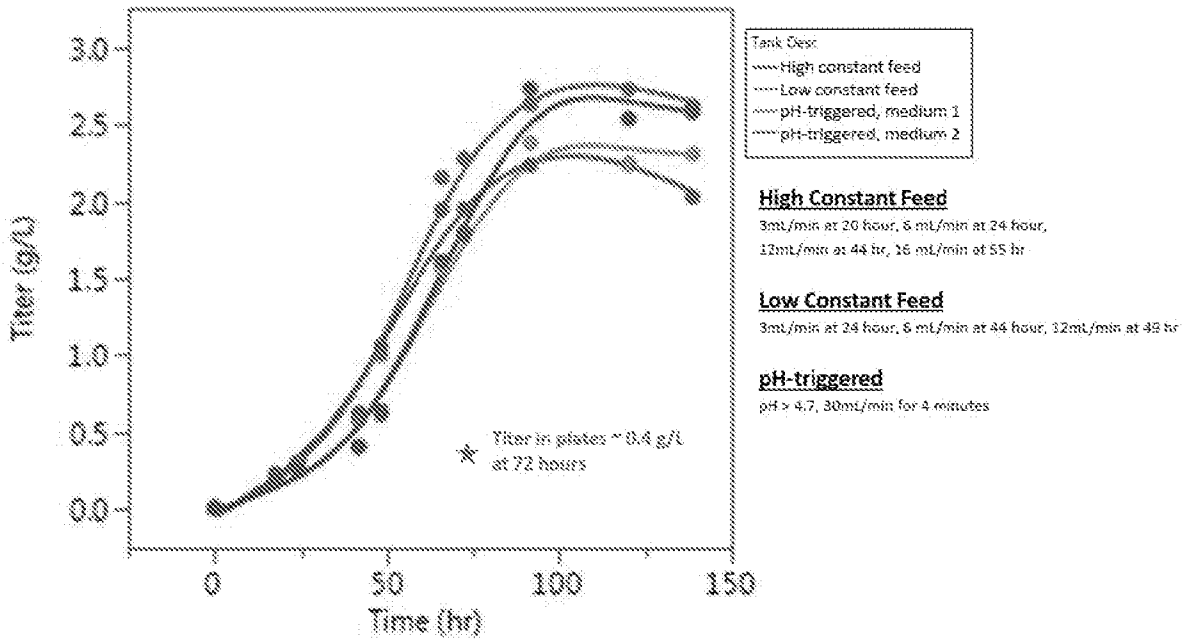
Titer (g/L)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
50
100
150
Time (hr)
Titer in plates ~ 0.4 g/L at 72 hours
Tank Desc
High constant feed
Low constant feed
pH-triggered, medium 1
pH-triggered, medium 2
High Constant Feed
3mL/min at 20 hour, 6 mL/min at 24 hour, 12mL/min at 44 hr, 16 mL/min at 55 hr
Low Constant Feed
3mL/min at 24 hour, 6 mL/min at 44 hour, 12mL/min at 48 hr
pH-triggered
pH > 4.7, 30mL/min for 4 minutes

Fig. 4B

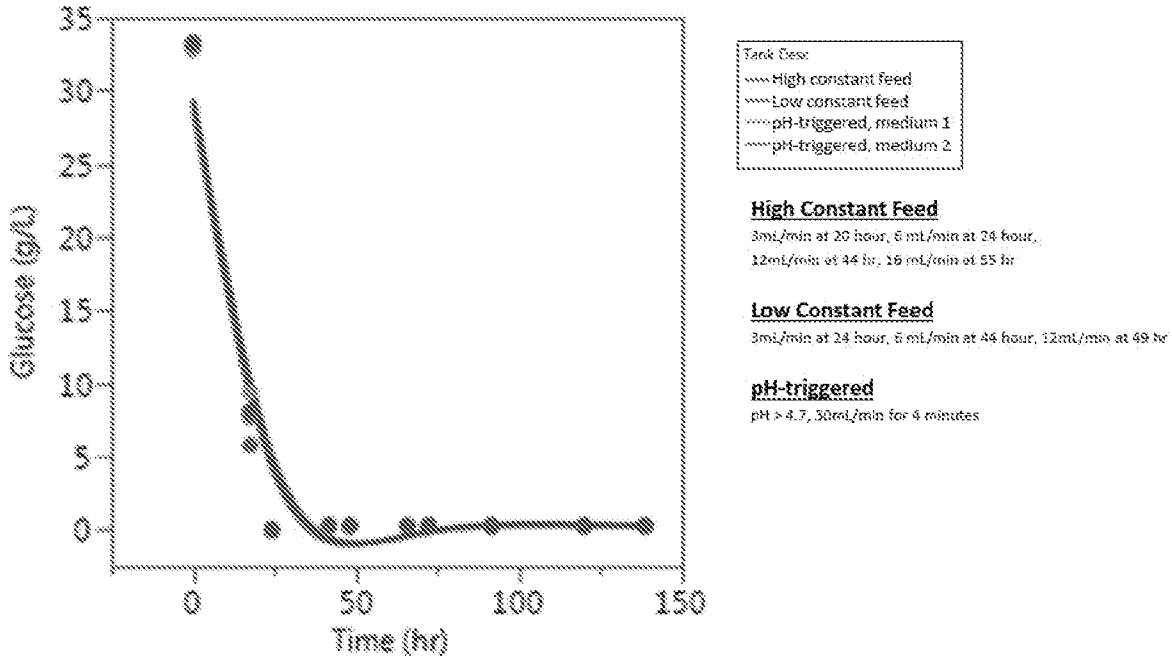
Glucose (g/L)
35
30
25
20
15
10
5
0
0
50
100
150
Time (hr)
Tank Desc
High constant feed
Low constant feed
pH-triggered, medium 1
pH-triggered, medium 2
High Constant Feed
3mL/min at 20 hour, 6 mL/min at 24 hour,
12mL/min at 44 hr, 16 mL/min at 55 hr
Low Constant Feed
3mL/min at 24 hour, 6 mL/min at 44 hour, 12mL/min at 49 hr
pH-triggered
pH > 4.7, 30mL/min for 4 minutes

Fig. 4C

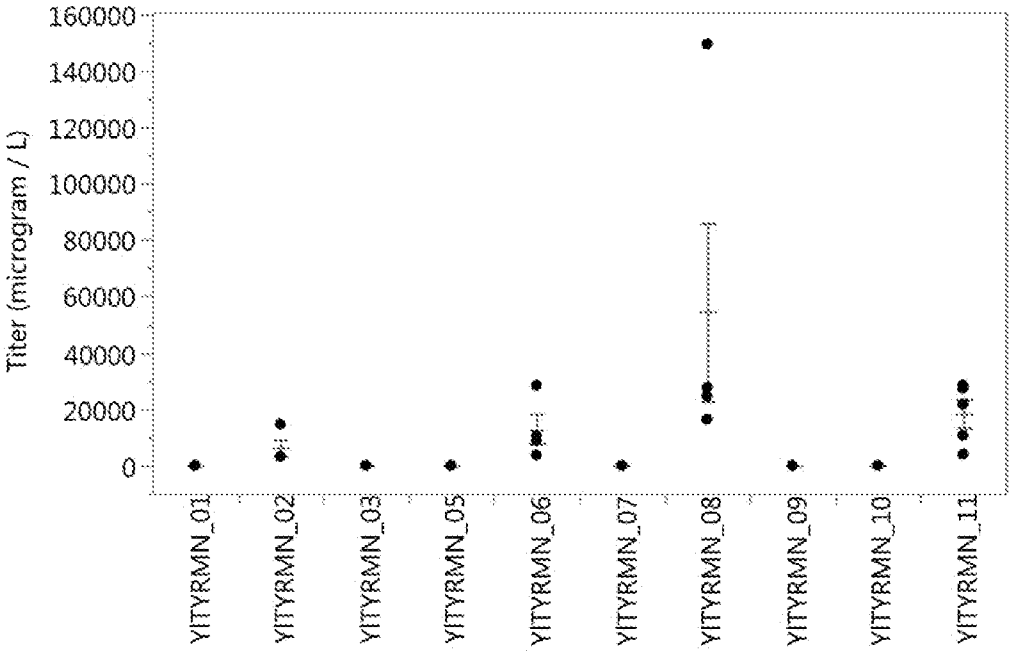
Titer (microgram / L)
160000
140000
120000
100000
80000
60000
40000
20000
0
YITYRMN_01
YITYRMN_02
YITYRMN_03
YITYRMN_05
YITYRMN_06
YITYRMN_07
YITYRMN_08
YITYRMN_09
YITYRMN_10
YITYRMN_11

Fig. 5

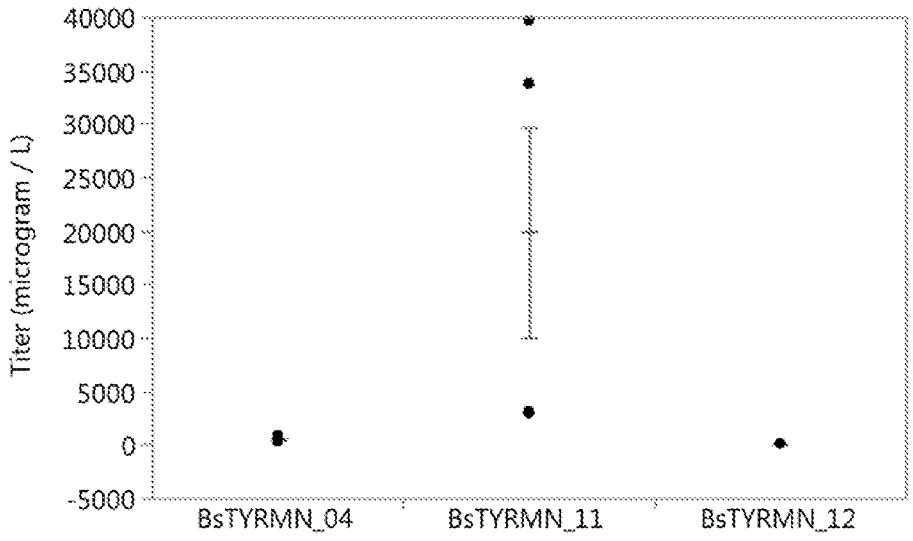
Titer (microgram / L)
40000
35000
30000
25000
20000
15000
10000
5000
0
-5000
BsTYRMN_04
BsTYRMN_11
BsTYRMN_12

Fig. 6

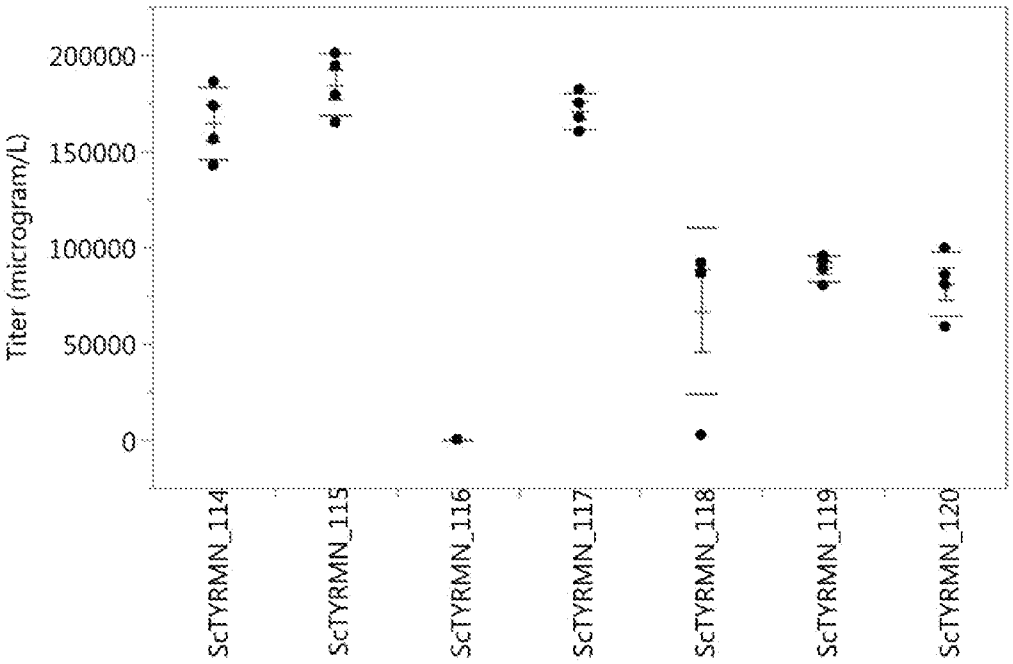
Titer (microgram/L)
200000
150000
100000
50000
0
ScTYRMN_114
ScTYRMN_115
ScTYRMN_116
ScTYRMN_117
ScTYRMN_118
ScTYRMN_119
ScTYRMN_120

Fig. 7

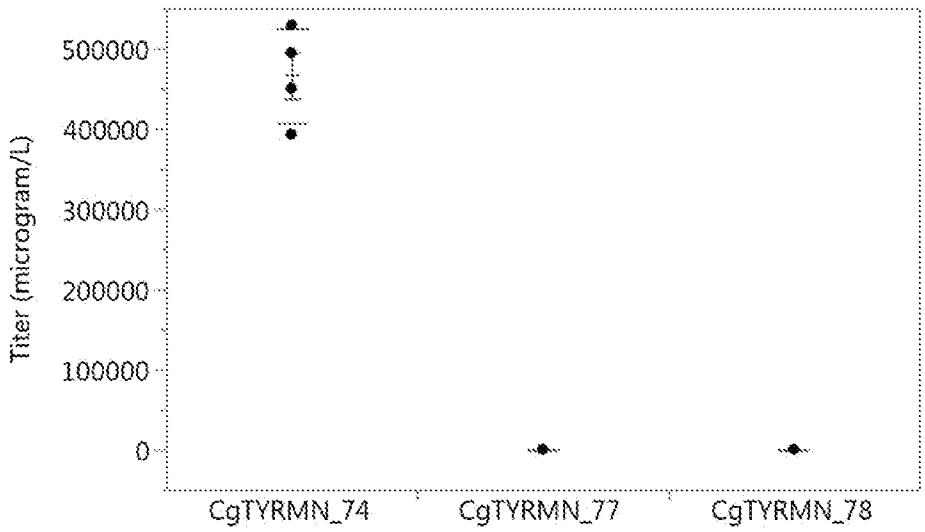
Titer (microgram/L)
500000
400000
300000
200000
100000
0
CgTYRMN_74
CgTYRMN_77
CgTYRMN_78

Fig. 8

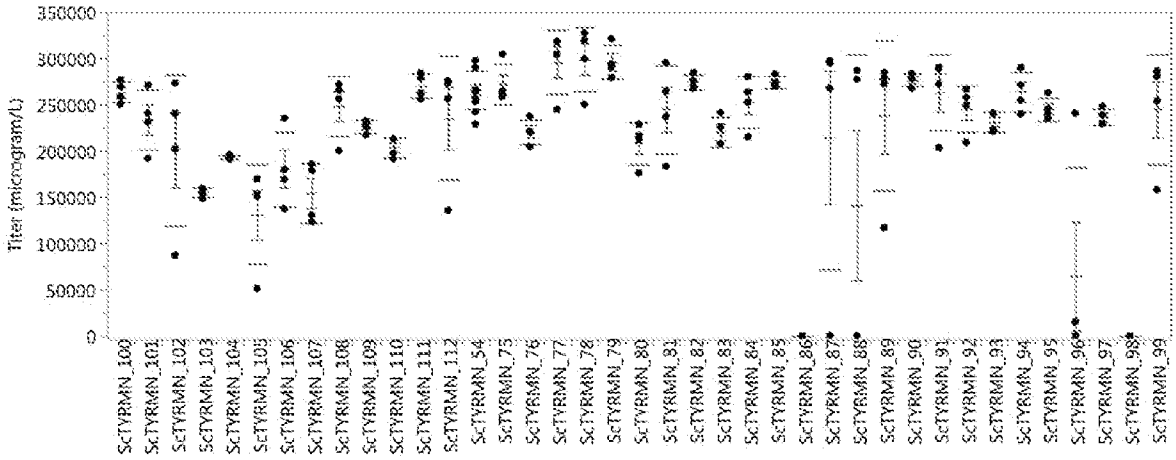
Titer (microgram/L)
350000
300000
250000
200000
150000
100000
50000
0
ScTYRMN_100
ScTYRMN_101
ScTYRMN_102
ScTYRMN_103
ScTYRMN_104
ScTYRMN_105
ScTYRMN_106
ScTYRMN_107
ScTYRMN_108
ScTYRMN_109
ScTYRMN_110
ScTYRMN_111
ScTYRMN_112
ScTYRMN_54
ScTYRMN_75
ScTYRMN_76
ScTYRMN_77
ScTYRMN_78
ScTYRMN_79
ScTYRMN_80
ScTYRMN_81
ScTYRMN_82
ScTYRMN_83
ScTYRMN_84
ScTYRMN_85
ScTYRMN_86
ScTYRMN_87
ScTYRMN_88
ScTYRMN_89
ScTYRMN_90
ScTYRMN_91
ScTYRMN_92
ScTYRMN_93
ScTYRMN_94
ScTYRMN_95
ScTYRMN_96
ScTYRMN_97
ScTYRMN_98
ScTYRMN_99

Fig. 9

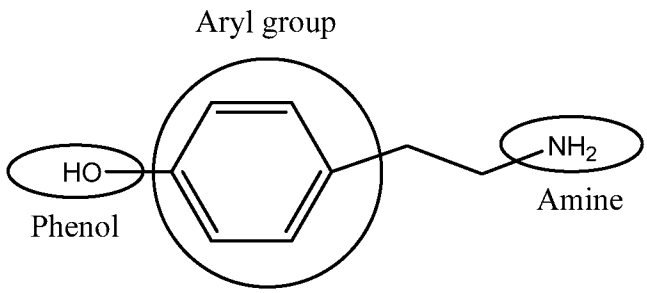
Aryl group
HO
Phenol
NH2
Amine

Fig. 10