(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
24 February 2022 (24.02.2022)

WIPO | PCT

(10) International Publication Number
WO 2022/040457 A1

(51) International Patent Classification:
C12P 13/00 (2006.01) *C12N 9/90* (2006.01)
C12N 15/81 (2006.01) *C12N 9/02* (2006.01)
C12N 9/10 (2006.01) *C12N 9/88* (2006.01)

(21) International Application Number:
PCT/US2021/046756

(22) International Filing Date:
19 August 2021 (19.08.2021)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
63/068,323 20 August 2020 (20.08.2020) US

(71) Applicant: **ZYMERGEN INC.** [US/US]; 5980 Horton St., #105, Emeryville, California 94608 (US).

(72) Inventors: **HAO, Zhangying**; 5980 Horton St., #105, Emeryville, California 94608 (US). **DESAI, Shuchi**; 5980 Horton St., #105, Emeryville, California 94608 (US). **EDGAR, Steven M.**; 5980 Horton St., #105, Emeryville, California 94608 (US). **GALLAGHER, Joseph**; 5980 Horton St., #105, Emeryville, California 94608 (US). **CHANDLER, James**; 5980 Horton St., #105, Emeryville, California 94608 (US). **AKIVA, Eyal**; 5980 Horton St., #105, Emeryville, California 94608 (US). **DAVIDSON, Rebecca**; 5980 Horton St., #105, Emeryville, California 94608 (US). **DAVIS, Matthew**; 5980 Horton St., #105, Emeryville, California 94608 (US).

(74) Agent: **HALIDAY, Emily M.** et al.; Weaver Austin Villeneuve & Sampson LLP, P.O. Box 70250, Oakland, California 94612-0250 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, IT, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*
— *with sequence listing part of description (Rule 5.2(a))*

(54) Title: ENGINEERED BIOSYNTHETIC PATHWAY FOR PRODUCTION OF 4-AMINOPHENYLETHYLAMINE BY FERMENTATION

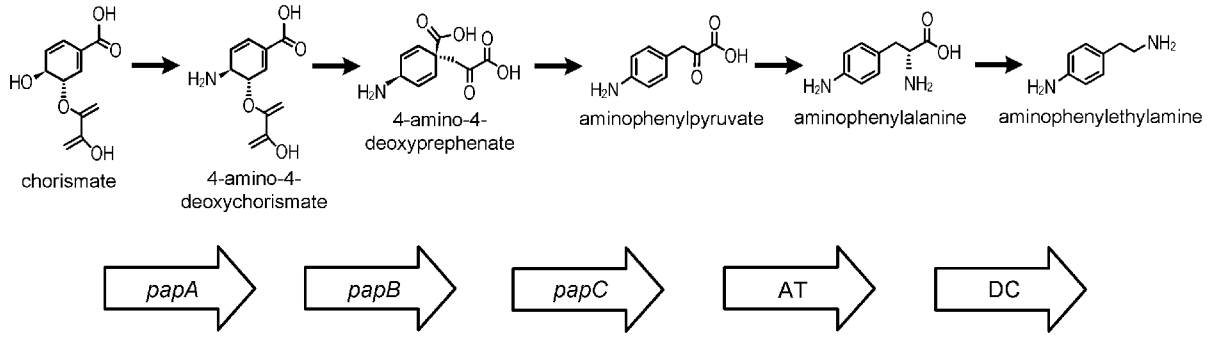

FIG. 2

(57) Abstract: The present disclosure describes the engineering of microbial cells for fermentative production of 4-APEA and related products and provides novel engineered microbial cells and cultures, as well as related 4-APEA production methods.

WO 2022/040457 A1

ENGINEERED BIOSYNTHETIC PATHWAY FOR PRODUCTION OF 4-AMINOPHENYLETHYLAMINE BY FERMENTATION

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. provisional application no. 63/068,323, filed August 20, 2020, which is hereby incorporated by reference in its entirety.

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

[0002] Not applicable.

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 August 19, 2021, is named ZMGNP043WO_SeqList_ST25.txt and is 448,733 bytes in size.

FIELD OF THE DISCLOSURE

[0004] The present disclosure relates generally to the area of engineering microbes for production of 4-aminophenylethylamine by fermentation.

BACKGROUND

[0005] 4-aminophenylethylamine (4-APEA) is an aromatic amine (AA). AAs are used in the production of advanced polymer materials including functional and/or high-performance plastics. The amine group and the aromatic moiety of AAs provide nucleophilic reactivity and excellent thermomechanical performance, respectively. AAs are often polycondensed with carbonyl compounds to generate aromatic polyamides, polyimides, polyazoles, polyurea, and polyazomethines. Polycondensation of AAs with aromatic acids generates super-engineering plastics with extremely high thermomechanical properties. These include poly(p-phenylene terephthalamide (KEVLAR™) and poly(4,4′-oxydiphenylene pyromellitimide) (KAPTON™), which are

used as thermostable materials in fabric for body armor and other flame-retardant materials, fiber-reinforced plastics for electronic devices, vehicle bodies, and anti-pressure cylinders.

[0006] Fermentative production of 4-APEA has been demonstrated in *Escherichia coli* (Masuo et al. (2016) Scientific Reports 6: 25764), but the poor tolerance of *E. coli* to high titers of 4-APEA makes it unsuitable for a large-scale fermentation host.

SUMMARY

[0007] The disclosure provides engineered microbial cells, cultures of the microbial cells, and methods for producing 4-aminophenylethylamine (4-APEA), including the following:

[0008] Various embodiments contemplated herein may include, but need not be limited to, one or more of the following:

[0009] Embodiment 1: An engineered microbial cell that produces 4-aminophenylethylamine (4-APEA), wherein the engineered microbial cell has a high tolerance to toxicity associated with the production of 4-APEA, as defined by a concentration at which the growth of engineered microbial cell is slowed by half (Ki) of at least 30 grams/liter.

[0010] Embodiment 2: The engineered microbial cell of embodiment 1, wherein the engineered microbial cell comprises a fungal cell.

[0011] Embodiment 3: The engineered microbial cell of embodiment 2, wherein the engineered microbial cell comprises a yeast cell.

[0012] Embodiment 4: The engineered microbial cell of embodiment 3, wherein the yeast cell is a cell of the genus *Komagataella*.

[0013] Embodiment 5: The engineered microbial cell of embodiment 4, wherein the yeast cell is a cell of the species *pastoris* or *phaffi*.

[0014] Embodiment 6: The engineered microbial cell of any one of embodiments 1-5, wherein the slope at which toxicity effects are observed over increasing 4-APEA concentrations is less than 6.

[0015] Embodiment 7: The engineered microbial cell of any one of embodiments 1-6, wherein the engineered microbial cell heterologously expresses each of the following

enzyme activities: 4-amino-4-deoxychorismate synthase; 4-amino-4-deoxychorismate mutase; 4-amino-4-deoxyprephenate dehydrogenase; aminotransferase (AT); and decarboxylase (DC); wherein the enzyme activities are provided by heterologously expressing genes encoding the enzymes, and at least one heterologously expressed enzyme is non-native to the engineered microbial cell.

[0016] Embodiment 8: The engineered microbial cell of embodiment 7, wherein at least two, three, four, or all of the heterologously expressed enzymes are non-native to the engineered microbial cell.

[0017] Embodiment 9: An engineered microbial cell of the genus *Komagataella* that produces 4-aminophenylpyruvate (4-APP).

[0018] Embodiment 10: The engineered microbial cell of embodiment 9, wherein the engineered microbial cell is a cell of the species *pastoris* or *phaffi*.

[0019] Embodiment 11: The engineered microbial cell of embodiment 9 or embodiment 10, wherein the engineered microbial cell heterologously expresses each of the following enzyme activities: 4-amino-4-deoxychorismate synthase; 4-amino-4-deoxychorismate mutase; and 4-amino-4-deoxyprephenate dehydrogenase, wherein each enzyme activity is provided by heterologously expressing genes encoding the enzymes, and at least one heterologously expressed enzyme is non-native to the engineered microbial cell.

[0020] Embodiment 12: The engineered microbial cell of any one of embodiments 9-11, wherein the engineered microbial cell additionally produces 4-aminophenylalanine (4-APhe).

[0021] Embodiment 13: The engineered microbial cell of embodiment 12, wherein the engineered microbial cell additionally heterologously expresses an aminotransferase (AT) activity.

[0022] Embodiment 14: The engineered microbial cell of any one of embodiments 9-11, wherein the engineered microbial cell additionally produces 4-aminophenylethanol.

[0023] Embodiment 15: The engineered microbial cell of embodiment 14, wherein the engineered microbial cell additionally heterologously expresses an alcohol dehydrogenase/acetaldehyde reductase enzyme.

[0024] Embodiment 16: The engineered microbial cell of any one of embodiments 9-15, wherein at least two, three, or all of the heterologously expressed enzymes are non-native to the engineered microbial cell.

[0025] Embodiment 17: The engineered microbial cell of any one of embodiments 7-16, wherein at least one of the heterologously expressed enzymes is expressed from a constitutive promoter.

[0026] Embodiment 18: The engineered microbial cell of any one of embodiments 7-16, wherein at least one of the heterologously expressed enzymes is expressed from a regulated promoter, optionally wherein the regulated promoter is a thiamine-repressed promoter.

[0027] Embodiment 19: The engineered microbial cell of any one of embodiments 1-18, wherein the engineered microbial cell comprises increased activity of one or more upstream chorismate pathway enzyme(s), said increased activity being increased relative to a control cell.

[0028] Embodiment 20: The engineered microbial cell of embodiment 19, wherein said increased activity is selected from the group consisting of glucokinase, transketolase, transaldolase, phospho-2-dehydro-3-deoxyheptonate aldolase, 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase, 3-dehydroquinate synthase, 3-dehydroquinate dehydratase, shikimate dehydrogenase, shikimate kinase, 3-phosphoshikimate 1-carboxyvinyltransferase, chorismate synthase activity, and any combination thereof.

[0029] Embodiment 21: The engineered microbial cell of embodiment 20, wherein said increased activity comprises increased 3-dehydroquinate synthase, 3-dehydroquinate dehydratase, shikimate dehydrogenase, shikimate kinase, and 3-phosphoshikimate 1-carboxyvinyltransferase activities, which are provided by heterologously expressing a pentafunctional enzyme.

[0030] Embodiment 22: The engineered microbial cell of any one of embodiments 1-21, wherein the engineered microbial cell comprises increased activity of one or more nitrogen assimilation and utilization pathway enzyme(s).

[0031] Embodiment 23: The engineered microbial cell of 22, wherein said increased activity is selected from the group consisting of isocitrate dehydrogenase,

glutamine synthetase, glutamate synthase, glutamate dehydrogenase, ammonium permease, and any combination thereof.

[0032] Embodiment 24: The engineered microbial cell of any one of embodiments 1-23, wherein the engineered microbial cell comprises reduced activity of one or more enzyme(s) that consume one or more chorismate pathway precursors, chorismate, and/or one or more intermediates in the pathway leading from chorismate to 4-APEA, and/or more enzymes that consume 4-APEA, said reduced activity being reduced relative to a control cell.

[0033] Embodiment 25: The engineered microbial cell of embodiment 24, wherein the one or more enzyme(s) that consume one or more chorismate pathway precursors are selected from the group consisting of dihydroxyacetone phosphatase, 3-dehydroshikimate dehydratase, shikimate dehydrogenase, and phosphoenolpyruvate phosphotransferase.

[0034] Embodiment 26: The engineered microbial cell of embodiment 24, wherein the one or more enzyme(s) that consume chorismate are selected from the group consisting of anthranilate synthase and chorismate mutase.

[0035] Embodiment 27: The engineered microbial cell of embodiment 24, wherein the one or more enzyme(s) that consume one or more intermediates in the pathway leading from chorismate to 4-APEA are selected from the group consisting of decarboxylase, aromatic amino acid decarboxylase, phenylpyruvate decarboxylase, pyruvate decarboxylase, aromatic amino acid ammonia lyase, and alcohol dehydrogenase/acetaldehyde reductase.

[0036] Embodiment 28: The engineered microbial cell of embodiment 24, wherein the one or more enzymes that consume 4-APEA are selected from the group consisting of phenylpyruvate dioxygenase, diamine oxidase, amine oxidase, and amino acid oxidase.

[0037] Embodiment 29: The engineered microbial cell of any one of embodiments 24-28, wherein the reduced activity is achieved by replacing a native promoter of a gene for said one or more enzymes with a less active promoter or by deleting or knocking out the gene.

[0038] Embodiment 30: The engineered microbial cell of any one of embodiments 1-29, wherein the engineered microbial cell additionally expresses a feedback-deregulated DAHP synthase.

[0039] Embodiment 31: The engineered microbial cell of any one of embodiments 1-30, wherein the engineered microbial cell comprises increased activity of one or more enzyme(s) that increase the supply of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH), said increased activity being increased relative to a control cell.

[0040] Embodiment 32: The engineered microbial cell of embodiment 31, wherein the one or more enzyme(s) that increase the supply of the reduced form of NADPH are selected from the group consisting of pentose phosphate pathway enzymes, NADP+-dependent glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and NADP+-dependent glutamate dehydrogenase.

[0041] Embodiment 33: The engineered microbial cell of embodiment 1, wherein the non-native enzymes comprise: a 4-amino-4-deoxychorismate synthase having at least 70% amino acid sequence identity with a 4-amino-4-deoxychorismate synthase from *Pseudomonas fluorescens* (strain SBW25); a 4-amino-4-deoxychorismate mutase having at least 70% amino acid sequence identity with a 4-amino-4-deoxychorismate mutase from *Photorhabdus laumondii subsp. laumondii* (strain DSM 15139 / CIP 105565 / TT01); a 4-amino-4-deoxyprephenate dehydrogenase having at least 70% amino acid sequence identity with a 4-amino-4-deoxyprephenate dehydrogenase from *Pseudomonas fluorescens* (strain SBW25); optionally, an aminotransferase (AT) having at least 70% amino acid sequence identity with an aminotransferase (AT) from Escherichia coli (strain K12); and optionally a decarboxylase (DC) having at least 70% amino acid sequence identity with a decarboxylase (DC) from *Papaver somniferum*.

[0042] Embodiment 34: The engineered microbial cell of embodiment 33, wherein the: 4-amino-4-deoxychorismate synthase from *Pseudomonas fluorescens* (strain SBW25) comprises SEQ ID NO:4; 4-amino-4-deoxychorismate mutase from *Photorhabdus laumondii subsp. laumondii* (strain DSM 15139 / CIP 105565 / TT01) comprises SEQ ID NO:6; 4-amino-4-deoxyprephenate dehydrogenase from *Pseudomonas fluorescens* (strain SBW25) comprises SEQ ID NO:8; aminotransferase (AT) from

Escherichia coli (strain K12), if present, comprises SEQ ID NO:(SEQ ID NO:13); and decarboxylase (DC) from *Papaver somniferum*, if present, comprises SEQ ID NO:9.

[0043] Embodiment 35: The engineered microbial cell of embodiment 1, wherein the non-native enzymes comprise: a 4-amino-4-deoxychorismate synthase having at least 70% amino acid sequence identity with a 4-amino-4-deoxychorismate synthase from *Streptomyces* sp. CB01635; a 4-amino-4-deoxychorismate mutase having at least 70% amino acid sequence identity with a 4-amino-4-deoxychorismate mutase from *Streptomyces pristinaespiralis*; a 4-amino-4-deoxyprephenate dehydrogenase having at least 70% amino acid sequence identity with a 4-amino-4-deoxyprephenate dehydrogenase from *Pseudomonas* sp. 2822; optionally, an aminotransferase (AT) having at least 70% amino acid sequence identity with an aminotransferase (AT) from *Petunia hybrida*; and optionally, a decarboxylase (DC) having at least 70% amino acid sequence identity with a decarboxylase (DC) from *Papaver somniferum*.

[0044] Embodiment 36: The engineered microbial cell of embodiment 35, wherein the: 4-amino-4-deoxychorismate synthase from *Streptomyces* sp. CB01635 comprises SEQ ID NO:3; 4-amino-4-deoxychorismate mutase from *Streptomyces pristinaespiralis* comprises SEQ ID NO:5; 4-amino-4-deoxyprephenate dehydrogenase from *Pseudomonas* sp. 2822 comprises SEQ ID NO:7; aminotransferase (AT) from *Petunia hybrida*, if present, comprises SEQ ID NO:14; and decarboxylase (DC) from *Papaver somniferum*, if present, comprises SEQ ID NO:9.

[0045] Embodiment 37: The engineered microbial cell of embodiment 1, wherein the non-native enzymes comprise: a 4-amino-4-deoxychorismate synthase having at least 70% amino acid sequence identity with a 4-amino-4-deoxychorismate synthase from *Streptomyces* sp. CB01635; a 4-amino-4-deoxychorismate mutase having at least 70% amino acid sequence identity with a 4-amino-4-deoxychorismate mutase from *Photorhabdus asymbiotica* subsp. *asymbiotica*; a 4-amino-4-deoxyprephenate dehydrogenase having at least 70% amino acid sequence identity with a 4-amino-4-deoxyprephenate dehydrogenase from *Xenorhabdus doucetiae*; optionally, an aminotransferase (AT) having at least 70% amino acid sequence identity with an aminotransferase (AT) from *Corynebacterium glutamicum*; and optionally, a decarboxylase (DC) having at least 70% amino acid sequence identity with a decarboxylase (DC) from *Papaver somniferum*.

[0046] Embodiment 38: The engineered microbial cell of embodiment 35, wherein the: 4-amino-4-deoxychorismate synthase from *Streptomyces* sp. CB01635 comprises SEQ ID NO:3; 4-amino-4-deoxychorismate mutase from *Photorhabdus asymbiotica* subsp. *asymbiotica* comprises SEQ ID NO:25; 4-amino-4-deoxyprephenate dehydrogenase from *Xenorhabdus doucetiae* comprises SEQ ID NO:29; aminotransferase (AT) from *Corynebacterium glutamicum*, if present, comprises SEQ ID NO:16; and decarboxylase (DC) from *Papaver somniferum*, if present, comprises SEQ ID NO:9.

[0047] Embodiment 39: The engineered microbial cell of any one of embodiments 7-38, wherein the engineered microbial cell additionally comprises a genotype change selected from the group consisting of: p9_pENO1_KPA:GLN1, p35_pKEX2_KPA:NUFM, p9_pENO1_KPA:NUFM, p115_pTHI11_KPA:PDC2, and p5_pTDH3_KPA:PDC2.

[0048] Embodiment 40: The engineered microbial cell of any one of embodiments 1-8 and 19-39, wherein, when cultured, the engineered microbial cell produces 4-APEA at a level of at least 11 gram/liter of culture medium.

[0049] Embodiment 41: The engineered microbial cell of any one of embodiments 9-16, wherein, when cultured, the engineered microbial cell produces 4-APP at a level of at least 20 milligram/liter of culture medium, optionally wherein, when cultured, the engineered microbial cell produces 4-APhe at a level of at least 5 milligram/liter of culture medium.

[0050] Embodiment 42: A culture of engineered microbial cells according to any one of embodiments 1-41.

[0051] Embodiment 43: The culture of embodiment 42, wherein the culture comprises 4-APP, 4-A-Phe, and/or 4-APEA.

[0052] Embodiment 44: A method of culturing engineered microbial cells according to any one of embodiments 1-41, the method comprising culturing the cells under conditions suitable for producing 4-APP, 4-APhe, and/or 4-APEA.

[0053] Embodiment 45: The method of embodiment 44, wherein the method comprises fed-batch culture, with an initial glucose level in the range of 1-100 g/L, followed by controlled sugar feeding.

[0054] Embodiment 46: The method of any one of embodiment 44 or embodiment 45, wherein the culture is pH-controlled during culturing.

[0055] Embodiment 47: The method of any one of embodiments 44-46, therein the concentration of thiamine is controlled during culturing.

[0056] Embodiment 48: The method of any one of embodiments 44-47, wherein the culture is aerated during culturing.

[0057] Embodiment 49: The culture of embodiment 42 or embodiment 43 or the method of any one of embodiments 44-48, wherein the culture comprises: 4-APP at a level of at least 20 milligram/liter of culture medium; 4-APhe at a level of at least 5 milligram/liter of culture medium; and/or 4-APEA at a level of at least 15 milligram/liter of culture medium.

[0058] Embodiment 50: The engineered microbial cell of embodiment 40 or the culture or method of embodiment 49, wherein, when cultured, the engineered microbial cell, or the culture comprises, 4-APEA at a level of at least 6 gram/liter of culture medium.

[0059] Embodiment 51: The engineered microbial cell of embodiment 40 or the culture or method of embodiment 49, wherein, when cultured, the engineered microbial cell, or the culture comprises, 4-APEA at a level of at least 11 gram/liter of culture medium.

[0060] Embodiment 52: The method of any one of embodiments 44-51, wherein the method additionally comprises recovering 4-APP, 4-APhe, and/or 4-APEA from the culture.

[0061] The disclosure also provides versions of the above embodiments wherein the embodiments consists of or consists essentially of the recited elements or actions. In the case of embodiments consisting essentially of the recited elements/actions, the "basic and novel characteristics" of the embodiment are the production characteristics of an engineered microbial cell, culture, or method (e.g., the yield of a particular product, optionally expressed in terms of titer in culture medium).

[0062] Also within the scope of the present disclosure are versions of the above embodiments wherein the embodiments are carried out using any means for providing the recited elements of composition claims and any means for carrying out the recited actions of the method claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0063] Figure 1: Toxicity impact of 4-APEA across a range of organisms tested in an Enzyscreen system. Various organisms are grown in media that is supplemented with the molecule of interest, and the growth of the organism is monitored over time. Analysis of the growth-time-course data allows determination of toxicity properties of the molecule of interest, such as the Minimal Inhibitory Concentration (MIC), the concentration at which growth is slowed to half (Ki), and the slope at which increased toxicity effects are observed (alpha). For Fig. 1, product toxicity was measured as a function of reduced substate uptake with increasing product concentration.

[0064] Figure 2: Biosynthesis of 4-APEA in five enzymatic steps from chorismate.

[0065] Figure 3: Pathway for production of chorismate.

[0066] Figure 4: Product profile of a 4-APEA production strain cultured in microtiter plates for 48 hours. See Example 1.

[0067] Figure 5: A "split-marker, double-crossover" genomic integration strategy, which was developed to engineer *K. pastoris* strains. Two plasmids with complementary 5' and 3' homology arms and overlapping halves of a selectable marker, such as an antibiotic marker or an auxotrophic marker (direct repeats shown by the hashed bars) were linearized by PCR or by digestion 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 selectable marker 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).

DETAILED DESCRIPTION

[0068] The present disclosure describes the engineering of microbial cells for fermentative production of 4-aminophenylethylamine (4-APEA) and provides novel engineered microbial cells and cultures, as well as related 4-APEA production methods.

Definitions

[0069] Terms used in the claims and specification are defined as set forth below unless otherwise specified.

[0070] 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 4-APEA) by means of one or more biological conversion steps, without the need for any chemical conversion step.

[0071] 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.

[0072] The term "native" is used herein to refer to a cellular component, such as a polynucleotide or polypeptide, that is naturally present in a particular cell. A native polynucleotide or polypeptide is endogenous to the cell.

[0073] When used with reference to a polynucleotide or polypeptide, the term "non-native" refers to a polynucleotide or polypeptide that is not naturally present in a particular cell.

[0074] When used with reference to the context in which a gene is expressed, the term "non-native" refers to a gene expressed in any context other than the genomic and cellular context in which it is naturally expressed. A gene expressed in a non-native manner may have the same nucleotide sequence as the corresponding gene in a host cell, but may be expressed from a vector or from an integration point in the genome that differs from the locus of the native gene.

[0075] The term "heterologous" is used herein to describe a polynucleotide or polypeptide introduced into a host cell. This term encompasses a polynucleotide or polypeptide, respectively, derived from a different organism, species, or strain than that of the host cell. In this case, the heterologous polynucleotide or polypeptide has a sequence that is different from any sequence(s) found in the same host cell. However, the term also encompasses a polynucleotide or polypeptide that has a sequence that is the same as a sequence found in the host cell, wherein the polynucleotide or polypeptide is present in a different context than the native sequence (e.g., a heterologous polynucleotide can be linked to a different promotor and/or inserted into a different genomic location than that of the native sequence). "Heterologous expression" thus encompasses expression of a sequence that is non-native to the host cell, as well as expression of a sequence that is native to the host cell in a non-native context.

[0076] "Heterologous expression" encompasses expressing a polynucleotide from a constitutive promoter or from a regulated promoter.

[0077] A "regulated promoter" is a promoter that is more or less active in response to one or more parameters. For example, a thiamine-repressed promoter is one whose activity increases in response to a reduction in thiamine concentration. Illustrative thiamine-repressed promoters are typically repressed at thiamine concentrations of 50 mg/L and above, with the degree of promoter repression decreasing as the thiamine concentration approaches zero.

[0078] As used with reference to polynucleotides or polypeptides, the term "wild-type" refers to any polynucleotide having a nucleotide sequence, or polypeptide having an amino acid, sequence present in a polynucleotide or 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.

[0079] 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 in the engineered cell. The control cell can include one or more specific modifications that are also present in the engineered cell being tested (i.e., genetic modifications that are not "being tested").

[0080] 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 native 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.

[0081] The term "feedback-deregulated" 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-deregulated" enzyme is a form of the enzyme that is less sensitive to feedback-inhibition than the enzyme native to the cell or a form of the enzyme that is native to the cell but is

naturally less sensitive to feedback inhibition than one or more other natural forms of the enzyme. A feedback-deregulated enzyme may be produced by introducing one or more mutations into a native enzyme. Alternatively, a feedback-deregulated enzyme may simply be a heterologous, native enzyme that, when introduced into a particular microbial cell, is not as sensitive to feedback-inhibition as the native, native enzyme. In some embodiments, the feedback-deregulated enzyme shows no feedback-inhibition in the microbial cell.

[0082] 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.

[0083] 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.

[0084] The term "titer," as used herein, refers to the mass of a product (e.g., 4-APEA) present in the cell culture medium in a culture of microbial cells divided by the culture volume.

[0085] The term "Ki," as used herein, refers to the concentration of a chemical (e.g., 4-APEA) at which the maximal growth rate of cells in culture is slowed by half. Cultures of the cells are grown in media supplemented with varying concentrations of a chemical, and the cell growth is monitored over time. Exponential growth curve data at each chemical concentration are used to determine a specific growth rate μ using the following equation:

$$X = X_0 e^{\mu t}$$

where:

X = cell concentration

X_0 = cell concentration at time (t) = 0

μ = specific growth rate.

The maximal growth rate for each chemical concentration is determined (μ_{obs}) and the Ki is the chemical concentration at which the maximal growth rate has been slowed to half of the maximal growth rate in the absence of the chemical (μ_{max}). Ki (denoted K_I below) can be determined from the equation:

$$\mu_{obs} = \frac{\mu_{max}}{1 + \left(\frac{C_I}{K_I}\right)^{\alpha}}$$

where:

C_I = concentration of inhibitor

K_I = inhibition constant – inhibitor concentration where growth rate is half of maximum

α = inhibition parameter to fit data

μ_{max} = the maximum growth rate of a culture in the absence of a chemical

μ_{obs} = the maximum growth rate of a culture at a given concentration of a chemical

[0086] The term "alpha" ("α"), as used herein, refers to the slope at which toxicity effects are observed. Cultures of the cells are grown in media supplemented with increasing concentrations of a chemical, and the cell growth is monitored over time. Alpha can be determined from the equation:

$$\mu_{obs} = \frac{\mu_{max}}{1 + \left(\frac{C_I}{K_I}\right)^{\alpha}}$$

where:

C_I = concentration of inhibitor

K_I = inhibition constant – inhibitor concentration where growth rate is half of maximum

α = inhibition parameter to fit data

μ_{max} = the maximum growth rate of a culture in the absence of a chemical

μ_{obs} = the maximum growth rate of a culture at a given concentration of a chemical

[0087] As used herein, the term "4-APEA pathway gene" refers to any gene encoding an enzyme that participates in the conversion of chorismate to 4-APEA, e.g., any

one of the following enzymes: 4-amino-4-deoxychorismate synthase, 4-amino-4-deoxychorismate mutase, 4-amino-4-deoxyprephenate dehydrogenase, aminotransferase (AT), and decarboxylase (DC).

[0088] As used herein, the term "upstream chorismate pathway enzyme" refers to any enzyme that participates in the conversion of glucose to chorismate.

[0089] As used herein with respect to recovering 4-APEA from a cell culture, "recovering" refers to separating the 4-APEA from at least one other component of the cell culture medium.

Engineering Microbes for Production of 4-Aminophenylethylamine and Precursors or Derivatives Thereof

[0090] One obstacle to efficient production of 4-aminophenylethylamine (4-APEA) by fermentation is that 4-APEA is toxic to many host microbes used conventionally for fermentation. Figure 1 depicts the concentration of 4-APEA at which an organism's growth is slowed by half (Ki) and the slope (alpha) at which toxicity effects are observed over increasing 4-APEA concentrations. A larger Ki and smaller alpha indicate higher tolerance and less susceptibility to inhibition, respectively. Fig. 1 shows that some species, such as *Saccharomyces cerevisiae* and *Escherichia coli*, suffer from significant toxicity in the presence of higher levels of 4APEA. Other fungi, including the yeasts *Komagataella pastoris* (also known as *Pichia pastoris*), *Komagataella phaffi*, and *Yarrowia lipolytica*, and other bacteria, such as *Bacillus licheniformis* provide better results. In particular, the substantial toxicity of 4-APEA on traditional metabolic engineering hosts such as *E. coli* and *S. cerevisiae*, shown in Fig. 1, in comparison to the more moderate effect on *K. pastoris* highlights the utility of *K. pastoris* as a production host for high-titer fermentation of 4-APEA. *K. phaffi* is expected to perform similarly to *K. pastoris* as a production host.

4-Aminophenylethylamine Biosynthesis Pathway

[0091] The metabolic pathway to 4-APEA is derived from the shikimate pathway metabolite, chorismate. Figure 2 depicts an assembled pathway for biosynthesis of 4-APEA from chorismate. This pathway is sequentially made up of the following enzyme activities: 4-amino-4-deoxychorismate synthase (e.g., encoded by a *papA* gene in some organisms and referred to herein as "papA," for short), 4-amino-4-deoxychorismate mutase (e.g., encoded by a *papB* gene in some organisms and referred to herein as "papB,"

for short), 4-amino-4-deoxyprephenate dehydrogenase (e.g., encoded by a *papC* gene in some organisms and referred to herein as "papC," for short), aminotransferase (referred to herein as "AT"), and decarboxylase (referred to herein as "DC"). In some cases, multiple enzyme activities may be performed by one enzyme. For example, in *Komagataella pastoris*, as well as other organisms, a native bifunctional enzyme that acts as a 4-amino-4-deoxychorismate synthase also has glutaminase activity.

[0092] Chorismate is derived from the aromatic branch of amino acid biosynthesis, based on the precursors phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P) (see Fig. 3). The first step of this aromatic biosynthesis pathway (carried out by 3-deoxy-D-arabinoheptulosonate 7-phosphate [DAHP] synthase) is subject to feedback inhibition by the aromatic amino acids tyrosine, tryptophan, and phenylalanine.

[0093] The production of 4-APEA by fermentation of a simple carbon source can be achieved by linking flux through the shikimate biosynthesis pathway to an introduced 4-APEA pathway including the five enzymes identified above, and optionally improving flux through this pathway, in a suitable microbial host.

Engineering for Microbial 4-Aminophenylethylamine Production

[0094] Any 4-APEA pathway enzyme that is active in the microbial cell being engineered may be introduced into the cell, typically by introducing and expressing the gene(s) encoding the enzyme(s) using standard genetic engineering techniques. Suitable 4-APEA pathway enzymes may be derived from any source, including plant, archaeal, fungal, gram-positive bacterial, and gram-negative bacterial sources (see, e.g., those described herein). In various embodiments, at least one, two, three, four, or all gene(s) introduced into the microbial cell is non-native to the cell.

[0095] One or more copies of any of these genes can be introduced into a selected microbial host cell. If more than one copy of a gene is introduced, the copies can have the same or different nucleotide sequences. In some embodiments, one or both (or all) of the heterologous gene(s) is/ are expressed from a strong, constitutive promoter. In some embodiments, the heterologous gene(s) is/are expressed from a regulable promoter (e.g., an inducible or repressible promoter). The heterologous gene(s) can optionally be codon-optimized to enhance expression in the selected microbial host cell. The codon-optimization table used in the Example is the *K. pastoris* Kazusa codon table at www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=4922.

[0096] In various embodiments, the 4-APEA titers achieved by expressing all five 4-APEA pathway enzymes are at least 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, or 900 mg/L or at least 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 10, 15, 20, 25, 30, or 35 gm/L. In various embodiments, the titer is in the range of 5 mg/L to 800 mg/L, 10 mg/L to 700 mg/L, 15 mg/L to 600 mg/L, 20 mg/L to 500 mg/L, 25 mg/L to 400 mg/L, 30 mg/L to 300 mg/L, 30 mg/L to 200 mg/L, 30 mg/L to 100 mg/L, 30 mg/L to 50 mg/L, or any range bounded by any of the values listed above.

Engineering for Microbial Production of 4-Aminophenylethylamine Precursors or Derivatives Thereof

[0097] In some embodiments, suitable microbial hosts, such as, e.g., *Komagataella* species like *K. pastoris* or *K. phaffi*, can be engineered to produce 4-aminophenylethylamine (4-APEA) precursors, such as 4-aminophenylpyruvate (4-APP) and/or 4-aminophenylalanine (4-APhe). To produce these precursors, a truncated version of the 4-APEA pathway described above can be introduced. To produce 4-APP, a microbial host cell can be engineered to heterologously express each of the following enzyme activities: 4-amino-4-deoxychorismate synthase, 4-amino-4-deoxychorismate mutase, and 4-amino-4-deoxyprephenate dehydrogenase, typically by introducing and expressing the gene(s) encoding the enzyme(s) using standard genetic engineering techniques. 4-APhe can be produced in such an engineered microbial cell by additionally engineering the cell to heterologously express an aminotransferase (AT) activity. The general considerations discussed above for introducing the full 4-APEA pathway also apply to introducing a truncated pathway. Likewise, the titers of 4-APP and/or 4-APhe achievable using the methods described herein are the same as those given above for 4-APEA.

[0098] In some embodiments, one or more enzymes other than those of the 4-APEA pathway can also be introduced to produce one or more derivatives of 4-APEA or a 4-APEA precursor. For example, an engineered microbial cell that is capable of producing 4-APP can be engineered to produce 4-aminophenylethanol by additionally engineering the cell to heterologously express the enzyme activities necessary to convert 4-APP to 4-aminophenylethanol. Conversion of aminophenylpyruvate to 4-aminophenylethanol is sequentially catalyzed by a phenylpyruvate decarboxylase (or a pyruvate decarboxylase), followed by an alcohol dehydrogenase/acetaldehyde reductase

(these enzymes are facilitate the interconversion between alcohols and aldehydes or ketones with the reduction of nicotinamide adenine dinucleotide (NAD+) to NADH and are thus termed "alcohol dehydrogenases," "acetaldehyde reductases," or "alcohol dehydrogenase/acetaldehyde reductases"). The general considerations discussed herein for introducing the full 4-APEA pathway also apply to introducing a truncated pathway with one or more additional enzyme activities. Likewise, the titers of a resultant derivative (such as 4-aminophenylethanol) that are achievable using the methods described herein are the same as those given above for 4-APEA.

[0099] Further genetic modifications can be used to increase the yield of the desired product (e.g., 4-APEA, 4-APP, 4-APhe, and or aminophenylethanol). These are described below. For ease of discussion, the modifications are described in terms of increasing 4-APEA production, but those of skill in the art understand that these further genetic modifications apply equally to increasing the yields of 4-APEA precursors or derivatives thereof.

Increasing the Activity of Upstream Enzymes

[0100] One approach to increasing 4-APEA production in a microbial cell that is capable of such production is to increase the activity of one or more upstream enzymes in the biosynthesis pathway. Upstream pathway enzymes include all enzymes involved in the conversions from a feedstock all the way to a metabolite that can be directly converted to 4-APEA (i.e., chorismate). These enzymes are referred to herein as "upstream chorismate pathway enzymes." Illustrative enzymes, for this purpose, include, but are not limited to, those shown in Fig. 1 in the pathway leading to this metabolite. In some embodiments, one or more upstream pathway enzymes whose activity is increased are selected from 3-deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) synthase and subsequent enzymes in the pathway leading to chorismate. Examples include glucokinase, transketolase, transaldolase, phospho-2-dehydro-3-deoxyheptonate aldolase, 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase, 3-dehydroquinate synthase, 3-dehydroquinate dehydratase, shikimate dehydrogenase, shikimate kinase, 3-phosphoshikimate 1-carboxyvinyltransferase, and chorismate synthase. In some embodiments, the activity of a pentafunctional enzyme that acts as a 3-dehydroquinate synthase, 3-dehydroquinate dehydratase, shikimate dehydrogenase, shikimate kinase, 3-phosphoshikimate 1-carboxyvinyltransferase can be increased. Suitable upstream pathway genes encoding these enzymes may be derived from any available source, including, for

example, those disclosed herein. For example, the activity of the native *Komagataella pastoris* pentafunctional enzyme can be increased, or a non-native pentafunctional enzyme can be introduced into an engineered microbial cell.

[0101] In some embodiments, the activity of one or more upstream pathway enzymes is increased by modulating the expression or activity of the native enzyme(s). For example, native regulators of the expression or activity of such enzymes can be exploited to increase the activity of suitable enzymes.

[0102] Alternatively, or in addition, one or more promoters can be substituted for native promoters. In certain embodiments, the replacement promoter is stronger than the native promoter and/or is a constitutive promoter. The replacement promoter can, if desired, be one that is regulable (e.g., inducible or repressible). In some embodiments a thiamine-repressed promoter can be employed, which can reduce the metabolic load on the cell.

[0103] 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 engineered microbial host cell. An introduced upstream pathway gene may be from an organism other than that of the host cell or may simply be an additional copy of a native gene. In some embodiments, one or more such genes are introduced into a microbial host cell capable of 4-APEA production and expressed from a strong constitutive promoter and/or can optionally be codon-optimized to enhance expression in the selected microbial host cell.

[0104] In various embodiments, the engineering of a 4-APEA-producing microbial cell to increase the activity of one or more upstream pathway enzymes increases the 4-APEA 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, 100-fold, 150-fold, 200-fold, 250-fold, 300-fold, 350-fold, 400-fold, 450-fold, 500-fold, 550-fold, 600-fold, 650-fold, 700-fold, 750-fold, 800-fold, 850-fold, 900-fold, 950-fold, or 1000-fold. In various embodiments, the increase in 4-APEA titer is in the range of 10-fold to 1000-fold, 20-fold to 500-fold, 50-fold to 400-fold, 10-

fold to 300-fold, or any range bounded by any of the values listed above. (Ranges herein include their endpoints.) These increases are determined relative to the 4-APEA titer observed in a 4-APEA-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 4-APEA production.

[0105] In various embodiments, the 4-APEA titers achieved by increasing the activity of one or more upstream pathway enzymes are at least 10, 20, 30, 40, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, or 900 mg/L or at least 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 10, 15, 20, 25, 30, 35 or 40 gm/L. In various embodiments, the titer is in the range of 10 mg/L to 900 mg/L, 15 mg/L to 800 mg/L, 20 mg/L to 700 mg/L, 25 mg/L to 600 mg/L, 30 mg/L to 500 mg/L, 30 mg/L to 400 mg/L, 30 mg/L to 300 mg/L, 30 mg/L to 200 mg/L, 30 mg/L to 100 mg/L, or any range bounded by any of the values listed above.

Increasing the Activity of Nitrogen Assimilation and Utilization Enzymes

[0106] One approach to increasing 4-APEA production in a microbial cell that is capable of such production is to increase the activity of one or more nitrogen assimilation and utilization pathway enzymes. Such enzymes include any enzyme that participates in nitrogen assimilation and utilization in a manner that increases production of 4-APEA. Illustrative enzymes, for this purpose, include, but are not limited to, isocitrate dehydrogenase, glutamine synthetase, glutamate synthase, glutamate dehydrogenase, and ammonium permease. The approaches to increasing activity of upstream pathway enzymes, discussed above, apply equally to nitrogen assimilation and utilization pathway enzymes.

[0107] In various embodiments, the engineering of a 4-APEA-producing microbial cell to increase the activity of one or more nitrogen assimilation and utilization pathway enzymes increases the 4-APEA 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, 100-fold, 150-fold, 200-fold, 250-fold, 300-fold, 350-fold, 400-fold, 450-fold, 500-fold, 550-fold, 600-fold, 650-fold, 700-fold,

750-fold, 800-fold, 850-fold, 900-fold, 950-fold, or 1000-fold. In various embodiments, the increase in 4-APEA titer is in the range of 10-fold to 1000-fold, 20-fold to 500-fold, 50-fold to 400-fold, 10-fold to 300-fold, or any range bounded by any of the values listed above. (Ranges herein include their endpoints.) These increases are determined relative to the 4-APEA titer observed in a 4-APEA-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 4-APEA production.

[0108] In various embodiments, the 4-APEA titers achieved by increasing the activity of one or more nitrogen assimilation and utilization pathway enzymes are at least 10, 20, 30, 40, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, or 900 mg/L or at least 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 10, 15, 20, 25, 30, 35 or 40 gm/L. In various embodiments, the titer is in the range of 10 mg/L to 900 mg/L, 15 mg/L to 800 mg/L, 20 mg/L to 700 mg/L, 25 mg/L to 600 mg/L, 30 mg/L to 500 mg/L, 30 mg/L to 400 mg/L, 30 mg/L to 300 mg/L, 30 mg/L to 200 mg/L, 30 mg/L to 100 mg/L, or any range bounded by any of the values listed above.

Introduction of Feedback-Deregulated Enzymes

[0109] Since aromatic amino acid biosynthesis is subject to feedback inhibition, another approach to increasing 4-APEA production in a microbial cell engineered for this purpose is to introduce feedback-deregulated forms of one or more enzymes that are normally subject to feedback inhibition in the pathway leading to chorismate. DAHP synthase is an example of such an enzyme. A feedback-deregulated 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-deregulated 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*, two from *E. coli*, and two from *K. pastoris*) that have known point mutations rendering them resistant to feedback inhibition, e.g., *S. cerevisiae ARO4*Q166K, *S. cerevisiae ARO4*K229L, *E. coli AroG*D146N, *E. coli AroG*P150L, *K. pastoris ARO4*K237L, and *K. pastoris ARO4*Q174K. 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.

[0110] In various embodiments, the engineering of a 4-APEA-producing microbial cell to express a feedback-deregulated enzymes increases the 4-APEA 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 4-APEA 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 4-APEA titer observed in a 4-APEA-producing microbial cell that does not express a feedback-deregulated enzyme. This reference cell may (but need not) have other genetic alterations aimed at increasing 4-APEA production, i.e., the cell may have increased activity of an upstream pathway enzyme resulting from some means other than feedback-insensitivity.

[0111] In various embodiments, the 4-APEA titers achieved by using a feedback-deregulated enzyme to increase flux though the 4-APEA biosynthetic pathway are at least 10, 20, 30, 40, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, or 900 mg/L or at least 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 10, 15, 20, 25, 30, 35, or 40 gm/L. In various embodiments, the titer is in the range of 10 mg/L to 900 mg/L, 15 mg/L to 800 mg/L, 20 mg/L to 700 mg/L, 25 mg/L to 600 mg/L, 30 mg/L to 500 mg/L, 30 mg/L to 400 mg/L, 30 mg/L to 300 mg/L, 30 mg/L to 200 mg/L, 30 mg/L to 100 mg/L, or any range bounded by any of the values listed above.

[0112] The approaches of supplementing the activity of one or more endogenous enzymes and/or introducing one or more feedback-deregulated enzymes can be combined in chorismate dehydratase-expressing microbial cells to achieve even higher 4-APEA production levels.

Reduction of Consumption of Chorismate, Its Precursors, and or Intermediates in the 4-APEA Pathway

[0113] Another approach to increasing 4-APEA production in a microbial cell that is capable of such production is to decrease the activity of one or more enzymes that consume one or more chorismate pathway precursors, that consume 4-chorismate itself, and/or one or more intermediates in the pathway leading from chorismate to 4-APEA. In an illustrative embodiment, the activity or expression of dihydroxyacetone phosphatase, which consumes the chorismate precursor dihydroxyacetone phosphate and converts it to dihydroxyacetone is reduced. Other illustrative enzymes that consume chorismate precursors include 3-dehydroshikimate dehydratase, shikimate dehydrogenase, and phosphoenolpyruvate phosphotransferase. Examples of enzymes that consume chorismate itself include anthranilate synthase and chorismate mutase. Illustrative enzymes that consume intermediates in the 4-APEA pathway include those that convert native aromatic amino acids to the corresponding monoamines, such as decarboxylases or aromatic amino acid decarboxylases. In embodiments not aimed at producing 4-aminophenylethanol, it can be advantageous to reduce the activity of the enzymes the covert 4-APP to this compound, namely phenylpyruvate decarboxylase (or pyruvate decarboxylase) and/or alcohol dehydrogenase/acetaldehyde reductase.

[0114] In some embodiments, the activity of one or more such enzymes is reduced by modulating the expression or activity of the native enzyme(s). The activity of such enzymes can be decreased, for example, by substituting the native promoter of the corresponding gene(s) with a less active or inactive promoter or by deleting the corresponding gene(s).

[0115] Another approach to increasing 4-APEA production in a microbial cell that is capable of such production is to increase the level of the chorismate precursor phosphoenolpyruvate (PEP) levels by uncoupling the uptake of glucose from the conversion of PEP to pyruvate which occurs by phosphoenolpyruvate phosphotransferase. Phosphoenolpyruvate phosphotransferase is also called the PTS system, and consists of three genes, *ptsG*, *ptsH*, and *ptsI*. Deletion or decreased expression of any one of the phosphoenolpyruvate phosphotransferase genes if present eliminates or decreases the activity of the PTS system and improves PEP availability for DAHP synthase. This approach can be used with any microbial host (typically bacterial) that has a PTS system.

[0116] In various embodiments, the engineering of a 4-APEA-producing microbial cell to reduce precursor, or chorismate, consumption by one or more side pathways increases the 4-APEA 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, 100-fold, 150-fold, 200-fold, 250-fold, 300-fold, 350-fold, 400-fold, 450-fold, 500-fold, 550-fold, 600-fold, 650-fold, 700-fold, 750-fold, 800-fold, 850-fold, 900-fold, 950-fold, or 1000-fold. In various embodiments, the increase in 4-APEA titer is in the range of 10-fold to 1000-fold, 20-fold to 500-fold, 50-fold to 400-fold, 10-fold to 300-fold, or any range bounded by any of the values listed above. These increases are determined relative to the 4-APEA titer observed in a 4-APEA-producing microbial cell that does not include genetic alterations to reduce precursor consumption. This reference cell may (but need not) have other genetic alterations aimed at increasing 4-APEA production, i.e., the cell may have increased activity of an upstream pathway enzyme.

[0117] In various embodiments, the 4-APEA titers achieved by reducing precursor, or chorismate, consumption are at least 10, 20, 30, 40, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, or 900 mg/L or at least 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 10, 15, 20, 25, 30, 35, and 40 gm/L. In various embodiments, the titer is in the range of 10 mg/L to 900 mg/L, 15 mg/L to 800 mg/L, 20 mg/L to 700 mg/L, 25 mg/L to 600 mg/L, 30 mg/L to 500 mg/L, 30 mg/L to 400 mg/L, 30 mg/L to 300 mg/L, 30 mg/L to 200 mg/L, 30 mg/L to 100 mg/L, or any range bounded by any of the values listed above.

Increasing the NADPH Supply

[0118] Another approach to increasing 4-APEA production in a microbial cell that is capable of such production is to increase the supply of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH), which provides the reducing equivalents for biosynthetic reactions. For example, the activity of one or more enzymes that increase the NADPH supply can be increased by means similar to those described above for upstream pathway enzymes, e.g., by modulating the expression or activity of the native enzyme(s), replacing the native promoter(s) with a stronger and/or constitutive promoter, and/or

introducing one or more gene(s) encoding enzymes that increase the NADPH supply. Illustrative enzymes, for this purpose, include, but are not limited to, pentose phosphate pathway enzymes, NADP+-dependent glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and NADP+-dependent glutamate dehydrogenase. Such enzymes may be derived from any available source, including any of those described herein with respect to other enzymes. Examples include the NADPH-dependent glyceraldehyde 3-phosphate dehydrogenase (GAPDH) encoded by *gapC* from *Clostridium acetobutylicum*, the NADPH-dependent GAPDH encoded by *gapB* from *Bacillus subtilis*, and the non-phosphorylating GAPDH encoded by *gapN* from *Streptococcus mutans*.

[0119] In various embodiments, the engineering of a 4-APEA-producing microbial cell to increase the activity of one or more of such enzymes increases the 4-APEA 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, 100-fold, 150-fold, 200-fold, 250-fold, 300-fold, 350-fold, 400-fold, 450-fold, 500-fold, 550-fold, 600-fold, 650-fold, 700-fold, 750-fold, 800-fold, 850-fold, 900-fold, 950-fold, or 1000-fold. In various embodiments, the increase in 4-APEA titer is in the range of 10-fold to 1000-fold, 20-fold to 500-fold, 50-fold to 400-fold, 10-fold to 300-fold, or any range bounded by any of the values listed above. (Ranges herein include their endpoints.) These increases are determined relative to the 4-APEA titer observed in a 4-APEA-producing microbial cell that lacks any increase in activity of such enzymes. This reference cell may have one or more other genetic alterations aimed at increasing 4-APEA production.

[0120] In various embodiments, the 4-APEA titers achieved by reducing precursor, or 4-APEA, consumption are at least 10, 20, 30, 40, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, or 900 mg/L or at least 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 10, 15, 20, 25, 30, 35, or 40 gm/L. In various embodiments, the titer is in the range of 10 mg/L to 900 mg/L, 15 mg/L to 800 mg/L, 20 mg/L to 700 mg/L, 25 mg/L to 600 mg/L, 30 mg/L to 500 mg/L, 30 mg/L to 400 mg/L, 30 mg/L to 300 mg/L, 30 mg/L to 200 mg/L, 30 mg/L to 100 mg/L, or any range bounded by any of the values listed above.

[0121] Any of the approaches for increasing 4-APEA production described above can be combined, in any combination, to achieve even higher 4-APEA production levels.

Illustrative Amino Acid and Nucleotide Sequences

[0122] The following table identifies amino acid and nucleotide sequences used in Example 1. The corresponding sequences are shown in the Sequence Listing.

Table A. SEQ ID NO Cross-Reference Table for Coding Sequences

Zymergen Gene Name	Gene_function	UniProt	Source Organism	AA SEQ ID NO:	NT SEQ ID NO:
aro4_Sc_K229L*	DAHP synthase		S. cerevisiae	1	56
aro1_Kpas	aro1		K. pastoris	2	57
papA_St	papA	A0A2M9JGB8	Streptomyces sp. CB01635	3	58
papA_Pf	papA	C3K4Z9	Pseudomonas fluorescens (strain SBW25)	4	59
papB_Sp	papB	D9UBV6	Streptomyces pristinaespiralis	5	60
papB_Pl	papB	Q7N1B4	Photorhabdus laumondii subsp. laumondii (strain DSM 15139 / CIP 105565 / TT01)	6	61
papC_Ps	papC	A0A2G5MIC2	Pseudomonas sp. 2822	7	62
papC_Pf	papC	C3K4Z8	Pseudomonas fluorescens (strain SBW25)	8	63
TYDC2_Ps	DC	P54769	Papaver somniferum (Opium poppy)	9	64
PheDC_Ef	DC	Q1JTV5	Enterococcus faecium (Streptococcus faecium)	10	65
AADC1A_Sl	DC	Q1KSC6	Solanum lycopersicum (Tomato) (Lycopersicon esculentum)	11	66
AADC1B_Sl	DC	Q1KSC5	Solanum lycopersicum (Tomato) (Lycopersicon esculentum)	12	67
tyrB_Ec	AT	P04693	Escherichia coli (strain K12)	13	68
AT_Ph	AT	V5M241	Petunia hybrida	14	69
TAT2_At	AT	Q9LVY1	Arabidopsis thaliana	15	70

aroT_Cg	AT	Q8NTT4	C. glutamicum	16	71
papA_Af	papA	B8ND45	Aspergillus flavus (strain ATCC 200026)	17	72
papA_As	papA	A0A0X4JQ12	Arthrobacter sp. ATCC 21022	18	73
papA_Fo	papA	A0A0J9VM93	Fusarium oxysporum f. sp. lycopersici (NRRL 34936)	19	74
papA_Sp	papA	A0A221P513	Streptomyces pluripotens	20	75
papA_Sc	papA	P37254	Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)	21	76
papB_Pp	papB	L1M7A5	Pseudomonas putida CSV86	22	77
papB_Pl48	papB	A0A0S8YVA0	Pseudomonas sp. Leaf48	23	78
papB_Ph	papB	A0A0F5VI64	Photobacterium halotolerans	24	79
papB_Pa	papB	B6VKG0	Photorhabdus asymbiotica subsp. asymbiotica (strain ATCC 43949)	25	80
papB_Sp_A127G*	papB	P72541	Streptomyces pristinaespiralis	26	81
papC_SCB	papC	A0A2M9JGH1	Streptomyces sp. CB01635	27	82
papC_Pwy	papC	A0A098MBF8	Paenibacillus wynnii	28	83
papC_Xdo	papC	A0A068QYW6	Xenorhabdus doucetiae	29	84
papC_Pfr	papC	A0A1H5DP46	Pseudomonas frederiksbergensis	30	85
papC_Pte	papC	T0P9D9	Photorhabdus temperata subsp. temperata M1021	31	86
ybdL_Eco	AT	P77806	Escherichia coli (strain K12)	32	87
hisC_Csu	AT	Q8R5Q4	Caldanaerobacter subterraneus subsp. Tengcongensis	33	88
aro9_Sce	AT	P38840	Saccharomyces cerevisiae	34	89

aatA_Ddi	AT	Q55F21	Dictyostelium discoideum	35	90
tyrB_Kpe	AT	O85746	Klebsiella pneumoniae	36	91
dapL_Ctr	AT	O84395	Chlamydia trachomatis	37	92
tyrB_Pde	AT	P95468	Paracoccus denitrificans	38	93
aro4_Sc_Q166K*	DAHP synthase		S. cerevisiae	39	94
aro4_Sc_Q166K*_K229L*	DAHP synthase		S. cerevisiae	40	95
DC_Aor	DC	Q2U3F8	Aspergillus oryzae (strain ATCC 42149)	41	96
mfn_Mja	DC	Q60358	Methanocaldococcus jannaschii	42	97
ATZ33_Esi	DC	A0A0S3K779	Enterococcus silesiacus	43	98
aro4_Kph_K219L*	DAHP synthase	F2QLN6	Komagataella phaffii (strain ATCC 76273 / CBS 7435 / CECT 11047 / NRRL Y-11430 / Wegner 21-1) (Yeast) (Pichia pastoris)	44	99
aro4_Kph_Q156K*	DAHP synthase	F2QLN6	Komagataella phaffii (strain ATCC 76273 / CBS 7435 / CECT 11047 / NRRL Y-11430 / Wegner 21-1) (Yeast) (Pichia pastoris)	45	100
aro4_Kph_Q156K*_K219L*	DAHP synthase	F2QLN6	Komagataella phaffii (strain ATCC 76273 / CBS 7435 / CECT 11047 / NRRL Y-11430 / Wegner 21-1) (Yeast) (Pichia pastoris)	46	101
aro4_Kpa_K237L*	DAHP synthase	A0A1B2J7W6	Komagataella pastoris (Yeast) (Pichia pastoris)	47	102
aro4_Kpa_Q174K*	DAHP synthase	A0A1B2J7W6	Komagataella pastoris (Yeast) (Pichia pastoris)	48	103
aro4_Kpa_Q174K*_K237L*	DAHP synthase	A0A1B2J7W6	Komagataella pastoris (Yeast) (Pichia pastoris)	49	104
aro3_Kpa_K222L*	DAHP synthase	A0A1B2JG33	Komagataella pastoris (Yeast) (Pichia pastoris)	50	105

Aro3_Kph_K230L*	DAHP synthase	A0A1G4KQ85	Komagataella phaffii (strain ATCC 76273 / CBS 7435 / CECT 11047 / NRRL Y-11430 / Wegner 21-1) (Yeast) (Pichia pastoris)	51	106
aro3_Scer_K222L*	DAHP synthase	P14843	Saccharomyces cerevisiae (strain ATCC 204508 / S288c) (Baker's yeast)	52	107
aroG_Eco_D146N*_P150L*	DAHP synthase	P0AB91	Escherichia coli (strain K12)	53	108
aroF_Eco_N8K*	DAHP synthase	P00888	Escherichia coli (strain K12)	54	109
aroH__Eco	DAHP synthase	P00887	Escherichia coli (strain K12)	55	110

The gene name follows the format XXX_YYY_ZZZ, where X is the gene name, Y is the organism identifier, and Z is mutation information if applicable.

*The encoded enzymes include the amino acid substitutions indicated using standard notation. Double mutants are indicated by the format XXX_YYY_ZZZ_1_ ZZZ_2_, where ZZZ_1 indicates a first amino acid substitution and ZZZ_2_ indicates a second amino acid substitution.

[0123] The following table identifies nucleotide sequences for regulable promoters that can be used to express any of the genes discussed herein. The corresponding sequences are shown in the Sequence Listing.

Table B. SEQ ID NO Cross-Reference Table for Regulable Promoters

Class of regulation	Promoter name	Gene name	Gene function	Source Organism	Inducer/repressor	NT SEQ ID NO:
Methanol induction	pAOX1	AOX1	alcohol oxidase 1	Komagataella pastoris	Methanol induced	111
Methanol induction	pAOX2	AOX2	alcohol oxidase 2	Komagataella pastoris	Methanol induced	112
Methanol induction	pDAS1	DAS1	Dihydroxyacetone synthase 1	Komagataella pastoris	Methanol induced	113
Methanol induction	pDAS2	DAS2	Dihydroxyacetone synthase 2	Komagataella pastoris	Methanol induced	114
Methanol induction	pFBA2	FBA2	fructose-1,6-bisphosphate aldolase 2	Komagataella pastoris	Methanol induced	115
Methanol induction	pTAL2	TAL2	transaldolase 2	Komagataella pastoris	Methanol induced	116
Methanol induction	pPMP20	PMP20	peroxiredoxin	Komagataella pastoris	Methanol induced	117
Thiamine repression	pTHI11	THI11	4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate synthase	Komagataella pastoris	Thiamine repressed	118
Thiamine repression	pTHI20	THI20	Hydroxymethylpyrimidine/phosphomethylpyrimidine kinase	Komagataella pastoris	Thiamine repressed	119
Thiamine repression	pTHI21	THI21	Hydroxymethylpyrimidine (HMP) and HMP-phosphate kinase	Komagataella pastoris	Thiamine repressed	120
Thiamine repression	pTHI5	THI5	4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate synthase	Komagataella pastoris	Thiamine repressed	121
Thiamine repression	pTHI13	THI13	4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate synthase	Komagataella pastoris	Thiamine repressed	122
Thiamine repression	pTHI4	THI4	Thiazole synthase	Komagataella pastoris	Thiamine repressed	123

Thiamine repression	pTHI6	THI6	Thiamine-phosphate diphosphorylase and hydroxyethylthiazole kinase	Komagataella pastoris	Thiamine repressed	124
Thiamine repression	pTHI80	THI80	Thiamine pyrophosphokinase	Komagataella pastoris	Thiamine repressed	125
Thiamine repression	pTHI72	THI72	Thiamine transporter	Komagataella pastoris	Thiamine repressed	126
Thiamine repression	pTHI73	THI73	Thiamine pathway transporter	Komagataella pastoris	Thiamine repressed	127
Methionine repression	pMET3	MET3	ATP sulfurylase (sulfate adenylyltransferase)	Komagataella pastoris	Methionine repressed	128
Methionine repression	pMET17	MET17	Homocysteine/cysteine synthase	Komagataella pastoris	Methionine repressed	129
Methanol induction	pAOX1	AOX1	alcohol oxidase 1	Komagataella phaffii	Methanol induced	130
Methanol induction	pAOX2	AOX2	alcohol oxidase 2	Komagataella phaffii	Methanol induced	131
Methanol induction	pDAS1	DAS1	Dihydroxyacetone synthase 1	Komagataella phaffii	Methanol induced	132
Methanol induction	pDAS2	DAS2	Dihydroxyacetone synthase 2	Komagataella phaffii	Methanol induced	133
Methanol induction	pFBA2	FBA2	fructose-1,6-bisphosphate aldolase 2	Komagataella phaffii	Methanol induced	134
Methanol induction	pTAL2	TAL2	transaldolase 2	Komagataella phaffii	Methanol induced	135
Methanol induction	pPMP20	PMP20	peroxiredoxin	Komagataella phaffii	Methanol induced	136
Thiamine repression	pTHI11	THI11	4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate synthase	Komagataella phaffii	Thiamine repressed	137
Methionine repression	pMET3	MET3	ATP sulfurylase (sulfate adenylyltransferase)	Komagataella phaffii	Methionine repressed	138
Methionine repression	pMET17	MET17	Homocysteine/cysteine synthase	Komagataella phaffii	Methionine repressed	139
Low glucose induction	pG1	GTH1	high-affinity glucose transporter	Komagataella pastoris	Low glucose induced	140
Low glucose induction	pG3	GTH1	high-affinity glucose transporter	Komagataella pastoris	Low glucose induced	141

Low glucose induction	pG4	GTH1	high-affinity glucose transporter	Komagataella pastoris	Low glucose induced	142
Low glucose induction	pG6	GTH1	high-affinity glucose transporter	Komagataella pastoris	Low glucose induced	143
Low glucose induction	pG7	GTH1	high-affinity glucose transporter	Komagataella pastoris	Low glucose induced	144
Low glucose induction	pG8	GTH1	high-affinity glucose transporter	Komagataella pastoris	Low glucose induced	145
Lysine repression	pLYS1	LYS1	Saccharopine dehydrogenase (NAD+, L-lysine-forming)	Komagataella pastoris	Lysine repressed	146
Lysine repression	pLYS9	LYS9	Saccharopine dehydrogenase (NADP+, L-glutamate-forming)	Komagataella pastoris	Lysine repressed	147
Lysine repression	pLYS1	LYS1	Saccharopine dehydrogenase (NAD+, L-lysine-forming)	Komagataella phaffii	Lysine repressed	148
Lysine repression	pLYS9	LYS9	Saccharopine dehydrogenase (NADP+, L-glutamate-forming)	Komagataella phaffii	Lysine repressed	149
Threonine repression	pTHR1	THR1	Homoserine kinase	Komagataella pastoris	Threonine repressed	150
Serine repression	pSER1	SER1	3-phosphoserine aminotransferase	Komagataella pastoris	Serine repressed	151
Zinc repression	pPIS1	PIS1	Phosphatidylinositol synthase	Komagataella pastoris	Zinc repressed	152
Phosphate repression	pPHO5	PHO5	Repressible acid phosphatase	Komagataella pastoris	Phosphate repressed	153
Phosphate repression	pPHO89	PHO89	Phosphate transporter	Komagataella pastoris	Phosphate repressed	154
Copper induction	pCUP1	CUP1	Copper metallothionein 1-1	Saccharomyces cerevisiae	Copper induced	155
Copper induction	pLCC1	LCC1	laccase	Pycnoporus coccineus	Copper induced	156
Copper repression	pCTR3	CTR3	Copper transport protein	Saccharomyces cerevisiae	Copper repressed	157
Nitrogen Catabolite Repression	pGAP1	GAP1	General amino acid permease	Komagataella pastoris	Low/ poor nitrogen conditions (proline, urea, low ammonia concentrations)	158

Ethanol inducible	pICL1	ICL1	Isocitrate lyase	Komagataella pastoris	Ethanol induced	159

Table C. SEQ ID NO Cross-Reference Table for Constitutive Promoters

Promoter name	Source organism	NT SEQ ID NO
pTEF_Ag	Ashbya gossypii	160
pAOX1	Komagataella pastoris	161
pILV5	Komagataella pastoris	162
pGCW14	Komagataella pastoris	163
pTEF1_Sc	Saccharomyces cerevisiae	164
pGAP	Komagataella pastoris	165
P0472	Komagataella pastoris	166
pTDH3_KP	Komagataella pastoris	167
pENO1_KP	Komagataella pastoris	168
pKEX2_KP	Komagataella pastoris	169
p1_pTEFI_KPA	Komagataella pastoris	170
p2_pADHI_KPA	Komagataella pastoris	171
p3_pPGKI_KPA	Komagataella pastoris	172
p4_pTPI1_KPA	Komagataella pastoris	173

p5_pTDH3_KPA	Komagataella pastoris	174
p6_pRPL3_KPA	Komagataella pastoris	175
p7_pSSB2_KPA	Komagataella pastoris	176
p8_pYEF3_KPA	Komagataella pastoris	177
p9_pENO1_KPA	Komagataella pastoris	178
p10_pHHF2_KPA	Komagataella pastoris	179
p11_pHTB2_KPA	Komagataella pastoris	180
p12_pRPL18B_KPA	Komagataella pastoris	181
p13_pALD6_KPA	Komagataella pastoris	182
p14_pALD4_KPA	Komagataella pastoris	183
p15_pALD5_KPA	Komagataella pastoris	184
p16_pPAB1_KPA	Komagataella pastoris	185
p17_pRNR1_KPA	Komagataella pastoris	186
p18_pSAC6_KPA	Komagataella pastoris	187
p19_pRNR2_KPA	Komagataella pastoris	188
p20_pPOP6_KPA	Komagataella pastoris	189
p21_pRAD27_KPA	Komagataella pastoris	190
p22_pPSP2_KPA	Komagataella pastoris	191

p23_pREV1_KPA	Komagataella pastoris	192
p24_pHXT7p_KPA	Komagataella pastoris	193
p25_pGPM1_KPA	Komagataella pastoris	194
p26_pGPD1_KPA	Komagataella pastoris	195
p27_pFBA1_KPA	Komagataella pastoris	196
p28_pPDC1_KPA	Komagataella pastoris	197
p29_pPYK1_KPA	Komagataella pastoris	198
p30_pPGI1_KPA	Komagataella pastoris	199
p31_pCYC1_KPA	Komagataella pastoris	200
p32_pHSP82_KPA	Komagataella pastoris	201
p33_pILV5_KPA	Komagataella pastoris	202
p34_pKAR2_KPA	Komagataella pastoris	203
p35_pKEX2_KPA	Komagataella pastoris	204
p36_pPET9_KPA	Komagataella pastoris	205
p37_pSSA4_KPA	Komagataella pastoris	206
p38_pTEFI_SC	Saccharomyces cerevisiae	207
p39_pADHI_SC	Saccharomyces cerevisiae	208
p40_pPGKI_SC	Saccharomyces cerevisiae	209

p41_pTPI1_SC	Saccharomyces cerevisiae	210
p42_pTDH3_SC	Saccharomyces cerevisiae	211
p43_pTEF2_SC	Saccharomyces cerevisiae	212
p44_pRPL3_SC	Saccharomyces cerevisiae	213
p45_pSSB1_SC	Saccharomyces cerevisiae	214
p46_pYEF3_SC	Saccharomyces cerevisiae	215
p47_pENO2_SC	Saccharomyces cerevisiae	216
p48_pCCW12_SC	Saccharomyces cerevisiae	217
p49_pHHF2_SC	Saccharomyces cerevisiae	218
p50_pHHF1_SC	Saccharomyces cerevisiae	219
p51_pHTB2_SC	Saccharomyces cerevisiae	220
p52_pRPL18B_SC	Saccharomyces cerevisiae	221
p53_pALD6_SC	Saccharomyces cerevisiae	222
p54_pPAB1_SC	Saccharomyces cerevisiae	223
p55_pRET2_SC	Saccharomyces cerevisiae	224
p56_pRNR1_SC	Saccharomyces cerevisiae	225
p57_pSAC6_SC	Saccharomyces cerevisiae	226
p58_pRNR2_SC	Saccharomyces cerevisiae	227

p59_pPOP6_SC	Saccharomyces cerevisiae	228
p60_pRAD27_SC	Saccharomyces cerevisiae	229
p61_pPSP2_SC	Saccharomyces cerevisiae	230
p62_pREV1_SC	Saccharomyces cerevisiae	231
p63_pHXT7p_SC	Saccharomyces cerevisiae	232
p64_pGPM1_SC	Saccharomyces cerevisiae	233
p65_pGPD1_SC	Saccharomyces cerevisiae	234
p66_pGPD2_SC	Saccharomyces cerevisiae	235
p67_pFBA_SC	Saccharomyces cerevisiae	236
p68_pPDC1_SC	Saccharomyces cerevisiae	237
p69_pPYK1_SC	Saccharomyces cerevisiae	238
p70_pTDH2_SC	Saccharomyces cerevisiae	239
p71_pPGI1_SC	Saccharomyces cerevisiae	240
p72_pCYC1_SC	Saccharomyces cerevisiae	241
p73_pHSP82_SC	Saccharomyces cerevisiae	242
p74_pILV5_SC	Saccharomyces cerevisiae	243
p75_pKAR2_SC	Saccharomyces cerevisiae	244
p76_pKEX2_SC	Saccharomyces cerevisiae	245

p77_pPET9_SC	Saccharomyces cerevisiae	246
p78_pSSA4_SC	Saccharomyces cerevisiae	247
p79_pGCW14	Komagataella pastoris	248
p80_pTEF1	Komagataella pastoris	249
p81_pPGK1	Komagataella pastoris	250
p83_pGAP	Komagataella pastoris	251
p91_pARG4	Komagataella pastoris	252
p92_pILV5	Komagataella pastoris	253
p194_pSPI1_KPA	Komagataella pastoris	254
p195_p41110_KPA	Komagataella pastoris	255
p196_pCPR1_KPA	Komagataella pastoris	256
p197_pTRX1_KPA	Komagataella pastoris	257
p198_pSDH4_KPA	Komagataella pastoris	258
p199_pBMH1_KPA	Komagataella pastoris	259
p200_pHHF2_KPA	Komagataella pastoris	260
p201_pRPS10_KPA	Komagataella pastoris	261
p202_pHSP12_KPA	Komagataella pastoris	262
p203_pGTH1_KPA	Komagataella pastoris	263

p204_p28050_KPA	Komagataella pastoris	264
p205_p06620_KPA	Komagataella pastoris	265
p206_p21610_KPA	Komagataella pastoris	266
p0472_v2	Komagataella pastoris	267
pGCW14_v2	Komagataella pastoris	268
pTEF1-EM7	Komagataella pastoris	269

Microbial Host Cells

[0124] Any microbe that can be used to express introduced genes and has a high tolerance to toxicity associated with the production of 4-APEA can be engineered for fermentative production of 4-APEA as described above. In certain embodiments, the microbe is one that is naturally incapable of fermentative production of 4-APEA. 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. In some embodiments, fungal cells, such as yeast cells, or bacterial cells can be engineered as described above. Examples of suitable yeast cells include yeast cells of the genus *Komagataella* (e.g., *K. pastoris*, as referred to as *Pinchia pastoris*) and of the genus *Yarrowia* (e.g., *Y. lipolytica*). Examples of suitable bacterial cells include bacterial cells of the genus *Bacillus* (e.g., *B. lichenformis*).

Genetic Engineering Methods

[0125] Microbial cells can be engineered for fermentative 4-APEA 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).

[0126] 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.

[0127] 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).

[0128] 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).

[0129] Example 1 describes illustrative integration approaches for introducing polynucleotides and other genetic alterations into the genomes of *K. pastoris* cells.

[0130] 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

[0131] The above-described methods can be used to produce engineered microbial cells that produce, and in certain embodiments, overproduce, 4-APEA. Engineered microbial cells can have at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 or more genetic alterations, such as 30-100 alterations, as compared to a native 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 native microbial cell. In various embodiments, microbial cells engineered for 4-APEA 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.

[0132] In some embodiments, an engineered microbial cell expresses at least one heterologous (e.g., non-native) gene, e.g., a 4-APEA pathway gene. In various embodiments, for each of the heterologous genes introduced, the microbial cell can include and express, for example: (1) a single copy of a given gene, (2) two or more copies of the gene, which can be the same or different (in other words, multiple copies of the same heterologous gene can be introduced or multiple, different genes encoding the same enzyme can be introduced), (3) a single heterologous gene that is not native to the cell and one or more additional copies of a native gene (if applicable), or (4) two or more non-native genes, which can be the same or different, and/or one or more additional copies of a native gene (if applicable).

[0133] In certain embodiments, this engineered host cell can include at least one additional genetic alteration that increases flux through any pathway leading to the

production of chorismate. As discussed above, this can be accomplished by one or more of the following: increasing the activity of upstream enzymes, e.g., by introducing a feedback-deregulated version of a DAHP synthase, alone or in combination with other means for increasing the activity of upstream enzymes.

[0134] The engineered microbial cells can contain introduced genes that have a native nucleotide sequence or that differ from native. For example, the native nucleotide sequence can be codon-optimized for expression in a particular host cell. Codon optimization for a particular host can, for example, be based on the codon usage tables found at www.kazusa.or.jp/codon/. The amino acid sequences encoded by any of these introduced genes can be native or can differ from native. In various embodiments, the amino acid sequences have at least 60 percent, 70 percent, 75 percent, 80 percent, 85 percent, 90 percent, 95 percent or 100 percent amino acid sequence identity with a native amino acid sequence.

[0135] The approach described herein has been carried out in yeast cells, namely *Komagataella pastoris* (See Example 1.)

Illustrative Engineered Yeast Cells

[0136] In certain embodiments, the engineered yeast (e.g., *K. pastoris*) cell expresses:

[0137] one or more non-native 4-amino-4-deoxychorismate synthase(s) having at least 70 percent, 75 percent, 80 percent, 85 percent, 90 percent, 95 percent or 100 percent amino acid sequence identity with a with a 4-amino-4-deoxychorismate synthase from *Pseudomonas fluorescens* (strain SBW25);

[0138] one or more non-native 4-amino-4-deoxychorismate mutase(s) having at least 70 percent, 75 percent, 80 percent, 85 percent, 90 percent, 95 percent or 100 percent amino acid sequence identity with a 4-amino-4-deoxychorismate mutase from *Photorhabdus laumondii subsp. laumondii* (strain DSM 15139 / CIP 105565 / TT01);

[0139] one or more non-native 4-amino-4-deoxyprephenate dehydrogenase(s) having at least 70 percent, 75 percent, 80 percent, 85 percent, 90 percent, 95 percent or 100 percent amino acid sequence identity with a 4-amino-4-deoxyprephenate dehydrogenase from *Pseudomonas fluorescens* (strain SBW25);

[0140] optionally, a non-native aminotransferase(s) having at least 70 percent, 75 percent, 80 percent, 85 percent, 90 percent, 95 percent or 100 percent amino acid sequence identity with an aminotransferase from *Escherichia coli* (strain K12);

[0141] optionally, one or more non-native decarboxylase(s) (DC) having at least 70 percent, 75 percent, 80 percent, 85 percent, 90 percent, 95 percent or 100 amino acid sequence identity with a decarboxylase (DC) from *Papaver somniferum.*

[0142] In particular embodiments, the:

[0143] 4-amino-4-deoxychorismate synthase (papA) from *Pseudomonas fluorescens* (strain SBW25) includes SEQ ID NO:4;

[0144] 4-amino-4-deoxychorismate mutase (papB) from *Photorhabdus laumondii subsp. laumondii* (strain DSM 15139 / CIP 105565 / TT01)includes SEQ ID NO:6;

[0145] 4-amino-4-deoxyprephenate dehydrogenase (papC) from *Pseudomonas fluorescens* (strain SBW25) includes SEQ ID NO:8;

[0146] aminotransferase (AT) from Escherichia coli (strain K12), if present, includes SEQ ID NO:13; and

[0147] decarboxylase (DC) from *Papaver somniferum*, if present, includes SEQ ID NO:9. In an illustrative embodiment, a titer of about 34 mg/L 4-APEA was achieved after engineering *K. pastoris* to express SEQ ID NOs:4, 6, 8, 9, and 13.

[0148] In certain embodiments, the engineered yeast (e.g., *K. pastoris*) cell expresses:

[0149] one or more non-native 4-amino-4-deoxychorismate synthase(s) having at least 70 percent, 75 percent, 80 percent, 85 percent, 90 percent, 95 percent or 100 percent amino acid sequence identity with a with a 4-amino-4-deoxychorismate synthase from *Streptomyces* sp. CB01635;

[0150] one or more non-native 4-amino-4-deoxychorismate mutase(s) having at least 70 percent, 75 percent, 80 percent, 85 percent, 90 percent, 95 percent or 100 percent amino acid sequence identity with a 4-amino-4-deoxychorismate mutase from *Streptomyces pristinaespiralis*;

[0151] one or more non-native 4-amino-4-deoxyprephenate dehydrogenase(s) having at least 70 percent, 75 percent, 80 percent, 85 percent, 90 percent, 95 percent or

100 percent amino acid sequence identity with a 4-amino-4-deoxyprephenate dehydrogenase from *Pseudomonas* sp. 2822;

[0152] optionally, one or more non-native aminotransferase(s) having at least 70 percent, 75 percent, 80 percent, 85 percent, 90 percent, 95 percent or 100 percent amino acid sequence identity with an aminotransferase from *Petunia hybrida*;

[0153] optionally, one or more non-native decarboxylase (DC) having at least 70 percent, 75 percent, 80 percent, 85 percent, 90 percent, 95 percent or 100 amino acid sequence identity with a decarboxylase (DC) from *Papaver somniferum*.

[0154] In particular embodiments, the:

[0155] 4-amino-4-deoxychorismate synthase (papA) from *Streptomyces* sp. CB01635 includes SEQ ID NO:3;

[0156] 4-amino-4-deoxychorismate mutase (papB) from *Streptomyces pristinaespiralis* includes SEQ ID NO:5;

[0157] 4-amino-4-deoxyprephenate dehydrogenase (papC) from *Pseudomonas* sp. 2822 includes SEQ ID NO:7;

[0158] aminotransferase (AT) from *Petunia hybrida*, if present, includes SEQ ID NO:14; and

[0159] decarboxylase (DC) from *Papaver somniferum*, if present, includes SEQ ID NO:9. In an illustrative embodiment, a titer of about 16 mg/L 4-APEA was achieved after engineering *K. pastoris* to express SEQ ID NOs:3, 5, 7, 9, and 14.

[0160] In embodiments aimed at producing, 4-APP, the illustrative engineered microbial cell can express just the first three of these five enzymes. To produce 4-APhe, the illustrative engineered microbial cell can express just the first four of these five enzymes. To produce 4-APEA, the illustrative engineered microbial cell can express all five of these enzymes.

Culturing of Engineered Microbial Cells

[0161] Any of the microbial cells described herein can be cultured, e.g., for maintenance, growth, and/or for production of 4-APEA or any of the above products described herein.

[0162] 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.

[0163] In various embodiments, the cultures have 4-APEA, 4-APP, 4-APhe, or 4-aminophenylethanol titers of at least 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, or 900 mg/L or at least 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 10, 15, 20, 25, 30, 35, or 40 gm/L. In various embodiments, the titer is in the range of 5 mg/L to 800 mg/L, 10 mg/L to 700 mg/L, 15 mg/L to 600 mg/L, 20 mg/L to 500 mg/L, 25 mg/L to 400 mg/L, 30 mg/L to 300 mg/L, 30 mg/L to 200 mg/L, 30 mg/L to 100 mg/L, or 30 mg/L to 50 mg/L, or any range bounded by any of the values listed above. In various embodiments in which 4-APEA (or other product) yield has been increased, e.g., by any of the means described herein, the titer is in the range of 10 mg/L to 900 mg/L, 15 mg/L to 800 mg/L, 20 mg/L to 700 mg/L, 25 mg/L to 600 mg/L, 30 mg/L to 500 mg/L, 30 mg/L to 400 mg/L, 30 mg/L to 300 mg/L, 30 mg/L to 200 mg/L, 30 mg/L to 100 mg/L, or any range bounded by any of the values listed above.

Culture Media

[0164] 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.

[0165] 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.

[0166] 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.

[0167] Minimal medium can be supplemented with one or more selective agents, such as antibiotics.

[0168] To produce 4-APEA or any of the other products described herein, 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

[0169] 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.

[0170] 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.

[0171] 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.

[0172] 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.

[0173] 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, 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).

[0174] 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).

[0175] Where regulated promoters are employed, the culture conditions can be adjusted to up- or down-regulate the promoter. For example, where thiamine-repressed promoters are employed, if thiamine is initially present in a sufficient amount for repression, the promoters become more active as thiamine is consumed during culturing. If it is advantageous to delay de-repression, thiamine can be added to the culture medium. In general, thiamine levels in this setting vary from 50 mg/L to 0 mg/L. Repression can occur at a thiamine concentration as low as 1 mg/L.

4-Aminophenylethylamine Production and Recovery

[0176] Any of the methods described herein may further include a step of recovering 4-APEA or any of the other products described herein. In some embodiments, the product 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 the desired product 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 desired product 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.

[0177] Further steps of separation and/or purification of the desired product 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, and/or chromatography. Further purification steps can include one or more of, e.g., concentration, crystallization, precipitation, washing and drying, treatment with activated carbon, ion exchange, nanofiltration, 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.

[0178] The following examples are given for the purpose of illustrating various embodiments of the disclosure and are 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 *Komagataella pastoris* Engineered to Produce 4-Aminophenylethylamine

Abstract

[0179] This example describes strains of *Komagataella pastoris* (also called *Pichia pastoris*) that have been engineered to produce 4-aminophenylethylamine (4-APEA), a diamine monomer useful for the production of polymers and polyimide films. The full pathway for production of 4-APEA is not known to exist in nature but could be assembled by five enzymatic steps downstream of the common metabolite chorismate. The pathway is sequentially made up the following enzymes: 4-amino-4-deoxychorismate synthase (papA), 4-amino-4-deoxychorismate mutase (papB), 4-amino-4-deoxyprephenate dehydrogenase (papC), aminotransferase (AT), and decarboxylase (DC). Some of these enzymatic activities are known to exist in *K. pastoris*, but efficient and specific production of 4-APEA is challenging as 4-APEA precursors are non-native substrates for some of the pathway enzymes, and expression or flux of native pathway enzymes is likely to be

suboptimal. Partial combinatorial search of diverse enzyme sequences and pathway designs, followed by testing in *K. pastoris*, identified multiple engineered strains that produce mg/L titers of 4-APEA. These strains and pathways may also be used to make chemicals related to 4-APEA, including 4-aminophenylalanine and 4-aminophenylpyruvate or derivatives of these. Finally, we have also demonstrated 4-APEA production in *Escherichia coli*, but the high toxicity of 4-APEA to *E. coli* makes it a poor host for large-scale fermentation host. In contrast, *K. pastoris* is tolerant of high concentrations of 4-APEA and is well-suited for large-scale production.

Plasmid/DNA Design

[0180] All strains tested for this work were transformed with plasmid DNA designed using proprietary software. Plasmid designs were specific to the *K. pastoris* host 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 a host-specific method described below.

***K. pastoris* Pathway Integration**

[0181] A "split-marker, double-crossover" genomic integration strategy has been developed to engineer *K. pastoris* strains. Fig. 2 illustrates genomic integration of complementary, split-marker plasmids and verification of correct genomic integration via colony PCR in *K. pastoris*. Two plasmids with complementary 5' and 3' homology arms and overlapping halves of a selectable marker, for example an antibiotic resistance marker such as KanMX or an auxotrophic marker such as URA3, were linearized and transformed as linear fragments. Linearization was achieved either by PCR or by digestion with meganucleases. Direct repeats present in the transformed DNA fragments are shown by the hashed bars. A triple-crossover event integrated the desired heterologous genes into the targeted locus and re-constituted the full selection marker 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 counter-selection media, for example 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. (Abbreviations: Primers: UF = upstream forward, DR = downstream reverse, IR = internal reverse, IF = internal forward.)

Cell Culture

[0182] The cell culture and metabolite production workflow is initiated by 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 eight replicates were tested from distinct colonies to test colony-to-colony variation and other process variation. If fewer than eight colonies were obtained, the existing colonies were replicated so that at least eight wells were tested from each desired genotype.

[0183] The colonies were consolidated into 96-well plates with selective medium (SD-ura or YPD with antibiotic) 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 primary seed stage in YPD media grown at 30°C for 16 hours at 1000 RPM. The primary seed plates were used to inoculate a secondary seed stage in Verduyn media grown at 30°C for 24 hours at 1000 RPM. The secondary seed plates were then used to inoculate a main cultivation plate with Verduyn media supplemented with 200 mM phthalate buffer and grown at 30°C for 24-48 hours at 1000 RPM. Plates were removed at the desired time points and tested for cell density (OD600), glucose, and supernatant samples stored for LC-MS or HPLC analysis for product of interest.

Cell Density

[0184] 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.

[0185] 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, but this is not generally observed

Glucose

[0186] 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

[0187] 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.

Genetic Engineering Approach and Results

[0188] Strains for 4-APEA production are described in the Tables below. Table 1 lists specific combinations of enzymes and promoters that were built. Tables 2A-C lists the titer of 4-APEA, as well as side products and intermediates, produced by strains that have been tested. A range of titers were detected, as well as the presence of side-products (including 4-aminophenylethanol and tyramine) and pathway intermediates (including 4-aminophenylpyruvate [4-APP] and 4-aminophenylalanine [4-APhe]). Tables 3A-B show further strain designs and results. Upon scaling up to production in a fermentation tank, a titer of 11.1 g/L APEA was achieved for strain 7001065091 (described below).

Table 1 - Strain Designs for Engineering *Komagataella pastoris* to Produce 4-APEA

Pro-duc-tion Strain ZID	Production Strain Genotype	Constuct Genotype
70008 39167	NS3::papA_Pf/papB_Pl/papC_Pf/tyrB_EC/TYDC2_Ps	pGCW14>papA_Pf<tTIF51_Scer/P0472>papB_Pl<tAOD_Scer/pGAP>papC_Pf<tCyc1_Sc tAOX1_Rev>TYDC2_Ps_Rev<pTEF1_Sc_Rev/tARG4_Rev>tyrB_Ec_Rev<pTDH3_Kp_Rev
70008 40665	NS3::papA_St/papB_Sp/papC_Ps/tyrB_EC/PheDC	pGCW14>papA_St<tTIF51_Scer/P0472>papB_Sp<tAOD_Scer/pGAP>papC_Ps<tCyc1_Sc tAOX1_Rev>PheDC_Ef_Rev<pTEF1_Sc_Rev/tARG4_Rev>tyrB_Ec_Rev<pTDH3_Kp_Rev
70008 40664	NS3::papA_Pf/papB_Pl/papC_Pf/tyrB_EC/Phe_DC	pGCW14>papA_Pf<tTIF51_Scer/P0472>papB_Pl<tAOD_Scer/pGAP>papC_Pf<tCyc1_Sc tAOX1_Rev>PheDC_Ef_Rev<pTEF1_Sc_Rev/tARG4_Rev>tyrB_Ec_Rev<pTDH3_Kp_Rev
70008 39175	NS3::papA_St/papB_Sp/papC_Ps/TAT2_At/PheDC	pGCW14>papA_St<tTIF51_Scer/P0472>papB_Sp<tAOD_Scer/pGAP>papC_Ps<tCyc1_Sc tAOX1_Rev>PheDC_Ef_Rev<pTEF1_Sc_Rev/tARG4_Rev>TAT2_At_Rev<pTDH3_Kp_Rev
70008 40676	NS3::papA_St/papB_Sp/papC_Ps/AT_Ph/TYDC2_Ps	pGCW14>papA_St<tTIF51_Scer/P0472>papB_Sp<tAOD_Scer/pGAP>papC_Ps<tCyc1_Sc tAOX1_Rev>TYDC2_Ps_Rev<pTEF1_Sc_Rev/tARG4_Rev>AT_Ph_Rev<pTDH3_Kp_Rev
70008 39664	NS3::papA_Pf/papB_Pl/papC_Pf/TAT2_At/PheDC	pGCW14>papA_Pf<tTIF51_Scer/P0472>papB_Pl<tAOD_Scer/pGAP>papC_Pf<tCyc1_Sc tAOX1_Rev>PheDC_Ef_Rev<pTEF1_Sc_Rev/tARG4_Rev>TAT2_At_Rev<pTDH3_Kp_Rev
70008 39164	NS3::papA_Pf/papB_Pl/papC_Pf/AT_Ph/TYDC2_Ps	pGCW14>papA_Pf<tTIF51_Scer/P0472>papB_Pl<tAOD_Scer/pGAP>papC_Pf<tCyc1_Sc
	NS3::papA_Pf/papB_Pl/papC_Pf/AT_Ph/TYDC2_Ps	tAOX1_Rev>TYDC2_Ps_Rev<pTEF1_Sc_Rev/tARG4_Rev>AT_Ph_Rev<pTDH3_Kp_Rev

Table 2A-2C – Titer of 4-APEA and Related Compounds in *Komagataella pastoris* Strains Engineered to Produce 4-APEA

2A:

		Metabolite Titer (mg/L) at 48 hours									
Production Strain ZID	**Production Strain Genotype**	**4-APE**	**StDev**	**4-APP**	**StDev**	**4-Aphe**	**StDev**	**4-APEA**	**StDev**	**Tyramine**	**StDev**
7000839167	NS3::papA_Pf/papB_Pl/papC_Pf/tyrB_EC/TYDC2_Ps	73.6	16.3	23.8	5.5	5.9	1.2	33.7	7.1	44.5	9.7
7000840665	NS3::papA_St/papB_Sp/papC_Ps/tyrB_EC/PheDC	78.3	32.3	64.3	42.0	5.0	4.2	16.2	6.1	96.1	35.1

2B:

		Metabolite titer at 24 hours (mg/L)									
Strain ID	**Strain genotype**	**4-APEA**	**Phenyl-ethyl-amine**	**Amino-phenyl-acetate**	**4-Amino-phenyl-ethanol + p-amino-benzoate**	**4-APhe**	**4-APP**	**Chorismate**	**Phenyl-alanine**	**Tyrosine**	**Tyramine**
7000840664	papA_Pf/papB_Pl/papC_Pf/PheDC_Ef_Rev/tyrB_Ec_Rev	67.97	11.01	1.25	64.39	6.62	14.21	10.27	4.51	0.26	26.98
7000840665	papA_St/papB_Sp/papC_Ps/PheDC_Ef_Rev/tyrB_Ec_Rev	28.86	6.21	1.67	80.21	6.03	47.38	11.76	3.22	0.18	10.97
7000839167	papA_Pf/papB_Pl/papC_Pf/TYDC2_Ps_Rev/tyrB_Ec_Rev	119.22	2.25	1.2	25.21	3.08	12.77	4.4	1.52	0.26	3.74

Strain ID	Strain genotype	4-APEA	Phenyl-ethyl-amine	Amino-phenyl-acetate	4-Amino-phenyl-ethanol + p-amino-benzoate	4-APhe	4-APP	Chorismate	Phenyl-alanine	Tyrosine	Tyramine
7000840676	papA_St/papB_Sp/papC_Ps/TYDC2_Ps_Rev/AT_Ph_Rev	151.75	2.18	5.03	156.31	11.35	87.27	15.53	5.38	0.27	0.83
7000839664	papA_Pf/papB_Pl/papC_Pf/PheDC_Ef_Rev/TAT2_At_Rev	117.2	2.31	1.92	44.44	2.01	18.32	1.59	1	0.24	4.24
7000839175	papA_St/papB_Sp/papC_Ps/PheDC_Ef_Rev/TAT2_At_Rev	49.98	10.89	4.48	181.26	12.5	105.02	19.84	6.02	0.25	8.99
7000839164	papA_Pf/papB_Pl/papC_Pf/TYDC2_Ps_Rev/AT_Ph_Rev	122.4	2.34	1.88	44.79	2.07	18.3	1.85	1.07	0.25	4.15

2C:

Fermentation tank production timecourse by strain 7000839167	
Time (hours)	APEA titer (g/L)
6	0
25	0.67
30	1.43
49	4.23
54	4.64
74	6.49

Table 3A-3B – Titer of 4-APEA in *Komagataella pastoris* Strains Engineered to Produce 4-APEA

3A: Specific strain genotypes conferring high-level constitutive and regulable production of 4-APEA

Strain ID	Strain type	APEA after 96 hour fermentation (mg/L)	Strain genotype
7001065091	constitutive production	11172	NS3::pGCW14 :papA_St/pTDH3:papB_Pa/pGAP:papC_Xdo/p0472:aroT_Cg/pTDH3:TYDC2_Ps
7001001659	regulated production	6665	NS3::pGCW14:papA_Pf/p0472:papB_Pl/pGAP:papC_Pf/pTDH3:tyrB_Ec/pTHI11:TYDC2_Ps; pENO1:GLN1
7000869673	constitutive production	4257	NS3::pGCW14:papA_Pf/p0472:papB_Pl/pGAP:papC_Pf/pTDH3:tyrB_Ec/pTHI11:TYDC2_Ps
7000931166	regulated production	4872	NS3::pGCW14:papA_Pf/p0472:papB_Pl/pGAP:papC_Pf/pTDH3:tyrB_Ec/pTEF1:TYDC2_Ps

5

3B: Increased 4-APEA production through promoter replacement or knockout of native genes

Strain ID	Parent Strain ID	Genotype change	Modification type	Promoter	Target locus	Gene function	Fold improvement over parent
7001023092	7000869673	KO:CYB2	gene deletion	n/a	CYB2	L-lactate dehydrogenase (cytochrome)	1.07
7001023104	7000869673	KO:GAP1	gene deletion	n/a	GAP1	yeast amino acid transporter	1.05
7001064504	7000931166	KO:PDC1	gene deletion	n/a	PDC1	pyruvate decarboxylase	1.12
7001023100	7000869673	KO:PFK26	gene deletion	n/a	PFK26	6-phosphofructo-2-kinase	1.06
7001022086	7000931166	KO:PHO13	gene deletion	n/a	PHO13	phosphoglycolate phosphatase	1.47
7001064486	7000931166	p115_pTHI11_KPA:ABZ1	promoter replacement	p115_pTHI11_KPA	ABZ1	para-aminobenzoate synthetase	1.29
7001064973	7000869673	p9_pENO1_KPA:ARO7	promoter replacement	p9_pENO1_KPA	ARO7	chorismate mutase	1.11
7001001784	7000931166	p115_pTHI11_KPA:ARP2	promoter replacement	p115_pTHI11_KPA	ARP2	hexokinase	1.13
7001002220	7000931166	p9_pENO1_KPA:ATP4	promoter replacement	p9_pENO1_KPA	ATP4	F-type H+-transporting ATPase subunit b	1.21

Strain ID	Parent Strain ID	Genotype change	Modification type	Promoter	Target locus	Gene function	Fold improvement over parent
7001001799	7000931166	p5_pTDH3_KPA:ATP4	promoter replacement	p5_pTDH3_KPA	ATP4	F-type H+-transporting ATPase subunit b	1.09
7001001658	7000931166	p9_pENO1_KPA:COX11	promoter replacement	p9_pENO1_KPA	COX11	cytochrome c oxidase assembly protein subunit 11	1.13
7001001646	7000931166	p5_pTDH3_KPA:COX11	promoter replacement	p5_pTDH3_KPA	COX11	cytochrome c oxidase assembly protein subunit 11	1.18
7001002197	7000869673	p9_pENO1_KPA:COX13	promoter replacement	p9_pENO1_KPA	COX13	cytochrome c oxidase subunit 6a	1.13
7000999114	7000869673	p35_pKEX2_KPA:COX5A	promoter replacement	p35_pKEX2_KPA	COX5A	cytochrome c oxidase subunit 4	1.17
7000999147	7000931166	p9_pENO1_KPA:CYT1	promoter replacement	p9_pENO1_KPA	CYT1	ubiquinol-cytochrome c reductase cytochrome c1 subunit	1.21
7001001659	7000931166	p9_pENO1_KPA:GLN1	promoter replacement	p9_pENO1_KPA	GLN1	glutamine synthetase	1.86
7001002203	7000931166	p5_pTDH3_KPA:GLN1	promoter replacement	p5_pTDH3_KPA	GLN1	glutamine synthetase	1.23
7000999152	7000931166	p115_pTHI11_KPA:MEP2	promoter replacement	p115_pTHI11_KPA	MEP2	ammonium transporter, Amt family	1.07
7001001647	7000931166	p9_pENO1_KPA:MEP2	promoter replacement	p9_pENO1_KPA	MEP2	ammonium transporter, Amt family	1.26
7001002668	7000931166	p35_pKEX2_KPA:MEP2	promoter replacement	p35_pKEX2_KPA	MEP2	ammonium transporter, Amt family	1.25
7001003167	7000931166	p35_pKEX2_KPA:NUFM	promoter replacement	p35_pKEX2_KPA	NUFM	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex subunit 5	1.76
7001001657	7000931166	p9_pENO1_KPA:NUFM	promoter replacement	p9_pENO1_KPA	NUFM	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex subunit 5	1.80
7000999120	7000869673	p5_pTDH3_KPA:NUFM	promoter replacement	p5_pTDH3_KPA	NUFM	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex subunit 5	1.15
7001001766	7000869673	p35_pKEX2_KPA:NUGM	promoter replacement	p35_pKEX2_KPA	NUGM	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex subunit 5	1.18
7001064492	7000931166	p115_pTHI11_KPA:PDC2	promoter replacement	p115_pTHI11_KPA	PDC2	transcription factor	2.03
7001065470	7000931166	p5_pTDH3_KPA:PDC2	promoter replacement	p5_pTDH3_KPA	PDC2	transcription factor	1.84

Strain ID	Parent Strain ID	Genotype change	Modification type	Promoter	Target locus	Gene function	Fold improvement over parent
7000999163	7000931166	p5_pTDH3_KPA:PFK1:PFK2	promoter replacement	p5_pTDH3_KPA	PFK1:PFK2	6-phosphofructokinase 1	1.17
7001001645	7000931166	p9_pENO1_KPA:PGI1	promoter replacement	p9_pENO1_KPA	PGI1	glucose-6-phosphate isomerase	1.52
7000999115	7000869673	p5_pTDH3_KPA:PGI1	promoter replacement	p5_pTDH3_KPA	PGI1	glucose-6-phosphate isomerase	1.14
7001002670	7000931166	p5_pTDH3_KPA:RPE1-1	promoter replacement	p5_pTDH3_KPA	RPE1-1	ribulose-phosphate 3-epimerase	1.15
7001001764	7000869673	p132_pPHO5_KPA:SHB17	promoter replacement	p132_pPHO5_KPA	SHB17	sedoheptulose-bisphosphatase	1.13
7001001786	7000931166	p5_pTDH3_KPA:SHB17	promoter replacement	p5_pTDH3_KPA	SHB17	sedoheptulose-bisphosphatase	1.24
7001064988	7000931166	p120_pTHI4_KPA:SHP1	promoter replacement	p120_pTHI4_KPA	SHP1	anthranilate synthase component I	1.35
7001064972	7000869673	p9_pENO1_KPA:SHP1	promoter replacement	p9_pENO1_KPA	SHP1	anthranilate synthase component I	1.15
7001064990	7000931166	p35_pKEX2_KPA:SHP1	promoter replacement	p35_pKEX2_KPA	SHP1	anthranilate synthase component I	1.15
7001063958	7000869673	p5_pTDH3_KPA:SHP1	promoter replacement	p5_pTDH3_KPA	SHP1	anthranilate synthase component I	1.17
7000999143	7000931166	p119_pTHI13_KPA:SOL1	promoter replacement	p119_pTHI13_KPA	SOL1	6-phosphogluconolactonase	1.24
7000999160	7000931166	p35_pKEX2_KPA:SOL1	promoter replacement	p35_pKEX2_KPA	SOL1	6-phosphogluconolactonase	1.17
7001002206	7000931166	p9_pENO1_KPA:SOL1	promoter replacement	p9_pENO1_KPA	SOL1	6-phosphogluconolactonase	1.20
7001001793	7000931166	p115_pTHI11_KPA:TAL1-2	promoter replacement	p115_pTHI11_KPA	TAL1-2	transaldolase	1.19
7001001648	7000931166	p9_pENO1_KPA:TAL1-2	promoter replacement	p9_pENO1_KPA	TAL1-2	transaldolase	1.29
7001001795	7000931166	p5_pTDH3_KPA:TAL1-2	promoter replacement	p5_pTDH3_KPA	TAL1-2	transaldolase	1.31
7001001656	7000931166	p35_pKEX2_KPA:TEX1	promoter replacement	p35_pKEX2_KPA	TEX1	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex subunit 9	1.22

Strain ID	Parent Strain ID	Genotype change	Modification type	Promoter	Target locus	Gene function	Fold improvement over parent
7000999156	7000931166	p9_pENO1_KPA:TEX1	promoter replacement	p9_pENO1_KPA	TEX1	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex subunit 9	1.14
7001001790	7000931166	p5_pTDH3_KPA:TEX1	promoter replacement	p5_pTDH3_KPA	TEX1	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex subunit 9	1.30
7001063953	7000869673	p35_pKEX2_KPA:TRP3	promoter replacement	p35_pKEX2_KPA	TRP3	anthranilate synthase / indole-3-glycerol phosphate synthase / phosphoribosylanthranilate isomerase	1.15
7001064498	7000931166	p35_pKEX2_KPA:TRP3	promoter replacement	p35_pKEX2_KPA	TRP3	anthranilate synthase / indole-3-glycerol phosphate synthase / phosphoribosylanthranilate isomerase	1.19
7001064488	7000931166	p5_pTDH3_KPA:TRP3	promoter replacement	p5_pTDH3_KPA	TRP3	anthranilate synthase / indole-3-glycerol phosphate synthase / phosphoribosylanthranilate isomerase	1.12
7001064494	7000869673	p9_pENO1_KPA:ZWF1	promoter replacement	p9_pENO1_KPA	ZWF1	glucose-6-phosphate 1-dehydrogenase	1.13
7001065469	7000869673	p5_pTDH3_KPA:ZWF1	promoter replacement	p5_pTDH3_KPA	ZWF1	glucose-6-phosphate 1-dehydrogenase	1.14

REFERENCES

[0189] 1. Masuo et al. (2016) Scientific Reports 6: 25764.

CLAIMS

What is claimed is:

1. An engineered microbial cell that produces 4-aminophenylethylamine (4-APEA), wherein the engineered microbial cell has a high tolerance to toxicity associated with the production of 4-APEA, as defined by a concentration at which the growth of engineered microbial cell is slowed by half (Ki) of at least 30 grams/liter, wherein the engineered microbial cell optionally comprises a yeast cell, optionally a cell of the genus *Komagataella*, optionally wherein the yeast cell is a cell of the species *pastoris* or *phaffi*.

2. The engineered microbial cell of claim 1, wherein the engineered microbial cell heterologously expresses each of the following enzyme activities:

4-amino-4-deoxychorismate synthase;

4-amino-4-deoxychorismate mutase;

4-amino-4-deoxyprephenate dehydrogenase;

aminotransferase (AT); and

decarboxylase (DC);

wherein the enzyme activities are provided by heterologously expressing genes encoding the enzymes, and at least one heterologously expressed enzyme is non-native to the engineered microbial cell, optionally wherein at least two, three, four, or all of the heterologously expressed enzymes are non-native to the engineered microbial cell.

3. An engineered microbial cell of the genus *Komagataella* that produces 4-aminophenylpyruvate (4-APP), optionally wherein the engineered microbial cell is a cell of the species *pastoris* or *phaffi*.

4. The engineered microbial cell of claim 3, wherein the engineered microbial cell heterologously expresses each of the following enzyme activities:

4-amino-4-deoxychorismate synthase;

4-amino-4-deoxychorismate mutase; and

4-amino-4-deoxyprephenate dehydrogenase,

wherein each enzyme activity is provided by heterologously expressing genes encoding the enzymes, and at least one heterologously expressed enzyme is non-native to the engineered microbial cell.

5. The engineered microbial cell of any one of claims 3-4, wherein the engineered microbial cell additionally produces 4-aminophenylalanine (4-APhe).

6. The engineered microbial cell of claim 5, wherein the engineered microbial cell additionally heterologously expresses an aminotransferase (AT) activity.

7. The engineered microbial cell of any one of claims 3-4, wherein the engineered microbial cell additionally produces 4-aminophenylethanol.

8. The engineered microbial cell of claim 7, wherein the engineered microbial cell additionally heterologously expresses an alcohol dehydrogenase/acetaldehyde reductase enzyme.

9. The engineered microbial cell of any one of claims 3-8, wherein at least two, three, or all of the heterologously expressed enzymes are non-native to the engineered microbial cell.

10. The engineered microbial cell of any one of claims 1-9, wherein the engineered microbial cell comprises increased activity of one or more upstream chorismate pathway enzyme(s), said increased activity being increased relative to a control cell, optionally wherein said increased activity is selected from the group consisting of glucokinase, transketolase, transaldolase, phospho-2-dehydro-3-deoxyheptonate aldolase, 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase, 3-dehydroquinate synthase, 3-dehydroquinate dehydratase, shikimate dehydrogenase, shikimate kinase, 3-phosphoshikimate 1-carboxyvinyltransferase, chorismate synthase activity, and any combination thereof.

11. The engineered microbial cell of any one of claims 1-10, wherein the engineered microbial cell comprises increased activity of one or more nitrogen assimilation and utilization pathway enzyme(s), optionally wherein said increased activity is selected from the group consisting of isocitrate dehydrogenase, glutamine synthetase,

glutamate synthase, glutamate dehydrogenase, ammonium permease, and any combination thereof.

12. The engineered microbial cell of any one of claims 1-11, wherein the engineered microbial cell comprises reduced activity of one or more enzyme(s) that consume one or more chorismate pathway precursors, chorismate, and/or one or more intermediates in the pathway leading from chorismate to 4-APEA, and/or more enzymes that consume 4-APEA, said reduced activity being reduced relative to a control cell, optionally wherein the one or more enzyme(s) that consume one or more chorismate pathway precursors are selected from the group consisting of dihydroxyacetone phosphatase, 3-dehydroshikimate dehydratase, shikimate dehydrogenase, and phosphoenolpyruvate phosphotransferase, optionally wherein the one or more enzyme(s) that consume chorismate are selected from the group consisting of anthranilate synthase and chorismate mutase, optionally wherein the one or more enzyme(s) that consume one or more intermediates in the pathway leading from chorismate to 4-APEA are selected from the group consisting of decarboxylase, aromatic amino acid decarboxylase, phenylpyruvate decarboxylase, pyruvate decarboxylase, aromatic amino acid ammonia lyase, and alcohol dehydrogenase/acetaldehyde reductase, optionally wherein the one or more enzymes that consume 4-APEA are selected from the group consisting of phenylpyruvate dioxygenase, diamine oxidase, amine oxidase, and amino acid oxidase.

13. The engineered microbial cell of any one of claims 1-12, wherein the engineered microbial cell additionally expresses a feedback-deregulated DAHP synthase.

14. The engineered microbial cell of any one of claims 1-13, wherein the engineered microbial cell comprises increased activity of one or more enzyme(s) that increase the supply of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH), said increased activity being increased relative to a control cell, optionally wherein the one or more enzyme(s) that increase the supply of the reduced form of NADPH are selected from the group consisting of pentose phosphate pathway enzymes, NADP+-dependent glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and NADP+-dependent glutamate dehydrogenase.

15. The engineered microbial cell of claim 1, wherein the non-native enzymes comprise:

a 4-amino-4-deoxychorismate synthase having at least 70% amino acid sequence identity with a 4-amino-4-deoxychorismate synthase from *Pseudomonas fluorescens* (strain SBW25);

a 4-amino-4-deoxychorismate mutase having at least 70% amino acid sequence identity with a 4-amino-4-deoxychorismate mutase from *Photorhabdus laumondii subsp. laumondii* (strain DSM 15139 / CIP 105565 / TT01);

a 4-amino-4-deoxyprephenate dehydrogenase having at least 70% amino acid sequence identity with a 4-amino-4-deoxyprephenate dehydrogenase from *Pseudomonas fluorescens* (strain SBW25);

optionally, an aminotransferase (AT) having at least 70% amino acid sequence identity with an aminotransferase (AT) from Escherichia coli (strain K12); and

optionally a decarboxylase (DC) having at least 70% amino acid sequence identity with a decarboxylase (DC) from *Papaver somniferum*.

16. The engineered microbial cell of claim 15, wherein the:

4-amino-4-deoxychorismate synthase from *Pseudomonas fluorescens* (strain SBW25) comprises SEQ ID NO:4;

4-amino-4-deoxychorismate mutase from *Photorhabdus laumondii subsp. laumondii* (strain DSM 15139 / CIP 105565 / TT01) comprises SEQ ID NO:6;

4-amino-4-deoxyprephenate dehydrogenase from *Pseudomonas fluorescens* (strain SBW25) comprises SEQ ID NO:8;

aminotransferase (AT) from Escherichia coli (strain K12), if present, comprises SEQ ID NO:(SEQ ID NO:13); and

decarboxylase (DC) from *Papaver somniferum*, if present, comprises SEQ ID NO:9.

17. The engineered microbial cell of claim 1, wherein the non-native enzymes comprise:

a 4-amino-4-deoxychorismate synthase having at least 70% amino acid sequence identity with a 4-amino-4-deoxychorismate synthase from *Streptomyces* sp. CB01635;

a 4-amino-4-deoxychorismate mutase having at least 70% amino acid sequence identity with a 4-amino-4-deoxychorismate mutase from *Streptomyces pristinaespiralis*;

a 4-amino-4-deoxyprephenate dehydrogenase having at least 70% amino acid sequence identity with a 4-amino-4-deoxyprephenate dehydrogenase from *Pseudomonas* sp. 2822;

optionally, an aminotransferase (AT) having at least 70% amino acid sequence identity with an aminotransferase (AT) from *Petunia hybrida*; and

optionally, a decarboxylase (DC) having at least 70% amino acid sequence identity with a decarboxylase (DC) from *Papaver somniferum*.

18. The engineered microbial cell of claim 17, wherein the:

4-amino-4-deoxychorismate synthase from *Streptomyces* sp. CB01635 comprises SEQ ID NO:3;

4-amino-4-deoxychorismate mutase from *Streptomyces pristinaespiralis* comprises SEQ ID NO:5;

4-amino-4-deoxyprephenate dehydrogenase from *Pseudomonas* sp. 2822 comprises SEQ ID NO:7;

aminotransferase (AT) from *Petunia hybrida*, if present, comprises SEQ ID NO:14; and

decarboxylase (DC) from *Papaver somniferum*, if present, comprises SEQ ID NO:9.

19. The engineered microbial cell of claim 1, wherein the non-native enzymes comprise:

a 4-amino-4-deoxychorismate synthase having at least 70% amino acid sequence identity with a 4-amino-4-deoxychorismate synthase from *Streptomyces* sp. CB01635;

a 4-amino-4-deoxychorismate mutase having at least 70% amino acid sequence identity with a 4-amino-4-deoxychorismate mutase from *Photorhabdus asymbiotica* subsp. *asymbiotica*;

a 4-amino-4-deoxyprephenate dehydrogenase having at least 70% amino acid sequence identity with a 4-amino-4-deoxyprephenate dehydrogenase from *Xenorhabdus doucetiae*;

optionally, an aminotransferase (AT) having at least 70% amino acid sequence identity with an aminotransferase (AT) from *Corynebacterium glutamicum*; and

optionally, a decarboxylase (DC) having at least 70% amino acid sequence identity with a decarboxylase (DC) from *Papaver somniferum*.

20. The engineered microbial cell of claim 17, wherein the:

4-amino-4-deoxychorismate synthase from *Streptomyces* sp. CB01635 comprises SEQ ID NO:3;

4-amino-4-deoxychorismate mutase from *Photorhabdus asymbiotica* subsp. *asymbiotica* comprises SEQ ID NO:25;

4-amino-4-deoxyprephenate dehydrogenase from *Xenorhabdus doucetiae* comprises SEQ ID NO:29;

aminotransferase (AT) from *Corynebacterium glutamicum*, if present, comprises SEQ ID NO:16; and

decarboxylase (DC) from *Papaver somniferum*, if present, comprises SEQ ID NO:9.

21. The engineered microbial cell of any one of claims 2-20, wherein the engineered microbial cell additionally comprises a genotype change selected from the group consisting of:

p9_pENO1_KPA:GLN1,

p35_pKEX2_KPA:NUFM,

p9_pENO1_KPA:NUFM,

p115_pTHI11_KPA:PDC2, and

p5_pTDH3_KPA:PDC2.

22. The engineered microbial cell of any one of claims 1-2 and 10-21, wherein, when cultured, the engineered microbial cell produces 4-APEA at a level of at least 11 gram/liter of culture medium, optionally wherein, when cultured, the engineered microbial cell produces 4-APP at a level of at least 20 milligram/liter of culture medium, optionally wherein, when cultured, the engineered microbial cell produces 4-APhe at a level of at least 5 milligram/liter of culture medium.

23. A method of culturing engineered microbial cells according to any one of claims 1-22, the method comprising culturing the cells under conditions suitable for producing 4-APP, 4-APhe, and/or 4-APEA, optionally wherein the culture comprises:

4-APP at a level of at least 20 milligram/liter of culture medium;

4-APhe at a level of at least 5 milligram/liter of culture medium; and/or

4-APEA at a level of at least 15 milligram/liter of culture medium, wherein the culture comprises 4-APEA at a level of at least 11 gram/liter of culture medium.

24. The method of claim 23, wherein the method additionally comprises recovering 4-APP, 4-APhe, and/or 4-APEA from the culture.

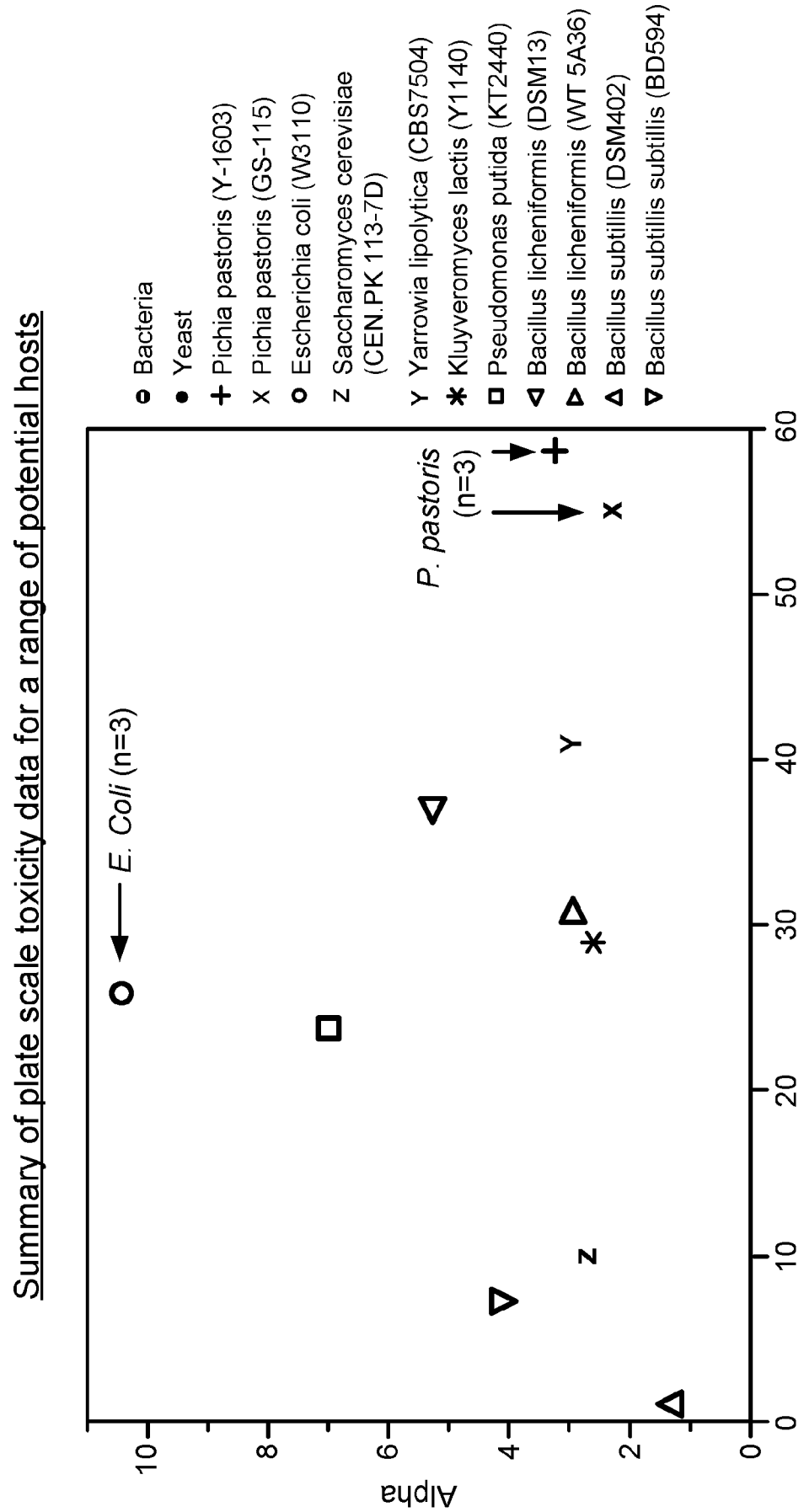
Summary of plate scale toxicity data for a range of potential hosts
Bacteria
Yeast
Pichia pastoris (Y-1603)
Pichia pastoris (GS-115)
Escherichia coli (W3110)
Saccharomyces cerevisiae (CEN.PK 113-7D)
Yarrowia lipolytica (CBS7504)
Kluyveromyces lactis (Y1140)
Pseudomonas putida (KT2440)
Bacillus licheniformis (DSM13)
Bacillus licheniformis (WT 5A36)
Bacillus subtillis (DSM402)
Bacillus subtillis subtillis (BD594)
E. Coli (n=3)
P. pastoris (n=3)
Alpha
Ki (g/L)
0
2
4
6
8
10
0
10
20
30
40
50
60

FIG. 1

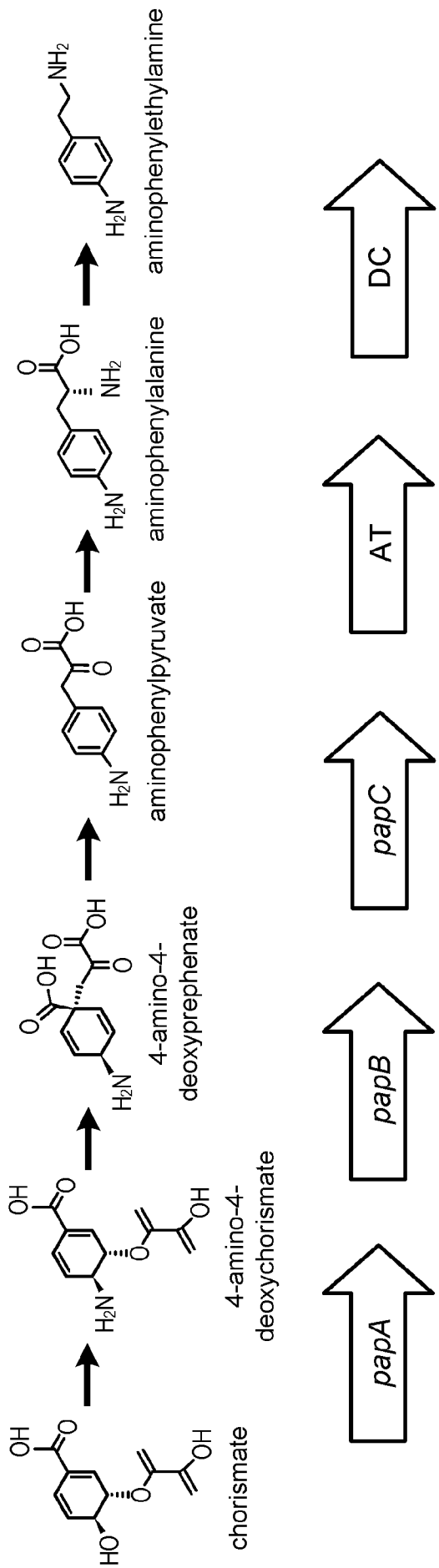
chorismate
4-amino-4-deoxychorismate
4-amino-4-deoxyprephenate
aminophenylpyruvate
aminophenylalanine
aminophenylethylamine
papA
papB
papC
AT
DC

FIG. 2

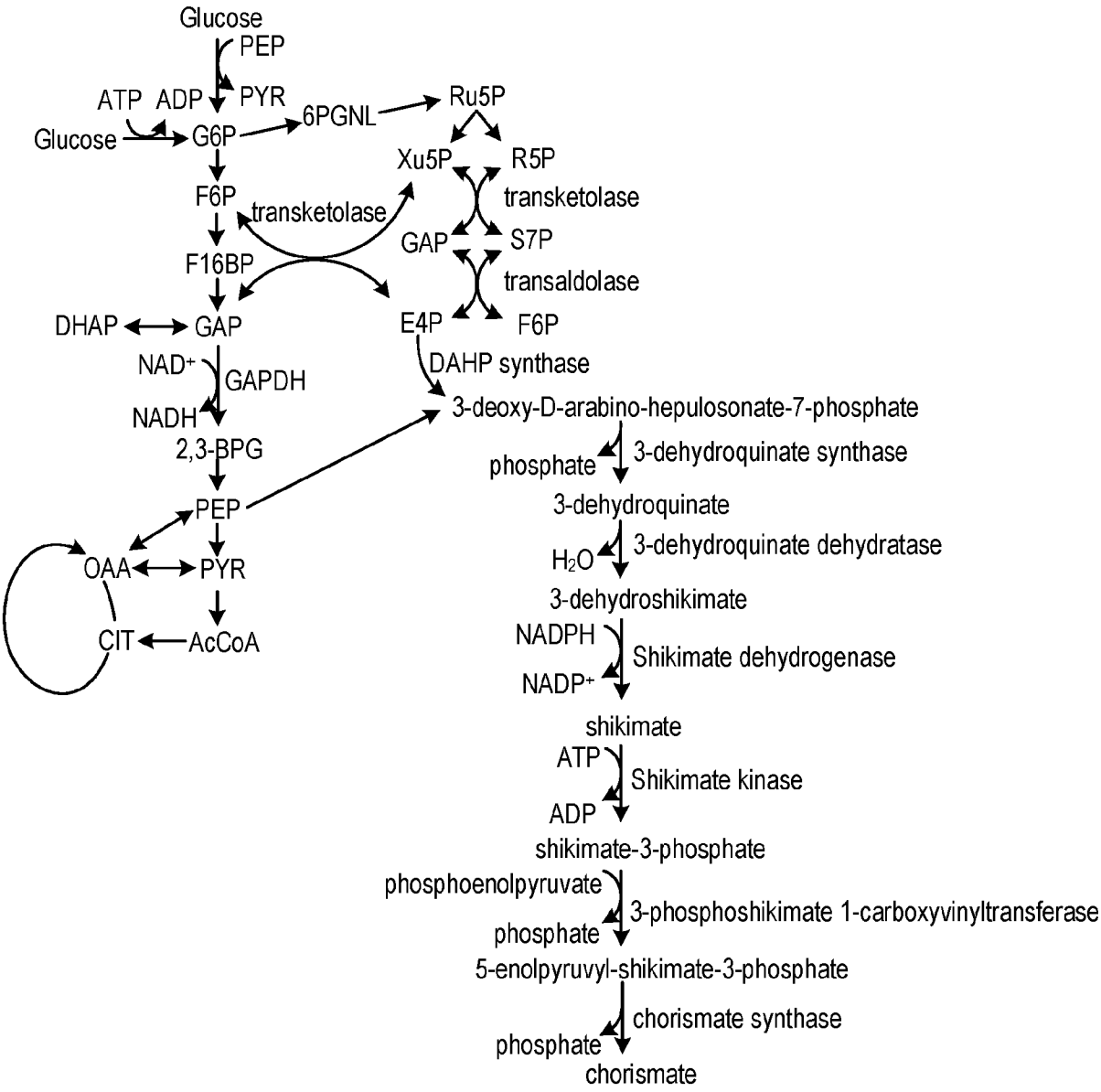
Glucose
PEP
ATP
ADP
PYR
6PGNL
Ru5P
Glucose
G6P
Xu5P
R5P
transketolase
F6P
transketolase
GAP
S7P
F16BP
transaldolase
DHAP
GAP
E4P
F6P
NAD+
GAPDH
DAHP synthase
NADH
3-deoxy-D-arabino-hepulosonate-7-phosphate
2,3-BPG
phosphate
3-dehydroquinate synthase
PEP
3-dehydroquinate
H2O
3-dehydroquinate dehydratase
OAA
PYR
3-dehydroshikimate
NADPH
Shikimate dehydrogenase
CIT
AcCoA
NADP+
shikimate
ATP
Shikimate kinase
ADP
shikimate-3-phosphate
phosphoenolpyruvate
3-phosphoshikimate 1-carboxyvinyltransferase
phosphate
5-enolpyruvyl-shikimate-3-phosphate
chorismate synthase
phosphate
chorismate

FIG. 3

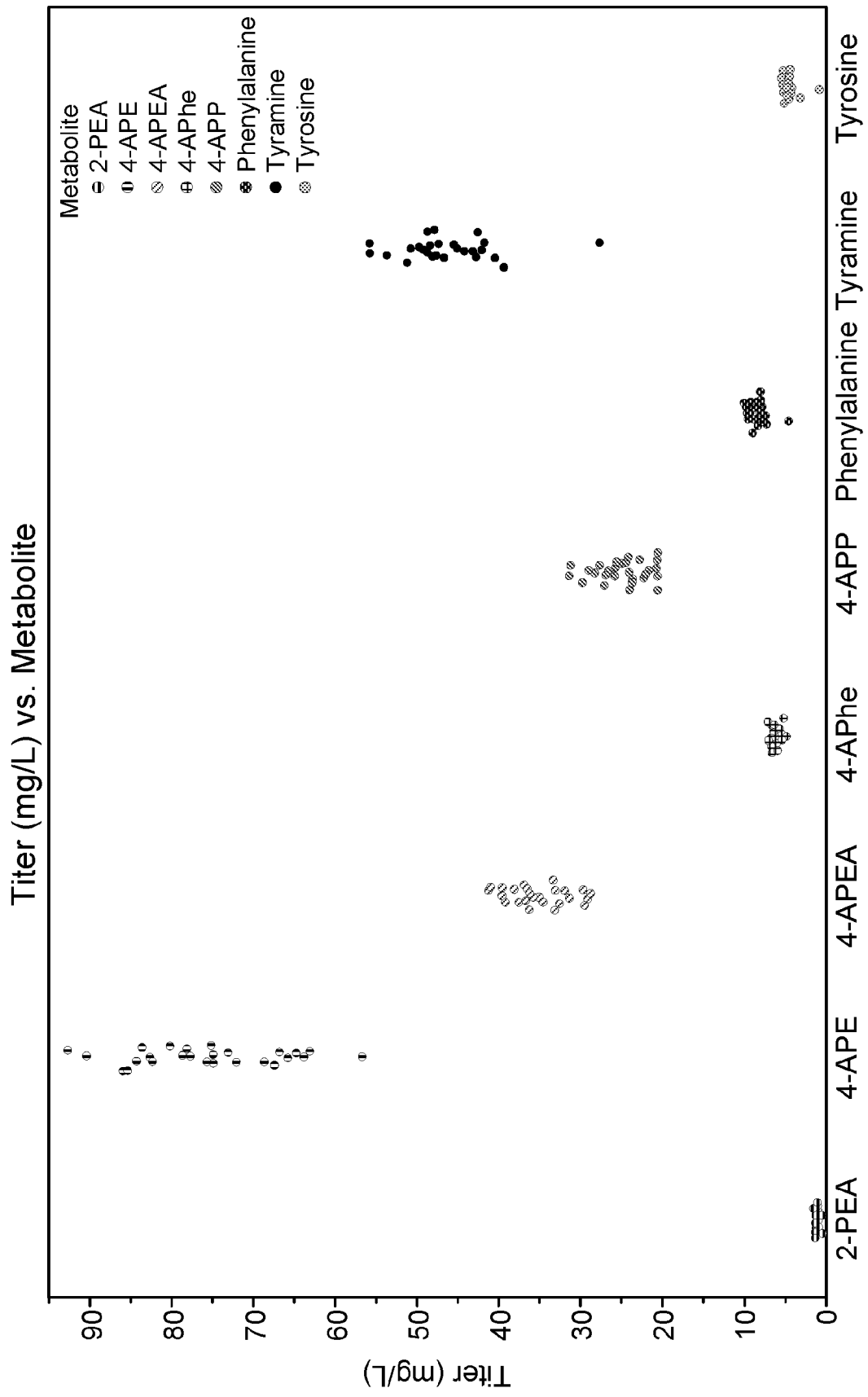
Titer (mg/L) vs. Metabolite
Metabolite
2-PEA
4-APE
4-APEA
4-APhe
4-APP
Phenylalanine
Tyramine
Tyrosine
Titer (mg/L)
0
10
20
30
40
50
60
70
80
90
2-PEA
4-APE
4-APEA
4-APhe
4-APP
Phenylalanine
Tyramine
Tyrosine
Metabolite

FIG. 4

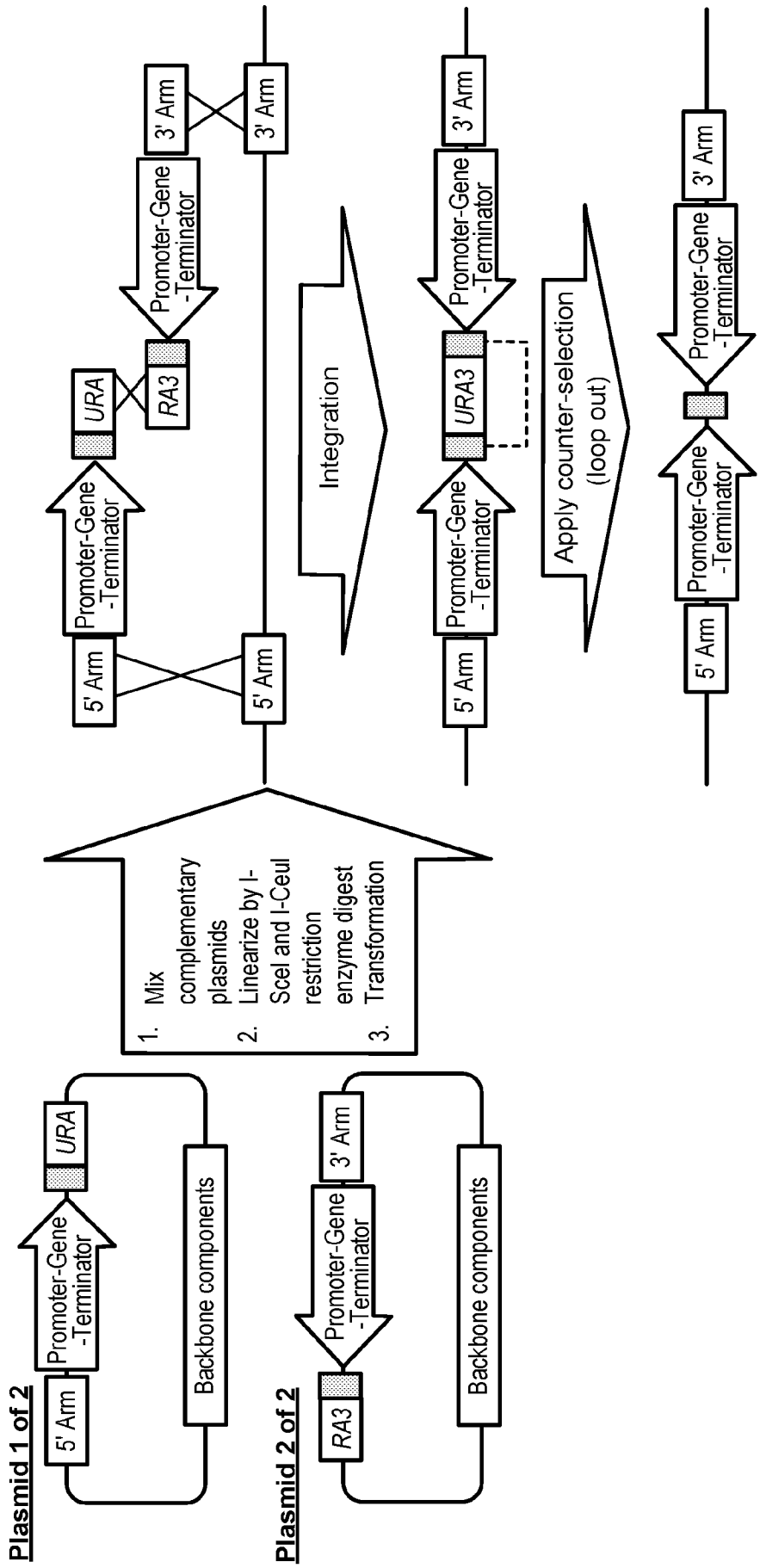
Plasmid 1 of 2
5' Arm
Promoter-Gene -Terminator
URA
Backbone components
Plasmid 2 of 2
RA3
Promoter-Gene -Terminator
3' Arm
Backbone components
1. Mix complementary plasmids
2. Linearize by I-SceI and I-CeuI restriction enzyme digest
3. Transformation
5' Arm
Promoter-Gene -Terminator
URA
RA3
Promoter-Gene -Terminator
3' Arm
3' Arm
Integration
5' Arm
Promoter-Gene -Terminator
URA3
Promoter-Gene -Terminator
3' Arm
Apply counter-selection (loop out)
5' Arm
Promoter-Gene -Terminator
Promoter-Gene -Terminator
3' Arm

FIG. 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2021/046756

A. CLASSIFICATION OF SUBJECT MATTER

C12P 13/00(2006.01)i; **C12N 15/81**(2006.01)i; **C12N 9/10**(2006.01)i; **C12N 9/90**(2006.01)i; **C12N 9/02**(2006.01)i; **C12N 9/88**(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12P 13/00(2006.01); C12P 13/22(2006.01)

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

Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKOMPASS(KIPO internal) & Keywords: 4-aminophenylethylamine, 4-aminophenylpyruvate, Komagataella, aromatic amine, toxicity, tolerance

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HUCCETOGULLARI, D. et al., 'Metabolic engineering of microorganisms for production of aromatic compounds', Microbial Cell Factories, 2019, Vol. 18, pp. 1-29 abstract; page 9, left column, 1st paragraph; figure 2; table 1	1-8,15-20
A	MASUO, S. et al., 'Bacterial fermentation platform for producing artificial aromatic amines', Scientific Reports, 11 May 2016, Vol. 6, Article No. 25764, pp. 1-9 the whole document	1-8,15-20
A	MOHAMMADI NARGESI, B. et al., 'Metabolic engineering of Escherichia coli for para-amino -phenylethanol and para-amino-phenylacetic acid biosynthesis', Frontiers in Bioengineering and Biotechnology, 04 January 2019, Vol. 6, Article No. 201, pp. 1-13 the whole document	1-8,15-20
A	US 2017-0022528 A1 (JAPAN SCIENCE AND TECHNOLOGY AGENCY) 26 January 2017 (2017-01-26) the whole document	1-8,15-20

☑ Further documents are listed in the continuation of Box C.

☑ See patent family annex.

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

Date of the actual completion of the international search	Date of mailing of the international search report
09 December 2021	**09 December 2021**

Name and mailing address of the ISA/KR	Authorized officer
Korean Intellectual Property Office **189 Cheongsa-ro, Seo-gu, Daejeon** **35208, Republic of Korea**	**Jung, Da Won**
Facsimile No. **+82-42-481-8578**	Telephone No. **+82-42-481-5373**

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2021/046756

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PEÑA, D. A. et al., 'Metabolic engineering of Pichia pastoris', Metabolic Engineering, Epub. 25 April 2018, Vol. 50, pp. 2-15 the whole document	1-8,15-20

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2021/046756

Box No. I **Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)**

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
   a. ☑ forming part of the international application as filed:
      ☑ in the form of an Annex C/ST.25 text file.
      ☐ on paper or in the form of an image file.
   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).
      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2021/046756

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☑ Claims Nos.: **24**
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Claim 24 is referring to the multiple dependent claims which do not comply with PCT Rule 6.4(a).

3. ☑ Claims Nos.: **9-14,21-23**
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/US2021/046756

Patent document cited in search report	Publication date (day/month/year)	Patent family member(s)	Publication date (day/month/year)
US 2017-0022528 A1	26 January 2017	CN 106471120 A	01 March 2017
		CN 106471120 B	03 July 2020
		EP 3121275 A1	25 January 2017
		JP 2017-141791 A1	13 April 2017
		JP 6570514 B2	04 September 2019
		US 9920343 B2	20 March 2018
		WO 2015-141791 A1	24 September 2015