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(71) Applicant: **BRASKEM S.A.** [BR/BR]; Rua Eteno, nº 1.561, Polo Petroquímico, Camaçari – BA, 42810-000 (BR).

(72) Inventors: **RADUAN ALEXANDRINO, Paulo Moises**; c/o Braskem S.A.,Rua Eteno, nº 1.561, Polo Petroquímico, Camaçari – BA, 42810-000 (BR). **ESTRADA GOUVEA, Iuri**; c/o Braskem S.A., Rua Eteno, nº 1.561, Polo Petroquímico, Camaçari – BA, 42810-000 (BR). **LEITE QUEIROZ, Veronica**; c/o Braskem S.A., Rua Eteno, nº 1.561, Polo Petroquímico, Camaçari – BA, 42810-000 (BR).

(74) Agent: **DANNEMANN, SIEMSEN, BIGLER & IPANEMA MOREIRA**; Rua Marquês de Olinda, 70 - Botafogo, 22251-040 Rio de Janeiro - RJ (BR).

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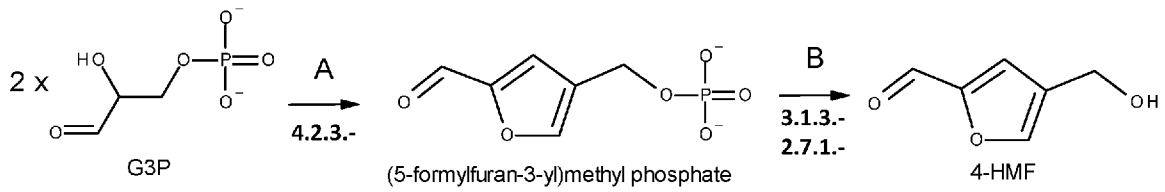
(54) Title: METHOD FOR THE IN VIVO SYNTHESIS OF 4-HYDROXYMETHYLFURFURAL AND DERIVATIVES THEREOF

FIG. 1



(57) **Abstract:** The present disclosure provides recombinant microorganisms and methods for the production of 4-HMF, 2,4-furandimethanol, furan-2,4-dicarbaldehyde, 4-(hydroxymethyl)furoic acid, 2-formylfuran-4-carboxylate, 4-formylfuran-2-carboxylate, and/or 2,4-FDCA from a carbon source. The method provides for engineered microorganisms that express endogenous and/or exogenous nucleic acid molecules that catalyze the conversion of a carbon source into 4-HMF, 2,4-furandimethanol, furan-2,4-dicarbaldehyde, 4-(hydroxymethyl)furoic acid, 2-formylfuran-4-carboxylate, 4-formylfuran-2-carboxylate, and/or 2,4-FDCA. The disclosure further provides methods of producing polymers derived from 4-HMF, 2,4-furandimethanol, furan-2,4-dicarbaldehyde, 4-(hydroxymethyl)furoic acid, 2-formylfuran-4-carboxylate, 4-formylfuran-2-carboxylate, and/or 2,4-FDCA.

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METHOD FOR THE IN VIVO SYNTHESIS OF 4-HYDROXYMETHYLFURFURAL AND DERIVATIVES THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application No. 62/812,904 filed March 1, 2019, entitled "METHOD FOR THE IN VIVO SYNTHESIS OF 4-HYDROXYMETHYLFURFURAL AND DERIVATIVES THEREOF", the disclosures of which are incorporated by reference herein.

FIELD OF THE INVENTION

[0002] This application relates to recombinant microorganisms for the biosynthesis of one or more of 4-HMF, 2,4-furandimethanol, furan-2,4-dicarbaldehyde, 4-(hydroxymethyl)furoic acid, 2-formylfuran-4-carboxylate, 4-formylfuran-2-carboxylate, and 2,4-FDCA and methods of producing the recombinant microorganisms. The application also relates to methods of producing one or more of 2,4-furandimethanol, furan-2,4-dicarbaldehyde, 4-(hydroxymethyl)furoic acid, 2-formylfuran-4-carboxylate, 4-formylfuran-2-carboxylate, and 2,4-FDCA with enzymatic catalysts in the absence of microorganisms or substantially free of microorganisms. The application further relates to methods of producing a polymer and a plasticizer agent from one or more of 2,4-furandimethanol, furan-2,4-dicarbaldehyde, 4-(hydroxymethyl)furoic acid, 2-formylfuran-4-carboxylate, 4-formylfuran-2-carboxylate, and 2,4-FDCA. The application further relates to compositions comprising one or more of these compounds and/or the recombinant microorganisms.

STATEMENT REGARDING SEQUENCE LISTING

[0003] The Sequence Listing associated with this application is provided in text format in lieu of a paper copy, and is hereby incorporated by reference into the specification. The name of the text file containing the Sequence Listing is BRSK-019_01WO_ST25.txt. The text file is about 240 KB, was created on February 27, 2020, and is being submitted electronically via EFS-Web.

BACKGROUND

[0004] 2,5-Furandicarboxylic acid (2,5-FDCA) has gained much attention due to its potential of substituting terephthalic acid in the synthesis of polyesters, specially polyethylene terephthalate

(PET) (Sousa, Andreia F., et al. "Biobased polyesters and other polymers from 2, 5-furandicarboxylic acid: a tribute to furan excellency." Polymer chemistry 6.33 (2015): 5961-5983). Substituting terephthalic acid to its furan analogue 2,5-FDCA in PET can lead to 2,5-furandicarboxylate (2,5-PEF) and this polymer has several advantages when compared to PET. In one aspect, 2,5-PEF has better thermal, barrier and mechanical properties when compared to its counterpart (PEP Report 294). Furthermore, as it is known that ethylene glycol could be produced from renewable resources, then 2,5-PEF could be 100% renewable as opposed to the semi renewable PET.

[0005] Despite all the aforementioned advantages of 2,5-FDCA in comparison to terephthalic acid, 2,5-FDCA production cost is still a current limitation in expanding the monomer usage. Existing technologies are not cost-competitive when compared to terephthalic acid. One of the possible reasons for this is related to the several sequential industrial steps required. One issue that could help reduce 2,5-FDCA production costs is finding a direct fermentation route from sugar to the desired molecule, but such a route has never been reported.

[0006] The present disclosure a direct fermentation pathway for 2,4-FDCA, an isomer of 2,5-FDCA. To our knowledge, besides the present disclosure, there is no described direct fermentation routes for any of FDCA isomers.

[0007] Significantly, the disclosed 2,4-FDCA molecule possesses unique properties compared to the well-studied 2,5-FDCA. Catalytically polymerizing 2,4-FDCA with a diol yields a polymer composed of 2,4-FDCA with valuable properties. In one study, Thiyagarajan and collaborators (2014) compare polyesters made of 2,4-FDCA, 3,4-FDCA, 2,5-FDCA and terephthalic acid and concluded that 2,4-FDCA and 3,4-FDCA polyesters can be made in sufficient molecular weights by industrially applicable methods (Thiyagarajan, Shanmugam, et al. "Biobased furandicarboxylic acids (FDCAs): effects of isomeric substitution on polyester synthesis and properties." Green Chemistry 16.4 (2014): 1957-1966). In another study, Thiyagarajan and colleagues concluded that structural analysis of 2,4-FDCA and 2,5-FDCA reveal that 2,4-FDCA possesses more linear characteristics resembling terephthalic acid than does 2,5-FDCA. These features make 2,4-FDCA an interesting monomer for synthetic polyesters (Thiyagarajan et al. “Concurrent formation of furan-2,5- and furan-2,4-dicarboxylic acid: unexpected aspects of the Henkel reaction” RSC Advances 3 (2013): 15678-15686). Further, these materials have properties unlike 2,5-FDCA polyesters (Bourdet et al. "Molecular Mobility in Amorphous Biobased Poly (ethylene 2, 5-furandicarboxylate) and Poly (ethylene 2, 4-furandicarboxylate)." Macromolecules 51.5 (2018): 1937-1945).

[0008] In certain cases, 2,4-FDCA polymers have been reported to have superior properties to those possessed by 2,5-FDCA polymers. Cui and collaborators (2016) report that the bond-angle between the double carboxyl groups linking with the central ring is a key factor that influences the stability of nematic liquid crystal molecules such as those utilized in LCD TVs, notebook computers, and other display elements (Cui, Min-Shu, et al. "Production of 4-hydroxymethylfurfural from derivatives of biomass-derived glycerol for chemicals and polymers." ACS Sustainable Chemistry & Engineering 4.3 (2016): 1707-1714). The first discovered liquid crystal, terephthalic acid diester molecules has a bond-angle between two carboxyl groups of 180°. In comparison, 2,5-furan dicarboxylic acid has a bond-angle between two carboxyl groups of 137°. Significantly, 2,4- furan dicarboxylic acid has a bond-angle between two carboxyl groups of 160° making it more suitable for synthesis of nematic liquid crystal molecules.

[0009] Despite these potential applications of 2,4-FDCA polymers, the production cost of 2,4-FDCA is also a current bottleneck in expanding this monomer applications (Cui MS, *et al.* (2016) Production of 4-Hydroxymethylfurfural from Derivatives of Biomass-Derived Glycerol for Chemicals and Polymers. ACS Sustainable Chem. Eng. 4(3):1707-1714 and WO2011003300A1). Previous synthesis of 2,4-substituted furans, including 2,4-FDCA, required multiple synthetic steps and therefore 2,4-FDCA-derived polymers are cost-prohibitive by currently available methodologies and industrial techniques.

[0010] The present disclosure provides, for the first time, a direct fermentation route to 2,4-FDCA in a recombinant microorganism. The novel direct fermentation of 2,4-FDCA from a glyceraldehyde-3-phosphate (G3P) from a carbon feedstock such as glucose, xylose, glycerol, or from any CO2 derived/capture technology will enable the production of novel polymers and materials with commercial applicability on an industrial scale. The present disclosure further provides, for the first time, direct fermentation routes for the production of one or more of 4-HMF, 2,4-furandimethanol, furan-2,4-dicarbaldehyde, 4-(hydroxymethyl)furoic acid, 2-formylfuran-4-carboxylate, and 4-formylfuran-2-carboxylate in a recombinant microorganism. The present disclosure also demonstrate, for the first time, that endogenous phosphatases from yeast and E.coli are able to dephosphorylate (5-formylfuran-3-yl)methyl phosphate to 4-HMF and that enzymes (oxidases, dehydrogenase and/or peroxigenase) are capable to oxidize the 4-HMF to 2,4 FDCA (directly or through the production of intermediates). While some of the enzymes candidates here deployed have been characterized as having activity on a 5-HMF isomer substrate,and intermediates, their activity against 4-HMF (and its intermediates) has nto been characterized. These novel direct fermentation routes will enable the production of 2,4-substituted furans with commercial applicability. *See* Deng *et al.* (2013. Linked Strategy for the

Production of Fuels via Formose Reaction. *Scientific Reports*, 3:1244) for exemplary applications of 4-HMF as a precursor to biofuels. *See* Zeng *et al.* (2013. Bio-based Furan Polymers with Self-Healing Ability. *Macromolecules*, 46.5:1794-1802) for exemplary applications of 2,4-furandimethanol in polymers with advanced properties.

SUMMARY OF THE DISCLOSURE

[0011] In certain cases, 2,4-FDCA polymers have been reported to have superior properties to those possessed by 2,5-FDCA polymers. Cui and collaborators (2016) report that the bond-angle between the double carboxyl groups linking with the central ring is a key factor that influences the stability of nematic liquid crystal molecules such as those utilized in LCD TVs, notebook computers, and other display elements (Cui, Min-Shu, et al. "Production of 4-hydroxymethylfurfural from derivatives of biomass-derived glycerol for chemicals and polymers." ACS Sustainable Chemistry & Engineering 4.3 (2016): 1707-1714). The first discovered liquid crystal, terephthalic acid diester molecules has a bond-angle between two carboxyl groups of 180°. In comparison, 2,5-furan dicarboxylic acid has a bond-angle between two carboxyl groups of 137°. Significantly, 2,4- furan dicarboxylic acid has a bond-angle between two carboxyl groups of 160° making it more suitable for synthesis of nematic liquid crystal molecules.

[0012] The disclosure provides a method of producing 2,4-furandicarboxylic acid (2,4-FDCA) by enzymatically converting glyceraldehyde 3-phosphate (G3P) to 2,4-furandicarboxylic acid (2,4-FDCA), the method comprising: (a) providing G3P in the presence of a methyl phosphate synthase that catalyzes the conversion of G3P to (5-formylfuran-3-yl)methyl phosphate; (b) providing the (5-formylfuran-3-yl)methyl phosphate from (a) a phosphatase that catalyzes the conversion of the (5-formylfuran-3-yl)methyl phosphate to 4-hydroxymethylfurfural (4-HMF); (c) providing the 4-HMF from (b) to a dehydrogenase and/or an oxidase that catalyzes independently or in synergy the oxidation of 4-HMF from (b) to 2,4 FDCA, directly or through the production of intermediates furan-2,4-dicarbaldehyde, 4-(hydroxymethyl)furoic acid, 4-formylfuran-2-carboxylate, 4-formylfuran-2-carboxylate, 2-formylfuran-4-carboxylate.

[0013] In some embodiments, the 2,4-FDCA is produced from furan-2,4-dicarbaldehyde, and/or-(hydroxymethyl)furoic acid intermediates, wherein: (a) a dehydrogenase, an oxidase, or a peroxigenase catalyzes the conversion of the 4-HMF to furan-2,4-dicarbaldehyde, and/or 4-(hydroxymethyl)furoic acid; and/or (b) a dehydrogenase, an oxidase, or a peroxigenase catalyzes the conversion of the furan-2,4-dicarbaldehyde from (a) to 4-formylfuran-2-carboxylate; and/or (c) a dehydrogenase, an oxidase, or a peroxigenase catalyzes the conversion of the 4-(hydroxymethyl)furoic acid from (a) to 4-formylfuran-2-carboxylate; and/or (d) a dehydrogenase,

an oxidase, or a peroxigenase catalyzes the conversion of the furan-2,4-dicarbaldehyde from (a) to 2-formylfuran-4-carboxylate; and or (e) a dehydrogenase, an oxidase, or a peroxigenase catalyzes the conversion of the the 4-formylfuran-2-carboxylate from (b) and/or (c) or the 2-formylfuran-4-carboxylate from (d) to 2,4-FDCA.

[0014] In some embodiments, the methyl phosphate synthase from (a) is classified as EC number 4.2.3.153. In some embodiments, the methyl phosphate synthase is (5-formylfuran-3-yl)methyl phosphate synthase. In some embodiments, the (5-formylfuran-3-yl)methyl phosphate synthase is selected from MfnB1, MfnB7, and MfnB14.

[0015] In some embodiments, the (5-formylfuran-3-yl)methyl phosphate synthase comprises an amino acid sequence comprising SEQ ID NO: 1, SEQ ID NO: 7, or SEQ ID NO: 14. In some embodiments, the phosphatase from (b) is classified as EC number 3.1.3. In some embodiments, the phosphatase is classified as a haloacid dehalogenase. In some embodiments, the phosphatase is endogenous to the host.

[0016] In some embodiments, the phosphatase enzyme endogenous to the host is overexpressed. In some embodiments, wherein the phosphatase is a 4-HMF phosphatase.

[0017] In some embodiments, the 4-HMF phosphatase is derived from Streptomyces coelicolor, Saccharomyces cerevisiae, or Escherichia coli.

[0018] In some embodiments, the 4-HMF phosphatase is encoded by an amino acid sequence comprising SEQ ID NO: 28, any one of SEQ ID NOs 40-52, or any one of SEQ ID NOs 53-68.

[0019] In some embodiments, the dehydrogenase from (c) is classified as EC number 1.1.1. or EC number 1.2.1. In some embodiments, the dehydrogenase is an alcohol dehydrogenase or an aldehyde dehydrogenase. In some embodiments, the oxidase from (c) is classified as EC number 1.1.3. In some embodiments, the oxidase is 5-hydroxymethylfurfural oxidase. In some embodiments, the dehydrogenase is classified as EC number 1.2.1. or EC number 1.1.1. In some embodiments, the dehydrogenase is an aldehyde dehydrogenase or and alcohol dehydrogenase.

[0020] In some embodiments, the oxidase is classified as EC number 1.1.3. In some embodiments, the oxidase is 5-hydroxymethylfurfural oxidase. In some embodiments, the oxidase is a 4-HMF oxidase. In some embodiments, the 4-HMF oxidase is selected from HmfH6 and HmfH7. In some embodiments, the 4-HMF oxidase comprises an amino acid sequence comprising SEQ ID NO: 85 or SEQ ID NO: 86.

[0021] In some embodiments, the dehydrogenase is classified as EC number 1.2.1. In some embodiments, the dehydrogenase is an aldehyde dehydrogenase.

[0022] The disclosure provides a recombinant microorganism capable of producing 2,4-furandicarboxylic acid (2,4-FDCA) from a feedstock comprising a carbon source, wherein the

recombinant microorganism expresses the following: (a) endogenous and/or exogenous nucleic acid molecules capable of converting a carbon source to glyceraldehyde 3-phosphate (G3P); (b) at least one endogenous or exogenous nucleic acid molecule encoding a (5-formylfuran-3-yl)methyl phosphate synthase that catalyzes the conversion of G3P from (a) to (5-formylfuran-3-yl)methyl phosphate; (c)at least one endogenous or exogenous nucleic acid molecule encoding a phosphatase that catalyzes the conversion of (5-formylfuran-3-yl)methyl phosphate from (b) to 4-hydroxymethylfurfural (4-HMF); (d) at least one endogenous or exogenous nucleic acid molecule encoding a peroxigenase, dehydrogenase, or a oxidase that catalyzes independently or in synergy the oxidation of 4-HMF from (c) to 2,4 FDCA, directly or through the production of intermediates furan-2,4-dicarbaldehyde, 4-(hydroxymethyl)furoic acid, 4-formylfuran-2-carboxylate, 4-formylfuran-2-carboxylate, 2-formylfuran-4-carboxylate.

[0023] In some embodiments, the carbon source comprises a hexose, a pentose, glycerol, C02, sucroses and/or combinations thereof. In some embodiments, the methyl phosphate synthase from (a) is classified as EC number 4.2.3.153. In some embodiments,wherein the synthase is (5-formylfuran-3-yl)methyl phosphate synthase.

[0024] In some embodiments, the phosphatase from (c) is classified as EC number 3.1.3. In some embodiments, the phosphatase is classified as haloacid dehalogenase. In some embodiments, the phosphatase is endogenous to the host. In some embodiments, phosphatase enzyme endogenous to the host is overexpressed.

[0025] In some embodiments, the oxidase from (d) is classified as EC number 1.1.3. In some embodiments, the oxidase from (d) is a 5-hydroxymethylfurfural oxidase.

[0026] In some embodiments, the dehydrogenase from (d) is classified as EC number 1.1.1. or EC number 1.2.1. In some embodiments, the dehydrogenase is an alcohol dehydrogenase or an aldehyde dehydrogenase.

[0027] In some embodiments, the 2,4-FDCA is produced from furan-2,4-dicarbaldehyde, and/or-(hydroxymethyl)furoic acid intermediates,wherein: (a) a dehydrogenase, an oxidase, or a peroxigenase catalyzes the conversion of the 4-HMF from (c) to furan-2,4-dicarbaldehyde, and/or 4-(hydroxymethyl)furoic acid; and/or (b) a dehydrogenase, an oxidase, or a peroxigenase catalyzes the conversion of the furan-2,4-dicarbaldehyde from (a) to 4-formylfuran-2-carboxylate; and/or (c) a dehydrogenase, an oxidase, or a peroxigenase catalyzes the conversion of the 4-(hydroxymethyl)furoic acid from (b) to 4-formylfuran-2-carboxylate; and/or (d) a dehydrogenase, an oxidase, or a peroxigenase catalyzes the conversion of the furan-2,4-dicarbaldehyde from (c) to 2-formylfuran-4-carboxylate; and/or (e) a dehydrogenase, an oxidase, or a peroxigenase

catalyzes the conversion of the 4-formylfuran-2-carboxylate from (b) and/or (c) or the 2-formylfuran-4-carboxylate from (d) to 2,4-FDCA.

[0028] In some embodiments, the dehydrogenase from (a), (b), (c), (d) and/or (e) is classified as EC number 1.2.1. or EC number 1.1.1 In some embodiments, the dehydrogenase is an aldehyde dehydrogenase or an alcohol dehydrogenase. In some embodiments, the oxidase from (a), (b), (c), (d) and/or (e) is classified as EC number 1.1.3. In some embodiments, the oxidase is 5-(hydroxymethyl)furfural oxidase.

[0029] In some embodiments, the one or more recombinant microorganisms are derived from a parental microorganism selected from the group consisting of Clostridium sp., Clostridium ljungdahlii, Clostridium autoethanogenum, Clostridium ragsdalei, Eubacterium limosum, Butyribacterium methylotrophicum, Moorella thermoacetica, Corynebacterium glutamicum, Clostridium aceticum, Acetobacterium woodii, Alkalibaculum bacchii, Clostridium drakei, Clostridium carboxidivorans, Clostridium formicoaceticum, Clostridium scatologenes, Moorella thermoautotrophica, Acetonema longum, Blautia producta, Clostridium glycolicum, Clostridium magnum, Candida krusei, Clostridium mayombei, Clostridium methoxybenzovorans, Clostridium acetobutylicum, Clostridium beijerinckii, Oxobacter pfennigii, Thermoanaerobacter kivui, Sporomusa ovata, Thermoacetogenium phaeum, Acetobacterium carbinolicum, Issatchenkia orientalis, Sporomusa termitida, Moorella glycerini, Eubacterium aggregans, Treponema azotonutricium, Pichia kudriavzevii, Escherichia coli, Saccharomyces cerevisiae, Pseudomonas putida, Bacillus sp, Corynebacterium sp., Yarrowia lipolytica, Scheffersomyces stipitis, and Terrisporobacter glycolicus.

[0030] In some embodiments, the one or more recombinant microorganisms are derived from a parental microorganism selected from the group consisting of Clostridium sp., Clostridium ljungdahlii, Clostridium autoethanogenum, Clostridium ragsdalei, Eubacterium limosum, Butyribacterium methylotrophicum, Moorella thermoacetica, Corynebacterium glutamicum, Clostridium aceticum, Acetobacterium woodii, Alkalibaculum bacchii, Clostridium drakei, Clostridium carboxidivorans, Clostridium formicoaceticum, Clostridium scatologenes, Moorella thermoautotrophica, Acetonema longum, Blautia producta, Clostridium glycolicum, Clostridium magnum, Candida krusei, Clostridium mayombei, Clostridium methoxybenzovorans, Clostridium acetobutylicum, Clostridium beijerinckii, Oxobacter pfennigii, Thermoanaerobacter kivui, Sporomusa ovata, Thermoacetogenium phaeum, Acetobacterium carbinolicum, Issatchenkia orientalis, Sporomusa termitida, Moorella glycerini, Eubacterium aggregans, Treponema azotonutricium, Pichia kudriavzevii, Escherichia coli, Saccharomyces cerevisiae, Pseudomonas

putida, Bacillus sp, Corynebacterium sp., Yarrowia lipolytica, Scheffersomyces stipitis, and Terrisporobacter glycolicus.

[0031] The disclosure provides a method of producing 2,4-FDCA using a recombinant microorganism of claim 27, the method comprising cultivating the recombinant microorganism in a culture medium containing a feedstock providing a carbon source until the 2,4-FDCA is produced.

[0032] The disclosure provides a method of producing a recombinant microorganism capable of producing 2,4-FDCA from a feedstock comprising a carbon source, the method comprising introducing into and/or overexpressing in the recombinant microorganism the following: (a) endogenous and/or exogenous nucleic acid molecules capable of converting glycerol or a monosaccharide to glyceraldehyde 3-phosphate (G3P); (b) at least one endogenous or exogenous nucleic acid molecule encoding a (5-formylfuran-3-yl)methyl phosphate synthase that catalyzes the conversion of G3P from (a) to (5-formylfuran-3-yl)methyl phosphate; (c) at least one endogenous or exogenous nucleic acid molecule encoding a phosphatase that catalyzes the conversion of (5-formylfuran-3-yl)methyl phosphate from (b) to 4-hydroxymethylfurfural (4-HMF); (d) at least one endogenous or exogenous nucleic acid molecule encoding a peroxigenase, dehydrogenase, or a oxidase that catalyzes independently or in synergy the oxidation of 4-HMF from (c) to 2,4 FDCA, directly or through the production of intermediates furan-2,4-dicarbaldehyde, 4-(hydroxymethyl)furoic acid, 4-formylfuran-2-carboxylate, 4-formylfuran-2-carboxylate, 2-formylfuran-4-carboxylate.

[0033] In some embodiments, the carbon source comprises a hexose, a pentose, glycerol, C02, sucroses and/or combinations thereof. In some embodiments, the methyl phosphate synthase from (a) is classified as EC number 4.2.3.153. In some embodiments, the synthase is (5-formylfuran-3-yl)methyl phosphate synthase. In some embodiments, the phosphatase from (c) is classified as EC number 3.1.3. In some embodiments, the phosphatase is classified as haloacid dehalogenase. In some embodiments, the phosphatase is endogenous to the host. In some embodiments, the phosphatase enzyme endogenous to the host is overexpressed.

[0034] In some embodiments, the dehydrogenase from (d) is classified as EC number 1.1.1. or EC number 1.2.1. In some embodiments, the dehydrogenase is an alcohol dehydrogenase or an aldehyde dehydrogenase. In some embodiments, the oxidase from (d) is classified as EC number 1.1.3. In some embodiments, the oxidase is (5-(hydroxymethyl)furfural oxidase.

[0035] In some embodiments, the 2,4-FDCA is produced from furan-2,4-dicarbaldehyde, and/or-(hydroxymethyl)furoic acid intermediates,wherein: (a) a dehydrogenase, an oxidase, or a peroxigenase catalyzes the conversion of the 4-HMF to furan-2,4-dicarbaldehyde, and/or 4-

(hydroxymethyl)furoic acid; and/or (b) a dehydrogenase, an oxidase, or a peroxigenase catalyzes the conversion of the furan-2,4-dicarbaldehyde from (a) to 4-formylfuran-2-carboxylate; and/or (c) a dehydrogenase, an oxidase, or a peroxigenase catalyzes the conversion of the 4-(hydroxymethyl)furoic acid from (a) to 4-formylfuran-2-carboxylate; and/or (d) a dehydrogenase, an oxidase, or a peroxigenase catalyzes the conversion of the furan-2,4-dicarbaldehyde from (a) to 2-formylfuran-4-carboxylate; and/or (e) a dehydrogenase, an oxidase, or a peroxigenase catalyzes the conversion of the the 4-formylfuran-2-carboxylate from (b) and/or (c) or the 2-formylfuran-4-carboxylate from (d) to 2,4-FDCA.

[0036] In some embodiments, the dehydrogenase from (a), (b), (c), (d) and/or (e) is classified as EC number 1.2.1. or EC number 1.1.1 In some embodiments, the dehydrogenase is an aldehyde dehydrogenase or an alcohol dehydrogenase. In some embodiments, the oxidase from (a), (b), (c), (d) and/or (e) is classified as EC number 1.1.3. In some embodiments, the oxidase is 5-(hydroxymethyl)furfural oxidase.

[0037] The disclosure provides a 2,4-FDCA produced according to the methods of the disclosure.

[0038] The disclosure provides a 2,4-FDCA produced according to the microorganisms of the disclosure.

[0039] The disclosure provides a polymer produced from the 2,4-FDCA of embodiments of the disclosure. In some embodiments, the polymer from 2,4-FDCA is formed in a non-biological process.

[0040] The disclosure provides a recombinant microorganism capable of producing 4-hydroxymethylfurfural (4-HMF) from a feedstock comprising an exogenous carbon source, wherein the recombinant microorganism expresses the following: (a) endogenous and/or exogenous nucleic acid molecules capable of converting the carbon source to glyceraldehyde 3-phosphate (G3P); (b) at least one endogenous or exogenous nucleic acid molecule encoding a (5-formylfuran-3-yl)methyl phosphate synthase that catalyzes the conversion of G3P from (a) to (5-formylfuran-3-yl)methyl phosphate; and (c) at least one endogenous or exogenous nucleic acid molecule encoding a phosphatase that catalyzes the conversion of (5-formylfuran-3-yl)methyl phosphate from (b) to 4-hydroxymethylfurfural (4-HMF).

[0041] In some embodiments, the methyl phosphate synthase from (a) is classified as EC number 4.2.3.153. In some embodiments, the synthase is (5-formylfuran-3-yl)methyl phosphate synthase. In some embodiments, the phosphatase from (c) is classified as EC number 3.1.3. In some embodiments, the phosphatase is classified as haloacid dehalogenase. In some embodiments, the phosphatase is endogenous to the host. In some embodiments, the phosphatase enzyme endogenous to the host is overexpressed.

[0042] The disclosure provides a recombinant microorganism capable of producing 2,4-furandicarboxylic acid (2,4-FDCA) from a feedstock comprising a carbon source, wherein the recombinant microorganism expresses one or more of the following: (a) endogenous and/or exogenous nucleic acid molecules capable of converting glycerol or a monosaccharide to glyceraldehyde 3-phosphate (G3P); (b) at least one endogenous or exogenous nucleic acid molecule encoding a (5-formylfuran-3-yl)methyl phosphate synthase that catalyzes the conversion of G3P from (a) to (5-formylfuran-3-yl)methyl phosphate; (c) at least one endogenous or exogenous nucleic acid molecule encoding a phosphatase that catalyzes the conversion of (5-formylfuran-3-yl)methyl phosphate from (b) to 4-hydroxymethylfurfural (4-HMF); (d) at least one endogenous or exogenous nucleic acid molecule encoding a dehydrogenases and/or an oxidase that catalyzes independently or in synergy the oxidation of 4-HMF from (b) to 2,4 FDCA, directly or through the production of intermediates furan-2,4-dicarbaldehyde, 4-(hydroxymethyl)furoic acid, 4-formylfuran-2-carboxylate, 4-formylfuran-2-carboxylate, 2-formylfuran-4-carboxylate.

[0043] In some aspects, the disclosure is generally drawn to a method of producing 2,4-furandicarboxylic acid (2,4-FDCA) by enzymatically converting glyceraldehyde 3-phosphate (G3P) to 2,4-furandicarboxylic acid (2,4-FDCA) in a recombinant microorganism, by enzymatic catalysts in the absence of microorganisms, the method comprising: (a) providing G3P in the presence of a methyl phosphate synthase or any enzyme able to catalyzes the conversion of G3P to (5-formylfuran-3-yl)methyl phosphate; (b) providing the (5-formylfuran-3-yl)methyl phosphate from (a) to a phosphatase or any enzyme able to catalyze the conversion of the (5-formylfuran-3-yl)methyl phosphate to 4-hydroxymethylfurfural (4-HMF); (c) providing the 4-HMF from (b) to oxidases, dehydrogenase or peroxigenase able to catalyze independently or in synergy the oxidation of 4-HMF to 2,4 FDCA, directly or through the production of intermediates furan-2,4-dicarbaldehyde, 4-(hydroxymethyl)furoic acid, 4-formylfuran-2-carboxylate, 4-formylfuran-2-carboxylate, 2-formylfuran-4-carboxylate.

[0044] In this sense, step C could be performed by providing the 4-HMF from (b) to a dehydrogenase or an oxidase or that catalyzes the conversion of the 4-HMF to: (i) furan-2,4-dicarbaldehyde, and/or (ii) 4-(hydroxymethyl)furoic acid; (d) providing the: (i) furan-2,4-dicarbaldehyde from (c)(i) to a dehydrogenase, an oxidase, or a peroxigenase that catalyzes the conversion of the furan-2,4-dicarbaldehyde to 4-formylfuran-2-carboxylate; (ii) 4-(hydroxymethyl)furoic acid from (c)(ii) to a dehydrogenase, an oxidase, or a peroxigenase that catalyzes the conversion of the 4-(hydroxymethyl)furoic acid to 4-formylfuran-2-carboxylate; and/or (iii) furan-2,4-dicarbaldehyde from I(i) to a dehydrogenase, an oxidase, or a peroxigenase that catalyzes the conversion of the furan-2,4-dicarbaldehyde to 2-formylfuran-4-carboxylate; and

(e) providing the 4-formylfuran-2-carboxylate from (d)(i) and/or (d)(ii) or the 2-formylfuran-4-carboxylate from (d)(iii) to a dehydrogenase or an oxidase that catalyzes the conversion of the 4-formylfuran-2-carboxylate from (d)(i) and/or (d)(ii) or the 2-formylfuran-4-carboxylate from (d)(iii) to 2,4-FDCA.

[0045] In some aspects, the methyl phosphate synthase from (a) is classified as EC number 4.2.3.153. In some aspects, the synthase is (5-formylfuran-3-yl)methyl phosphate synthase.

[0046] In some aspects, the phosphatase from (b) is a Phosphoric monoester hydrolase classified as EC number 3.1.3. In some aspects, the phosphatase is classified as haloacid dehalogenase (Koonin *et al*. J. Mol. Biol. 244(1). 1994). In some aspects, the phosphatase of reaction b is endogenous to the host (Offley *et al.* Curr. Gen. 65. 2019). In some aspects, the phosphatase enzyme endogenous to the host is overexpressed. In some cases a heterologous phosphatase able to perform the desired reaction is used and is selected from an alkaline phosphatase, acid phosphatase, fructose-bisphosphatase, sugar-phosphatase, or sugar-terminal-phosphatase.

[0047] In some aspects, the dehydrogenase from (c) is classified as EC number 1.1.1. when oxidizing an alcohol to a carbonyl or EC number 1.2.1. when oxidizing a carbonyl to an acid. In some aspects, the dehydrogenase is an alcohol dehydrogenase or an aldehyde dehydrogenase.

[0048] In some aspects, the oxidase from (c) is classified as EC number 1.1.3. In some aspects, the oxidase is 5-(hydroxymethylfurfural oxidase. In some aspects the 5-hydroxymethylfurfural oxidase convert the 4-hydroxymethylfurfural (4-HMF) into 2,4 FDCA in a three-step reaction.

[0049] In some aspects, the disclosure is generally drawn to a recombinant microorganism capable of producing 2,4-furandicarboxylic acid (2,4-FDCA) from a feedstock comprising a carbon source, wherein the recombinant microorganism expresses the following: (a) endogenous and/or exogenous nucleic acid molecules capable of converting a carbon source to glyceraldehyde 3-phosphate (G3P); (b) at least one endogenous or exogenous nucleic acid molecule encoding a (5-formylfuran-3-yl)methyl phosphate synthase that catalyzes the conversion of G3P from (a) to (5-formylfuran-3-yl)methyl phosphate; (c) at least one endogenous or exogenous nucleic acid molecule encoding a phosphatase that catalyzes the conversion of (5-formylfuran-3-yl)methyl phosphate from (b) to 4-hydroxymethylfurfural (4-HMF); (d) at least one endogenous or exogenous nucleic acid molecule encoding a peroxigenase, dehydrogenase, or a oxidase that catalyzes the conversion of 4-HMF from (c) to 2,4 FDCA directly or through the production of intermediates furan-2,4-dicarbaldehyde, 4-(hydroxymethyl)furoic acid, 4-formylfuran-2-carboxylate, 4-formylfuran-2-carboxylate, 2-formylfuran-4-carboxylate.

[0050] In some aspects, 2,4-furandicarboxylic acid (2,4-FDCA) can be produced by providing the 4-HMF from (c) to a dehydrogenase or an oxidase or peroxidase that catalyzes the conversion of the 4-HMF to: (i) furan-2,4-dicarbaldehyde, and/or (ii) 4-(hydroxymethyl)furoic acid; (d) providing the: (i) furan-2,4-dicarbaldehyde from (c)(i) to a dehydrogenase, an oxidase, or a peroxigenase that catalyzes the conversion of the furan-2,4-dicarbaldehyde to 4-formylfuran-2-carboxylate; (ii) 4-(hydroxymethyl)furoic acid from (c)(ii) to a dehydrogenase, an oxidase, or a peroxigenase that catalyzes the conversion of the 4-(hydroxymethyl)furoic acid to 4-formylfuran-2-carboxylate; and/or (iii) furan-2,4-dicarbaldehyde from I(i) to a dehydrogenase, an oxidase, or a peroxigenase that catalyzes the conversion of the furan-2,4-dicarbaldehyde to 2-formylfuran-4-carboxylate; and (e) providing the 4-formylfuran-2-carboxylate from (d)(i) and/or (d)(ii) or the 2-formylfuran-4-carboxylate from (d)(iii) to a dehydrogenase or an oxidase that catalyzes the conversion of the 4-formylfuran-2-carboxylate from (d)(i) and/or (d)(ii) or the 2-formylfuran-4-carboxylate from (d)(iii) to 2,4-FDCA.

[0051] In some aspects, the host microorganism is genetically modified to improve G3P availability to the (5-formylfuran-3-yl)methyl phosphate synthase that catalyzes the conversion of G3P to (5-formylfuran-3-yl)methyl phosphate. Different metabolic engineering strategies can be performed to achieve varied levels of gene expression through modification of regulation of transcription (Alper *et al.* PNAS 102(36). 2005), mRNA stability and translation (Ferreira *et al.* PNAS 110(28). 2013)(Salis *et al.* Nat. Biotech. 27. 2009), protein stability (Cameron *et al.*Nat. Biotech. 32. 2014) or genes substitution for a less or more efficient orthologue.

[0052] In some aspects, the carbon source comprises a hexose, a pentose, glycerol, C02, sucroses and/or combinations thereof.

[0053] In some aspects, the methyl phosphate synthase from (b) is classified as EC number 4.2.3.153. In some aspects, the synthase is (5-formylfuran-3-yl)methyl phosphate synthase (Table 1).

[0054] In some aspects, the phosphatase from (c) is a Phosphoric monoester hydrolases classified as EC number 3.1.3 .In some aspects, the phosphatase is classified as haloacid dehalogenase (Koonin *et al.* J. Mol. Biol. 244(1). 1994). In some aspects, the phosphatase of reaction c is endogenous to the host (Offley *et al.* Curr. Gen. 65. 2019). In some aspects, the phosphatase enzyme endogenous to the host is overexpressed. In some cases, a heterologous phosphatase able to perform the desired reaction is used and is selected from an alkaline phosphatase, acid phosphatase, fructose-bisphosphatase, sugar-phosphatase, or sugar-terminal-phosphatase.

[0055] In some aspects, the oxidase from (d) is classified as EC number 1.1.3. In some aspects, the oxidase is 5-hydroxymethylfurfural oxidase. In some aspects the 5-hydroxymethylfurfural oxidase convert the 4-hydroxymethylfurfural (4-HMF) into 2,4 FDCA in a three-step reaction.

[0056] In some aspects, the dehydrogenase from (d) is classified as EC number 1.1.1. when oxidizing an alcohol to a carbonyl or EC number 1.2.1. when oxidizing an carbonyl to acid. In some aspects, the dehydrogenase is an alcohol dehydrogenase or an aldehyde dehydrogenase.

[0057] In some aspects, the dehydrogenase from (e) is classified as EC number 1.2.1. or EC number 1.1.1 In some aspects, the dehydrogenase is an aldehyde dehydrogenase or an alcohol dehydrogenase. In some aspects, the oxidase from (e) is classified as EC number 1.1.3. In some aspects, the oxidase is (5-(hydroxymethyl)furfuraloxidase. In some aspects, the dehydrogenase from (f) is classified as EC number 1.2.1. In some aspects, the dehydrogenase is an aldehyde dehydrogenase. In some aspects, the oxidase from (f) is classified as EC number 1.1.3. In some aspects, the oxidase is (5-(hydroxymethyl)furfural oxidase.

[0058] In some aspects, the one or more recombinant microorganisms are derived from a parental microorganism selected from the group consisting of *Clostridium sp., Clostridium ljungdahlii, Clostridium autoethanogenum, Clostridium ragsdalei, Eubacterium limosum, Butyribacterium methylotrophicum, Moorella thermoacetica, Clostridium aceticum, Acetobacterium woodii, Alkalibaculum bacchii, Clostridium drakei, Clostridium carboxidivorans, Clostridium formicoaceticum, Clostridium scatologenes, Moorella thermoautotrophica, Acetonema longum, Blautia producta, Clostridium glycolicum, Clostridium magnum, Clostridium mayombei, Clostridium methoxybenzovorans, Clostridium acetobutylicum, Clostridium beijerinckii, Oxobacter pfennigii, Thermoanaerobacter kivui, Sporomusa ovata, Thermoacetogenium phaeum, Acetobacterium carbinolicum, Sporomusa termitida, Moorella glycerini, Eubacterium aggregans, Treponema azotonutricium, Escherichia coli*, *Saccharomyces cerevisiae, Pseudomonas putida, Bacillus sp., Corynebacterium sp., Yarrowia lipolytica, Scheffersomyces stipitis*, and *Terrisporobacter glycolicus.*

[0059] In some aspects, the disclosure is generally drawn to a method of producing 2,4-FDCA using a recombinant microorganism of the disclosure, the method comprising cultivating the recombinant microorganism in a culture medium containing a feedstock providing a carbon source until the 2,4-FDCA is produced.

[0060] In some aspects, the disclosure is generally drawn to a method of producing a recombinant microorganism capable of producing 2,4-FDCA from a feedstock comprising a carbon source, the method comprising introducing into and/or overexpressing in the recombinant microorganism the following: (a) endogenous and/or exogenous nucleic acid molecules capable

of converting glycerol or a monosaccharide to glyceraldehyde 3-phosphate (G3P); (b) at least one endogenous or exogenous nucleic acid molecule encoding a (5-formylfuran-3-yl)methyl phosphate synthase that catalyzes the conversion of G3P from (a) to (5-formylfuran-3-yl)methyl phosphate; (c) at least one endogenous or exogenous nucleic acid molecule encoding a phosphatase that catalyzes the conversion of (5-formylfuran-3-yl)methyl phosphate from (b) to 4-hydroxymethylfurfural (4-HMF); (d) at least one endogenous or exogenous nucleic acid molecule encoding a peroxigenase, dehydrogenase, or an oxidase that catalyzes the conversion of 4-HMF from (c) to: (i) furan-2,4-dicarbaldehyde and/or (ii) 4-(hydroxymethyl)furoic acid; (e) at least one endogenous or exogenous nucleic acid molecule encoding a peroxigenase, dehydrogenase, or a oxidase that catalyzes the conversion of: (i) furan-2,4-dicarbaldehyde from (d)(i) to 4-formylfuran-2-carboxylate and/or (ii) 4-(hydroxymethyl)furoic acid from (d)(ii) to 4-formylfuran-2-carboxylate; and/or (iii) furan-2,4-dicarbaldehyde from (c)(i) to 2-formylfuran-4-carboxylate; and (f) at least one endogenous or exogenous nucleic acid molecule encoding a peroxigenase, dehydrogenase, or an oxidase that catalyzes the conversion of 4-formylfuran-2-carboxylate from (e)(i) and (e)(ii) or 2-formylfuran-4-carboxylate from (e)(iii)to 2,4-FDCA.

[0061] In some aspects, the carbon source comprises a hexose, a pentose, glycerol, and/or combinations thereof. In some aspects, the methyl phosphate synthase from (b) is classified as EC number 4.2.3.153. In some aspects, the synthase is (5-formylfuran-3-yl)methyl phosphate synthase. In some aspects, the phosphatase from (c) is a Phosphoric monoester hydrolase classified as EC number 3.1.3. In some aspects, the phosphatase is classified as haloacid dehalogenase (Koonin *et al*. J. Mol. Biol. 244(1). 1994). In some aspects, the phosphatase of reaction c is endogenous to the host (Offley *et al.* Curr. Gen. 65. 2019). In some aspects, the phosphatase enzyme endogenous to the host is overexpressed. In some cases, a heterologous phosphatase able to perform the desired reaction is used and is selected from an alkaline phosphatase, acid phosphatase, fructose-bisphosphatase, sugar-phosphatase, or sugar-terminal-phosphatase.

[0062] In some aspects, the dehydrogenase from (d) is classified as EC number 1.1.1. when oxidizing an alcohol to a carbonyl or EC number 1.2.1. when oxidizing a carbonyl to an acid. In some aspects, the dehydrogenase is an alcohol dehydrogenase or an aldehyde dehydrogenase.

[0063] In some aspects, the oxidase from (d) is classified as EC number 1.1.3. In some aspects, the oxidase is 5-(hydroxymethylfurfural oxidase. In some aspects the 5-hydroxymethylfurfural oxidase convert the 4-hydroxymethylfurfural (4-HMF) into 2,4 FDCA in a three-step reaction.

[0064] In some aspects, the oxidase from (e) is classified as EC number 1.1.3. In some aspects, the oxidase is (5-(hydroxymethyl)furfural oxidase. In some aspects, the dehydrogenase from (f)

is classified as EC number 1.2.1. In some aspects, the dehydrogenase is aldehyde dehydrogenase. In some aspects, the oxidase from (f) is classified as EC number 1.1.3. In some aspects, the oxidase is (5-(hydroxymethyl)furfural oxidase.

[0065] In some aspects, the disclosure is drawn to a method of producing a polymer from 2,4-FDCA produced by the microorganism wherein the 2,4-FDCA and a diol are catalytically polymerized in a non-biological process. In some aspects the 2,4-FDCA is part of a plasticizer agent composition and where the plasticizer agent is part of a plasticized polymer composition.

[0066] In some aspects, the disclosure is generally drawn to a recombinant microorganism capable of producing 4-hydroxymethylfurfural (4-HMF) from a feedstock comprising an exogenous carbon source, wherein the recombinant microorganism expresses the following: (a) endogenous and/or exogenous nucleic acid molecules capable of converting the carbon source to glyceraldehyde 3-phosphate (G3P); (b) at least one endogenous or exogenous nucleic acid molecule encoding a (5-formylfuran-3-yl)methyl phosphate synthase that catalyzes the conversion of G3P from (a) to (5-formylfuran-3-yl)methyl phosphate; and (c) at least one endogenous or exogenous nucleic acid molecule encoding a phosphatase that catalyzes the conversion of (5-formylfuran-3-yl)methyl phosphate from (b) to 4-hydroxymethylfurfural (4-HMF).

[0067] In some aspects, the phosphatase is classified as haloacid dehalogenase (Koonin *et al.* J. Mol. Biol. 244(1). 1994). In some aspects, the phosphatase of reaction b is endogenous to the host (Offley *et al.* Curr. Gen. 65. 2019). In some aspects, the phosphatase enzyme endogenous to the host is overexpressed. In some cases, a heterologous phosphatase able to perform the desired reaction is used and is selected from an alkaline phosphatase, acid phosphatase, fructose-bisphosphatase, sugar-phosphatase, or sugar-terminal-phosphatase.

[0068] In some aspects, the disclosure is generally drawn to a recombinant microorganism capable of producing 2,4-furandicarboxylic acid (2,4-FDCA) from a feedstock comprising a carbon source, wherein the recombinant microorganism expresses one or more of the following: (a) endogenous and/or exogenous nucleic acid molecules capable of converting glycerol or a monosaccharide to glyceraldehyde 3-phosphate (G3P); (b) at least one endogenous or exogenous nucleic acid molecule encoding a (5-formylfuran-3-yl)methyl phosphate synthase that catalyzes the conversion of G3P from (a) to (5-formylfuran-3-yl)methyl phosphate; (c) at least one endogenous or exogenous nucleic acid molecule encoding a phosphatase that catalyzes the conversion of (5-formylfuran-3-yl)methyl phosphate from (b) to 4-hydroxymethylfurfural (4-HMF); (d) that catalyzes the conversion of 4-HMF from (c) to 2,4 FDCA directly or through the production of intermediates furan-2,4-dicarbaldehyde, 4-(hydroxymethyl)furoic acid, 4-formylfuran-2-carboxylate, 4-formylfuran-2-carboxylate, 2-formylfuran-4-carboxylate.

[0069] In some aspects, 2,4-furandicarboxylic acid (2,4-FDCA) can be produced by providing the 4-HMF from (c) to at least one endogenous or exogenous nucleic acid molecule encoding a peroxigenase, dehydrogenase, or an oxidase that catalyzes the conversion of 4-HMF from (c) to: (i) furan-2,4-dicarbaldehyde and/or (ii) 4-(hydroxymethyl)furoic acid; (e) at least one endogenous or exogenous nucleic acid molecule encoding a peroxigenase, dehydrogenase, or an oxidase that catalyzes the conversion of: (i) furan-2,4-dicarbaldehyde from (d)(i) to 4-formylfuran-2-carboxylate and/or (ii) 4-(hydroxymethyl)furoic acid from (d)(ii) to 4-formylfuran-2-carboxylate; and (f) at least one endogenous or exogenous nucleic acid molecule encoding a peroxigenase, dehydrogenase, or an oxidase that catalyzes the conversion of 4-formylfuran-2-carboxylate from (e) to 2,4-FDCA.

BRIEF DESCRIPTION OF THE DRAWINGS

[0070] FIG. 1 is a schematic overview of the biosynthetic pathway utilized by recombinant microorganisms of the disclosure for the novel conversion of G3P to 4-HMF. The numbers below the enzymatic reaction rows indicate the 3-digit EC number for the corresponding enzymes.

[0071] FIG. 2 is a schematic overview of the biosynthetic production of products contemplated, utilizing 4-HMF as a substrate. The products include, but are not limited to, 2,4-furandimethanol, furan-2,4-dicarbaldehyde, 4-(hydroxymethyl)furoic acid, 2-formylfuran-4-carboxylate, 4-formylfuran-2-carboxylate, and 2,4-FDCA. The numbers near the enzymatic reaction rows indicate the 3-digit EC number for the corresponding enzymes.

[0072] FIG. 3 is a schematic overview of possible biosynthetic pathways for the conversion of a carbon source (in this case glucose or glycerol) to G3P.

[0073] FIG. 4 is an illustrative SDS-PAGE image of expressed and purified 5-formylfuran-3-yl)methyl phosphate synthase candidate, MfnB1.

[0074] FIG. 5 is a representative UV spectra showing a negative control sample (grey) and methyl phosphate synthase reaction (black) showing (5-formylfuran-3-yl)methyl phosphate produced from G3P.

[0075] FIG. 6 is a representative UV spectra showing (5-formylfuran-3-yl)methyl phosphate production from G3P by methyl phosphate synthases at t_0 (Upper panel) and t_{2h} (Lower panel).

[0076] FIG. 7 is a representative UV spectra showing 4-HMF production from (5-formylfuran-3-yl)methyl phosphate by phosphatase (Upper Panel), *E. coli* lysates (Middle Panel), and yeast lysates (Lower Panel).

[0077] FIG. 8 is an illustrative SDS-PAGE image of the expressed the 4-HMF oxidase candidate, HmfH1, in purified form (PE), soluble phase before purification (SP), in the insoluble phase (IP), and the flow through (FT) after purification.

[0078] FIG. 9 is a representative UV spectra showing 2,4 FDCA production from 4-HMF by 4-HMF oxidase candidates HmfH1 (Upper Panel), HmfH6 (Middle Panel), and HmfH7 (Lower Panel) after 16 hours incubation. Reaction intermediates 4-formylfuran-2-carboxylate (2,4-FFCA) and furan-2,4-dicarbaldehyde (2,4-DFF) were also identified and quantified. The chromatographic separation was performed by HPLC-DAD.

[0079] FIG. 10 is a representative GC-MS chromatogram (Upper Panel) and mass spectrum (Lower Panel) showing identification of 2,4-FDCA produced from 4-HMF with hMFh7.

[0080] FIG. 11 is a representation of silylated 2,4-FDCA.

[0081] FIG. 12 is a representative plot showing NAD(P)H depletion due to its oxidation during the reduction of 2,4-HMF to 2,4-furandimethanol by 4-HMF dehydrogenase candidates DH1, DH2, or DH6.

[0082] FIG. 13 is a representative plot showing NAD(P)H formation due to reduction of the cofactor and oxidation of the 2,4-HMF substrate to furan-2,4-dicaraldehyde.

[0083] FIG. 14 is a representative plot showing NAD(P)H formation due to reduction of the cofactor and oxidation of the 2,4-HMF substrate to 4-(hydroxymethyl)furoic acid by aldehyde dehydrogenase candidates, DH8, DH9, DH10, and DH11.

[0084] FIG. 15 is a representative chromatogram showing 2,4-FDCA production from 4-HMF by the combination of an aldehyde dehydrogenase (DH8) and an alcohol dehydrogenase (DH6). Negative control reaction (Upper Panel) performed without 4-HMF substrate. Reaction with DH8, DH6, and 4-HMF substrate (Middle Panel). Negative control reaction (Lower Panel) performed without DH6 and DH8 enzymes.

[0085] FIG. 16 is a representative chromatogram showing the 2,4-FDCA production *in vivo* from glucose fermentation at t_0 (Upper Panel) and t_{48h} (Lower Panel).

DETAILED DESCRIPTION

Definitions

[0086] The following definitions and abbreviations are to be used for the interpretation of the disclosure.

[0087] As used herein and in the appended claims, the singular forms “a,” “an,” and “the” include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to “an enzyme” includes a plurality of such enzymes and reference to “the microorganism” includes reference to one or more microorganisms, and so forth.

[0088] As used herein, the terms “comprises,” “comprising,” “includes,” “including,” “has,” “having, “contains,” “containing,” or any other variation thereof, are intended to cover a non-exclusive inclusion. A composition, mixture, process, method, article, or apparatus that comprises a list of

elements is not necessarily limited to only those elements but may include other elements not expressly listed or inherent to such composition, mixture, process, method, article, or apparatus. Further, unless expressly stated to the contrary, "or" refers to an inclusive "or" and not to an exclusive "or."

[0089] The terms "about" and "around," as used herein to modify a numerical value, indicate a close range surrounding that explicit value. If "X" were the value, "about X" or "around X" would indicate a value from 0.9X to 1.1X, or, in some embodiments, a value from 0.95X to 1.05X. Any reference to "about X" or "around X" specifically indicates at least the values X, 0.95X, 0.96X, 0.97X, 0.98X, 0.99X, 1.01X, 1.02X, 1.03X, 1.04X, and 1.05X. Thus, "about X" and "around X" are intended to teach and provide written description support for a claim limitation of, e.g., "0.98X."

[0090] As used herein, the terms "microbial," "microbial organism," and "microorganism" include any organism that exists as a microscopic cell that is included within the domains of archaea, bacteria or eukarya, the latter including yeast and filamentous fungi, protozoa, algae, or higher Protista. Therefore, the term is intended to encompass prokaryotic or eukaryotic cells or organisms having a microscopic size and includes bacteria, archaea, and eubacteria of all species as well as eukaryotic microorganisms such as yeast and fungi. Also included are cell cultures of any species that can be cultured for the production of a chemical.

[0091] As described herein, in some embodiments, the recombinant microorganisms are prokaryotic microorganism. In some embodiments, the prokaryotic microorganisms are bacteria. "Bacteria", or "eubacteria", refers to a domain of prokaryotic organisms. Bacteria include at least eleven distinct groups as follows: (1) Gram-positive (gram+) bacteria, of which there are two major subdivisions: (1) high G+C group (Actinomycetes, Mycobacteria, Micrococcus, others) (2) low G+C group (Bacillus, Clostridia, Lactobacillus, Staphylococci, Streptococci, Mycoplasmas); (2) Proteobacteria, e.g., Purple photosynthetic +non-photosynthetic Gram-negative bacteria (includes most "common" Gram-negative bacteria); (3) Cyanobacteria, e.g., oxygenic phototrophs; (4) Spirochetes and related species; (5) Planctomyces; (6) Bacteroides, Flavobacteria; (7) Chlamydia; (8) Green sulfur bacteria; (9) Green non-sulfur bacteria (also anaerobic phototrophs); (10) Radioresistant micrococci and relatives; (11) Thermotoga and Thermosipho thermophiles.

[0092] "Gram-negative bacteria" include cocci, nonenteric rods, and enteric rods. The genera of Gram-negative bacteria include, for example, Neisseria, Spirillum, Pasteurella, Brucella, Yersinia, Francisella, Haemophilus, Bordetella, Escherichia, Salmonella, Shigella, Klebsiella, Proteus, Vibrio, Pseudomonas, Bacteroides, Acetobacter, Aerobacter, Agrobacterium, Azotobacter, Spirilla, Serratia, Vibrio, Rhizobium, Chlamydia, Rickettsia, Treponema, and Fusobacterium.

[0093] "Gram positive bacteria" include cocci, nonsporulating rods, and sporulating rods. The genera of gram positive bacteria include, for example, Actinomyces, Bacillus, Clostridium, Corynebacterium, Erysipelothrix, Lactobacillus, Listeria, Mycobacterium, Myxococcus, Nocardia, Staphylococcus, Streptococcus, and Streptomyces.

[0094] The term "recombinant microorganism" and "recombinant host cell" are used interchangeably herein and refer to microorganisms that have been genetically modified to express or to overexpress endogenous enzymes, to express heterologous enzymes, such as those included in a vector, in an integration construct, or which have an alteration in expression of an endogenous gene. By "alteration" it is meant that the expression of the gene, or level of a RNA molecule or equivalent RNA molecules encoding one or more polypeptides or polypeptide subunits, or activity of one or more polypeptides or polypeptide subunits is up regulated or down regulated, such that expression, level, or activity is greater than or less than that observed in the absence of the alteration. It is understood that the terms "recombinant microorganism" and "recombinant host cell" refer not only to the particular recombinant microorganism but to the progeny or potential progeny of such a microorganism.

[0095] The term "expression" with respect to a gene sequence refers to transcription of the gene and, as appropriate, translation of the resulting mRNA transcript to a protein. Thus, as will be clear from the context, expression of a protein results from transcription and translation of the open reading frame sequence. The level of expression of a desired product in a host cell may be determined on the basis of either the amount of corresponding mRNA that is present in the cell, or the amount of the desired product encoded by the selected sequence. For example, mRNA transcribed from a selected sequence can be quantitated by qRT-PCR or by Northern hybridization (see Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press (1989)). Protein encoded by a selected sequence can be quantitated by various methods, e.g., by ELISA, by assaying for the biological activity of the protein, or by employing assays that are independent of such activity, such as western blotting or radioimmunoassay, using antibodies that recognize and bind the protein. See Sambrook et al., 1989, supra.

[0096] The term “decreasing” or “reducing” the level of expression of a gene or an enzyme activity refers to the partial or complete suppression of the expression of a gene or enzyme activity. This suppression of expression or activity can be either an inhibition of the expression of the gene, a deletion of all or part of the promoter region necessary for the gene expression, a deletion in the coding region of the gene, or the replacement of the wild-type promoter by a weaker natural or synthetic promoter. For example, a gene may be completely deleted and may be replaced by a selection marker gene that facilitates the identification, isolation and purification of the strains

according to the present disclosure. Alternatively, endogenous genes may be knocked out or deleted to favor the new metabolic pathway. In yet another embodiment, the expression of the gene may be decreased or reduced by using a weak promoter or by introducing certain mutations.

[0097] As used herein, the term "non-naturally occurring," when used in reference to a microorganism organism or enzyme activity of the disclosure, is intended to mean that the microorganism organism or enzyme has at least one genetic alteration not normally found in a naturally occurring strain of the referenced species, including wild-type strains of the referenced species. Genetic alterations include, for example, modifications introducing expressible nucleic acids encoding metabolic polypeptides, other nucleic acid additions, nucleic acid deletions and/or other functional disruption of the microorganism's genetic material. Such modifications include, for example, coding regions and functional fragments thereof, for heterologous, homologous, or both heterologous and homologous polypeptides for the referenced species. Additional modifications include, for example, non-coding regulatory regions in which the modifications alter expression of a gene or operon. Exemplary non-naturally occurring microorganism or enzyme activity includes the hydroxylation activity described above.

[0098] The term "exogenous" as used herein with reference to various molecules, e.g., polynucleotides, polypeptides, enzymes, etc., refers to molecules that are not normally or naturally found in and/or produced by a given yeast, bacterium, organism, microorganism, or cell in nature.

[0099] On the other hand, the term "endogenous" or "native" as used herein with reference to various molecules, e.g., polynucleotides, polypeptides, enzymes, etc., refers to molecules that are normally or naturally found in and/or produced by a given yeast, bacterium, organism, microorganism, or cell in nature.

[00100] The term "heterologous" as used herein in the context of a modified host cell refers to various molecules, e.g., polynucleotides, polypeptides, enzymes, etc., wherein at least one of the following is true: (a) the molecule(s) is/are foreign ("exogenous") to (i.e., not naturally found in) the host cell; (b) the molecule(s) is/are naturally found in (e.g., is "endogenous to") a given host microorganism or host cell but is either produced in an unnatural location or in an unnatural amount in the cell; and/or (c) the molecule(s) differ(s) in nucleotide or amino acid sequence from the endogenous nucleotide or amino acid sequence(s) such that the molecule differing in nucleotide or amino acid sequence from the endogenous nucleotide or amino acid as found endogenously is produced in an unnatural (e.g., greater than naturally found) amount in the cell.

[00101] The term "homolog," as used herein with respect to an original enzyme or gene of a first family or species, refers to distinct enzymes or genes of a second family or species which

are determined by functional, structural, or genomic analyses to be an enzyme or gene of the second family or species which corresponds to the original enzyme or gene of the first family or species. Homologs most often have functional, structural, or genomic similarities. Techniques are known by which homologs of an enzyme or gene can readily be cloned using genetic probes and PCR. Identity of cloned sequences as homologs can be confirmed using functional assays and/or by genomic mapping of the genes.

[00102] A protein has “homology” or is “homologous” to a second protein if the amino acid sequence encoded by a gene has a similar amino acid sequence to that of the second gene. Alternatively, a protein has homology to a second protein if the two proteins have “similar” amino acid sequences. Thus, the term “homologous proteins” is intended to mean that the two proteins have similar amino acid sequences. In certain instances, the homology between two proteins is indicative of its shared ancestry, related by evolution. The terms "homologous sequences" or "homologs" are thought, believed, or known to be functionally related. A functional relationship may be indicated in any one of a number of ways, including, but not limited to: (a) degree of sequence identity and/or (b) the same or similar biological function. Preferably, both (a) and (b) are indicated. The degree of sequence identity may vary, but in one embodiment, is at least 50% (when using standard sequence alignment programs known in the art), at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least 98.5%, or at least about 99%, or at least 99.5%, or at least 99.8%, or at least 99.9%. Homology can be determined using software programs readily available in the art, such as those discussed in Current Protocols in Molecular Biology (F.M. Ausubel et al., eds., 1987) Supplement 30, section 7.718, Table 7.71. Some alignment programs are MacVector (Oxford Molecular Ltd, Oxford, U.K.) and ALIGN Plus (Scientific and Educational Software, Pennsylvania). Other non-limiting alignment programs include Sequencher (Gene Codes, Ann Arbor, Michigan), AlignX, and Vector NTI (Invitrogen, Carlsbad, CA). A similar biological function may include, but is not limited to: catalyzing the same or similar enzymatic reaction; having the same or similar selectivity for a substrate or co-factor; having the same or similar stability; having the same or similar tolerance to various fermentation conditions (temperature, pH, etc.); and/or having the same or similar tolerance to various metabolic substrates, products, by-products, intermediates, etc. The degree of similarity in biological function may vary, but in one embodiment, is at least 1%, at least 2%, at least 3%, at least 4%, at least 5%, at least 6%, at least 7%, at least 8%, at least 9%, at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 65%, at least 70%, at least 75%, at least

80%, at least 85%, at least 90%, at least about 91%, at least about 92%, at least about 93%, at least about 94%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least 98.5%, or at least about 99%, or at least 99.5%, or at least 99.8%, or at least 99.9%, according to one or more assays known to one skilled in the art to determine a given biological function.

[00103] The term "variant" refers to any polypeptide or enzyme described herein. A variant also encompasses one or more components of a multimer, multimers comprising an individual component, multimers comprising multiples of an individual component (e.g., multimers of a reference molecule), a chemical breakdown product, and a biological breakdown product. In particular, non-limiting embodiments, an enzyme may be a “variant” relative to a reference enzyme by virtue of alteration(s) in any part of the polypeptide sequence encoding the reference enzyme. A variant of a reference enzyme can have enzyme activity of at least 10%, at least 30%, at least 50%, at least 80%, at least 90%, at least 100%, at least 105%, at least 110%, at least 120%, at least 130% or more in a standard assay used to measure enzyme activity of a preparation of the reference enzyme. In some embodiments, a variant may also refer to polypeptides having at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to the full-length, or unprocessed enzymes of the present disclosure. In some embodiments, a variant may also refer to polypeptides having at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to the mature, or processed enzymes of the present disclosure.

[00104] The term "yield potential" or as used herein refers to a yield of a product from a biosynthetic pathway. In one embodiment, the yield potential may be expressed as a percent by weight of end product per weight of starting compound.

[00105] The term "thermodynamic maximum yield" as used herein refers to the maximum yield of a product obtained from fermentation of a given feedstock, such as glucose, based on the energetic value of the product compared to the feedstock. In a normal fermentation, without use of additional energy sources such as light, hydrogen gas or methane or electricity, for instance, the product cannot contain more energy than the feedstock. The thermodynamic maximum yield signifies a product yield at which all energy and mass from the feedstock is converted to the product. This yield can be calculated and is independent of a specific pathway. If a specific pathway towards a product has a lower yield than the thermodynamic maximum yield, then it

loses mass and can most likely be improved upon or substituted with a more efficient pathway towards the product.

[00106] The term "redox balance" refers to the overall amount of redox cofactors in a given set of reactions. When there is a shortage of redox cofactors, the redox balance is negative and the yield of such pathway would not be realistic since there is a need to burn feedstock to fulfill the cofactor demand. When there is a surplus of redox cofactors, the redox balance is said to be positive and the yield of such pathway is lower than the maximum yield (Dugar et al. "Relative potential of biosynthetic pathways for biofuels and bio-based products" Nature biotechnology 29.12 (2011): 1074). In addition, when the pathway produces the same amount of redox cofactors as it consumes, the redox balance is zero and one can refer to this pathway as "redox balanced." Designing metabolic pathways and engineering an organism such that the redox cofactors are balanced or close to being balanced usually results in a more efficient, higher yield production of the desired compounds when compared to an unbalanced pathway. Redox reactions always occur together as two half-reactions happening simultaneously, one being an oxidation reaction and the other a reduction reaction. In redox processes, the reductant transfers electrons to the oxidant. Thus, in the reaction, the reductant or reducing agent loses electrons and is oxidized, and the oxidant or oxidizing agent gains electrons and is reduced. In one embodiment, the redox reactions take place in a biological system. The term redox state is often used to describe the balance of NAD+/NADH and NADP+/NADPH of natural or non-natural metabolic pathways in a biological system such as a microbial cell. The redox state is reflected in the balance of several sets of metabolites (e.g., lactate and pyruvate, beta-hydroxybutyrate, and acetoacetate), whose interconversion is dependent on these ratios. In one embodiment, an external source of hydrogen or electrons, combined or not with the use of hydrogenase enzymes able to convert hydrogen to NAD(P)H, may be beneficial to increase product yield in metabolic pathways with negative redox balance, i.e., when there is a shortage in redox cofactors, such as NAD(P)H.

[00107] The terms "polynucleotide", "nucleotide", "nucleotide sequence", "nucleic acid" and "oligonucleotide" are used interchangeably. They refer to a polymeric form of nucleotides of any length, either deoxyribonucleotides or ribonucleotides, or analogs thereof. Polynucleotides may have any three dimensional structure, and may perform any function, known or unknown. The following are non-limiting examples of polynucleotides: coding or non-coding regions of a gene or gene fragment, loci (locus) defined from linkage analysis, exons, introns, messenger RNA (mRNA), transfer RNA (tRNA), ribosomal RNA (rRNA), short interfering RNA (siRNA), short-hairpin RNA (shRNA), micro-RNA (miRNA), ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any

sequence, nucleic acid probes, and primers. A polynucleotide may comprise one or more modified nucleotides, such as methylated nucleotides and nucleotide analogs. If present, modifications to the nucleotide structure may be imparted before or after assembly of the polymer. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified after polymerization, such as by conjugation with a labeling component.

[00108] "Complementarity" refers to the ability of a nucleic acid to form hydrogen bond(s) with another nucleic acid sequence by either traditional Watson-Crick or other non-traditional types. A percent complementarity indicates the percentage of residues in a nucleic acid molecule which can form hydrogen bonds (e.g., Watson-Crick base pairing) with a second nucleic acid sequence (e.g., 5, 6, 7, 8, 9, 10 out of 10 being 50%, 60%, 70%, 80%, 90%, and 100% complementary, respectively). "Perfectly complementary" means that all the contiguous residues of a nucleic acid sequence will hydrogen bond with the same number of contiguous residues in a second nucleic acid sequence. "Substantially complementary" as used herein refers to a degree of complementarity that is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% over a region of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, or more nucleotides, or refers to two nucleic acids that hybridize under stringent conditions. Sequence identity, such as for the purpose of assessing percent complementarity, may be measured by any suitable alignment algorithm, including but not limited to the Needleman-Wunsch algorithm (see e.g. the EMBOSS Needle aligner available at www.ebi.ac.uk/Tools/psa/emboss_needle/nucleotide.html, optionally with default settings), the BLAST algorithm (see e.g. the BLAST alignment tool available at blast.ncbi.nlm.nih.gov/Blast.cgi, optionally with default settings), or the Smith-Waterman algorithm (see e.g. the EMBOSS Water aligner available at www.ebi.ac.uk/Tools/psa/emboss_water/nucleotide.html, optionally with default settings). Optimal alignment may be assessed using any suitable parameters of a chosen algorithm, including default parameters.

[00109] The term "biologically pure culture" or "substantially pure culture" refers to a culture of a bacterial species described herein containing no other bacterial species in quantities sufficient to interfere with the replication of the culture or be detected by normal bacteriological techniques.

[00110] As used herein, a "control sequence" refers to an operator, promoter, silencer, or terminator.

[00111] As used herein, "introduced" refers to the introduction by means of modern biotechnology, and not a naturally occurring introduction.

[00112] As used herein, a "constitutive promoter" is a promoter, which is active under most conditions and/or during most development stages. There are several advantages to using constitutive promoters in expression vectors used in biotechnology, such as: high level of production of proteins used to select transgenic cells or organisms; high level of expression of reporter proteins or scorable markers, allowing easy detection and quantification; high level of production of a transcription factor that is part of a regulatory transcription system; production of compounds that requires ubiquitous activity in the organism; and production of compounds that are required during all stages of development.

[00113] As used herein, a "non-constitutive promoter" is a promoter which is active under certain conditions, in certain types of cells, and/or during certain development stages. For example, inducible promoters, and promoters under development control are non-constitutive promoters.

[00114] As used herein, "inducible" or "repressible" promoter is a promoter which is under chemical or environmental factors control. Examples of environmental conditions that may affect transcription by inducible promoters include anaerobic conditions, certain chemicals, the presence of light, acidic or basic conditions, etc.

[00115] As used herein, the term "operably linked" refers to the association of nucleic acid sequences on a single nucleic acid fragment so that the function of one is regulated by the other. For example, a promoter is operably linked with a coding sequence when it is capable of regulating the expression of that coding sequence (i.e., that the coding sequence is under the transcriptional control of the promoter). Coding sequences can be operably linked to regulatory sequences in a sense or antisense orientation. In another example, the complementary RNA regions of the disclosure can be operably linked, either directly or indirectly, 5′ to the target mRNA, or 3′ to the target mRNA, or within the target mRNA, or a first complementary region is 5′ and its complement is 3′ to the target mRNA.

[00116] The term "catalytically polymerized" as used herein refers to polymerization process wherein monomers of the disclosure are polymerized in a non-biological or non-*in vivo* context.

[00117] The term "signal sequence" as used herein refers to an amino acid sequence that targets peptides and polypeptides to cellular locations or to the extracellular environment. Signal sequences are typically at the N-terminal portion of a polypeptide and are typically removed enzymatically. Polypeptides that have their signal sequences are referred to as being full-length and/or unprocessed. Polypeptides that have had their signal sequences removed are referred to as being mature and/or processed.

[00118] As used herein, “microbial composition” refers to a composition comprising one or more microbes of the present disclosure.

[00119] As used herein, “carrier,” “acceptable carrier,” “commercially acceptable carrier,” or “industrial acceptable carrier” refers to a diluent, adjuvant, excipient, or vehicle with which the microbe can be administered, stored, or transferred, which does not detrimentally effect the microbe.

[00120] As used herein, the term "productivity" refers to the total amount of bioproduct, such as (2,4-FDCA), produced per hour.

[00121] As used herein, the term “biosynthesis products” refers to any one or more of the following products contemplated herein: 4-HMF, 2,4-furandimethanol, furan-2,4-dicarbaldehyde, 4-(hydroxymethyl)furoic acid, 2-formylfuran-4-carboxylate, 4-formylfuran-2-carboxylate, and 2,4-FDCA.

Recombinant Microorganisms

[00122] In one embodiment, the present disclosure provides a recombinant microorganism capable of producing any one or more of the biosynthetic products contemplated herein. In one embodiment, a recombinant microorganism produces a 4-HMF. In one embodiment, a recombinant microorganism produces a 2,4,furandimethanol. In one embodiment, a recombinant microorganism produces a furan-2,4-dicarbaldehyde. In one embodiment, a recombinant microorganism produces a 4-(hydroxymethyl)furoic acid. In one embodiment, a recombinant microorganism produces a 2-formylfuran-4-carboxylate. In one embodiment, a recombinant microorganism produces a 4-formylfuran-2-carboxylate. In one embodiment, a recombinant microorganism produces a 2,4-FDCA.

[00123] In one embodiment, a recombinant microorganism produces any six of the biosynthetic products. In one embodiment, a recombinant microorganism produces any five of the biosynthetic products. In one embodiment, a recombinant microorganism produces any four of the biosynthetic products. In one embodiment, a recombinant microorganism produces any three of the biosynthetic products.

[00124] In one embodiment, the carbon source is converted to glyceraldehyde 3-phosphate (G3P). G3P is a common natural intermediary metabolite. In some embodiments, it can be produced from glucose via the glycolysis pathway or from xylose (like from the pentose phosphate pathway but not limited) or from glycerol. In some embodiments, G3P can be derived from CO2 capture technologies. In one embodiment, the recombinant microorganism capable of producing any one or more of the biosynthetic products utilizing a carbon source that comprises

a hexose, a pentose, glycerol, or from CO2 capture technologies. In certain embodiments, the carbon source is glycerol.

[00125] In one embodiment, the recombinant microorganism comprises the novel capacity to convert G3P to any one or more of the biosynthetic products via several enzymatically-catalyzed successive steps.

[00126] In one embodiment, the host microorganism is genetically modified to improve G3P availability to the (5-formylfuran-3-yl)methyl phosphate synthase that catalyzes the conversion of G3P to (5-formylfuran-3-yl)methyl phosphate.

[00127] In one embodiment, the recombinant microorganisms are derived from a parental microorganism selected from the group consisting of *Clostridium sp., Clostridium ljungdahlii, Clostridium autoethanogenum, Clostridium ragsdalei, Eubacterium limosum, Butyribacterium methylotrophicum, Moorella thermoacetica, Clostridium aceticum, Acetobacterium woodii, Alkalibaculum bacchii, Clostridium drakei, Clostridium carboxidivorans, Clostridium formicoaceticum, Clostridium scatologenes, Moorella thermoautotrophica, Acetonema longum, Blautia producta, Clostridium glycolicum, Clostridium magnum, Clostridium mayombei, Clostridium methoxybenzovorans, Clostridium acetobutylicum, Clostridium beijerinckii, Oxobacter pfennigii, Thermoanaerobacter kivui, Sporomusa ovata, Thermoacetogenium phaeum, Acetobacterium carbinolicum, Sporomusa termitida, Moorella glycerini, Eubacterium aggregans, Treponema azotonutricium, Escherichia coli*, *Saccharomyces cerevisiae, Pseudomonas putida, Bacillus sp., Corynebacterium sp., Yarrowia lipolytica, Scheffersomyces* stipitis, *Methylovorus sp., Cupriavidus sp., Methanocaldococcus sp.* and *Terrisporobacter glycolicus.*

4-HMF

[00128] In one embodiment, the present disclosure comprises converting one or more carbon sources to glyceraldehyde 3-phosphate (G3P); converting G3P to (5-formylfuran-3-yl)methyl phosphate (Step A); converting (5-formylfuran-3-yl)methyl phosphate to 4-hydroxymethylfurfural (4-HMF) (Step B).

[00129] In one embodiment, the recombinant microorganism of any one of the embodiments disclosed herein comprises an endogenous and/or exogenous nucleic acid molecules capable of converting a carbon source to glyceraldehyde 3-phosphate (G3P). In one embodiment, glycerol is converted to glycerol-3-phopshate by at least one endogenous or exogenous nucleic acid molecule encoding a glycerol kinase. In one embodiment, glycerol-3-phosphate is converted to dihydroxyacetone phosphate (DHAP) by at least one endogenous or exogenous nucleic acid molecule encoding a glycerol-3-phosphate dehydrogenase. In one

embodiment, glycerol is converted to dihydroxyacetone by at least one endogenous or exogenous nucleic acid molecule encoding a glycerol dehydrogenase. In one embodiment, dihydroxyacetone is converted to dihydroxyacetone phosphate (DHAP) by at least one endogenous or exogenous nucleic acid molecule encoding a dihydroxyacetone kinase. In one embodiment, DHAP is converted to G3P by at least one endogenous or exogenous nucleic acid molecule encoding a triose phosphate isomerase. *See* Zhang *et al.* (2010. Applied and Environmental Microbiology, 76.8:2397-2401) for exemplary, but non-limiting, glycerol assimilation pathways contemplated herein.

[00130] In one embodiment, the recombinant microorganism of any one of the embodiments of disclosed herein comprises at least one endogenous or exogenous nucleic acid molecule encoding a (5-formylfuran-3-yl)methyl phosphate synthase that catalyzes the conversion of G3P to (5-formylfuran-3-yl)methyl phosphate. In one embodiment, the (5-formylfuran-3-yl)methyl phosphate synthase is classified as EC number 4.2.3.153. In some embodiments the EC 4.2.3.153 (5-formylfuran-3-yl)methyl phosphate synthase can be derived from the gene mfnB. In some embodiments, mfnB can be derived from *Methanocaldococcus jannaschii.* In some embodiments, the (5-formylfuran-3-yl)methyl phosphate synthase can be derived from enzyme candidates listed at Table 1. In some embodiments the (5-formylfuran-3-yl)methyl phosphate synthase is encoded by an amino acid sequence listed in Table 1. In some embodiments, the (5-formylfuran-3-yl)methyl phosphate synthase is homologous or similar to the enzymes listed at Table 1. In some embodiments, an (5-formylfuran-3-yl)methyl phosphate synthase enzyme is evolved or engineered to improve its catalytic efficiency, markedly kcat.

Table 1. (5-formylfuran-3-yl)methyl phosphate synthases enzymes

Name	Organism	Sequence
MfnB 1	*Methanocaldococcus jannaschii*	MILLVSPIDVEEAKEAIAGGADIIDVKNPKEGSLGANFPWMI KAIREVTPKDLLVSATVGDVPYKPGTISLAAVGAAISGADYI KVGLYGVKNYYQAVELMKNVVRAVKDIDENKIVVAAGYAD AYRVGAVEPLIVPKIARDAGCDVAMLDTAIKDGKTLFDFQS KEILAEFVDEAHSYGLKCALAGSIKKEHIPILKEIGTDIVGVR GAACKGGDRNNGRIDRELVKELKELCK (SEQ ID NO: 1)
MfnB 2	*Methanocaldococcus fervens*	MILLVSPIDVEEAKEAIAGGADIIDVKNPKEGSLGANFPWMI KAIREVTPKELLVSATVGDVPFKPGTISLAAVGAAISGADYI KVGLYGVKNYYEGVELMKNVVRAVKDIDENKIVVAAGYAD AHRVGAVEPLIIPKIARDAGCDVAMLDTAVKDGKTLFDFQS KEILEEFVQESHDYGLKCALAGSIKKEHIPILKEIGTDIVGVR GAVCKGGDRNNGRIDRELVRELKELCK (SEQ ID NO: 2)
MfnB 3	*Methanocaldococcus vulcanius*	MILLVSPIDVDEAREAIAGGADIIDVKNPKEGSLGANFPWMI KAIREITPKELLVSATVGDVPYKPGTVSLASVGAAMSGAD YIKVGLYGVKNYYEAVELMKNVVRAVKDVDENKIVVAAGY ADAHRVGAVDPLIIPKIARDADCDVAMLDTAIKDGKTLFDF

		QSKEILEEFVEETHSYGLKCALAGSIKKEHIPILKEIGTDIVG VRGAVCKGGDRNKGRIDRNLVKELKELV (SEQ ID NO: 3)
MfnB 4	*Methanocaldococcus infernus*	MLLLVSPIDVEEAKEAIEGGADIIDVKNPKEGSLGANFPWVI REVRKITPKSLLVSATVGDVPYKPGTVSLAALGAGMSGAD YIKVGLYGVKNYNQAVELMKSVVKAVKDFDDNKIVVAAGY ADAYRVGAVDPLVIPKIARDSGADVAMLDTAIKDGKTLFDF LSKEILEEFVSEVHDYGLKCALAGTIKKDHIPILKEIGTDIVG VRGAACKGGDRNKGRIDRNLVRELKELC (SEQ ID NO: 4)
MfnB 5	*Methanothermococcus okinawensis*	MILLVSPKDVNEAIETIKGGADIVDVKNPPEGSLGANFPWII KEIREITPKNLFVSAAIGDVPYKPGTVALAALGAAMSGADY IKVGLYGTKSYNEAVDLMEKVVKAVKGVDENKIVVAAGYA DAHRVGAVEPLIVPKIARDAGCDVAMLDTAVKDGKTLFDH LNEKILAEFVEETHSYGLKCALAGSIKKEEIPILKDINCDIVG VRGAACTKGDRNNGTIKSELVKELSKLCK (SEQ ID NO: 5)
MfnB 6	*Methanococcales archaeon HHB*	MRILISPKDIEEAKEAIEGGADIIDVKNPLEGSLGANFPWVI REIRNITPKDRLVSATVGDVPYKPGTVALAAVGAAISGADY IKVGLYGTKSYREAVDVMNKVVKAVKEIDENKIVVAAGYA DAYRVGAVDPLIIPKVARDSGCDVAMLDTAVKDGKRLFDH LNRELISEFVEEVHNYGLECALAGSIRKEDIPVLKEIGCDIV GIRGAACTKGDRNNGKIKKELVEELVKLCKNGDK (SEQ ID NO: 6)
MfnB 7	*Methanobrevibacter smithii*	MLLLISPINHEEALESIKGGADIVDVKNPKEGSLGANFPWVI RDIREITPEDKLVSATLGDVPYKPGTVSLAAMGAHVSGAD YIKVGLYGTKDYDEAVEVMENVAKTIKDVDNDTIVVAAGY ADAHRVGAVDPMEIPKVAKDAGCDLAMLDTAVKDGHTLF DYLSIEDLEKFVNEAHSYGLKTALAGSVKKEQLKPLNDIGC DVVGIRGAACVGGDRNTGKIHHSAVAELKELCDSF (SEQ ID NO: 7)
MfnB 8	*Methanobacterium sp. PtaB.Bin024*	MLLLISPINTQEAREAIDGGADIVDVKNPKEGSLGANFPWV IRNIREITPKNMKVSATLGDVPYKPGTVALAAAGAIVSGAD YIKVGLYGTTNYSEALEVMENVVKTVDEFNSDAIVVAAGY ADAHRVGAVDPMEIPKIAADSGSDLAMVDTAVKDGKTLFD FMNEETLSQFTEQTHEYGLKSALAGSVTEEQLPILAELGC DVVGIRGAACIGGDRNSGSIHHEAVARLKQIV (SEQ ID NO: 8)
MfnB 9	*Methanopyrus sp. KOL6*	MRPRLLVSPVNRDEALEAVEGGAHIIDVKNPEEGSLGANF PWVIREIMEVVPEDREVSATVGDVPYKPGTVAQAVLGVA AVGVDYAKVGLYGTKTEEEALEVMRACSRAVREFGYDTR VVAAGYADAHRVDSIDPMSVPEVAAEAECDVAMVDTAVK DGKRLFDFLREEEVGEFVDLAHEHGLEVALAGSLRHEDM PIVRDLGADIVGIRGAACERGDRNRGAIRSHLVRKLAEALA (SEQ ID NO: 9)
MfnB 10	*Candidatus Argoarchaeum ethanivorans*	MTMKLLVSPISVEEARIALDGGADIIDVKNPKEGSLGANFP DVIQSVKRVITKPMSVAIGDFNYKPGTASLAALGASVAGA DYIKIGLFDVQTREQASEMTERVTKAVKQYDSKKKVVICG YSDYNRINSISPFELPGIVSDAGADVVMMDTGVKDGRSTL EFLNLEKLESFIGSAHQYGLLAAIAGSLTFEDIEALKEVAPD IIGVRGCVCGGDRNSSIKLELVRELKERIHH (SEQ ID NO: 10)

MfnB 11	*Methanobacterium congolense*	MLLLISPINTEEAREAIEGGADIVDVKNPKEGSLGANFPWVI KSISELTPEGMYVSATLGDVPYKPGTVSLAAAGAVVSGAD YIKVGLYGTKNYEEALEVMKNVVKTVKDFNEDAVVVAAGY ADAHRVGAVDPMEIPRVAADAGADLAMVDTAVKDGKTLF DFMDEDTLTKFNNTIHDYGLKSALAGSVKKEQLEMLYNIG CDVVGIRGAACVGGDRNTGKIHRSAVGELKKMIENF (SEQ ID NO: 11)
MfnB 12	*Methanobrevibacter arboriphilus*	MLLLISPINNEEALESIEGGADIVDVKNPKEGSLGANFPWVI SEIRKMTPDDMLVSATLGDVPYKPGTVSLAAMGALTSGA DYIKVGLYGTSNYDEALEVMTNVVKTVKSNNPNATVVASG YGDAHRVGAVSPWDIPKVAKESGSDLAMLDTAVKDGKTL FDYLNIDDLKKFVEETHSYGLKSALAGSVKKEQLKPLYDIG CDVVGVRGAACTGGDRNNGKISRTAVAELKELVNSFD (SEQ ID NO: 12)
MfnB 13	*Methanococcus maripaludis*	MILLVSPKDVAEAHEAIEGGADIIDVKNPPEGSLGANFPWV IKETREATPEGMLVSAAIGDVPYKPGTVTLAALGAAISGAD YIKVGLYGTRSYQEALDVMKNVTKAVKDSGENKIVVAAGY ADAYRVGGVDPLIIPRVARDAGCDVAMLDTAVKDGKTLFD HMSIELLKEFVEETHKYGMKCALAGSIKIEEIPMLKEINCDI VGVRGAACTKGDRNEGRIQKDLVKEIVKVCRQ (SEQ ID NO: 13)
MfnB 14	*Methanococcus vannielii*	MILLVSPKDVAEAYEAINGGADIIDVKNPPEGSLGANFPWV IKEIRSATPNGMLVSAAIGDVHYKPGTVTLAALGATISGAD YIKIGLYGTRSYQEAVDVMKNVSNAVKSEDPKKIVVAAGY ADAYRVGAVDPLIIPKIARDSGCDVAMLDTAVKDGKTLFD HLSIDLLKEFVEETHKYGMKCALAGSIKKEEIPMLKEIGCDI VGIRGAACTKGDRNEGKIQKDLVKEIVKICKE (SEQ ID NO: 14)
MfnB 15	*Methanosarcina acetivorans*	MKLLVSPINREEAIIASLGGADIVDVKNPKEGSLGANFPWV IRDVKEVVNGRQPISATIGDFNYKPGTASLAALGAAVAGA DYIKVGLYDIQTEAQALELLTKITLAVKDYDPSKKVVASGY SDYKRINSISPLLLPAVAAEAGVDVVMVDTGIKDGKSTFEF MDEQELKEFTDLAHEHGLENAIAGSLKFEDLPVLERIGPDII GVRGMVCGGDRRTAIRQELVEKLVAECQI (SEQ ID NO: 15)
MfnB 16	*Methanosarcina barkeri*	MKLLISPINKEEAIIASRGGADIVDVKNPKEGSLGANFPWVI RDVKGAVNGRQPISATIGDFNYKPGTASLAAFGAAVAGAD YIKVGLYDIQTEDQALELITKITQAVKDYDSTKKVVASGYSD YKRINSISPLLLPSIAAKAGADVVMVDTGIKDGKSTFEFMD EEELKKFTGLAHECGLENAIAGSLKFEDLPVLERIGPDIIGV RGMVCGGDRTNSIRQELVEKLVAECQA (SEQ ID NO: 16)
MfnB 17	*Methylorubrum extorquens*	MSDIVSISSARPRLLVSVRGPDEALTALRAGADLIDAKDPE RGALGALPPETVRAIVAGVGGRAVTSAVAGDGTGREIAAA IATIAATGVDFIKIAVGGADDAALAEAAAQAPGRVIGVLFAE DDVAEDGPARLAAAGFVGAMIDTRGKSGTTLTSLMAAPQ LAAFVAGCRTHGLMSGLAGSLGLGDIPVLARLDPDYLGFR GGLCRASDRRQALDGARVAQAVEAMRAGPRADAA (SEQ ID NO: 17)
MfnB 18	*Methylobacterium sp.*	MTRPEPHLSVRAAPRLLVSVRDAAEAEVARAAGADLVDA KDPARGALGALDPALVRAMVARIGDRATTSAVAGEPREA

		GDLVAKVAAMAATGVDYVKVALPPGLRSGRDGLREAADA ARGRLIAVLFAEDGLDLAVLPTLADAGFVGAMIDTNTKDG RRLTDRIAVPALSAFTAACRAEGLVSGLAGSLALADIPALS DLGAGYLGFRGGLCRGGDRRGDLDPARIAEAARLLRAGG RRDAA(SEQ ID NO: 18)
MfnB 19	*Methanosarcina mazei*	MKLLVSPINSEEAIIASIGGADIVDVKNPKEGSLGANFPWVI REVKAVVNGRQPISATIGDFNYKPGTAALAALGAAVAGAD YIKVGLYDIQTESQALELLTKITRAVKDYNPLKKVVASGYS DYKRINSISPLLLPAVAAEAGVDVVMVDTGVKDGKSTFEF MDEKELKEFTDLAHSYGLENAIAGSLKFEDIPLLERIGPDII GVRGMVCGGDRSTSIRQELVEKLVAECQA (SEQ ID NO: 19)
MfnB 20	*Methyloversatilis universalis*	MIRMLASVRNLDEARIVLEAGVDLIDLKQPADGALGALPAE VIREVVDFVAGRTLTSATAGNVEPDAQAVQSAMARIAATG VDYVKAGLFPGNWQQGGRDYAAVRACLRGLTPLAGARRI AVMFADLSPPLALVDAVADAGFDGVMVDTALKTGHSLPD VASTEWLSGFVERARARGLLCGLAGSLRVTHIPALAQRCP DYLGFRGALCAGQARAQALDARAVLAVREALEKVQRLAA (SEQ ID NO: 20)
MfnB 21	*Nitrosococcus watsonii*	MSCWLASVRNLEEISCLLAEGPDIIDFKEPKEGVLGALPLE TVREAVALIGRRCQTSAAIGDFPVDSPQIYQRVLEMAATG VDYVKIGLPSNIQQAAACLLSLRPLADQGVSMVGVIFADK RPDFSWTYLIGQAGFKGIMLDTAIKDDFGLLSHLSLSELNN FVKLARSVRLISGLAGSLSIQDIPKLLPLRADYLGFRSALCV AARNRCSRLDPKAVLLIKQAMRENLRIFEI (SEQ ID NO: 21)
MfnB 22	*Streptomyces cattleya NRRL 8057*	MKEPTLLLLISPDSVEEALDCAKAAEHLDIVDVKKPDEGSL GANYPWVIREIRDAIPADKPVSATVGDVPYKPGTVAQAAL GAVVSGATYIKVGLYGCTTPDQVVEVMRGVVRAVKDHRP DALVVASGYADAHRIGCVNPLAIPGVAQRSGCDAAMLDT AVKDGTRLFDHVPPDVCGEFVRLAHEGGLLAALAGSVKA EDLGALTRIGTDIVGVRGAVCEGGDRNAGRIQPHLVAAFR AEMDRHAREHAAVVTPTG (SEQ ID NO: 22)
MfnB 23	*Streptomyces coelicolor*	MLLLISPDGVDEALDCAKAAEHLDIVDVKKPDEGSLGANY PWVIREIRAAVPADKPVSATVGDVPYKPGTVAQAALGAAV SGATYIKVGLYGCATPEQAVEVMRGVVRAVKDHRADAFV VASGYADAHRIGCVNPLSLPDIARRSGSDAAMLDTAIKDG TRLFDHVPPDVCAEFVRRAHDCGLLAALAGSVRSGDLGE LARIQTDIVGVRGAVCEGGDRTTGRIRPHLVAAFRAEMDR HVREHAAAAAQS (SEQ ID NO: 23)
MfnB 24	*Streptomyces EFF88969*	MLLISPDSVEEALECAKAAQHLDIVDVKKPDEGSLGANHP WVIRAVRDAVPADKPVSATVGDVPYKPGTVAQAALGATV SGATYIKVGLYGCTTPDQAVEVMRGVVRAVKDFRPDALV VASGYADAHRIGCVNPLALPDIARRSGSDGAMLDTAVKD GTRLFDHTPPQVCAEFVRLAHEAGLLAALAGSVKAGDLAE LAGMGTDIVGVRGAVCEGGDRNAGRIRPELVAAFRAEMD RCVQQHGGQGAAVAAAS (SEQ ID NO: 24)
MfnB 25	*Streptomyces griseus*	MLLLISPDGVEEALACATAAEHLDIVDVKKPDEGSLGANFP WVIREIRAAVPADKPVSATVGDVPYKPGTVAQAALGAAVS GATYIKVGLYGCATPDQAIDVMRGVVRAVKDFRADAFVVA SGYADAHRIGCVNPLALPDIARRAGADAAMLDTAIKDGTR

		LFDHVPPEGCAEFVRLAHEAGLLAALAGSVKAADLATLTRI GTDIVGVRGAVCEGGDRDAGRIQPRLVAAFRAEMDRHAR AFAAAPAAS (SEQ ID NO: 25)
MfnB 26	*Streptomyces sp. DH-12*	MLLLISPDGVEEALDCAKAAEHLDIVDVKKPDEGSLGANF PWVIREIREAVPADKPVSATVGDVPYKPGTVAQAALGAVV SGATYIKVGLYGCTTPDQGIDVMRAVVRAVKEHNPDALVV ASGYADAHRIGCVNPLAVPDIAARSGADAAMLDTAVKDGT RLFDHVPPDVCAEFVRLAHASGRLAALAGSVRQDDLGEL TRIGTDIVGVRGAVCEGGDRNAGRIQPHLVAAFRAEMDR YDRERTAGLPAAR (SEQ ID NO: 26)
MfnB 27	*Streptomyces venezuelae*	MLLLISPDSVEEALDCVKAAEHLDIVDVKKPDEGSLGANFP WVIREIRDAVPADKPVSATVGDVPYKPGTVAQAALGAVVS GATYIKVGLYGCTTPEQGIEVMRAVVRAVKDHRPDALVVA SGYADAHRVGCVNPLAVPDIAARSGADAAMLDTAIKDGT RLFDHVPPDACAEFVRRAHASGLLAALAGSITQADLGPLT RMGTDIVGVRGAVCAGGDRNAGRIQPHLITAFRAEMDRQ GREYAVGIPAAN (SEQ ID NO: 27)

[00131] In one embodiment, the recombinant microorganism of any one of the embodiments disclosed herein comprises at least one endogenous or exogenous nucleic acid molecule encoding a phosphatase or a kinase that catalyzes the conversion of (5-formylfuran-3-yl)methyl phosphate to (4-HMF). In one embodiment, the phosphatase is classified as haloacid dehalogenase (Koonin *et al*. J. Mol. Biol. 244(1). 1994). In some aspects, the phosphatase of reaction b is endogenous to the host (Offley *et al.* Curr. Gen. 65. 2019). In some aspects, the phosphatase enzyme endogenous to the host is overexpressed. In some cases a heterologous phosphatase able to perform the desired reaction is used and is selected from an alkaline phosphatase, acid phosphatase, fructose-bisphosphatase, sugar-phosphatase, or sugar-terminal-phosphatase. In some embodiments, the phosphatase can be derived from enzyme candidates listed at Table 2. In some embodiments, the phosphatase is homologous or similar to the enzymes listed at Table 2. In some embodiments the 4-HMF phosphatase enzyme is encoded by an amino acid sequence listed in Table 2. In some embodiments, a phosphatase enzyme is evolved or engineered to improve its catalytic efficiency and or specificity for the conversion of (5-formylfuran-3-yl)methyl phosphate to (4-HMF).

Table 2. 4-HMF phosphatase enzymes

Name	**Organism**	**Sequence**
PH1	*Streptomyces coelicolor*	MMPEPPRERRTAANRSPAIRPIAFFDVDETLITAKSMLDFA RQAPHSLRDDITAQASGQRHSADADLTAMRRRGASRVE MNRVYYRRYAGVSLARLQEAGRDWYHAYRTRPDGYVRA GLAALARHRRAGHTIVLISGSARPLLTPLAQDLGADRILCT EQFADAQGVLTGEVNRPMIGEAKAEAVTEVMAKRGVVPA DCFAYGDHESDFGMLQAVGNPVVVGTDLVLVRHAQGSN

		WPVLPADAGPRCACARRPGPLGHDDPSAIG (SEQ ID NO: 28)
PH2	*Streptomyces sp. E5N91*	MMPEPPRERRTAANRSPAIRPIAFFDVDETLITAKSMLDFA RQAPHSLRDDITAQASGQRHSADADLTAMRRRGASRVE MNRVYYRRYAGVSLARLQEAGRDWYHAYRTRPDGYVRA GLAALARHRRAGHTIVLISGSARPLLTPLAQDLGADRILCT EQFADAQGVLTGEVDRPMIGEAKAEAVTEVMAKRGVVSA DCFAYGDHESDFGMLQAVGNPVVVGTDLVLVRHAQASN WPVLPADAGPRCACARRPGPLGHDDPSAIG (SEQ ID NO: 29)
PH3	*Streptomyces sp. NRRL S-31*	MSALRHERRAAVSRPVVIRHIAFFDVDETLITAKSLLDFAQ RVPHGLWEDETGQPIERLRSGEIDLAALQRSGASRAEMN RAYYRRYAGVPLERLQKAGRDWYHAYRMRPDGYITAGL AALARHRRAGHMIVLISGSARPLLTPLSEDLGADRILCTEQ LDDAQGVLTGEVAHPMVGEAKAEAVTEVMAQLRVPTTDC FAYGDHGSDLDMLQAVGSPVVVGTDPVLARHAQASNWP MLPADAGPRIARAQHHDTSAQYGPQVIALASGRGAAPRR QERW (SEQ ID NO: 30)
PH4	*Streptomyces aureus*	MNASIAPAAFFDVDETLVNTKSMFHFLRFWMARQGDDGS GHEAVMAGVRRAAASGVHRSEINRAYYRRFAGVPYAALL EAGRDWWQEYRRGSDAVVVPAWAAATRHRKAGHLVVL VSGSFRGCLEPLAQDLGAHRILCSEPLVDTDGRLTGEVVR PMIGSVKADAVRETVAELGLTAADCSCYGDHSSDLDMLG AVGNPVVVGGDRVLLEHAQRLDWPVLPATPGHLPSPDAS PARLLTAAERR (SEQ ID NO: 31)
PH5	*Saccharothrix syringae*	MSTPPAVAFFDVDETVIKVKSMFEFLRHWMTAQGDDGSA YESFMAGVRELADAGVPRAEVNRHYYRRYAGASAADVR AAGEDWYASYRRRPDGFLTATVAAVAAHRAAGNRVVLVS GSFLPVLGPLMADVGADEALCGDPEVGPDGRYTGAIAVP MIGENKTAAVRARMAELGVDPADCYAYGDHQSDLGMLE AVGNPVVVGEDPVLVGKAEAGGWRRLPATTGPLGVPPR VLSVVE (SEQ ID NO: 32)
PH6	*Rhodococcus sp. MTM3W5.2*	MTHTGSRPVQVAFFDVDETLITVKSMFAFLEHWLRERGD DGSEYSRLLAALRRASDEGAPREEVNRSYYRTFRGVPLV ELEESGRRWYREFESTAAPYYADTLAALRDHRDAGAAIVL LSGSFAPALGPIGEAVCADRIVASRPVTDGHGVLTGEVER PMIGKAKAEAVTSVLEELGIDTGNSYGYGDHDSDLAFLEA VGHPGLRGSDPVLRAHAARNRWRVLGSATTGLAGAVPLL AATSTGQRGLR (SEQ ID NO: 33)
PH7	*Rhodococcus sp. UNC363MFTsu5.1*	MTGTGPRPGQVAFFDVDETLITVKSMFAFLEHWLWERGD DGSEYARLLGALRRQSDEGAPREEVNRSYYRTFRGVPLV ELEESGRRWYREFESTNAPYYAATLAALHAHREAGAAIVL LSGSFAPALVPIGEAVGADRIVASRPVTDQGGVLTGEVER PMIGQAKAEAVTSVQAELGVDAENSYGYGDHESDLAFLE AVGHPGLRGDDQVLLARAARDRWRSLGSETTGLAGAGP LAGSASAGLAQRGIL (SEQ ID NO: 34)
PH8	*Buttiauxella warmboldiae*	MHTSAAFFDVDETLITVKSMFDFYDFWCRENNEYDKLQR YMTDFRSAVKNGTPREQLNREYYRQFAGVNYKDLEEAG KNWFRGKKLDSELFISSAVAALKKHQANNMFIVFISGSMH PVLSPVANYLGVTDILCTPLELTGEGIITGEIGTPQTIGIGKK

		EALINFCSQKKISAADCYAYGDDLSDIPMLESVGYPVCVG KYTELARHAINQRWPVI (SEQ ID NO: 35)
PH9	*Chania multitudinisentens*	MRQTAFYDVDDTLINIKSMFDFFQFWASENGLISQQEQFD SQFSVLARKMSSREELNRAYYRFFKGVPLLKIEQCAERW FKNSFSNTEIFISYTLKSILAHRVLGHNIVLVSGSMTPLLKPI AQLLGITDILCTKLATDQSGVVTGEILETQTIGEGKAIVIRQY ALENDINLSACFAYGDDVSDIPMLACVGHPICIGEGTALSH YASNNNWPIVRVE (SEQ ID NO: 36)
PH10	*Methylosinus sporium*	MMEHRSFAFFDVDETLISIKSMFDFFPFWCKWIGAAPEAY SRFETEIASAIARHATREELNRLYYRSFRGAQLPVLEAAGA AWFLQRFGRSPPYRKHVVARLEKHRQEGVVPVLVSGSM RPLLRPIARELQAEHCLCTQLVVDESGRLTGEIGSPQTIGE GKAEAIRAFLREQGGRPADCLAYGDDISDLAMLELVGAPV VVGAQPDLLSICRQRDWPYLPL (SEQ ID NO: 37)
PH11	*Klebsiella oxytoca*	MQQAAAFFDVDETLINIKSMFDFFDFWCKENNEPIKLHKY MANFQSEVKKGIPREHLNREYYRQFAGISYKALEEAGEK WFRFKLNSELFIGSAVSALKKHQAENMDIVFISGSMLPVLS PVARYLGVKDILCTPLKFTAAGEMTGEIGYPQTIGDGKKD ALLQFCEQRNINPSDCYAYGDDLSDIPMLASTGHPVCVGK HSALARHAITHRWQVI (SEQ ID NO: 38)
PH12	*Serratia*	MTSAAAFFDVDETLIKMKSMFHFYHYWSNVRGNQKAYEE FIKRFQQAVAEGVPREVLNRMYYRQFSGIDIDDVYQVAED WFHKYLHEKEAYIASAVDRFQRHKISGHLTVFISGSMLPLL KPLGQRLGADAILCTQLLLDAKGKLTGEIGEPQTIGQGKQ RALLSFSQSHHIDLAKSFAYGDDLSDIPMLAATGNPVCVG EHSNLAEYARRNNWNMLAENATN (SEQ ID NO: 39)
PH13	Saccharomyces cerevisiae ycr015c	MKTIIISDFDETITRVDTICTIAKLPYLLNPRLKPEWGHFTKT YMDGYHKYKYNGTRSLPLLSSGVPTIISQSNFNKLFADEL KYQNHNRVVELNSVNEITKQQIFKSISLDQMKTFARDQNH EDCLLRDGFKTFCSSVVKNFESDFYVLSINWSKEFIHEVIG DRRLKNSHIFCNDLKKVSDKCSQSYNGEFDCRLLTGSDK VKILGEILDKIDSGCNKEGNSCSYWYIGDSETDLLSILHPST NGVLLINPQENPSKFIKITEKIIGIPKDKISSFEADNGPAWLQ FCEKEGGKGAYLVKSWDSLKDLIMQVTKM (SEQ ID NO: 40)
PH14	Saccharomyces cerevisiae ydl236w	MTAQQGVPIKITNKEIAQEFLDKYDTFLFDCDGVLWLGSQ ALPYTLEILNLLKQLGKQLIFVTNNSTKSRLAYTKKFASFGI DVKEEQIFTSGYASAVYIRDFLKLQPGKDKVWVFGESGIG EELKLMGYESLGGADSRLDTPFDAAKSPFLVNGLDKDVS CVIAGLDTKVNYHRLAVTLQYLQKDSVHFVGTNVDSTFPQ KGYTFPGAGSMIESLAFSSNRRPSYCGKPNQNMLNSIISA FNLDRSKCCMVGDRLNTDMKFGVEGGLGGTLLVLSGIET EERALKISHDYPRPKFYIDKLGDIYTLTNNEL (SEQ ID NO: 41)
PH15	Saccharomyces cerevisiae ydl236w	MTIAKDYRTIYRNQIKKQIRLNQEHLQSLTHLGSQINFEVD PPKLPDPDPARKVFFFDIDNTLYRKSTKVQLLMQQSLSNF FKYELGFDDDEAERLIESYYQEYGLSVKGLIKNKQIDDVLQ YNTFIDDSLPLQDYLKPDWKLRELLINLKKKKLGKFDKLWL FTNSYKNHAIRCVKILGIADLFDGITYCHYDRPIEEEFICKP DPKFFETAKLQSGLSSFANAWFIDDNESNVRSALSMGMG

		HVIHLIEDYQYESENIVTKDHKNKQQFSILKDILEIPLIMDVE VYRPSSIAIKEMEELEEEGEAVNWSNQQINVQSS (SEQ ID NO: 42)
PH16	Saccharomyces cerevisiae yer062c	MGLTTKPLSLKVNAALFDVDGTIIISQPAIAAFWRDFGKDK PYFDAEHVIQVSHGWRTFDAIAKFAPDFANEEYVNKLEAEI PVKYGEKSIEVPGAVKLCNALNALPKEKWAVATSGTRDM AQKWFEHLGIRRPKYFITANDVKQGKPHPEPYLKGRNGL GYPINEQDPSKSKVVVFEDAPAGIAAGKAAGCKIIGIATTF DLDFLKEKGCDIIVKNHESIRVGGYNAETDEVEFIFDDYLY AKDDLLKW (SEQ ID NO: 43)
PH17	Saccharomyces cerevisiae yfl045c	MSIAEFAYKEKPETLVLFDVDGTLTPARLTVSEEVRKTLAK LRNKCCIGFVGGSDLSKQLEQLGPNVLDEFDYSFSENGLT AYRLGKELASQSFINWLGEEKYNKLAVFILRYLSEIDLPKR RGTFLEFRNGMINVSPIGRNASTEERNEFERYDKEHQIRA KFVEALKKEFPDYGLTFSIGGQISFDVFPAGWDKTYCLQH VEKDGFKEIHFFGDKTMVGGNDYEIFVDERTIGHSVQSPD DTVKILTELFNL (SEQ ID NO: 44)
PH18	Saccharomyces cerevisiae ygl224c	MTVEYTASDLATYQNEVNEQIAKNKAHLESLTHPGSKVTF PIDQDISATPQNPNLKVFFFDIDNCLYKSSTRIHDLMQQSIL RFFQTHLKLSPEDAHVLNNSYYKEYGLAIRGLVMFHKVNA LEYNRLVDDSLPLQDILKPDIPLRNMLLRLRQSGKIDKLWL FTNAYKNHAIRCLRLLGIADLFDGLTYCDYSRTDTLVCKPH VKAFEKAMKESGLARYENAYFIDDSGKNIETGIKLGMKTCI HLVENEVNEILGQTPEGAIVISDILELPHVVSDLF (SEQ ID NO: 45)
PH19	Saccharomyces cerevisiae yhr043c	MPQFSVDLCLFDLDGTIVSTTTAAESAWKKLCRQHGVDP VELFKHSHGARSQEMMKKFFPKLDNTDNKGVLALEKDMA DNYLDTVSLIPGAENLLLSLDVDTETQKKLPERKWAIVTSG SPYLAFSWFETILKNVGKPKVFITGFDVKNGKPDPEGYSR ARDLLRQDLQLTGKQDLKYVVFEDAPVGIKAGKAMGAITV GITSSYDKSVLFDAGADYVVCDLTQVSVVKNNENGIVIQV NNPLTRD (SEQ ID NO: 46)
PH20	Saccharomyces cerevisiae yhr044c	MAEFSADLCLFDLDGTIVSTTVAAEKAWTKLCYEYGVDPS ELFKHSHGARTQEVLRRFFPKLDDTDNKGVLALEKDIAHS YLDTVSLIPGAENLLLSLDVDTETQKKLPERKWAIVTSGSP YLAFSWFETILKNVGKPKVFITGFDVKNGKPDPEGYSRAR DLLRQDLQLTGKQDLKYVVFEDAPVGIKAGKAMGAITVGIT SSYDKSVLFDAGADYVVCDLTQVSVVKNNENGIVIQVNNP LTRA (SEQ ID NO: 47)
PH21	Saccharomyces cerevisiae yil053w	MPLTTKPLSLKINAALFDVDGTIIISQPAIAAFWRDFGKDKP YFDAEHVIHISHGWRTYDAIAKFAPDFADEEYVNKLEGEIP EKYGEHSIEVPGAVKLCNALNALPKEKWAVATSGTRDMA KKWFDILKIKRPEYFITANDVKQGKPHPEPYLKGRNGLGF PINEQDPSKSKVVVFEDAPAGIAAGKAAGCKIVGIATTFDL DFLKEKGCDIIVKNHESIRVGEYNAETDEVELIFDDYLYAK DDLLKW(SEQ ID NO: 48)
PH22	Saccharomyces cerevisiae ykr070w	MIGKRFFQTTSKKIAFAFDIDGVLFRGKKPIAGASDALKLLN RNKIPYILLTNGGGFSERARTEFISSKLDVDVSPLQIIQSHT PYKSLVNKYSRILAVGTPSVRGVAEGYGFQDVVHQTDIVR YNRDIAPFSGLSDEQVMEYSRDIPDLTTKKFDAVLVFNDP

		HDWAADIQIISDAINSENGMLNTLRNEKSGKPSIPIYFSNQ DLLWANPYKLNRFGQGAFRLLVRRLYLELNGEPLQDYTL GKPTKLTYDFAHHVLIDWEKRLSGKIGQSVKQKLPLLGTK PSTSPFHAVFMVGDNPASDIIGAQNYGWNSCLVKTGVYN EGDDLKECKPTLIVNDVFDAVTKTLEKYA(SEQ ID NO: 49)
PH23	Saccharomyces cerevisiae ynl010w	MVKAVIFTDFDGTVTLEDSNDYLTDTLGFGKEKRLKVFEG VLDDTKSFRQGFMEMLESIHTPFPECIKILEKKIRLDPGFK DTFEWAQENDVPVIVVSSGMKPIIKVLLTRLVGQESIHKIDI VSNEVEIDAHDQWKIIYKDESPFGHDKSRSIDAYKKKFEST LKAGEQRPVYFYCGDGVSDLSAAKECDLLFAKRGKDLVT YCKKQNVPFHEFDTFKDILASMKQVLAGEKTVAELMEN (SEQ ID NO: 50)
PH24	Saccharomyces cerevisiae yor131c	MTKLQGLQGLKHIKAVVFDMDGTLCLPQPWMFPAMRNAI GLEDKSIDILHFIDTLPTEKEKKEAHDRIELVEAKAMKEMQ PQPGLVDIMRYLTKNGISKNICTRNVGAPVETFVKRFIPSE LSRFDYIVTREFRPTKPQPDPLLHIASKLNIRPLEMIMVGDS FDDMKSGRSAGCFTVLLKNHVNGHLLLEHKELVDVSVED LSEIIELIQNMNKESF (SEQ ID NO: 51)
PH25	Saccharomyces cerevisiae yor155c	MSSRYRVEYHLKSHRKDEFIDWVKGLLASPFVLHAVSHE GDYNDDLATTQRVRSQYADIFKDIEGLIKDKIEFDSRNMSQ DEIEDGASSQSLNILGQSRLNLLVPSIGTFFTELPLEQAFL WEDSQRAISARRMVAPSFNDIRHILNTAQIFHFKKQENLH NGKVLRLVTFDGDVTLYEDGGSLVYTNPVIPYILKLLRCGI NVGIVTAAGYDEAGTYENRLKGLIVALHDSTDIPVSQKQNL TIMGGESSYLFRYYEDPEEDNFGFRQIDKEEWLLPRMKA WSLEDVEKTLDFAERTLNRLRKRLNLPSEISIIRKVRAVGIV PGERYDEASKRQVPVKLDREQLEEIVLTLQNTLESFAPSR RIQFSCFDGGSDVWCDIGGKDLGVRSLQQFYNPESPIQP SETLHVGDQFAPVGSANDFKARLAGCTLWIASPQETVNY LHRLLETD (SEQ ID NO: 52)
PH26	*Escherichia coli* **YniC**	MSTPRQILAAIFDMDGLLIDSEPLWDRAELDVMASLGVDIS RRNELPDTLGLRIDMVVDLWYARQPWNGPSRQEVVERVI ARAISLVEETRPLLPGVREAVALCKEQGLLVGLASASPLH MLEKVLTMFDLRDSFDALASAEKLPYSKPHPQVYLDCAAK LGVDPLTCVALEDSVNGMIASKAARMRSIVVPAPEAQNDP RFVLANVKLSSLTELTAKDLLG (SEQ ID NO: 53)
PH27	*Escherichia coli* **YfbT**	MRCKGFLFDLDGTLVDSLPAVERAWSNWARRHGLAPEE VLAFIHGKQAITSLRHFMAGKSEADIAAEFTRLEHIEATETE GITALPGAIALLSHLNKAGIPWAIVTSGSMPVARARHKIAGL PAPEVFVTAERVKRGKPEPDAYLLGAQLLGLAPQECVVV EDAPAGVLSGLAAGCHVIAVNAPADTPRLNEVDLVLHSLE QITVTKQPNGDVIIQ (SEQ ID NO: 54)
PH28	*Escherichia coli* **YieH**	MSTPRQILAAIFDMDGLLIDSEPLWDRAELDVMASLGVDIS RRNELPDTLGLRIDMVVDLWYARQPWNGPSRQEVVERVI ARAISLVEETRPLLPGVREAVALCKEQGLLVGLASASPLH MLEKVLTMFDLRDSFDALASAEKLPYSKPHPQVYLDCAAK LGVDPLTCVALEDSVNGMIASKAARMRSIVVPAPEAQNDP RFVLADVKLSSLTELTAKDLLG (SEQ ID NO: 55)
PH29	*Escherichia coli* **YihX**	MLYIFDLGNVIVDIDFNRVLGAWSDLTRIPLASLKKSFHMG EAFHQHERGEISDEAFAEALCHEMALPLSYEQFSHGWQA

		VFVALRPEVIAIMHKLREQGHRVVVLSNTNRLHTTFWPEE YPEIRDAADHIYLSQDLGMRKPEARIYQHVLQAEGFSPSD TVFFDDNADNIEGANQLGITSILVKDKTTIPDYFAKVLC (SEQ ID NO: 56)
PH31	*Escherichia coli* **YjjG**	MRILLSNDDGVHAPGIQTLAKALREFADVQVVAPDRNRSG ASNSLTLESSLRTFTFENGDIAVQMGTPTDCVYLGVNALM RPRPDIVVSGINAGPNLGDDVIYSGTVAAAMEGRHLGFPA LAVSLDGHKHYDTAAAVTCSILRALCKEPLRTGRILNINVP DLPLDQIKGIRVTRCGTRHPADQVIPQQDPRGNTLYWIGP PGGKCDAGPGTDFAAVDEGYVSITPLHVDLTAHSAQDVV SDWLNSVGVGTQW (SEQ ID NO: 57)
PH32	*Escherichia coli* **YqaB**	MYERYAGLIFDMDGTILDTEPTHRKAWREVLGHYGLQYDI QAMIALNGSPTWRIAQAIIELNQADLDPHALAREKTEAVRS MLLDSVEPLPLVDVVKSWHGRRPMAVGTGSESAIAEALL AHLGLRHYFDAVVAADHVKHHKPAPDTFLLCAQRMGVQP TQCVVFEDADFGIQAARAAGMDAVDVRLL (SEQ ID NO: 58)
PH33	*Escherichia coli* **YigB**	MRFYRPLGRISALTFDLDDTLYDNRPVILRTEREALTFVQN YHPALRSFQNEDLQRLRQAVREAEPEIYHDVTRWRFRSIE QAMLDAGLSAEEASAGAHAAMINFAKWRSRIDVPQQTHD TLKQLAKKWPLVAITNGNAQPELFGLGDYFEFVLRAGPHG RSKPFSDMYFLAAEKLNVPIGEILHVGDDLTTDVGGAIRSG MQACWIRPENGDLMQTWDSRLLPHLEISRLASLTSLI (SEQ ID NO: 59)
PH34	*Escherichia coli* **YrfG**	MHINIAWQDVDTVLLDMDGTLLDLAFDNYFWQKLVPETW GAKNGVTPQEAMEYMRQQYHDVQHTLNWYCLDYWSEQ LGLDICAMTTEMGPRAVLREDTIPFLEALKASGKQRILLTN AHPHNLAVKLEHTGLDAHLDLLLSTHTFGYPKEDQRLWH AVAEATGLKAERTLFIDDSEAILDAAAQFGIRYCLGVTNPD SGIAEKQYQRHPSLNDYRRLIPSLM (SEQ ID NO: 60)
PH35	*Escherichia coli* **Gph**	MSTPRQILAAIFDMDGLLIDSEPLWDRAELDVMASLGVDIS RRNELPDTLGLRIDMVVDLWYARQPWNGPSRQEVVERVI ARAISLVEETRPLLPGVREAVALCKEQGLLVGLASASPLH MLEKVLTMFDLRDSFDALASAEKLPYSKPHPQVYLDCAAK LGVDPLTCVALEDSVNGMIASKAARMRSIVVPAPEAQNDP RFVLADVKLSSLTELTAKDLLG (SEQ ID NO: 61)
PH36	*Escherichia coli* **YbiV**	MSVKVIVTDMDGTFLNDAKTYNQPRFMAQYQELKKRGIK FVVASGNQYYQLISFFPELKDEISFVAENGALVYEHGKQLF HGELTRHESRIVIGELLKDKQLNFVACGLQSAYVSENAPE AFVALMAKHYHRLKPVKDYQEIDDVLFKFSLNLPDEQIPLV IDKLHVALDGIMKPVTSGFGFIDLIIPGLHKANGISRLLKRW DLSPQNVVAIGDSGNDAEMLKMARYSFAMGNAAENIKQIA RYATDDNNHEGALNVIQAVLDNTSPFNS (SEQ ID NO: 62)
PH37	*Escherichia coli* **YidA**	MAIKLIAIDMDGTLLLPDHTISPAVKNAIAAARARGVNVVLT TGRPYAGVHNYLKELHMEQPGDYCITYNGALVQKAADGS TVAQTALSYDDYRFLEKLSREVGSHFHALDRTTLYTANRD ISYYTVHESFVATIPLVFCEAEKMDPNTQFLKVMMIDEPAIL DQAIARIPQEVKEKYTVLKSAPYFLEILDKRVNKGTGVKSL ADVLGIKPEEIMAIGDQENDIAMIEYAGVGVAMDNAIPSVK EVANFVTKSNLEDGVAFAIEKYVLN (SEQ ID NO: 63)

PH38	*Escherichia coli* **YbhA**	MTTRVIALDLDGTLLTPKKTLLPSSIEALARAREAGYRLIIVT GRHHVAIHPFYQALALDTPAICCNGTYLYDYHAKTVLEAD PMPVNKALQLIEMLNEHHIHGLMYVDDAMVYEHPTGHVIR TSNWAQTLPPEQRPTFTQVASLAETAQQVNAVWKFALTH DDLPQLQHFGKHVEHELGLECEWSWHDQVDIARGGNSK GKRLTKWVEAQGWSMENVVAFGDNFNDISMLEAAGTGV AMGNADDAVKARANIVIGDNTTDSIAQFIYSHLI (SEQ ID NO: 64)
PH39	*Escherichia coli* **YbjI**	MRFYRPLGRISALTFDLDDTLYDNRPVILRTEREALTFVQN YHPALRSFQNEDLQRLRQAVREAEPEIYHDVTRWRFRSIE QAMLDAGLSAEEASAGAHAAMINFAKWRSRIDVPQQTHD TLKQLAKKWPLVAITNGNAQPELFGLGDYFEFVLRAGPHG RSKPFSDMYFLAAEKLNVPIGEILHVGDDLTTDVGGAIRSG MQACWIRPENGDLMQTWDSRLLPHLEISRLASLTSLI (SEQ ID NO: 65)
PH40	*Escherichia coli* **YigL**	MYQVVASDLDGTLLSPDHTLSPYAKETLKLLTARGINFVFA TGRHHVDVGQIRDNLEIKSYMITSNGARVHDLDGNLIFAH NLDRDIASDLFGVVNDNPDIITNVYRDDEWFMNRHRPEE MRFFKEAVFQYALYEPGLLEPEGVSKVFFTCDSHEQLLPL EQAINARWGDRVNVSFSTLTCLEVMAGGVSKGHALEAVA KKLGYSLKDCIAFGDGMNDAEMLSMAGKGCIMGSAHQRL KDLHPELEVIGTNADDAVPHYLRKLYLS (SEQ ID NO: 66)
PH41	*Escherichia coli* **OtsB**	MTEPLTETPELSAKYAWFFDLDGTLAEIKPHPDQVVVPDN ILQGLQLLATASDGALALISGRSMVELDALAKPYRFPLAGV HGAERRDINGKTHIVHLPDAIARDISVQLHTVIAQYPGAEL EAKGMAFALHYRQAPQHEDALMTLAQRITQIWPQMALQQ GKCVVEIKPRGTSKGEAIAAFMQEAPFIGRTPVFLGDDLT DESGFAVVNRLGGMSVKIGTGATQASWRLAGVPDVWSW LEMITTALQQKRENNRSDDYESFSRSI (SEQ ID NO: 67)
PH42	*Escherichia coli* **YaeD**	MAKSVPAIFLDRDGTINVDHGYVHEIDNFEFIDGVIDAMRE LKKMGFALVVVTNQSGIARGKFTEAQFETLTEWMDWSLA DRDVDLDGIYYCPHHPQGSVEEFRQVCDCRKPHPGMLLS ARDYLHIDMAASYMVGDKLEDMQAAVAANVGTKVLVRTG KPITPEAENAADWVLNSLADLPQAIKKQQKPAQ (SEQ ID NO: 68)

[00132] Accordingly, in one embodiment, provided herein is a recombinant microorganism that comprises an endogenous and/or exogenous nucleic acid molecules capable of converting a carbon source to glyceraldehyde 3-phosphate (G3P); at least one endogenous or exogenous nucleic acid molecule encoding a (5-formylfuran-3-yl)methyl phosphate synthase that catalyzes the conversion of G3P to (5-formylfuran-3-yl)methyl phosphate; at least one endogenous or exogenous nucleic acid molecule encoding a phosphatase that catalyzes the conversion of (5-formylfuran-3-yl)methyl phosphate to 4-HMF.

2,4-FDCA

[00133] In one embodiment, the present disclosure provides a recombinant microorganism capable of producing 2,4-furandicarboxylic acid (2,4-FDCA) from a carbon source. Some embodiments of the present disclosure are presented in **FIG. 1**, **FIG. 2**, **and FIG. 3,** which collectively detail the biosynthetic conversion of a carbon feedstock to 2,4-FDCA.

[00134] In one embodiment, the recombinant microorganism comprises the novel capacity to convert G3P to 2,4-FDCA via several enzymatically-catalyzed successive steps described herein. In one embodiment, the present disclosure comprises converting 4-HMF to 2,4 FDCA directly or through the production of intermediates furan-2,4-dicarbaldehyde, 4-(hydroxymethyl)furoic acid, 4-formylfuran-2-carboxylate, 4-formylfuran-2-carboxylate, 2-formylfuran-4-carboxylate.

[00135] In one embodiment, the present disclosure comprises converting 4-HMF to furan-2,4-dicarbaldehyde (Step D) and/or 4-(hydroxymethyl)furoic acid (Step E); converting furan-2,4-dicarbaldehyde to 4-formylfuran-2-carboxylate (Step G) and/or 2-formylfuran-4-carboxylate (Step F) and/or converting 4-(hydroxymethyl)furoic acid to 4-formylfuran-2-carboxylate (Step H); converting 4-formylfuran-2-carboxylate to 2,4-FDCA (Step J) and/or converting 2-formylfuran-4-carboxylate to 2,4-FDCA (Step I).

[00136] In one embodiment, the dehydrogenase is classified as EC number 1.1.1. when oxidizing an alcohol to a carbonyl group or EC number 1.2.1. when oxidizing an carbonyl to acid. In some aspects, the dehydrogenase is an alcohol dehydrogenase or an aldehyde dehydrogenase.

[00137] In some aspects, the oxidase from (c) is classified as EC number 1.1.3. In some aspects, the oxidase is 5-hydroxymethylfurfural oxidase. In some aspects the 5-hydroxymethylfurfural oxidase convert the 4-hydroxymethylfurfural (4-HMF) into 2,4 FDCA in a three-step reaction.

[00138] In a further embodiment, the one or more carbon sources may include glycerol or a monosaccharide.

[00139] In one embodiment, a microorganism comprises at least one endogenous or exogenous nucleic acid molecule encoding a dehydrogenase, an oxidase, or a peroxygenase that catalyzes the conversion of 4-HMF to furan-2,4-dicarbaldehyde and/or 4-(hydroxymethyl)furoic acid; at least one endogenous or exogenous nucleic acid molecule encoding a dehydrogenase, an oxidase, or a peroxygenase that catalyzes the conversion of furan-2,4-dicarbaldehyde to 4-formylfuran-2-carboxylate and/or 2-formylfuran-4-carboxylate and/or the conversion of 4-(hydroxymethyl)furoic acid to 4-formylfuran-2-carboxylate; at least one endogenous or exogenous nucleic acid molecule encoding a dehydrogenase, an oxidase, or a peroxygenase that catalyzes the conversion of 2-formylfuran-4-carboxylate to 2,4-FDCA and/or 4-formylfuran-2-carboxylate to 2,4-FDCA.

[00140] In one embodiment, the recombinant microorganism of any one of the embodiments disclosed herein comprises at least one endogenous or exogenous nucleic acid molecule encoding a dehydrogenase, an oxidase, or a peroxygenase that catalyzes the conversion of 4-HMF to furan-2,4-dicarbaldehyde. In one embodiment, the dehydrogenase is classified as EC number 1.1.1. In one embodiment, the dehydrogenase EC number 1.1.1 selected from alcohol dehydrogenase (EC number 1.1.1.1), or alcohol dehydrogenase (NADP+) (EC number 1.1.1.2), or D-xylose reductase (EC number 1.1.1.307), or aryl-alcohol dehydrogenase (EC number 1.1.1.90), or aryl-alcohol dehydrogenase (NADP+) (EC number 1.1.1.91). In one embodiment the dehydrogenases can be derived from enzyme candidates listed at Table 3. In some embodiments, the dehydrogenases are homologous or similar to the enzymes listed at Table 3. In some embodiments the 4-HMF dehydrogenase enzyme is encoded by an amino acid sequence listed in Table 3. In some embodiments, a dehydrogenase is evolved or engineered to improve its catalytic efficiency against its desirable substrate.

Table 3. 4 HMF Dehydrogenases enzymes

Name	Organism	Sequence
DH1	*Zymomonas mobilis*	MLNFDYYNPTHIVFGKGRIAQLDTLLSKDARVLVLYGGSS AQKTGTLDEVRKALGDRTYFEFGGIEPNPSYETLMKAVEQ VKQEKVDFLLAVGGGSVIDGTKFVAAAVPYEGEPWEILET DGKKIKEALPVGTVLTLPATGSEMNRNSVVTRKSIKSKRG FHNDHVFPVFSILDPTKVYTLPPRQLANGVVDSFIHITEQY LTYPVDGMVQDEFAEGLLRTLIKIGPELLKDQKNYDLAANF MWTATLALNGLIGAGVPQDWATHMVGHELTAAFGIDHGR TLAIILPSLLQNQREAKKGKLLQYAKNVWHIDQGSDDERID AAIEKTRHFFESLGIPTHLKDYDVGEESIDMLVKELEAHGM SQLGEHKAITPEVSRAILLASL (SEQ ID NO: 69)

DH2	*Zymomonas mobilis subsp. pomaceae ATCC 29192*	MLNFDYYNPTHIAFGKDSIAKLDTLIPQDACVMVLYGGSSA KKTGTLDEVKTALGSRKIHEFGGIEPNPSYETLMQAVEQV KKEKIDFLLAVGGGSVIDGTKFVAAAVPYEGEPWEILETDG KKIKKALPLGTVLTLPATGSEMNPNSVVTRKSIKAKRAFHN KIVFPLFSILDPTKVYTLPPRQIANGIVDSFVHITEQYLTYPV EGMVQDEFAEGLLRILINIGPKLLKDQKNYDLAANFMWTA TLALNGLIGAGVPQDWATHMIGHEITAAFGVDHGRTLAIIL PSLLQNQRQVKKDKLLQYAKNVWHIESGSEKERIDAVIAK TRSFFEEMGIPTHLSDYNIGKESIDMLIHELEAHGMTKLGE HNAITPDVSRAILIASL (SEQ ID NO: 70)
DH3	*Shewanella baltica*	MLNFNYYNPTRIRFGKDTIAEIDTLVPSDAKVMILFGGSSA RKTGTLDEVKQSLGNRFIVEFDGIEPNPTYETLMKAVAQV REQKIDFLLAVGGGSVIDGTKFVAAAAVFEGEPWDILTSW GAKVTQAMPFGSVLTLPATGSEMNNASVVTRKSLQAKLP FRNDLVYPQFSILDPTKTFTLPERQVANGVVDAFVHITEQY LTYPVNAAVQDRFAEGLLQTLIELGPQVLAQPEDYDIRANL MWVATMALNGTIGVGVPHDWATHMIGHELTALYDIDHAR TLAIVLPALLQCTKEAKREKLLQYADRVWHINTGTDDERID AAIAKTKAFFEAMGIPTHLSAYDLDASHVDTLVKQLELHGM VALGEHGNINPAMSRDILTLAL (SEQ ID NO: 71)
DH4	*Burkholderia pseudomallei*	MLNFDFYNPTRIVFGEKTAARLNDLLPAAARVLVLYGGES ARSNGTLDEVRAALGARDVREFGGIEPNPAYETLMRAVE LARRERVDFLLAVGGGSVIDGTKFVAAAVPFEGDPWTILE THGANVAAALPFGCVLTLPATGSEMNNGAVLTRRATRAK LAFRHPLVFPTFSILDPTKTYTLPPRQVANGVVDAFTHIVE QYLTYPADGLAQDRFAEGLLQTLIEIGPKALAEPRDYATRA NLMWVATLALNGLIGAGVPQDRATHMVGHELTARYDIDH ARTLAVVLPSMLDVRRDAKRAKLLQYAARVWNIVDGPED ARIDAAIARTRAFFESLGVKTRLADYGVGADAIDGLIAQLE AHGMTRLGERKDVTLDVSRRVLEASL (SEQ ID NO: 72)
DH5	*Saccharomyces cerevisiae*	MSIPETQKGVIFYESHGKLEYKDIPVPKPKANELLINVKYS GVCHTDLHAWHGDWPLPTKLPLVGGHEGAGVVVGMGE NVKGWKIGDYAGIKWLNGSCMACEYCELGNEPNCPHAD SSGYTHDGSFQQYATADAVQAAHIPQGTDLAEVAPVLCA GITVYKALKSANLMAGHWVAISGAAGGLGSLAVQYAKAM GYRVLGIDGGEGKEELFRSIGGEVFIDFTKEKDIVGAVLKA TDGGAHGVINVSVSEAAIEASTRYVRANGTTVLVGMPAGA KCCSDVFNQVVKSISIVGSCVGNRADTREALDFFARGLVK SPIKVVGLSTLPEIYEKMEKGQIVGRYVVDTSK (SEQ ID NO: 73)
DH6	*Saccharomyces cerevisiae*	MSYPEKFEGIAIQSHEDWKNPKKTKYDPKPFYDHDIDIKIE ACGVCGSDIHCAAGHWGNMKMPLVVGHEIVGKVVKLGP KSNSGLKVGQRVGVGAQVFSCLECDRCKNDNEPYCTKF VTTYSQPYEDGYVSQGGYANYVRVHEHFVVPIPENIPSHL AAPLLCGGLTVYSPLVRNGCGPGKKVGIVGLGGIGSMGTL ISKAMGAETYVISRSSRKREDAMKMGADHYIATLEEGDW GEKYFDTFDLIVVCASSLTDIDFNIMPKAMKVGGRIVSISIP EQHEMLSLKPYGLKAVSISYSALGSIKELNQLLKLVSEKDIK IWVETLPVGEAGVHEAFERMEKGDVRYRFTLVGYDKEFS D (SEQ ID NO: 74)

DH7	*Pseudomonas putida*	MSIEHRLNHIAGQLSGNGEVLLNSVDAHTGEPLPYAFHQA TSDEVDAAVQAAEAAYPAYRSTSPAQRAAFLDAIANELDA LGDDFVQHVMRETALPEARIRGERARTSNQLRLFADVVR RGDFLGARIDRAQPERTPLPRPDLRQYRIGVGPVAVFGAS NFPLAFSTAGGDTASALAAGCPVVFKAHSGHMLTAAHVA AAIDRAVAGSGMPAGVFNMIYGAGVGEVLVKHPAIQAVGF TGSLRGGRALCDMAAARPQPIPVFAEMSSINPVIVLPQAL QARGEQVAGELAASVVLGCGQFCTNPGLVVGIKSPQFER FVHTLVARMADQAPQTMLNAGTLRSYQSGVQHLLAHPGI QHLAGQPQAGKQAQPQLFKADVSLLLDSDPLLQEEVFGP TTVVVEVADAQQLAEALRHLQGQLTATLIAEPDDLRAFAA LVPLLERKAGRLLLNGYPTGVEVSDAMVHGGPYPATSDA RGTSVGTLAIDRFLRPVCFQNYPDALLPEALKSANPLGIAR LVDGVASRGAV (SEQ ID NO: 75)
DH8	*Pseudomonas putida*	MSIEHRLNHIAGQLSGNGDVLLNSVDAHTGEPLPYAFHQA TGDEVEAAVQAADAAYPAYRSTSPAQRAAFLDAIANELDA LGDDFIQHVMRETALPEARIRGERSRTSNQLRLFAEVVRR GDFYAARIDRALPQRTPLPRPDLRQYRIGVGPVAVFGASN FPLAFSTAGGDTASALAAGCPVVFKAHSGHMLTAAHVAG AIDRAVATSGMPAGVFNLIYGAGVGEALVKHPAIQAVGFT GSLRGGRALCDMAAARPQPIPVFAEMSSINPVIVLPQALQ ARGEQVAGELAASVVMGCGQFCTNPGLVVGIQSPQFEHF VQTLVARMADQGPQTMLNAGTLRSYQNGVQHLLAHPGIQ HLAGQPHTGNQAQPQLFKADVSLLLNGDPLLQEEVFGPT TVVVEVADAEQLAEALRHLQGQLTATLIAEPDDLRAFASLV PLLERKAGRLLLNGYPTGVEVSDAMVHGGPYPATSDARG TSVGTLAIDRFLRPVCFQNYPDALLPDALKNANPLGIARLL DGVNSRDAV (SEQ ID NO: 76)
DH9	*Pseudomonas sp. NBRC 111139*	MSIEHRLNHIAGQLSGHGDVLLHSLDAHTGEALPYAFHQA TGDEVEAAAQAAEVAYPSYRSTRPDQRAAFLDAIASELDA LGDDFIQDVMRETALPEARIRGERSRTSNQLRLFAEVVRR GDFYAARIDRALPQRTPLPRPDLRQYRIGVGPVAVFGASN FPLAFSTAGGDTASALAAGCPVVFKAHSGHMLTAAHVAA AIDRAVTGSGMPAGVFNMIYGAGVGEALVKHPAIQAVGFT GSLRGGRALCDMAAARPQPIPVFAEMSSINPVIVLPQALQ ARGEQVATELAASVVLGCGQFCTNPGLVVGIRSPHFEHFL QTLVARMADQGPQTMLNAGTLRSYQNAVQHLLAHPGIQH LAGQPQTGNQAQPQLFKADVSLLLNGDPLLQEEVFGPCT VVVEVADAQQLAEALRHLQGQLTATLIAEPDDLRAFASLV PLLERKAGRLLLNGYPTGVEVSDAMVHGGPYPATSDARG TSVGTLAIDRFLRPVCFQNYPDALLPDALKNANPLGIARLL EGVSSREAV (SEQ ID NO: 77)
DH10	*Pseudomonas sp. JUb52*	MQIQGKNYIGGARSGEGEVRVYSIDATTGEKLPYEFFQAS TAEVDAAARAAEQAAPLYRKLSAEQRATFLDAIADELDAL GDDFVQLVCQETALPAGRIQGERGRTSGQMRLFAKVLRR GDFHGARIDTALPERKPLPRPDLRQYRIGLGPVAVFGASN FPLAFSTAGGDTAAALAAGCPVVFKAHSGHMVTAEYVAD AIIRAAEKTGMPKGVFNMIYGGGVGEQLVKHPAIQAVGFT GSLRGGRALCDMAAARPQPIPVFAEMSSINPVVVLPEALK ARGDAITGELAASVVLGCGQFCTNPGLVIGLRSPEFSTFL EGLAAAMNEQAPQTMLNPGTLKSYEKGVAALLAHSGVQH

		LAGANQEGNQARPQLFKADVSLLLENDELLQEEVFGPTT VVVEVADEAQLHQALQGLHGQLTATLLAEPADLQRFEAII GLLEQKAGRLLLNGYPTGVEVCDAMVHGGPYPATSDAR GTSVGTLAIDRFLRPVCYQNYPDAFLPEALQNANPLGIQR LVNGENTKAAI (SEQ ID NO: 78)
DH11	*Pseudomonas citronellolis*	MFGHNFIGGARTAQGNLTLQSLDAGTGEALPYSFHQATP EEVDAAALAAEAAFPAYRALPDARRAEFLDAIAAELDALG EDFIAIVCRETALPAARIQGERARTSNQLRLFAQVLRRGDY HGARIDRALPERQPLPRPDLRQCRIGVGPVAVFGASNFPL AFSTAGGDTAAALAAGCPVVFKAHSGHMATAEHVASAIV RAAQATGMPAGVFNMIYGGGVGERLVKHPAIQAVGFTGS LKGGRALCDLAAARPQPIPVFAEMSSINPVLALPAALAAR GEQVAADLAASVVLGCGQFCTNPGMVIGIASAEFSAFVAS LTGRMADQPAQTMLNAGTLKSYERGIAALHAHPGIRHLAG QPQKGRQALPQLFQADARLLIEGDELLQEEVFGPVTVVVE VADAAELQRALQGLRGQLTATLIAEPEDLSCFAALVPLLER KAGRLLLNGYPTGVEVCDAMVHGGPYPATSDARGTSVG TLAIDRFLRPVCYQNYPDALLPPALKDANPLGIARLVDGVA SREPL (SEQ ID NO: 79)

[00141] In one embodiment, the oxidase is classified as EC number 1.1.3. In one embodiment, the oxidase EC number 1.1.3 is 5-(hydroxymethylfurfural oxidase (EC number 1.1.3.47). In some embodiments the HMF oxidase can be derived from the gene hmfH. In some embodiments, HMF oxidase can be derived from *Methylovorus sp*. MP688 or Cupriavidus basilensis. *See* Dijkman and Fraaije (2014. Applied Environmental Microbiology, 80.3:1082-1090) and Koopman *et al.* (2010. PNAS, 107(11):4919-4924). In one embodiment, the HMF oxidase EC number 1.1.3 is aryl-alcohol oxidase (EC number 1.1.3.7). *See* Carro et al. (2015). In one embodiment, the peroxygenase is classified as EC number 1.11.2. In one embodiment, the peroxygenase EC number 1.11.2 is unspecific peroxygenase (EC number 1.11.2.1). *See* Carro *et al.* (2015). In some embodiments, the HMF oxidase can be derived from enzyme candidates listed at Table 4. In some embodiments, the HMF oxidase is homologous or similar to the enzymes listed at Table 4. In some embodiments the 4-HMF oxidaze enzyme is encoded by an amino acid sequence listed in Table 4. In some embodiments, the HMF oxidase enzyme is evolved or engineered to improve its catalytic efficiency (See Martin et al. Biotechnology for Biofuels. (2018) 11, Article number: 56).

Table 4. 4-HMF oxidases enzymes

Name	**Organism**	**Sequence**
HmfH 1	*Methylovorus sp*	MTDTIFDYVIVGGGTAGSVLANRLSARPENRVLLIEAGIDT PENNIPPEIHDGLRPWLPRLSGDKFFWPNLTIHRAAEHPGI TREPQFYEQGRLLGGGSSVNMVVSNRGLPRDYDEWQAL

		GADGWDWQGVLPYFIKTERDADYGDDPLHGNAGPIPIGR VDSRHWSDFTVAATQALEAAGLPNIHDQNARFDDGYFPP AFTLKGEERFSAARGYLDASVRVRPNLSLWTESRVLKLLT TGNAITGVSVLRGRETLQVQAREVILTAGALQSPAILLRTGI GPAADLHALGIPVLADRPGVGRNLWEHSSIGVVAPLTEQA RADASTGKAGSRHQLGIRASSGVDPATPSDLFLHIGADPV SGLASAVFWVNKPSSTGWLKLKDADPFSYPDVDFNLLSD PRDLGRLKAGLRLITHYFAAPSLAKYGLALALSRFAAPQP GGPLLNDLLQDEAALERYLRTNVGGVWHASGTARIGRAD DSQAVVDKAGRVYGVTGLRVADASIMPTVPTANTNLPTL MLAEKIADAILTQA (SEQ ID NO: 80)
HmfH 2	*Cupriavidus basilensis*	MDTPRERFDYVIVGGGSAGCVLANRLSQDPAIRVALIEAG VDTPPDAVPAEILDSYPMPLFFGDRYIWPSLQARAVAGGR SKVYEQGRVMGGGSSINVQAANRGLPRDYDEWAASGAS GWSWQDVLPYFRHLERDVDYGNSPLHGSHGPVPIRRILP QAWPPFCTEFAHAMGRSGLSALADQNAEFGDGWFPAAF SNLDDKRVSTAIAYLDADTRRRANLRIYAETTVRKLVVSGR EARGVIAMRADGSRLALDAGEVIVSAGALQSPAILMRAGI GDAGALQALGIEVVADRPGVGRNLQDHPALTFCQFLAPQ YRMPLSRRRASMTAARFSSGVPGGEASDMYLSSSTRAG WHALGNRLGLFFLWCNRPFSRGQVSLAGAQPDVPPMVE LNLLDDERDLRRMVAGVRKLVQIVGASALHQHPGDFFPA TFSPRVKALSRVSRGNVLLTELLGAVLDVSGPLRRSLIARF VTGGANLASLLTDESALEGFVRQSVFGVWHASGTCRMG AHADRSAVTDAAGRVHDVGRLRVIDASLMPRLPTANTNIP TIMLAEKIADTMQAERRAVRPASSEVAHPS (SEQ ID NO: 81)
HmfH 3	*Cupriavidus necator*	MDTPRERFDYVIVGGGSAGCVLANRLSQDPAIRVALIEGG VDTPPDAVPVEILDSYPMPLFFGDRYIWPSLQARAVAGGR SKVYEQGRVMGGGSSINVQAANRGLPRDYDEWAASGAP GWSWQDVLPYFRNLERDVDYGNSPLHGSHGPVPIRRILP QAWPPFCTEFAHAMGLSGLSALADQNAEFGDGWFPAAF SNLDDKRVSTAIAYLDADTRRRANLRIYAETTVRKLVVSGR EARGVIAIRADGSRLALDAGEVIVSAGALQSPAILMRAGIG DAGALQALGIEVVADRPGVGRNLQDHPALTFCQFLAPQY RMPLSRRRASMTAARFSSGVPGGEASDMYLSSSTRAGW HALGNRLGLFFLWCNRPFSRGQVSLAGAQPDVPPMVEL NLLDDERDLRRMVAGVRKLVQIVGASALHQHPGDFFPAT FSPRVKALSRLSRGNALLTELLGALLDVSGPLRRSLIARFV TGGANLASLLVEESALEGFVRQSVFGVWHASGTCRMGA HADRSAVTDAAGRVHDVGRLRVVDASLMPRLPTANTNIP TIMLAEKIADTMQAERRAVRLASSEVAHQS (SEQ ID NO: 82)
HmfH 4	*Cupriavidus pinatubonensis*	MGTPRDRFDYVIVGGGSAGCVLANRLSRDPGIRVALIEGG VDTPPGAVPAEILDSYPMPLFFGDRYLWPSLQARAVAGG RARLYEQGRVMGGGSSINVQAANRGLPRDYDEWAASGA PGWSWQEVLPYFRKLERDVDFASSPMHGSDGPVPIRRIL PPAWPPFCTAFAQAMGRSGLSALDDQNAEFGDGWFPAA FSNLDGKRVSTAIAYLDANTRKRTNLRIFAETTVKELVVSG REARGVIAVRADGARLALEAAEVIVSAGALQSPAILMRAGI GDAAALQALGIEVVADRPGVGRNLQDHPALTFCQFLAPE

		YRMPLARRRSSMTAARFSSEVPGGEASDMYLSSSTRAG WHALGNRLGLFFLWCNRPFSRGQVSLAGAQPEVSPLVEL NLLDDERDLRRMVAGVRRLVRIVGASALHQHPDDFFPAIF SPRVKAMSRVSPGNALLTALLGALLDVSGPLRRSLIARFV TGGANLASLLADESALEGFVRQSVFGVWHASGTCRMGA HADRSAVTDTTGRVHDVGRLRVVDASLMPRLPTANTNIPT IMLAEKIADAMLAERRATRRALSEVADPG (SEQ ID NO: 83)
HmfH 5	*Pandoraea sp. B-6*	MPRGHAHRRIRRHSVQNVRERFDYVIIGGGSAGCVLAHR LSANRELRVALIEAGSDTPPGAIPAEILDSYPMPVFCGDRY IWPELKAKATAASPLKVYEQGKVMGGGSSINVQAANRGL PRDYDDWAEQGASGWAWKDVLPYFRKLERDADYGGSA LHGADGPVAIRRIKPDAWPRFCHAFAEGLQRNGLPMLED QNAEFGDGMFPAAFSNLDDKRVSTAVAYLDAATRARTNL RIYSNTTVERLIVTGQRAHGVVAMSAGGERLQIDAAEVIVS AGALQSPALLLRAGIGAGSELQALGIPVVADRPGVGRNLQ DHPSLTFCHFLDPEFRMPLSRRRASMTAARFSSGLDGCD NADMYLSSATRAAWHALGNRLGLFFLWCNRPFSRGRVQ LTSADPFTPPRVDLNLLDDERDARRMAIGVRRVAQIVQQT ALHRHPDDFFPAAFSPRVKALSRFSAGNAALTKVLGLALD TPAPLRRWIIDTFVTGGIRMSALLADDKELDAFIRKYVFGV WHASGTCRMGPASDRMAVTNQEGLVHDVANLRVVDASL MPKLPSANTNIPTIMMAEKIADAILARRKAPPGVLVSSEA (SEQ ID NO: 84)
HmfH 6	*Methylovorus sp*	MTDTIFDYVIVGGGTAGSVLANRLSARPENRVLLIEAGIDT PENNIPPEIHDGLRPWLPRLSGDKFFWPNLTIHRAAEHPGI TREPQFYEQGRLLGGGSSVNMVVSNRGLPRDYDEWQAL GADGWDWQGVLPYFIKTERDADYGDDPLHGNAGPIPIGR VDSRHWSDFTVAATQALEAAGLPNIHDQNARFDDGYFPP AFTLKGEERFSAARGYLDASVRVRPNLSLWTESRVLKLLT TGNAITGVSVLRGRETLQVQAREVILTAGALQSPAILLRTGI GPAADLHALGIPVLADRPGVGRNLWEHSSIGVVAPLTEQA RADASTGKAGSRHQLGIRASSGVDPATPSDLFLHIGADPV SGLASARFWVNKPSSTGWLKLKDADPFSYPDVDFNLLSD PRDLGRLKAGLRLITHYFAAPSLAKYGLALALSRFAAPQP GGPLLNDLLQDEAALERYLRTNVGGVFHASGTARIGRAD DSQAVVDKAGRVYGVTGLRVADASIMPTVPTANTNLPTL MLAEKIADAILTQA (SEQ ID NO: 85)
HmfH 7	*Methylovorus sp MUT*	MTDTIFDYVIVGGGTAGSVLANRLSARPENRVLLIEAGIDT PENNIPPEIHDGLRPWLPRLSGDKFFWPNLTVYRAAEHPG ITREPQFYEQGRLLGGGSSVNMVVSNRGLPRDYDEWQA LGADGWDWQGVLPYFIKTERDADYGDDPLHGNAGPIPIG RVDSRHWSDFTVAATQALEAAGLPNIHDQNARFDDGYFP PAFTLKGEERFSAARGYLDASVRVRPNLSLWTESRVLKLL TTGNAITGVSVLRGRETLQVQAREVILTAGALQSPAILLRT GIGPAADLHALGIPVLADRPGVGRNLWEHSSIGVVAPLTE QARADASTGKAGSRHQLGIRASSGVDPATPSDLFLHIHAD PVSGLASARFWVNKPSSTGWLKLKDADPFSYPDVDFNLL SDPRDLGRLKAGLRLIKHYFAYPSLAKYGLALALSRFEAP QPGGPLLNDLLQDEAALERYLRTNVGGVFHASGTARIGR ADDSQAVVDKAGRVYGVTGLRVADASIMPTVPTANTNLP TLMLAEKIADAILTQA (SEQ ID NO: 86)

[00142] In one embodiment, the recombinant microorganism of any one of the embodiments disclosed herein comprises at least one endogenous or exogenous nucleic acid molecule encoding a dehydrogenase, or a oxidase, or a peroxygenase that catalyzes the conversion of 4-HMF to 4-(hydroxymethyl)furoic acid. In one embodiment, the dehydrogenase is classified as EC number 1.2.1. In one embodiment, the dehydrogenase EC number 1.2.1 selected from aldehyde dehydrogenase (NAD+) (EC number 1.2.1.3) or aldehyde dehydrogenase (NADP+) (EC number 1.2.1.4) or aldehyde dehydrogenase [NAD(P)+] (EC number 1.2.1.5) or 4-(γ-glutamylamino)butanal dehydrogenase (EC number 1.2.1.99). In one embodiment, the oxidase is classified as EC number 1.1.3. In one embodiment, the oxidase EC number 1.1.3 is 5-(hydroxymethylfurfural oxidase (EC number 1.1.3.47). In some embodiments the EC 1.1.3.47 oxidase can be derived from the gene hmfH. In some embodiments, hmfH can be derived from *Methylovorus sp.* MP688 or Cupriavidus basilensis. In one embodiment, the oxidase EC number 1.1.3 is aryl-alcohol oxidase (EC number 1.1.3.7). In one embodiment, the peroxygenase is classified as EC number 1.11.2. In one embodiment, the peroxygenase EC number 1.11.2 is unspecific peroxygenase (EC number 1.11.2.1).

[00143] In one embodiment, the recombinant microorganism of any one of the embodiments disclosed herein comprises at least one endogenous or exogenous nucleic acid molecule encoding a dehydrogenase, or a oxidase, or a peroxygenase that catalyzes the conversion of furan-2,4-dicarbaldehyde to 4-formylfuran-2-carboxylate and/or to 2-formylfuran-4-carboxylate. In one embodiment, the dehydrogenase is classified as EC number 1.2.1. In one embodiment, the dehydrogenase EC number 1.2.1 selected from aldehyde dehydrogenase (NAD+) (EC number 1.2.1.3) or aldehyde dehydrogenase (NADP+) (EC number 1.2.1.4) or aldehyde dehydrogenase [NAD(P)+] (EC number 1.2.1.5) or 4-(γ-glutamylamino)butanal dehydrogenase (EC number 1.2.1.99). In one embodiment, the oxidase is classified as EC number 1.1.3. In one embodiment, the oxidase EC number 1.1.3 is 5-(hydroxymethylfurfural oxidase (EC number 1.1.3.47). In some embodiments the EC 1.1.3.47 oxidase can be derived from the gene hmfH. In some embodiments, hmfH can be derived from *Methylovorus sp.* MP688 or Cupriavidus basilensis. In one embodiment, the oxidase EC number 1.1.3 is aryl-alcohol oxidase (EC number 1.1.3.7). In one embodiment, the peroxygenase is classified as EC number 1.11.2. In one embodiment, the peroxygenase EC number 1.11.2 is unspecific peroxygenase (EC number 1.11.2.1).

[00144] In one embodiment, the recombinant microorganism of any one of the embodiments disclosed herein comprises at least one endogenous or exogenous nucleic acid

molecule encoding a dehydrogenase, or a oxidase, or a peroxygenase that catalyzes the conversion of 4-(hydroxymethyl)furoic acid to 4-formylfuran-2-carboxylate. In one embodiment, the dehydrogenase is classified as EC number 1.1.1. In one embodiment, the dehydrogenase EC number 1.1.1 selected from alcohol dehydrogenase (EC number 1.1.1.1), or alcohol dehydrogenase (NADP+) (EC number 1.1.1.2), or D-xylose reductase (EC number 1.1.1.307), or aryl-alcohol dehydrogenase (EC number 1.1.1.90), or aryl-alcohol dehydrogenase (NADP+) (EC number 1.1.1.91). In one embodiment, the dehydrogenase EC number 1.1.1 is. In one embodiment, the oxidase is classified as EC number 1.1.3. In one embodiment, the oxidase EC number 1.1.3 is 5-(hydroxymethylfurfural oxidase (EC number 1.1.3.47). In some embodiments the EC 1.1.3.47 oxidase can be derived from the gene hmfH. In some embodiments, hmfH can be derived from *Methylovorus sp.* MP688 or Cupriavidus basilensis. In one embodiment, the oxidase EC number 1.1.3 is aryl-alcohol oxidase (EC number 1.1.3.7). In one embodiment, the peroxygenase is classified as EC number 1.11.2. In one embodiment, the peroxygenase EC number 1.11.2 is unspecific peroxygenase (EC number 1.11.2.1).

[00145] In one embodiment, the recombinant microorganism of any one of the embodiments disclosed herein comprises at least one endogenous or exogenous nucleic acid molecule encoding a dehydrogenase, or a oxidase, or a peroxygenase that catalyzes the conversion of 4-formylfuran-2-carboxylate and/or 2-formylfuran-4-carboxylate to 2,4-FDCA. In one embodiment, the dehydrogenase is classified as EC number 1.2.1. In one embodiment, the dehydrogenase EC number 1.2.1 selected from aldehyde dehydrogenase (NAD+) (EC number 1.2.1.3) or aldehyde dehydrogenase (NADP+) (EC number 1.2.1.4) or aldehyde dehydrogenase [NAD(P)+] (EC number 1.2.1.5) or 4-(γ-glutamylamino)butanal dehydrogenase (EC number 1.2.1.99). In one embodiment, the oxidase is classified as EC number 1.1.3. In one embodiment, the oxidase EC number 1.1.3 is 5-(hydroxymethylfurfural oxidase (EC number 1.1.3.47). In some embodiments the EC 1.1.3.47 oxidase can be derived from the gene hmfH. In some embodiments, hmfH can be derived from *Methylovorus sp.* MP688 or Cupriavidus basilensis. In one embodiment, the oxidase EC number 1.1.3 is aryl-alcohol oxidase (EC number 1.1.3.7). In one embodiment, the peroxygenase is classified as EC number 1.11.2. In one embodiment, the peroxygenase EC number 1.11.2 is unspecific peroxygenase (EC number 1.11.2.1).

[00146] In some aspects, 2,4-FDCA is produced enzymatically, in the absence of microbes. In some aspects, 2,4-FDCA is produced enzymatically in one or more vessels. In some aspects, the one or more vessels are substantially free of microbes. In some aspects, the enzymatic production of 2,4-FDCA is performed in the same step-wise fashion as described with in the methods utilizing recombinant microorganisms, but substantially free of microorganisms or

in the absence of microorganisms. In some aspects, the enzymes utilized in the enzymatic production of 2,4-FDCA are isolated from microbes, recombinant or otherwise, and provided to their corresponding substrates for the stepwise production of the intermediates utilized to produce 2,4-FDCA. In some aspects, one or more of the steps of the methods are performed in the same vessel. In some aspects, once the desired product is produced as a result of the individual method steps described herein, the product is isolated and purified and then utilized as the substrate in the next step of the method of producing 2,4-FDCA.

2,4-furandimethanol

[00147] In one embodiment, the present disclosure provides a recombinant microorganism capable of producing 2,4-furandimethanol from a carbon source. Some embodiments of the present disclosure are presented in **FIG. 1**, **FIG. 2**, **and FIG. 3**, which collectively detail the biosynthetic conversion of a carbon feedstock to 2,4-furandimethanol.

[00148] In one embodiment, the bioproduction of 2,4-furandimethanol from 4-HMF is catalyzed by a dehydrogenase encoded by the microorganism. In one embodiment, the dehydrogenase is classified as EC number 1.1.1. In one embodiment, the dehydrogenase EC number 1.1.1 is selected from alcohol dehydrogenase (EC number 1.1.1.1). In one embodiment, the dehydrogenase EC number 1.1.1 is selected from alcohol dehydrogenase (NADP+) (EC number 1.1.1.2). In one embodiment, the dehydrogenase EC number 1.1.1 is selected from D-xylose reductase (EC number 1.1.1.90). In one embodiment, the dehydrogenase EC number 1.1.1 is selected from aryl-alcohol dehydrogenase (EC number 1.1.1.91). In one embodiment the dehydrogenases can be derived from enzyme candidates listed at Table 5. In some embodiments, the dehydrogenases are homologous or similar to the enzymes listed at Table 5. In some embodiments the 4-HMF reductase enzyme is encoded by an amino acid sequence listed in Table 5. In some embodiments, a dehydrogenases is evolved or engineered to improve its catalytic efficiency for 4-HMF reduction to 2,4-furandimethanol.

Table 5. 4-HMF reductase enzymes (4-HMF reduction to 2,4-furandimethanol)

Name	Organism	Sequence
DH1	*Zymomonas mobilis*	MLNFDYYNPTHIVFGKGRIAQLDTLLSKDARVLVLYGGSS AQKTGTLDEVRKALGDRTYFEFGGIEPNPSYETLMKAVEQ VKQEKVDFLLAVGGGSVIDGTKFVAAAVPYEGEPWEILET DGKKIKEALPVGTVLTLPATGSEMNRNSVVTRKSIKSKRG FHNDHVFPVFSILDPTKVYTLPPRQLANGVVDSFIHITEQY LTYPVDGMVQDEFAEGLLRTLIKIGPELLKDQKNYDLAANF MWTATLALNGLIGAGVPQDWATHMVGHELTAAFGIDHGR TLAIILPSLLQNQREAKKGKLLQYAKNVWHIDQGSDDERID AAIEKTRHFFESLGIPTHLKDYDVGEESIDMLVKELEAHGM SQLGEHKAITPEVSRAILLASL (SEQ ID NO: 87)

DH2	*Zymomonas mobilis subsp. pomaceae ATCC 29192*	MLNFDYYNPTHIAFGKDSIAKLDTLIPQDACVMVLYGGSSA KKTGTLDEVKTALGSRKIHEFGGIEPNPSYETLMQAVEQV KKEKIDFLLAVGGGSVIDGTKFVAAAVPYEGEPWEILETDG KKIKKALPLGTVLTLPATGSEMNPNSVVTRKSIKAKRAFHN KIVFPLFSILDPTKVYTLPPRQIANGIVDSFVHITEQYLTYPV EGMVQDEFAEGLLRILINIGPKLLKDQKNYDLAANFMWTA TLALNGLIGAGVPQDWATHMIGHEITAAFGVDHGRTLAIIL PSLLQNQRQVKKDKLLQYAKNVWHIESGSEKERIDAVIAK TRSFFEEMGIPTHLSDYNIGKESIDMLIHELEAHGMTKLGE HNAITPDVSRAILIASL (SEQ ID NO: 88)
DH3	*Shewanella baltica*	MLNFNYYNPTRIRFGKDTIAEIDTLVPSDAKVMILFGGSSA RKTGTLDEVKQSLGNRFIVEFDGIEPNPTYETLMKAVAQV REQKIDFLLAVGGGSVIDGTKFVAAAAVFEGEPWDILTSW GAKVTQAMPFGSVLTLPATGSEMNNASVVTRKSLQAKLP FRNDLVYPQFSILDPTKTFTLPERQVANGVVDAFVHITEQY LTYPVNAAVQDRFAEGLLQTLIELGPQVLAQPEDYDIRANL MWVATMALNGTIGVGVPHDWATHMIGHELTALYDIDHAR TLAIVLPALLQCTKEAKREKLLQYADRVWHINTGTDDERID AAIAKTKAFFEAMGIPTHLSAYDLDASHVDTLVKQLELHGM VALGEHGNINPAMSRDILTLAL (SEQ ID NO: 89)
DH4	*Burkholderia pseudomallei*	MLNFDFYNPTRIVFGEKTAARLNDLLPAAARVLVLYGGES ARSNGTLDEVRAALGARDVREFGGIEPNPAYETLMRAVE LARRERVDFLLAVGGGSVIDGTKFVAAAVPFEGDPWTILE THGANVAAALPFGCVLTLPATGSEMNNGAVLTRRATRAK LAFRHPLVFPTFSILDPTKTYTLPPRQVANGVVDAFTHIVE QYLTYPADGLAQDRFAEGLLQTLIEIGPKALAEPRDYATRA NLMWVATLALNGLIGAGVPQDRATHMVGHELTARYDIDH ARTLAVVLPSMLDVRRDAKRAKLLQYAARVWNIVDGPED ARIDAAIARTRAFFESLGVKTRLADYGVGADAIDGLIAQLE AHGMTRLGERKDVTLDVSRRVLEASL (SEQ ID NO: 90)
DH5	*Saccharomyces cerevisiae*	MSIPETQKGVIFYESHGKLEYKDIPVPKPKANELLINVKYS GVCHTDLHAWHGDWPLPTKLPLVGGHEGAGVVVGMGE NVKGWKIGDYAGIKWLNGSCMACEYCELGNEPNCPHAD SSGYTHDGSFQQYATADAVQAAHIPQGTDLAEVAPVLCA GITVYKALKSANLMAGHWVAISGAAGGLGSLAVQYAKAM GYRVLGIDGGEGKEELFRSIGGEVFIDFTKEKDIVGAVLKA TDGGAHGVINVSVSEAAIEASTRYVRANGTTVLVGMPAGA KCCSDVFNQVVKSISIVGSCVGNRADTREALDFFARGLVK SPIKVVGLSTLPEIYEKMEKGQIVGRYVVDTSK (SEQ ID NO: 91)
DH6	*Saccharomyces cerevisiae*	MSYPEKFEGIAIQSHEDWKNPKKTKYDPKPFYDHDIDIKIE ACGVCGSDIHCAAGHWGNMKMPLVVGHEIVGKVVKLGP KSNSGLKVGQRVGVGAQVFSCLECDRCKNDNEPYCTKF VTTYSQPYEDGYVSQGGYANYVRVHEHFVVPIPENIPSHL AAPLLCGGLTVYSPLVRNGCGPGKKVGIVGLGGIGSMGTL ISKAMGAETYVISRSSRKREDAMKMGADHYIATLEEGDW GEKYFDTFDLIVVCASSLTDIDFNIMPKAMKVGGRIVSISIP EQHEMLSLKPYGLKAVSISYSALGSIKELNQLLKLVSEKDIK IWVETLPVGEAGVHEAFERMEKGDVRYRFTLVGYDKEFS D (SEQ ID NO: 92)

[00149] In some aspects, 2,4-furandimethanol is produced enzymatically, in the absence of microbes. In some aspects, 2,4-furandimethanol is produced enzymatically in one or more vessels. In some aspects, the one or more vessels are substantially free of microbes. In some aspects, the enzymatic production of 2,4-furandimethanol is performed in the same step-wise fashion as described with in the methods utilizing recombinant microorganisms, but substantially free of microorganisms or in the absence of microorganisms. In some aspects, the enzymes utilized in the enzymatic production of 2,4-furandimethanol are isolated from microbes, recombinant or otherwise, and provided to their corresponding substrates for the stepwise production of the intermediates utilized to produce 2,4-furandimethanol. In some aspects, one or more of the steps of the methods are performed in the same vessel. In some aspects, once the desired product is produced as a result of the individual method steps described herein, the product is isolated and purified and then utilized as the substrate in the next step of the method of producing 2,4-furandimethanol.

Furan-2,4-dicarbaldehyde

[00150] In one embodiment, the present disclosure provides a recombinant microorganism capable of producing furan-2,4-dicarbaldehyde from a carbon source. Some embodiments of the present disclosure are presented in **FIG. 1**, **FIG. 2, and FIG. 3**, which collectively detail the biosynthetic conversion of a carbon feedstock to furan-2,4-dicarbaldehyde.

[00151] In one embodiment, step D in **FIG. 2** is a single step reaction utilizing 4-HMF as a substrate. In one embodiment, the bioproduction of furan-2,4-dicarbaldehyde from 4-HMF is catalyzed by one or more enzymes represented by EC numbers 1.1.1.-, 1.1.3.-, and 1.11.2.-.

[00152] In one embodiment, the recombinant microorganism of any one of the embodiments disclosed herein comprises at least one endogenous or exogenous nucleic acid molecule encoding a dehydrogenase, an oxidase, or a peroxygenase that catalyzes the conversion of 4-HMF to furan-2,4-dicarbaldehyde. In one embodiment, the dehydrogenase is classified as EC number 1.1.1. In one embodiment, the dehydrogenase EC number 1.1.1 selected from alcohol dehydrogenase (EC number 1.1.1.1), or alcohol dehydrogenase (NADP+) (EC number 1.1.1.2), or D-xylose reductase (EC number 1.1.1.307), or aryl-alcohol dehydrogenase (EC number 1.1.1.90), or aryl-alcohol dehydrogenase (NADP+) (EC number 1.1.1.91). In one embodiment, the oxidase is classified as EC number 1.1.3. In one embodiment, the oxidase EC number 1.1.3 is 5-(hydroxymethylfurfural oxidase (EC number 1.1.3.47). In some embodiments the EC 1.1.3.47 oxidase can be derived from the gene hmfH. In some embodiments, hmfH can

be derived from *Methylovorus sp.* MP688 or Cupriavidus basilensis. *See* Dijkman and Fraaije (2014) and Koopman *et al.* (2010). In one embodiment, the oxidase EC number 1.1.3 is aryl-alcohol oxidase (EC number 1.1.3.7). *See* Carro et al. (2015). In one embodiment, the peroxygenase is classified as EC number 1.11.2. In one embodiment, the peroxygenase EC number 1.11.2 is unspecific peroxygenase (EC number 1.11.2.1). *See* Carro *et al.* (2015).

[00153] In some aspects, furan-2,4-dicarbaldehyde is produced enzymatically, in the absence of microbes. In some aspects, furan-2,4-dicarbaldehyde is produced enzymatically in one or more vessels. In some aspects, the one or more vessels are substantially free of microbes. In some aspects, the enzymatic production of furan-2,4-dicarbaldehyde is performed in the same step-wise fashion as described with in the methods utilizing recombinant microorganisms, but substantially free of microorganisms or in the absence of microorganisms. In some aspects, the enzymes utilized in the enzymatic production of furan-2,4-dicarbaldehyde are isolated from microbes, recombinant or otherwise, and provided to their corresponding substrates for the stepwise production of the intermediates utilized to produce furan-2,4-dicarbaldehyde. In some aspects, one or more of the steps of the methods are performed in the same vessel. In some aspects, once the desired product is produced as a result of the individual method steps described herein, the product is isolated and purified and then utilized as the substrate in the next step of the method of producing furan-2,4-dicarbaldehyde.

4-(hydroxymethyl)furoic acid

[00154] In one embodiment, the present disclosure provides a recombinant microorganism capable of producing 4-(hydroxymethyl)furoic acid from a carbon source. Some embodiments of the present disclosure are presented in **FIG. 1**, **FIG. 2, and FIG. 3**, which collectively detail the biosynthetic conversion of a carbon feedstock to 4-(hydroxymethyl)furoic acid.

[00155] In one embodiment, step E in **FIG. 2** is a single step reaction utilizing 4-HMF as a substrate. In one embodiment, the bioproduction of 4-(hydroxymethyl)furoic acid from 4-HMF is catalyzed by one or more enzymes represented by EC numbers 1.1.1.-, 1.1.3.-, and 1.11.2.-.

[00156] In one embodiment, the recombinant microorganism of any one of the embodiments disclosed herein comprises at least one endogenous or exogenous nucleic acid molecule encoding a dehydrogenase, or a oxidase, or a peroxygenase that catalyzes the conversion of 4-HMF to 4-(hydroxymethyl)furoic acid. In one embodiment, the dehydrogenase is classified as EC number 1.2.1. In one embodiment, the dehydrogenase EC number 1.2.1 selected from aldehyde dehydrogenase (NAD+) (EC number 1.2.1.3) or aldehyde dehydrogenase (NADP+) (EC number 1.2.1.4) or aldehyde dehydrogenase [NAD(P)+] (EC number 1.2.1.5) or 4-

(γ-glutamylamino)butanal dehydrogenase (EC number 1.2.1.99). In one embodiment, the oxidase is classified as EC number 1.1.3. In one embodiment, the oxidase EC number 1.1.3 is 5-(hydroxymethylfurfural oxidase (EC number 1.1.3.47). In some embodiments the EC 1.1.3.47 oxidase can be derived from the gene hmfH. In some embodiments, hmfH can be derived from *Methylovorus sp.* MP688 or Cupriavidus basilensis. In one embodiment, the oxidase EC number 1.1.3 is aryl-alcohol oxidase (EC number 1.1.3.7). In one embodiment, the peroxygenase is classified as EC number 1.11.2. In one embodiment, the peroxygenase EC number 1.11.2 is unspecific peroxygenase (EC number 1.11.2.1).

[00157] In some aspects, 4-(hydroxymethyl)furoic acid is produced enzymatically, in the absence of microbes. In some aspects, 4-(hydroxymethyl)furoic acid is produced enzymatically in one or more vessels. In some aspects, the one or more vessels are substantially free of microbes. In some aspects, the enzymatic production of 4-(hydroxymethyl)furoic acid is performed in the same step-wise fashion as described with in the methods utilizing recombinant microorganisms, but substantially free of microorganisms or in the absence of microorganisms. In some aspects, the enzymes utilized in the enzymatic production of 4-(hydroxymethyl)furoic acid are isolated from microbes, recombinant or otherwise, and provided to their corresponding substrates for the stepwise production of the intermediates utilized to produce 4-(hydroxymethyl)furoic acid. In some aspects, one or more of the steps of the methods are performed in the same vessel. In some aspects, once the desired product is produced as a result of the individual method steps described herein, the product is isolated and purified and then utilized as the substrate in the next step of the method of producing 4-(hydroxymethyl)furoic acid.

2-formylfuran-4-carboxylate

[00158] In one embodiment, the present disclosure provides a recombinant microorganism capable of producing 2-formylfuran-4-carboxylate from a carbon source. Some embodiments of the present disclosure are presented in **FIG. 1**, **FIG. 2, and FIG. 3**, which collectively detail the biosynthetic conversion of a carbon feedstock to 2-formylfuran-4-carboxylate.

[00159] In one embodiment, step F in **FIG. 2** is a single step reaction utilizing furan-2,4-dicarbaldehyde as a substrate. In one embodiment, the bioproduction of 2-formylfuran-4-carboxylate from furan-2,4-dicarbaldehyde is catalyzed by one or more enzymes represented by EC numbers 1.2.1.-, 1.1.3.-, and 1.11.2.-.

[00160] In one embodiment, the recombinant microorganism of any one of the embodiments disclosed herein comprises at least one endogenous or exogenous nucleic acid molecule encoding a dehydrogenase, or a oxidase, or a peroxygenase that catalyzes the conversion of

furan-2,4-dicarbaldehyde to 2-formylfuran-4-carboxylate. In one embodiment, the dehydrogenase is classified as EC number 1.2.1. In one embodiment, the dehydrogenase EC number 1.2.1 selected from aldehyde dehydrogenase (NAD+) (EC number 1.2.1.3) or aldehyde dehydrogenase (NADP+) (EC number 1.2.1.4) or aldehyde dehydrogenase [NAD(P)+] (EC number 1.2.1.5) or 4-(γ-glutamylamino)butanal dehydrogenase (EC number 1.2.1.99). In one embodiment, the oxidase is classified as EC number 1.1.3. In one embodiment, the oxidase EC number 1.1.3 is 5-(hydroxymethylfurfural oxidase (EC number 1.1.3.47). In some embodiments the EC 1.1.3.47 oxidase can be derived from the gene hmfH. In some embodiments, hmfH can be derived from *Methylovorus sp.* MP688 or Cupriavidus basilensis. In one embodiment, the oxidase EC number 1.1.3 is aryl-alcohol oxidase (EC number 1.1.3.7). In one embodiment, the peroxygenase is classified as EC number 1.11.2. In one embodiment, the peroxygenase EC number 1.11.2 is unspecific peroxygenase (EC number 1.11.2.1).

[00161] In some aspects, 2-formylfuran-4-carboxylate is produced enzymatically, in the absence of microbes. In some aspects, 2-formylfuran-4-carboxylate is produced enzymatically in one or more vessels. In some aspects, the one or more vessels are substantially free of microbes. In some aspects, the enzymatic production of 2-formylfuran-4-carboxylate is performed in the same step-wise fashion as described with in the methods utilizing recombinant microorganisms, but substantially free of microorganisms or in the absence of microorganisms. In some aspects, the enzymes utilized in the enzymatic production of 2-formylfuran-4-carboxylate are isolated from microbes, recombinant or otherwise, and provided to their corresponding substrates for the stepwise production of the intermediates utilized to produce 2-formylfuran-4-carboxylate. In some aspects, one or more of the steps of the methods are performed in the same vessel. In some aspects, once the desired product is produced as a result of the individual method steps described herein, the product is isolated and purified and then utilized as the substrate in the next step of the method of producing 2-formylfuran-4-carboxylate.

4-formylfuran-2-carboxylate

[00162] In one embodiment, the present disclosure provides a recombinant microorganism capable of producing 4-formylfuran-2-carboxylate from a carbon source. Some embodiments of the present disclosure are presented in **FIG. 1**, **FIG. 2, and FIG. 3**, which collectively detail the biosynthetic conversion of a carbon feedstock to 4-formylfuran-2-carboxylate.

[00163] In one embodiment, step G in **FIG. 2** is a single step reaction utilizing furan-2,4-dicarbaldehyde as a substrate. In one embodiment, the bioproduction of 4-formylfuran-2-

carboxylate from furan-2,4-dicarbaldehyde is catalyzed by one or more enzymes represented by EC numbers 1.2.1.-, 1.1.3.-, and 1.11.2.-.

[00164] In one embodiment, the recombinant microorganism of any one of the embodiments disclosed herein comprises at least one endogenous or exogenous nucleic acid molecule encoding a dehydrogenase, or a oxidase, or a peroxygenase that catalyzes the conversion of furan-2,4-dicarbaldehyde to 4-formylfuran-2-carboxylate. In one embodiment, the dehydrogenase is classified as EC number 1.2.1. In one embodiment, the dehydrogenase EC number 1.2.1 selected from aldehyde dehydrogenase (NAD+) (EC number 1.2.1.3) or aldehyde dehydrogenase (NADP+) (EC number 1.2.1.4) or aldehyde dehydrogenase [NAD(P)+] (EC number 1.2.1.5) or 4-(γ-glutamylamino)butanal dehydrogenase (EC number 1.2.1.99). In one embodiment, the oxidase is classified as EC number 1.1.3. In one embodiment, the oxidase EC number 1.1.3 is 5-(hydroxymethylfurfural oxidase (EC number 1.1.3.47). In some embodiments the EC 1.1.3.47 oxidase can be derived from the gene hmfH. In some embodiments, hmfH can be derived from *Methylovorus sp.* MP688 or Cupriavidus basilensis. In one embodiment, the oxidase EC number 1.1.3 is aryl-alcohol oxidase (EC number 1.1.3.7). In one embodiment, the peroxygenase is classified as EC number 1.11.2. In one embodiment, the peroxygenase EC number 1.11.2 is unspecific peroxygenase (EC number 1.11.2.1).

[00165] In one embodiment, step H in **FIG. 2** is a single step reaction utilizing 4-(hydroxymethyl)furoic acid as a substrate. In one embodiment, the bioproduction of 4-formylfuran-2-carboxylate from 4-(hydroxymethyl)furoic acid is catalyzed by one or more enzymes represented by EC numbers 1.1.1.-, 1.1.3.-, and 1.11.2.-.

[00166] In one embodiment, the recombinant microorganism of any one of the embodiments disclosed herein comprises at least one endogenous or exogenous nucleic acid molecule encoding a dehydrogenase, or a oxidase, or a peroxygenase that catalyzes the conversion of 4-(hydroxymethyl)furoic acid to 4-formylfuran-2-carboxylate. In one embodiment, the dehydrogenase is classified as EC number 1.1.1. In one embodiment, the dehydrogenase EC number 1.1.1 selected from alcohol dehydrogenase (EC number 1.1.1.1), or alcohol dehydrogenase (NADP+) (EC number 1.1.1.2), or D-xylose reductase (EC number 1.1.1.307), or aryl-alcohol dehydrogenase (EC number 1.1.1.90), or aryl-alcohol dehydrogenase (NADP+) (EC number 1.1.1.91). In one embodiment, the oxidase is classified as EC number 1.1.3. In one embodiment, the oxidase EC number 1.1.3 is 5-(hydroxymethylfurfural oxidase (EC number 1.1.3.47). In some embodiments the EC 1.1.3.47 oxidase can be derived from the gene hmfH. In some embodiments, hmfH can be derived from *Methylovorus sp.* MP688 or Cupriavidus basilensis. *See* Dijkman and Fraaije (2014) and Koopman *et al.* (2010). In one embodiment, the

oxidase EC number 1.1.3 is aryl-alcohol oxidase (EC number 1.1.3.7). *See* Carro et al. (2015). In one embodiment, the peroxygenase is classified as EC number 1.11.2. In one embodiment, the peroxygenase EC number 1.11.2 is unspecific peroxygenase (EC number 1.11.2.1). *See* Carro *et al.* (2015).

[00167] In some aspects, 4-formylfuran-2-carboxylate is produced enzymatically, in the absence of microbes. In some aspects, 4-formylfuran-2-carboxylate is produced enzymatically in one or more vessels. In some aspects, the one or more vessels are substantially free of microbes. In some aspects, the enzymatic production of 4-formylfuran-2-carboxylate is performed in the same step-wise fashion as described with in the methods utilizing recombinant microorganisms, but substantially free of microorganisms or in the absence of microorganisms. In some aspects, the enzymes utilized in the enzymatic production of 4-formylfuran-2-carboxylate are isolated from microbes, recombinant or otherwise, and provided to their corresponding substrates for the stepwise production of the intermediates utilized to produce 4-formylfuran-2-carboxylate. In some aspects, one or more of the steps of the methods are performed in the same vessel. In some aspects, once the desired product is produced as a result of the individual method steps described herein, the product is isolated and purified and then utilized as the substrate in the next step of the method of producing 4-formylfuran-2-carboxylate.

Generation of Microbial Populations

Genetic Modification

[00168] The genetic modification introduced into one or more microbes of the present disclosure may alter or abolish a regulatory sequence of a target gene. In some aspects, the genetic modification introduced into one or more microbes of the present disclosure may introduce a new trait or phenotype into the one or more microbes. One or more regulatory sequences may also be inserted, including heterologous regulatory sequences and regulatory sequences found within a genome of an animal, plant, fungus, yeast, bacteria, or virus corresponding to the microbe into which the genetic variation is introduced. Moreover, regulatory sequences may be selected based on the expression level of a gene in a microbial culture. The genetic variation may be a pre-determined genetic variation that is specifically introduced to a target site. In some aspects the genetic variation is a nucleic acid sequence that is introduced into one or more microbial chromosomes. In some aspects, the genetic variation is a nucleic acid sequence that is introduced into one or more extrachromosomal nucleic acid sequence. The genetic variation may be a random mutation within the target site. The genetic variation may be an insertion or deletion of one or more nucleotides. In some cases, a plurality of different genetic variations (e.g. 2, 3, 4, 5,

10, or more) are introduced into one or more of the isolated bacteria. The plurality of genetic variations can be any of the above types, the same or different types, and in any combination. In some cases, a plurality of different genetic variations are introduced serially, introducing a first genetic variation after a first isolation step, a second genetic variation after a second isolation step, and so forth so as to accumulate a plurality of desired modifications in the microbes.

[00169] In some aspects, one or more of the substrates set forth in the production of the 2,4-FDCA monomers and polymers are biosynthesized from a carbon feedstock (e.g., glucose or glycerol).

[00170] In general, the term "genetic variation" refers to any change introduced into a polynucleotide sequence relative to a reference polynucleotide, such as a reference genome or portion thereof, or reference gene or portion thereof. A genetic variation may be referred to as a "mutation," and a sequence or organism comprising a genetic variation may be referred to as a "genetic variant" or "mutant". Genetic variations can have any number of effects, such as the increase or decrease of some biological activity, including gene expression, metabolism, and cell signaling.

[00171] Genetic variations can be specifically introduced to a target site, or introduced randomly. A variety of molecular tools and methods are available for introducing genetic variation. For example, genetic variation can be introduced via polymerase chain reaction mutagenesis, oligonucleotide-directed mutagenesis, saturation mutagenesis, fragment shuffling mutagenesis, homologous recombination, recombineering, lambda red mediated recombination, CRISPR/Cas9 systems, chemical mutagenesis, and combinations thereof. Chemical methods of introducing genetic variation include exposure of DNA to a chemical mutagen, e.g., ethyl methanesulfonate (EMS), methyl methanesulfonate (MMS), N-nitrosourea (EN U), N-methyl-N-nitro-N′-nitrosoguanidine, 4-nitroquinoline N-oxide, diethylsulfate, benzopyrene, cyclophosphamide, bleomycin, triethylmelamine, acrylamide monomer, nitrogen mustard, vincristine, diepoxyalkanes (for example, diepoxybutane), ICR-170, formaldehyde, procarbazine hydrochloride, ethylene oxide, dimethylnitrosamine, 7,12 dimethylbenz(a)anthracene, chlorambucil, hexamethylphosphoramide, bisulfan, and the like. Radiation mutation-inducing agents include ultraviolet radiation, γ-irradiation, X-rays, and fast neutron bombardment. Genetic variation can also be introduced into a nucleic acid using, e.g., trimethylpsoralen with ultraviolet light. Random or targeted insertion of a mobile DNA element, e.g., a transposable element, is another suitable method for generating genetic variation.

[00172] Genetic variations can be introduced into a nucleic acid during amplification in a cell-free in vitro system, e.g., using a polymerase chain reaction (PCR) technique such as error-prone

PCR. Genetic variations can be introduced into a nucleic acid *in vitro* using DNA shuffling techniques (e.g., exon shuffling, domain swapping, and the like). Genetic variations can also be introduced into a nucleic acid as a result of a deficiency in a DNA repair enzyme in a cell, e.g., the presence in a cell of a mutant gene encoding a mutant DNA repair enzyme is expected to generate a high frequency of mutations (i.e., about 1 mutation/100 genes-1 mutation/10,000 genes) in the genome of the cell. Examples of genes encoding DNA repair enzymes include but are not limited to Mut H, Mut S, Mut L, and Mut U, and the homologs thereof in other species (e.g., MSH 1 6, PMS 1 2, MLH 1, GTBP, ERCC-1, and the like). Example descriptions of various methods for introducing genetic variations are provided in e.g., Stemple (2004) Nature 5:1-7; Chiang et al. (1993) PCR Methods Appl 2(3): 210-217; Stemmer (1994) Proc. Natl. Acad. Sci. USA 91:10747-10751; and U.S. Pat. Nos. 6,033,861, and 6,773,900.

[00173] Genetic variations introduced into microbes may be classified as transgenic, cisgenic, intragenomic, intrageneric, intergeneric, synthetic, evolved, rearranged, or SNPs.

[00174] CRISPR/Cas9 (Clustered regularly interspaced short palindromic repeats) /CRISPR-associated (Cas) systems can be used to introduce desired mutations. CRISPR/Cas9 provide bacteria and archaea with adaptive immunity against viruses and plasmids by using CRISPR RNAs (crRNAs) to guide the silencing of invading nucleic acids. The Cas9 protein (or functional equivalent and/or variant thereof, i.e., Cas9-like protein) naturally contains DNA endonuclease activity that depends on the association of the protein with two naturally occurring or synthetic RNA molecules called crRNA and tracrRNA (also called guide RNAs). In some cases, the two molecules are covalently link to form a single molecule (also called a single guide RNA ("sgRNA"). Thus, the Cas9 or Cas9-like protein associates with a DNA-targeting RNA (which term encompasses both the two-molecule guide RNA configuration and the single-molecule guide RNA configuration), which activates the Cas9 or Cas9-like protein and guides the protein to a target nucleic acid sequence. If the Cas9 or Cas9-like protein retains its natural enzymatic function, it will cleave target DNA to create a double-stranded break, which can lead to genome alteration (i.e., editing: deletion, insertion (when a donor polynucleotide is present), replacement, etc.), thereby altering gene expression. Some variants of Cas9 (which variants are encompassed by the term Cas9-like) have been altered such that they have a decreased DNA cleaving activity (in some cases, they cleave a single strand instead of both strands of the target DNA, while in other cases, they have severely reduced to no DNA cleavage activity). Further exemplary descriptions of CRISPR systems for introducing genetic variation can be found in, e.g. US8795965.

[00175] Oligonucleotide-directed mutagenesis, also called site-directed mutagenesis, typically utilizes a synthetic DNA primer. This synthetic primer contains the desired mutation and is

complementary to the template DNA around the mutation site so that it can hybridize with the DNA in the gene of interest. The mutation may be a single base change (a point mutation), multiple base changes, deletion, or insertion, or a combination of these. The single-strand primer is then extended using a DNA polymerase, which copies the rest of the gene. The gene thus copied contains the mutated site, and may then be introduced into a host cell as a vector and cloned. Finally, mutants can be selected by DNA sequencing to check that they contain the desired mutation.

[00176] Genetic variations can be introduced using error-prone PCR. In this technique, the gene of interest is amplified using a DNA polymerase under conditions that are deficient in the fidelity of replication of sequence. The result is that the amplification products contain at least one error in the sequence. When a gene is amplified and the resulting product(s) of the reaction contain one or more alterations in sequence when compared to the template molecule, the resulting products are mutagenized as compared to the template. Another means of introducing random mutations is exposing cells to a chemical mutagen, such as nitrosoguanidine or ethyl methanesulfonate (Nestmann, Mutat Res 1975 June; 28(3):323-30), and the vector containing the gene is then isolated from the host.

[00177] Homologous recombination mutagenesis involves recombination between an exogenous DNA fragment and the targeted polynucleotide sequence. After a double-stranded break occurs, sections of DNA around the 5' ends of the break are cut away in a process called resection. In the strand invasion step that follows, an overhanging 3' end of the broken DNA molecule then "invades" a similar or identical DNA molecule that is not broken. The method can be used to delete a gene, remove exons, add a gene, and introduce point mutations. Homologous recombination mutagenesis can be permanent or conditional. Typically, a recombination template is also provided. A recombination template may be a component of another vector, contained in a separate vector, or provided as a separate polynucleotide. In some aspects, a recombination template is designed to serve as a template in homologous recombination, such as within or near a target sequence nicked or cleaved by a site-specific nuclease. A template polynucleotide may be of any suitable length, such as about or more than about 10, 15, 20, 25, 50, 75, 100, 150, 200, 500, 1000, or more nucleotides in length. In some aspects, the template polynucleotide is complementary to a portion of a polynucleotide comprising the target sequence. When optimally aligned, a template polynucleotide might overlap with one or more nucleotides of a target sequences (e.g. about or more than about 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100 or more nucleotides). In some aspects, when a template sequence and a polynucleotide comprising a target sequence are optimally aligned, the nearest nucleotide of the template

polynucleotide is within about 1, 5, 10, 15, 20, 25, 50, 75, 100, 200, 300, 400, 500, 1000, 5000, 10000, or more nucleotides from the target sequence. Non-limiting examples of site-directed nucleases useful in methods of homologous recombination include zinc finger nucleases, CRISPR nucleases, TALE nucleases, and meganuclease. For a further description of the use of such nucleases, see e.g. US8795965 and US20140301990.

[00178] Introducing genetic variation may be an incomplete process, such that some bacteria in a treated population of bacteria carry a desired mutation while others do not. In some cases, it is desirable to apply a selection pressure so as to enrich for bacteria carrying a desired genetic variation. Traditionally, selection for successful genetic variants involved selection for or against some functionality imparted or abolished by the genetic variation, such as in the case of inserting antibiotic resistance gene or abolishing a metabolic activity capable of converting a non-lethal compound into a lethal metabolite. It is also possible to apply a selection pressure based on a polynucleotide sequence itself, such that only a desired genetic variation need be introduced (e.g. without also requiring a selectable marker). In this case, the selection pressure can comprise cleaving genomes lacking the genetic variation introduced to a target site, such that selection is effectively directed against the reference sequence into which the genetic variation is sought to be introduced. Typically, cleavage occurs within 100 nucleotides of the target site (e.g. within 75, 50, 25, 10, or fewer nucleotides from the target site, including cleavage at or within the target site). Cleaving may be directed by a site-specific nuclease selected from the group consisting of a Zinc Finger nuclease, a CRISPR nuclease, a TALE nuclease (TALEN), or a meganuclease. Such a process is similar to processes for enhancing homologous recombination at a target site, except that no template for homologous recombination is provided. As a result, bacteria lacking the desired genetic variation are more likely to undergo cleavage that, left unrepaired, results in cell death. Bacteria surviving selection may then be isolated for assessing conferral of an improved trait.

[00179] A CRISPR nuclease may be used as the site-specific nuclease to direct cleavage to a target site. An improved selection of mutated microbes can be obtained by using Cas9 to kill non-mutated cells. Microbes can then be re-isolated from tissues. CRISPR nuclease systems employed for selection against non-variants can employ similar elements to those described above with respect to introducing genetic variation, except that no template for homologous recombination is provided. Cleavage directed to the target site thus enhances death of affected cells.

[00180] Other options for specifically inducing cleavage at a target site are available, such as zinc finger nucleases, TALE nuclease (TALEN) systems, and meganuclease. Zinc-finger

nucleases (ZFNs) are artificial DNA endonucleases generated by fusing a zinc finger DNA binding domain to a DNA cleavage domain. ZFNs can be engineered to target desired DNA sequences and this enables zinc-finger nucleases to cleave unique target sequences. When introduced into a cell, ZFNs can be used to edit target DNA in the cell (e.g., the cell's genome) by inducing double stranded breaks. Transcription activator-like effector nucleases (TALENs) are artificial DNA endonucleases generated by fusing a TAL (Transcription activator-like) effector DNA binding domain to a DNA cleavage domain. TALENS can be quickly engineered to bind practically any desired DNA sequence and when introduced into a cell, TALENs can be used to edit target DNA in the cell (e.g., the cell's genome) by inducing double strand breaks. Meganucleases (homing endonuclease) are endodeoxyribonucleases characterized by a large recognition site (double-stranded DNA sequences of 12 to 40 base pairs. Meganucleases can be used to replace, eliminate or modify sequences in a highly targeted way. By modifying their recognition sequence through protein engineering, the targeted sequence can be changed. Meganucleases can be used to modify all genome types, whether bacterial, plant or animal and are commonly grouped into four families: the LAGLIDADG family, the GIY-YIG family, the His-Cyst box family and the HNH family. Exemplary homing endonucleases include I-SceI, I-CeuI, PI-PspI, PI-Sce, I-SceIV, I-CsmI, I-PanI, I-SceII, I-PpoI, I-SceIII, I-CreI, I-TevI, I-TevII and I-TevIII.

Microbes

[00181] As described herein, in some aspects, recombinant microorganisms are capable of producing 4-HMF, 2,4-furandimethanol, furan-2,4-dicarbaldehyde, 4-(hydroxymethyl)furoic acid, 2-formylfuran-4-carboxylate, 4-formylfuran-2-carboxylate, or 2,4-FDCA, and any combination thereof.

[00182] As described herein, in some aspects, the recombinant microorganisms are prokaryotic microorganism. In some aspects, the prokaryotic microorganisms are bacteria. "Bacteria", or "eubacteria", refers to a domain of prokaryotic organisms. Bacteria include at least eleven distinct groups as follows: (1) Gram-positive (gram+) bacteria, of which there are two major subdivisions: (1) high G+C group (Actinomycetes, Mycobacteria, Micrococcus, others) (2) low G+C group (Bacillus, Clostridia, Lactobacillus, Staphylococci, Streptococci, Mycoplasmas); (2) Proteobacteria, e.g., Purple photosynthetic +non-photosynthetic Gram-negative bacteria (includes most "common" Gram-negative bacteria); (3) Cyanobacteria, e.g., oxygenic phototrophs; (4) Spirochetes and related species; (5) Planctomyces; (6) Bacteroides, Flavobacteria; (7) Chlamydia; (8) Green sulfur bacteria; (9) Green non-sulfur bacteria (also anaerobic phototrophs); (10) Radioresistant micrococci and relatives; (11) Thermotoga and Thermosipho thermophiles.

[00183] "Gram-negative bacteria" include cocci, nonenteric rods, and enteric rods. The genera of Gram-negative bacteria include, for example, Neisseria, Spirillum, Pasteurella, Brucella, Yersinia, Francisella, Haemophilus, Bordetella, Escherichia, Salmonella, Shigella, Klebsiella, Proteus, Vibrio, Pseudomonas, Bacteroides, Acetobacter, Aerobacter, Agrobacterium, Azotobacter, Spirilla, Serratia, Vibrio, Rhizobium, Chlamydia, Rickettsia, Treponema, and Fusobacterium.

[00184] "Gram positive bacteria" include cocci, nonsporulating rods, and sporulating rods. The genera of gram positive bacteria include, for example, Actinomyces, Bacillus, Clostridium, Corynebacterium, Erysipelothrix, Lactobacillus, Listeria, Mycobacterium, Myxococcus, Nocardia, Staphylococcus, Streptococcus, and Streptomyces.

[00185] In some aspects, the microorganisms of the present disclosure are fungi.

[00186] In some aspects, the recombinant microorganism is a eukaryotic microorganism. In some aspectsts, the eukaryotic microorganism is a yeast. In exemplary aspects, the yeast is a member of a genus selected from the group consisting of Yarrowia, Candida, Saccharomyces, Pichia, Hansenula, Kluyveromyces, Issatchenkia, Zygosaccharomyces, Debaryomyces, Schizosaccharomyces, Pachysolen, Cryptococcus, Trichosporon, Rhodotorula, and Myxozyma.

[00187] In some aspects, the recombinant microorganism is a prokaryotic microorganism. In exemplary aspects, the prokaryotic microorganism is a member of a genus selected from the group consisting of Escherichia, Clostridium, Zymomonas, Salmonella, Rhodococcus, Pseudomonas, Bacillus, Lactobacillus, Enterococcus, Alcaligenes, Klebsiella, Paenibacillus, Arthrobacter, Corynebacterium, and Brevibacterium.

[00188] In some aspects, microorganism for use in the methods of the present disclosure can be selected from the group consisting of Yarrowia, Candida, Saccharomyces, Pichia, Hansenula, Kluyveromyces, Issatchenkia, Zygosaccharomyces, Debaryomyces, Schizosaccharomyces, Pachysolen, Cryptococcus, Trichosporon, Rhodotorula, Myxozyma, Escherichia, Clostridium, Zymomonas, Salmonella, Rhodococcus, Pseudomonas, Bacillus, Lactobacillus, Enterococcus, Alcaligenes, Klebsiella, Paenibacillus, Arthrobacter, Corynebacterium, and Brevibacterium.

[00189] In some aspects, a microbe resulting from the methods described herein may be a species selected from any of the following genera: Neisseria, Spirillum, Pasteurella, Brucella, Yersinia, Francisella, Haemophilus, Bordetella, Candida, Escherichia, Salmonella, Shigella, Klebsiella, Proteus, Vibrio, Pseudomonas, Bacteroides, Acetobacter, Aerobacter, Agrobacterium, Azotobacter, Spirilla, Serratia, Vibrio, Rhizobium, Chlamydia, Rickettsia, Treponema, Fusobacterium, Actinomyces, Bacillus, Clostridium, Corynebacterium, Erysipelothrix, Lactobacillus, Listeria, Mycobacterium, Myxococcus, Nocardia, Issatchenkia, Staphylococcus, Streptococcus, Streptomyces, Saccharomyces, Pichia, and Aspergillus.

[00190] In some aspects, microorganisms for use in the methods of the present disclosure include Clostridium sp., Clostridium ljungdahlii, Clostridium autoethanogenum, Clostridium ragsdalei, Eubacterium limosum, Butyribacterium methylotrophicum, Moorella thermoacetica, Corynebacterium glutamicum, Clostridium aceticum, Acetobacterium woodii, Alkalibaculum bacchii, Clostridium drakei, Clostridium carboxidivorans, Clostridium formicoaceticum, Clostridium scatologenes, Moorella thermoautotrophica, Acetonema longum, Blautia producta, Clostridium glycolicum, Clostridium magnum, Candida krusei, Clostridium mayombei, Clostridium methoxybenzovorans, Clostridium acetobutylicum, Clostridium beijerinckii, Oxobacter pfennigii, Thermoanaerobacter kivui, Sporomusa ovata, Thermoacetogenium phaeum, Acetobacterium carbinolicum, Issatchenkia orientalis, Sporomusa termitida, Moorella glycerini, Eubacterium aggregans, Treponema azotonutricium, Pichia kudriavzevii, Escherichia coli, Saccharomyces cerevisiae, Pseudomonas putida, Bacillus sp, Corynebacterium sp., Yarrowia lipolytica, Scheffersomyces stipitis, and Terrisporobacter glycolicus.

[00191] The term "recombinant microorganism" and "recombinant host cell" are used interchangeably herein and refer to microorganisms that have been genetically modified to express or to overexpress endogenous enzymes, to express heterologous enzymes, such as those included in a vector, in an integration construct, or which have an alteration in expression of an endogenous gene. By "alteration" it is meant that the expression of the gene, or level of a RNA molecule or equivalent RNA molecules encoding one or more polypeptides or polypeptide subunits, or activity of one or more polypeptides or polypeptide subunits is up regulated or down regulated, such that expression, level, or activity is greater than or less than that observed in the absence of the alteration. For example, the term "alter" can mean "inhibit," but the use of the word "alter" is not limited to this definition. It is understood that the terms "recombinant microorganism" and "recombinant host cell" refer not only to the particular recombinant microorganism but to the progeny or potential progeny of such a microorganism. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

[00192] Culturing of the microorganisms used in the methods of the disclosure may be conducted using any number of processes known in the art for culturing and fermenting substrates using the microorganisms of the present disclosure.

[00193] The fermentation may be carried out in any suitable bioreactor, such as Continuous Stirred Tank Bioreactor, Bubble Column Bioreactor, Airlift Bioreactor, Fluidized Bed Bioreactor, Packed Bed Bioreactor, Photo-Bioreactor, Immobilized Cell Reactor, Trickle Bed Reactor, Moving

Bed Biofilm Reactor, Bubble Column, Gas Lift Fermenter, Membrane Reactors such as Hollow Fiber Membrane Bioreactor. In some aspects, the bioreactor comprises a first, growth reactor in which the microorganisms are cultured, and a second, fermentation reactor, to which fermentation broth from the growth reactor is fed and in which most of the fermentation product is produced. In some aspects, the bioreactor simultaneously accomplishes the culturing of microorganism and the producing the fermentation product from carbon sources such substrates and/or feedstocks provided.

[00194] In some aspects, the disclosure is drawn to a method of recovering/isolating a 2,4-FDCA monomer. In some aspects, the disclosure is drawn to a method of recovering/isolating 4-HMF, 2,4-furandimethanol, furan-2,4-dicarbaldehyde, 4-(hydroxymethyl)furoic acid, 2-formylfuran-4-carboxylate, 4-formylfuran-2-carboxylate, or 2,4-FDCA, and any combination thereof. In some aspects, the disclosure is drawn to a method of recovering/isolating a 2,4-furandimethanol, furan-2,4-dicarbaldehyde, 4-(hydroxymethyl)furoic acid, 2-formylfuran-4-carboxylate, and/or 4-formylfuran-2-carboxylate monomer or polymer. In some aspects, the disclosure is drawn to a method of recovering/isolating 4-HMF, 2,4-furandimethanol, furan-2,4-dicarbaldehyde, 4-(hydroxymethyl)furoic acid, 2-formylfuran-4-carboxylate, 4-formylfuran-2-carboxylate, or 2,4-FDCA, and any combination thereof. The recovery/collection/isolation can be by methods known in the art, such as distillation, membrane-based separation gas stripping, precipitation, solvent extraction, and expanded bed adsorption.

Feedstock

[00195] In some aspects, the feedstock comprises a carbon source. In some aspects, the carbon source may be selected from sugars, glycerol, alcohols, organic acids, alkanes, fatty acids, lignocellulose, proteins, carbon dioxide, and carbon monoxide. In one aspect, the carbon source is a sugar. In one aspect, the sugar is a monosaccharide. In one aspect, the sugar is a polysaccharide. In one aspect, the sugar is glucose or oligomers of glucose thereof. In one aspect, the oligomers of glucose are selected from fructose, sucrose, starch, cellobiose, maltose, lactose and cellulose. In one aspect, the sugar is a five carbon sugar. In one aspect, the sugar is a six carbon sugar. In some aspects, the feedstock comprises one or more five carbon sugars and/or one or more six carbon sugars. In some aspects, the feedstock comprises one or more of xylose, glucose, arabinose, galactose, maltose, fructose, mannose, sucrose, and/or combinations thereof. In some aspects, the feedstock comprises one or more of xylose and/or glucose. In some aspects, the feedstock comprises one or more of arabinose, galactose, maltose, fructose, mannose, sucrose, and/or combinations thereof.

[00196] In some aspects, the microbes utilize one or more five carbon sugars (pentoses) and/or one or more six carbon sugars (hexoses). In some aspects, the microbes utilize one or more of xylose and/or glucose. In some aspects, the microbes utilize one or more of arabinose, galactose, maltose, fructose, mannose, sucrose, and/or combinations thereof. In some aspects, the microbes utilize one or more of xylose, glucose, arabinose, galactose, maltose, fructose, mannose, sucrose, and/or combinations thereof

[00197] In some aspects, hexoses may be selected from D-allose, D-altrose, D-glucose, D-mannose, D-gulose, D-idose, D-galactose, D-talose, D-tagtose, D-sorbose, D-fructose, D-psicose, and other hexoses known in the art. In some aspects, pentoses may be selected from D-xylose, D-ribose, D-arabinose, D-lyxose, D-xylulose, D-ribulose, and other pentoses known in the art. In some aspects, the hexoses and pentoses may be selected from the levorotary or dextrorotary enantiomer of any of the hexoses and pentoses disclosed herein.

Microbial Compositions

[00198] In some aspects, the microbes of the disclosure are combined into microbial compositions.

[00199] In some aspects, the microbial compositions of the present disclosure are solid. Where solid compositions are used, it may be desired to include one or more carrier materials including, but not limited to: mineral earths such as silicas, talc, kaolin, limestone, chalk, clay, dolomite, diatomaceous earth; calcium sulfate; magnesium sulfate; magnesium oxide; zeolites, calcium carbonate; magnesium carbonate; trehalose; chitosan; shellac; albumins; starch; skim milk powder; sweet whey powder; maltodextrin; lactose; inulin; dextrose; and products of vegetable origin such as cereal meals, tree bark meal, wood meal, and nutshell meal.

[00200] In some aspects, the microbial compositions of the present disclosure are liquid. In further aspects, the liquid comprises a solvent that may include water or an alcohol or a saline or carbohydrate solution. In some aspects, the microbial compositions of the present disclosure include binders such as polymers, carboxymethylcellulose, starch, polyvinyl alcohol, and the like.

[00201] In some aspects, microbial compositions of the present disclosure comprise saccharides (e.g., monosaccharides, disaccharides, trisaccharides, polysaccharides, oligosaccharides, and the like), polymeric saccharides, lipids, polymeric lipids, lipopolysaccharides, proteins, polymeric proteins, lipoproteins, nucleic acids, nucleic acid polymers, silica, inorganic salts and combinations thereof. In further aspect, microbial compositions comprise polymers of agar, agarose, gelrite, gellan gum, and the like. In some aspects, microbial compositions comprise plastic capsules, emulsions (e.g., water and oil), membranes, and artificial membranes. In some

aspects, emulsions or linked polymer solutions comprise microbial compositions of the present disclosure. See Harel and Bennett (US Patent 8,460,726 B2).

[00202] In some aspects, microbial compositions of the present disclosure occur in a solid form (e.g., dispersed lyophilized spores) or a liquid form (microbes interspersed in a storage medium). In some aspects, microbial compositions of the present disclosure are added in dry form to a liquid to form a suspension immediately prior to use.

Methods of Producing Biosynthesis Products

[00203] The present disclosure provides a method of producing one or more biosynthesis products using a recombinant microorganisms. The biosynthesis products include: 4-HMF, 2,4-furandimethanol, furan-2,4-dicarbaldehyde, 4-(hydroxymethyl)furoic acid, 2-formylfuran-4-carboxylate, 4-formylfuran-2-carboxylate, and 2,4-FDCA. In one embodiment, the method comprises cultivating the recombinant microorganism in a culture medium. In one embodiment, the culture medium contains a feedstock comprising a carbon source that the recombinant microorganism can utilize to produce the one or more biosynthesis products. In one embodiment, the carbon source in the culture medium is selected from the group that comprises a hexose, a pentose, or glycerol. In certain embodiments, the carbon source is glycerol. Some embodiments of the present disclosure are presented in **FIG. 1, FIG. 2, and FIG. 3,** which collectively detail the biosynthetic conversion of a carbon feedstock to one or more of the biosynthesis products.

[00204] The present disclosure provides a method of producing a recombinant microorganism that produces 4-HMF, 2,4-furandimethanol, furan-2,4-dicarbaldehyde, 4-(hydroxymethyl)furoic acid, 2-formylfuran-4-carboxylate, 4-formylfuran-2-carboxylate, and 2,4-FDCA from a feedstock comprising an exogenous carbon source. In one embodiment, the method comprises introducing into and/or overexpressing in the recombinant microorganism endogenous and/or exogenous nucleic acid molecules capable of converting a carbon source into 4-HMF, 2,4-furandimethanol, furan-2,4-dicarbaldehyde, 4-(hydroxymethyl)furoic acid, 2-formylfuran-4-carboxylate, 4-formylfuran-2-carboxylate, and 2,4-FDCA. In one embodiment, the carbon source may include glycerol and/or monosaccharides.

[00205] In one embodiment, endogenous and/or exogenous nucleic acid molecules convert glycerol or a monosaccharide into glyceraldehyde 3-phosphate (G3P). G3P is a common natural intermediary metabolite. In some embodiments, it can be produced from glucose via the glycolysis pathway or from xylose via the pentose phosphate pathway, or from glycerol. In one embodiment, the recombinant microorganism capable of producing 4-HMF, 2,4-furandimethanol, furan-2,4-dicarbaldehyde, 4-(hydroxymethyl)furoic acid, 2-formylfuran-4-carboxylate, 4-formylfuran-2-

carboxylate, and 2,4-FDCA utilizes a carbon source that comprises a hexose, a pentose, or glycerol. In certain embodiments, the carbon source is glycerol.

[00206] In one embodiment, the present disclosure contemplates methods of producing 2,4-FDCA and the multiple steps and processes for producing 2,4-FDCA. In some embodiments, the present disclosure contemplates the individual methods for producing one or more of 4-HMF, 2,4-furandimethanol, furan-2,4-dicarbaldehyde, 4-(hydroxymethyl)furoic acid, 2-formylfuran-4-carboxylate, and 4-formylfuran-2-carboxylate, that are described in the process of making 2,4-FDCA.

[00207] In one embodiment, the recombinant microorganisms of the method are derived from a parental microorganism selected from the group consisting of Clostridium sp., Clostridium ljungdahlii, Clostridium autoethanogenum, Clostridium ragsdalei, Eubacterium limosum, Butyribacterium methylotrophicum, Moorella thermoacetica, Corynebacterium glutamicum, Clostridium aceticum, Acetobacterium woodii, Alkalibaculum bacchii, Clostridium drakei, Clostridium carboxidivorans, Clostridium formicoaceticum, Clostridium scatologenes, Moorella thermoautotrophica, Acetonema longum, Blautia producta, Clostridium glycolicum, Clostridium magnum, Candida krusei, Clostridium mayombei, Clostridium methoxybenzovorans, Clostridium acetobutylicum, Clostridium beijerinckii, Oxobacter pfennigii, Thermoanaerobacter kivui, Sporomusa ovata, Thermoacetogenium phaeum, Acetobacterium carbinolicum, Issatchenkia orientalis, Sporomusa termitida, Moorella glycerini, Eubacterium aggregans, Treponema azotonutricium, Pichia kudriavzevii, Escherichia coli, Saccharomyces cerevisiae, Pseudomonas putida, Bacillus sp, Corynebacterium sp., Yarrowia lipolytica, Scheffersomyces stipitis, and Terrisporobacter glycolicus.

4-HMF

[00208] In one embodiment, the present disclosure comprises converting one or more carbon sources to glyceraldehyde 3-phosphate (G3P); converting G3P to (5-formylfuran-3-yl)methyl phosphate (Step A); converting (5-formylfuran-3-yl)methyl phosphate to 4-hydroxymethylfurfural (4-HMF) (Step B).

[00209] In one embodiment, the disclosure is drawn to a method of producing a recombinant microorganism of any one of the embodiments disclosed herein comprising an endogenous and/or exogenous nucleic acid molecules capable of converting a carbon source to glyceraldehyde 3-phosphate (G3P). In one embodiment, glycerol is converted to glycerol-3-phopshate by at least one endogenous or exogenous nucleic acid molecule encoding a glycerol kinase. In one embodiment, glycerol-3-phosphate is converted to dihydroxyacetone phosphate

(DHAP) by at least one endogenous or exogenous nucleic acid molecule encoding a glycerol-3-phosphate dehydrogenase. In one embodiment, glycerol is converted to dihydroxyacetone by at least one endogenous or exogenous nucleic acid molecule encoding a glycerol dehydrogenase. In one embodiment, dihydroxyacetone is converted to dihydroxyacetone phosphate (DHAP) by at least one endogenous or exogenous nucleic acid molecule encoding a dihydroxyacetone kinase. In one embodiment, DHAP is converted to G3P by at least one endogenous or exogenous nucleic acid molecule encoding a triose phosphate isomerase. *See* Zhang *et al.* (2010).

[00210] In one embodiment, the disclosure is drawn to a method of producing a recombinant microorganism of any one of the embodiments of disclosed herein comprising at least one endogenous or exogenous nucleic acid molecule encoding a (5-formylfuran-3-yl)methyl phosphate synthase that catalyzes the conversion of G3P to (5-formylfuran-3-yl)methyl phosphate. In one embodiment, the (5-formylfuran-3-yl)methyl phosphate synthase is classified as EC number 4.2.3.153. In some embodiments the EC 4.2.3.153 (5-formylfuran-3-yl)methyl phosphate synthase can be derived from the gene mfnB. In some embodiments, mfnB can be derived from *Methanocaldococcus jannaschii.* In some embodiments, the (5-formylfuran-3-yl)methyl phosphate synthase can be derived from enzyme candidates listed at Table 1. In some embodiments, the (5-formylfuran-3-yl)methyl phosphate synthase is homologous or similar to the enzymes listed at Table 1. In some embodiments, an (5-formylfuran-3-yl)methyl phosphate synthase enzyme is evolved or engineered to improve its catalytic efficiency, markedly kcat.

[00211] In one embodiment, the disclosure is drawn to a method of producing a recombinant microorganism of any one of the embodiments disclosed herein comprising at least one endogenous or exogenous nucleic acid molecule encoding a phosphatase that catalyzes the conversion of (5-formylfuran-3-yl)methyl phosphate to (4-HMF). In one embodiment, the phosphatase is classified as haloacid dehalogenase (Koonin *et al.* J. Mol. Biol. 244(1). 1994). In some aspects, the phosphatase of reaction b is endogenous to the host (Offley *et al.* Curr. Gen. 65. 2019). In some aspects, the phosphatase enzyme endogenous to the host is overexpressed. In some cases a heterologous phosphatase able to perform the desired reaction is used and is selected from an alkaline phosphatase, acid phosphatase, fructose-bisphosphatase, sugar-phosphatase, or sugar-terminal-phosphatase. In some embodiments , the phosphatase can be derived from enzyme candidates listed at Table 2. In some embodiments, the phosphatase is homologous or similar to the enzymes listed at Table 2. In some embodiments, an phosphatase enzyme is evolved or engineered to improve its catalytic efficiency and or specificity for the conversion of (5-formylfuran-3-yl)methyl phosphate to (4-HMF).

[00212] Accordingly, in one embodiment, the disclosure is drawn to a method of producing a recombinant microorganism that comprises endogenous and/or exogenous nucleic acid molecules capable of converting a carbon source to glyceraldehyde 3-phosphate (G3P); at least one endogenous or exogenous nucleic acid molecule encoding a (5-formylfuran-3-yl)methyl phosphate synthase that catalyzes the conversion of G3P to (5-formylfuran-3-yl)methyl phosphate; at least one endogenous or exogenous nucleic acid molecule encoding a phosphatase that catalyzes the conversion of (5-formylfuran-3-yl)methyl phosphate to 4-HMF.

2,4-FDCA

[00213] In one embodiment, methods of the disclosure convert G3P to 2,4-FDCA via several enzymatically-catalyzed successive steps. In one embodiment, the present disclosure comprises converting one or more carbon sources to glyceraldehyde 3-phosphate (G3P); converting G3P to (5-formylfuran-3-yl)methyl phosphate (Step A); converting (5-formylfuran-3-yl)methyl phosphate to 4-hydroxymethylfurfural (4-HMF) (Step B); converting 4-HMF to 2,4 FDCA directly (Step C) or or through the production of intermediates, as converting 4-HMF to furan-2,4-dicarbaldehyde (Step D) and/or 4-(hydroxymethyl)furoic acid (Step E); converting furan-2,4-dicarbaldehyde to 4-formylfuran-2-carboxylate (Step G) and/or 2-formylfuran-4-carboxylate (Step F) and/or converting 4-(hydroxymethyl)furoic acid to 4-formylfuran-2-carboxylate (Step H); converting 4-formylfuran-2-carboxylate to 2,4-FDCA (Step J) and/or converting 2-formylfuran-4-carboxylate to 2,4-FDCA (Step I). In a further embodiment, the one or more carbon sources may include glycerol or a monosaccharide.

[00214] Accordingly, in one embodiment, provided herein is a method of producing a recombinant microorganism that comprises an endogenous and/or exogenous nucleic acid molecules capable of converting a carbon source to glyceraldehyde 3-phosphate (G3P); at least one endogenous or exogenous nucleic acid molecule encoding a (5-formylfuran-3-yl)methyl phosphate synthase that catalyzes the conversion of G3P to (5-formylfuran-3-yl)methyl phosphate; at least one endogenous or exogenous nucleic acid molecule encoding a phosphatase that catalyzes the conversion of (5-formylfuran-3-yl)methyl phosphate to (4-HMF); at least one endogenous or exogenous nucleic acid molecule encoding a dehydrogenase or an oxidase or a peroxygenase that catalyzes the conversion of 4-HMF to 2,4 FDCA directly or through the production of intermediates, as furan-2,4-dicarbaldehyde and/or 4-(hydroxymethyl)furoic acid; at least one endogenous or exogenous nucleic acid molecule encoding a dehydrogenase or an oxidase or a peroxygenase that catalyzes the conversion of furan-2,4-dicarbaldehyde to 4-formylfuran-2-carboxylate and/or 2-formylfuran-4-carboxylate and/or the conversion of 4-(hydroxymethyl)furoic

acid to 4-formylfuran-2-carboxylate; at least one endogenous or exogenous nucleic acid molecule encoding a dehydrogenase or an oxidase or a peroxygenase that catalyzes the conversion of 2-formylfuran-4-carboxylate to 2,4-FDCA and/or 4-formylfuran-2-carboxylate to 2,4-FDCA.

[00215] In one embodiment, the recombinant microorganism of any one of the embodiments of the method disclosed herein comprise an endogenous and/or exogenous nucleic acid molecules capable of converting a carbon source to glyceraldehyde 3-phosphate (G3P). In one embodiment, glycerol is converted to glycerol-3-phopshate by at least one endogenous or exogenous nucleic acid molecule encoding a glycerol kinase. In one embodiment, glycerol-3-phosphate is converted to dihydroxyacetone phosphate (DHAP) by at least one endogenous or exogenous nucleic acid molecule encoding a glycerol-3-phosphate dehydrogenase. In one embodiment, glycerol is converted to dihydroxyacetone by at least one endogenous or exogenous nucleic acid molecule encoding a glycerol dehydrogenase. In one embodiment, dihydroxyacetone is converted to dihydroxyacetone phosphate (DHAP) by at least one endogenous or exogenous nucleic acid molecule encoding a dihydroxyacetone kinase. In one embodiment, DHAP is converted to G3P by at least one endogenous or exogenous nucleic acid molecule encoding a triose phosphate isomerase.

[00216] In one embodiment, the recombinant microorganism of any one of the embodiments of the method disclosed herein comprise at least one endogenous or exogenous nucleic acid molecule encoding a (5-formylfuran-3-yl)methyl phosphate synthase that catalyzes the conversion of G3P to (5-formylfuran-3-yl)methyl phosphate. In one embodiment, the (5-formylfuran-3-yl)methyl phosphate synthase is classified as EC number 4.2.3.153. In some embodiments the EC 4.2.3.153 (5-formylfuran-3-yl)methyl phosphate synthase can be derived from the gene mfnB. In some embodiments, mfnB can be derived from *Methanocaldococcus jannaschii.* In some embodiments, EC 4.2.3.153 can be derived from homologs of mfnB

[00217] In one embodiment, the recombinant microorganism of any one of the embodiments of the method disclosed herein comprise at least one endogenous or exogenous nucleic acid molecule encoding a phosphatase that catalyzes the conversion of (5-formylfuran-3-yl)methyl phosphate to (4-HMF). In one embodiment, the phosphatase is classified as EC number 3.1.3. In one embodiment, the phosphatase EC number 3.1.3 phosphatase is selected from an alkaline phosphatase (EC number 3.1.3.1), acid phosphatase (EC number 3.1.3.2), fructose-bisphosphatase (EC number 3.1.3.11), sugar-phosphatase (EC number 3.1.3.23), or sugar-terminal-phosphatase (EC number 3.1.3.58). In one embodiment, the kinase is classified as EC number 2.7.1. In one embodiment, the kinase EC number 2.7.1 is selected from fructokinase (EC

number 2.7.1.4), ribokinase (EC number 2.7.1.15), ribulokinase (EC number 2.7.1.16), xylulokinase (EC number 2.7.1.17), or D-ribulokinase (EC number 2.7.1.47).

[00218] In one embodiment, the recombinant microorganism of any one of the embodiments of the method disclosed herein comprise at least one endogenous or exogenous nucleic acid molecule encoding a dehydrogenase, or a oxidase, or a peroxygenase that catalyzes the conversion of 4-HMF to furan-2,4-dicarbaldehyde. In one embodiment, the dehydrogenase is classified as EC number 1.1.1. In one embodiment, the dehydrogenase EC number 1.1.1 selected from alcohol dehydrogenase (EC number 1.1.1.1), or alcohol dehydrogenase (NADP+) (EC number 1.1.1.2), or D-xylose reductase (EC number 1.1.1.307), or aryl-alcohol dehydrogenase (EC number 1.1.1.90), or aryl-alcohol dehydrogenase (NADP+) (EC number 1.1.1.91). In one embodiment, the oxidase is classified as EC number 1.1.3. In one embodiment, the oxidase EC number 1.1.3 is 5-(hydroxymethylfurfural oxidase (EC number 1.1.3.47). In some embodiments the EC 1.1.3.47 oxidase can be derived from the gene hmfH. In some embodiments, hmfH can be derived from *Methylovorus sp.* MP688 or Cupriavidus basilensis. In one embodiment, the oxidase EC number 1.1.3 is aryl-alcohol oxidase (EC number 1.1.3.7). In one embodiment, the peroxygenase is classified as EC number 1.11.2. In one embodiment, the peroxygenase EC number 1.11.2 is unspecific peroxygenase (EC number 1.11.2.1).

[00219] In one embodiment, the recombinant microorganism of any one of the embodiments of the method disclosed herein comprise at least one endogenous or exogenous nucleic acid molecule encoding a dehydrogenase, or a oxidase, or a peroxygenase that catalyzes the conversion of 4-HMF to 4-(hydroxymethyl)furoic acid. In one embodiment, the dehydrogenase is classified as EC number 1.2.1. In one embodiment, the dehydrogenase EC number 1.2.1 selected from aldehyde dehydrogenase (NAD+) (EC number 1.2.1.3) or aldehyde dehydrogenase (NADP+) (EC number 1.2.1.4) or aldehyde dehydrogenase [NAD(P)+] (EC number 1.2.1.5) or 4-(γ-glutamylamino)butanal dehydrogenase (EC number 1.2.1.99). In one embodiment, the oxidase is classified as EC number 1.1.3. In one embodiment, the oxidase EC number 1.1.3 is 5-(hydroxymethylfurfural oxidase (EC number 1.1.3.47). In some embodiments the EC 1.1.3.47 oxidase can be derived from the gene hmfH. In some embodiments, hmfH can be derived from *Methylovorus sp.* MP688 or Cupriavidus basilensis. In one embodiment, the oxidase EC number 1.1.3 is aryl-alcohol oxidase (EC number 1.1.3.7). In one embodiment, the peroxygenase is classified as EC number 1.11.2. In one embodiment, the peroxygenase EC number 1.11.2 is unspecific peroxygenase (EC number 1.11.2.1).

[00220] In one embodiment, the recombinant microorganism of any one of the embodiments of the method disclosed herein comprise at least one endogenous or exogenous nucleic acid

molecule encoding a dehydrogenase, or a oxidase, or a peroxygenase that catalyzes the conversion of furan-2,4-dicarbaldehyde to 4-formylfuran-2-carboxylate and/or to 2-formylfuran-4-carboxylate. In one embodiment, the dehydrogenase is classified as EC number 1.2.1. In one embodiment, the dehydrogenase EC number 1.2.1 selected from aldehyde dehydrogenase (NAD+) (EC number 1.2.1.3) or aldehyde dehydrogenase (NADP+) (EC number 1.2.1.4) or aldehyde dehydrogenase [NAD(P)+] (EC number 1.2.1.5) or 4-(γ-glutamylamino)butanal dehydrogenase (EC number 1.2.1.99). In one embodiment, the oxidase is classified as EC number 1.1.3. In one embodiment, the oxidase EC number 1.1.3 is 5-(hydroxymethylfurfural oxidase (EC number 1.1.3.47). In some embodiments the EC 1.1.3.47 oxidase can be derived from the gene hmfH. In some embodiments, hmfH can be derived from *Methylovorus sp.* MP688 or Cupriavidus basilensis. In one embodiment, the oxidase EC number 1.1.3 is aryl-alcohol oxidase (EC number 1.1.3.7). In one embodiment, the peroxygenase is classified as EC number 1.11.2. In one embodiment, the peroxygenase EC number 1.11.2 is unspecific peroxygenase (EC number 1.11.2.1).

[00221] In one embodiment, the recombinant microorganism of any one of the embodiments of the method disclosed herein comprise at least one endogenous or exogenous nucleic acid molecule encoding a dehydrogenase, or a oxidase, or a peroxygenase that catalyzes the conversion of 4-(hydroxymethyl)furoic acid to 4-formylfuran-2-carboxylate. In one embodiment, the dehydrogenase is classified as EC number 1.1.1. In one embodiment, the dehydrogenase EC number 1.1.1 selected from alcohol dehydrogenase (EC number 1.1.1.1), or alcohol dehydrogenase (NADP+) (EC number 1.1.1.2), or D-xylose reductase (EC number 1.1.1.307), or aryl-alcohol dehydrogenase (EC number 1.1.1.90), or aryl-alcohol dehydrogenase (NADP+) (EC number 1.1.1.91). In one embodiment, the dehydrogenase EC number 1.1.1 is. In one embodiment, the oxidase is classified as EC number 1.1.3. In one embodiment, the oxidase EC number 1.1.3 is 5-(hydroxymethylfurfural oxidase (EC number 1.1.3.47). In some embodiments the EC 1.1.3.47 oxidase can be derived from the gene hmfH. In some embodiments, hmfH can be derived from *Methylovorus sp.* MP688 or Cupriavidus basilensis. In one embodiment, the oxidase EC number 1.1.3 is aryl-alcohol oxidase (EC number 1.1.3.7). In one embodiment, the peroxygenase is classified as EC number 1.11.2. In one embodiment, the peroxygenase EC number 1.11.2 is unspecific peroxygenase (EC number 1.11.2.1).

[00222] In one embodiment, the recombinant microorganism of any one of the embodiments of the method disclosed herein comprise at least one endogenous or exogenous nucleic acid molecule encoding a dehydrogenase, or a oxidase, or a peroxygenase that catalyzes the conversion of 4-formylfuran-2-carboxylate and/or 2-formylfuran-4-carboxylate to 2,4-FDCA.

In one embodiment, the dehydrogenase is classified as EC number 1.1.1. In one embodiment, the dehydrogenase EC number 1.1.1 selected from alcohol dehydrogenase (EC number 1.1.1.1), or alcohol dehydrogenase (NADP+) (EC number 1.1.1.2), or D-xylose reductase (EC number 1.1.1.307), or aryl-alcohol dehydrogenase (EC number 1.1.1.90), or aryl-alcohol dehydrogenase (NADP+) (EC number 1.1.1.91). In one embodiment the dehydrogenases can be derived from enzyme candidates listed at Table 3. In some embodiments, the dehydrogenases are homologous or similar to the enzymes listed at Table 3. In some embodiments, a dehydrogenases is evolved or engineered to improve its catalytic efficiency against its desirable substrate.

[00223] In one embodiment, the oxidase is classified as EC number 1.1.3. In one embodiment, the oxidase EC number 1.1.3 is 5-(hydroxymethylfurfural oxidase (EC number 1.1.3.47). In some embodiments the HMF oxidase can be derived from the gene hmfH. In some embodiments, HMF oxidase can be derived from *Methylovorus sp.* MP688 or Cupriavidus basilensis. *See* Dijkman and Fraaije (2014. Applied Environmental Microbiology, 80.3:1082-1090) and Koopman *et al.* (2010. PNAS, 107(11):4919-4924). In one embodiment, the HMF oxidase EC number 1.1.3 is aryl-alcohol oxidase (EC number 1.1.3.7). *See* Carro et al. (2015). In one embodiment, the peroxygenase is classified as EC number 1.11.2. In one embodiment, the peroxygenase EC number 1.11.2 is unspecific peroxygenase (EC number 1.11.2.1). *See* Carro *et al.* (2015). In some embodiments, the HMF oxidase can be derived from enzyme candidates listed at Table 4. In some embodiments, the HMF oxidase is homologous or similar to the enzymes listed at Table 4. In some embodiments, the HMF oxidase enzyme is evolved or engineered to improve its catalytic efficiency.

2,4-furandimethanol

[00224] In one embodiment, the present disclosure is drawn to a method of producing a recombinant microorganism capable of producing 2,4-furandimethanol from a carbon source. Some embodiments of the present disclosure are presented in **FIG. 1**, **FIG. 2**, **and FIG. 3**, which collectively detail the biosynthetic conversion of a carbon feedstock to 2,4-furandimethanol.

[00225] In one embodiment, the bioproduction of 2,4-furandimethanol from 4-HMF is catalyzed by a dehydrogenase encoded by the microorganism. In one embodiment, the dehydrogenase is classified as EC number 1.1.1. In one embodiment, the dehydrogenase EC number 1.1.1 is selected from alcohol dehydrogenase (EC number 1.1.1.1). In one embodiment, the dehydrogenase EC number 1.1.1 is selected from alcohol dehydrogenase (NADP+) (EC number 1.1.1.2). In one embodiment, the dehydrogenase EC number 1.1.1 is selected from D-xylose

reductase (EC number 1.1.1.90). In one embodiment, the dehydrogenase EC number 1.1.1 is selected from aryl-alcohol dehydrogenase (EC number 1.1.1.91). In one embodiment the dehydrogenases can be derived from enzyme candidates listed at Table 5. In some embodiments, the dehydrogenases are homologous or similar to the enzymes listed at Table 5. In some embodiments, a dehydrogenases is evolved or engineered to improve its catalytic efficiency for 4-HMF reduction to 2,4-furandimethanol.

[00226] In some aspects, 2,4-furandimethanol is produced enzymatically, in the absence of microbes. In some aspects, 2,4-furandimethanol is produced enzymatically in one or more vessels. In some aspects, the one or more vessels are substantially free of microbes. In some aspects, the enzymatic production of 2,4-furandimethanol is performed in the same step-wise fashion as described with in the methods utilizing recombinant microorganisms, but substantially free of microorganisms or in the absence of microorganisms. In some aspects, the enzymes utilized in the enzymatic production of 2,4-furandimethanol are isolated from microbes, recombinant or otherwise, and provided to their corresponding substrates for the stepwise production of the intermediates utilized to produce 2,4-furandimethanol. In some aspects, one or more of the steps of the methods are performed in the same vessel. In some aspects, once the desired product is produced as a result of the individual method steps described herein, the product is isolated and purified and then utilized as the substrate in the next step of the method of producing 2,4-furandimethanol.

Furan-2,4-dicarbaldehyde

[00227] In one embodiment, the present disclosure is drawn to a method of producing a recombinant microorganism capable of producing furan-2,4-dicarbaldehyde from a carbon source. Some embodiments of the present disclosure are presented in **FIG. 1**, **FIG. 2, and FIG. 3**, which collectively detail the biosynthetic conversion of a carbon feedstock to furan-2,4-dicarbaldehyde.

[00228] In one embodiment, step D in **FIG. 2** is a single step reaction utilizing 4-HMF as a substrate. In one embodiment, the bioproduction of furan-2,4-dicarbaldehyde from 4-HMF is catalyzed by one or more enzymes represented by EC numbers 1.1.1.-, 1.1.3.-, and 1.11.2.-.

[00229] In one embodiment, the recombinant microorganism of any one of the embodiments disclosed herein comprises at least one endogenous or exogenous nucleic acid molecule encoding a dehydrogenase, an oxidase, or a peroxygenase that catalyzes the conversion of 4-HMF to furan-2,4-dicarbaldehyde. In one embodiment, the dehydrogenase is classified as EC number 1.1.1. In one embodiment, the dehydrogenase EC number 1.1.1 selected from alcohol

dehydrogenase (EC number 1.1.1.1), or alcohol dehydrogenase (NADP+) (EC number 1.1.1.2), or D-xylose reductase (EC number 1.1.1.307), or aryl-alcohol dehydrogenase (EC number 1.1.1.90), or aryl-alcohol dehydrogenase (NADP+) (EC number 1.1.1.91). In one embodiment, the oxidase is classified as EC number 1.1.3. In one embodiment, the oxidase EC number 1.1.3 is 5-(hydroxymethylfurfural oxidase (EC number 1.1.3.47). In some embodiments the EC 1.1.3.47 oxidase can be derived from the gene hmfH. In some embodiments, hmfH can be derived from *Methylovorus sp.* MP688 or Cupriavidus basilensis. *See* Dijkman and Fraaije (2014) and Koopman *et al.* (2010). In one embodiment, the oxidase EC number 1.1.3 is aryl-alcohol oxidase (EC number 1.1.3.7). *See* Carro et al. (2015). In one embodiment, the peroxygenase is classified as EC number 1.11.2. In one embodiment, the peroxygenase EC number 1.11.2 is unspecific peroxygenase (EC number 1.11.2.1). *See* Carro *et al.* (2015).

[00230] In some aspects, furan-2,4-dicarbaldehyde is produced enzymatically, in the absence of microbes. In some aspects, furan-2,4-dicarbaldehyde is produced enzymatically in one or more vessels. In some aspects, the one or more vessels are substantially free of microbes. In some aspects, the enzymatic production of furan-2,4-dicarbaldehyde is performed in the same step-wise fashion as described with in the methods utilizing recombinant microorganisms, but substantially free of microorganisms or in the absence of microorganisms. In some aspects, the enzymes utilized in the enzymatic production of furan-2,4-dicarbaldehyde are isolated from microbes, recombinant or otherwise, and provided to their corresponding substrates for the stepwise production of the intermediates utilized to produce furan-2,4-dicarbaldehyde. In some aspects, one or more of the steps of the methods are performed in the same vessel. In some aspects, once the desired product is produced as a result of the individual method steps described herein, the product is isolated and purified and then utilized as the substrate in the next step of the method of producing furan-2,4-dicarbaldehyde.

4-(hydroxymethyl)furoic acid

[00231] In one embodiment, the present disclosure is drawn to a method of producing a recombinant microorganism capable of producing 4-(hydroxymethyl)furoic acid from a carbon source. Some embodiments of the present disclosure are presented in **FIG. 1**, **FIG. 2, and FIG. 3**, which collectively detail the biosynthetic conversion of a carbon feedstock to 4-(hydroxymethyl)furoic acid.

[00232] In one embodiment, step E in **FIG. 2** is a single step reaction utilizing 4-HMF as a substrate. In one embodiment, the bioproduction of 4-(hydroxymethyl)furoic acid from 4-HMF is catalyzed by one or more enzymes represented by EC numbers 1.1.1.-, 1.1.3.-, and 1.11.2.-.

[00233] In one embodiment, the recombinant microorganism of any one of the embodiments disclosed herein comprises at least one endogenous or exogenous nucleic acid molecule encoding a dehydrogenase, or a oxidase, or a peroxygenase that catalyzes the conversion of 4-HMF to 4-(hydroxymethyl)furoic acid. In one embodiment, the dehydrogenase is classified as EC number 1.2.1. In one embodiment, the dehydrogenase EC number 1.2.1 selected from aldehyde dehydrogenase (NAD+) (EC number 1.2.1.3) or aldehyde dehydrogenase (NADP+) (EC number 1.2.1.4) or aldehyde dehydrogenase [NAD(P)+] (EC number 1.2.1.5) or 4-(γ-glutamylamino)butanal dehydrogenase (EC number 1.2.1.99). In one embodiment, the oxidase is classified as EC number 1.1.3. In one embodiment, the oxidase EC number 1.1.3 is 5-(hydroxymethylfurfural oxidase (EC number 1.1.3.47). In some embodiments the EC 1.1.3.47 oxidase can be derived from the gene hmfH. In some embodiments, hmfH can be derived from *Methylovorus sp.* MP688 or Cupriavidus basilensis. In one embodiment, the oxidase EC number 1.1.3 is aryl-alcohol oxidase (EC number 1.1.3.7). In one embodiment, the peroxygenase is classified as EC number 1.11.2. In one embodiment, the peroxygenase EC number 1.11.2 is unspecific peroxygenase (EC number 1.11.2.1).

[00234] In some aspects, 4-(hydroxymethyl)furoic acid is produced enzymatically, in the absence of microbes. In some aspects, 4-(hydroxymethyl)furoic acid is produced enzymatically in one or more vessels. In some aspects, the one or more vessels are substantially free of microbes. In some aspects, the enzymatic production of 4-(hydroxymethyl)furoic acid is performed in the same step-wise fashion as described with in the methods utilizing recombinant microorganisms, but substantially free of microorganisms or in the absence of microorganisms. In some aspects, the enzymes utilized in the enzymatic production of 4-(hydroxymethyl)furoic acid are isolated from microbes, recombinant or otherwise, and provided to their corresponding substrates for the stepwise production of the intermediates utilized to produce 4-(hydroxymethyl)furoic acid. In some aspects, one or more of the steps of the methods are performed in the same vessel. In some aspects, once the desired product is produced as a result of the individual method steps described herein, the product is isolated and purified and then utilized as the substrate in the next step of the method of producing 4-(hydroxymethyl)furoic acid.

2-formylfuran-4-carboxylate

[00235] In one embodiment, the present disclosure is drawn to a method of producing a recombinant microorganism capable of producing 2-formylfuran-4-carboxylate from a carbon source. Some embodiments of the present disclosure are presented in **FIG. 1**, **FIG. 2, and FIG.**

3, which collectively detail the biosynthetic conversion of a carbon feedstock to 2-formylfuran-4-carboxylate.

[00236] In one embodiment, step F in **FIG. 2** is a single step reaction utilizing furan-2,4-dicarbaldehyde as a substrate. In one embodiment, the bioproduction of 2-formylfuran-4-carboxylate from furan-2,4-dicarbaldehyde is catalyzed by one or more enzymes represented by EC numbers 1.2.1.-, 1.1.3.-, and 1.11.2.-.

[00237] In one embodiment, the recombinant microorganism of any one of the embodiments disclosed herein comprises at least one endogenous or exogenous nucleic acid molecule encoding a dehydrogenase, or a oxidase, or a peroxygenase that catalyzes the conversion of furan-2,4-dicarbaldehyde to 2-formylfuran-4-carboxylate. In one embodiment, the dehydrogenase is classified as EC number 1.2.1. In one embodiment, the dehydrogenase EC number 1.2.1 selected from aldehyde dehydrogenase (NAD+) (EC number 1.2.1.3) or aldehyde dehydrogenase (NADP+) (EC number 1.2.1.4) or aldehyde dehydrogenase [NAD(P)+] (EC number 1.2.1.5) or 4-(γ-glutamylamino)butanal dehydrogenase (EC number 1.2.1.99). In one embodiment, the oxidase is classified as EC number 1.1.3. In one embodiment, the oxidase EC number 1.1.3 is 5-(hydroxymethylfurfural oxidase (EC number 1.1.3.47). In some embodiments the EC 1.1.3.47 oxidase can be derived from the gene hmfH. In some embodiments, hmfH can be derived from *Methylovorus sp.* MP688 or Cupriavidus basilensis. In one embodiment, the oxidase EC number 1.1.3 is aryl-alcohol oxidase (EC number 1.1.3.7). In one embodiment, the peroxygenase is classified as EC number 1.11.2. In one embodiment, the peroxygenase EC number 1.11.2 is unspecific peroxygenase (EC number 1.11.2.1).

[00238] In some aspects, 2-formylfuran-4-carboxylate is produced enzymatically, in the absence of microbes. In some aspects, 2-formylfuran-4-carboxylate is produced enzymatically in one or more vessels. In some aspects, the one or more vessels are substantially free of microbes. In some aspects, the enzymatic production of 2-formylfuran-4-carboxylate is performed in the same step-wise fashion as described with in the methods utilizing recombinant microorganisms, but substantially free of microorganisms or in the absence of microorganisms. In some aspects, the enzymes utilized in the enzymatic production of 2-formylfuran-4-carboxylate are isolated from microbes, recombinant or otherwise, and provided to their corresponding substrates for the stepwise production of the intermediates utilized to produce 2-formylfuran-4-carboxylate. In some aspects, one or more of the steps of the methods are performed in the same vessel. In some aspects, once the desired product is produced as a result of the individual method steps described herein, the product is isolated and purified and then utilized as the substrate in the next step of the method of producing 2-formylfuran-4-carboxylate.

4-formylfuran-2-carboxylate

[00239] In one embodiment, the present disclosure is drawn to a method of producing a recombinant microorganism capable of producing 4-formylfuran-2-carboxylate from a carbon source. Some embodiments of the present disclosure are presented in **FIG. 1**, **FIG. 2, and FIG. 3**, which collectively detail the biosynthetic conversion of a carbon feedstock to 4-formylfuran-2-carboxylate.

[00240] In one embodiment, step G in **FIG. 2** is a single step reaction utilizing furan-2,4-dicarbaldehyde as a substrate. In one embodiment, the bioproduction of 4-formylfuran-2-carboxylate from furan-2,4-dicarbaldehyde is catalyzed by one or more enzymes represented by EC numbers 1.2.1.-, 1.1.3.-, and 1.11.2.-.

[00241] In one embodiment, the recombinant microorganism of any one of the embodiments disclosed herein comprises at least one endogenous or exogenous nucleic acid molecule encoding a dehydrogenase, or a oxidase, or a peroxygenase that catalyzes the conversion of furan-2,4-dicarbaldehyde to 4-formylfuran-2-carboxylate. In one embodiment, the dehydrogenase is classified as EC number 1.2.1. In one embodiment, the dehydrogenase EC number 1.2.1 selected from aldehyde dehydrogenase (NAD+) (EC number 1.2.1.3) or aldehyde dehydrogenase (NADP+) (EC number 1.2.1.4) or aldehyde dehydrogenase [NAD(P)+] (EC number 1.2.1.5) or 4-(γ-glutamylamino)butanal dehydrogenase (EC number 1.2.1.99). In one embodiment, the oxidase is classified as EC number 1.1.3. In one embodiment, the oxidase EC number 1.1.3 is 5-(hydroxymethylfurfural oxidase (EC number 1.1.3.47). In some embodiments the EC 1.1.3.47 oxidase can be derived from the gene hmfH. In some embodiments, hmfH can be derived from *Methylovorus sp.* MP688 or Cupriavidus basilensis. In one embodiment, the oxidase EC number 1.1.3 is aryl-alcohol oxidase (EC number 1.1.3.7). In one embodiment, the peroxygenase is classified as EC number 1.11.2. In one embodiment, the peroxygenase EC number 1.11.2 is unspecific peroxygenase (EC number 1.11.2.1).

[00242] In one embodiment, step H in **FIG. 2** is a single step reaction utilizing 4-(hydroxymethyl)furoic acid as a substrate. In one embodiment, the bioproduction of 4-formylfuran-2-carboxylate from 4-(hydroxymethyl)furoic acid is catalyzed by one or more enzymes represented by EC numbers 1.1.1.-, 1.1.3.-, and 1.11.2.-.

[00243] In one embodiment, the recombinant microorganism of any one of the embodiments disclosed herein comprises at least one endogenous or exogenous nucleic acid molecule encoding a dehydrogenase, or a oxidase, or a peroxygenase that catalyzes the conversion of 4-(hydroxymethyl)furoic acid to 4-formylfuran-2-carboxylate. In one embodiment, the

dehydrogenase is classified as EC number 1.1.1. In one embodiment, the dehydrogenase EC number 1.1.1 selected from alcohol dehydrogenase (EC number 1.1.1.1), or alcohol dehydrogenase (NADP+) (EC number 1.1.1.2), or D-xylose reductase (EC number 1.1.1.307), or aryl-alcohol dehydrogenase (EC number 1.1.1.90), or aryl-alcohol dehydrogenase (NADP+) (EC number 1.1.1.91). In one embodiment, the oxidase is classified as EC number 1.1.3. In one embodiment, the oxidase EC number 1.1.3 is 5-(hydroxymethylfurfural oxidase (EC number 1.1.3.47). In some embodiments the EC 1.1.3.47 oxidase can be derived from the gene hmfH. In some embodiments, hmfH can be derived from *Methylovorus sp.* MP688 or Cupriavidus basilensis. *See* Dijkman and Fraaije (2014) and Koopman *et al.* (2010). In one embodiment, the oxidase EC number 1.1.3 is aryl-alcohol oxidase (EC number 1.1.3.7). *See* Carro et al. (2015). In one embodiment, the peroxygenase is classified as EC number 1.11.2. In one embodiment, the peroxygenase EC number 1.11.2 is unspecific peroxygenase (EC number 1.11.2.1). *See* Carro *et al.* (2015).

[00244] In some aspects, 4-formylfuran-2-carboxylate is produced enzymatically, in the absence of microbes. In some aspects, 4-formylfuran-2-carboxylate is produced enzymatically in one or more vessels. In some aspects, the one or more vessels are substantially free of microbes. In some aspects, the enzymatic production of 4-formylfuran-2-carboxylate is performed in the same step-wise fashion as described with in the methods utilizing recombinant microorganisms, but substantially free of microorganisms or in the absence of microorganisms. In some aspects, the enzymes utilized in the enzymatic production of 4-formylfuran-2-carboxylate are isolated from microbes, recombinant or otherwise, and provided to their corresponding substrates for the stepwise production of the intermediates utilized to produce 4-formylfuran-2-carboxylate. In some aspects, one or more of the steps of the methods are performed in the same vessel. In some aspects, once the desired product is produced as a result of the individual method steps described herein, the product is isolated and purified and then utilized as the substrate in the next step of the method of producing 4-formylfuran-2-carboxylate.

[00245] The present disclosure provides methods and recombinant microorganisms capable of producing high yields of one or more of 4-HMF, 2,4-furandimethanol, furan-2,4-dicarbaldehyde, 4-(hydroxymethyl)furoic acid, 2-formylfuran-4-carboxylate, 4-formylfuran-2-carboxylate, and 2,4-FDCA. In one embodiment one molecule of glucose and two molecules of ATP are converted into one molecule of 2,4-FDCA and three molecules of NAD(P)H according to the net equation 1:

Equation 1: 1 glucose + 2 ATP → 1 2,4-FDCA + 3 NAD(P)H

[00246] The net reaction results in a mass yield of about 0.87 grams of 2,4-FDCA per gram of glucose. This yield is equivalent to 75% of the maximal thermodynamic yield of 1.16 grams of 2,4-

FDCA per gram of glucose. In some embodiments, the yield of 2,4-FDCA can be about 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.16 grams per gram of glucose.

[00247] In one embodiment one molecule of glucose, two molecules of ATP and three molecules of oxygen are converted into one molecule of 2,4-FDCA and three molecules of hydrogen peroxide (H_2O_2) according to the net equation 2:

Equation 2: 1 glucose + 2 ATP + 3 O_2 → 1 2,4-FDCA + 3 H_2O_2

[00248] In one embodiment two molecules of glycerol and two molecules of ATP are converted into one molecule of 2,4-FDCA and five molecules of NAD(P)H according to the net equation 3:

Equation 3: 2 glycerol + 2 ATP → 1 2,4-FDCA + 5 NAD(P)H

[00249] The net reaction results in a mass yield of about 0.85 grams of 2,4-FDCA per gram of glycerol. This yield is equivalent to 64% of the maximal thermodynamic yield of 1.32 grams of 2,4-FDCA per gram of glycerol. In some embodiments, the yield of 2,4-FDCA can be about 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.32 grams per gram of glycerol.

[00250] In one embodiment two molecules of glycerol, two molecules of ATP and three molecules of oxygen are converted into one molecule of 2,4-FDCA and three molecules of hydrogen peroxide according to the net equation 4:

Equation 4: 2 glycerol + 2 ATP + 3 O_2 → 1 2,4-FDCA + 3 H_2O_2

Methods of Producing and Isolating Biosynthesis Product Monomers and/or Polymers

[00251] In some aspects, modified microbes of the present disclosure are modified such that the microbes produce 4-HMF, 2,4-furandimethanol, furan-2,4-dicarbaldehyde, 4-(hydroxymethyl)furoic acid, 2-formylfuran-4-carboxylate, 4-formylfuran-2-carboxylate, and 2,4-FDCA monomers. In some aspects, modified microbes of the present disclosure are modified such that the microbes produce polymers derived from 4-HMF, 2,4-furandimethanol, furan-2,4-dicarbaldehyde, 4-(hydroxymethyl)furoic acid, 2-formylfuran-4-carboxylate, 4-formylfuran-2-carboxylate, and 2,4-FDCA. In some aspects, a method of producing the biosynthesis product monomers and/or polymers comprises growing/fermenting one or more microbes of the present disclosure under conditions sufficient to produce the biosynthesis product monomers and/or polymers, and isolating/collecting the resulting 4-HMF, 2,4-furandimethanol, furan-2,4-dicarbaldehyde, 4-(hydroxymethyl)furoic acid, 2-formylfuran-4-carboxylate, 4-formylfuran-2-carboxylate, and 2,4-FDCA and/or polymers thereof. In some aspects, the biosynthesis monomers will polymerize into polymers *in vivo*. In some aspects, the production of the biosynthesis monomers and polymers is proportional to the number of bacteria utilized in the microbial fermentation process. In some aspects, the bacteria are grown in a reaction chamber.

Once a desired number of bacteria have been achieved, the spent media is subjected to a process for the isolating the biosynthesis product monomers and/or polymers. In some aspects, the microbes are lysed and the cellular debris is pelleted out of solution in a centrifuge. In some aspects, the biosynthesis product monomers and/or polymers are collected from the cell pellet fraction or the liquid fraction with the aid of a solvent extraction process or a gradient ultra-centrifugation process. In some aspects, the biosynthesis product polymer can be isolated by filtration.

[00252] In some aspects, a biosynthesis product monomer is produced by cultivating the recombinant microorganism in a culture medium containing a feedstock providing a carbon source until the monomer is produced. In some aspects, the feedstock comprises one or more hexose, one or more pentose, or a combination thereof. In some aspects, the monomer is extracted from the culture medium and polymerized in the presence of a catalyst. The present disclosure provides a method of producing a polymer from biosynthesis product produced by the recombinant microorganisms and methods of the disclosure. In one embodiment the one or more biosynthesis products are catalytically polymerized with a diol to form a polymer.

[00253] In some aspects, the biosynthesis product monomer is catalyzed in the presence of a catalyst selected from a titanium-based catalyst, germanium-based catalyst, magnesium-based catalyst, silicon-based catalyst, aluminum-based catalyst, or an antimony-based catalyst. In some aspects, the catalyst is selected from: antimony acetate, antimony trioxide, germanium dioxide, tetra-isopropyl titanate, and tetra-n-butyl titanate.

[00254] In some aspects, the biosynthesis product-derived polymer is polymerized *in vivo* by a pha synthase. In some aspects, the biosynthesis product-derived monomer is polymerized *ex vivo* by a pha synthase.

[00255] In some aspects, the biosynthesis product 4-HMF is extracted from the culture medium and transformed, in the presence of a catalyst, into one or more of the other biosynthesis products as reported in the state of the art. *See* Van Putten *et al.* (2013. Hydroxymethylfurfural, a Versatile Platform Chemical Made from Renewable Resources. *Chemical Reviews*, 113.3:1499-1597).

[00256] In some aspects, the biosynthesis product 4-HMF is extracted from the culture medium and transformed, in the presence of a catalyst, into 2,4-dimethylfuran. *See* Deng *et al.* (2013. Linked Strategy for the Production of Fuels via Formose Reaction. *Scientific Reports*, 3:1244).

[00257] In some aspects, any one or more of the biosynthesized products produced by the methods and compositions described herein are extracted from the culture medium in which they are biosynthesized and are transformed in the presence of a chemical or biological catalyst(s).

[00258] In some aspects, the transformation of the biosynthesized products in the presence of a chemical or biological catalyst(s) is performed in the absence of microorganisms. In some aspects, the the transformation of the biosynthesized products in the presence of a biological catalyst(s) is performed in the absence of microorganisms and in the presence of one or more enzymes isolated and purified from one or more microorganisms.

[00259] In some aspects, the chemical catalyst or catalysts are any one or more of the chemicals that are known to be utilized in non-biological synthesis of 2,4-furandimethanol, furan-2,4-dicarbaldehyde, 4-(hydroxymethyl)furoic acid, 2-formylfuran-4-carboxylate, 4-formylfuran-2-carboxylate, and/or 2,4-FDCA.

[00260] In some aspects, the biological catalyst or catalysts are any one or more of the enzymes described herein for reaction steps C, D, E, F, G, H, I, or J in **FIG. 2**.

[00261] In some aspects, the transformation of biosynthesized 4-HMF into 2,4-furandimethanol occurs in a composition substantially free of microorganisms and in the presence of a chemical or biological catalyst described herein, such as the biological catalysts of EC 1.1.1.-.

[00262] In some aspects, the transformation of biosynthesized 4-HMF into furan-2,4-dicarbaldehyde occurs in a composition substantially free of microorganisms and in the presence of a chemical or biological catalyst described herein, such as the biological catalysts of EC 1.1.1.-, EC 1.1.3.-, and/or EC 1.11.2.-.

[00263] In some aspects, the transformation of biosynthesized 4-HMF into 4-(hydroxymethyl)furoic acid) occurs in a composition substantially free of microorganisms and in the presence of a chemical or biological catalyst described herein, such as the biological catalysts of EC 1.1.1.-, EC 1.1.3.-, and/or EC 1.11.2.-.

[00264] In some aspects, the transformation of biosynthesized 2,4-furandimethanol into 2-formylfuran-4-carboxylate occurs in a composition substantially free of microorganisms and in the presence of a chemical or biological catalyst described herein, such as the biological catalysts of EC 1.2.1.-, EC 1.1.3.-, and/or EC 1.11.2.-.

[00265] In some aspects, the transformation of biosynthesized furan-2,4-dicarbaldehyde into 2-formylfuran-4-carboxylate occurs in a composition substantially free of microorganisms and in the presence of a chemical or biological catalyst described herein, such as the biological catalysts of EC 1.2.1.-, EC 1.1.3.-, and/or EC 1.11.2.-.

[00266] In some aspects, the transformation of biosynthesized furan-2,4-dicarbaldehyde into 4-formylfuran-2-carboxylate occurs in a composition substantially free of microorganisms and in the presence of a chemical or biological catalyst described herein, such as the biological catalysts of EC 1.2.1.-, EC 1.1.3.-, and/or EC 1.11.2.-.

[00267] In some aspects, the transformation of biosynthesized 4-(hydroxymethyl)furoic acid into 4-formylfuran-3-carboxylate occurs in a composition substantially free of microorganisms and in the presence of a chemical or biological catalyst described herein, such as the biological catalysts of EC 1.2.1.-, EC 1.1.3.-, and/or EC 1.11.2.-.

[00268] In some aspects, the transformation of biosynthesized 2-formylfuran-4-carboxylate into 2,4-FDCA occurs in a composition substantially free of microorganisms and in the presence of a chemical or biological catalyst described herein, such as the biological catalysts of EC 1.2.1.-, EC 1.1.3.-, and/or EC 1.11.2.-.

[00269] In some aspects, the transformation of biosynthesized 4-formylfuran-2-carboxylate into 2,4-FDCA occurs in a composition substantially free of microorganisms and in the presence of a chemical or biological catalyst described herein, such as the biological catalysts of EC 1.2.1.-, EC 1.1.3.-, and/or EC 1.11.2.-.

[00270] In some aspects, any one or more of the transformations described above can be combined with another transformation such that the product of the first transformation is the substrate for the product of the second transformation.

[00271] In some aspects, any one or more of the transformations described above can be combined with another transformation such that the product of the first transformation is the substrate for the product of the second transformation, whose product is the substrate for the product of the third transformation.

Biological processes for producing the biosynthesis products

[00272] The present disclosure provides a biological process for producing one or more of the biosynthesis products described herein; 4-HMF, 2,4-furandimethanol, furan-2,4-dicarbaldehyde, 4-(hydroxymethyl)furoic acid, 2-formylfuran-4-carboxylate, 4-formylfuran-2-carboxylate, and 2,4-FDCA. In some embodiments, the process comprises: providing to at least one bioreactor one or more recombinant microorganisms engineered to express one or more enzymes involved in the biosynthesis of glyceraldehyde 3-phosphate (G3P) from one or more biosynthesis pathways and one or more of the biosynthesis products from G3P and a feedstock comprising an exogenous carbon source; cultivating the one or more recombinant microorganisms in one or more stages in a culture medium comprising the feedstock; fermenting the resulting culture in one or more stages under aerobic, microaerobic and/or anaerobic conditions; and recovering from the bioreactor the one or more biosynthesis products after the fermentation step.

[00273] In some embodiments of the biological process, the one or more biosynthesis products are recovered continuously prior to exhaustion of the culture medium or the feedstock. In some embodiments, the biosynthesis products are recovered in batches prior to exhaustion of the

culture medium or the feedstock. In some embodiments, the one or more recombinant microorganisms are derived from a parental microorganism selected from the group consisting of Clostridium sp., Clostridium ljungdahlii, Clostridium autoethanogenum, Clostridium ragsdalei, Eubacterium limosum, Butyribacterium methylotrophicum, Moorella thermoacetica, Corynebacterium glutamicum, Clostridium aceticum, Acetobacterium woodii, Alkalibaculum bacchii, Clostridium drakei, Clostridium carboxidivorans, Clostridium formicoaceticum, Clostridium scatologenes, Moorella thermoautotrophica, Acetonema longum, Blautia producta, Clostridium glycolicum, Clostridium magnum, Candida krusei, Clostridium mayombei, Clostridium methoxybenzovorans, Clostridium acetobutylicum, Clostridium beijerinckii, Oxobacter pfennigii, Thermoanaerobacter kivui, Sporomusa ovata, Thermoacetogenium phaeum, Acetobacterium carbinolicum, Issatchenkia orientalis, Sporomusa termitida, Moorella glycerini, Eubacterium aggregans, Treponema azotonutricium, Pichia kudriavzevii, Escherichia coli, Saccharomyces cerevisiae, Pseudomonas putida, Bacillus sp, Corynebacterium sp., Yarrowia lipolytica, Scheffersomyces stipitis, and Terrisporobacter glycolicus.

[00274] In some embodiments of the biological process, the feedstock comprises C6 carbohydrates and/or C5 carbohydrates. In some embodiments, the feedstock comprises monosaccharides, disaccharides, oligosaccharides, polysaccharides, or combinations thereof.

[00275] In some embodiments of the biological process, the cultivating and fermenting steps occur in the same stage. In some embodiments, the cultivating and fermenting steps occur in separate stages. In some embodiments, the cultivating and fermenting steps occur in separate bioreactors. In some embodiments, the cultivating and fermenting steps occurs in the same bioreactor. In some embodiments, the bioreactor operates under aerobic, microaerobic, or anaerobic conditions; or a combination thereof.

[00276] In some embodiments, the one or more stages receive the culture and/or culture media as a batch, a fed-batch, or a continuous mode feed. In some embodiments, the cultivating stage receives the culture and/or culture media as a batch, a fed-batch, or a continuous mode feed, and any subsequent stages operate as a batch, a fed-batch, or a continuous mode feed.

[00277] In some embodiments, the culture medium comprises carbon (C) that is provided from C5 carbohydrates, C6 carbohydrates, and/or disaccharides. In some embodiments, the culture medium comprises essential nutrients including nitrogen (N), phosphorus (P), magnesium (Mg), and iron (Fe).

[00278] In some embodiments, wherein a ratio of C:N in the cultivating step is at least 10:1. In some embodiments, wherein a ratio of C:P in the cultivating step is at least 5:1. In some

embodiments, a ratio of C:Mg in the cultivating step is at least 50:1. In some embodiments, a ratio of C:Fe in the cultivating step is at least 300:1.

[00279] In some embodiments, the cultivating step operates from 5 up to 100 hours for the cultivation of the cells of the one or more recombinant microorganisms. In some embodiments, the culture in the fermenting step comprises about 1% to about 30% of the cell mass, which is transferred from the cultivating step in the culture medium with the one or more substrates. In some embodiments, a total amount of the feedstock provided to the fermenting step ranges from about 100 kg/m^3 to about 800 kg/m^3.

[00280] In some embodiments, a ratio of C:N in the fermenting step is at least 50:1. In some embodiments, a ratio of C:P in the fermenting step is at least 20:1. In some embodiments, a ratio of C:Mg in the fermenting step is at least 200:1. In some embodiments, a ratio of C:Fe in the fermenting step is at least 800:1. In some embodiments, the fermenting step operates from 10 up to 300 hours for fed-batch operation and up to 300 hours for continuous operation.

[00281] EXAMPLES

[00282] Example 1: Expression and purification of methyl phosphate synthase

[00283] The expression and purification of enzymes used in enzymatic assays was carried out under the following conditions: Genes coding 27 (5-formylfuran-3-yl)methyl phosphate synthases candidates (Table 1) were synthetized by GenScript and cloned in expression vector pET28a in NdeI and BamHI restriction sites. The expression vector was transformed into *E. coli* BL21 (DE3) and the transformant was stored in 15% glycerol until use for enzyme expression.

[00284] The stored transformant was inoculated into 50 mL of TB broth containing kanamycin at 37°C with agitation for 16h to prepare a seed culture. The seed culture was added to 300 mL of TB broth containing kanamycin with initial OD (600 nm) of 0.2, the culture was then incubated at 37°C with agitation until OD (600 nm) reached 0.6-0.8 at which point 1 mM IPTG was added to induce expression overnight at 18°C with agitation.

[00285] Following overnight expression, the cells were centrifuged at 6000x rpm for 30 min and the pellet cell was suspended in cold lysis buffer (20 mM phosphate buffer and 500 mM NaCl pH 7.4) before ultrasonic disruption. The cell lysate was again centrifuged at 8000 rpm for 30 min at 4°C and filtered before purification with affinity chromatography. The column utilized was a HisTrap FF Crude (GE Healthcare) for his-tagged protein purification. The purified protein was bound and washed in the column with binding buffer A (20 mM phosphate buffer, 20 mM imidazole, 500 mM NaCl, 1 mM PMSF and beta-mercaptoethanol, pH 7.4) and eluted in a gradient

of elution buffer B (20 mM phosphate buffer, 500 mM imidazole, 500 mM NaCl, 1 mM PMSF and beta-mercaptoethanol, pH 7.4). Then using a PD-10 column the buffer was changed to a 50 mM Tris-HCl pH 7.4. Candidates expression and purification were analyzed on 12% polyacrylamide gel by electrophoresis, as illustrated at FIG. 4.

[00286] Example 2: (5-formylfuran-3-yl)methyl phosphate production from G3P

[00287] The (5-formylfuran-3-yl)methyl phosphate production from glyceraldehyde-3-phosphate (G3P) by enzyme candidates described in Table 1 was demonstrated *in vitro* by incubating approximately 450 µg of purified candidates with a 1 mL solution containing 5 mM of glyceraldehyde-3-phosphate (Sigma) in 20 mM Tris-HCl, 200 mM NaCl (pH 7.4) buffer. The reaction was incubated at 37°C for 2 hours. Reaction vessels without synthases or substrate (G3P) were used as negative controls. The reaction was monitored by UV-Vis using a spectrophotometer (SpectraMax M5, Molecular Devices), accordingly to the 5-formylfuran-3-yl)methyl phosphate Molar absorption coefficient (ε) 280 nm. Product formation was also confirmed by HPLC analysis.

[00288] The chromatographic quantitative analysis of (5-formylfuran-3-yl)methyl phosphate production was performed in a HPLC-DAD (Thermo Ultimate 3000) equipped with an Aminex HPX-87H Biorad column (300 x 7.8 mm). The column was maintained at 50°C and the mobile phase used was a 5 mM H_2SO_4 solution with flow rate of 0.75 mL/min (isocratic gradient mode).

[00289] As shown in Table 6, FIG. 5, and FIG. 6, G3P was successfully converted into (5-formylfuran-3-yl)methyl phosphate by methyl phosphate synthases. In FIG. 5, the negative control sample (grey line) shows a low absorbance at 280, indicating little to no presence of the (5-formylfuran-3-yl)methyl phosphate in comparison to the reaction containing the methyl phosphoate synthase (black line), indicating that the synthase catalyzed the formation of the (5-formylfuran-3-yl)methyl phosphate from G3P; these results are summarized in Table 6. FIG. 6 shows detectable (5-formylfuran-3-yl)methyl phosphate in the reaction containing synthase (Lower Panel) but not in the negative control reaction (Upper Panel). Table 7 contains a list of methyl phosphate synthase candidates that positively tested for the production of (5-formylfuran-3-yl)methyl phosphate from G3P.

[00290] **Table 6.** Absorbance obtained at 280 nm for methyl phosphate synthase production of (5-formylfuran-3-yl)methyl phosphate from G3P.

	Absorbance at 280 nm

Methyl phosphate synthase positive reaction (MfnB1 candidate)	2.97
Negative control	0.26

[00291] **Table 7**. (5-formylfuran-3-yl)methyl phosphate synthases candidates that positively tested for catalyzing the production of (5-formylfuran-3-yl)methyl phosphate production from G3P.

Name	**Organism**
MfnB 1	*Methanocaldococcus jannaschii*
MfnB 2	*Methanocaldococcus fervens*
MfnB 3	*Methanocaldococcus vulcanius*
MfnB 4	*Methanocaldococcus infernos*
MfnB 5	*Methanothermococcus okinawensis*
MfnB 6	*Methanococcales archaeon HHB*
MfnB 7	*Methanobrevibacter smithii*
MfnB 8	*Methanobacterium sp. PtaB.Bin024*
MfnB 9	*Methanopyrus sp. KOL6*
MfnB 10	*Candidatus Argoarchaeum ethanivorans*
MfnB 12	*Methanobrevibacter arboriphilus*
MfnB 13	*Methanococcus maripaludis*
MfnB 14	*Methanococcus vannielii*
MfnB 15	*Methanosarcina acetivorans*
MfnB 16	*Methanosarcina barkeri*
MfnB 17	*Methylorubrum extorquens*
MfnB 18	*Methylobacterium sp.*
MfnB 19	*Methanosarcina mazei*
MfnB 20	*Methyloversatilis universalis*
MfnB 22	*Streptomyces cattleya NRRL 8057*
MfnB 23	*Streptomyces coelicolor*
MfnB 24	*Streptomyces EFF88969*
MfnB 25	*Streptomyces griséus*
MfnB 26	*Streptomyces sp. DH-12*
MfnB 27	*Streptomyces venezuelae*

[00292] Example 3: Production of 4-hydroxymethylfurfural (4-HMF) from (5-formylfuran-3-yl)methyl phosphate

[00293] The production of 4-HMF from (5-formylfuran-3-yl)methyl phosphate using phosphatases was demonstrated using commercially available phosphatase, *E. coli* lysates, and yeast lysates to demonstrate their capability to produce 2,4-HMF from (5-formylfuran-3-yl)methyl phosphate. The substrate (5-formylfuran-3-yl)methyl phosphate was produced by (5-formylfuran-3-yl)methyl phosphate synthases as described at Example 2.

[00294] The chromatographic quantitative analysis of (5-formylfuran-3-yl)methyl phosphate and 4-HMF production was performed in a HPLC-DAD (Thermo Ultimate 3000) equipped with an Aminex HPX-87H Biorad column (300 x 7.8 mm). The column was maintained at 50°C and the mobile phase used was a 5 mM H_2SO_4 solution with flow rate of 0.75 mL/min (isocratic gradient mode). Both compounds were detected at 280 nm.

[00295] To carry out the reaction demonstrating the production of 4-HMF from (5-formylfuran-3-yl)methyl phosphate using a commercially available phosphatase, 2 µL of alkaline phosphatase from bovine intestinal mucosa (Sigma) was added to 1 mL of reaction vessel from Example 2, containing approximately 1-2 mM of (5-formylfuran-3-yl)methyl phosphate. The reaction was incubated at 37°C for 1h and initial and final samples were analyzed by HPLC-DAD. As shown in FIG. 7 (Upper Panel) and Table 8, the commercially available phosphatase was able to perform the full conversion of (5-formylfuran-3-yl)methyl phosphate to 4-HMF.

Table 8. Peak area of (5-formylfuran-3-yl)methyl phosphate produced using methyl phosphate synthase.

	Area (mAU*min)
Methyl phosphate synthase positive reaction	62,1544
Negative control	0

[00296] To carry out the reaction demonstrating the production of 4-HMF from (5-formylfuran-3-yl)methyl phosphate using phosphatases in an *E. coli* lysate, a strain of *E. coli* MG1655 was inoculated into 200 mL of LB broth at 37°C with agitation overnight. The culture was centrifuged at 4000 rpm for 15 min and the pellet suspended in 20 mL of 20 mM HEPES buffer pH 7.4 resulting in an OD of 70. The lysis was performed by ultrasonic disruption. 1 mL of the *E. coli* lysate was mixed with 1 mL of reaction from Example 2 and incubated overnight at 37°C with agitation. Samples were analyzed by HPLC-DAD at 280 nm for production of 4-HMF.

[00297] To carry out the reaction demonstrating the production of 4-HMF from (5-formylfuran-3-yl)methyl phosphate using phosphatases in a yeast lysate, a strain of

Saccharomyces cerevisiae was inoculated into 200 mL of YPD broth at 30°C with agitation overnight. The culture was centrifuged at 4000 rpm for 15 min and the pellet suspended in 20 mL of 20 mM HEPES buffer pH 7.4 resulting in an OD of 120. Cell lysis was performed by ultrasonic disruption. 1 mL of the yeast lysate was mixed with 1 mL of reaction from example 2 and incubated overnight at 30°C with agitation. Samples were analyzed by HPLC-DAD at 280 nm for production of 4-HMF.

[00298] As shown in FIG. 7 (Middle Panel) and FIG. 7 (Lower Panel) and Table 9, both *E. coli* and yeast lysates showed endogenous phosphatase activity able to perform the conversion of (5-formylfuran-3-yl)methyl phosphate to 4-HMF.

[00299] **Table 9.** 4-HMF production from (5-formylfuran-3-yl)methyl phosphate with commercially available phosphatase after 1 hour incubation and *E. coli* l and yeast lysates after overnight incubation at 37°C and 30°C,respectively.

Sample	**(5-formylfuran-3-yl)methyl phosphate area (mAU*min)**	**4-HMF area (mAU*min)**
Sigma phosphatase	n.a.	385,3242
E. coli lysate reaction	57,2574	5,6535
Yeast lysate reaction	187,9746	67,0542
E. coli negative control reaction (absence of 5-formylfuran-3-yl)methyl phosphate substrate)	n.a.	n.a.
Yeast negative control reaction (absence of 5-formylfuran-3-yl)methyl phosphate substrate)	22,1085	4,0767

[00300] **Example 4: Expression of 4-HMF oxidases enzymes**

[00301] Genes coding 7 4-HMF oxidases enzymes candidates (Table 4) were synthesized by GenScript and cloned in expression vector pET28a in NdeI and BamHI restriction sites. The expression vector was transformed into *E. coli* BL21 (DE3) and the transformant was stored in 15% glycerol until use for enzyme expression.

[00302] The stored transformant was inoculated into 50 mL of TB broth containing kanamycin at 37°C with agitation for 16h to prepare a seed culture. The seed culture was added to 300 mL of TB broth containing kanamycin with initial OD (600 nm) of 0.2, the culture was then

incubated at 37°C with agitation until OD (600 nm) reached 0.6-0.8 at which point 1 mM IPTG was added to induce expression overnight at 18°C with agitation.

[00303] Following overnight expression, the cells were centrifuged at 6000x rpm for 30 min, the cell pellet was suspended in cold lysis buffer (20 mM phosphate buffer and 500 mM NaCl pH 7.4) before ultrasonic disruption. The cell lysate was again centrifuged at 8000x rpm for 30 min at 4°C and filtered before purification with affinity chromatography. The column utilized was a HisTrap FF Crude (GE Healthcare) for the his-tagged protein purification. The purified protein was bound and washed in the column with binding buffer A (20 mM phosphate buffer, 20 mM imidazole, 500 mM NaCl, 1 mM PMSF and beta-mercaptoethanol, pH 7.4) and eluted in a gradient of elution buffer B (20 mM phosphate buffer, 500 mM imidazole, 500 mM NaCl, 1 mM PMSF and beta-mercaptoethanol, pH 7.4). Then, using a PD-10 column, the buffer was changed to a 50 mM Tris-HCl pH 7.4. Candidates expression and purification were analyzed on 12% polyacrylamide gel by electrophoresis, as illustrated at FIG. 8.

[00304] **Example 5: Production of 2,4-FDCA from 2,4-HMF by HmfH oxidases**

[00305] The 2,4-FDCA production from 2,4-HMF by enzyme candidates described in Table 4 was demonstrated *in vitro* by incubating approximately 100 µg of purified HmfH oxidase candidates with a 1 mL of reaction vessel from Example 3 (using the commercially available phosphatase), containing approximately 1 mM 4-HMF. The reaction was incubated at 30°C for 16 hours and both initial and final samples analyzed by HPLC-DAD. Samples were injected in HPLC-DAD and the production of 2,4-FDCA and its intermediates confirmed by GC-MS.

[00306] The quantitative analysis of 2,4-FDCA was performed using HPLC-DAD (Thermo Ultimate 3000) equipped with an Aminex HPX-87H Biorad column (300 x 7.8 mm). The column was maintained at 50 °C. The mobile phase used was a 5 mM H_2SO_4 solution with flow rate of 0.6 mL/min with isocratic gradient mode. The molecule was detected at 245 nm.

[00307] For GC-MS identification, initial and final samples were stopped by adding 6M HCl to reduce pH to 2-3. The products were liquid/liquid extracted using ethyl acetate and dried with Na_2SO_4 to remove water traces. The extracted material was then evaporated in a speedvac and derivatized using bis-(trimethylsilyl)trifluoroacetamide at 60°C for 2h. The samples were injected in a gas chromatograph with HP-5MS column (Agilent, 30 m x 0.25 mm ID, 0.25 um film thickness) coupled with a quadrupole mass detector (ISQ, Thermo). The oven program started at 110°C for 2 min with increasing ramp of 20°C/min until 300°C that was held for 3 min. Helium was used as carrier gas at a flow rate of 1.2 mL/min. 2,4-FDCA was identified by comparing their mass spectra with those in literature. (Ref: Carro, Juan, et al. "5-hydroxymethylfurfural conversion by fungal aryl-alcohol oxidase and unspecific peroxygenase." The FEBS journal 282.16 (2015): 3218-

3229.) 4-formylfuran-2-carboxylate (2,4-FFCA) and furan-2,4-dicarbaldehyde (2,4-DFF) were also identified by their mass spectra.

[00308] As shown in Table 10 and FIG. 9, FIG. 10, and FIG. 11, the conversion of 4-HMF into 2,4-FDCA was successfully demonstrated with 4-HMF oxidases, especially with enzyme HmfH7 that was able to fully convert 2,4-HMF into 2,4-FDCA.

[00309] FIG. 9 (Upper Panel) shows the chromatogram of HmfH1, FIG. 9 (Middle Panel) the chromatogram of HmfH6 and FIG. 9 (Lower Panel) the chromatogram of HmfH7. FIG. 10 (Upper Pannel) shows the relative abundance of the products obtained in GC-MS and the mass spectra (Lower Panel) of silylated 2,4-FDCA (FIG. 11).

Table 10. 2,4 FDCA production from 4-HMF with 4-HMF oxidases candidates after 16 hours incubation. The reaction intermediates 4-formylfuran-2-carboxylate (2,4-FFCA) and furan-2,4-dicarbaldehyde (2,4-DFF) were also identified and quantified[a].

Reaction Condition	**2,4-HMF area (mAU*min)**	**2,4-FDCA area (mAU*min)**	**2,4-FFCA area (mAU*min)**	**2,4-DFF area (mAu*min)**
negative control reaction	100	n.a.	n.a.	n.a.
HmfH1	n.a.	34,2154	42,5550	n.a.
HmfH6	1,4416	63,9778	8,1581	1,5700
HmfH7	n.a.	93,0784	n.a.	n.a.

[a] Negative control reaction was performed in similar assay condition but in absence of HMF-oxidase enzymes.

[00310] **Example 6: Production of 2,4-furandimethanol from 4-HMF – Reaction C**

[00311] Purified enzymes were produced as described at Example 1.

[00312] Production of 2,4-furandimethanol from 4-HMF by enzyme candidates described in Table 5 was demonstrated *in vitro* by incubating approximately 20 µg of purified enzyme candidates in 100 mM potassium phosphate buffer (pH 7) with 0.5 mM NAD(P)H or NADH. The reactions were started by the addition of 0.5 mM 4-HMF obtained as shown in Example 3. The decrease of NAD(P)H was monitored at 340 nm during 40 min at 37°C on a UV-Vis spectrophotometer (SpectraMax M5, Molecular Devices). Product formation was also confirmed by HPLC and GC-MS analysis (Data not shown). Reaction vessels without enzymes or substrate (4-HMF) were used as negative controls.

[00313] As demonstrated in FIG. 12, enzymes candidates DH1, DH2 and DH6 promoted reduction of 2,4-HMF to 2,4-furandimethanol, measured by its oxidation of NAD(P)H to NAD(P)+.

[00314] Example 7: Production of furan-2,4-dicarbaldehyde from 4-HMF – Reaction D

[00315] Purified enzymes were produced as described in Example 1. The furan-2,4-dicarbaldehyde production from 4-HMF by enzyme candidates described at Table 3 was demonstrated in vitro by incubating approximately 20 µg of purified enzyme candidates in 100 mM potassium phosphate buffer (pH 7) with 0.5 mM NAD(P)+ or NAD+. The reactions started by the addition of 0.5 mM 4-HMF obtained as shown in Example 3. The increase of NAD(P)H was monitored at 340 nm during 40 min at 37°C on a UV-Vis spectrophotometer (SpectraMax M5, Molecular Devices). Product formation was also confirmed by HPLC and GC-MS analysis (Data not shown). Reaction vessels without enzymes or substrate (4-HMF) were used as negative controls.

[00316] As demonstrated for enzyme DH2, selected dehydrogenases are able to oxidate 4-HMF to furan-2,4-dicarbaldehyde *in vitro*. The data shown in FIG. 13 was plotted after subtraction of the baseline signal and highlights the absorbance increase and consequently the reduction of NAD(P)H and oxidation of 2,4-HMF to furan-2,4-dicarbaldehyde when using the enzyme DH2.

[00317] Example 8: Production of 4-(hydroxymethyl)furoic acid from 2,4-HMF – Reaction E

[00318] Purified aldehyde dehydrogenase enzymes were produced as described at Example 1. The 4-(hydroxymethyl)furoic acid production from 4-HMF by aldehyde dehydrogenase candidates described at Table 3 was demonstrated *in vitro* by incubating approximately 20 µg of purified enzyme candidates in 100 mM potassium phosphate buffer (pH 7) with 0.5 mM NAD(P)+ or NAD+. The reactions started by the addition of 0.5 mM 4-HMF obtained as shown in example 3. The increase of NAD(P)H was monitored at 340 nm during 40 min at 37 °C on a UV-Vis spectrophotometer (SpectraMax M5, Molecular Devices). Product formation was also confirmed by HPLC and GC-MS analysis (Data not shown). Reaction vessels without enzymes or substrate (4-HMF) were used as negative controls.

[00319] As representatively demonstrated for enzymes DH8, DH9, DH10 and DH11, selected aldehyde dehydrogenases are able to oxidate 4-HMF to 4-(hydroxymethyl)furoic acid in vitro (FIG. 14). The data shown in FIG. 14 was plotted after subtraction of the baseline signal and highlights the absorbance increase and consequently the reduction of NAD(P)H and oxidation of 2,4-HMF to 4-(hydroxymethyl)furoic acid when using the respected aldehyde dehydrogenases.

[00320] Example 9: One pot reaction for the production of 2,4-FDCA from 4-HMF

[00321] Purified aldehyde dehydrogenase enzymes and alcohol dehydrogenase enzymes were produced as described at Example 1. The one pot oxidative reaction for 2,4-FDCA production from 4- HMF was performed using DH8 as the representative aldehyde dehydrogenase and DH6 as the representative alcohol dehydrogenase.

[00322] To carry out the reaction, 2 mL of a reaction mixture from Example 3 containing 0.5 mM of 2,4-HMF and 1mM of NAD(P)H were added 20 uM of purified enzyme candidates DH8 and DH6. Positive control reactions were prepared as shown in Table 11. Two negative controls were prepared one without the enzymes and another one without the substrate. The reaction was incubated at 30°C for 16 hours and both initial and final samples analyzed by HPLC-DAD. Samples were injected in HPLC-DAD and the production of 2,4-FDCA was confirmed in GC-MS using the following method.

[00323] The quantitative analysis of 2,4-FDCA was performed using HPLC-DAD (Thermo Ultimate 3000) equipped with an Aminex HPX-87H Biorad column (300 x 7.8 mm). The column was maintained at 50°C. The mobile phase used was a 5 mM H_2SO_4 solution with flow rate of 0.6 mL/min with isocratic gradient mode. The molecule was detected at 245 nm.

[00324] For GC-MS identification, initial and final samples were stopped by adding 6M HCl to reduce pH to 2-3. The products were liquid/liquid extracted using ethyl acetate and dried with Na_2SO_4 to remove water traces. The extracted material was then evaporated in a speedvac and derivatized using bis-(trimethylsilyl)trifluoroacetamide at 60°C for 2h. The samples were injected in a gas chromatograph with HP-5MS column (Agilent, 30 m x 0.25 mm ID, 0.25 um film thickness) coupled with a quadrupole mass detector (ISQ, Thermo). The oven program started at 110°C for 2 min with increasing ramp of 20°C/min until 300°C that was hold for 3 min. Helium was used as carrier gas at a flow rate of 1.2 mL/min. 2,4-FDCA was identified by comparing their mass spectra with those in literature. (Ref: Carro, Juan, et al. "5-hydroxymethylfurfural conversion by fungal aryl-alcohol oxidase and unspecific peroxygenase." The FEBS journal 282.16 (2015): 3218-3229.) 4-formylfuran-2-carboxylate (2,4-FFCA) and furan-2,4-dicarbaldehyde (2,4-DFF) were also identified by their mass spectra.

[00325] As shown in Table 11 and FIG. 15 (Middle Panel), the conversion of 4-HMF into 2,4-FDCA was successfully demonstrated with the synergic action/combination of an aldehyde dehydrogenase (DH8) and an alcohol dehydrogenase (DH6).

[00326] Table 11. 2,4 FDCA production (2,4 FDCA peak area) from 4-HMF by the synergic action/combination of an aldehyde dehydrogenase (DH8) and an alcohol dehydrogenase (DH6) after 16 hours incubation.

Reaction Condition	**2,4-FDCA area**

	(mAU*min)
Positive reaction with enzymes DH8+DH6	26,1823
Negative control - No enzyme	0,3357
Negative control - No substrate	n.a.

Example 10. *In vivo* production of 2,4-FDCA from glucose

[00327] A plasmid containing the MfnB 1 gene (Table 1) under the control of the OXB20 promoter was constructed in a pET28a backbone. A second plasmid containing two 4-HMF oxidase genes (HmfH6 and HmfH7 (Table 4)) under the control of OXB20 promoter was constructed in a pZS*13 backbone. The plasmids were constructed using In-fusion commercial kit and were confirmed by sequencing. An E. coli K12 strain MG1655 (F-, λ-, rph-1, ilvG-, rfb-50, ΔgapA::gapN (UniProtKB - Q59931), ΔglcDEFGB, ΔaraFGH, ΔxylFGH, ΔfucO was used as production host.

[00328] The *in vivo* production of 2,4-FDCA from glucose was evaluated in shake flask fermentations in triplicate, using a defined media composed by 2.2 $g.L^{-1}$ KH_2PO_4, 9.4 $g.L^{-1}$ K_2HPO_4, 1.3 $g.L^{-1}$ $(NH_4)_2SO_4$, 10 $mg.L^{-1}$ thiamine, 320 $mg.L^{-1}$ EDTA-NaOH, 2 $mg.L^{-1}$ $CoCl_2.6H_2O$, 10 $mg.L^{-1}$ $MnSO_4.H_2O$, 5 $mg.L^{-1}$ $CuSO_4.5H_2O$, 2$mg.L^{-1}$ H_3BO_3, 2$mg.L^{-1}$ $Na_2MoO_4.2H_2O$, 54 $mg.L^{-1}$ $ZnSO_4.7H_2O$, 1 $mg.L^{-1}$ $NiSO_4.6H_2O$, 100 $mg.L^{-1}$ citrate Fe (III), 100 $mg.L^{-1}$ $CaCl_2.2H_2O$, 0.3 $g.L^{-1}$ $MgSO_4.H_2O$. Carbon source was provided by 10 g/L glucose and nitrogen sulphate was used as nitrogen source. Erlenmeyer flasks were inoculated with the recombinant strain to an initial OD of 0.1, and incubated at 37°C, 225rpm for 48 hours. Analysis of supernatant in 48h by HPLC indicated the production of 14 ± 2 mg/L 2,4-FDCA (FIG. 16).

CLAIMS

1. A method of producing 2,4-furandicarboxylic acid (2,4-FDCA) by enzymatically converting glyceraldehyde 3-phosphate (G3P) to 2,4-furandicarboxylic acid (2,4-FDCA), the method comprising:

(a) providing G3P in the presence of a methyl phosphate synthase that catalyzes the conversion of G3P to (5-formylfuran-3-yl)methyl phosphate;

(b) providing the (5-formylfuran-3-yl)methyl phosphate from (a) a phosphatase that catalyzes the conversion of the (5-formylfuran-3-yl)methyl phosphate to 4-hydroxymethylfurfural (4-HMF);

(c) providing the 4-HMF from (b) to a dehydrogenase and/or an oxidase that catalyzes independently or in synergy the oxidation of 4-HMF from (b) to 2,4 FDCA, directly or through the production of intermediates furan-2,4-dicarbaldehyde, 4-(hydroxymethyl)furoic acid, 4-formylfuran-2-carboxylate, 4-formylfuran-2-carboxylate, 2-formylfuran-4-carboxylate.

2. The method of claim 1, wherein the 2,4-FDCA is produced from furan-2,4-dicarbaldehyde, and/or-(hydroxymethyl)furoic acid intermediates, wherein:

(a) a dehydrogenase, an oxidase, or a peroxigenase catalyzes the conversion of the 4-HMF to furan-2,4-dicarbaldehyde, and/or 4-(hydroxymethyl)furoic acid; and/or

(b) a dehydrogenase, an oxidase, or a peroxigenase catalyzes the conversion of the furan-2,4-dicarbaldehyde from (a) to 4-formylfuran-2-carboxylate; and/or

(c) a dehydrogenase, an oxidase, or a peroxigenase catalyzes the conversion of the 4-(hydroxymethyl)furoic acid from (a) to 4-formylfuran-2-carboxylate; and/or

(d) a dehydrogenase, an oxidase, or a peroxigenase catalyzes the conversion of the furan-2,4-dicarbaldehyde from (a) to 2-formylfuran-4-carboxylate; and or

(e) a dehydrogenase, an oxidase, or a peroxigenase catalyzes the conversion of the the 4-formylfuran-2-carboxylate from (b) and/or (c) or the 2-formylfuran-4-carboxylate from (d) to 2,4-FDCA.

3. The method of claim 1, wherein the methyl phosphate synthase from (a) is classified as EC number 4.2.3.153.

4.The method of claim 3, wherein the methyl phosphate synthase is (5-formylfuran-3-yl)methyl phosphate synthase.

5. The method of claim 4, wherein the (5-formylfuran-3-yl)methyl phosphate synthase is selected from MfnB1, MfnB7, and MfnB14.

6. The method of claim 5, wherein the (5-formylfuran-3-yl)methyl phosphate synthase comprises an amino acid sequence comprising SEQ ID NO: 1, SEQ ID NO: 7, or SEQ ID NO: 14.

7. The method of claim 1, wherein the phosphatase from (b) is classified as EC number 3.1.3.

8. The method of claim 5, wherein the phosphatase is classified as a haloacid dehalogenase.

9. The method of claim 1, wherein the phosphatase is endogenous to the host.

10. The method of claim 11, wherein the phosphatase enzyme endogenous to the host is overexpressed.

11. The method of any one of claims 5-8, wherein the phosphatase is a 4-HMF phosphatase.

12. The method of claim 11, wherein the 4-HMF phosphatase is derived from Streptomyces coelicolor, Saccharomyces cerevisiae, or Escherichia coli.

13. The method of claim 11 or 12, wherein the 4-HMF phosphatase is encoded by an amino acid sequence comprising SEQ ID NO: 28, any one of SEQ ID NOs 40-52, or any one of SEQ ID NOs 53-68.

14. The method of claim 1, wherein the dehydrogenase from (c) is classified as EC number 1.1.1. or EC number 1.2.1.

15. The method of claim 14, wherein the dehydrogenase is an alcohol dehydrogenase or an aldehyde dehydrogenase.

16. The method of claim 1, wherein the oxidase from (c) is classified as EC number 1.1.3.

17. The method of claim 16, wherein the oxidase is 5-hydroxymethylfurfural oxidase.

18. The method of claim 2, wherein the dehydrogenase is classified as EC number 1.2.1. or EC number 1.1.1.

19. The method of claim 18, wherein the dehydrogenase is an aldehyde dehydrogenase or and alcohol dehydrogenase.

20. The method of claim 2, wherein the oxidase is classified as EC number 1.1.3.

21. The method of claim 20, wherein the oxidase is 5-hydroxymethylfurfural oxidase.

22. The method of claim 1, wherein the oxidase is a 4-HMF oxidase.

23. The method of claim 22, wherein the 4-HMF oxidase is selected from HmfH6 and HmfH7.

24. The method of claim 23, wherein the 4-HMF oxidase comprises an amino acid sequence comprising SEQ ID NO: 85 or SEQ ID NO: 86.

25. The method of claim 2, wherein the dehydrogenase is classified as EC number 1.2.1.

26. The method of claim 25, wherein the dehydrogenase is an aldehyde dehydrogenase.

27. A recombinant microorganism capable of producing 2,4-furandicarboxylic acid (2,4-FDCA) from a feedstock comprising a carbon source, wherein the recombinant microorganism expresses the following:

(a) endogenous and/or exogenous nucleic acid molecules capable of converting a carbon source to glyceraldehyde 3-phosphate (G3P);
(b) at least one endogenous or exogenous nucleic acid molecule encoding a (5-formylfuran-3-yl)methyl phosphate synthase that catalyzes the conversion of G3P from (a) to (5-formylfuran-3-yl)methyl phosphate;
(c)at least one endogenous or exogenous nucleic acid molecule encoding a phosphatase that catalyzes the conversion of (5-formylfuran-3-yl)methyl phosphate from (b) to 4-hydroxymethylfurfural (4-HMF);
(d) at least one endogenous or exogenous nucleic acid molecule encoding a peroxigenase, dehydrogenase, or a oxidase that catalyzes independently or in synergy the oxidation of 4-HMF from (c) to 2,4 FDCA, directly or through the production of intermediates furan-2,4-dicarbaldehyde, 4-(hydroxymethyl)furoic acid, 4-formylfuran-2-carboxylate, 4-formylfuran-2-carboxylate, 2-formylfuran-4-carboxylate.

28. The recombinant microorganism of claim 27, wherein the carbon source comprises a hexose, a pentose, glycerol, C02, sucroses and/or combinations thereof.

29. The recombinant microorganism of claim 27, wherein the methyl phosphate synthase from (a) is classified as EC number 4.2.3.153.

30. The recombinant microorganism of claim 29, wherein the synthase is (5-formylfuran-3-yl)methyl phosphate synthase.

31. The recombinant microorganism of claim 27, wherein the phosphatase from (c) is classified as EC number 3.1.3.

32. The recombinant microorganism of claim 31, wherein the phosphatase is classified as haloacid dehalogenase.

33. The recombinant microorganism of claim 31, wherein the phosphatase is endogenous to the host.

34. The recombinant microorganism of claim 33, wherein phosphatase enzyme endogenous to the host is overexpressed.

35. The recombinant microorganism of claim 27, wherein the oxidase from (d) is classified as EC number 1.1.3.

36. The recombinant microorganism of claim 35, wherein the oxidase from (d) is a 5-hydroxymethylfurfural oxidase

37. The recombinant microorganism of claim 36, wherein the dehydrogenase from (d) is classified as EC number 1.1.1. or EC number 1.2.1.

38. The recombinant microorganism of claim 37, wherein the dehydrogenase is an alcohol dehydrogenase or an aldehyde dehydrogenase.

39. The recombinant microorganism of claim 27, wherein the 2,4-FDCA is produced from furan-2,4-dicarbaldehyde, and/or-(hydroxymethyl)furoic acid intermediates,wherein:
(a) a dehydrogenase, an oxidase, or a peroxigenase catalyzes the conversion of the 4-HMF from (c) to furan-2,4-dicarbaldehyde, and/or 4-(hydroxymethyl)furoic acid; and/or

(b) a dehydrogenase, an oxidase, or a peroxigenase catalyzes the conversion of the furan-2,4-dicarbaldehyde from (a) to 4-formylfuran-2-carboxylate; and/or

(c) a dehydrogenase, an oxidase, or a peroxigenase catalyzes the conversion of the 4-(hydroxymethyl)furoic acid from (b) to 4-formylfuran-2-carboxylate; and/or

(d) a dehydrogenase, an oxidase, or a peroxigenase catalyzes the conversion of the furan-2,4-dicarbaldehyde from (c) to 2-formylfuran-4-carboxylate; and or

(e) a dehydrogenase, an oxidase, or a peroxigenase catalyzes the conversion of the 4-formylfuran-2-carboxylate from (b) and/or (c) or the 2-formylfuran-4-carboxylate from (d) to 2,4-FDCA.

40. The recombinant microorganism of claim 39, wherein the dehydrogenase from (a), (b), (c), (d) and/or (e) is classified as EC number 1.2.1. or EC number 1.1.1

41. The recombinant microorganism of claim 40, wherein the dehydrogenase is an aldehyde dehydrogenase or an alcohol dehydrogenase.

42. The recombinant microorganism of claim 39, wherein the oxidase from (a), (b), (c), (d) and/or (e) is classified as EC number 1.1.3.

43. The recombinant microorganism of claim 42, wherein the oxidase is 5-(hydroxymethyl)furfural oxidase.

44. The recombinant microorganism of claim 27, wherein the one or more recombinant microorganisms are derived from a parental microorganism selected from the group consisting of Clostridium sp., Clostridium ljungdahlii, Clostridium autoethanogenum, Clostridium ragsdalei, Eubacterium limosum, Butyribacterium methylotrophicum, Moorella thermoacetica, Corynebacterium glutamicum, Clostridium aceticum, Acetobacterium woodii, Alkalibaculum bacchii, Clostridium drakei, Clostridium carboxidivorans, Clostridium formicoaceticum, Clostridium scatologenes, Moorella thermoautotrophica, Acetonema longum, Blautia producta, Clostridium glycolicum, Clostridium magnum, Candida krusei, Clostridium mayombei, Clostridium methoxybenzovorans, Clostridium acetobutylicum, Clostridium beijerinckii, Oxobacter pfennigii, Thermoanaerobacter kivui, Sporomusa ovata, Thermoacetogenium phaeum, Acetobacterium carbinolicum, Issatchenkia orientalis, Sporomusa termitida, Moorella glycerini, Eubacterium aggregans, Treponema azotonutricium, Pichia kudriavzevii, Escherichia coli, Saccharomyces cerevisiae, Pseudomonas putida, Bacillus sp, Corynebacterium sp., Yarrowia lipolytica, Scheffersomyces stipitis, and Terrisporobacter glycolicus.

45. The recombinant microorganism of claim 44, wherein the one or more recombinant microorganisms are derived from a parental microorganism selected from the group consisting of Clostridium sp., Clostridium ljungdahlii, Clostridium autoethanogenum, Clostridium ragsdalei, Eubacterium limosum, Butyribacterium methylotrophicum, Moorella thermoacetica,

Corynebacterium glutamicum, Clostridium aceticum, Acetobacterium woodii, Alkalibaculum bacchii, Clostridium drakei, Clostridium carboxidivorans, Clostridium formicoaceticum, Clostridium scatologenes, Moorella thermoautotrophica, Acetonema longum, Blautia producta, Clostridium glycolicum, Clostridium magnum, Candida krusei, Clostridium mayombei, Clostridium methoxybenzovorans, Clostridium acetobutylicum, Clostridium beijerinckii, Oxobacter pfennigii, Thermoanaerobacter kivui, Sporomusa ovata, Thermoacetogenium phaeum, Acetobacterium carbinolicum, Issatchenkia orientalis, Sporomusa termitida, Moorella glycerini, Eubacterium aggregans, Treponema azotonutricium, Pichia kudriavzevii, Escherichia coli, Saccharomyces cerevisiae, Pseudomonas putida, Bacillus sp, Corynebacterium sp., Yarrowia lipolytica, Scheffersomyces stipitis, and Terrisporobacter glycolicus.

46. A method of producing 2,4-FDCA using a recombinant microorganism of claim 27, the method comprising cultivating the recombinant microorganism in a culture medium containing a feedstock providing a carbon source until the 2,4-FDCA is produced.

47. A method of producing a recombinant microorganism capable of producing 2,4-FDCA from a feedstock comprising a carbon source, the method comprising introducing into and/or overexpressing in the recombinant microorganism the following:

(a) endogenous and/or exogenous nucleic acid molecules capable of converting glycerol or a monosaccharide to glyceraldehyde 3-phosphate (G3P);

(b) at least one endogenous or exogenous nucleic acid molecule encoding a (5-formylfuran-3-yl)methyl phosphate synthase that catalyzes the conversion of G3P from (a) to (5-formylfuran-3-yl)methyl phosphate;

(c) at least one endogenous or exogenous nucleic acid molecule encoding a phosphatase that catalyzes the conversion of (5-formylfuran-3-yl)methyl phosphate from (b) to 4-hydroxymethylfurfural (4-HMF);

(d) at least one endogenous or exogenous nucleic acid molecule encoding a peroxigenase, dehydrogenase, or a oxidase that catalyzes independently or in synergy the oxidation of 4-HMF from (c) to 2,4 FDCA, directly or through the production of intermediates furan-2,4-dicarbaldehyde, 4-(hydroxymethyl)furoic acid, 4-formylfuran-2-carboxylate, 4-formylfuran-2-carboxylate, 2-formylfuran-4-carboxylate.

48. The method of claim 47, wherein the carbon source comprises a hexose, a pentose, glycerol, C02, sucroses and/or combinations thereof.

49. The method of claim 47, wherein the methyl phosphate synthase from (a) is classified as EC number 4.2.3.153.

50. The method of claim 49, wherein the synthase is (5-formylfuran-3-yl)methyl phosphate synthase

51. The method of claim 47, wherein the phosphatase from (c) is classified as EC number 3.1.3.

52. The method of claim 51, wherein the phosphatase is classified as haloacid dehalogenase.

53. The method of claim 47, wherein the phosphatase is endogenous to the host.

54. The method of claim 53, wherein phosphatase enzyme endogenous to the host is overexpressed.

55. The method of claim 39 wherein the dehydrogenase from (d) is classified as EC number 1.1.1. or EC number 1.2.1.

56. The method of claim 47, wherein the dehydrogenase is an alcohol dehydrogenase or an aldehyde dehydrogenase.

57. The method of claim 39, wherein the oxidase from (d) is classified as EC number 1.1.3.

58. The method of claim 49, wherein the oxidase is (5-(hydroxymethyl)furfural oxidase.

59. The method of claim 39, wherein the 2,4-FDCA is produced from furan-2,4-dicarbaldehyde, and/or-(hydroxymethyl)furoic acid intermediates,wherein:

(a) a dehydrogenase, an oxidase, or a peroxigenase catalyzes the conversion of the 4-HMF to furan-2,4-dicarbaldehyde, and/or 4-(hydroxymethyl)furoic acid; and/or

(b) a dehydrogenase, an oxidase, or a peroxigenase catalyzes the conversion of the furan-2,4-dicarbaldehyde from (a) to 4-formylfuran-2-carboxylate; and/or

(c) a dehydrogenase, an oxidase, or a peroxigenase catalyzes the conversion of the 4-(hydroxymethyl)furoic acid from (a) to 4-formylfuran-2-carboxylate; and/or

(d) a dehydrogenase, an oxidase, or a peroxigenase catalyzes the conversion of the furan-2,4-dicarbaldehyde from (a) to 2-formylfuran-4-carboxylate; and or

(e) a dehydrogenase, an oxidase, or a peroxigenase catalyzes the conversion of the the 4-formylfuran-2-carboxylate from (b) and/or (c) or the 2-formylfuran-4-carboxylate from (d) to 2,4-FDCA.

60. The method of claim 59, wherein the dehydrogenase from (a), (b), (c), (d) and/or (e) is classified as EC number 1.2.1. or EC number 1.1.1

61. The method of claim 60, wherein the dehydrogenase is an aldehyde dehydrogenase or an alcohol dehydrogenase.

62. The method of claim 59, wherein the oxidase from (a), (b), (c), (d) and/or € is classified as EC number 1.1.3.

63. The method of claim 62, wherein the oxidase is 5-(hydroxymethyl)furfural oxidase.

64. A 2,4-FDCA produced according to the method of any one of claims 1-26 or 46-63.

65. A 2,4-FDCA produced according to the recombinant microorganism of any one of claims 27-45 or 66-73.

66. A polymer produced from the 2,4-FDCA of claim 64 or 65.

67. The polymer of claim 66, wherein the polymer from 2,4-FDCA is formed in a non-biological process.

68. A recombinant microorganism capable of producing 4-hydroxymethylfurfural (4-HMF) from a feedstock comprising an exogenous carbon source, wherein the recombinant microorganism expresses the following:

(a) endogenous and/or exogenous nucleic acid molecules capable of converting the carbon source to glyceraldehyde 3-phosphate (G3P);
(b) at least one endogenous or exogenous nucleic acid molecule encoding a (5-formylfuran-3-yl)methyl phosphate synthase that catalyzes the conversion of G3P from (a) to (5-formylfuran-3-yl)methyl phosphate; and
(c) at least one endogenous or exogenous nucleic acid molecule encoding a phosphatase that catalyzes the conversion of (5-formylfuran-3-yl)methyl phosphate from (b) to 4-hydroxymethylfurfural (4-HMF).

69. The recombinant microorganism of claim 68, wherein the methyl phosphate synthase from (a) is classified as EC number 4.2.3.153.

70. The recombinant microorganism of claim 69, wherein the synthase is (5-formylfuran-3-yl)methyl phosphate synthase.

71. The recombinant microorganism of claim 68, wherein the phosphatase from (c) is classified as EC number 3.1.3.

72. The recombinant microorganism of claim 71, wherein the phosphatase is classified as haloacid dehalogenase.

73. The recombinant microorganism of claim 68, wherein the phosphatase is endogenous to the host.

74. The recombinant microorganism of claim 73, wherein the phosphatase enzyme endogenous to the host is overexpressed.

75. A recombinant microorganism capable of producing 2,4-furandicarboxylic acid (2,4-FDCA) from a feedstock comprising a carbon source, wherein the recombinant microorganism expresses one or more of the following:
(a) endogenous and/or exogenous nucleic acid molecules capable of converting glycerol or a monosaccharide to glyceraldehyde 3-phosphate (G3P);

(b) at least one endogenous or exogenous nucleic acid molecule encoding a (5-formylfuran-3-yl)methyl phosphate synthase that catalyzes the conversion of G3P from (a) to (5-formylfuran-3-yl)methyl phosphate;

(c) at least one endogenous or exogenous nucleic acid molecule encoding a phosphatase that catalyzes the conversion of (5-formylfuran-3-yl)methyl phosphate from (b) to 4-hydroxymethylfurfural (4-HMF);

(d) at least one endogenous or exogenous nucleic acid molecule encoding a dehydrogenases and/or an oxidase that catalyzes independently or in synergy the oxidation of 4-HMF from (b) to 2,4 FDCA, directly or through the production of intermediates furan-2,4-dicarbaldehyde, 4-(hydroxymethyl)furoic acid, 4-formylfuran-2-carboxylate, 4-formylfuran-2-carboxylate, 2-formylfuran-4-carboxylate.

FIG. 1

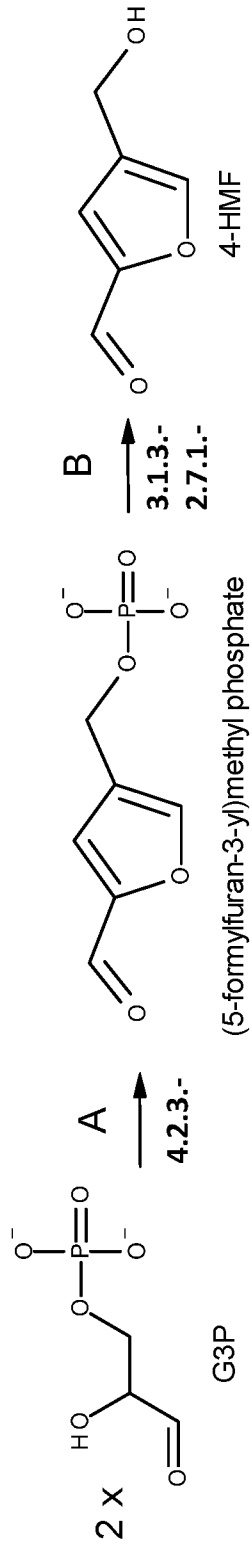

2 x
G3P
A
4.2.3.-
(5-formylfuran-3-yl)methyl phosphate
B
3.1.3.-
2.7.1.-
4-HMF

FIG. 2

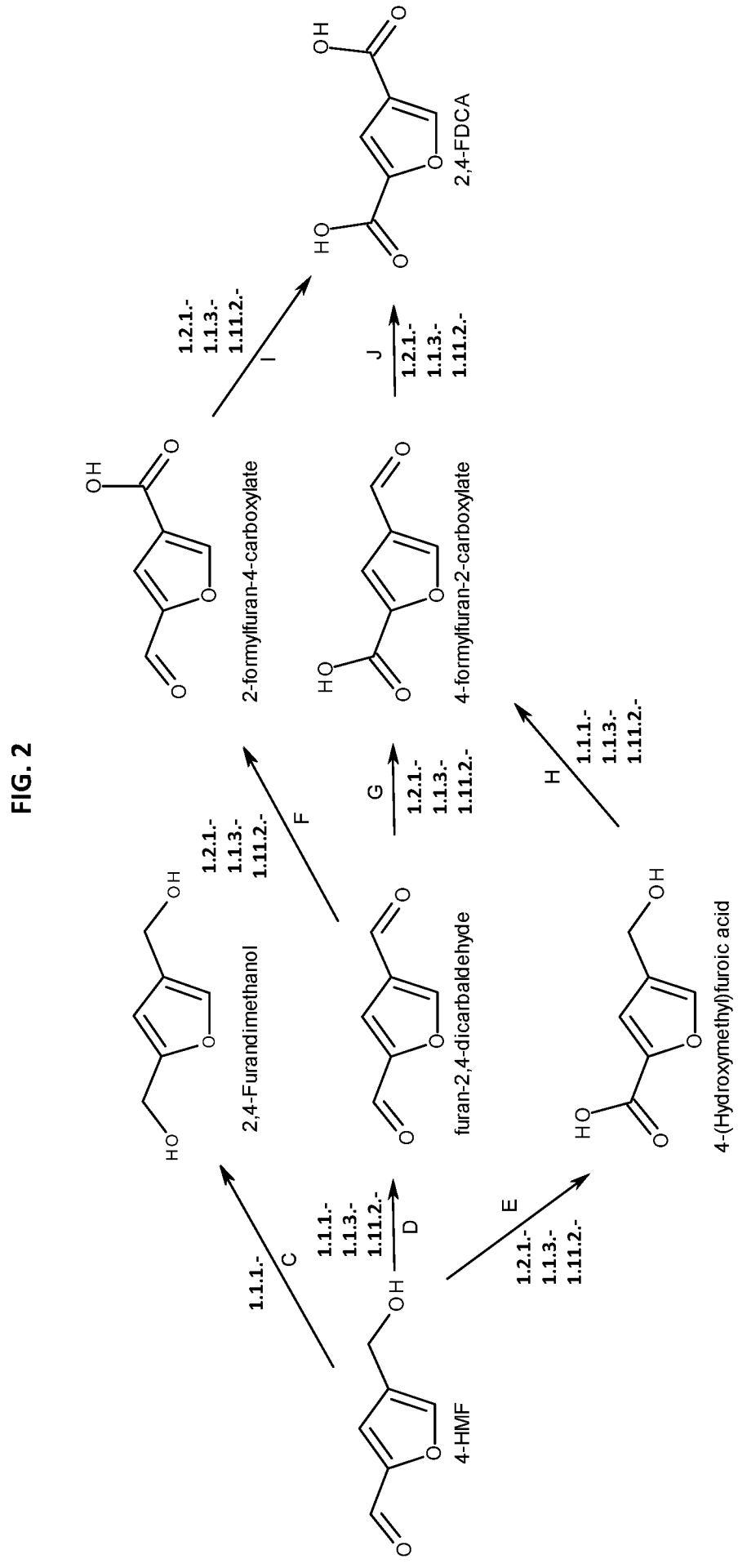

4-HMF
2,4-Furandimethanol
furan-2,4-dicarbaldehyde
4-(Hydroxymethyl)furoic acid
2-formylfuran-4-carboxylate
4-formylfuran-2-carboxylate
2,4-FDCA
1.1.1.-
1.1.3.-
1.11.2.-
C
D
E
F
G
H
I
J
1.2.1.-
1.1.3.-
1.11.2.-

FIG. 3

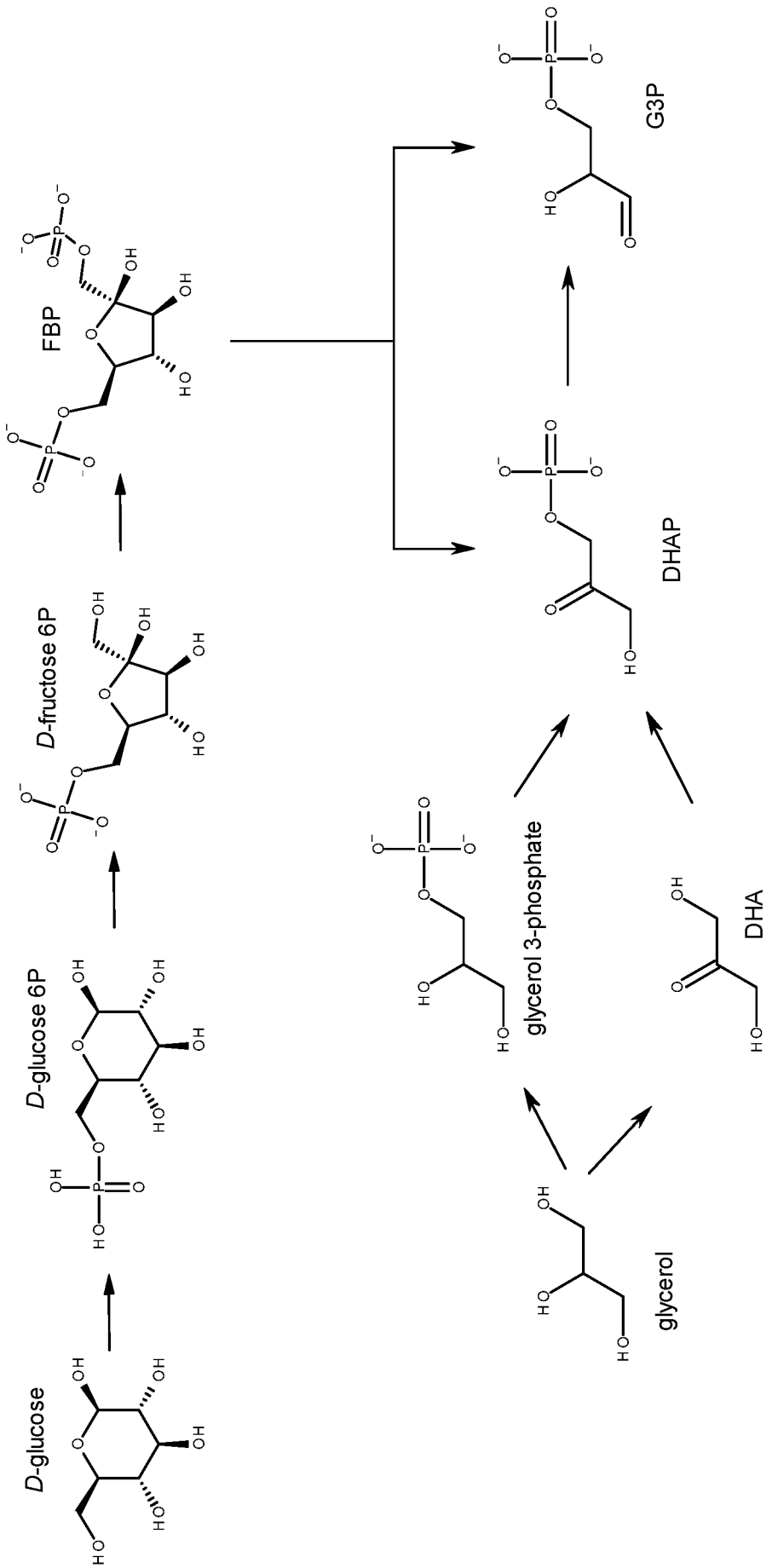

D-glucose
D-glucose 6P
D-fructose 6P
FBP
G3P
DHAP
glycerol 3-phosphate
glycerol
DHA

FIG. 4

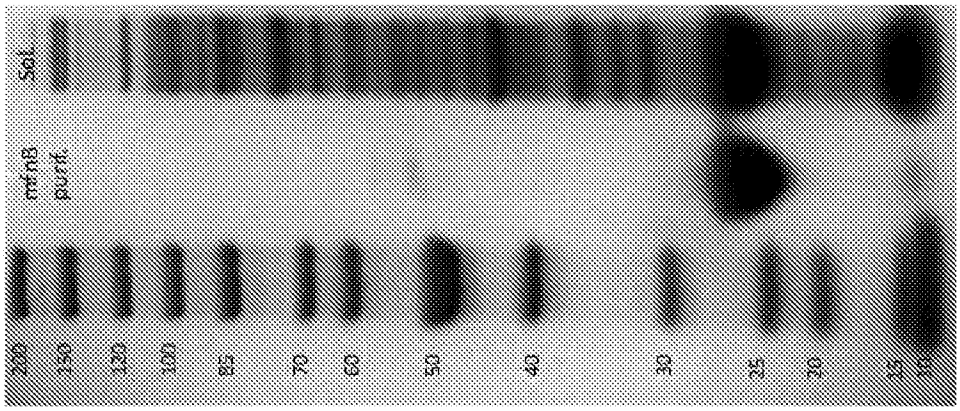


FIG. 5

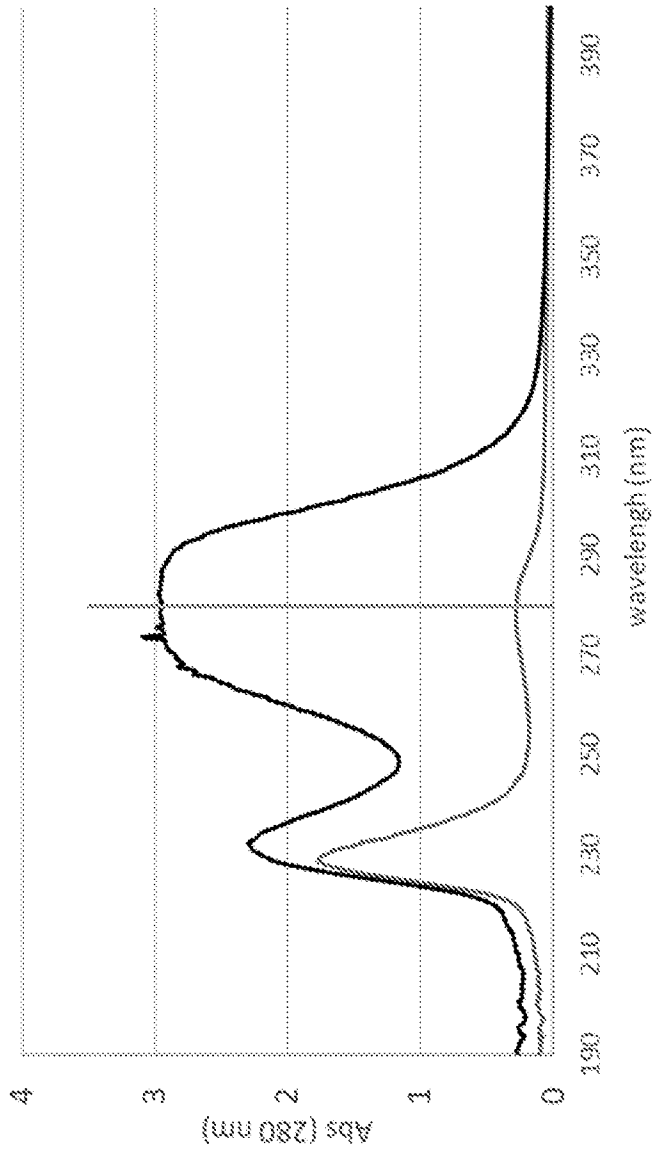

Abs (280 nm)
0
1
2
3
4
190
210
230
250
270
290
310
330
350
370
390
wavelength (nm)

FIG. 6

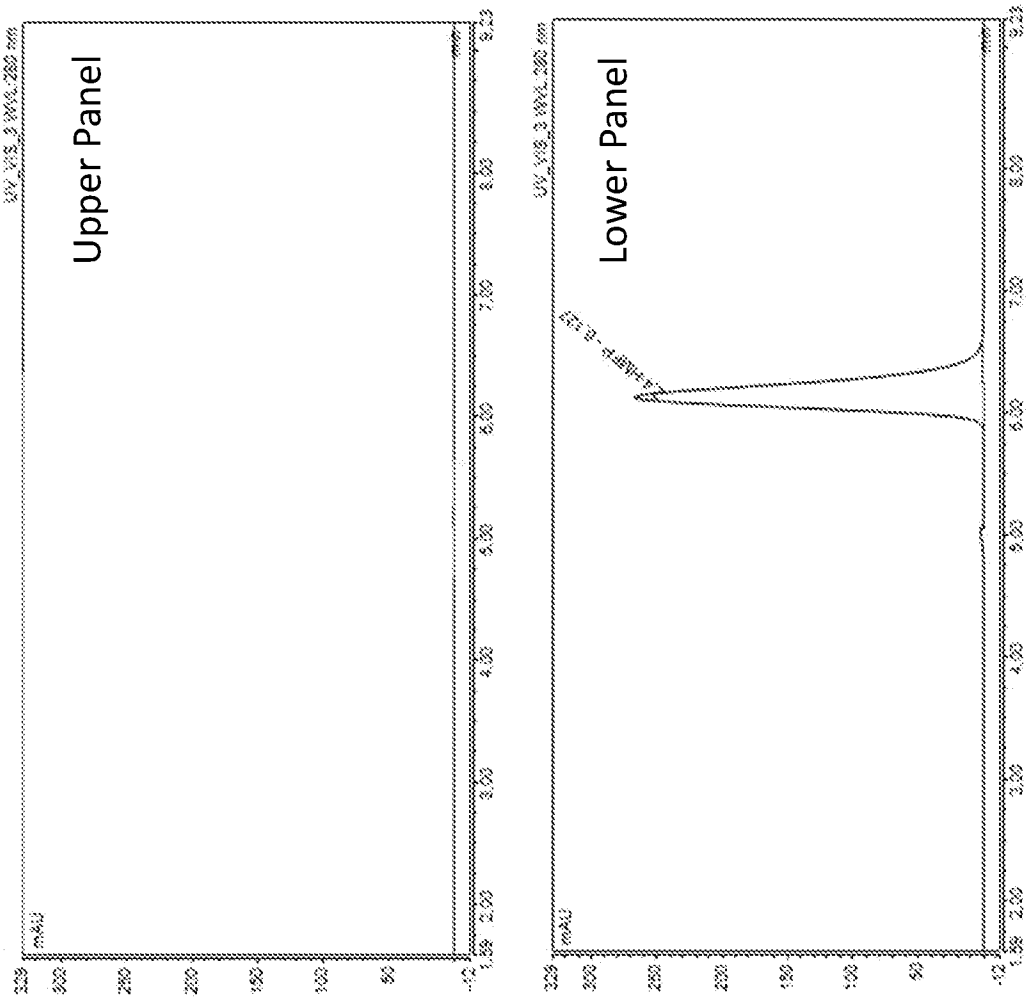


FIG. 7

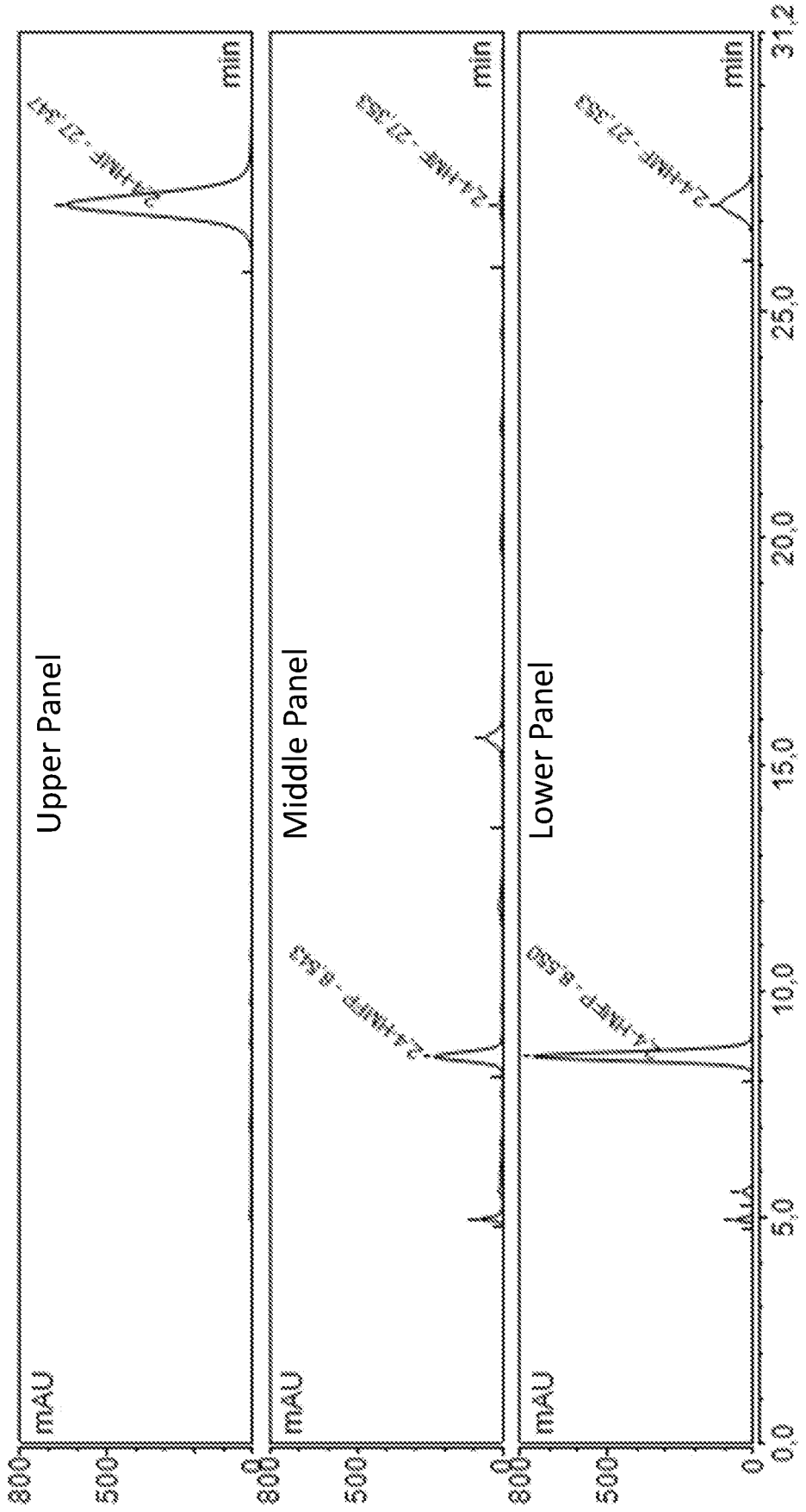

Upper Panel
Middle Panel
Lower Panel
mAU
min
0,0
5,0
10,0
15,0
20,0
25,0
31,2
800
500
0

FIG. 8

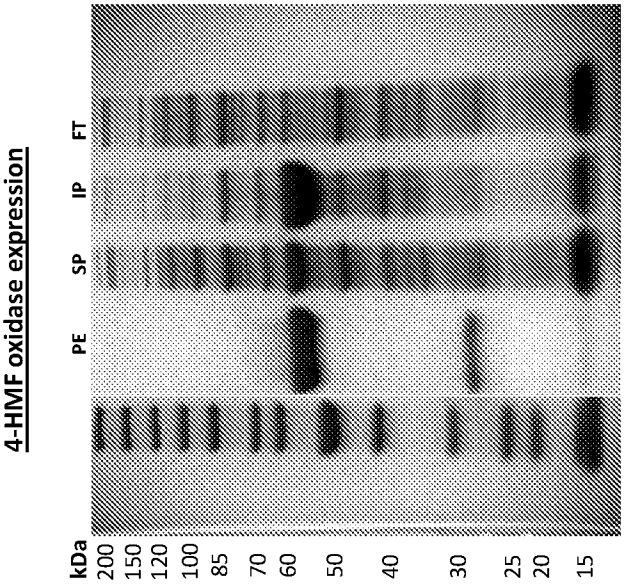

4-HMF oxidase expression
PE
SP
IP
FT
kDa
200
150
120
100
85
70
60
50
40
30
25
20
15

FIG. 9

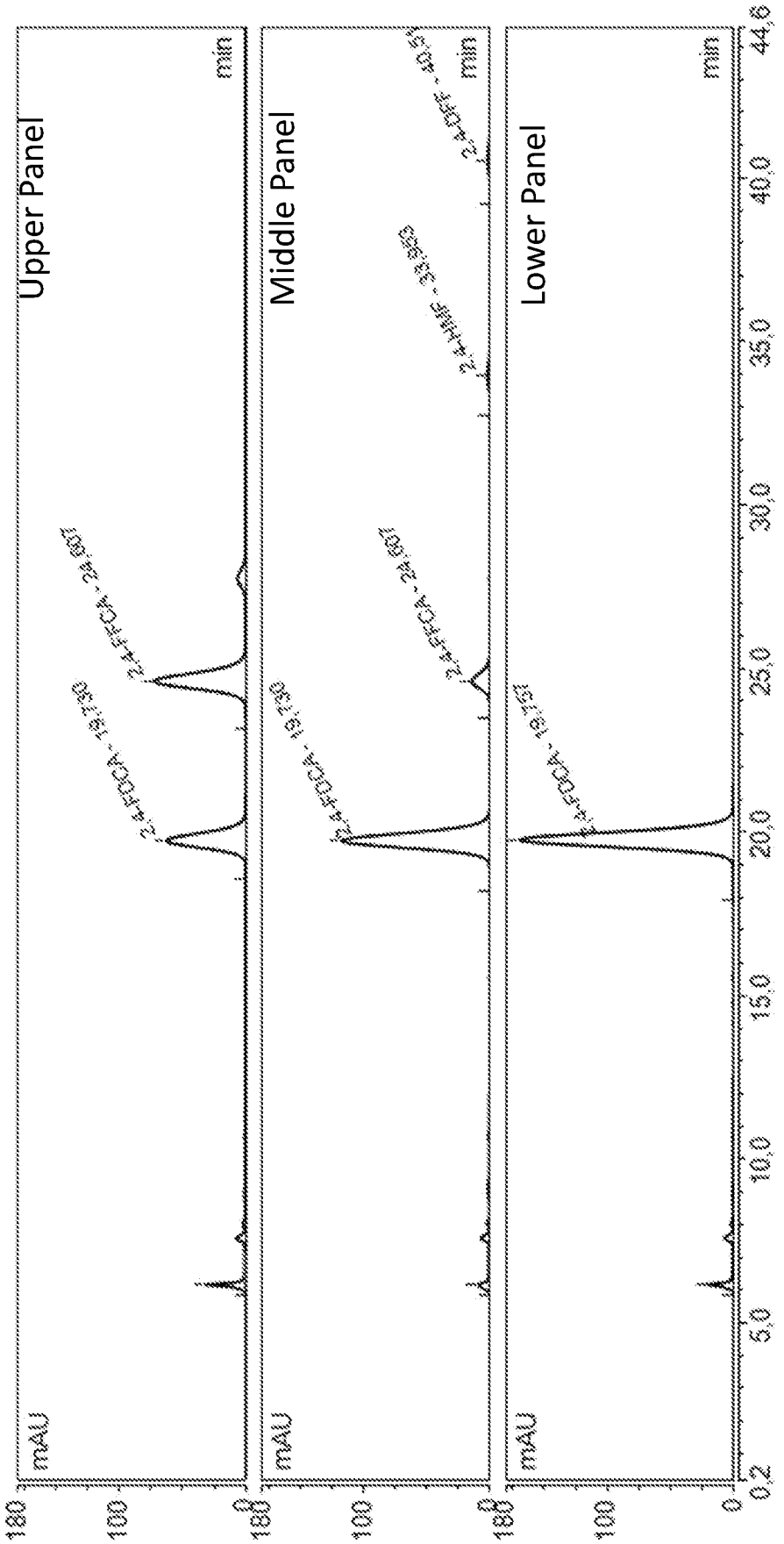

Upper Panel
Middle Panel
Lower Panel
mAU
min
180
100
0
0,2
5,0
10,0
15,0
20,0
25,0
30,0
35,0
40,0
44,6

FIG. 10

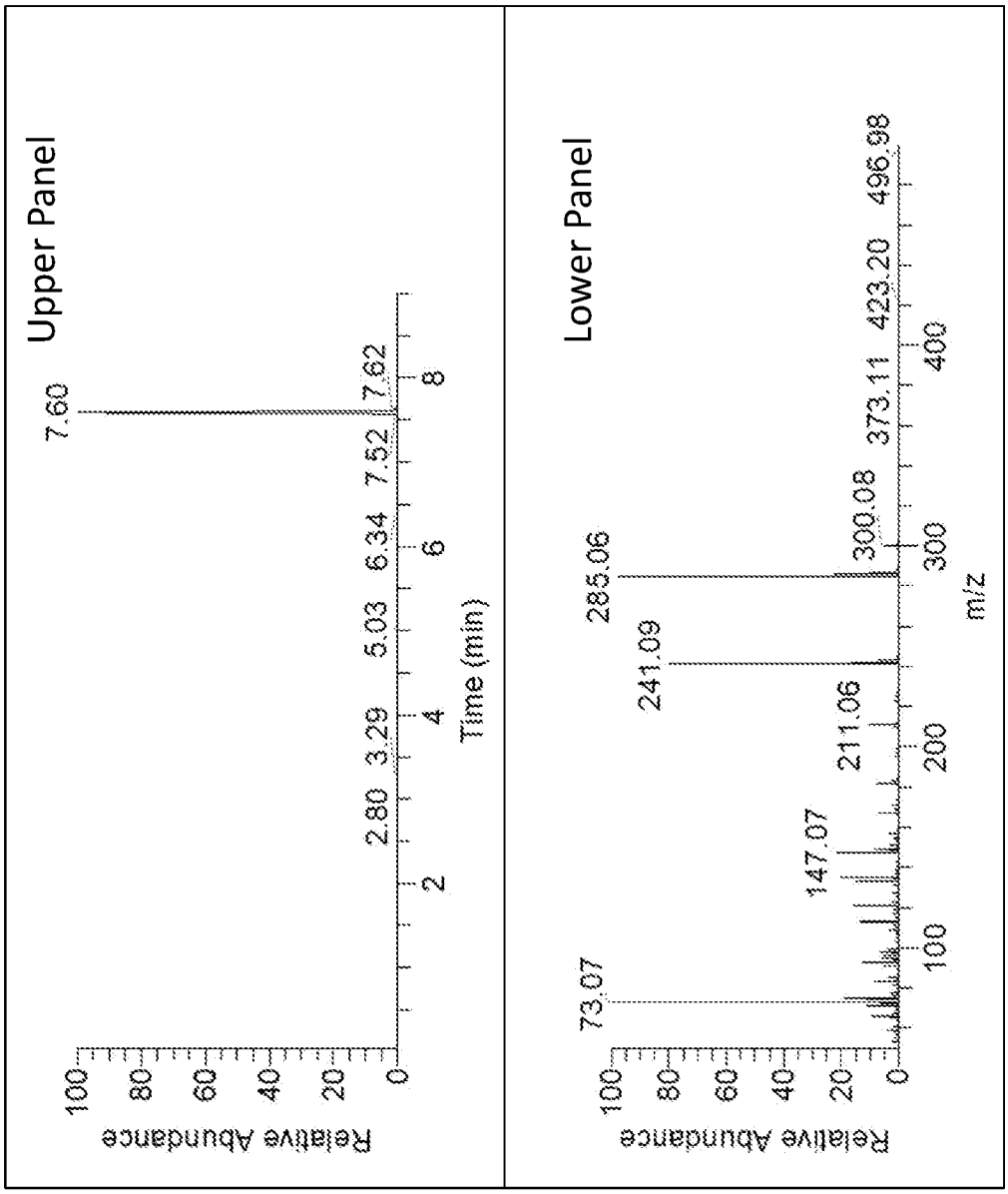

Upper Panel
Relative Abundance
Time (min)
7.60
7.62
7.52
6.34
5.03
3.29
2.80
Lower Panel
Relative Abundance
m/z
73.07
147.07
211.06
241.09
285.06
300.08
373.11
423.20
496.98

FIG. 11

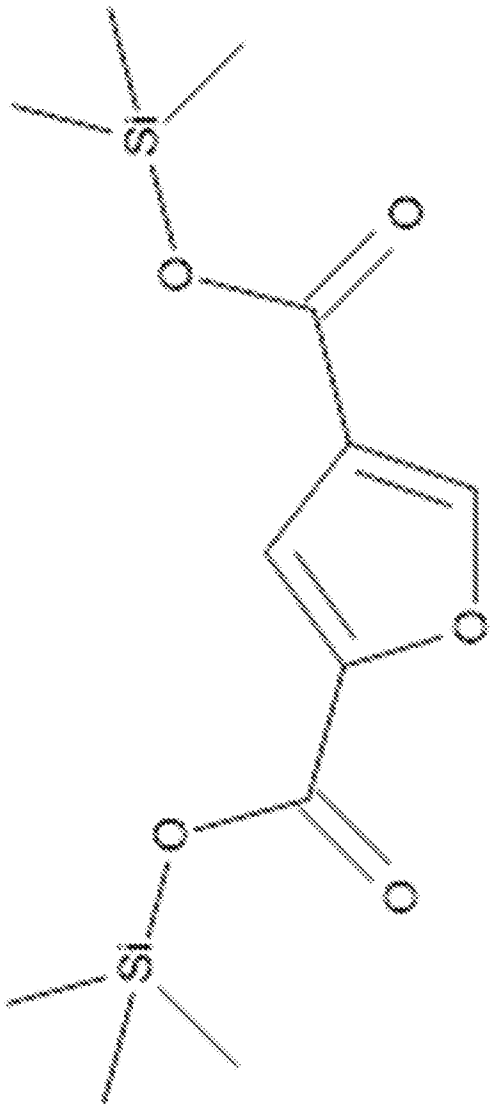


FIG. 12

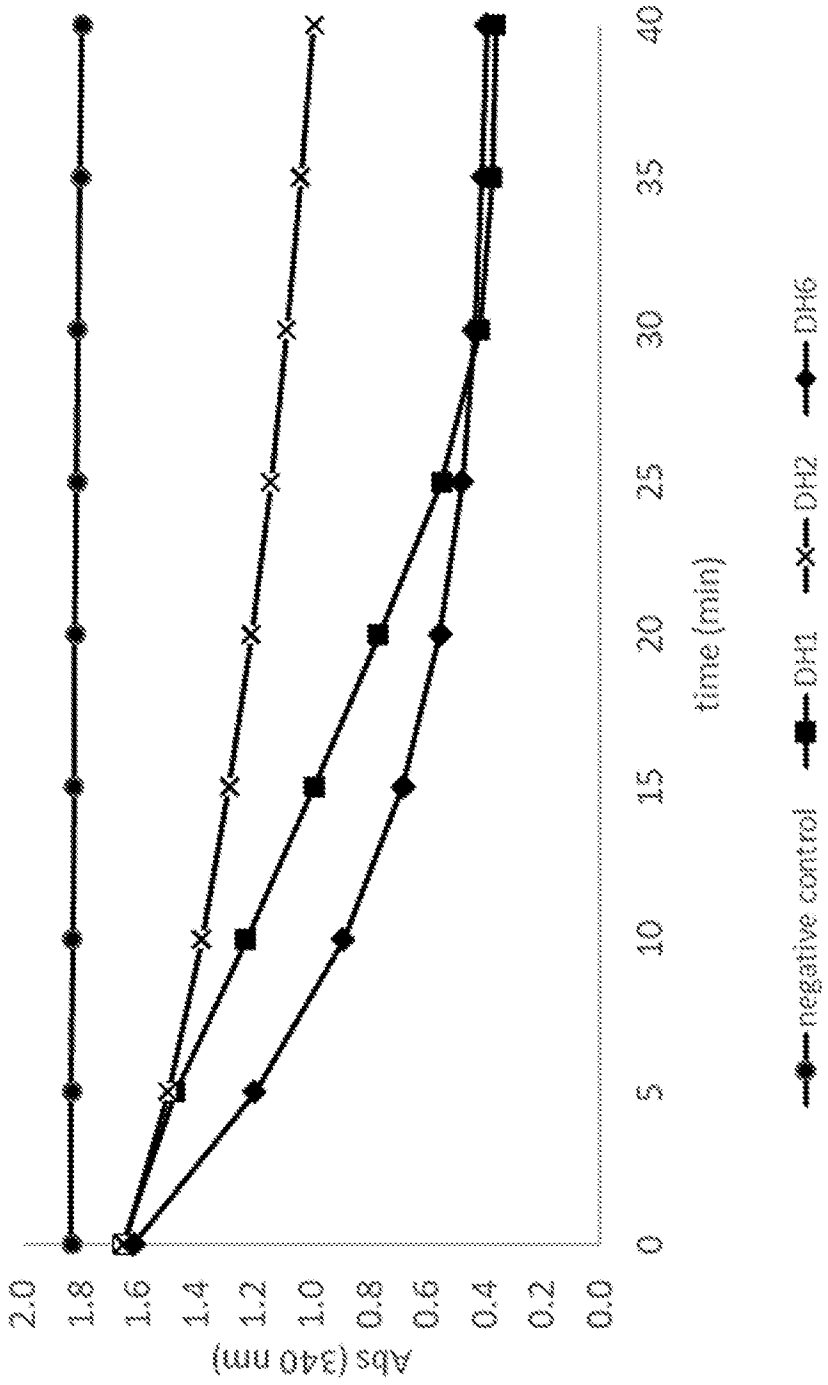

Abs (340 nm)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
30
35
40
time (min)
negative control
DH1
DH2
DH6

FIG. 13

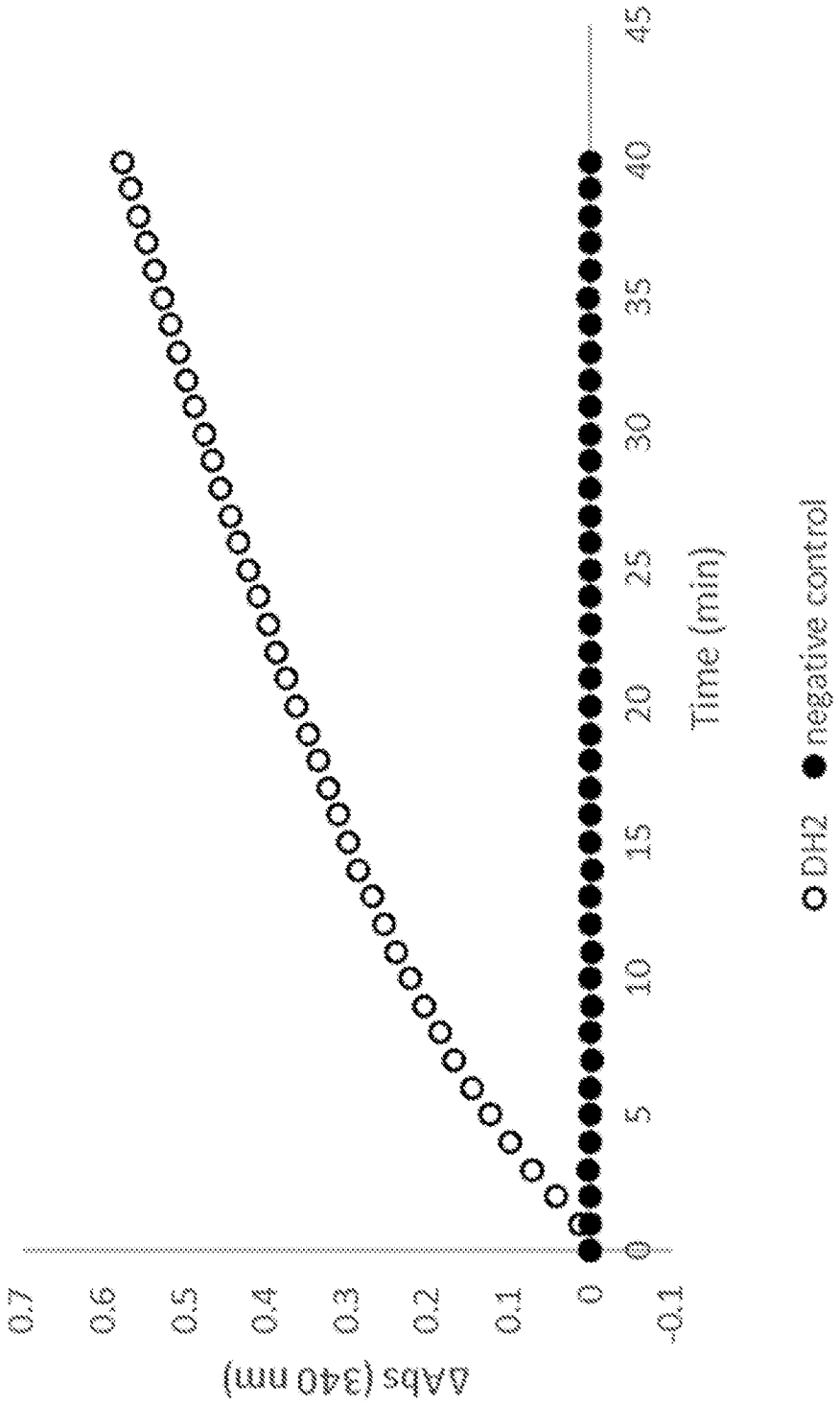

ΔAbs (340 nm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
0
5
10
15
20
25
30
35
40
45
Time (min)
DH2
negative control

FIG. 14

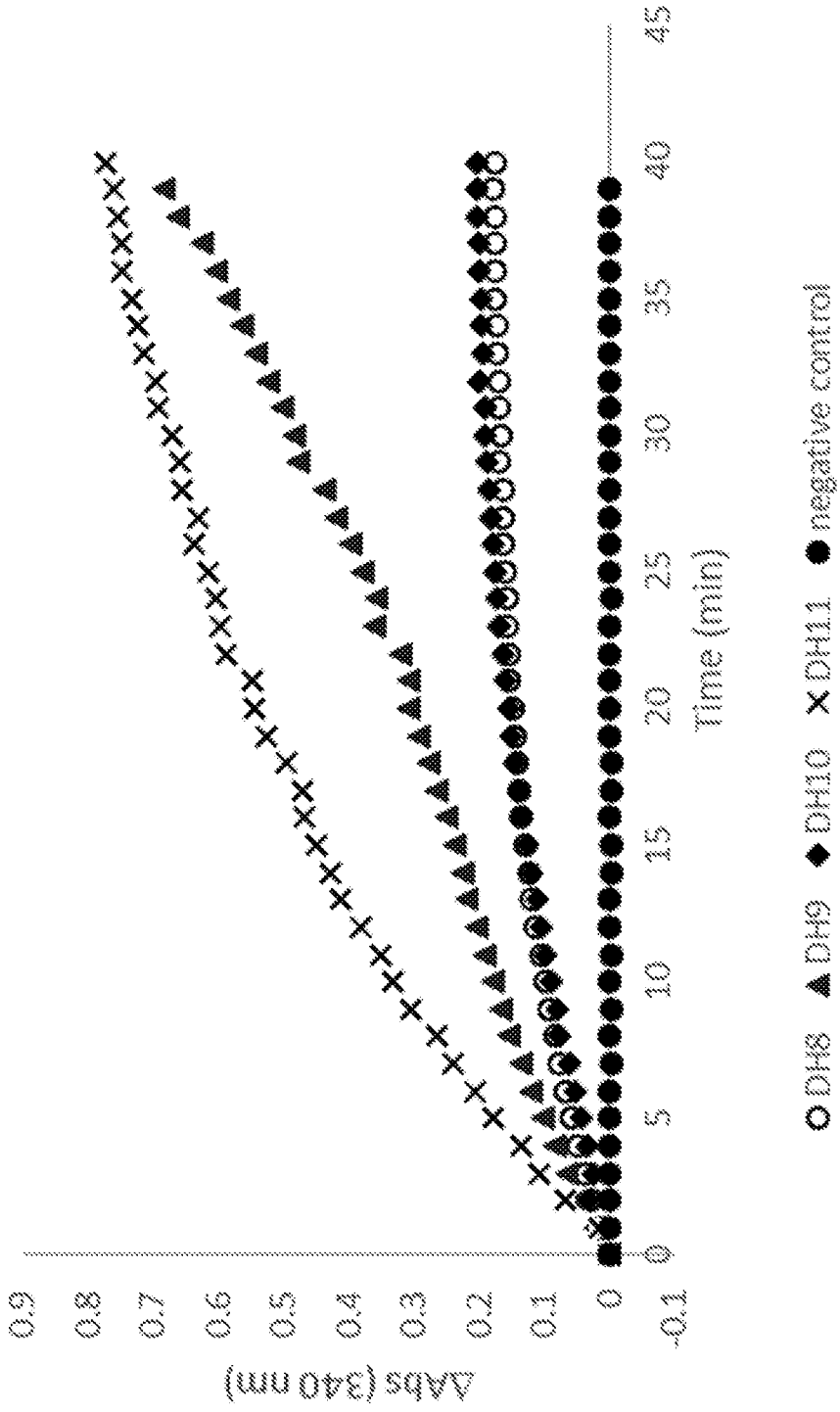

ΔAbs (340 nm)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
0
5
10
15
20
25
30
35
40
45
Time (min)
DH8
DH9
DH10
DH11
negative control

FIG. 15

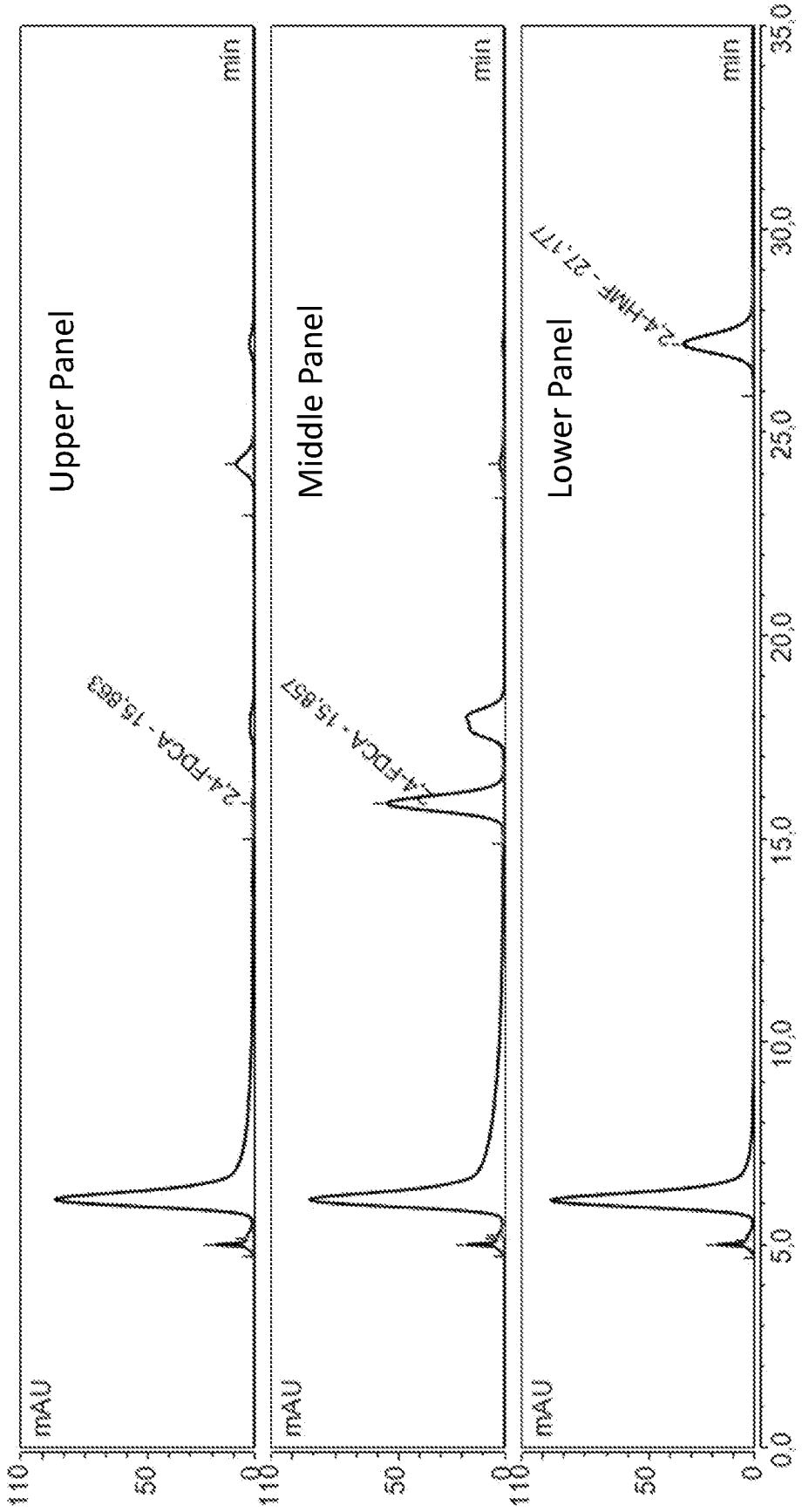
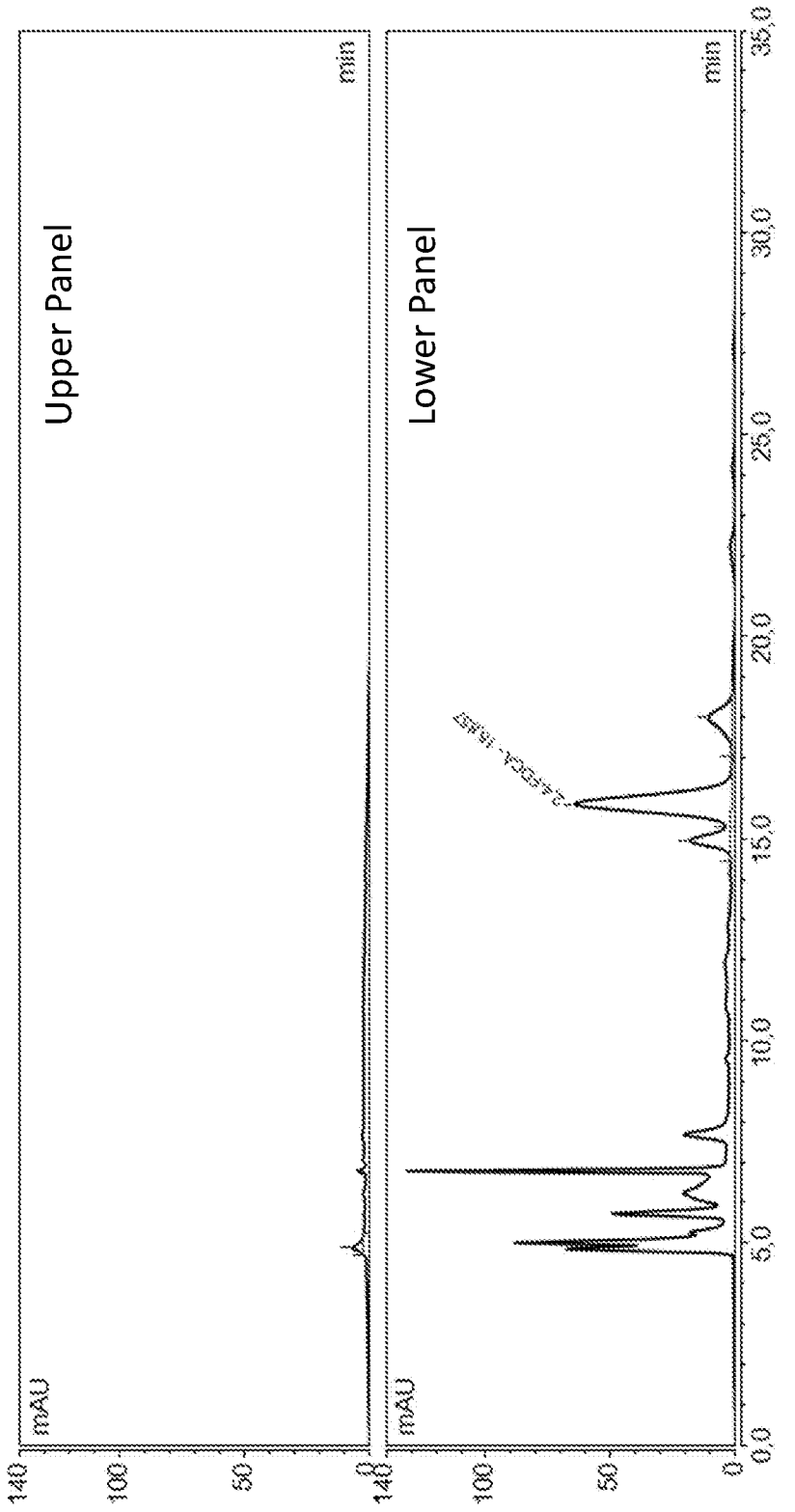

Upper Panel
Middle Panel
Lower Panel
2,4-FDCA - 15,803
2,4-FDCA - 15,857
2,4-HMF - 21,177
mAU
min
110
50
0
0,0
5,0
10,0
15,0
20,0
25,0
30,0
35,0

FIG. 16


Upper Panel
Lower Panel
mAU
min
140
100
50
0
0,0
5,0
10,0
15,0
20,0
25,0
30,0
35,0

INTERNATIONAL SEARCH REPORT

International application No
PCT/BR2020/050064

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12P17/04 C07D307/46 C07D307/50 C12N9/04 C12N9/02
C12N9/16 C12N9/88
ADD.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12P C12N C07D

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	YU WANG ET AL: "Mechanism of the Enzymatic Synthesis of 4-(Hydroxymethyl)-2-furancarboxaldehyde-phosphate (4-HFC-P) from Glyceraldehyde-3-phosphate Catalyzed by 4-HFC-P Synthase", BIOCHEMISTRY, vol. 54, no. 19, 4 May 2015 (2015-05-04), pages 2997-3008, XP055700125, US ISSN: 0006-2960, DOI: 10.1021/acs.biochem.5b00176 abstract page 3001, left-hand column, paragraph 1 - page 3002, right-hand column, paragraph 1; figures 2,6	1-63, 68-75

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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"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
4 June 2020	17/06/2020

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Mateo Rosell, A

1

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Caroline Brown: "The Mechanism of the Enzymatic Synthesis of 2,4-hydroxymethfufural phosphate", University of North Georgia. Honor Theses. 44, 1 January 2019 (2019-01-01), pages 1-39, XP055700512, Retrieved from the Internet: URL:https://digitalcommons.northgeorgia.edu/cgi/viewcontent.cgi?article=1044&context=honors_theses [retrieved on 2020-06-03] page 6 - page 14; figures 2,4,5	1-63, 68-75
Y	WO 2016/133384 A1 (PURAC BIOCHEM BV [NL]) 25 August 2016 (2016-08-25) page 2, line 20 - page 3, line 26 page 4, line 29 - page 5, line 20 page 12, line 20 - page 14, line 26 page 19, line 3 - page 21, line 21 page 22, line 13 - page 23, line 13; examples IV, V	1-63, 68-75
Y	WILLEM P. DIJKMAN ET AL: "Discovery and Characterization of a 5-Hydroxymethylfurfural Oxidase from Methylovorus sp. Strain MP688", APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 80, no. 3, 1 February 2014 (2014-02-01), pages 1082-1090, XP055700609, US ISSN: 0099-2240, DOI: 10.1128/AEM.03740-13 abstract; figure 1 page 1084, right-hand column, paragraph 4 - page 1087, right-hand column, paragraph 1; table 1	1-63, 68-75
Y	JUAN CARRO ET AL: "5-hydroxymethylfurfural conversion by fungal aryl-alcohol oxidase and unspecific peroxygenase", FEBS JOURNAL, vol. 282, no. 16, 8 January 2015 (2015-01-08), pages 3218-3229, XP055555047, GB ISSN: 1742-464X, DOI: 10.1111/febs.13177 cited in the application abstract page 3219, right-hand column, paragraph 2 - page 3222, right-hand column, paragraph 3; figures 1-4,7; tables 1,2	1-63, 68-75

-/--

1

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SHANMUGAM THIYAGARAJAN ET AL: "Concurrent formation of furan-2,5- and furan-2,4-dicarboxylic acid: unexpected aspects of the Henkel reaction", RSC ADVANCES,, vol. 3, 1 January 2013 (2013-01-01), pages 15678-15686, XP007923218, ISSN: 2046-2069, DOI: 10.1039/C3RA42457J cited in the application abstract; figure 1 Scheme 4	64-67
X	YU WANG ET AL: "Industrial production, application, microbial biosynthesis and degradation of furanic compound, hydroxymethylfurfural (HMF)", AIMS MICROBIOLOGY, vol. 4, no. 2, 1 January 2018 (2018-01-01) , pages 261-273, XP055700144, ISSN: 2471-1888, DOI: 10.3934/microbiol.2018.2.261 abstract page 263, paragraph 1; figures 1,3,4	64-67
X	SAMI ZAIDI ET AL: "Highly transparent films of new copolyesters derived from terephthalic and 2,4-furandicarboxylic acids", POLYMER CHEMISTRY, vol. 10, no. 39, 1 January 2019 (2019-01-01), pages 5324-5332, XP055700937, ISSN: 1759-9954, DOI: 10.1039/C9PY00844F abstract schema 1 and 2; tables 1,2	64-67

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/BR2020/050064

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2016133384 A1	25-08-2016	AU 2016220612 A1	17-08-2017
		BR 112017017525 A2	17-04-2018
		CA 2975226 A1	25-08-2016
		CN 107250368 A	13-10-2017
		EP 3259349 A1	27-12-2017
		JP 2018504904 A	22-02-2018
		KR 20170116051 A	18-10-2017
		TW 201638336 A	01-11-2016
		US 2018030488 A1	01-02-2018
		WO 2016133384 A1	25-08-2016