

METHANOL DEHYDROGENASE FUSION PROTEINS

Priority Claim

This application claims the benefit of U.S. Provisional Patent Application Serial Number 62/249,032 filed October 30, 2015, and U.S. Provisional Patent Application Serial
5 Number 62/260,189 filed November 25, 2015 the disclosures of which are incorporated in their entireties herein by reference. Also, the entire content of the ASCII text file entitled “GNO0027WO_Sequence_Listing.txt” created on October 26, 2016, having a size of 28 kilobytes is incorporated herein by reference.

Field of Invention

10 The disclosure is directed to fusion proteins containing methanol dehydrogenase and engineered cells expressing these fusions and methods for using methanol for production of a bioproduct.

Background

In an intracellular metabolic pathway, metabolic intermediates are molecules which are
15 the precursors of the pathway products, which are typically biologically significant molecules.

Various factors can affect the processing of metabolic intermediates in a product pathway. When there is a rate limiting event in the conversion of an intermediate into a subsequent metabolite, that intermediate may accumulate in the cell. In some cases the accumulation of an intermediate may adversely affect the cell. For example, accumulation of
20 an intermediate may indirectly affect the cell by diverting intracellular energy resources, or by reducing the production of a biologically important product. In other cases, the accumulation of metabolic intermediates may have a direct adverse effect on the cell. For example, at elevated concentrations, small molecules such as methylglyoxal or formaldehyde can non-specifically modify intracellular proteins and have an adverse effect on cell health.

25 Alcohol dehydrogenases (ADHs; EC 1.1.1.1) promote the conversion of alcohols to and aldehydes or ketones, typically along with the reduction of nicotinamide adenine dinucleotide (NAD⁺ to NADH). ADHs are instrumental in the generation of important compounds having aldehyde, ketone, and alcohol groups during biosynthesis of various metabolites. However, some of these compounds, such as aldehyde-containing compounds,
30 include formaldehyde which, when accumulated in the cell, can be problematic.

One class of alcohol dehydrogenase is methanol dehydrogenases (MeDHs). MeDHs, converts methanol (MeOH) to formaldehyde (Fald), may be used in an enzymatic pathway engineered into a microbe to enable MeOH as a sole carbon source or as a co-carbon source

with other feed stocks such as, for example, glucose, dextrose, plant biomass or syngas, to produce valuable products. Microorganisms have been that metabolize methanol, and in some instances do so via methanol dehydrogenase, and in even fewer instances produce valuable products. Increasing MeDH activity will enable improved use of MeOH, improving MeOH as a sole carbon source, decreasing production costs, decreasing amounts of any more expensive secondary or co-carbon source, e.g. glucose, increasing product yields, and providing faster rate of MeOH use.

Summary

The current disclosure is directed towards fusion proteins that include methanol dehydrogenase activity and at least one other activity that promotes formaldehyde fixation. For example, the fusion can include activities which promote the conversion of methanol to formaldehyde and then from formaldehyde to a ketose phosphate such as hexulose 6-phosphate, or fructose-6-phosphate. Alternatively, for example, the fusion can include activities which promote the conversion of methanol to formaldehyde and then from formaldehyde to dihydroxyacetone (DHA) and glyceraldehyde-3-phosphate (G3P), and then to fructose-6-phosphate. The current disclosure is also directed to engineered cells that express such fusion proteins, as well as engineered cells having a heterologous methanol dehydrogenase in combination with a fusion protein that promotes formaldehyde fixation. The engineered cells can display increased methanol uptake, through increased expression of the methanol dehydrogenase fusion and/or by using active variants, or through increased efficiency of fixation of formaldehyde into ketose phosphate compounds as a result of the fusion. And while this promotes increased formation of formaldehyde, the formaldehyde is more rapidly fixed into ketose phosphate compounds such as hexulose 6-phosphate, or fructose-6-phosphate, or to DHA and G3P, due to the localization of the enzymatic activities because of the fusion proteins. Therefore, undesirable levels of formaldehyde do not build up in the cell and cause adverse effects on growth.

Further, the increased flux and formation of ketose phosphate compounds, or to DHA and G3P, can provide precursors to the glycolysis pathway. In turn, increased glycolysis can provide increases in the pools of subsequent intermediate compounds such as glycerol 3-phosphate, pyruvate, and acetyl-CoA. These intermediate compounds can be used by product pathways for forming desired (target) bioproducts such as butanediols (BDOs) like 1,3-butanediol (1,3-BDO) and 1,4-butanediol (1,4-BDO), 4-hydroxybutyrate (4-HB), butadiene, 6-amino caproic acid (6ACA), hexamethylenediamine (HMDA), adipic acid, crotyl alcohol, methyl vinyl carbinol, 3-buten-1-ol, 1,2-propanediol (propylene glycol), n-propanol, 1,3-

propanediol, etc. Therefore, the fusions can be expressed in cells that naturally have such product pathways, or cells engineered to express the fusion proteins can further be engineered to include target product pathway enzymes that use the increased amounts of intermediate products available when the fusion proteins of the disclosure are expressed.

5 The fusion proteins of the disclosure improve the ability of a microorganism to grow on methanol as a carbon source. The improved growth on methanol using methanol pathway fusions is likely due to several benefits the fusions provide: 1) rapid conversion of formaldehyde, a potentially toxic product of the MeDH reaction, to hexulose-6-phosphate (or alternatively to DHA and G3P) due to the localized presence of fused HPS; 2) increased
10 efficiency of the fusion protein(s) in the methanol pathway reduces the amount of total methanol pathway protein required for efficient methanol utilization; 3) the presence of methanol pathway fusion proteins pushes the reaction in the forward, desired direction with respect to methanol utilization.

 In one aspect, the disclosure provides a fusion protein comprising: (5a) a first region
15 comprising methanol dehydrogenase or an enzymatically active portion thereof, (5b) a second region comprising 3-hexulose-6-phosphate dehydrogenase or an enzymatically active portion thereof; and (5c) a third region comprising 6-phospho-3-hexuloisomerase or an enzymatically active portion thereof; or (6a) a first region comprising methanol dehydrogenase or an enzymatically active portion thereof, (6b) a second region comprising 3-hexulose-6-phosphate
20 dehydrogenase or an enzymatically active portion thereof; (6c) a third region comprising 6-phospho-3-hexuloisomerase or an enzymatically active portion thereof; and (6d) a fourth region comprising methanol dehydrogenase activator or an enzymatically active portion thereof; wherein the fusion protein optionally comprises one or more linker amino acid

sequence(s) positioned between two or more of the first, second, and third regions of the fusion protein.

5 In another aspect, the disclosure provides a fusion protein comprising: first and second portions of a methanol dehydrogenase, 3-hexulose-6-phosphate dehydrogenase or an enzymatically active portion thereof, and 6-phospho-3-hexuloisomerase or an enzymatically active portion thereof, and optionally methanol dehydrogenase activator or an enzymatically active portion thereof, positioned between the first and second portions of a methanol dehydrogenase.

10 In yet another aspect, the disclosure provides a nucleic acid encoding the fusion protein as described herein.

In yet another aspect, the disclosure provides an expression construct comprising the nucleic acid as described herein.

In yet another aspect, the disclosure provides an engineered cell comprising the fusion protein as described herein, the nucleic acid as described herein, or the expression construct as described herein, or an engineered cell that heterologously expresses methanol dehydrogenase, 3-hexulose-6-phosphate dehydrogenase, and 6-phospho-3-hexuloisomerase, wherein heterologous expression includes expression of a fusion protein comprising (1a) a first region comprising methanol dehydrogenase or an enzymatically active portion thereof, and (1b) a second region comprising 3-hexulose-6-phosphate dehydrogenase or an enzymatically active portion thereof; (2a) a first region comprising methanol dehydrogenase or an enzymatically active portion thereof, (2b) a second region comprising 3-hexulose-6-phosphate dehydrogenase or an enzymatically active portion thereof; and (2c) a third region comprising methanol dehydrogenase activator or an enzymatically active portion thereof; (3a) a first region comprising methanol dehydrogenase or an enzymatically active portion thereof, and (3b) a second region comprising 6-phospho-3-hexuloisomerase or an enzymatically active portion thereof; (4a) a first region comprising methanol dehydrogenase or an enzymatically active portion thereof, (4b) a second region comprising 6-phospho-3-hexuloisomerase or an enzymatically active portion thereof; and (4c) a third region comprising methanol dehydrogenase activator or an enzymatically active portion thereof; (5a) a first region comprising methanol dehydrogenase or an enzymatically active portion thereof, (5b) a second region comprising 3-hexulose-6-phosphate dehydrogenase or an enzymatically active portion thereof; and (5c) a third region comprising 6-phospho-3-hexuloisomerase or an enzymatically active portion thereof; (6a) a first region comprising methanol dehydrogenase or an enzymatically active portion thereof, (6b) a second region comprising 3-hexulose-6-phosphate dehydrogenase or an enzymatically active portion thereof; (6c) a third region comprising 6-phospho-3-hexuloisomerase or an enzymatically active portion thereof; and (6d) a fourth region comprising methanol dehydrogenase activator or an enzymatically active portion thereof; or (7a) first and second portions of a methanol dehydrogenase, and 3-hexulose-6-phosphate dehydrogenase or an enzymatically active portion thereof, and/or 6-phospho-3-hexuloisomerase or an enzymatically active portion thereof, and optionally methanol dehydrogenase activator or an enzymatically active portion thereof, positioned between the first and second portions of a methanol dehydrogenase.

In yet another aspect, the disclosure provides an engineered cell comprising (i) a fusion protein comprising a first region comprising methanol dehydrogenase and a second region comprising methanol dehydrogenase activator, or enzymatically active portions thereof, and (ii) and a fusion protein comprising a first region comprising 3-hexulose-6-phosphate dehydrogenase or an enzymatically active portion thereof; and a second region comprising 6-phospho-3-hexuloisomerase or an enzymatically active portion thereof.

In yet another aspect, the disclosure provides a composition comprising the engineered cell as described herein.

In yet another aspect, the disclosure provides a method of producing a target product or its intermediate comprising culturing the engineered cell as disclosed herein in a culture medium comprising methanol to produce the target product or an intermediate of the target product.

In examples the disclosure provides a fusion protein comprising: (1a) a first region comprising methanol dehydrogenase or an enzymatically active portion thereof, and (1b) a second region comprising 3-hexulose-6-phosphate dehydrogenase or an enzymatically active portion thereof; (2a) a first region comprising methanol dehydrogenase or an enzymatically active portion thereof, (2b) a second region comprising 3-hexulose-6-phosphate dehydrogenase or an enzymatically active portion thereof; and (2c) a third region comprising methanol dehydrogenase activator or an enzymatically active portion thereof; (3a) a first region comprising methanol dehydrogenase or an enzymatically active portion thereof, and (3b) a second region comprising 6-phospho-3-hexuloisomerase or an enzymatically active portion thereof; (4a) a first region comprising methanol dehydrogenase or an enzymatically active portion thereof, (4b) a second region comprising 3-hexulose-6-phosphate dehydrogenase or an enzymatically active portion thereof; and (4c) a third region comprising methanol dehydrogenase activator or an enzymatically active portion thereof; (5a) a first region comprising methanol dehydrogenase or an enzymatically active portion thereof, (5b) a second region comprising 3-hexulose-6-phosphate dehydrogenase or an enzymatically active portion thereof; and (5c) a third region comprising 6-phospho-3-hexuloisomerase or an enzymatically active portion thereof; or (6a) a first region comprising methanol dehydrogenase or an enzymatically active portion thereof, (6b) a second region comprising 3-hexulose-6-phosphate dehydrogenase or an enzymatically active portion thereof; (6c) a third region comprising 6-phospho-3-hexuloisomerase or an enzymatically active portion thereof; and (6d) a fourth region comprising methanol dehydrogenase activator or an enzymatically active portion thereof; wherein the fusion protein optionally comprises one or more linker amino acid sequence(s) positioned between two or more of the first, second, and third regions of the fusion protein.

In an alternative example directed to MeDH with one or more DHA pathway enzyme(s) fusion proteins, the disclosure provides a fusion protein comprising: (7a) a first region comprising methanol dehydrogenase or an enzymatically active portion thereof and (7b) a second region comprising DHA synthase or an enzymatically active portion thereof; (8a) a first region comprising methanol dehydrogenase or an enzymatically active portion thereof, (8b) a second region comprising DHA synthase or an enzymatically active portion thereof; and (8c) a third region comprising methanol dehydrogenase activator or an enzymatically active portion thereof; (9a) a first region comprising methanol dehydrogenase or an enzymatically active portion thereof, and (9b) a second region comprising a F6P aldolase or an enzymatically active portion thereof; (10a) a first region comprising methanol dehydrogenase or an enzymatically active portion thereof, (10b) a second region comprising

DHA synthase or an enzymatically active portion thereof; and (10c) a third region comprising methanol dehydrogenase activator or an enzymatically active portion thereof; (11a) a first region comprising methanol dehydrogenase or an enzymatically active portion thereof, (11b) a second region comprising DHA synthase or an enzymatically active portion thereof; and (11c) a third region comprising F6P aldolase or an enzymatically active portion thereof; or (12a) a first region comprising methanol dehydrogenase or an enzymatically active portion thereof, (12b) a second region comprising DHA synthase or an enzymatically active portion thereof; (12c) a third region comprising F6P aldolase or an enzymatically active portion thereof; and (12d) a fourth region comprising methanol dehydrogenase activator or an enzymatically active portion thereof; wherein the fusion protein optionally comprises one or more linker amino acid sequence(s) positioned between two or more of the first, second, and third regions of the fusion protein

Examples of the disclosure also include a nucleic acid encoding the MeDH-containing fusion protein (1-6) or (7-12), expression constructs comprising the nucleic acid, engineered cells comprising the MeDH-containing fusion protein (1-6) or (7-12), such as engineered cells comprising exogenous nucleic acids that express the fusion protein (1-6) or (7-12), and methods for producing a target product or an intermediate thereof comprising culturing the engineered cell comprising the MeDH-containing fusion protein (1-6) or (7-12).

The disclosure also provides fusion proteins where the methanol dehydrogenase is split into at least two portions separated by at least one regions of 3-hexulose-6-phosphate dehydrogenase, 6-phospho-3-hexuloisomerase, or/and methanol dehydrogenase activator, or alternatively separated by at least one region(s) of DHAS, F6P aldolase, or/and methanol dehydrogenase activator. In this example the disclosure provides a fusion protein comprising first and second portions of a methanol dehydrogenase, and one or more of 3-hexulose-6-phosphate dehydrogenase or an enzymatically active portion thereof, 6-phospho-3-hexuloisomerase or an enzymatically active portion thereof, or methanol dehydrogenase activator or an enzymatically active portion thereof, positioned between the first and second portions of a methanol dehydrogenase; or alternatively in this example the disclosure provides a fusion protein comprising first and second portions of a methanol dehydrogenase, and one or more of DHAS or an enzymatically active portion thereof, F6P aldolase or an enzymatically active portion thereof, or methanol dehydrogenase activator or an enzymatically active portion thereof, positioned between the first and second portions of a methanol dehydrogenase. Optionally, the fusion protein includes one or more linker amino acid sequence(s) positioned between two or more of the first, second, and third regions of the fusion protein

Examples of the disclosure also include a nucleic acid encoding the fusion protein with a split MeDH sequence, expression constructs comprising the nucleic acid, engineered cells comprising the fusion protein with a split MeDH sequence, such as engineered cells comprising exogenous nucleic acids that express the fusion protein, and methods for producing a target product or an intermediate thereof comprising culturing the engineered cell comprising the fusion protein with a split MeDH sequence.

Other examples of the disclosure are directed to products made from the target product obtained from methods using the engineered cell with the fusion protein. Exemplary products include polymers made with a target bioproducts, such as polymers made from diol target products combined with diacids.

Description of the Drawings

Figures 1A – 1F are schematic diagrams of fusion proteins with a MeDH region that is at the N-terminal portion of the protein.

Figures 1G-1H are schematic diagrams of fusion proteins with a MeDH region that is at the C-terminal portion of the protein.

Figures 2A - 2H are schematic diagrams of fusion proteins with a MeDH region that is between N- and C-terminal portions of the protein.

Figures 3A-3C are schematic diagrams of fusion proteins with a MeDH region that is split into two portions in the fusion protein.

Figures 4A and 4B are schematic illustrations of HPS-PHI and MeDH-HPS-PHI fusion proteins in a methanol pathway for formation of fructose-6-phosphate.

Figure 5 illustrates alternative formaldehyde assimilation pathways (RuMP and DHA) and metabolic intermediates from each that can be used in a product pathway. Enzymes catalyzing the reactions are (1) methanol dehydrogenase, e.g. EC 1.1.1, (2) hexulose-6-phosphate synthase, e.g. EC 4.1.2.43, (3) 6-phospho-3-hexuloisomerase, e.g. EC 5.3.1.27, (6) DHA (dihydroxyacetone) synthase, e.g. EC 2.2.1.3, (7) F6P (fructose-6-phosphate) aldolase, e.g. EC 4.1.2, (8) DHA kinase, e.g. EC 2.7.1.121, (9) fructose-bisphosphate aldolase, e.g. EC 4.1.2.13. The fusions described herein can catalyze two or more of the reactions.

Figure 6 illustrates an exemplary target product pathway, a 1,4-BDO product pathway, which can exploit acetyl-CoA available from methanol assimilation as disclosed herein.

Enzymes catalyzing the biosynthetic reactions are: (1) succinyl-CoA synthetase; (2) CoA-independent succinic semialdehyde dehydrogenase; (3) α -ketoglutarate dehydrogenase; (4) glutamate:succinate semialdehyde transaminase; (5) glutamate decarboxylase; (6) CoA-dependent succinic semialdehyde dehydrogenase; (7) 4-hydroxybutanoate dehydrogenase; (8) α -ketoglutarate decarboxylase; (9) 4-hydroxybutyryl CoA: acetyl-CoA transferase; (10) butyrate kinase; (11) phosphotransbutyrylase; (12) aldehyde dehydrogenase; (13) alcohol dehydrogenase.

Figures 7A – 7F are schematic diagrams of fusion proteins of DHA (dihydroxyacetone) synthase (DHAS) or F6P (fructose-6-phosphate) aldolase (F6P Ald) with a MeDH region that is at the N-terminal portion of the fusion protein. Figures 7G-7H are schematic diagrams of fusion proteins with a MeDH region that is at the C-terminal portion of the fusion protein.

Figures 8A - 8H are schematic diagrams of fusion proteins of DHA (dihydroxyacetone) synthase and F6P (fructose-6-phosphate) aldolase with a MeDH region that is between N- and C-terminal portions of the protein.

Figures 9A-9C are schematic diagrams of fusion proteins of DHA (dihydroxyacetone) synthase (DHAS) and/or F6P (fructose-6-phosphate) aldolase with a MeDH region that is split into two portions in the fusion protein.

Figures 10A and 10B are schematic illustrations of a methanol pathway for formation of fructose-6-phosphate comparing a non-fusion (10A) and an exemplary fusion (10B) MeDH-DHAS-F6PALd proteins in which less diffusion of an intermediate (Fald, DHA, G3P) occurs.

Detailed Description

The embodiments of the description described herein are not intended to be exhaustive or to limit the disclosure to the precise forms disclosed in the following detailed description. Rather, the embodiments are chosen and described so that others skilled in the art can appreciate and understand the principles and practices of the description.

All publications and patents mentioned herein are hereby incorporated by reference. The publications and patents disclosed herein are provided solely for their disclosure. Nothing herein is to be construed as an admission that the inventors are not entitled to antedate any publication and/or patent, including any publication and/or patent cited herein.

5 In methanotrophic bacteria formaldehyde made from methane and methanol oxidation is used to form metabolic intermediates in pathways leading to the formation of cellular products (Anthony, C. (1991) *Biotechnology* 18:79-109). The serine and D-ribulose 5-phosphate (RuMP) pathways use formaldehyde to produce carbon-containing intermediate compounds which are subsequently converted into other downstream products.

10 The RuMP pathway hexulose-6-phosphate synthase (HPS) enzymatically condenses formaldehyde and D-ribulose 5-phosphate (RuMP) to form hexulose 6-phosphate (HuMP). 6-phospho-3-hexuloisomerase (HPI) enzymatically converts HuMP to β -D-fructofuranose 6-phosphate (FMP). HPS and HPI are unique to natural organisms that have the RuMP pathway. For every one molecule of formaldehyde assimilated, one molecule of FMP is created.

15 FMP can then be cleaved to 3-carbon compounds by either of two routes. Enzymes of these other routes are not exclusive to those methanotrophic bacteria expressing HPS and HPI

In one route 6-phosphofructokinase (EC 2.7.1.11) phosphorylates FMP to fructose 1,6-bisphosphate (FDP). Fructose-bisphosphate aldolase (EC 4.1.2.13) then cleaves FDP into dihydroxy acetone phosphate (DHAP) and glyceraldehyde 3-phosphate.

20 In another route glucose-6-phosphate isomerase (EC 5.3.1.9) isomerizes FMP to glucose 6-phosphate (GMP). Glucose-6-phosphate 1-dehydrogenase (EC 1.1.1.49) then dehydrogenates GMP to D-glucono-1,5-lactone 6-phosphate which is further dehydrogenated to 6-phospho-gluconate by 6-phosphogluconolactonase (EC 3.1.1.31). Phosphogluconate dehydratase (EC 4.2.1.12) then converts 6-phospho-gluconate to 2-keto-3-deoxy-6-phospho-D-gluconate (KDPG). Subsequently, KDPG aldolase (EC 4.1.2.14) cleaves KDPG into
25 glyceraldehyde 3-phosphate and pyruvate. Pyruvate and DHAP formed through this pathway can be used in cellular pathways for the synthesis of biomolecules.

In engineered cells of the disclosure, methanol consumption minimally requires the heterologous expression of three genes: methanol dehydrogenase (MeDH), 3-hexulose-6-phosphate dehydrogenase (HPS or *rmpA*), and 6-phospho-3-hexuloisomerase (PHI or *rmpB*).
30 In another embodiment the engineered cells of the disclosure, methanol consumption minimally requires the heterologous expression of three genes: methanol dehydrogenase (MeDH), DHA (dihydroxyacetone) synthase (DHAS) and F6P (fructose-6-phosphate) aldolase (F6P Ald) (e.g., see DHA Route of Figure 5) or alternatively, less preferably, MeDH, DHA
35 (dihydroxyacetone) synthase, and DHA kinase optionally with fructose-bisphosphate aldolase

(Figure 5, DHA Route 2). The MeDH protein catalyzes the inter-conversion of methanol and formaldehyde. As noted herein, the HPS protein catalyzes bond formation between formaldehyde and D-ribulose 5-phosphate, forming hexulose-6-phosphate and the PHI protein catalyzes isomerization of hexulose-6-phosphate to fructose-6-phosphate, which is an intermediate in glycolysis. In addition, the presence of an accessory protein, the MeDH Activator (ACT), may increase the activity of MeDH and improve methanol uptake. It is generally recognized that the activity of this heterologous pathway limits the ability of the non-native organism to consume methanol and grow on methanol as a carbon source. One of the issues associated with converting an organism to methylotrophy, is the potential toxic build-up of formaldehyde, the product of MeDH.

The fusion proteins of the disclosure provide approaches to avoid unwanted accumulation of formaldehyde by increasing the efficiency of the HPS reaction that uses formaldehyde and D-ribulose 5-phosphate as substrates, forming hexulose-6-phosphate. Increasing HPS efficiency could be accomplished such as by co-localization of the MeDH and HPS so HPS has easy access to higher local concentrations of formaldehyde. The fusion proteins of MeDH with a DHA Route 1 or Route 2 enzyme or enzymes avoid unwanted accumulation of formaldehyde by providing increased efficiency of the DHA synthase reaction that uses formaldehyde and xylulose-5-phosphate, forming DHA and G3P. Increased rate of MeOH use is also obtained.

Generally, the disclosure provides fusion proteins having at least (i) methanol dehydrogenase activity and (ii) 3-hexulose-6-phosphate dehydrogenase activity and/or 6-phospho-3-hexuloisomerase activity. Generally, in another embodiment the disclosure provides fusion proteins having at least (i) methanol dehydrogenase activity and (ii) DHA synthase activity and/or fructose-6-phosphate aldolase activity; or in yet another embodiment at least (i) methanol dehydrogenase activity and (ii) DHA synthase activity and/or DHA kinase activity. The fusion proteins can also include methanol dehydrogenase activator activity. Therefore, fusion proteins of the disclosure can include two, three, or four or more regions each providing a different activity. The regions can include the full (e.g., native) sequence of the enzyme, or can include an active portion thereof. Optionally, one or more linker sequences can bond one region to another in the fusion protein.

Methanol dehydrogenase activity, 3-hexulose-6-phosphate dehydrogenase activity, 6-phospho-3-hexuloisomerase activity, and methanol dehydrogenase activator activity can be provided by methanol dehydrogenase, 3-hexulose-6-phosphate dehydrogenase, 6-phospho-3-hexuloisomerase activity, and methanol dehydrogenase activator enzymes and active portions thereof, including those polypeptide sequence that are known in the art, as well as those

described herein. Methanol dehydrogenase activity, DHA synthase, F6P aldolase, and DHA kinase activity and methanol dehydrogenase activator activity can be provided by methanol dehydrogenase, DHA synthase, F6P aldolase and DHA kinase and methanol dehydrogenase activator enzymes and active portions thereof, including those polypeptide sequence that are known in the art, as well as those described herein. In exemplary fusion protein diagrams the acronym “MeDH” can indicate a polypeptide region having methanol dehydrogenase activity; the acronym “HPS” can indicate a polypeptide region having 3-hexulose-6-phosphate dehydrogenase activity; the acronym “PHI” can indicate a polypeptide region having 6-phospho-3-hexuloisomerase activity; and the acronym “ACT” can indicate a polypeptide region having methanol dehydrogenase activator activity. The acronym “DHAS” can indicate a polypeptide region having DHA synthase activity; the acronym “F6P Ald” can indicate a polypeptide region having F6P aldolase activity; the acronym “DHAK” can indicate a polypeptide region having DHA kinase activity. The fusion proteins can include one or more polypeptide linkers, positions between polypeptide regions of the fusion proteins.

Nucleic acids encoding the fusion proteins, as well as expression constructs including the nucleic acids, are described.

Also described are engineered cells expressing the fusion proteins, optionally including one or more additional metabolic pathway transgene(s), methanol metabolic pathway genes, and/or target product pathway genes; cell culture compositions including the cells; methods for promoting production of the target product or intermediate thereof from the cells; compositions including the target product or intermediate; and products made from the target product or intermediate.

The term “non-naturally occurring”, when used in reference to an organism (e.g., microbial) is intended to mean that the organism has at least one genetic alteration not normally found in a naturally occurring organism of the referenced species. Naturally-occurring organisms can be referred to as “wild-type” such as wild type strains of the referenced species. Likewise, a “non-natural” polypeptide or nucleic acid can include at least one genetic alteration not normally found in a naturally-occurring polypeptide or nucleic acid. Fusion proteins of the current disclosure are considered non-natural polypeptides. Naturally-occurring organisms, nucleic acids, and polypeptides can be referred to as “wild-type” or “original” such as wild type strains of the referenced species. Likewise, amino acids found in the wild type organism can be referred to as “original” with regards to any amino acid position.

A genetic alteration that makes an organism non-natural can 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

organism'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.

In the fusion protein, the location of regions having 3-hexulose-6-phosphate dehydrogenase activity (HPS), 6-phospho-3-hexuloisomerase activity (PHI), and methanol dehydrogenase activator activity (ACT) relative to the polypeptide having methanol dehydrogenase activity (MeDH) can be explained relative to the amino (N) and/or carboxyl (C) terminus of the fusion protein. In another embodiment the fusion protein, the location of regions having DHAS, F6P Ald (or DHAK), and methanol dehydrogenase activator activity (ACT) relative to the polypeptide having methanol dehydrogenase activity (MeDH) can be explained relative to the amino (N) and/or carboxyl (C) terminus of the fusion protein. For example, regions of the fusion proteins may be described relative to one another as "at the N-terminal portion of the protein" or "at the C-terminal portion of the protein" of the fusion protein if desired. Regions of the fusion proteins may also alternatively be described as "extending inwards from the N-terminus" or "extending inwards from the C-terminus" of the fusion protein if desired. Some regions of the fusion proteins may be described as "between one region and the N-terminal portion of the protein," "between one region and the C-terminal portion of the protein," or, "between the N-terminal portion of the protein and the C-terminal portion of the protein."

The N- terminal and C-terminal portions of the fusion proteins, which, in various embodiments, can include MeDH, HPS, PHI, or ACT regions, or alternatively MeDH, DHAS, F6P ALd (or DHAK) or ACT regions, can optionally be described in terms of the number of amino acid residues extending inward from either N- or C-terminus of the fusion protein. For example, the N- terminal and C-terminal portions of the fusion proteins may encompass up to 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 amino acids inwards in the fusion protein from either N- or C-terminus, respectively.

Likewise, one or more regions between the N- terminal and C-terminal portions which may encompass one or more of MeDH, HPS, PHI, or ACT regions, can be described in terms of an amount of amino acid residues. For example, a region of the fusion protein between the N-terminal and C-terminal portions may encompass up to 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, or 500 amino acids.

Embodiments of the disclosure include, but are not limited to the following fusion protein arrangements: (a) a fusion protein having a MeDH region and PHI region, where the

PHI region is between MeDH region and the C-terminus of the fusion protein; (b) a fusion protein having a MeDH region and HPS region, where the HPS region is between MeDH region and the C-terminus of the fusion protein; (c) a fusion protein having a MeDH region and PHI region, where the PHI region is between MeDH region and the N-terminus of the fusion protein; (d) a fusion protein having a MeDH region and HPS region, where the HPS region is between MeDH region and the N-terminus of the fusion protein; (e) a fusion protein having MeDH, HPS and PHI regions, where the MeDH region is between HPS and PHI regions, and where the PHI region is between the MeDH region and the N-terminus of the fusion protein and where the HPS region is between the MeDH region and the C-terminus of the fusion protein; (f) a fusion protein having MeDH, HPS and PHI regions, where the MeDH region is between HPS and PHI regions, and where the HPS region is between the MeDH region and the N-terminus of the fusion protein and where the PHI region is between the MeDH region and the C-terminus of the fusion protein; (g) a fusion protein having MeDH, HPS and PHI regions, where the HPS region is between MeDH and PHI regions, and where the MeDH region is between the HPS region and the N-terminus of the fusion protein and where the PHI region is between the HPS region and the C-terminus of the fusion protein; (h) a fusion protein having MeDH, HPS and PHI regions, where the HPS region is between MeDH and PHI regions, and where the PHI region is between the HPS region and the N-terminus of the fusion protein and where the MeDH region is between the HPS region and the C-terminus of the fusion protein; and (i) a fusion protein having ACT, MeDH, HPS and PHI regions, where the MeDH and HPS regions are between ACT and PHI regions, and where the ACT region is between the MeDH region and the N-terminus of the fusion protein and where the PHI region is between the HPS region and the C-terminus of the fusion protein. Linker sequences can be present between any one or more regions of the fusion proteins of (a)-(i).

For example, Figure 1A is a schematic diagram of a fusion protein with MeDH region that extends from the N-terminus and a PHI region that extends from the C-terminus. Such a fusion protein can be designated MeDH-PHI. Optionally, if a polypeptide linker is between the two regions the fusion protein designated MeDH-L¹-PHI.

As another embodiment, Figure 1B is a schematic diagram of a fusion protein with a MeDH region that is at the N-terminal portion of the protein and a HPS region that is at the C-terminal portion of the protein. Such a fusion protein can be designated MeDH-HPS. Optionally, if a polypeptide linker is between the two regions the fusion protein designated MeDH-L¹-HPS.

As another embodiment, Figure 1C is a schematic diagram of a fusion protein with a MeDH region that is at the N-terminal portion of the protein and a PHI region that is at the C-

terminal portion of the protein, and a HPS region that is between the MeDH and PHI regions. Such a fusion protein can be designated MeDH-HPS-PHI. Optionally, if polypeptide linker(s) is/are between the different region(s) the fusion protein can be designated MeDH-L¹-HPS-PHI, MeDH-HPS-L¹-PHI, or MeDH-L¹-HPS-L²-PHI.

5 As another embodiment, Figure 1D is a schematic diagram of a fusion protein with a MeDH region that is at the N-terminal portion of the protein and a HPS region that is at the C-terminal portion of the protein, and a PHI region that is between the MeDH and HPS regions. Such a fusion protein can be designated MeDH-PHI-HPS. Optionally, if polypeptide linker(s) is/are between the different region(s) the fusion protein can be designated MeDH-L¹-PHI-HPS,
10 MeDH-PHI-L¹-HPS, or MeDH-L¹-PHI-L²-HPS.

As another embodiment, Figure 1E is a schematic diagram of a fusion protein with a MeDH region that is at the N-terminal portion of the protein and a PHI region that is at the C-terminal portion of the protein, and an ACT region that is between the MeDH and PHI regions. Such a fusion protein can be designated MeDH-ACT-PHI. Optionally, if polypeptide linker(s)
15 is/are between the different region(s) the fusion protein can be designated MeDH-L¹-ACT-PHI, MeDH-ACT-L¹-PHI, or MeDH-L¹-ACT-L²-PHI.

As another embodiment, Figure 1F a is a schematic diagram of a fusion protein with a MeDH region that is at the N-terminal portion of the protein and a HPS region that is at the C-terminal portion of the protein, and an ACT region that is between the MeDH and HPS
20 regions. Such a fusion protein can be designated MeDH-ACT-HPS. Optionally, if polypeptide linker(s) is/are between the different region(s) the fusion protein can be designated MeDH-L¹-ACT-HPS, MeDH-ACT-L¹-HPS, or MeDH-L¹-ACT-L²-HPS.

As another embodiment, Figure 1G is a schematic diagram of a fusion protein with MeDH region that is at the C-terminal portion of the protein and a HPS region that is at the N-terminal portion of the protein. Such a fusion protein can be designated HPS-MeDH.
25 Optionally, if a polypeptide linker is between the two regions the fusion protein designated HPS-L¹-MeDH.

As another embodiment, Figure 1H is a schematic diagram of a fusion protein with MeDH region that is at the C-terminal portion of the protein and a PHI region that is at the N-terminal portion of the protein. Such a fusion protein can be designated PHI-MeDH.
30 Optionally, if a polypeptide linker is between the two regions the fusion protein designated PHI-L¹-MeDH.

In other embodiments MeDH is positioned between two other regions of amino acid sequence. As another embodiment, Figure 2A is a schematic diagram of a fusion protein with
35 HPS region that is at the N-terminal portion of the protein and a PHI region that is at the C-

terminal portion of the protein, and a MeDH region between the HPS and PHI regions. Such a fusion protein can be designated HPS-MeDH-PHI. Optionally, if polypeptide linker(s) is/are between the different region(s) the fusion protein can be designated HPS-MeDH-L¹-PHI, HPS-L¹-MeDH-PHI, or HPS-L¹-MeDH-L²-PHI.

5 As another embodiment, Figure 2B is a schematic diagram of a fusion protein with PHI region that is at the N-terminal portion of the protein and a HPS region that is at the C-terminal portion of the protein, and a MeDH region between the PHI and HPS regions. Such a fusion protein can be designated PHI-MeDH-HPS. Optionally, if polypeptide linker(s) is/are between the different region(s) the fusion protein can be designated PHI-MeDH-L¹-HPS, PHI-L¹-
10 MeDH-HPS, or PHI-L¹-MeDH-L²-HPS.

As another embodiment, Figure 2C is a schematic diagram of a fusion protein with PHI region that is at the N-terminal portion of the protein and an ACT region that is at the C-terminal portion of the protein, and a MeDH region between the PHI and ACT regions,. Such a fusion protein can be designated PHI-MeDH-ACT. Optionally, if polypeptide linker(s) is/are
15 between the different region(s) the fusion protein can be designated PHI-MeDH-L¹-ACT, PHI-L¹-MeDH-ACT, or PHI-L¹-MeDH-L²-ACT.

As another embodiment, Figure 2D is a schematic diagram of a fusion protein with HPS region that is at the N-terminal portion of the protein and an ACT region that is at the C-terminal portion of the protein, and a MeDH region between the HPS and ACT regions,. Such
20 a fusion protein can be designated HPS-MeDH-ACT. Optionally, if polypeptide linker(s) is/are between the different region(s) the fusion protein can be designated HPS-MeDH-L¹-ACT, HPS-L¹-MeDH-ACT, or HPS-L¹-MeDH-L²-ACT.

As another embodiment, Figure 2E is a schematic diagram of a fusion protein with ACT region that is at the N-terminal portion of the protein and a PHI region that is at the C-terminal portion of the protein, and MeDH and HPS regions between the ACT and PHI
25 regions, with MeDH being closer to the ACT region and HPS being closer to the PHI region. Such a fusion protein can be designated ACT-MeDH-HPS-PHI. Optionally, if polypeptide linker(s) is/are between the different region the fusion protein can be designated ACT-L¹-MeDH-HPS-PHI, ACT-MeDH-L¹-HPS-PHI, ACT-MeDH-HPS-L¹-PHI, ACT-L¹-MeDH-L²-HPS-PHI, ACT-L¹-MeDH-HPS-L²-PHI, ACT-MeDH-L¹-HPS-L²-PHI, and ACT-L¹-MeDH-L²-HPS-L³-PHI.
30

As another embodiment, Figure 2F is a schematic diagram of a fusion protein with ACT region that is at the N-terminal portion of the protein and a HPS region that is at the C-terminal portion of the protein, and MeDH and PHI regions between the ACT and HPS
35 regions, with MeDH being closer to the ACT region and PHI being closer to the HPS region.

Such a fusion protein can be designated ACT-MeDH-PHI-HPS. Optionally, if polypeptide linker(s) is/are between the different region the fusion protein can be designated ACT-L¹-MeDH-PHI-HPS, ACT-MeDH-L¹-PHI-HPS, ACT-MeDH-PHI-L¹-HPS, ACT-L¹-MeDH-L²-PHI-HPS, ACT-L¹-MeDH-PHI-L²-HPS, ACT-MeDH-L¹-PHI-L²-HPS, and ACT-L¹-MeDH-L²-PHI-L³-HPS.

As another embodiment, Figure 2G is a schematic diagram of a fusion protein with HPS region that is at the N-terminal portion of the protein and an ACT region that is at the C-terminal portion of the protein, and PHI and MeDH regions between the HPS and ACT regions, with PHI being closer to the HPS region and MeDH being closer to the ACT region.

Such a fusion protein can be designated HPS-PHI-MeDH-ACT. Optionally, if polypeptide linker(s) is/are between the different region the fusion protein can be designated HPS-L¹-PHI-MeDH-ACT, HPS-PHI-L¹-MeDH-ACT, HPS-PHI-MeDH-L¹-ACT, HPS-L¹-PHI-L²-MeDH-ACT, HPS-L¹-PHI-MeDH-L²-ACT, HPS-PHI-L¹-MeDH-L²-ACT, and HPS-L¹-PHI-L²-MeDH-L³-ACT.

As another embodiment, Figure 2H is a schematic diagram of a fusion protein with PHI region that is at the N-terminal portion of the protein and an ACT region that is at the C-terminal portion of the protein, and HPS and MeDH regions between the PHI and ACT regions, with HPS being closer to the PHI region and MeDH being closer to the ACT region. Such a fusion protein can be designated PHI-HPS-MeDH-ACT. Optionally, if polypeptide linker(s) is/are between the different region the fusion protein can be designated PHI-L¹-HPS-MeDH-ACT, PHI-HPS-L¹-MeDH-ACT, PHI-HPS-MeDH-L¹-ACT, PHI-L¹-HPS-L²-MeDH-ACT, PHI-L¹-HPS-MeDH-L²-ACT, PHI-HPS-L¹-MeDH-L²-ACT, and PHI-L¹-HPS-L²-MeDH-L³-ACT.

In other embodiments, in the fusion protein the methanol dehydrogenase is interrupted by at least one different region of amino acid sequence, such as a PHI or a HPS region. For example, the nucleic acid will encode, in frame, a polypeptide with a first portion of a MeDH, a PHI or a HPS region, and then a second portion of a MeDH. Although the MeDH sequence is interrupted, the fusion protein still maintains methanol dehydrogenase activity.

For example, Figure 3A is a schematic diagram of a fusion protein having a first portion of a MeDH that is at the N-terminal portion of the protein, a second portion of a MeDH that is at the C-terminal portion of the protein, and PHI region between the first and second methanol dehydrogenase portions. Such fusion proteins can be designated MeDH(P1)-PHI-MeDH(P2). Optionally, if polypeptide linker(s) is/are between the different portions and region(s) the fusion protein can be designated MeDH(P1)-L¹-PHI-MeDH(P2), MeDH(P1)-PHI-L¹-MeDH(P2), or MeDH(P1)-L¹-PHI-L²-MeDH(P2).

As another embodiment, Figure 3B is a schematic diagram of a fusion protein having a first portion of a MeDH that is at the N-terminal portion of the protein, a second portion of a MeDH that is at the C-terminal portion of the protein, and HPS and PHI regions between the first and second MeDH portions, with HPS being closer to the first portion of the MeDH and PHI being closer to the second portion of the MeDH. Such a fusion protein can be designated MeDH(P1)-HPS-PHI-MeDH(P2). Optionally, if polypeptide linker(s) is/are between the different portions and region(s) the fusion protein can be designated MeDH(P1)-L¹-HPS-PHI-MeDH(P2), MeDH(P1)-HPS-L¹-PHI-MeDH(P2), MeDH(P1)-HPS-PHI-L¹-MeDH(P2), MeDH(P1)-L¹-HPS-L²-PHI-MeDH(P2), MeDH(P1)-L¹-HPS-PHI-L²-MeDH(P2), MeDH(P1)-HPS-L¹-PHI-L²-MeDH(P2), or MeDH(P1)-L¹-HPS-L²-PHI-L³-MeDH(P2).

As another embodiment, Figure 3C is a schematic diagram of a fusion protein having an ACT region that is at the N-terminal portion of the protein, a second portion of a MeDH that is at the C-terminal portion of the protein, a first portion of the MeDH and a HPS region between the ACT region and the second portion of a MeDH, with HPS being in between the first and second MeDH portions. Such a fusion protein can be designated ACT-MeDH(P1)-HPS-MeDH(P2). Optionally, if polypeptide linker(s) is/are between the different portions and region(s) the fusion protein can be designated ACT-L¹-MeDH(P1)-HPS-MeDH(P2), ACT-MeDH(P1)-L¹-HPS-MeDH(P2), ACT-MeDH(P1)-HPS-L¹-MeDH(P2), ACT-L¹-MeDH(P1)-L²-HPS-MeDH(P2), ACT-MeDH(P1)-L¹-HPS-L²-MeDH(P2), ACT-L¹-MeDH(P1)-HPS-L²-MeDH(P2), or ACT-L¹-MeDH(P1)-L²-HPS-L³-MeDH(P2).

Additional embodiments of the disclosure include, but are not limited to the following fusion protein arrangements of MeDH with one or more DHA Pathway enzymes: (a) a fusion protein having a MeDH region and DHAS region, where the DHAS region is between MeDH region and the C-terminus of the fusion protein; (b) a fusion protein having a MeDH region and F6PALD region (alternatively written as "F6P Ald"), where the F6PALD region is between MeDH region and the C-terminus of the fusion protein; (c) a fusion protein having a MeDH region and DHAS region, where the DHAS region is between MeDH region and the N-terminus of the fusion protein; (d) a fusion protein having a MeDH region and F6PALD region, where the F6PALD region is between MeDH region and the N-terminus of the fusion protein; (e) a fusion protein having MeDH, F6PALD and DHAS regions, where the MeDH region is between DHAS and DHAS regions, and where the DHAS region is between the MeDH region and the N-terminus of the fusion protein and where the F6PALD region is between the MeDH region and the C-terminus of the fusion protein; (f) a fusion protein having MeDH, F6PALD and DHAS regions, where the MeDH region is between F6PALD and DHAS regions, and where the F6PALD region is between the MeDH region and the N-terminus of the

fusion protein and where the DHAS region is between the MeDH region and the C-terminus of the fusion protein; (g) a fusion protein having MeDH, F6PALD and DHAS regions, where the F6PALD region is between MeDH and DHAS regions, and where the MeDH region is between the F6PALD region and the N-terminus of the fusion protein and where the DHAS region is between the F6PALD region and the C-terminus of the fusion protein; (h) a fusion protein having MeDH, F6PALD and DHAS regions, where the F6PALD region is between MeDH and DHAS regions, and where the DHAS region is between the F6PALD region and the N-terminus of the fusion protein and where the MeDH region is between the F6PALD region and the C-terminus of the fusion protein; and (i) a fusion protein having ACT, MeDH, F6PALD and DHAS regions, where the MeDH and F6PALD regions are between ACT and DHAS regions, and where the ACT region is between the MeDH region and the N-terminus of the fusion protein and where the DHAS region is between the F6PALD region and the C-terminus of the fusion protein. Linker sequences can be present between any one or more regions of the fusion proteins of (a)-(i).

For example, Figure 7A is a schematic diagram of a fusion protein with MeDH region that extends from the N-terminus and a DHAS region that extends from the C-terminus. Such a fusion protein can be designated MeDH-DHAS. Optionally, if a polypeptide linker is between the two regions the fusion protein designated MeDH-L¹-DHAS.

As another embodiment, Figure 7B is a schematic diagram of a fusion protein with a MeDH region that is at the N-terminal portion of the protein and a F6PALD region that is at the C-terminal portion of the protein. Such a fusion protein can be designated MeDH-F6PALD. Optionally, if a polypeptide linker is between the two regions the fusion protein is designated MeDH-L¹-F6PALD.

As another embodiment, Figure 7C is a schematic diagram of a fusion protein with a MeDH region that is at the N-terminal portion of the protein and a DHAS region that is at the C-terminal portion of the protein, and a F6PALD region that is between the MeDH and DHAS regions. Such a fusion protein can be designated MeDH-F6PALD-DHAS. Optionally, if polypeptide linker(s) is/are between the different region(s) the fusion protein can be designated MeDH-L¹-F6PALD-DHAS, MeDH-F6PALD-L¹-DHAS, or MeDH-L¹-F6PALD-L²-DHAS.

As another embodiment, Figure 7D is a schematic diagram of a fusion protein with a MeDH region that is at the N-terminal portion of the protein and a F6PALD region that is at the C-terminal portion of the protein, and a DHAS region that is between the MeDH and F6PALD regions. Such a fusion protein can be designated MeDH-DHAS-F6PALD. Optionally, if polypeptide linker(s) is/are between the different region(s) the fusion protein can

be designated MeDH-L¹-DHAS-F6PALD, MeDH-DHAS-L¹-F6PALD, or MeDH-L¹-DHAS-L²-F6PALD.

As another embodiment, Figure 7E is a schematic diagram of a fusion protein with a MeDH region that is at the N-terminal portion of the protein and a DHAS region that is at the C-terminal portion of the protein, and an ACT region that is between the MeDH and DHAS regions. Such a fusion protein can be designated MeDH-ACT-DHAS. Optionally, if polypeptide linker(s) is/are between the different region(s) the fusion protein can be designated MeDH-L¹-ACT-DHAS, MeDH-ACT-L¹-DHAS, or MeDH-L¹-ACT-L²-DHAS.

As another embodiment, Figure 7F is a schematic diagram of a fusion protein with a MeDH region that is at the N-terminal portion of the protein and a F6PALD region that is at the C-terminal portion of the protein, and an ACT region that is between the MeDH and F6PALD regions. Such a fusion protein can be designated MeDH-ACT-F6PALD. Optionally, if polypeptide linker(s) is/are between the different region(s) the fusion protein can be designated MeDH-L¹-ACT-F6PALD, MeDH-ACT-L¹-F6PALD, or MeDH-L¹-ACT-L²-F6PALD.

As another embodiment, Figure 7G is a schematic diagram of a fusion protein with a MeDH region that is at the C-terminal portion of the protein and a F6PALD region that is at the N-terminal portion of the protein. Such a fusion protein can be designated F6PALD-MeDH. Optionally, if a polypeptide linker is between the two regions the fusion protein designated F6PALD-L¹-MeDH.

As another embodiment, Figure 7H is a schematic diagram of a fusion protein with a MeDH region that is at the C-terminal portion of the protein and a DHAS region that is at the N-terminal portion of the protein. Such a fusion protein can be designated DHAS-MeDH. Optionally, if a polypeptide linker is between the two regions the fusion protein designated DHAS-L¹-MeDH.

In other embodiments MeDH is positioned between two other regions of amino acid sequence. As another embodiment, Figure 8A is a schematic diagram of a fusion protein with a F6PALD region that is at the N-terminal portion of the protein and a DHAS region that is at the C-terminal portion of the protein, and a MeDH region between the F6PALD and DHAS regions. Such a fusion protein can be designated F6PALD-MeDH-DHAS. Optionally, if polypeptide linker(s) is/are between the different region(s) the fusion protein can be designated F6PALD-MeDH-L¹-DHAS, F6PALD-L¹-MeDH-DHAS, or F6PALD-L¹-MeDH-L²-DHAS.

As another embodiment, Figure 8B is a schematic diagram of a fusion protein with a DHAS region that is at the N-terminal portion of the protein and a F6PALD region that is at the C-terminal portion of the protein, and a MeDH region between the DHAS and F6PALD

regions. Such a fusion protein can be designated DHAS-MeDH-F6PALD. Optionally, if polypeptide linker(s) is/are between the different region(s) the fusion protein can be designated DHAS-MeDH-L¹-F6PALD, DHAS-L¹-MeDH-F6PALD, or DHAS-L¹-MeDH-L²-F6PALD.

As another embodiment, Figure 8C is a schematic diagram of a fusion protein with DHAS region that is at the N-terminal portion of the protein and an ACT region that is at the C-terminal portion of the protein, and a MeDH region between the DHAS and ACT regions,. Such a fusion protein can be designated DHAS-MeDH-ACT. Optionally, if polypeptide linker(s) is/are between the different region(s) the fusion protein can be designated DHAS-MeDH-L¹-ACT, DHAS-L¹-MeDH-ACT, or DHAS-L¹-MeDH-L²-ACT.

As another embodiment, Figure 8D is a schematic diagram of a fusion protein with F6PALD region that is at the N-terminal portion of the protein and an ACT region that is at the C-terminal portion of the protein, and a MeDH region between the F6PALD and ACT regions. Such a fusion protein can be designated F6PALD-MeDH-ACT. Optionally, if polypeptide linker(s) is/are between the different region(s) the fusion protein can be designated F6PALD-MeDH-L¹-ACT, F6PALD-L¹-MeDH-ACT, or F6PALD-L¹-MeDH-L²-ACT.

As another embodiment, Figure 8E is a schematic diagram of a fusion protein with ACT region that is at the N-terminal portion of the protein and a DHAS region that is at the C-terminal portion of the protein, and MeDH and F6PALD regions between the ACT and DHAS regions, with MeDH being closer to the ACT region and F6PALD being closer to the DHAS region. Such a fusion protein can be designated ACT-MeDH-F6PALD-DHAS. Optionally, if polypeptide linker(s) is/are between the different region the fusion protein can be designated ACT-L¹-MeDH-F6PALD-DHAS, ACT-MeDH-L¹-F6PALD-DHAS, ACT-MeDH-F6PALD-L¹-DHAS, ACT-L¹-MeDH-L²-F6PALD-DHAS, ACT-L¹-MeDH-F6PALD-L²-DHAS, ACT-MeDH-L¹-F6PALD-L²-DHAS, and ACT-L¹-MeDH-L²-F6PALD-L³-DHAS.

As another embodiment, Figure 8F is a schematic diagram of a fusion protein with ACT region that is at the N-terminal portion of the protein and a F6PALD region that is at the C-terminal portion of the protein, and MeDH and DHAS regions between the ACT and F6PALD regions, with MeDH being closer to the ACT region and DHAS being closer to the F6PALD region. Such a fusion protein can be designated ACT-MeDH-DHAS-F6PALD. Optionally, if polypeptide linker(s) is/are between the different region the fusion protein can be designated ACT-L¹-MeDH-DHAS-F6PALD, ACT-MeDH-L¹-DHAS-F6PALD, ACT-MeDH-DHAS-L¹-F6PALD, ACT-L¹-MeDH-L²-DHAS-F6PALD, ACT-L¹-MeDH-DHAS-L²-F6PALD, ACT-MeDH-L¹-DHAS-L²-F6PALD, and ACT-L¹-MeDH-L²-DHAS-L³-F6PALD.

As another embodiment, Figure 8G is a schematic diagram of a fusion protein with F6PALD region that is at the N-terminal portion of the protein and an ACT region that is at the

C-terminal portion of the protein, and DHAS and MeDH regions between the F6PALD and ACT regions, with DHAS being closer to the F6PALD region and MeDH being closer to the ACT region. Such a fusion protein can be designated F6PALD-DHAS-MeDH-ACT.

Optionally, if polypeptide linker(s) is/are between the different region the fusion protein can be designated F6PALD-L¹-DHAS-MeDH-ACT, F6PALD-DHAS-L¹-MeDH-ACT, F6PALD-DHAS-MeDH-L¹-ACT, F6PALD-L¹-DHAS-L²-MeDH-ACT, F6PALD-L¹-DHAS-MeDH-L²-ACT, F6PALD-DHAS-L¹-MeDH-L²-ACT, and F6PALD-L¹-DHAS-L²-MeDH-L³-ACT.

As another embodiment, Figure 8H is a schematic diagram of a fusion protein with DHAS region that is at the N-terminal portion of the protein and an ACT region that is at the C-terminal portion of the protein, and F6PALD and MeDH regions between the DHAS and ACT regions, with F6PALD being closer to the DHAS region and MeDH being closer to the ACT region. Such a fusion protein can be designated DHAS-F6PALD-MeDH-ACT.

Optionally, if polypeptide linker(s) is/are between the different region the fusion protein can be designated DHAS-L¹-F6PALD-MeDH-ACT, DHAS-F6PALD-L¹-MeDH-ACT, DHAS-F6PALD-MeDH-L¹-ACT, DHAS-L¹-F6PALD-L²-MeDH-ACT, DHAS-L¹-F6PALD-MeDH-L²-ACT, DHAS-F6PALD-L¹-MeDH-L²-ACT, and DHAS-L¹-F6PALD-L²-MeDH-L³-ACT.

In other embodiments, in the fusion protein the methanol dehydrogenase is interrupted by at least one different region of amino acid sequence, such as a DHAS or a F6PALD region. For example, the nucleic acid will encode, in frame, a polypeptide with a first portion of a MeDH and a DHAS and/or a F6PALD region, and then a second portion of a MeDH. Although the MeDH sequence is interrupted, the fusion protein still maintains methanol dehydrogenase activity.

For example, Figure 9A is a schematic diagram of a fusion protein having a first portion of a MeDH that is at the N-terminal portion of the protein, a second portion of a MeDH that is at the C-terminal portion of the protein, and DHAS region between, and in frame with, the first and second methanol dehydrogenase portions. Such fusion proteins can be designated MeDH(P1)-DHAS-MeDH(P2). Optionally, if polypeptide linker(s) is/are between the different portions and region(s) the fusion protein can be designated MeDH(P1)-L¹-DHAS-MeDH(P2), MeDH(P1)-DHAS-L¹-MeDH(P2), or MeDH(P1)-L¹-DHAS-L²-MeDH(P2).

As another embodiment, Figure 9B is a schematic diagram of a fusion protein having a first portion of a MeDH that is at the N-terminal portion of the protein, a second portion of a MeDH that is at the C-terminal portion of the protein, and F6PALD and DHAS regions between the first and second MeDH portions, with DHAS being closer to the first portion of the MeDH and F6PALD being closer to the second portion of the MeDH. Such a fusion protein can be designated MeDH(P1)-DHAS-F6PALD-MeDH(P2). Optionally, if polypeptide

linker(s) is/are between the different portions and region(s) the fusion protein can be designated MeDH(P1)-L¹-DHAS-F6PALD-MeDH(P2), MeDH(P1)-DHAS-L¹-F6PALD-MeDH(P2), MeDH(P1)-DHAS-F6PALD-L¹-MeDH(P2), MeDH(P1)-L¹-DHAS-L²-F6PALD-MeDH(P2), MeDH(P1)-L¹-DHAS-F6PALD-L²-MeDH(P2), MeDH(P1)-DHAS-L¹-F6PALD-L²-MeDH(P2), or MeDH(P1)-L¹-DHAS-L²-F6PALD-L³-MeDH(P2).

As another embodiment, Figure 9C is a schematic diagram of a fusion protein having an ACT region that is at the N-terminal portion of the protein, a second portion of a MeDH that is at the C-terminal portion of the protein, and a first portion of the MeDH and a F6PALD region between the ACT region and the second portion of a MeDH, with F6PALD being in between the first and second MeDH portions. Such a fusion protein can be designated ACT-MeDH(P1)-F6PALD-MeDH(P2). Optionally, if polypeptide linker(s) is/are between the different portions and region(s) the fusion protein can be designated ACT-L¹-MeDH(P1)-F6PALD-MeDH(P2), ACT-MeDH(P1)-L¹-F6PALD-MeDH(P2), ACT-MeDH(P1)-F6PALD-L¹-MeDH(P2), ACT-L¹-MeDH(P1)-L²-F6PALD-MeDH(P2), ACT-MeDH(P1)-L¹-F6PALD-L²-MeDH(P2), or ACT-L¹-MeDH(P1)-F6PALD-L²-MeDH(P2).

In specifically contemplated fusion protein embodiments above and elsewhere herein DHA Kinase (DHAK) replaces F6P Aldolase.

Table 1 includes a list of amino acid domains in the MeDH sequence *Bacillus methanolicus* MGA3 MeDH (SEQ ID NO: 1) predicted to have a secondary structure consistent with a coil. These domains represent sites for splitting the MeDH sequence and insertion of another open reading frame (with or without flanking linker sequences), such as shown in Figures 3A-3C. For example, the sequence AGTGSETT corresponding to amino acids 139-146 in *Bacillus methanolicus* MGA3 MeDH (SEQ ID NO: 1) represent a first domain having a predicted coil. A fusion protein of the disclosure could have a sequence with a split in the MeDH sequence anywhere within this domain, and insertion of one or more sequences selected from PHI, HPS, and ACT, or alternatively DHAS, F6Pald (or alternatively DHAK) and ACT. For example, for this domain the MeDH sequence can be split between amino acids 139 and 140, 140 and 141, 141 and 142, 142 and 143, 143 and 144, 144 and 145, or 145-146.

Table 1

Type	Sequence	Amino Acid positions
coil	AGTGSETT	139-146
coil	GIPSGY	334-339
coil	HSTGL	42-46
coil	AQPDP	67-71
coil	GSSHD	97-101
coil	VNSV	122-125
coil	IPP	7-9
coil	ITP	167-169

The secondary structure of any MeDH can be predicted using EMBOSS (EMBOSS: The European Molecular Biology Open Software Suite (2000) Rice, P. Longden, I. and Bleasby, A. Trends in Genetics 16, (6) pp276—277). The residues that constitute a predicted coil (residues which are not in any of the conformations such as helix, turn, or sheet) are proposed as sites where the MeDH sequence can be split and a second open reading frame inserted.

Embodiments of the invention include fusion proteins having a region with an amino acid sequence that provides methanol dehydrogenase (MeDH) activity. In the fusion protein, the MeDH amino acid sequence can be based on a full-length protein sequence, a partial sequence, such as one having N- and or C-terminal deletions, or a variant sequence, such as one having one or more amino acid substitutions that differ from the wild type sequence.

In some embodiments the fusion protein has first and second portions of a methanol dehydrogenase which are interrupted by another amino acid sequence that is different than the methanol dehydrogenase, such as a 3-hexulose-6-phosphate dehydrogenase or 6-phospho-3-hexuloisomerase (or alternatively DHAS or F6PAld; or in a further alternative DHAS or DHAK) amino acid sequence or an enzymatically active portion thereof, or methanol dehydrogenase activator or an enzymatically active portion thereof, positioned between the first and second portions of a methanol dehydrogenase.

The fusion protein can include a polypeptide sequence based on the NAD(P)+-dependent methanol dehydrogenase from *Bacillus methanolicus* MGA3 (Genbank Accession number EIJ77596.1, GI number: 387585261; designated herein as MeDH 2315, 382 amino acids long; SEQ ID NO: 1). MeDH 2315 is reported in the literature as an NAD(P)-dependent methanol dehydrogenase from *Bacillus methanolicus* MGA3 and its sequence was described in Brautaset *et al.*, “Plasmid-Dependent Methylo-trophy in Thermotolerant *Bacillus methanolicus*”, Journal of Bacteriology, vol. 186, pp1229-1238 (2004). It is also referred to as MeDH MGA3 in WO2013/110797 to Brautaset and MeDH “M” in Krog *et al.*,

“Methylotrophic *Bacillus methanolicus* Encodes Two Chromosomal and One Plasmid Born NAD⁺ Dependent Methanol Dehydrogenase Paralogs with Different Catalytic and Biochemical Properties”, PLOS ONE, pp. 1-11, (2013), which report additional wild-type *Bacillus* MeDHs. SEQ ID NO: 2 is a nucleic acid sequence encoding SEQ ID NO: 1.

The fusion protein of the disclosure can optionally have a methanol dehydrogenase sequence that is less than 100% identical to *Bacillus methanolicus* MGA3 (SEQ ID NO: 1). For example, the methanol dehydrogenase region in the fusion polypeptide can have 50% or greater, 60% or greater, 70% or greater, 80% or greater, 85% or greater, 90% or greater, 95% or greater, 96% or greater, 97% or greater, 98% or greater, or 99% or greater identity to (SEQ ID NO: 1).

In some cases, variants having less than 100% identity of SEQ ID NO:1 can be generated by sequence alignment of SEQ ID NO:1 with other known methanol dehydrogenases or alcohol dehydrogenases to identify regions that are conserved and/or important for enzymatic functioning of the protein. Once these regions are identified, the methanol dehydrogenase can be modified at one or more amino acid locations outside of these conserved regions. Therefore, the methanol dehydrogenase region of the fusion protein can have one or more amino acid substitutions, deletions, or additions which cause the sequence to vary from a native methanol dehydrogenase while retaining certain sequence features. Table 2 is a table of pairwise sequence identity of various methanol dehydrogenases including *Bacillus* *methanolicus* MGA3 MeDH (SEQ ID NO: 1).

Table 2

Pairwise alignment (%ID)					
MeDH	<i>Bacillus methanolicus</i> MGA3 ¹	<i>Bacillus methanolicus</i> MGA3 ²	<i>Bacillus methanolicus</i> PB1	<i>Lysinibacillus fusiformis</i>	<i>Clostridium perfringens</i> str. 13
<i>Bacillus methanolicus</i> MGA3 ¹	100	62	60.7	58.4	48.7
<i>Bacillus methanolicus</i> MGA3 ²	62	100	92.7	72.2	53.2
<i>Bacillus methanolicus</i> PB1	60.7	92.7	100	72.2	53.5
<i>Lysinibacillus fusiformis</i>	58.4	72.2	72.2	100	51.2
<i>Clostridium perfringens</i> str. 13	48.7	53.2	53.5	51.2	100

1: EIJ77596.1

2: EIJ83020.1

In other cases, variants having less than 100% identity of SEQ ID NO:1 can be generated by known variants of methanol dehydrogenases, such as described in the art. Such variants may provide increased catalytic activity in the fusion protein, such as increased conversion of methanol to formaldehyde, which is then subsequently converted into a ketose

phosphate compound such as hexulose 6-phosphate, or fructose 6-phosphate due to the localization of HPS and/or PSI in the fusion protein.

Exemplary variants of *Bacillus methanolicus* MGA3 MeDH (SEQ ID NO: 1) are described in International Patent Application No. PCT/US2014/059135, the disclosure of which is incorporated herein. A fusion protein including a MeDH region of the current disclosure can include one or more amino acid substitutions based on these variants.

Exemplary amino acid substitutions of SEQ ID NO: 1 include, but are not limited to, those as follows: S11T, D38N, H42Q, E48D, N53I, E56K, D60E, V61A, I63F, P65Q, D70N, P71I, P71T, P71V, T74S, D81G, K84R, E86K, N87K, I94V, S99P, S99T, A103V, I106L, G107S, L108V, L108W, V109Y, N112K, N112R, R115H, I116F, N117D, N117Q, N117Y, Q120H, Q120R, G121A, G121D, G121E, G121L, G121M, G121R, G121S, G121T, G121V, G121W, G121Y, V122A, V122P, N123D, N123I, N123L, N123R, N123Y, S124I, S124L, S124R, V125C, V125G, V125W, E126G, E126V, K127C, K127R, P128A, P128R, P128S, V129A, V129M, V129P, V129S, V130F, V130I, V130Y, A134T, S143T, T146N, S147R, L148A, L148F, L148G, L148I, L148T, L148V, L148W, A149L, A149M, A149T, A149V, V150A, V150I, T152M, A155V, K157N, V158E, V158H, V158K, V158W, P161A, P161G, P161Q, P161S, P161V, I163F, I163N, I163Q, I163T, D164G, D164N, E165G, K181R, A184T, L186M, T190A, T190S, I199V, Q217K, L226M, G256C, Q267H, G269S, G270M, G270S, G270Y, T296S, R298H, A300T, I302V, G312V, A316V, I323M, F333L, P336L, S337C, G343D, V344A, V344G, K345E, E350K, K354M, N355D, N355I, N355K, E358G, V360A, V360G, V360K, V360R, V360S, C361N, C361R, Q363K, and K379M.

Other exemplary amino acid substitutions of SEQ ID NO: 1 include: D38N, D60E, P71I, P71V, N87K, S99T, A103V, G107S, L108V, L108W, V109Y, R115H, I116F, N117D, N117Q, G121D, G121E, G121L, G121M, G121R, G121S, G121T, G121V, G121W, G121Y, V122P, N123D, N123I, N123L, N123R, N123Y, S124I, S124L, V125C, V125G, V125W, E126G, K127C, K127R, P128A, P128R, P128S, V129A, V129M, V129P, V129S, V130F, V130I, V130Y, A134T, S143T, T146N, A149L, A149M, A149T, A149V, V150A, K157N, V158E, V158H, V158K, V158W, I163Q, D164N, Q267H, G270M, G270S, G270Y, K345E, N355D, V360G, V360K, V360R, V360S, C361R.

In exemplary embodiments a fusion protein including a MeDH region of the current disclosure includes one, two, three, or all four amino acid substitutions selected from the following group: V129M, V130I, G270S, and N355D. An exemplary MeDH variant is SEQ ID NO: 8 which is encoded by the nucleic acid sequence SEQ ID NO: 9.

Corresponding amino acid substitutions can also be made in other methanol dehydrogenase sequences based on alignment of SEQ ID NO: 1.

SEQ ID NO: 1, or an amino acid sequence with at least 50% or greater identity to SEQ ID NO: 1, can be used in any of the fusion proteins of the disclosure, including, but not limited to MeDH-PHI, MeDH-HPS, MeDH-HPS-PHI, MeDH-ACT-PHI, MeDH-ACT-HPS, HPS-MeDH, PHI-MeDH, HPS-MeDH-PHI, PHI-MeDH-HPS, PHI-MeDH-ACT, HPS-MeDH-
 5 ACT, ACT-MeDH-PHI-HPS, ACT-MeDH-HPS-PHI, HPS-PHI-MeDH-ACT, PHI-HPS-MeDH-ACT, MeDH(P1)-PHI-MeDH(P2), MeDH(P1)-HPS-PHI-MeDH(P2), and ACT-MeDH(P1)-HPS-MeDH(P2), including those fusion proteins having one or more linker sequences between regions/portions of the fusion protein. In another embodiment SEQ ID NO: 1, or an amino acid sequence with at least 50% or greater identity to SEQ ID NO: 1, can
 10 be used in any of the fusion proteins of the disclosure, including, but not limited to MeDH-DHAS, MeDH-F6PALD, MeDH-F6PALD-DHAS, MeDH-ACT-DHAS, MeDH-ACT-F6PALD, F6PALD-MeDH, DHAS-MeDH, F6PALD-MeDH-DHAS, DHAS-MeDH-F6PALD, DHAS-MeDH-ACT, F6PALD-MeDH-ACT, ACT-MeDH-DHAS-F6PALD, ACT-MeDH-F6PALD-DHAS, F6PALD-DHAS-MeDH-ACT, DHAS-F6PALD-MeDH-ACT, MeDH(P1)-
 15 DHAS-MeDH(P2), MeDH(P1)-F6PALD-DHAS-MeDH(P2), and ACT-MeDH(P1)-F6PALD-MeDH(P2), including those fusion proteins having one or more linker sequences between regions/portions of the fusion protein.

An exemplary MeDH having at least 50% or greater identity to SEQ ID NO: 1 is SEQ ID NO: 10 which is encoded by the nucleic acid sequence SEQ ID NO: 11.

20 The fusion protein can include a polypeptide sequence based on other methanol dehydrogenase sequences, including those known in the art. Methanol dehydrogenases are of the enzyme class (EC) 1.1.1. Other MeDH sequences include *Bacillus methanolicus* MGA3 MeDH (3EIJ77596.1); *Bacillus methanolicus* C1 MeDH (AAA22593.1); *Bacillus methanolicus* PB1 MeDH (EIJ77618.1); *Bacillus methanolicus* PB1 MeDH (EIJ78790.1);
 25 *Bacillus methanolicus* MGA3 MeDH (EIJ80770.1); *Bacillus methanolicus* PB1 MeDH (EIJ78397.1); *Bacillus methanolicus* MGA3 MeDH (EIJ83020.1); *Lysinibacillus fusiformis* MeDH (EFI69743.1); *Bacillus coagulans* 36D1 MeDH (YP_004860127.1); *Lysinibacillus sphaericus* MeDH (YP_001699778.1); *Bacillus azotoformans* LMG 9581 MeDH (ZP_11313277.1); *Burkholderia thailandensis* E264 MeDH (ZP_05587334.1); *Cupriavidus necator* N-1 MeDH (YP_004681552.1); uncultured organism MeDH (AGF87161); *Geobacter bemidjiensis* Bem MeDH (YP_002138168.1); *Carboxydotherrmus hydrogenoformans* Z-2901 MeDH (YP_359772.1); *Actinobacillus succinogenes* 130Z MeDH (YP_001343716.1);
 30 *Acinetobacter baumannii* Naval-82 MeDH (ZP_16224338.1); *Clostridium pasteurianum* DSM 525 MeDH (AAC45651.1); *Methanosarcina mazei* Tuc01 MeDH (YP_007491369.1);
 35 *Desulfovibrio vulgaris* str. 'Miyazaki F' MeDH (YP_002434746); *Desulfovibrio africanus* str.

Walvis Bay MeDH (YP_005052855); *Clostridium perfringens* str. 13 MeDH (NP_561852.1); *Vibrio campbellii* ATCC BAA-1116 MeDH (YP_001447544); *Desulfotomaculum reducens* MI-1 MeDH (YP_001113612.1); *Desulfovibrio vulgaris* str. Hildenborough MeDH (YP_011618); *Photobacterium profundum* 3TCK MeDH (ZP_01220157.1); *Geobacillus* sp. Y4.1MC1 MeDH (YP_003990729.1); *Desulfovibrio fructosovorans* JJ MeDH (ZP_07335453.1); *Shewanella oneidensis* MR-1 MeDH (NP_717107); *Sebaldella termitidis* ATCC 33386 MeDH (YP_003310546.1); *Paenibacillus peoriae* KCTC 3763 MeDH (ZP_10241531.1); *Klebsiella pneumoniae* subsp. *pneumoniae* MGH 78578 MeDH (YP_001337153.1); *Escherichia coli* MeDH (YP_026233.1); *Clostridium perfringens* ATCC 13124 MeDH (YP_694908); *Ralstonia eutropha* H16 MeDH (YP_725376.1); *Thermoanaerobacter* sp. X514 MeDH (YP_001663549); human gut metagenome MeDH (EKC54576); or *Geobacillus themodenitrificans* NG80-2 MeDH (YP_001126968.1). One of skill can modify the MeDH with one or more amino acid substitutions, deletions, and/or additions based on alignment of MeDH sequences, including those known in the art.

Any of these MeDH sequences, or an amino acid sequence with at least 50% or greater identity to these sequences, can be used in any of the fusion proteins of the disclosure, including, but not limited to MeDH-PHI, MeDH-HPS, MeDH-HPS-PHI, MeDH-ACT-PHI, MeDH-ACT-HPS, HPS-MeDH., PHI-MeDH, HPS-MeDH-PHI, PHI-MeDH-HPS, PHI-MeDH-ACT, HPS-MeDH-ACT, ACT-MeDH-PHI-HPS, ACT-MeDH-HPS-PHI, HPS-PHI-MeDH-ACT, PHI-HPS-MeDH-ACT, MeDH(P1)-PHI-MeDH(P2), MeDH(P1)-HPS-PHI-MeDH(P2), and ACT-MeDH(P1)-HPS-MeDH(P2), including those fusion proteins having one or more linker sequences between regions/portions of the fusion protein. In embodiments of MeDH with DHA Pathway enzymes, any of these MeDH sequences, or an amino acid sequence with at least 50% or greater identity to these sequences, can be used in any of the fusion proteins of the disclosure, including, but not limited to MeDH-DHAS, MeDH-F6PALD, MeDH-F6PALD-DHAS, MeDH-ACT-DHAS, MeDH-ACT-F6PALD, F6PALD-MeDH., DHAS-MeDH, F6PALD-MeDH-DHAS, DHAS-MeDH-F6PALD, DHAS-MeDH-ACT, F6PALD-MeDH-ACT, ACT-MeDH-DHAS-F6PALD, ACT-MeDH-F6PALD-DHAS, F6PALD-DHAS-MeDH-ACT, DHAS-F6PALD-MeDH-ACT, MeDH(P1)-DHAS-MeDH(P2), MeDH(P1)-F6PALD-DHAS-MeDH(P2), and ACT-MeDH(P1)-F6PALD-MeDH(P2), including those fusion proteins having one or more linker sequences between regions/portions of the fusion protein.

In some embodiments of the disclosure, an engineered cell can express one or more fusion proteins that include a MeDH region, in addition to a MeDH protein that is not fused to another protein (e.g., a MeDH protein in wild-type form). Expression of a non-fused MeDH

protein, in combination with MeDH fusion proteins that include a 3-hexulose-6-phosphate dehydrogenase activity or 6-phospho-3-hexuloisomerase region can allow expression of desired amounts of polypeptide conferring MeDH, HPS, PHI, and ACT in the cell. As discussed herein, MeDH and MeDH fusion proteins can form a multi-protein complex and predetermined amounts of MeDH, HPS, PHI, and ACT can be present in the multi-protein complex to provide optimal conversion of methanol through d-arabino-3-hexulose-6-phosphate to fructose 6-phosphate. In embodiments of MeDH with the DHA Pathway enzymes of the disclosure, an engineered cell can express one or more fusion proteins that include a MeDH region, in addition to a MeDH protein that is not fused to another protein (e.g., a MeDH protein in wild-type form). Expression of a non-fused MeDH protein, in combination with MeDH fusion proteins that include a DHAS or F6PAld (or DHAK) region can allow expression of desired amounts of polypeptide conferring MeDH, DHAS, F6PAld (or DHAK), and ACT in the cell. As discussed herein, MeDH and MeDH fusion proteins can form a multi-protein complex and predetermined amounts of MeDH, DHAS, F6PAld (or DHAK), and ACT can be present in the multi-protein complex to provide optimal conversion of methanol through DHA and G3P to fructose 6-phosphate.

Other suitable methanol dehydrogenase sequences that can be used in the fusion protein of the disclosure include those that are “orthologs” of known methanol dehydrogenase sequences, such as SEQ ID NO: 1, can be identified and part or all of its sequence can be used to prepare the fusion proteins of the disclosure. An ortholog is a gene or genes that are related by vertical descent and are responsible for substantially the same or identical functions in different organisms. Genes are related by vertical descent when, for example, they share sequence similarity of sufficient amount to indicate they are homologous, or related by evolution from a common ancestor. Genes can also be considered orthologs if they share three-dimensional structure but not necessarily sequence similarity, of a sufficient amount to indicate that they have evolved from a common ancestor to the extent that the primary sequence similarity is not identifiable. Genes that are orthologous can encode proteins with sequence similarity of about 45% to 100% amino acid sequence identity, and more preferably about 60% to 100% amino acid sequence identity.

For example, genes sharing a desired amount of identity (e.g., 45%, 50%, 55%, or 60% or greater) to the NAD(P)-dependent methanol dehydrogenase from *Bacillus methanolicus* MGA3 (2315A), including orthologs and paralogs, can be determined by methods well known to those skilled in the art. For example, inspection of nucleic acid or amino acid sequences for two polypeptides will reveal sequence identity and similarities between the compared sequences. Based on such similarities, one skilled in the art can determine if the similarity is

sufficiently high to indicate the proteins are related through evolution from a common ancestor.

Computational approaches to sequence alignment and generation of sequence identity include global alignments and local alignments. Global alignment uses global optimization to forces alignment to span the entire length of all query sequences. Local alignments, by contrast, identify regions of similarity within long sequences that are often widely divergent overall. For understanding the identity of a target sequence to a known methanol dehydrogenase, such as

For the *Bacillus methanolicus* MGA3 (2315A) template, a global alignment can be used. Optionally, amino terminal and/or carboxyl-terminal sequences of the target sequence that share little or no identity with the template sequence can be excluded for a global alignment and generation of an identity score.

Algorithms well known to those skilled in the art, such as Align, BLAST, Clustal W and others compare and determine a raw sequence similarity or identity, and also determine the presence or significance of gaps in the sequence which can be assigned a weight or score. Default parameters can be used for the alignment and BLOSUM62 as the scoring matrix.

Such algorithms also are known in the art and are similarly applicable for determining nucleotide sequence similarity or identity. Parameters for sufficient similarity to determine relatedness are computed based on well-known methods for calculating statistical similarity, or the chance of finding a similar match in a random polypeptide, and the significance of the match determined. A computer comparison of two or more sequences can, if desired, also be optimized visually by those skilled in the art. Related gene products or proteins can be expected to have a high similarity, for example, 45% to 100% sequence identity. Proteins that are unrelated can have an identity which is essentially the same as would be expected to occur by chance, if a database of sufficient size is scanned (about 5%).

In some cases, it can be useful to use the Basic Local Alignment Search Tool (BLAST) algorithm to understand the sequence identity between an amino acid motif in a template sequence and a target sequence. Therefore, in preferred modes of practice, BLAST is used to identify or understand the identity of a shorter stretch of amino acids (e.g. a sequence motif between a template and a target protein. BLAST finds similar sequences using a heuristic method that approximates the Smith-Waterman algorithm by locating short matches between the two sequences. The (BLAST) algorithm can identify library sequences that resemble the query sequence above a certain threshold. Exemplary parameters for determining relatedness of two or more sequences using the BLAST algorithm, for example, can be as set forth below. Briefly, amino acid sequence alignments can be performed using BLASTP version 2.0.8 (Jan-

05-1999) and the following parameters: Matrix: 0 BLOSUM62; gap open: 11; gap extension: 1; x_dropoff: 50; expect: 10.0; wordsize: 3; filter: on. Nucleic acid sequence alignments can be performed using BLASTN version 2.0.6 (Sept-16-1998) and the following parameters: Match: 1; mismatch: -2; gap open: 5; gap extension: 2; x_dropoff: 50; expect: 10.0; wordsize: 11; filter: off. Those skilled in the art will know what modifications can be made to the above parameters to either increase or decrease the stringency of the comparison, for example, and determine the relatedness of two or more sequences.

The enzyme 3-hexulose-6-phosphate synthase (HPS) can carry out the fixation of formaldehyde with ribulose 5-phosphate (Ru5P) to form d-arabino-3-hexulose-6-phosphate (Hu6P). The fusion protein can include a polypeptide sequence based on *Bacillus methanolicus* MGA HPS (Genbank Accession number AAR39392.1, 211 amino acids long; SEQ ID NO: 3).

The fusion protein of the disclosure can optionally have a 3-hexulose-6-phosphate synthase sequence that is less than 100% identical to *Bacillus methanolicus* MGA HPS (SEQ ID NO: 3). For example, the methanol dehydrogenase region in the fusion polypeptide can have 50% or greater, 60% or greater, 70% or greater, 80% or greater, 85% or greater, 90% or greater, 95% or greater, 96% or greater, 97% or greater, 98% or greater, or 99% or greater identity to SEQ ID NO: 3.

In some cases, variants having less than 100% identity of SEQ ID NO: 3 can be generated by sequence alignment of SEQ ID NO: 3 with other known 3-hexulose-6-phosphate synthase to identify regions that are conserved and/or important for enzymatic functioning of the protein. Once these regions are identified, the 3-hexulose-6-phosphate synthase can be modified at one or more amino acid locations outside of these conserved regions. Therefore, the 3-hexulose-6-phosphate synthase region of the fusion protein can have one or more amino acid substitutions, deletions, or additions which cause the sequence to vary from a native methanol dehydrogenase while retaining certain sequence features.

SEQ ID NO: 3, or an amino acid sequence with at least 50% or greater identity to SEQ ID NO: 3, can be used in fusion proteins of the disclosure, including, but not limited to MeDH-PHI, MeDH-HPS, MeDH-HPS-PHI, MeDH-ACT-PHI, MeDH-ACT-HPS, HPS-MeDH, PHI-MeDH, HPS-MeDH-PHI, PHI-MeDH-HPS, PHI-MeDH-ACT, HPS-MeDH-ACT, ACT-MeDH-PHI-HPS, ACT-MeDH-HPS-PHI, HPS-PHI-MeDH-ACT, PHI-HPS-MeDH-ACT, MeDH(P1)-HPS-PHI-MeDH(P2), ACT-MeDH(P1)-HPS-MeDH(P2), including those fusion proteins having one or more linker sequences between regions/portions of the fusion protein.

The fusion protein can include a polypeptide sequence based on other 3-hexulose-6-phosphate dehydrogenase sequences, including those known in the art. 3-hexulose-6-

phosphate dehydrogenases are of the enzyme class (EC) 4.1.2.43. Other 3-hexulose-6-phosphate dehydrogenases include, but are not limited to, *Bacillus methanolicus* PB1 HPS (EIJ81375.1); *Methylobacillus flagellatus* HPS (YP 544362.1); *Methylobacillus flagellatus* HPS (YP 544363.1); *Bacillus subtilis* HPS (NP_388228.1); *Methylophilus methylotrophus* HPS (WP_018986666.1); *Methylophilus methylotrophus* ATCC 53528 HPS (WP_018985298.1); *Aminomonas aminovorans* HPS (AAG29505.1), *Amycolatopsis methanolica* 239 HPS (AIJ24611.1); *Geobacillus sp.* GHH01 HPS (YP_007402409.1); *Geobacillus sp.* M10EXG HPS (AAR91478.1); *Geobacillus sp.* Y4.1MC1 HPS (YP_003990382.1); *Geobacillus thermodenitrificans* NG80-2 HPS (WP_008879217.1); *Methylomonas aminofaciens* HPS (BAA83096.1); *Methylovorus glucosetrophus* SIP3-4 HPS (YP_003050044.1); *Methylovorus sp.* MP688 HPS (YP_004038706.1); and *Mycobacterium gastri* HPS (BAA90546.1).

Any of these HPS sequences, or an amino acid sequence with at least 50% or greater identity to these sequences, can be used in fusion proteins of the disclosure, including, but not limited to MeDH-PHI, MeDH-HPS, MeDH-HPS-PHI, MeDH-ACT-PHI, MeDH-ACT-HPS, HPS-MeDH., PHI-MeDH, HPS-MeDH-PHI, PHI-MeDH-HPS, PHI-MeDH-ACT, HPS-MeDH-ACT, ACT-MeDH-PHI-HPS, ACT-MeDH-HPS-PHI, HPS-PHI-MeDH-ACT, PHI-HPS-MeDH-ACT, MeDH(P1)-HPS-PHI-MeDH(P2), ACT-MeDH(P1)-HPS-MeDH(P2), including those fusion proteins having one or more linker sequences between regions/portions of the fusion protein.

Other suitable 3-hexulose-6-phosphate dehydrogenase sequences that can be used in the fusion protein of the disclosure include those that are “orthologs” of known 3-hexulose-6-phosphate dehydrogenase sequences, such as orthologs of SEQ ID NO: 3, can be identified and part or all of its sequence can be used to prepare the fusion proteins of the disclosure.

One of skill can modify the HPS with one or more amino acid substitutions, deletions, and/or additions based on alignment of HPS sequences, including those known in the art. Tables 3A and 3B are tables of pairwise sequence identity of various 3 hexulose 6 phosphate dehydrogenase sequences including *Bacillus methanolicus* MGA HPS (SEQ ID NO: 3).

Table 3A

Pairwise alignment (% ID)							
HPS	<i>Geobacillus thermodenitrificans</i> NG80-2	<i>Bacillus methanolicus</i> MGA3	<i>Bacillus methanolicus</i> PB1	<i>Geobacillus</i> sp. GHH01	<i>Geobacillus</i> sp. M10EXG	<i>Geobacillus</i> sp. Y4.1MC1	<i>Methylophilus methylotrophus</i>
<i>Geobacillus thermodenitrificans</i> NG80-2	100	30.8	30.8	30.3	29.9	29.9	31.8
<i>Bacillus methanolicus</i> MGA3	30.8	100	98.1	76.8	76.8	77.3	38.4
<i>Bacillus methanolicus</i> PB1	30.8	98.1	100	76.3	76.3	76.8	37.9
<i>Geobacillus</i> sp. GHH01	30.3	76.8	76.3	100	97.6	98.1	38.9
<i>Geobacillus</i> sp. M10EXG	29.9	76.8	76.3	97.6	100	99.5	38.4
<i>Geobacillus</i> sp. Y4.1MC1	29.9	77.3	76.8	98.1	99.5	100	38.4
<i>Methylophilus methylotrophus</i>	31.8	38.4	37.9	38.9	38.4	38.4	100
<i>Methylophilus methylotrophus</i> ATCC 53528	32.1	34.4	34	38.3	37.8	37.8	81.9
<i>Methylobacillus flagellatus</i>	33.2	37.5	37	40.9	40.4	40.4	83.7
<i>Methylomonas aminofaciens</i>	32.7	36.5	36.1	39.9	39.4	39.4	84.7
<i>Methylovorus glucosetrophus</i> SIP3-4	31.5	37.2	36.7	38.2	37.7	37.7	80.4
<i>Aminomonas aminovorans</i>	31.3	37	36.5	37.9	37.4	37.4	76.1
<i>Methylobacillus flagellatus</i>	31.3	37	36.5	37.9	37.4	37.4	76.5
<i>Amycolatopsis methanolica</i> 239	33.8	40	38.6	37.6	38.1	38.1	46.2
<i>Mycobacterium gastri</i>	32.7	39.4	39.9	39.4	38.9	38.9	46.6

Table 3B

Pairwise alignment (% ID)								
HPS	<i>Methylophilus methylotrophus</i> ATCC 53528	<i>Methylobacillus flagellatus</i>	<i>Methylomonas aminofaciens</i>	<i>Methylovorus glucosetrophus</i> SIP3-4	<i>Aminomonas aminovorans</i>	<i>Methylobacillus flagellatus</i>	<i>Amycolatopsis methanolica</i> 239	<i>Mycobacterium gastri</i>
<i>Geobacillus thermodenitrificans</i> NG80-2	32.1	33.2	32.7	31.5	31.3	31.3	33.8	32.7
<i>Bacillus methanolicus</i> MGA3	34.4	37.5	36.5	37.2	37	37	40	39.4
<i>Bacillus methanolicus</i> PB1	34	37	36.1	36.7	36.5	36.5	38.6	39.9
<i>Geobacillus</i> sp. GHH01	38.3	40.9	39.9	38.2	37.9	37.9	37.6	39.4
<i>Geobacillus</i> sp. M10EXG	37.8	40.4	39.4	37.7	37.4	37.4	38.1	38.9
<i>Geobacillus</i> sp. Y4.1MC1	37.8	40.4	39.4	37.7	37.4	37.4	38.1	38.9
<i>Methylophilus methylotrophus</i>	81.9	83.7	84.7	80.4	76.1	76.5	46.2	46.6
<i>Methylophilus methylotrophus</i> ATCC 53528	100	87.1	87.6	82	83.3	83.8	47.1	47.1
<i>Methylobacillus flagellatus</i>	87.1	100	97.1	88.2	87.6	86.6	47.4	47.1
<i>Methylomonas aminofaciens</i>	87.6	97.1	100	89.2	86.1	86.1	46.9	45.7
<i>Methylovorus glucosetrophus</i> SIP3-4	82	88.2	89.2	100	90.5	90.5	46.6	45.6
<i>Aminomonas aminovorans</i>	83.3	87.6	86.1	90.5	100	97.4	47.6	47.1
<i>Methylobacillus flagellatus</i>	83.8	86.6	86.1	90.5	97.4	100	47.6	46.2
<i>Amycolatopsis methanolica</i> 239	47.1	47.4	46.9	46.6	47.6	47.6	100	60.1
<i>Mycobacterium gastri</i>	47.1	47.1	45.7	45.6	47.1	46.2	60.1	100

6-phospho-3-hexuloisomerase activity (PHI), can carry out the isomerization of d-arabino-3-hexulose-6-phosphate (Hu6P) to fructose 6-phosphate (F6P). The fusion protein can include a polypeptide sequence based on *Bacillus methanolicus* MGA3 PHI (Genbank Accession number AAR39393.1, 184 amino acids long; SEQ ID NO: 4).

The fusion protein of the disclosure can optionally have a 6-phospho-3-hexuloisomerase sequence that is less than 100% identical to *Bacillus methanolicus* MGA3 PHI (SEQ ID NO: 4). For example, the methanol dehydrogenase region in the fusion polypeptide can have 50% or greater, 60% or greater, 70% or greater, 80% or greater, 85% or greater, 90% or greater, 95% or greater, 96% or greater, 97% or greater, 98% or greater, or 99% or greater identity to (SEQ ID NO: 4).

In some cases, variants having less than 100% identity of SEQ ID NO: 4 can be generated by sequence alignment of SEQ ID NO: 4 with other known 6-phospho-3-hexuloisomerase to identify regions that are conserved and/or important for enzymatic functioning of the protein. Once these regions are identified, the 6-phospho-3-hexuloisomerase can be modified at one or more amino acid locations outside of these conserved regions. Therefore, the 6-phospho-3-hexuloisomerase region of the fusion protein can have one or more amino acid substitutions, deletions, or additions which cause the sequence to vary from a native 6-phospho-3-hexuloisomerase while retaining certain sequence features.

SEQ ID NO: 4, or an amino acid sequence with at least 50% or greater identity to SEQ ID NO: 4, can be used in fusion proteins of the disclosure, including, but not limited to MeDH-PHI, MeDH-HPS-PHI, ACT-MeDH-HPS-PHI, PHI-MeDH, HPS-MeDH-PHI, PHI-MeDH-HPS, MeDH(P1)-PHI-MeDH(P2), MeDH(P1)-HPS-PHI-MeDH(P2), including those fusion proteins having one or more linker sequences between regions/portions of the fusion protein.

The fusion protein can include a polypeptide sequence based on other 6-phospho-3-hexuloisomerase activity sequences, including those known in the art. 6-phospho-3-hexuloisomerases are of the enzyme class (EC) 5.3.1.27. Other PHI sequences include, but are not limited to *Bacillus methanolicus* PB1 PHI (EIJ81376.1); *Mycobacterium gastri* PB1 PHI (BAA90545.1); *Methylobacillus flagellatus* KT PHI (YP_545762.1); *Bacillus subtilis* PHI (NP_388227.1); *Methylophilus methylotrophus* ATCC 53528 HPS (WP_018985297.1); *Amycolatopsis methanolica* 239 PHI (AIJ24609.1); *Geobacillus sp.* GHH01 PHI (YP_007402408.1); *Geobacillus sp.* Y4.1MC1 PHI (YP_003990383.1); *Geobacillus thermodenitrificans* NG80-2 PHI (WP_011887353.1); *Methylomonas aminofaciens* PHI (BAA83098.1); *Methylovorus glucosetrophus* SIP3-4 PHI (YP_003051269.1); and *Methylovorus sp.* MP688 PHI (ADQ84715.1).

Any of these PHI sequences, or an amino acid sequence with at least 50% or greater identity to these sequences, can be used in fusion proteins of the disclosure, including, but not limited to MeDH-PHI, MeDH-HPS-PHI, ACT-MeDH-HPS-PHI, PHI-MeDH, HPS-MeDH-

PHI, PHI-MeDH-HPS, MeDH(P1)-PHI-MeDH(P2), MeDH(P1)-HPS-PHI-MeDH(P2), including those fusion proteins having one or more linker sequences between regions/portions of the fusion protein.

One of skill can modify the PHI with one or more amino acid substitutions, deletions, and/or additions based on alignment of PHI sequences, including those known in the art. Tables 4A and 4B are tables of pairwise sequence identity of various 6-phospho-3-hexuloisomerase sequences including *Bacillus methanolicus* MGA3 PHI (SEQ ID NO: 4).

Table 4A

Pairwise alignment (% ID)					
PHI	<i>Methylobacillus flagellatus</i> KT	<i>Methylomonas aminofaciens</i>	<i>Methylophilus methylotrophus</i> ATCC 53528	<i>Methylovorus glucosetrophus</i> SIP3-4	<i>Geobacillus thermodenitrificans</i> NG80-2
<i>Methylobacillus flagellatus</i> KT	100	95.6	58.9	64.4	31.1
<i>Methylomonas aminofaciens</i>	95.6	100	59.4	65	30.6
<i>Methylophilus methylotrophus</i> ATCC 53528	58.9	59.4	100	86.5	28
<i>Methylovorus glucosetrophus</i> SIP3-4	64.4	65	86.5	100	29.7
<i>Geobacillus thermodenitrificans</i> NG80-2	31.1	30.6	28	29.7	100
<i>Bacillus methanolicus</i> MGA3	32.4	33	32	33.1	37.2
<i>Bacillus methanolicus</i> PB1	32.4	33	32	33.1	37.2
<i>Geobacillus</i> sp. GHHD1	35	34.4	32.4	32.4	41.5
<i>Geobacillus</i> sp. Y4.1MC1	33.9	34.4	33	33.5	39.9
<i>Amycolatopsis methanolica</i> 239	31.3	31.9	27.1	27.6	36.6
<i>Mycobacterium gastri</i>	33	33	31.5	33.1	37.7

Table 4B

Pairwise alignment (% ID)						
PHI	<i>Bacillus methanolicus</i> MGA3	<i>Bacillus methanolicus</i> PB1	<i>Geobacillus</i> sp. GH401	<i>Geobacillus</i> sp. Y4.1MC1	<i>Amycolatopsis methanolica</i> 239	<i>Mycobacterium</i> <i>gastri</i>
<i>Methylobacillus flagellatus</i> KT	32.4	32.4	35	33.9	31.3	33
<i>Methylobacterium aminofaciens</i>	33	33	34.4	34.4	31.9	33
<i>Methylophilus methylotrophus</i> ATCC 53528	32	32	32.4	33	27.1	31.5
<i>Methylovorus glucosetrophus</i> SIP3-4	33.1	33.1	32.4	33.5	27.6	33.1
<i>Geobacillus thermodenitrificans</i> NG80-2	37.2	37.2	41.5	39.9	36.6	37.7
<i>Bacillus methanolicus</i> MGA3	100	98.9	75.1	76.8	32.6	37.5
<i>Bacillus methanolicus</i> PB1	98.9	100	74.6	76.2	32.6	38.6
<i>Geobacillus</i> sp. GH401	75.1	74.6	100	93	33	36.2
<i>Geobacillus</i> sp. Y4.1MC1	76.8	76.2	93	100	32.6	38
<i>Amycolatopsis methanolica</i> 239	32.6	32.6	33	32.6	100	47.7
<i>Mycobacterium gastri</i>	37.5	38.6	36.2	38	47.7	100

Engineered cells of the disclosure can also express MeDH-containing fusion proteins that include sequences of naturally-occurring HPS-PHI fusion proteins, or variants thereof.

The engineered fusion protein can include a polypeptide sequence based on natural HPS-PHI fusions, such as, a fusion from *Methylococcus capsulatus* (YP_115138.1); *Methylomicrobium album* BG8 (EIC30826.1); *Pyrococcus abyssi* (NP_127388.1); *Pyrococcus furiosus* (NP_577949.1); *Pyrococcus horikoshii* OT3 (NP_143767.1), or *Thermococcus kodakaraensis* (YP_182888.1). An exemplary HPS-PHI fusion is SEQ ID NO: 5.

Any of these HPS-PHI sequences, or an amino acid sequence with at least 50% or greater identity to these sequences, can be used in the fusion proteins of the disclosure, such as MeDH-PHI, MeDH-HPS-PHI, ACT-MeDH-HPS-PHI, and MeDH(P1)-HPS-PHI-MeDH(P2), including those fusion proteins having one or more linker sequences between regions/portions of the fusion protein.

Engineered cells of the disclosure (whether using HPA and PHI or using DHAS or F6PALd, or DHAS and DHAK) can also express MeDH-containing fusion proteins that include a MeDH activator sequence. A MeDH activator sequence can activate a MeDH enzyme by providing hydrolytic removal of a nicotinamide mononucleotide (NMN) moiety of the NAD cofactor. MeDH activator is active in the presence of magnesium ions and is also able to use ADP-ribose. (Kloosterman, H., et al. (2002) J Biol Chem. 277:34785-34792).

The engineered fusion protein can include a polypeptide sequence based on MeDH activator sequences, such as, activators of *Bacillus methanolicus* MGA3 (WP_004435441.1; SEQ ID NO: 12) and *Bacillus methanolicus* PB1 (WP_004437560.1).

These MeDH activator sequences, or an amino acid sequence with at least 50% or greater identity to SEQ ID NO: 12, can be used in the fusion proteins of the disclosure, such as MeDH-ACT-PHI, MeDH-ACT-HPS, HPS-MeDH-ACT, ACT-MeDH-PHI-HPS, ACT-MeDH-HPS-PHI, HPS-PHI-MeDH-ACT, PHI-HPS-MeDH-ACT, and ACT-MeDH(P1)-HPS-MeDH(P2).

One or more linker amino acid sequences can be present between regions in the fusion proteins of the disclosure. Linker sequence can provide desired functionality in the fusion protein. Some fusion proteins of the disclosure can be expressed as a single open reading frame without the introduction of linker(s), and some fusion proteins of the disclosure can include a linker sequence between one or more regions of the fusion protein, wherein the linker can allow the region to exhibit desired enzymatic activity. Linker sequences can generically be described as “rigid” or “flexible,” with linker sequences of longer length generally considered to be more flexible. Rigid linkers frequently form alpha-helical structures. Linker sequences can be prepared to provide desired properties such as length, solubility, and protease sensitivity.

Exemplary linker sequences are from 1 to 150, 1 to 100, or 1 to 50 amino acids in length. Some linker sequences are short, such as from one to ten, or from two to six amino acids in length. Linker sequences may be referred to as “linkers,” “linker peptides,” “linker peptide sequences.” The linker may include any amino acid, but preferably includes one or more amino acids that are selected from the group consisting of glycine, alanine, serine, and threonine. In some linker sequences the majority (> 50%) of the amino acids residues are glycine. Some linker sequences may include acidic residues such as Asp or Glu, or basic residues, such as Lys, Arg. Linker sequences can be chosen to impart one or more particular properties to the fusion protein, such as improved stability or solubility properties. The amino acids may provide regions of flexibility between the MeDH, PHI, HPS, and/or ACT regions in the fusion proteins.

Exemplary linker sequence include, but are not limited to $(G)_n$ where n is 4-8; $(GS)_n$, $(GGS)_n$, $(GGGS)_n$, $(GGGGS)_n$, and $(GGGGGS)_n$, where n is 1-6, such as 1, 2, 3, 4, 5, or 6; $[(GGS)_x(GS)_y]_n$ where x is 1-6, y is 1-6, and n is 1-4; $(GE)_n$, $(GGE)_n$, $(GGGE)_n$, and $(GGGGE)_n$, where n is 1-10; $(GD)_n$, $(GGD)_n$, $(GGGD)_n$, and $(GGGGD)_n$, where n is 1-10; $(GK)_n$, $(GGK)_n$, $(GGGK)_n$, and $(GGGGK)_n$, where n is 1-10; $(GR)_n$, $(GGR)_n$, $(GGGR)_n$, and $(GGGGR)_n$, where n is 1-10; $(EAAAK)_n$ where n is 1-10; $A(EAAAK)_nA$ where n is 1-10;

A(EAAAK)₄ALEA(EAAAK)₄A; (PAPAP)_n where n is 1-4; (AEAAAKEAAKA)_n where n is 1-4; (AP)_n where n is 1-35; (KP)_n where n is 1-35; (EP)_n where n is 1-35; (LE)_n where n is 1-35; (GA₂PA₃PAKQEA₃PAPA₂KAEAPA₃PA₂KA)_n where n is 1-5; (KESGVSSEQLAQFRSLD)_n where n is 1-5; (EGKSSGSGSESKST)_n where n is 1-5; (GSAGSAAGSGEF)_n where n is 1-4;
 5 [A(EAAAK)_nA]_y where n is 1-5 and y is 1-4; (GGSGSGSGSGSGSGSGSGGS)_n where n is 1 or 2; and SSGWGS GG.

Preferably the linker is [(GGS)_x(GS)_y(GGS)_z]_n where x is 1-6, y is 1-6, z is 0-3, and n is 1-4, preferably where x is 1-3, y is 1-3, z is 1-2, and n is 1-2.

Fusion protein formulas may optionally be described with reference to one or more
 10 SEQ ID NO(s). For example, a fusion protein of the general formula MeDH-L¹-HPS-L²-PHI, may be more specifically described as SEQ ID NO:1-L¹-SEQ ID NO:3-L²-SEQ ID NO:4. If the fusion protein has a sequences with less than 100% identity to referenced sequences (e.g., 90% or greater identity), the fusion protein may described as follows: [SEQ ID NO:1, ≥90%]-L¹-[SEQ ID NO:3, ≥90%]-L²-[SEQ ID NO:4, ≥90%]. Or for example, a fusion protein of the
 15 general formula MeDH-L¹-DHAS-L²-F6PAId, may be more specifically described as SEQ ID NO:1-L¹-SEQ ID NO:X-L²-SEQ ID NO:Y, where X and Y are a SEQ ID Number of a DHAS or F6PAId sequence, respectively, of which are exemplary sequences are described herein and below. If the fusion protein has a sequences with less than 100% identity to referenced sequences (e.g., 90% or greater identity), the fusion protein may described as follows:
 20 ID NO:1 ≥90%]-L¹-[SEQ ID NO:X ≥90%]-L²-[SEQ ID NO:Y ≥90%].

As another example, a fusion protein of the general formula ACT-L¹-MeDH-L²-PHI-L³-HPS, may be more specifically described as SEQ ID NO:12-L¹-SEQ ID NO:1-L²-SEQ ID NO:4-L³-SEQ ID NO:3. If certain regions of the fusion protein have sequences with less than 100% identity (e.g., 90% or greater identity, 85% or greater identity) to referenced sequences,
 25 the fusion protein may described as follows: SEQ ID NO:12-L¹-[SEQ ID NO:1 ≥90%]-L²-SEQ ID NO:4-L³-[SEQ ID NO:3 ≥85%].

As another example, a fusion protein of the general formula MeDH-L¹-HPS-PHI, wherein HPS-PHI is from a natural fusion protein, may be more specifically described as SEQ ID NO:1-L¹-SEQ ID NO:5, SEQ ID NO:6-L¹-SEQ ID NO:5, SEQ ID NO:8-L¹-SEQ ID NO:5,
 30 or SEQ ID NO:10-L¹-SEQ ID NO:5. In some arrangements L¹ is SEQ ID NO:7. In some embodiments the fusion protein has 50% or greater, 60% or greater, 70% or greater, 80% or greater, 85% or greater, 90% or greater, 95% or greater, 96% or greater, 97% or greater, 98% or greater, or 99% or greater identity to SEQ ID NO:1-SEQ ID NO:7-SEQ ID NO:5, SEQ ID NO:6-SEQ ID NO:7-SEQ ID NO:5, SEQ ID NO:8-SEQ ID NO:7-SEQ ID NO:5, or SEQ ID
 35 NO:10-SEQ ID NO:7-SEQ ID NO:5.

In some embodiments of the invention, fusion proteins are expressed in an engineered cell to provide a multi-protein complex including the fusion proteins. Formation of a multi-protein complex can be achieved through MeDH-MeDH interactions. MeDH is believed to be a homo-multimeric protein, frequently composed of 10 monomer subunits. It has been shown that MeDH from *Methylococcus capsulatus* (Bath) is primarily a dimer in solution, and an oligomeric species with a molecular mass of ~450-560 kDa forms at higher protein concentrations. (Culpepper M.A., Rosenzweig, A.C. (2014) Biochemistry 53:6211-6219). Therefore, a multi-protein complex can be formed in the cell by interactions between MeDH portions of fusion proteins, or between MeDH portions of fusion proteins and a non-fused MeDH, HPS, PHI, and/or ACT (e.g., wild type) proteins, or between MeDH portions of fusion proteins and a non-fused MeDH, DHAS, F6PALd (or DHAK) and/or ACT (e.g., wild type) proteins. The expression of desired fusion proteins, or combination of fusion proteins and unfused proteins, can produce a variety of heterodecamers or heteromultimers with activity optimized for methanol utilization, in some cases, subsequent bioproduct formation. For example, the cell can be engineered to provide a multi-protein complex that efficiently converts methanol to d-arabino-3-hexulose-6-phosphate to fructose 6-phosphate, which then can be further used as a precursor to a desired bioproduct.

A cell can be engineered in such a way to provide desired ratios of MeDH, HPS, PHI, and ACT sequences or to provide desired ratios of MeDH, DHAS, F6PALd (or DHAK) and ACT sequences. For example, the cell can include exogenous nucleic acids encoding the following fusion proteins: MeDH-PHI, MeDH-HPS, and MeDH-ACT, (or alternately MeDH-DHAS, MeDH-F6PALd and MeDH-ACT) and if the cell is engineered so that each of these fusion proteins is expressed in equal numbers, then the amount of MeDH sequence would be stoichiometrically greater (three times) greater than PHI, HPS, and ACT sequences (or alternately DHAS, F6PALd and ACT sequences). Optionally, the cell can be engineered to express a MeDH (non-fusion) protein. The fusion proteins can assemble along with the non-fusion MeDH protein to form a multi-protein complex having a desired ratio of protein components which in turn can provide an optimal balance of methanol pathway activities to maximize methanol flux.

In some embodiments, the cell is engineered to provide a stoichiometric ratio of MeDH that is greater than any one of HPS, PHI, or ACT (MeDH > HPS, PHI, or ACT). For example, the ratio of MeDH to any one of HPS, PHI, or ACT can be in the range of 10:1 to 0.5:1, 6:1 to 1:1, or 3:1 to 1:1. Alternatively in some embodiments, the cell is engineered to provide a stoichiometric ratio of MeDH that is greater than any one of DHAS, F6PALd or ACT (MeDH

> DHAS, F6PAld or ACT). For example, the ratio of MeDH to any one of DHAS, F6PAld or ACT can be in the range of 10:1 to 0.5:1, 6:1 to 1:1, or 3:1 to 1:1.

An expression vector or vectors can be constructed to include one or more fusion protein-encoding nucleic acids as exemplified herein, operably linked to expression control sequences functional in the host organism. Expression vectors applicable for use in the microbial host organisms provided include, for example, plasmids, phage vectors, viral vectors, episomes and artificial chromosomes, including vectors and selection sequences or markers operable for stable integration into a host chromosome. Additionally, the expression vectors can include one or more selectable marker genes and appropriate expression control sequences. Selectable marker genes also can be included that, for example, provide resistance to antibiotics or toxins, complement auxotrophic deficiencies, or supply critical nutrients not in the culture media. Expression control sequences can include constitutive and inducible promoters, transcription enhancers, transcription terminators, and the like which are well known in the art.

The fusion protein can be co-expressed with one or more additional nucleic acids that may encode enzyme(s) useful for converting intermediates, such as fructose 6-phosphate (F6P) and other compounds downstream of F6P, to desired bioproducts. When two or more exogenous encoding nucleic acids are to be co-expressed, both nucleic acids can be inserted, for example, into a single expression vector or in separate expression vectors. For single vector expression, the encoding nucleic acids can be operationally linked to one common expression control sequence or linked to different expression control sequences, such as one inducible promoter and one constitutive promoter. The transformation of exogenous nucleic acid sequences involved in a metabolic or synthetic pathway can be confirmed using methods well known in the art. Such methods include, for example, nucleic acid analysis such as Northern blots or polymerase chain reaction (PCR) amplification of mRNA, or immunoblotting for expression of gene products, or other suitable analytical methods to test the expression of an introduced nucleic acid sequence or its corresponding gene product. It is understood by those skilled in the art that the exogenous nucleic acid is expressed in a sufficient amount to produce the desired product, and it is further understood that expression levels can be optimized to obtain sufficient expression using methods well known in the art and as disclosed herein.

The term "exogenous" is intended to mean that the referenced molecule or the referenced activity is introduced into the host microbial organism. The molecule can be introduced, for example, by introduction of an encoding nucleic acid into the host genetic material such as by integration into a host chromosome or as non-chromosomal genetic

material such as a plasmid. Therefore, the term as it is used in reference to expression of an encoding nucleic acid refers to introduction of the encoding nucleic acid in an expressible form into the microbial organism. When used in reference to a biosynthetic activity, the term refers to an activity that is introduced into the host reference organism. The source can be, for example, a homologous or heterologous encoding nucleic acid that expresses the referenced activity following introduction into the host microbial organism. Therefore, the term “endogenous” refers to a referenced molecule or activity that is present in the host. Similarly, the term when used in reference to expression of an encoding nucleic acid refers to expression of an encoding nucleic acid contained within the microbial organism. The term “heterologous” refers to a molecule or activity derived from a source other than the referenced species whereas “homologous” refers to a molecule or activity derived from the host microbial organism. Accordingly, exogenous expression of an encoding nucleic acid can utilize either or both a heterologous or homologous encoding nucleic acid.

It is understood that when more than one exogenous nucleic acid is included in a microbial organism, the more than one exogenous nucleic acid refers to the referenced encoding nucleic acid or biosynthetic activity, as discussed above. It is further understood, as disclosed herein, that more than one exogenous nucleic acid can be introduced into the host microbial organism on separate nucleic acid molecules, on polycistronic nucleic acid molecules, or a combination thereof, and still be considered as more than one exogenous nucleic acid. For example, as disclosed herein a microbial organism can be engineered to express two or more exogenous nucleic acids encoding a desired pathway enzyme or protein, such as an exogenous nucleic acid that expresses a fusion protein of the disclosure, and one or more other enzymes that convert an intermediate from the RuMP pathway (or alternatively a DHA Pathway, preferably DHA Route 1) to a desired bioproduct.

In the case where two exogenous nucleic acids encoding a desired activity are introduced into a host microbial organism, it is understood that the two exogenous nucleic acids can be introduced as a single nucleic acid, for example, on a single plasmid, on separate plasmids, can be integrated into the host chromosome at a single site or multiple sites, and still be considered as two exogenous nucleic acids. Similarly, it is understood that more than two exogenous nucleic acids can be introduced into a host organism in any desired combination, for example, on a single plasmid, on separate plasmids, can be integrated into the host chromosome at a single site or multiple sites, and still be considered as two or more exogenous nucleic acids, for example three exogenous nucleic acids. Thus, the number of referenced exogenous nucleic acids or biosynthetic activities refers to the number of encoding nucleic

acids or the number of biosynthetic activities, not the number of separate nucleic acids introduced into the host organism.

Exogenous fusion protein-encoding nucleic acid sequences can be introduced stably or transiently into a host cell using techniques well known in the art including, but not limited to, conjugation, electroporation, chemical transformation, transduction, transfection, and ultrasound transformation. Optionally, for exogenous expression in *E. coli* or other prokaryotic cells, some nucleic acid sequences in the genes or cDNAs of eukaryotic nucleic acids can encode targeting signals such as an N-terminal mitochondrial or other targeting signal, which can be removed before transformation into prokaryotic host cells, if desired. For example, removal of a mitochondrial leader sequence led to increased expression in *E. coli* (Hoffmeister et al., *J. Biol. Chem.* 280:4329-4338 (2005)). For exogenous expression in yeast or other eukaryotic cells, genes can be expressed in the cytosol without the addition of leader sequence, or can be targeted to mitochondrion or other organelles, or targeted for secretion, by the addition of a suitable targeting sequence such as a mitochondrial targeting or secretion signal suitable for the host cells. Thus, it is understood that appropriate modifications to a nucleic acid sequence to remove or include a targeting sequence can be incorporated into an exogenous nucleic acid sequence to impart desirable properties. Furthermore, genes can be subjected to codon optimization with techniques well known in the art to achieve optimized expression of the proteins.

The terms “microbial,” “microbial organism” or “microorganism” are intended to mean any organism that exists as a microscopic cell that is included within the domains of archaea, bacteria or eukarya. 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. The term also includes cell cultures of any species that can be cultured for the production of a biochemical.

The term “isolated” when used in reference to a microbial organism is intended to mean an organism that is substantially free of at least one component as the referenced microbial organism is found in nature. The term includes a microbial organism that is removed from some or all components as it is found in its natural environment. The term also includes a microbial organism that is removed from some or all components as the microbial organism is found in non-naturally occurring environments.

In some aspects a nucleic acid encoding the fusion protein is introduced into a cell with a gene disruption. The term “gene disruption,” or grammatical equivalents thereof, is intended to mean a genetic alteration that renders the encoded gene product inactive or attenuated. The genetic alteration can be, for example, deletion of the entire gene, deletion of a regulatory

sequence required for transcription or translation, deletion of a portion of the gene which results in a truncated gene product, or by any of various mutation strategies that inactivate or attenuate the encoded gene product. One particularly useful method of gene disruption is complete gene deletion because it reduces or eliminates the occurrence of genetic reversions.

5 The phenotypic effect of a gene disruption can be a null mutation, which can arise from many types of mutations including inactivating point mutations, entire gene deletions, and deletions of chromosomal segments or entire chromosomes. Specific antisense nucleic acid compounds and enzyme inhibitors, such as antibiotics, can also produce null mutant phenotype, therefore being equivalent to gene disruption.

10 A metabolic modification refers to a biochemical reaction that is altered from its naturally occurring state. Therefore, microorganisms may have genetic modifications to nucleic acids encoding metabolic polypeptides, or functional fragments thereof. Exemplary metabolic modifications are disclosed herein.

The microorganisms provided herein can contain stable genetic alterations, which
15 refers to microorganisms that can be cultured for greater than five generations without loss of the alteration. Generally, stable genetic alterations include modifications that persist greater than 10 generations, particularly stable modifications will persist more than about 25 generations, and more particularly, stable genetic modifications will be greater than 50 generations, including indefinitely.

20 Those skilled in the art will understand that the genetic alterations, including metabolic modifications exemplified herein, are described with reference to a suitable host organism such as *E. coli* and their corresponding metabolic reactions or a suitable source organism for desired genetic material such as genes for a desired metabolic pathway. However, given the complete genome sequencing of a wide variety of organisms and the high level of skill in the area of
25 genomics, those skilled in the art will readily be able to apply the teachings and guidance provided herein to essentially all other organisms. For example, the *E. coli* metabolic alterations exemplified herein can readily be applied to other species by incorporating the same or analogous encoding nucleic acid from species other than the referenced species. Such genetic alterations include, for example, genetic alterations of species homologs, in general,
30 and in particular, orthologs, paralogs or nonorthologous gene displacements.

A variety of microorganism may be suitable for incorporating nucleic acid encoding the fusion protein, optionally with one or more other transgenes. Such organisms include both prokaryotic and eukaryotic organisms including, but not limited to, bacteria, including archaea and eubacteria, and eukaryotes, including yeast, plant, insect, animal, and mammal, including
35 human. Exemplary species are reported in U.S. Patent Pub No. 2014/0058056 (Burgard *et al.*),

which is incorporated herein by reference, and include, for example, *Escherichia coli*, *Saccharomyces cerevisiae*, *Saccharomyces kluyveri*, *Candida boidinii*, *Clostridium kluyveri*, *Clostridium acetobutylicum*, *Clostridium beijerinckii*, *Clostridium*
saccharoperbutylacetonicum, *Clostridium perfringens*, *Clostridium difficile*, *Clostridium*
5 *botulinum*, *Clostridium tyrobutyricum*, *Clostridium tetanomorphum*, *Clostridium tetani*,
Clostridium propionicum, *Clostridium aminobutyricum*, *Clostridium subterminale*,
Clostridium sticklandii, *Ralstonia eutropha*, *Mycobacterium bovis*, *Mycobacterium*
tuberculosis, *Porphyromonas gingivalis*, *Arabidopsis thaliana*, *Thermus thermophilus*,
Pseudomonas species, including *Pseudomonas aeruginosa*, *Pseudomonas putida*,
10 *Pseudomonas stutzeri*, *Pseudomonas fluorescens*, *Homo sapiens*, *Oryctolagus cuniculus*,
Rhodobacter spaeroides, *Thermoanaerobacter brockii*, *Metallosphaera sedula*, *Leuconostoc*
mesenteroides, *Chloroflexus aurantiacus*, *Roseiflexus castenholzii*, *Erythrobacter*, *Simmondsia*
chinensis, *Acinetobacter* species, including *Acinetobacter calcoaceticus* and *Acinetobacter*
baylyi, *Porphyromonas gingivalis*, *Sulfolobus tokodaii*, *Sulfolobus solfataricus*, *Sulfolobus*
15 *acidocaldarius*, *Bacillus subtilis*, *Bacillus cereus*, *Bacillus megaterium*, *Bacillus brevis*,
Bacillus pumilus, *Rattus norvegicus*, *Klebsiella pneumonia*, *Klebsiella oxytoca*, *Euglena*
gracilis, *Treponema denticola*, *Moorella thermoacetica*, *Thermotoga maritima*, *Halobacterium*
salinarum, *Geobacillus stearothermophilus*, *Aeropyrum pernix*, *Sus scrofa*, *Caenorhabditis*
elegans, *Corynebacterium glutamicum*, *Acidaminococcus fermentans*, *Lactococcus lactis*,
20 *Lactobacillus plantarum*, *Streptococcus thermophilus*, *Enterobacter aerogenes*, *Candida*,
Aspergillus terreus, *Pedicoccus pentosaceus*, *Zymomonas mobilis*, *Acetobacter pasteurians*,
Kluyveromyces lactis, *Eubacterium barkeri*, *Bacteroides capillosus*, *Anaerotruncus*
colihominis, *Natranaerobius thermophilus*, *Campylobacter jejuni*, *Haemophilus influenzae*,
Serratia marcescens, *Citrobacter amalonaticus*, *Myxococcus xanthus*, *Fusobacterium*
25 *nucleatum*, *Penicillium chrysogenum*, marine gamma proteobacterium, butyrate-producing
bacterium, *Nocardia iowensis*, *Nocardia farcinica*, *Streptomyces griseus*,
Schizosaccharomyces pombe, *Geobacillus thermoglucosidasius*, *Salmonella typhimurium*,
Vibrio cholera, *Helicobacter pylori*, *Nicotiana tabacum*, *Oryza sativa*, *Haloferax mediterranei*,
Agrobacterium tumefaciens, *Achromobacter denitrificans*, *Fusobacterium nucleatum*,
30 *Streptomyces clavuligenus*, *Acinetobacter baumannii*, *Mus musculus*, *Lachancea kluyveri*,
Trichomonas vaginalis, *Trypanosoma brucei*, *Pseudomonas stutzeri*, *Bradyrhizobium*
japonicum, *Mesorhizobium loti*, *Bos taurus*, *Nicotiana glutinosa*, *Vibrio vulnificus*,
Selenomonas ruminantium, *Vibrio parahaemolyticus*, *Archaeoglobus fulgidus*, *Haloarcula*
marismortui, *Pyrobaculum aerophilum*, *Mycobacterium smegmatis* MC2 155, *Mycobacterium*
35 *avium* subsp. *paratuberculosis* K-10, *Mycobacterium marinum* M, *Tsukamurella*

paurometabola DSM 20162, *Cyanobium* PCC7001, *Dictyostelium discoideum* AX4, as well as other exemplary species disclosed herein or available as source organisms for corresponding genes.

In certain embodiments, suitable organisms include *Acinetobacter baumannii* Naval-
 5 82, *Acinetobacter* sp. ADP1, *Acinetobacter* sp. strain M-1, *Actinobacillus succinogenes* 130Z, *Allochromatium vinosum* DSM 180, *Amycolatopsis methanolica*, *Arabidopsis thaliana*, *Atopobium parvulum* DSM 20469, *Azotobacter vinelandii* DJ, *Bacillus alcalophilus* ATCC 27647, *Bacillus azotoformans* LMG 9581, *Bacillus coagulans* 36D1, *Bacillus megaterium*, *Bacillus methanolicus* MGA3, *Bacillus methanolicus* PB1, *Bacillus methanolicus* PB-1,
 10 *Bacillus selenitireducens* MLS10, *Bacillus smithii*, *Bacillus subtilis*, *Burkholderia cenocepacia*, *Burkholderia cepacia*, *Burkholderia multivorans*, *Burkholderia pyrrocinia*, *Burkholderia stabilis*, *Burkholderia thailandensis* E264, *Burkholderiales bacterium* Joshi_001, *Butyrate-producing bacterium* L2-50, *Campylobacter jejuni*, *Candida albicans*, *Candida boidinii*, *Candida methylica*, *Carboxydotherrmus hydrogenoformans*, *Carboxydotherrmus*
 15 *hydrogenoformans* Z-2901, *Caulobacter* sp. AP07, *Chloroflexus aggregans* DSM 9485, *Chloroflexus aurantiacus* J-10-fl, *Citrobacter freundii*, *Citrobacter koseri* ATCC BAA-895, *Citrobacter youngae*, *Clostridium*, *Clostridium acetobutylicum*, *Clostridium acetobutylicum* ATCC 824, *Clostridium acidurici*, *Clostridium aminobutyricum*, *Clostridium asparagiforme* DSM 15981, *Clostridium beijerinckii*, *Clostridium beijerinckii* NCIMB 8052, *Clostridium*
 20 *bolteae* ATCC BAA-613, *Clostridium carboxidivorans* P7, *Clostridium cellulovorans* 743B, *Clostridium difficile*, *Clostridium hiranonis* DSM 13275, *Clostridium hylemonae* DSM 15053, *Clostridium kluyveri*, *Clostridium kluyveri* DSM 555, *Clostridium ljungdahli*, *Clostridium* *ljungdahlii* DSM 13528, *Clostridium methylpentosum* DSM 5476, *Clostridium pasteurianum*, *Clostridium pasteurianum* DSM 525, *Clostridium perfringens*, *Clostridium perfringens* ATCC
 25 13124, *Clostridium perfringens* str. 13, *Clostridium phytofermentans* ISDg, *Clostridium saccharobutylicum*, *Clostridium saccharoperbutylacetonicum*, *Clostridium* *saccharoperbutylacetonicum* N1-4, *Clostridium tetani*, *Corynebacterium glutamicum* ATCC 14067, *Corynebacterium glutamicum* R, *Corynebacterium* sp. U-96, *Corynebacterium* *variabile*, *Cupriavidus necator* N-1, *Cyanobium* PCC7001, *Desulfatibacillum alkenivorans*
 30 *AK-01*, *Desulfotobacterium hafniense*, *Desulfotobacterium metallireducens* DSM 15288, *Desulfotomaculum reducens* MI-1, *Desulfovibrio africanus* str. Walvis Bay, *Desulfovibrio* *fructosovorans* JJ, *Desulfovibrio vulgaris* str. Hildenborough, *Desulfovibrio vulgaris* str. 'Miyazaki F', *Dictyostelium discoideum* AX4, *Escherichia coli*, *Escherichia coli* K-12,
 35 *Escherichia coli* K-12 MG1655, *Eubacterium hallii* DSM 3353, *Flavobacterium frigidis*, *Fusobacterium nucleatum* subsp. *polymorphum* ATCC 10953, *Geobacillus* sp. Y4.1MC1,

- Geobacillus themodenitrificans* NG80-2, *Geobacter bemidjiensis* Bem, *Geobacter sulfurreducens*, *Geobacter sulfurreducens* PCA, *Geobacillus stearothermophilus* DSM 2334, *Haemophilus influenzae*, *Helicobacter pylori*, *Homo sapiens*, *Hydrogenobacter thermophilus*, *Hydrogenobacter thermophilus* TK-6, *Hyphomicrobium denitrificans* ATCC 51888,
- 5 *Hyphomicrobium zavarzinii*, *Klebsiella pneumoniae*, *Klebsiella pneumoniae* subsp. *pneumoniae* MGH 78578, *Lactobacillus brevis* ATCC 367, *Leuconostoc mesenteroides*, *Lysinibacillus fusiformis*, *Lysinibacillus sphaericus*, *Mesorhizobium loti* MAFF303099, *Metallosphaera sedula*, *Methanosarcina acetivorans*, *Methanosarcina acetivorans* C2A, *Methanosarcina barkeri*, *Methanosarcina mazei* Tuc01, *Methylobacter marinus*,
- 10 *Methylobacterium extorquens*, *Methylobacterium extorquens* AM1, *Methylococcus capsulatus*, *Methylomonas aminofaciens*, *Moorella thermoacetica*, *Mycobacter* sp. strain JC1 DSM 3803, *Mycobacterium avium* subsp. *paratuberculosis* K-10, *Mycobacterium bovis* BCG, *Mycobacterium gastri*, *Mycobacterium marinum* M, *Mycobacterium smegmatis*, *Mycobacterium smegmatis* MC2 155, *Mycobacterium tuberculosis*, *Nitrosopumilus salaria*
- 15 *BD31*, *Nitrososphaera gargensis* Ga9.2, *Nocardia farcinica* IFM 10152, *Nocardia iowensis* (sp. NRRL 5646), *Nostoc* sp. PCC 7120, *Ogataea angusta*, *Ogataea parapolyomorpha* DL-1 (*Hansenula polymorpha* DL-1), *Paenibacillus peoriae* KCTC 3763, *Paracoccus denitrificans*, *Penicillium chrysogenum*, *Photobacterium profundum* 3TCK, *Phytofermentans* ISDg, *Pichia pastoris*, *Picrophilus torridus* DSM9790, *Porphyromonas gingivalis*, *Porphyromonas gingivalis* W83, *Pseudomonas aeruginosa* PA01, *Pseudomonas denitrificans*, *Pseudomonas knackmussii*, *Pseudomonas putida*, *Pseudomonas* sp, *Pseudomonas syringae* pv. *syringae* B728a, *Pyrobaculum islandicum* DSM 4184, *Pyrococcus abyssi*, *Pyrococcus furiosus*, *Pyrococcus horikoshii* OT3, *Ralstonia eutropha*, *Ralstonia eutropha* H16, *Rhodobacter capsulatus*, *Rhodobacter sphaeroides*, *Rhodobacter sphaeroides* ATCC 17025,
- 25 *Rhodopseudomonas palustris*, *Rhodopseudomonas palustris* CGA009, *Rhodopseudomonas palustris* DX-1, *Rhodospirillum rubrum*, *Rhodospirillum rubrum* ATCC 11170, *Ruminococcus obeum* ATCC 29174, *Saccharomyces cerevisiae*, *Saccharomyces cerevisiae* S288c, *Salmonella enterica*, *Salmonella enterica* subsp. *enterica* serovar *Typhimurium* str. LT2, *Salmonella enterica* *typhimurium*, *Salmonella typhimurium*, *Schizosaccharomyces pombe*, *Sebaldella termitidis* ATCC 33386, *Shewanella oneidensis* MR-1, *Sinorhizobium meliloti* 1021, *Streptomyces coelicolor*, *Streptomyces griseus* subsp. *griseus* NBRC 13350, *Sulfolobus acidocalarius*, *Sulfolobus solfataricus* P-2, *Synechocystis* str. PCC 6803, *Syntrophobacter fumaroxidans*, *Thauera aromatica*, *Thermoanaerobacter* sp. X514, *Thermococcus kodakaraensis*, *Thermococcus litoralis*, *Thermoplasma acidophilum*, *Thermoproteus*
- 35 *neutrophilus*, *Thermotoga maritima*, *Thiocapsa roseopersicina*, *Tolomonas auensis* DSM

9187, *Trichomonas vaginalis* G3, *Trypanosoma brucei*, *Tsukamurella paurometabola* DSM 20162, *Vibrio cholera*, *Vibrio harveyi* ATCC BAA-1116, *Xanthobacter autotrophicus* Py2, *Yersinia intermedia*, and *Zea mays*.

Therefore, the engineered cell including the fusion protein having at least (i) methanol
 5 dehydrogenase activity and (ii) 3-hexulose-6-phosphate dehydrogenase activity and/or 6-phospho-3-hexuloisomerase activity, or alternatively including the fusion protein having at least (i) methanol dehydrogenase activity and (ii) DHAS activity and/or F6PAld activity, can include one or more further genetic alterations, such as inserted transgenes, deletions, attenuation, mutations, etc., desired to increase levels of one or more intermediates or a product
 10 thereof, and include those genetic modifications as described in U.S. Patent Pub No. 2014/0058056 (Burgard *et al.*), which is incorporated herein by reference.

Target products obtained from, and product pathways suitable for producing in, host cells expressing the fusion protein having at least (i) methanol dehydrogenase activity and (ii) 3-hexulose-6-phosphate dehydrogenase activity and/or 6-phospho-3-hexuloisomerase activity,
 15 or alternatively including the fusion protein having at least (i) methanol dehydrogenase activity and (ii) DHAS activity and/or F6PAld activity, described herein include the following.

Of particular interest are a target product obtained using pyruvate and acetyl-CoA as entry point or precursor to its product pathway(s), in part because the methanol metabolic pathway using the novel enzymes enables fixing the carbon of methanol into pathways to
 20 pyruvate and acetyl-CoA. Target products include (a) 1,4-butanediol and intermediates thereto, such as 4-hydroxybutanoic acid (4-hydroxybutanoate, 4-hydroxybutyrate, 4-HB), (b) butadiene and intermediates thereto, such as 1,4-butanediol, 1,3-butanediol, crotyl alcohol, 3-buten-2-ol (methyl vinyl carbinol) and 3-buten-1-ol, (c) 1,3-butanediol and intermediates thereto, such as 2,4-pentadienoate, crotyl alcohol or 3-buten-1-ol, (d) adipate, 6-aminocaproic
 25 acid, caprolactam, hexamethylenediamine and levulinic acid and their intermediates, e.g. 4-aminobutyryl-CoA, (e) methacrylic acid (2-methyl-2-propenoic acid) and its esters known collectively as methacrylates, such as methyl methacrylate, methyl methacrylate, 3-hydroxyisobutyrate and/or 2-hydroxyisobutyrate and their intermediates, (f) 1,2-propanediol (propylene glycol), n-propanol, 1,3-propanediol and glycerol, and their intermediates and (g)
 30 succinic acid and intermediates thereto.

In some aspects, a gene encoding a fusion protein having at least (i) methanol dehydrogenase activity and (ii) 3-hexulose-6-phosphate dehydrogenase activity and/or 6-phospho-3-hexuloisomerase activity, or alternatively including the fusion protein having at least (i) methanol dehydrogenase activity and (ii) DHAS activity and/or F6PAld activity, is
 35 introduced into a cell engineered with increased levels of 1,4-butanediol (BDO) or

hydroxylbutyrate (4-HB) biosynthetic capability, those skilled in the art will understand with applying the teaching and guidance provided herein to a particular species that the identification of metabolic modifications can include identification and inclusion or inactivation of orthologs. To the extent that paralogs and/or nonorthologous gene
5 displacements are present in the referenced microorganism that encode an enzyme catalyzing a similar or substantially similar metabolic reaction, those skilled in the art also can utilize these evolutionally related genes.

With the complete genome sequence available for now more than 550 species (with more than half of these available on public databases such as the NCBI), including 395
10 microorganism genomes and a variety of yeast, fungi, plant, and mammalian genomes, the identification of genes encoding the requisite BDO or 4-HB biosynthetic pathway as well as other known biosynthetic pathways for 1,3-butanediol (13BDO), butadiene, 6-amino caproic acid (6ACA), hexamethyldiamine (HMDA), adipic acid or derivatives thereof, coryl alcohol, methyl vinyl carbinol, 3-buten-1-ol, succinic acid or derivatives thereof, n-propanol,
15 isopropanol, propylene, methacrylic acid or derivatives thereof, methanol metabolic and/or formaldehyde assimilation activity for one or more genes in related or distant species, including for example, homologues, orthologs, paralogs and nonorthologous gene displacements of known genes, and the interchange of genetic alterations between organisms is routine and well known in the art. Accordingly, the metabolic alterations allowing
20 biosynthesis of various target products including 1,3-butanediol (13BDO), 1, 4-butanediol (BDO), 4-HB, butadiene, 6-amino caproic acid (6ACA), hexamethyldiamine (HMDA), adipic acid or derivatives thereof, coryl alcohol, methyl vinyl carbinol, 3-buten-1-ol, succinic acid or derivatives thereof, n-propanol, isopropanol, propylene, methacrylic acid or derivatives thereof, metabolism of methanol and/or assimilation of formaldehyde described herein with
25 reference to a particular organism such as *E. coli* can be readily applied to other microorganisms, including prokaryotic and eukaryotic organisms alike. Given the teachings and guidance provided herein, those skilled in the art will know that a metabolic alteration exemplified in one organism can be applied equally to other organisms.

Exemplary alcohol metabolic pathway gene(s), such as described in U.S. Patent Pub
30 No. 2014/0058056, encode a protein selected from the group consisting of: a formate dehydrogenase, a formaldehyde activating enzyme, a formaldehyde dehydrogenase, a S-(hydroxymethyl)glutathione synthase, a glutathione-dependent formaldehyde dehydrogenase, a S-formylglutathione hydrolase, a formate hydrogen lyase, and a hydrogenase, any or more can be coexpressed with the fusion protein having at least (i) methanol dehydrogenase activity and
35 (ii) 3-hexulose-6-phosphate dehydrogenase activity and/or 6-phospho-3-hexuloisomerase

activity, or alternatively including the fusion protein having at least (i) methanol dehydrogenase activity and (ii) DHAS activity and/or F6PAld activity, in the engineered cell.

Other exemplary alcohol metabolic pathway gene(s), such as described in U.S. Patent Pub No. 2014/0058056, encode an alcohol metabolic pathway gene(s) encoding a protein

5 selected from the group consisting of a succinyl-CoA reductase (aldehyde forming), a 4-hydroxybutyrate (4-HB) dehydrogenase, a 4-HB kinase, a phosphotrans-4-hydroxybutyrylase, a 4-hydroxybutyryl-CoA reductase (aldehyde forming), a 1,4-butanediol dehydrogenase; a succinate reductase, a succinyl-CoA reductase (alcohol forming), 4-hydroxybutyryl-CoA transferase, a 4-hydroxybutyryl-CoA synthetase, a 4-HB reductase, and a 4-hydroxybutyryl-CoA reductase (alcohol forming), a succinyl-CoA transferase, and a succinyl-CoA synthetase,
10 any or more can be co-expressed with the fusion protein having at least (i) methanol dehydrogenase activity and (ii) 3-hexulose-6-phosphate dehydrogenase activity and/or 6-phospho-3-hexuloisomerase activity, or alternatively including the fusion protein having at least (i) methanol dehydrogenase activity and (ii) DHAS activity and/or F6PAld activity, in the
15 engineered cell.

1,4-butanediol and intermediates thereto, such as 4-hydroxybutanoic acid (4-hydroxybutanoate, 4-hydroxybutyrate, 4-HB), are target products that can be made by co-expressing the fusion protein having at least (i) methanol dehydrogenase activity and (ii) 3-hexulose-6-phosphate dehydrogenase activity and/or 6-phospho-3-hexuloisomerase activity, or
20 alternatively including the fusion protein having at least (i) methanol dehydrogenase activity and (ii) DHAS activity and/or F6PAld activity, described herein with a product pathway described herein as well as in the following documents. Suitable product pathways and enzymes, methods for screening and methods for isolating are found herein as well as in the following documents, incorporated herein by reference: WO2008115840A2 published 25
25 September 2008 entitled "Compositions and Methods for the Biosynthesis of 1, 4-Butanediol and Its Precursors"; WO2010141780A1 published 9 December 2010 entitled "Process of Separating Components of A Fermentation Broth"; WO2010141920A2 published 9 December 2010 entitled "Microorganisms for the Production of 1, 4-Butanediol and Related Methods"; WO2010030711A2 published 18 March 2010 entitled "Microorganisms for the Production of
30 1, 4-Butanediol"; WO2010071697A1 published 24 June 2010 entitled "Microorganisms and Methods for Conversion of Syngas and Other Carbon Sources to Useful Products"; WO2009094485A1 published 30 July 2009 entitled "Methods and Organisms for Utilizing Synthesis Gas or Other Gaseous Carbon Sources and Methanol"; WO2009023493A1 published 19 February 2009 entitled "Methods and Organisms for the Growth-Coupled
35 Production of 1,4-Butanediol"; WO2008115840A2 published 25 September 2008 entitled

“Compositions and Methods for the Biosynthesis of 1,4-Butanediol and Its Precursors”; and International Application No. PCT/US13/56725 filed 27 August 2013 entitled “Microorganisms and Methods for Enhancing the Availability of Reducing Equivalents in the Presence of Methanol, and for Producing 1,4-Butanediol Related Thereto”.

5 Butadiene and intermediates thereto, such as 1,4-butanediol, 1,3-butanediol, crotyl alcohol, 3-buten-2-ol (methyl vinyl carbinol) and 3-buten-1-ol, are target products that can be made by co-expressing the fusion protein having at least (i) methanol dehydrogenase activity and (ii) 3-hexulose-6-phosphate dehydrogenase activity and/or 6-phospho-3-hexuloisomerase activity, or alternatively including the fusion protein having at least (i) methanol
10 dehydrogenase activity and (ii) DHAS activity and/or F6PAld activity, described herein with a product pathway described in the following documents. In addition to direct fermentation to produce butadiene, 1,3-butanediol, 1,4-butanediol, crotyl alcohol, 3-buten-2-ol (methyl vinyl carbinol) and 3-buten-1-ol can be separated, purified (for any use), and then dehydrated to butadiene in a second step involving metal-based catalysis. Suitable product pathways and
15 enzymes, methods for screening and methods for isolating are found in the following documents, incorporated herein by reference: WO2011140171A2 published 10 November 2011 entitled “Microorganisms and Methods for the Biosynthesis of Butadiene”; WO2012018624A2 published 9 February 2012 entitled “Microorganisms and Methods for the Biosynthesis of Aromatics, 2,4-Pentadienoate and 1,3-Butadiene”; WO2011140171A2
20 published 10 November 2011 entitled “Microorganisms and Methods for the Biosynthesis of Butadiene”; WO2013040383A1 published 21 March 2013 entitled “Microorganisms and Methods for Producing Alkenes”; WO2012177710A1 published 27 December 2012 entitled “Microorganisms for Producing Butadiene and Methods Related thereto”; WO2012106516A1 published 9 August 2012 entitled “Microorganisms and Methods for the Biosynthesis of
25 Butadiene”; WO2013028519A1 published 28 February 2013 entitled “Microorganisms and Methods for Producing 2,4-Pentadienoate, Butadiene, Propylene, 1,3-Butanediol and Related Alcohols”; and U.S. Patent Pub No.2015/0050708.

1,3-butanediol and intermediates thereto, such as 2,4-pentadienoate, crotyl alcohol or 3-buten-1-ol, are target products that can be made by co-expressing the fusion protein having at
30 least (i) methanol dehydrogenase activity and (ii) 3-hexulose-6-phosphate dehydrogenase activity and/or 6-phospho-3-hexuloisomerase activity, or alternatively including the fusion protein having at least (i) methanol dehydrogenase activity and (ii) DHAS activity and/or F6PAld activity, described herein with a product pathway described herein as well as in the following documents. Suitable product pathways and enzymes, methods for screening and
35 methods for isolating are found herein as well as in the following documents, incorporated

herein by reference: WO2011071682A1 published 16 June 2011 entitled “Methods and Organisms for Converting Synthesis Gas or Other Gaseous Carbon Sources and Methanol to 1, 3-Butanediol”; WO2011031897A published 17 March 2011 entitled “Microorganisms and Methods for the Co-Production of Isopropanol with Primary Alcohols, Diols and Acids”;

- 5 WO2010127319A2 published 4 November 2010 entitled “Organisms for the Production of 1,3-Butanediol”; WO2013071226A1 published 16 May 2013 entitled “Eukaryotic Organisms and Methods for Increasing the Availability of Cytosolic Acetyl-CoA, and for Producing 1,3-Butanediol”; WO2013028519A1 published 28 February 2013 entitled “Microorganisms and Methods for Producing 2,4-Pentadienoate, Butadiene, Propylene, 1,3-Butanediol and Related
- 10 Alcohols”; WO2013036764A1 published 14 March 2013 entitled “Eukaryotic Organisms and Methods for Producing 1,3-Butanediol”; WO2013012975A1 published 24 January 2013 entitled “Methods for Increasing Product Yields”; WO2012177619A2 published 27 December 2012 entitled “Microorganisms for Producing 1, 3-Butanediol and Methods Related Thereto”; and U.S. Patent Pub No.2015/0050708.

- 15 Adipate, 6-aminocaproic acid, caprolactam, hexamethylenediamine and levulinic acid, and their intermediates, e.g. 4-aminobutyryl-CoA, are target products, useful for example for making nylon polymers, that can be made by co-expressing the fusion protein having at least (i) methanol dehydrogenase activity and (ii) 3-hexulose-6-phosphate dehydrogenase activity and/or 6-phospho-3-hexuloisomerase activity, or alternatively including the fusion protein
- 20 having at least (i) methanol dehydrogenase activity and (ii) DHAS activity and/or F6PAld activity, described herein with a product pathway described herein as well as in the following documents. Suitable product pathways and enzymes, methods for screening and methods for isolating are found herein as well as in the following documents, incorporated herein by reference: WO2010129936A1 published 11 November 2010 entitled “Microorganisms and
- 25 Methods for the Biosynthesis of Adipate, Hexamethylenediamine and 6-Aminocaproic Acid”; WO2013012975A1 published 24 January 2013 entitled “Methods for Increasing Product Yields”; WO2012177721A1 published 27 December 2012 entitled “Microorganisms for Producing 6-Aminocaproic Acid”; WO2012099621A1 published 26 July 2012 entitled “Methods for Increasing Product Yields”; and U.S. Patent Pub No. 2014/0329916
- 30 entitled “Microorganisms an Methods for Enhancing the Availability of Reducing Equivalents in the Presence of Methanol, and for Producing Adipate, 6-Aminocaproate, Hexamethylenediamine or Caprolactam Related Thereto”.

- Methacrylic acid (2-methyl-2-propenoic acid; used in the preparation of its esters known collectively as methacrylates, such as methyl methacrylate, which is used most notably
- 35 in the manufacture of polymers), methacrylate ester such as methyl methacrylate, 3-

hydroxyisobutyrate and/or 2-hydroxyisobutyrate and their intermediates are target products, useful for example for making polymers, that can be made by co-expressing the fusion protein having at least (i) methanol dehydrogenase activity and (ii) 3-hexulose-6-phosphate dehydrogenase activity and/or 6-phospho-3-hexuloisomerase activity, or alternatively

5 including the fusion protein having at least (i) methanol dehydrogenase activity and (ii) DHAS activity and/or F6PAld activity, described herein with a product pathway described herein as well as in the following documents. Suitable product pathways and enzymes, methods for screening and methods for isolating are found herein as well as in the following documents, incorporated herein by reference: WO2012135789A2 published 4 October 2012 entitled
10 “Microorganisms for Producing Methacrylic Acid and Methacrylate Esters and Methods Related Thereto”; WO2009135074A2 published 5 November 2009 entitled “Microorganisms for the Production of Methacrylic Acid”; and U.S. Patent Pub No. 2014/0288254 entitled “Microorganisms an Methods for Enhancing the Availability of Reducing Equivalents in the Presence of Methanol, and for Producing 3-Hydroxyisobutyrate or Methacrylic Acid Related
15 Thereto”.

1,2-propanediol (propylene glycol), n-propanol, 1,3-propanediol and glycerol, and their intermediates are target products, useful for example for making polymers, that can be made by co-expressing the fusion protein having at least (i) methanol dehydrogenase activity and (ii) 3-hexulose-6-phosphate dehydrogenase activity and/or 6-phospho-3-hexuloisomerase activity, or
20 alternatively including the fusion protein having at least (i) methanol dehydrogenase activity and (ii) DHAS activity and/or F6PAld activity, described herein with a product pathway described herein as well as in the following documents. Suitable product pathways and enzymes, methods for screening and methods for isolating are found herein as well as in the following documents, incorporated herein by reference: WO2009111672A1 published 9
25 November 2009 entitled “Primary Alcohol Producing Organisms”; WO2011031897A1 17 March 2011 entitled “Microorganisms and Methods for the Co-Production of Isopropanol with Primary Alcohols, Diols and Acids”; WO2012177599A2 published 27 December 2012 entitled “Microorganisms for Producing N-Propanol 1, 3-Propanediol, 1, 2-Propanediol or Glycerol and Methods Related Thereto”; and U.S. Patent Pub No. 2014/0302575 entitled “Microorganisms
30 an Methods for Enhancing the Availability of Reducing Equivalents in the Presence of Methanol, and for Producing 1,2-Propanediol, n-Propanol, 1,3-Propanediol, or Glycerol Related Thereto”.

Succinic acid and intermediates thereto (useful to produce products including polymers, e.g. PBS, 1,4-butanediol, tetrahydrofuran, pyrrolidone, solvents, paints, deicers, plastics, fuel
35 additives, fabrics, carpets, pigments, and detergents) are target products that can be made by

co-expressing the fusion protein having at least (i) methanol dehydrogenase activity and (ii) 3-hexulose-6-phosphate dehydrogenase activity and/or 6-phospho-3-hexuloisomerase activity, or alternatively including the fusion protein having at least (i) methanol dehydrogenase activity and (ii) DHAS activity and/or F6PAld activity, described herein with a product pathway
5 described herein as well as in the following documents. Suitable product pathways and enzymes, methods for screening and methods for isolating are found herein as well as in the following documents, incorporated herein by reference: EP1937821A2 published 2 July 2008 entitled "Methods and Organisms for the Growth-Coupled Production of Succinate"; and U.S. Patent Pub No. 2014/0302575 entitled "Microorganisms and Methods for Enhancing the
10 Availability of Reducing Equivalents in the Presence of Methanol, and for Producing Succinate Related Thereto".

Target products obtained from, and product pathways suitable for producing in, host cells co-expressing the fusion protein having at least (i) methanol dehydrogenase activity and (ii) 3-hexulose-6-phosphate dehydrogenase activity and/or 6-phospho-3-hexuloisomerase
15 activity, or alternatively including the fusion protein having at least (i) methanol dehydrogenase activity and (ii) DHAS activity and/or F6PAld activity, described herein include the following. Butadiene and intermediates thereto, such as 1,4-butanediol, 1,3-butanediol, crotyl alcohol, 3-buten-2-ol (methyl vinyl carbinol) and 3-buten-1-ol, are target products that can be made by co-expressing the fusion protein having at least (i) methanol
20 dehydrogenase activity and (ii) 3-hexulose-6-phosphate dehydrogenase activity and/or 6-phospho-3-hexuloisomerase activity described herein with a product pathway described in the following documents. In addition to direct fermentation to produce butadiene, 1,3-butanediol, 1,4-butanediol, crotyl alcohol, 3-buten-2-ol (methyl vinyl carbinol) and 3-buten-1-ol can be separated, purified (for any use), and then dehydrated to butadiene in a second step involving
25 metal-based catalysis. Suitable product pathways and enzymes, methods for screening and methods for isolating are found in: WO2011140171A2 published 10 November 2011 entitled "Microorganisms and Methods for the Biosynthesis of Butadiene"; WO2012018624A2 published 9 February 2012 entitled "Microorganisms and Methods for the Biosynthesis of Aromatics, 2, 4-Pentadienoate and 1, 3-Butadiene"; WO2011140171A2 published 10
30 November 2011 entitled "Microorganisms and Methods for the Biosynthesis of Butadiene"; WO2013040383A1 published 21 March 2013 entitled "Microorganisms and Methods for Producing Alkenes"; WO2012177710A1 published 27 December 2012 entitled "Microorganisms for Producing Butadiene and Methods Related thereto"; WO2012106516A1 published 9 August 2012 entitled "Microorganisms and Methods for the Biosynthesis of
35 Butadiene"; WO2013028519A1 published 28 February 2013 entitled "Microorganisms and

Methods for Producing 2,4-Pentadienoate, Butadiene, Propylene, 1,3-Butanediol and Related Alcohols"; and U.S. Patent Pub No. 2015/0050708.

Enzymes, genes and methods for engineering pathways from acetyl-CoA, succinate and succinyl-CoA to various products, such as BDO, into a microorganism, are now known in the art (*see, e.g.*, U.S. Publ. No. 2011/0201089). A set of BDO pathway enzymes represents a group of enzymes that can convert succinate or alpha-ketoglutarate to BDO as shown in Figure 6. For example, BDO can be produced from succinyl-CoA via previously disclosed pathways (see for example, Burk et al., WO 2008/115840). Figure 6 presents exemplary pathways which can use the primary metabolites, e.g. acetyl-CoA, made available by the use of methanol as a carbon source as described herein. In Figure 6, the organism comprises at least one exogenous nucleic acid encoding a BDO pathway enzyme expressed in a sufficient amount to produce BDO. In certain embodiments, the BDO pathway enzyme is selected from the group consisting of (1) a succinyl-CoA synthetase; (2) a CoA-independent succinic semialdehyde dehydrogenase; (3) a α -ketoglutarate dehydrogenase; (4) a glutamate:succinate semialdehyde transaminase; (5) a glutamate decarboxylase; (6) a CoA-dependent succinic semialdehyde dehydrogenase; (7) a 4-hydroxybutanoate dehydrogenase; (8) a α -ketoglutarate decarboxylase; (9) a 4-hydroxybutyryl CoA:acetyl-CoA transferase; (10) a butyrate kinase; (11) a phosphotransbutyrylase; (12) an aldehyde dehydrogenase; and (13) an alcohol dehydrogenase. Preferred pathways include those from alpha-ketoglutarate, e.g. steps 8, 7, 9, 12 and 13; steps 3, 6, 7, 9, 12 and 13; and steps 1, 6, 7, 9, 12, 13. In an alternative, a single protein can comprise the activities of steps 12 and 13. Specific enzymes, classes of enzymes and sources of enzymes and their genes can be found in WO2008115840, for example.

In some embodiments, the disclosure provides organisms comprising a fusion protein having at least (i) methanol dehydrogenase activity and (ii) 3-hexulose-6-phosphate dehydrogenase activity and/or 6-phospho-3-hexuloisomerase activity, or alternatively including the fusion protein having at least (i) methanol dehydrogenase activity and (ii) DHAS activity and/or F6PAld activity, and that are engineered to improve the availability of reducing equivalents, which can be used for the production of target product molecules. It will be recognized by one skilled in the art that any product molecule that utilizes reducing equivalents in its production can exhibit enhanced production through other biosynthetic pathways.

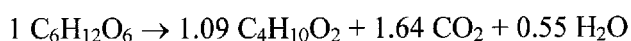
In numerous engineered pathways, realization of maximum product yields based on carbohydrate feedstock is hampered by insufficient reducing equivalents or by loss of reducing equivalents to byproducts. Methanol is a relatively inexpensive organic feedstock that can be used to generate reducing equivalents by using the fusion protein having at least (i) methanol dehydrogenase activity and (ii) 3-hexulose-6-phosphate dehydrogenase activity and/or 6-

phospho-3-hexuloisomerase activity, or alternatively including the fusion protein having at least (i) methanol dehydrogenase activity and (ii) DHAS activity and/or F6PAld activity, and one or more metabolic enzymes. The reducing equivalents produced by the metabolism of methanol can then be used to power the glucose to BDO production pathways, for example, as shown in Figure 6.

Figure 6 presents exemplary pathways which can use the primary metabolites, e.g. acetyl-CoA, made available by the use of methanol as a carbon source as described herein. In Figure 6, the organism comprises at least one exogenous nucleic acid encoding a BDO pathway enzyme expressed in a sufficient amount to produce BDO. In certain embodiments, the BDO pathway enzyme is selected from the group consisting of (1) a succinyl-CoA synthetase; (2) a CoA-independent succinic semialdehyde dehydrogenase; (3) a α -ketoglutarate dehydrogenase; (4) a glutamate:succinate semialdehyde transaminase; (5) a glutamate decarboxylase; (6) a CoA-dependent succinic semialdehyde dehydrogenase; (7) a 4-hydroxybutanoate dehydrogenase; (8) a α -ketoglutarate decarboxylase; (9) a 4-hydroxybutyryl CoA:acetyl-CoA transferase; (10) a butyrate kinase; (11) a phosphotransbutyrylase; (12) an aldehyde dehydrogenase; and (13) an alcohol dehydrogenase. Preferred pathways include those from alpha-ketoglutarate, e.g. steps 8, 7, 9, 12 and 13; steps 3, 6, 7, 9, 12 and 13; and steps 1, 6, 7, 9, 12, 13. In an alternative, a single protein can comprise the activities of steps 12 and 13. Specific enzymes, classes of enzymes and sources of enzymes and their genes can be found in WO2008115840A2, for example.

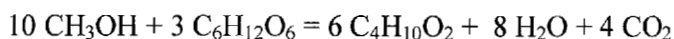
Enzymes, genes and methods for engineering pathways from succinate and succinyl-CoA to various products, such as BDO, into a microorganism, are now known in the art (*see, e.g.,* U.S. Publ. No. 2011/0201089). A set of BDO pathway enzymes represents a group of enzymes that can convert succinate or alpha-ketoglutarate to BDO as shown in Figure 6. The additional reducing equivalents obtained from the MeDH pathway, as disclosed herein, improve the yields of all these products when utilizing carbohydrate-based feedstock. For example, BDO can be produced from succinyl-CoA via previously disclosed pathways (*see for example, Burk et al., WO 2008/115840*).

The maximum theoretical yield of BDO via the pathway shown in Figure 6 supplemented with the reactions of the oxidative TCA cycle (e.g., citrate synthase, aconitase, isocitrate dehydrogenase, alpha-ketoglutarate dehydrogenase) is 1.09 mol/mol.

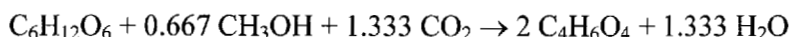
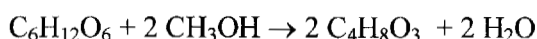


When both feedstocks of sugar and methanol are available, the methanol can be utilized to generate reducing equivalents by employing one or more of enzymes. The reducing equivalents generated from methanol can be utilized to power the glucose to BDO production

pathways, *e.g.*, as shown in Figure 6. Theoretically, all carbons in glucose will be conserved, thus resulting in a maximal theoretical yield to produce BDO from glucose at 2 mol BDO per mol of glucose under either aerobic or anaerobic conditions:



In a similar manner, the maximum theoretical yields of succinate and 4-HB can reach 2 mol/mol glucose:



In other embodiments, the organism having a fusion protein having at least (i) methanol dehydrogenase activity and (ii) 3-hexulose-6-phosphate dehydrogenase activity and/or 6-phospho-3-hexuloisomerase activity, or alternatively including the fusion protein having at least (i) methanol dehydrogenase activity and (ii) DHAS activity and/or F6PAld activity, either alone or in combination with a BDO pathway, as provided herein, may further comprise a second formaldehyde assimilation pathway (FAP). The second FAP can also utilize formaldehyde, for example, formaldehyde that not utilized by the fusion protein, in the formation of intermediates of certain central metabolic pathways that can be used, for example, in the formation of biomass.

With reference to Figure 5, in the second formaldehyde assimilation pathway, the organism comprises at least one exogenous nucleic acid encoding a formaldehyde assimilation pathway enzyme that is different than the MeDH, PSI, HPS, and ACT proteins. Enzymes of the second formaldehyde assimilation pathway can be expressed in a sufficient amount to produce an intermediate of glycolysis and/or a metabolic pathway that can be used in the formation of biomass. In one embodiment, the second formaldehyde assimilation pathway enzyme is expressed in a sufficient amount to produce an intermediate of glycolysis. In another embodiment, the second formaldehyde assimilation pathway enzyme is expressed in a sufficient amount to produce an intermediate of a metabolic pathway that can be used in the formation of biomass. In some of the embodiments, the second formaldehyde assimilation pathway comprises a dihydroxyacetone (DHA) synthase or a DHA kinase. The preferred DHA pathway is DHA Route 1 in Figure 5, which is a combination of DHA (dihydroxyacetone) synthase, *e.g.* EC 2.2.1.3 (Step 6) and F6P (fructose-6-phosphate) aldolase (Step 7). In one embodiment, the intermediate is a DHA, a DHA phosphate, or a combination thereof. In certain embodiments, the organism comprises two exogenous nucleic acids, each encoding a second formaldehyde assimilation pathway enzyme.

In addition to the fusion protein having at least (i) methanol dehydrogenase activity and (ii) 3-hexulose-6-phosphate dehydrogenase activity and/or 6-phospho-3-hexuloisomerase

activity, the cell can also possess a pathway that proceeds through dihydroxyacetone (DHA). Both the fusion protein of the RuMP pathway and the DHA pathway can be for the detoxification and assimilation of formaldehyde. As shown in Figure 5, a transketolase first transfers a glycoaldehyde group from xylulose-5-phosphate to formaldehyde (Step 6, Figure 5), resulting in the formation of DHA and G3P, which is an intermediate in glycolysis. The DHA obtained from DHA synthase is then further phosphorylated to form DHA phosphate (DHAP) by a DHA kinase. DHAP can be assimilated into glycolysis and several other pathways. Alternatively, DHA and G3P can be converted by fructose-6-phosphate aldolase to form fructose-6-phosphate (F6P) (Figure 5, step 7).

In some embodiments, in addition to the fusion protein having at least (i) methanol dehydrogenase activity and (ii) DHAS and/or F6PAld activity, the cell can also possess a pathway that proceeds through hexose-6-phosphate (H6P) as depicted in Figure 5. The pathway that proceeds through hexose-6-phosphate (H6P) can optionally use a MeDH fusion protein. For example, an engineered cell of the disclosure can include a MeDH fusion protein of the DHA pathway and a MeDH fusion protein of the RuMP pathway, which can be for the detoxification and assimilation of formaldehyde.

The DHA synthase in the engineered cell, which in some embodiments can be present as a fusion protein with MeDH, can be an exogenous DHA synthase, such as a DHA synthase from *Mycobacter sp. strain JC1 DSM 3803* (AAG12171.2); *Ogataea parapolymorpha DL-1* (*Hansenula polymorpha DL-1*) (EFW95760.1); or *Candida boidinii* (AAC83349.1). DHA synthases are of the enzyme class (EC) 2.2.1.3. Table 5 is a table of pairwise sequence identity of various DHA synthase sequences.

Table 5

Pairwise alignment (% ID)			
	<i>Mycobacter sp. strain JC1 DSM 3803</i>	<i>Ogataea parapolymorpha DL-1 (Hansenula polymorpha DL-1)</i>	<i>Candida boidinii</i>
DHA Synthase			
<i>Mycobacter sp. strain JC1 DSM 3803</i>	100	29.8	30.9
<i>Ogataea parapolymorpha DL-1 (Hansenula polymorpha DL-1)</i>	29.8	100	70.7
<i>Candida boidinii</i>	30.9	70.7	100

The DHA kinase in the engineered cell, which in some embodiments can be present as a fusion protein with MeDH, can be an exogenous DHA kinase, such as a DHA kinase from *Pichia angusta* (AAC27705.1); *Saccharomyces cerevisiae* S288c (P54838.1); *Saccharomyces cerevisiae* S288c (P43550.1); *Citrobacter freundii* ATCC 8090 (ZP_16280678.1); *Klebsiella pneumoniae subsp. pneumoniae* WGLW3 (ZP_18488498.1); *Escherichia coli* K-12 MG1655

(NP_415718.6); *Escherichia coli* K-12 MG1655 (NP_415717.1); or *Escherichia coli* K-12 MG1655 (NP_415716.4). DHA kinases are of the enzyme class (EC) 2.7.1.121. Table 6 is a table of pairwise sequence identity of various DHA kinase sequences.

Table 6

Pairwise alignment (% ID)								
DHA kinase	<i>Escherichia coli</i> K-12 MG1655	<i>Escherichia coli</i> K-12 MG1655	<i>Citrobacter freundii</i> ATCC 8090	<i>Klebsiella pneumoniae</i> subsp. <i>pneumoniae</i> WGLW3	<i>Escherichia coli</i> K-12 MG1655	<i>Saccharomyces cerevisiae</i> S288c	<i>Pichia angusta</i>	<i>Saccharomyces cerevisiae</i> S288c
<i>Escherichia coli</i> K-12 MG1655	100	5.5	9.9	10.6	11.1	11	10.1	9.5
<i>Escherichia coli</i> K-12 MG1655	5.5	100	11.4	11.2	5.2	9.4	9.2	10.3
<i>Citrobacter freundii</i> ATCC 8090	9.9	11.4	100	86.2	26.9	31.7	31.3	29
<i>Klebsiella pneumoniae</i> subsp. <i>pneumoniae</i> WGLW3	10.6	11.2	86.2	100	27.2	30.8	30.1	29.1
<i>Escherichia coli</i> K-12 MG1655	11.1	5.2	26.9	27.2	100	32.7	33.1	32.6
<i>Saccharomyces cerevisiae</i> S288c	11	9.4	31.7	30.8	32.7	100	42.6	43.1
<i>Pichia angusta</i>	10.1	9.2	31.3	30.1	33.1	42.6	100	50.2
<i>Saccharomyces cerevisiae</i> S288c	9.5	10.3	29	29.1	32.6	43.1	50.2	100

The fructose-6-phosphate aldolase in the engineered cell, which in some embodiments can be present as a fusion protein with MeDH, can be an exogenous fructose-6-phosphate aldolase, such as a fructose-6-phosphate aldolase from *Amycolatopsis methanolica* 239 (AIJ24623.1); *Bacillus methanolicus* MGA3 plasmid pBM19 (WP_003349819.1); *Bacillus methanolicus* PB1 plasmid pBM20 (WP_003352247.1); *Geobacillus thermodenitrificans* NG80-2 (WP_008880720.1); *Geobacillus thermodenitrificans* NG80-2 (WP_008880032.1); *Methylophilus methylotrophus* ATCC 53528 (WP_018986971.1); and *Bacillus methanolicus* MGA3 (WP_003346852.1). Fructose-6-phosphate aldolases are of the enzyme class (EC) 4.1.2.

The fructose biphosphate aldolase in the engineered cell, which in some embodiments can be present as a fusion protein with MeDH, can be an exogenous fructose biphosphate aldolase. Fructose biphosphate aldolases are of the enzyme class (EC) 4.1.2.13.

Suitable purification and/or assays to test, e.g., for the production of BDO can be performed using well known methods. Suitable replicates such as triplicate cultures can be grown for each engineered strain to be tested. For example, product and byproduct formation in the engineered production host can be monitored. The final product and intermediates, and other organic compounds, can be analyzed by methods such as HPLC (High Performance Liquid Chromatography), GC-MS (Gas Chromatography-Mass Spectroscopy) and LC-MS (Liquid Chromatography-Mass Spectroscopy) or other suitable analytical methods using routine procedures well known in the art. The release of product in the fermentation broth can also be tested with the culture supernatant. Byproducts and residual glucose can be quantified

by HPLC using, for example, a refractive index detector for glucose and alcohols, and a UV detector for organic acids (Lin *et al.*, *Biotechnol. Bioeng.* 90:775-779 (2005)), or other suitable assay and detection methods well known in the art. The individual enzyme or protein activities from the exogenous DNA sequences can also be assayed using methods well known in the art.

5 The BDO or other target molecules may be separated from other components in the culture using a variety of methods well known in the art. Such separation methods include, for example, extraction procedures as well as methods that include continuous liquid-liquid extraction, pervaporation, evaporation, filtration, membrane filtration (including reverse osmosis, nanofiltration, ultrafiltration, and microfiltration), membrane filtration with
10 diafiltration, membrane separation, reverse osmosis, electrodialysis, distillation, extractive distillation, reactive distillation, azeotropic distillation, crystallization and recrystallization, centrifugation, extractive filtration, ion exchange chromatography, size exclusion chromatography, adsorption chromatography, carbon adsorption, hydrogenation, and ultrafiltration. All of the above methods are well known in the art.

15 Examples of target molecule isolation processes include distillation for 13BDO, 14BDO, butadiene, methyl vinyl carbinol, 3-buten-1-ol, n-propanol, isopropanol, propylene, and crotyl alcohol; crystallization for 6ACA (alternatively it can be converted to caprolactam and then purified via distillation as a final step), HMDA, adipic acid or derivatives thereof, succinic acid or derivatives thereof, or any of crystallization, distillation, or extraction for
20 methacrylic acid or derivatives thereof.

Target molecules such as 13BDO, 14BDO, butadiene, methyl vinyl carbinol n-propanol, isopropanol, propylene, crotyl alcohol; 3-buten-1-ol, 6ACA, HMDA, adipic acid or derivatives thereof, succinic acid or derivatives thereof, or methacrylic acid or derivatives thereof are chemicals used in commercial and industrial applications. In some embodiments, BDO and/or
25 4-HB are used in various commercial and industrial applications. Non-limiting examples of such applications include production of plastics, elastic fibers, polyurethanes, polyesters, including polyhydroxyalkanoates such as P4HB or co-polymers thereof, PTMEG and polyurethane-polyurea copolymers, referred to as spandex, elastane or LycraTM, nylons, and the like. Moreover, BDO and/or 4-HB are also used as a raw material in the production of a
30 wide range of products including plastics, elastic fibers, polyurethanes, polyesters, including polyhydroxyalkanoates such as P4HB or co-polymers thereof, PTMEG and polyurethane-polyurea copolymers, referred to as spandex, elastane or LycraTM, nylons, and the like.

Accordingly, in some embodiments, provided are biobased plastics, elastic fibers, polyurethanes, polyesters, including polyhydroxyalkanoates such as P4HB or co-polymers
35 thereof, PTMEG and polyurethane-polyurea copolymers, referred to as spandex, elastane or

Lycra™, nylons, and the like, comprising one or more bioderived BDO and/or 4-HB or bioderived BDO and/or 4-HB intermediate thereof produced by an organism provided herein or produced using a method disclosed herein.

As used herein, the term "bioderived" means derived from or synthesized by a biological organism and can be considered a renewable resource since it can be generated by a biological organism. Such a biological organism, in particular the microbial organisms disclosed herein, can utilize feedstock or biomass, such as, sugars or carbohydrates obtained from an agricultural, plant, bacterial, or animal source. Alternatively, the biological organism can utilize atmospheric carbon. As used herein, the term "biobased" means a product as described above that is composed, in whole or in part, of a bioderived compound of the disclosure. A biobased or bioderived product is in contrast to a petroleum derived product, wherein such a product is derived from or synthesized from petroleum or a petrochemical feedstock.

In some embodiments, provided herein is a culture medium comprising bioderived BDO. In some embodiments, the bioderived BDO is produced by culturing an organism having a fusion protein and BDO pathway, as provided herein. In certain embodiments, the bioderived BDO has a carbon-12, carbon-13 and carbon-14 isotope ratio that reflects an atmospheric carbon dioxide uptake source. In one embodiment, the culture medium is separated from an organism having a fusion protein and BDO pathway.

In other embodiments, provided herein is a bioderived BDO. In some embodiments, the bioderived BDO is produced by culturing an organism having a fusion protein and BDO pathway, as provided herein. In some embodiments, the bioderived BDO has an Fm value of at least 80%, at least 85%, at least 90%, at least 95% or at least 98%. In certain embodiments, the bioderived BDO is a component of culture medium.

In certain embodiments, provided herein is a composition comprising a bioderived BDO provided herein, for example, a bioderived BDO produced by culturing an organism having a MeDH fusion protein and BDOP (BDO pathway), as provided herein. In some embodiments, the composition further comprises a compound other than said bioderived BDO. In certain embodiments, the compound other than said bioderived BDO is a trace amount of a cellular portion of an organism having a fusion protein and a BDO pathway, as provided herein.

In some embodiments, provided herein is a biobased product comprising a bioderived BDO provided herein. In certain embodiments, the biobased product is a plastic, elastic fiber, polyurethane, polyester, polyhydroxyalkanoate, poly-4-HB, co-polymer of poly-4-HB, poly(tetramethylene ether) glycol, polyurethane-polyurea copolymer, spandex, elastane,

Lycra™, or nylon. In certain embodiments, the biobased product comprises at least 5% bioderived BDO. In certain embodiments, the biobased product is (i) a polymer, THF or a THF derivative, or GBL or a GBL derivative; (ii) a plastic, elastic fiber, polyurethane, polyester, polyhydroxyalkanoate, poly-4-HB, co-polymer of poly-4-HB, poly(tetramethylene ether) glycol, polyurethane-polyurea copolymer, spandex, elastane, Lycra™, or nylon; (iii) a polymer, a resin, a fiber, a bead, a granule, a pellet, a chip, a plastic, a polyester, a thermoplastic polyester, a molded article, an injection-molded article, an injection-molded part, an automotive part, an extrusion resin, an electrical part and a casing; and optionally where the biobased product is reinforced or filled and further where the biobased product is glass-reinforced or –filled or mineral-reinforced or –filled; (iv) a polymer, wherein the polymer comprises polybutylene terephthalate (PBT); (v) a polymer, wherein the polymer comprises PBT and the biobased product is a resin, a fiber, a bead, a granule, a pellet, a chip, a plastic, a polyester, a thermoplastic polyester, a molded article, an injection-molded article, an injection-molded part, an automotive part, an extrusion resin, an electrical part and a casing; and optionally where the biobased product is reinforced or filled and further where the biobased product is glass-reinforced or –filled or mineral-reinforced or –filled; (vi) a THF or a THF derivative, wherein the THF derivative is polytetramethylene ether glycol (PTMEG), a polyester ether (COPE) or a thermoplastic polyurethane; (viii) a THF derivative, wherein the THF derivative comprises a fiber; or (ix) a GBL or a GBL derivative, wherein the GBL derivative is a pyrrolidone. In certain embodiments, the biobased product comprises at least 10% bioderived BDO. In some embodiments, the biobased product comprises at least 20% bioderived BDO. In other embodiments, the biobased product comprises at least 30% bioderived BDO. In some embodiments, the biobased product comprises at least 40% bioderived BDO. In other embodiments, the biobased product comprises at least 50% bioderived BDO. In one embodiment, the biobased product comprises a portion of said bioderived BDO as a repeating unit. In another embodiment, provided herein is a molded product obtained by molding the biobased product provided herein. In other embodiments, provided herein is a process for producing a biobased product provided herein, comprising chemically reacting said bioderived-BDO with itself or another compound in a reaction that produces said biobased product. In certain embodiments, provided herein is a polymer comprising or obtained by converting the bioderived BDO. In other embodiments, provided herein is a method for producing a polymer, comprising chemically or enzymatically converting the bioderived BDO to the polymer. In yet other embodiments, provided herein is a composition comprising the bioderived BDO, or a cell lysate or culture supernatant thereof.

BDO is a valuable chemical for the production of high performance polymers, solvents, and fine chemicals. It is the basis for producing other high value chemicals such as tetrahydrofuran (THF) and gamma-butyrolactone (GBL). The value chain is comprised of three main segments including: (1) polymers, (2) THF derivatives, and (3) GBL derivatives. In the case of polymers, BDO is a comonomer for polybutylene terephthalate (PBT) production. PBT is a medium performance engineering thermoplastic used in automotive, electrical, water systems, and small appliance applications. Conversion to THF, and subsequently to polytetramethylene ether glycol (PTMEG), provides an intermediate used to manufacture spandex products such as LYCRA[®] fibers. PTMEG is also combined with BDO in the production of specialty polyester ethers (COPE). COPEs are high modulus elastomers with excellent mechanical properties and oil/environmental resistance, allowing them to operate at high and low temperature extremes. PTMEG and BDO also make thermoplastic polyurethanes processed on standard thermoplastic extrusion, calendaring, and molding equipment, and are characterized by their outstanding toughness and abrasion resistance. The GBL produced from BDO provides the feedstock for making pyrrolidones, as well as serving the agrochemical market. The pyrrolidones are used as high performance solvents for extraction processes of increasing use, including for example, in the electronics industry and in pharmaceutical production. Accordingly, provided herein is bioderived BDO produced according to the methods described herein and biobased products comprising or obtained using the bioderived BDO.

In some embodiments, the carbon feedstock and other cellular uptake sources such as phosphate, ammonia, sulfate, chloride and other halogens can be chosen to alter the isotopic distribution of the atoms present in BDO and/or 4-HB or any BDO and/or 4-HB pathway intermediate. The various carbon feedstock and other uptake sources enumerated above will be referred to herein, collectively, as “uptake sources.” Uptake sources can provide isotopic enrichment for any atom present in the product BDO and/or 4-HB or BDO and/or 4-HB pathway intermediate, or for side products generated in reactions diverging away from a BDO and/or 4-HB pathway. Isotopic enrichment can be achieved for any target atom including, for example, carbon, hydrogen, oxygen, nitrogen, sulfur, phosphorus, chloride or other halogens. The same holds true for the MMPs and FAPs, as well as intermediates thereof, provided herein.

In some embodiments, the uptake sources can be selected to alter the carbon-12, carbon-13, and carbon-14 ratios. In some embodiments, the uptake sources can be selected to alter the oxygen-16, oxygen-17, and oxygen-18 ratios. In some embodiments, the uptake sources can be selected to alter the hydrogen, deuterium, and tritium ratios. In some

embodiments, the uptake sources can be selected to alter the nitrogen-14 and nitrogen-15 ratios. In some embodiments, the uptake sources can be selected to alter the sulfur-32, sulfur-33, sulfur-34, and sulfur-35 ratios. In some embodiments, the uptake sources can be selected to alter the phosphorus-31, phosphorus-32, and phosphorus-33 ratios. In some embodiments, the uptake sources can be selected to alter the chlorine-35, chlorine-36, and chlorine-37 ratios.

In some embodiments, the isotopic ratio of a target atom can be varied to a desired ratio by selecting one or more uptake sources. An uptake source can be derived from a natural source, as found in nature, or from a man-made source, and one skilled in the art can select a natural source, a man-made source, or a combination thereof, to achieve a desired isotopic ratio of a target atom. An example of a man-made uptake source includes, for example, an uptake source that is at least partially derived from a chemical synthetic reaction. Such isotopically enriched uptake sources can be purchased commercially or prepared in the laboratory and/or optionally mixed with a natural source of the uptake source to achieve a desired isotopic ratio. In some embodiments, a target isotopic ratio of an uptake source can be obtained by selecting a desired origin of the uptake source as found in nature. For example, as discussed herein, a natural source can be a biobased derived from or synthesized by a biological organism or a source such as petroleum-based products or the atmosphere. In some such embodiments, a source of carbon, for example, can be selected from a fossil fuel-derived carbon source, which can be relatively depleted of carbon-14, or an environmental or atmospheric carbon source, such as CO₂, which can possess a larger amount of carbon-14 than its petroleum-derived counterpart.

Isotopic enrichment is readily assessed by mass spectrometry using techniques known in the art such as Stable Isotope Ratio Mass Spectrometry (SIRMS) and Site-Specific Natural Isotopic Fractionation by Nuclear Magnetic Resonance (SNIF-NMR). Such mass spectral techniques can be integrated with separation techniques such as liquid chromatography (LC) and/or high performance liquid chromatography (HPLC).

Accordingly, in some embodiments, provided are BDO and/or 4-HB or a BDO and/or 4-HB pathway intermediate thereof that has a carbon-12, carbon-13, and carbon-14 ratio that reflects an atmospheric carbon, also referred to as environmental carbon, uptake source.

Further, the disclosure relates, in part, to biologically produced BDO and/or 4-HB or BDO and/or 4-HB intermediate thereof as disclosed herein, and to the products derived therefrom, wherein the BDO and/or 4-HB or a BDO and/or 4-HB intermediate thereof has a carbon-12, carbon-13, and carbon-14 isotope ratio of about the same value as the CO₂ that occurs in the environment.

Those skilled in the art will understand that an organism can be engineered that secretes the biosynthesized compounds when grown on a carbon source such as a methanol alone or combined with other carbohydrates. Such compounds include, for example, BDO and any of the intermediate metabolites in the BDOP. All that is required is to engineer in one or more of the required enzyme or protein activities to achieve biosynthesis of the desired compound or intermediate including, for example, inclusion of some or all of the BDO biosynthetic pathways. Accordingly, provided herein is an organism that produces and/or secretes BDO when grown on a carbohydrate or other carbon source and produces and/or secretes any of the intermediate metabolites shown in the BDOP when grown on a carbohydrate or other carbon source. The BDO producing microbial organisms provided herein can initiate synthesis from an intermediate. The same holds true for intermediates in the formaldehyde assimilation.

In one embodiment, the carbon source is methanol or formate. In certain embodiments, methanol is used as a carbon source. In other embodiments, formate is used as a carbon source. In specific embodiments, methanol is used as a carbon source in the organisms provided herein, either alone or in combination with the product pathways provided herein.

In one embodiment, the carbon source comprises methanol, and sugar (*e.g.*, glucose) or a sugar-containing biomass. In another embodiment, the carbon source comprises formate, and sugar (*e.g.*, glucose) or a sugar-containing biomass. In one embodiment, the carbon source comprises methanol, formate, and sugar (*e.g.*, glucose) or a sugar-containing biomass. In specific embodiments, the methanol or formate, or both, in the fermentation feed is provided as a mixture with sugar (*e.g.*, glucose) or sugar-comprising biomass. In certain embodiments, sugar is provided for sufficient strain growth. In some embodiments, the sugar (*e.g.*, glucose) is provided at a molar concentration ratio of methanol to sugar of from 200:1 to 1:200. In certain embodiments, the carbon source comprises formate and a sugar (*e.g.*, glucose). In some embodiments, the sugar (*e.g.*, glucose) is provided at a molar concentration ratio of formate to sugar of from 200:1 to 1:200. In certain embodiments, the carbon source comprises a mixture of methanol and formate, and a sugar (*e.g.*, glucose). In certain embodiments, sugar is provided for sufficient strain growth. In some embodiments, the sugar (*e.g.*, glucose) is provided at a molar concentration ratio of methanol and formate to sugar of from 200:1 to 1:200.

Example 1: Preparation of MeDH fusion protein constructs

Nucleic acid constructs for expression of various MeDH-containing fusion proteins were prepared, as well as control constructs without a fusion and a no insert control. Table 7 is a list of the nucleic acid constructs and Table 8 provides details of the genes used in the fusion protein constructs.

Table 7

pZS13S-p100-2315LS-linker-hps-phi
pZS13S-p100-2435A-linker-hps-phi
pZS13S-p100-2451A-linker-hps-phi
pZS13S-p108-2315LS-linker-hps-phi
pZS13S-p108-2435A-linker-hps-phi
pZS13S-p108-2451A-linker-hps-phi
pZS13S-p100-2435A-hps-phi
pZS13S-p100-2451A-hps-phi
pZS13S-p108-2315LS-hps-phi
pZS13S-p108-2435A-hps-phi
pZS13S-p108-2451A-hps-phi

Table 8

Gene/region	CDS number	Identifier/SEQ ID NO	Source
MeDH	2315LS	Variant of MGA3_17392 SEQ ID NO:8	Evolved variant of MeDH from <i>Bacillus Methanolicus</i>
MeDH	2435A	ADH_2277 SEQ ID NO: 6	<i>Desulfotomaculum reducens</i> MI-1
MeDH	2451A	ADH/KC157637 SEQ ID NO: 10	Metalibrary sp
HPS-PHI	2616A	rmpAB fusion SEQ ID NO: 5	<i>Mycobacterium gastri</i> MB19
Linker			
GGSGGSGSGSGSGSGSGGS (SEQ ID NO: 7)			

- 5 Nucleic acid constructs that express these MeDH-GGSGGSGSGSGSGSGSGGS-2616A fusions were transformed into *E.coli* as described in Example 2.

Example 2: Preparation of engineered *E. coli* expressing**MeDH fusion proteins and analysis**

Nucleic acid constructs as listed in Table 7 were transformed into *E. coli*. *E. coli* transformants that express MeDH-2616A fusions were assessed for expression of soluble protein, methanol dehydrogenase activity in lysates of *E. coli* cells, and ability to confer an engineered *E. coli* strain (ECKh-8665) the ability to grow on methanol as a carbon source. Several variables were tested to identify combinations that confer improved activity, including: promoter strength, different MeDH variants, and peptide linkers in MeDH and 2616 fusions.

Nucleic acid constructs were transformed into *E. coli* strain 7539 and grown overnight at 37°C in 5 mL of LB + carb100 broth.

In vitro assay conditions were as follows. Lysis: 1 mL of cells harvested and lysed in 200ul of BugBuster with lysozyme and benzonase (samples were all OD normalized during data normalization). Assay: 10 uL of lysate added to 100ul of assay buffer, rate of NADH production monitored at 340nm over 2 hours. Assay buffer: 100mM Tris 8.5, 500mM Methanol, 5mM MgCl₂, 500mM NAD⁺.

Growth in Bioscreen instrument of *E. coli* host ECKh-8665 containing the denoted plasmid. The media was MM9 minimal media containing 2g/L glycerol and 500 mM methanol. 3 replicates. See Table 9.

Table 9

plasmid (pZS*13S-)	Growth rate estimation
P108_2451A-linker-2616A	0.11
P100_2435A	0.11
P100_2315LS	0.10
P107_2435A	0.10
P108_2435A-linker-2616A	0.09
P108_2315LS	0.09
P108_2315LS-linker-2616A	0.08
P119_2435A	0.04
P108_2315LS-2616A	no growth
Empty Vector	no growth

What is claimed is:

1. A fusion protein comprising:

(5a) a first region comprising methanol dehydrogenase or an enzymatically active portion thereof, (5b) a second region comprising 3-hexulose-6-phosphate dehydrogenase or an enzymatically active portion thereof; and (5c) a third region comprising 6-phospho-3-hexuloisomerase or an enzymatically active portion thereof; or

(6a) a first region comprising methanol dehydrogenase or an enzymatically active portion thereof, (6b) a second region comprising 3-hexulose-6-phosphate dehydrogenase or an enzymatically active portion thereof; (6c) a third region comprising 6-phospho-3-hexuloisomerase or an enzymatically active portion thereof; and (6d) a fourth region comprising methanol dehydrogenase activator or an enzymatically active portion thereof;

wherein the fusion protein optionally comprises one or more linker amino acid sequence(s) positioned between two or more of the first, second, and third regions of the fusion protein.

2. A fusion protein comprising:

first and second portions of a methanol dehydrogenase, 3-hexulose-6-phosphate dehydrogenase or an enzymatically active portion thereof, and 6-phospho-3-hexuloisomerase or an enzymatically active portion thereof, and optionally methanol dehydrogenase activator or an enzymatically active portion thereof, positioned between the first and second portions of a methanol dehydrogenase.

3. The fusion protein of claim 1 having a structure as follows:

(v) 5a-L¹-5b-L²-5c, 5a-L¹-5c-L²-5b, 5b-L¹-5a-L²-5c, 5b-L¹-5c-L²-5a, 5c-L¹-5a-L²-5b, or 5c-L¹-5b-L²-5a; or

(vi) 6a-L¹-6b-L²-6c-L³-6d, 6a-L¹-6b-L²-6d-L³-6c, 6a-L¹-6c-L²-6b-L³-6d, 6a-L¹-6c-L²-6d-L³-6b, 6a-L¹-6d-L²-6b-L³-6c, 6a-L¹-6d-L²-6c-L³-6b, 6b-L¹-6a-L²-6c-L³-6d, 6b-L¹-6a-L²-6c-L³-6d, 6b-L¹-6c-L²-6a-L³-6d, 6b-L¹-6c-L²-6d-L³-6a, 6b-L¹-6d-L²-6a-L³-6c, 6b-L¹-6d-L²-6c-L³-6a, 6c-L¹-6a-L²-6b-L³-6d, 6c-L¹-6a-L²-6d-L³-6b, 6c-L¹-6b-L²-6a-L³-6d, 6c-L¹-6b-L²-6d-L³-6a, 6c-L¹-6d-L²-6a-L³-6b, 6c-L¹-6d-L²-6b-L³-6a, 6d-L¹-6a-L²-6b-L³-6c, 6d-L¹-6a-L²-6c-L³-6b, 6d-L¹-6b-L²-6a-L³-6c, 6d-L¹-6b-L²-6c-L³-6a, 6d-L¹-6c-L²-6b-L³-6a, and 6d-L¹-6c-L²-6a-L³-6b;

wherein L¹, L², and L³ are independently selected from (-) (a covalent bond) and a linker amino acid sequence.

4. The fusion protein of claim 1, 2 or 3 wherein one or more of the methanol dehydrogenase, methanol dehydrogenase activator, the 3-hexulose-6-phosphate dehydrogenase, the 6-phospho-3-hexuloisomerase, or enzymatically active portions thereof, is from bacteria.
- 5 5. The fusion protein of claim 4 wherein one or more of the methanol dehydrogenase, the methanol dehydrogenase activator, the 3-hexulose-6-phosphate dehydrogenase, the 6-phospho-3-hexuloisomerase, or enzymatically active portions thereof, is from *Bacillus*.
6. The fusion protein of claim 5 wherein one or more of the methanol dehydrogenase, the
10 methanol dehydrogenase activator, the 3-hexulose-6-phosphate dehydrogenase, the 6-phospho-3-hexuloisomerase, or enzymatically active portions thereof, is from *Bacillus methanolicus* MGA3 or *Bacillus methanolicus* PBI.
7. The fusion protein of claim 1, 2 or 3 wherein the methanol dehydrogenase (MeDH) or an
15 enzymatically active portion thereof is from *Bacillus methanolicus* MGA3 MeDH (3EIJ77596.1); *Bacillus methanolicus* C1 MeDH (AAA22593.1); *Bacillus methanolicus* PB1 MeDH (EIJ77618.1); *Bacillus methanolicus* PB1 MeDH (EIJ78790.1); *Bacillus methanolicus* MGA3 MeDH (EIJ80770.1); *Bacillus methanolicus* PB1 MeDH (EIJ78397.1); *Bacillus methanolicus* MGA3 MeDH (EIJ83020.1); *Lysinibacillus fusiformis* MeDH (EFI69743.1);
20 *Bacillus coagulans* 36D1 MeDH (YP_004860127.1); *Lysinibacillus sphaericus* MeDH (YP_001699778.1); *Bacillus azotoformans* LMG 9581 MeDH (ZP_11313277.1); *Burkholderia thailandensis* E264 MeDH (ZP_05587334.1); *Cupriavidus necator* N-1 MeDH (YP_004681552.1); uncultured organism MeDH (AGF87161); *Geobacter bemidjiensis* Bem MeDH (YP_002138168.1); *Carboxydotherrmus hydrogenoformans* Z-2901 MeDH (YP_359772.1); *Actinobacillus succinogenes* 130Z MeDH (YP_001343716.1); *Acinetobacter baumannii* Naval-82 MeDH (ZP_16224338.1); *Clostridium pasteurianum* DSM 525 MeDH (AAC45651.1); *Methanosarcina mazei* Tuc01 MeDH (YP_007491369.1); *Desulfovibrio vulgaris* str. 'Miyazaki F' MeDH (YP_002434746); *Desulfovibrio africanus* str. Walvis Bay MeDH (YP_005052855); *Clostridium perfringens* str. 13 MeDH (NP_561852.1); *Vibrio campbellii* ATCC BAA-1116 MeDH (YP_001447544); *Desulfotomaculum reducens* MI-1 MeDH (YP_001113612.1); *Desulfovibrio vulgaris* str. Hildenborough MeDH (YP_011618); *Photobacterium profundum* 3TCK MeDH (ZP_01220157.1); *Geobacillus* sp. Y4.1MC1 MeDH (YP_003990729.1); *Desulfovibrio fructosovorans* JJ MeDH (ZP_07335453.1); *Shewanella oneidensis* MR-1 MeDH (NP_717107); *Sealdella termitidis* ATCC 33386 MeDH
30 (YP_003310546.1); *Paenibacillus peoriae* KCTC 3763 MeDH (ZP_10241531.1); *Klebsiella*

- pneumoniae* subsp. *pneumoniae* MGH 78578 MeDH (YP_001337153.1); *Escherichia coli* MeDH (YP_026233.1); *Clostridium perfringens* ATCC 13124 MeDH (YP_694908); *Ralstonia eutropha* H16 MeDH (YP_725376.1); *Thermoanaerobacter* sp. X514 MeDH (YP_001663549); human gut metagenome MeDH (EKC54576); or *Geobacillus* 5 *thiodenitrificans* NG80-2 MeDH (YP_001126968.1).
8. The fusion protein of claim 1, 2 or 3 wherein the methanol dehydrogenase or an enzymatically active portion thereof is a polypeptide having 50% or greater, 60% or greater, 70% or greater, 80% or greater, 85% or greater, 90% or greater, 95% or greater, 96% or 10 greater, 97% or greater, 98% or greater, or 99% or greater identity to *Bacillus methanolicus* MGA3MeDH (3EIJ77596.1) or to any other MeDH according to claim 7.
9. The fusion protein of claim 8 comprising one or more amino acid substitutions that differ from a native sequence of the methanol dehydrogenase, 3-hexulose-6-phosphate 15 dehydrogenase, 6-phospho-3-hexuloisomerase, or methanol dehydrogenase activator.
10. The fusion protein of claim 1, 2 or 3 wherein the region comprising 3-hexulose-6-phosphate dehydrogenase or an enzymatically active portion thereof is from *Bacillus methanolicus* MGA3 HPS (AAR39392.1); *Bacillus methanolicus* PB1 HPS (EIJ81375.1); 20 *Methylobacillus flagellatus* HPS (YP 544362.1); *Methylobacillus flagellatus* HPS (YP 544362.1); *Bacillus subtilis* HPS (NP_388228.1); *Methylophilus methylotrophus* HPS (WP_018986666.1); or *Methylophilus methylotrophus* ATCC 53528 HPS (WP_018985298.1).
11. The fusion protein of claim 1, 2 or 3, wherein 3-hexulose-6-phosphate dehydrogenase or an 25 enzymatically active portion thereof is a polypeptide having 50% or greater, 60% or greater, 70% or greater, 80% or greater, 85% or greater, 90% or greater, 95% or greater, 96% or greater, 97% or greater, 98% or greater, or 99% or greater identity to *Bacillus methanolicus* MGA3 HPS (AAR39392.1) or to any other HPS according to claim 10.
- 30 12. The fusion protein of claim 1, 2 or 3, wherein 6-phospho-3-hexuloisomerase or an enzymatically active portion thereof is from *Bacillus methanolicus* MGA3 PHI (AAR39393.1); *Bacillus methanolicus* PB1 PHI (EIJ81376.1); *Mycobacterium gastri* PB1 PHI (BAA90545.1); *Methylobacillus flagellatus* KT PHI (YP 545762.1); *Bacillus subtilis* PHI (NP_388227.1); or *Methylophilus methylotrophus* ATCC 53528 HPS (WP_018985297.1).

13. The fusion protein of claim 1, 2 or 3, wherein the region comprising 6-phospho-3-hexuloisomerase or an enzymatically active portion thereof is a polypeptide having 50% or greater, 60% or greater, 70% or greater, 80% or greater, 85% or greater, 90% or greater, 95% or greater, 96% or greater, 97% or greater, 98% or greater, or 99% or greater identity to
5 *Bacillus methanolicus* MGA3 PHI (AAR39393.1) or to any other PHI according to claim 12;.
14. The fusion protein of claim 1, 2 or 3, wherein the region comprising the methanol dehydrogenase activator or an enzymatically active portion thereof is a polypeptide having 50% or greater, 60% or greater, 70% or greater, 80% or greater, 85% or greater, 90% or
10 greater, 95% or greater, 96% or greater, 97% or greater, 98% or greater, or 99% or greater identity to *Bacillus methanolicus* MGA3 ACT (Q8KP10) or a paralog or ortholog of MGA3ACT.
15. The fusion protein of any one of the previous claims having one linker amino acid
15 sequence.
16. The fusion protein of any of claims 1-15 having two linker amino acid sequences, wherein the two linker amino acid sequences are the same or different.
- 20 17. The fusion protein of any one of the previous claims, wherein the linker amino acid sequence comprises one or more amino acids selected from the group consisting of glycine, alanine, serine, and threonine.
18. The fusion protein of any one of the previous claims, wherein the linker amino acid
25 sequence has a length in the range of 1-50 amino acid residues, or 1-20 amino acid residues.
19. The fusion protein of any one of the previous claims, wherein the linker amino acid sequence is $[(\text{GGS})_x(\text{GS})_y(\text{GGS})_z]_n$ where x is 1-6, y is 1-6, z is 0-3, and n is 1-4, preferably where x is 1-3, y is 1-3, z is 1-2, and n is 1-2.
30
20. A nucleic acid encoding the fusion protein of any of the previous claims.
21. An expression construct comprising the nucleic acid of claim 20.

22. An engineered cell comprising the fusion protein of any one of claims 1-19, the nucleic acid of claim 20, or the expression construct of claim 21, or

an engineered cell that heterologously expresses methanol dehydrogenase, 3-hexulose-6-phosphate dehydrogenase, and 6-phospho-3-hexuloisomerase, wherein heterologous

5 expression includes expression of a fusion protein comprising

(1a) a first region comprising methanol dehydrogenase or an enzymatically active portion thereof, and (1b) a second region comprising 3-hexulose-6-phosphate dehydrogenase or an enzymatically active portion thereof;

10 (2a) a first region comprising methanol dehydrogenase or an enzymatically active portion thereof, (2b) a second region comprising 3-hexulose-6-phosphate dehydrogenase or an enzymatically active portion thereof; and (2c) a third region comprising methanol dehydrogenase activator or an enzymatically active portion thereof;

15 (3a) a first region comprising methanol dehydrogenase or an enzymatically active portion thereof, and (3b) a second region comprising 6-phospho-3-hexuloisomerase or an enzymatically active portion thereof;

20 (4a) a first region comprising methanol dehydrogenase or an enzymatically active portion thereof, (4b) a second region comprising 6-phospho-3-hexuloisomerase or an enzymatically active portion thereof; and (4c) a third region comprising methanol dehydrogenase activator or an enzymatically active portion thereof;

(5a) a first region comprising methanol dehydrogenase or an enzymatically active portion thereof, (5b) a second region comprising 3-hexulose-6-phosphate dehydrogenase or an enzymatically active portion thereof; and (5c) a third region comprising 6-phospho-3-hexuloisomerase or an enzymatically active portion thereof;

25 (6a) a first region comprising methanol dehydrogenase or an enzymatically active portion thereof, (6b) a second region comprising 3-hexulose-6-phosphate dehydrogenase or an enzymatically active portion thereof; (6c) a third region comprising 6-phospho-3-hexuloisomerase or an enzymatically active portion thereof; and (6d) a fourth region comprising methanol dehydrogenase activator or an
30 enzymatically active portion thereof; or

(7a) first and second portions of a methanol dehydrogenase, and 3-hexulose-6-phosphate dehydrogenase or an enzymatically active portion thereof, and/or 6-phospho-

3-hexuloisomerase or an enzymatically active portion thereof, and optionally methanol dehydrogenase activator or an enzymatically active portion thereof, positioned between the first and second portions of a methanol dehydrogenase.

5 23. The engineered cell of claim 22 which is a microbial cell.

24. The engineered cell of claim 23 which is a bacterial cell.

25. The engineered cell of claim 24 which is *E. coli*.

10

26. The engineered cell of any one of claims 22-25 further comprising one or more transgene(s) encoding a protein of a metabolic pathway that promotes production of a target product or intermediate thereof, wherein the metabolic pathway uses a compound resulting from methanol consumption by the cell.

15

27. The engineered cell of any one of claims 22-25 wherein expression of the fusion provides an increased amount of reducing equivalents for an increase in a target product and/or for increased fixation of carbon from formaldehyde into a target product.

20 28. The engineered cell of any one of claims 22-25 further comprising a transgene encoding an enzyme to convert the formaldehyde to formate thereby generating reducing equivalents useful to product the target product and/or able to fix carbon of formate into the target product.

25 29. The engineered cell of any one of claims 22-25 capable of producing a greater amount of acetyl CoA in the presence of methanol as compared to a cell that does not express the fusion protein.

30 30. The engineered cell of any one of claims 22-25 comprising a metabolic pathway capable of producing a target product selected from the group consisting of 1,4-butanediol, 1,3-butanediol, butadiene, succinate, adipate, HMDA, or 6-aminocaproic acid (6ACA).

31. An engineered cell comprising (i) a fusion protein comprising a first region comprising methanol dehydrogenase and a second region comprising methanol dehydrogenase activator, or enzymatically active portions thereof, and (ii) and a fusion protein comprising a first region

comprising 3-hexulose-6-phosphate dehydrogenase or an enzymatically active portion thereof; and a second region comprising 6-phospho-3-hexuloisomerase or an enzymatically active portion thereof.

- 5 32. The engineered cell of any one of claims 22-31 comprising any one or more enzymes selected from the group consisting of DHA (dihydroxyacetone) synthase, F6P (fructose-6-phosphate) aldolase, DHA kinase, fructose-bisphosphate aldolase, wherein any one or more of the enzymes is optionally expressed from an exogenous nucleic acid.
- 10 33. The engineered cell of any one of claims 22-32 comprising any one or more enzymes selected from the group consisting of succinyl-CoA synthetase; CoA-independent succinic semialdehyde dehydrogenase; α -ketoglutarate dehydrogenase; glutamate:succinate semialdehyde transaminase; glutamate decarboxylase; CoA-dependent succinic semialdehyde dehydrogenase; 4-hydroxybutanoate dehydrogenase; α -ketoglutarate decarboxylase; 4-
- 15 hydroxybutyryl CoA: acetyl-CoA transferase; butyrate kinase; phosphotransbutyrylase; aldehyde dehydrogenase; alcohol dehydrogenase, wherein any one or more of the enzymes is optionally expressed from an exogenous nucleic acid.
34. A composition comprising the engineered cell of any of claims 22-33.
- 20 35. The composition of claim 34 wherein the composition is a cell culture composition, optionally comprising a target product or intermediate thereof.
36. A method of producing a target product or its intermediate comprising culturing the
- 25 engineered cell of any of claims 22-33 in a culture medium comprising methanol to produce the target product or an intermediate of the target product.
37. The method of claim 36 wherein the culture medium comprises methanol and a second carbon source.
- 30 38. The method of claim 36 or 37 further comprising a step of isolating or purifying the target product or an intermediate of the target product.