# CELL-FREE SYSTEM FOR CONVERTING METHANE INTO FUEL PYRUVATE OR ISOBUTANOL

## RELATED APPLICATIONS

This application claims priority under 35 U.S.C. § 119(e) to U.S. provisional application, U.S.S.N. 61/740,972, filed December 21, 2012, which is incorporated by reference herein in its entirety.

## BACKGROUND OF INVENTION

Isobutanol is produced naturally during the fermentation of carbohydrates and may also be a byproduct of the decay process of organic matter (Atsumi *et al.* 2008 *Nature* 451:86–89). The biosynthetic pathway used to produce isobutanol was first discovered in species of bacteria from the genus *Clostridium*. This pathway has been genetically engineered into several species of microorganisms which are more easily manipulated by current scientific methods than microorganisms of the genus *Clostridium* (Peralta-Yahya *et al.* 2012 *Nature* 488: 320–328).

## SUMMARY ~~OF INVENTION~~

The present invention provides in one aspect, a cell-free method for conversion of methane to isobutanol, comprising: combining, in a bioreactor at a pressure of at least one bar (a) methane, (b) oxygen, and (c) cell lysates containing a methane monooxygenase, a methanol dehydrogenase, and each of the following enzymes that catalyze the conversion of formaldehyde to isobutanol: a hexulose-6-phosphate synthase, a 6-phospho-3-hexuloisomerase, a 6-phosphofructokinase, a fructose bisphosphate aldolase, a triose phosphate isomerase, a transketolase, a ribose-5-phosphate isomerase or a ribulose-5-phosphate 3-epimerase, a glyceraldehyde 3-phosphate dehydrogenase, a phosphoglycerate kinase, a phosphoglycerate mutase, an enolase, a pyruvate kinase, an acetolactate synthase, a acetohydroxy acid isomeroreductase, a dihydroxy acid dehydratase, an alpha-ketoisovalerate decarboxylase, and an isobutanol dehydrogenase, to form a cell-free reaction mixture; and incubating under suitable conditions the cell-free reaction to convert methane to isobutanol. In another aspect, the present invention refers to a cell-free method for conversion of methane to pyruvate, comprising: combining, in a bioreactor at a pressure of at least one bar (a) methane,

(b) oxygen, and (c) cell lysates containing a methane monooxygenase, a methanol dehydrogenase, and each of the following enzymes that catalyze the conversion of formaldehyde to pyruvate: a hexulose-6-phosphate synthase, a 6-phospho-3-hexuloisomerase, a 6-phosphofructokinase, a fructose bisphosphate aldolase, a triose phosphate isomerase, a transketolase, a ribose-5-phosphate isomerase or a ribulose-5-phosphate 3-epimerase, a glyceraldehyde 3-phosphate dehydrogenase, a phosphoglycerate kinase, a phosphoglycerate mutase, an enolase, and a pyruvate kinase, to form a cell-free reaction mixture; and incubating under suitable conditions the cell-free reaction to convert methane to pyruvate. In still another aspect, the present invention refers to a cell-free system for conversion of methane to isobutanol, the system comprising a bioreactor that comprises: a gas phase comprising methane and oxygen; and an aqueous phase comprising a cell lysate containing (a) a methane monooxygenase, (b) a methanol dehydrogenase, and (c) each of the following enzymes that catalyze the conversion of formaldehyde to isobutanol: a hexulose-6-phosphate synthase, a 6-phospho-3-hexuloisomerase, a 6-phosphofructokinase, a fructose bisphosphate aldolase, a triose phosphate isomerase, a transketolase, a ribose-5-phosphate isomerase or a ribulose-5-phosphate 3-epimerase, a glyceraldehyde 3-phosphate dehydrogenase, a phosphoglycerate kinase, a phosphoglycerate mutase, an enolase, a pyruvate kinase, an acetolactate synthase, a acetohydroxy acid isomeroreductase, a dihydroxy acid dehydratase, an alpha-ketoisovalerate decarboxylase, and an isobutanol dehydrogenase. In a further aspect, the present invention refers to a cell-free system for conversion of methane to pyruvate, the system comprising a bioreactor that comprises: a gas phase comprising methane and oxygen; and an aqueous phase comprising a cell lysate containing (a) a methane monooxygenase, (b) a methanol dehydrogenase, and (c) each of the following enzymes that catalyze the conversion of formaldehyde to pyruvate: a hexulose-6-phosphate synthase, a 6-phospho-3-hexuloisomerase, a 6-phosphofructokinase, a fructose bisphosphate aldolase, a triose phosphate isomerase, a transketolase, a ribose-5-phosphate isomerase or a ribulose-5-phosphate 3-epimerase, a glyceraldehyde 3-phosphate dehydrogenase, a phosphoglycerate kinase, a phosphoglycerate mutase, an enolase, and a pyruvate kinase. In yet another aspect, the present invention refers to a cell lysate as defined by the present invention. Also disclosed herein is a cell-free system for the conversion of natural gas, in particular, methane, to target biofuels (*e.g.*, isobutanol) and other chemical compounds. Natural gas is a hydrocarbon gas mixture of primarily methane, but also includes varying amounts of other higher alkanes and a lesser percentage of carbon

dioxide, nitrogen, and hydrogen sulfide. ~~Aspects of the invention~~Examples disclosed herein uniquely combine biosynthetic and chemical engineering processes to provide a cell-free biosynthetic system capable of producing from natural gas greater than, for example, 10 g to 25 g biofuel per liter-hr. For example, an industrial or commercial plant sized to use a 155 $m^3$ reactor can produce greater than 30,000 metric tons of biofuel (*e.g.*, isobutanol) per year (*e.g.*, >500 BPD scale), in some instances with raw material costs of less than 20% of the product's value.

The cell-free process provided herein, in some ~~embodiments~~examples, combines (i) a cell-free lysate from methane-metabolizing methanotrophs (*e.g.*, *Methylococcus capsulatus* Bath) that express enzymes (*e.g.*, methane monooxygenase, MMO) for the conversion of methane to methanol or formaldehyde with (ii) a cell-free lysate(s) from recombinant bacterial cells (*e.g.*,

*Escherichia coli*) that express enzymes for the conversion of methanol or formaldehyde to biofuel or other chemical compound. The cell lysates are combined with methane and oxygen, both required for MMO activation, in a pressurized bioreactor (*e.g.*, at least 1 bar) to drive the transformation of methane to biofuel or other chemical compound. The cell-free process of the present disclosure typically uses a multiphase reaction system that is particularly useful for delivering methane and oxygen to exposed lipid bilayer membranes (e.g., of intracellular membrane vesicles of the cell lysate(s)) by first diffusing the gases into an aqueous phase. In some ~~embodiments~~examples, enzymes for the conversion of methane to formaldehyde and/or for the conversion of formaldehyde to biofuel or other chemical compound are added exogenously to the cell-free system. Such exogenously added enzymes may be purified or partially purified.

It should be understood that while the present disclosure describes the use of methanotrophs for the expression/production of enzymes for the conversion of methane to formaldehyde, other organisms for use in producing enzymes for the conversion of methane to formaldehyde are contemplated herein.

In some ~~aspects~~examples, ~~provided~~ disclosed herein are cell-free methods for large-scale conversion of methane to a biofuel or other chemical compound comprising: combining, in a bioreactor at elevated pressure, one or more cell lysates containing enzyme(s) that catalyze the conversion of methane to formaldehyde, and enzymes that catalyze the conversion of formaldehyde to a biofuel or another chemical compound, methane, and oxygen to form a cell-free reaction mixture; and incubating under suitable conditions (*e.g.*, high pressure, 37 °C) the cell-free reaction to convert methane to a biofuel or other chemical compound. In some ~~embodiments~~examples, the bioreactor may further comprise an organic solvent (*e.g.*, decane). In some examples~~embodiments~~, the organic phase is saturated or supersaturated with methane. "Suitable conditions" include, without limitation, a pressure of at least 1 bar (*e.g.*, 1 bar to 10 bar, or 5 bar to 10 bar) and/or a temperature of 35 °C to 40 °C (*e.g.*, 37 °C).

In some examples~~embodiments~~, ~~provided~~ disclosed herein are cell-free methods for large-scale conversion of methane to isobutanol comprising: combining, in a bioreactor at elevated pressure, one or more cell lysates containing methane monooxygenase and methanol dehydrogenase (*e.g.*, a recombinant NAD-linked methanol dehydrogenase), which catalyze the conversion of methane to formaldehyde, and one or more of hexulose-6-phosphate synthase, 6-phospho-3-hexuloisomerase, 6-phosphofructokinase, fructose biphosphate aldolase, triose phosphate isomerase, transketolase, ribose-5-phosphate isomerase, ribose-5-phosphate 3-epimerase,

glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, enolase, pyruvate kinase, acetolactate synthase, acetohydroxy acid isomeroreductase, dihydroxy acid dehydratase, alpha-ketoisovalerate decarboxylase, and isobutanol dehydrogenase, which catalyze the conversion of formaldehyde to isobutanol, methane, and oxygen to form a cell-free reaction mixture; and incubating under suitable conditions the cell-free reaction to convert methane to isobutanol. In some examples~~embodiments~~, the bioreactor may further comprise an organic solvent (*e.g.*, decane).

One of the intermediate products of the conversion formaldehyde to biofuel or other chemical compound is pyruvate. Thus, in some examples~~aspects~~, ~~provided~~ disclosed herein are cell-free methods for large-scale conversion of methane to pyruvate comprising: combining, in a bioreactor at elevated pressure, one or more cell lysates containing enzyme(s) that catalyze the conversion of methane to formaldehyde, and enzymes that catalyze the conversion of formaldehyde to pyruvate, methane, and oxygen to form a cell-free reaction mixture; and incubating under suitable conditions the cell-free reaction to convert methane to pyruvate. In some examples~~embodiments~~, the bioreactor may further comprise an organic solvent (*e.g.*, decane).

In some examples~~embodiments~~, provided herein are cell-free methods for large-scale conversion of methane to pyruvate comprising: combining, in a bioreactor at elevated pressure, one or more cell lysates containing methane monooxygenase and methanol dehydrogenase (*e.g.*, a recombinant NAD-linked methanol dehydrogenase), which catalyze the conversion of methane to formaldehyde, and one or more of hexulose-6-phosphate synthase, 6-phospho-3-hexuloisomerase, 6-phosphofructokinase, fructose biphosphate aldolase, triose phosphate isomerase, transketolase, ribose-5-phosphate isomerase, ribose-5-phosphate 3-epimerase, glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, enolase, and pyruvate kinase, which catalyze the conversion of formaldehyde to pyruvate, methane, and oxygen to form a cell-free reaction mixture; and incubating under suitable conditions the cell-free reaction to convert methane to pyruvate. In some examples~~embodiments~~, the bioreactor may further comprise an organic solvent (*e.g.*, decane).

In other examples~~aspects~~, ~~provided~~ disclosed herein are cell-free systems and compositions for large-scale conversion of methane to a biofuel or other chemical compound comprising a bioreactor that comprises: a gas phase comprising methane and oxygen; and an aqueous phase comprising a cell lysate containing enzyme(s) that catalyze the conversion of methane to formaldehyde and enzymes that catalyze the conversion of formaldehyde to a biofuel or chemical compound. In

some examplesembodiments, the bioreactor further comprises an organic phase comprising an organic solvent (*e.g.*, decane).

In some examplesembodiments, provided herein are cell-free systems and compositions for large-scale conversion of methane to isobutanol comprising a bioreactor that comprises: a gas phase comprising methane and oxygen; and an aqueous phase comprising one or more cell lysates containing methane monooxygenase, and methanol dehydrogenase (*e.g.*, a recombinant NAD-linked methanol dehydrogenase), which catalyze the conversion of methane to formaldehyde and hexulose-6-phosphate synthase, 6-phospho-3-hexuloisomerase, 6-phosphofructokinase, fructose biphosphate aldolase, triose phosphate isomerase, transketolase, ribose-5-phosphate isomerase, ribose-5-phosphate 3-epimerase, glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, enolase, pyruvate kinase, acetolactate synthase, acetohydroxy acid isomeroreductase, dihydroxy acid dehydratase, alpha-ketoisovalerate decarboxylase, and isobutanol dehydrogenase, which catalyze the conversion of formaldehyde to isobutanol. In some examplesembodiments, the bioreactor further comprises an organic phase comprising an organic solvent (*e.g.*, decane).

In some examplesembodiments, ~~provided~~ disclosed herein are cell-free systems and compositions for large-scale conversion of methane to pyruvate comprising a bioreactor that comprises: a gas phase comprising methane and oxygen; and an aqueous phase comprising one or more cell lysates containing methane monooxygenase and methanol dehydrogenase (*e.g.*, a recombinant NAD-linked methanol dehydrogenase), which catalyze the conversion of methane to formaldehyde, and hexulose-6-phosphate synthase, 6-phospho-3-hexuloisomerase, 6-phosphofructokinase, fructose biphosphate aldolase, triose phosphate isomerase, transketolase, ribose-5-phosphate isomerase, ribose-5-phosphate 3-epimerase, glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, enolase, and pyruvate kinase, which catalyze the conversion of formaldehyde to pyruvate. In some examplesembodiments, the bioreactor further comprises an organic phase comprising an organic solvent (*e.g.*, decane).

In some examplesaspects, ~~provided~~ disclosed herein are cell-free methods for large-scale conversion of methane to pyruvate, comprising: (a) expressing in methanotrophs one or more enzymes for the conversion of methane to formaldehyde, (b) expressing in recombinant bacteria one or more enzymes for the conversion of formaldehyde to pyruvate, (c) combining cell lysate from the methanotrophs and the recombinant bacteria with methane, an organic solvent (*e.g.*, decane), and oxygen to form a cell-free reaction mixture, and (d) incubating under suitable

conditions the cell-free reaction to convert methane to pyruvate. In some examplesembodiments, the one or more enzymes for the conversion of methane to formaldehyde are selected from methane monooxygenase and methanol dehydrogenase. In some examplesembodiments, the one or more enzymes for the conversion of formaldehyde to pyruvate are selected from hexulose-6-phosphate synthase, 6-phospho-3-hexuloisomerase, 6-phosphofructokinase, fructose biphosphate aldolase, triose phosphate isomerase, transketolase, ribose-5-phosphate isomerase, ribose-5-phosphate 3-epimerase, glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, enolase, and pyruvate kinase.

In some examplesembodiments, the method further comprises lysing the methanotroph and/or recombinant bacterial cells (*e.g.*, prior to the combining step).

In some examplesembodiments, the method further comprises expressing in the recombinant bacterial cells a protease that cleaves and inactivates an enzyme. For example, the protease may cleave and inactivate pyruvate dehydrogenase and/or formaldehyde dehydrogenase. Alternatively, the protease may be added exogenously.

Also provided disclosed herein are cell lysates that comprise one or more enzymes for the conversion of methane to formaldehyde, as well as cell lysates comprising one or more enzymes for the conversion of formaldehyde to a biofuel or other chemical compound. In some examplesembodiments, the cell lysates comprise one or more enzymes for the conversion of formaldehyde to pyruvate, or one or more enzymes for the conversion of formaldehyde to isobutanol. Cell lysates may further comprise small molecules and cofactors (*e.g.*, adenosine triphosphate, NAD(PH)), and salts, such as magnesium, for enzyme function.

Further provided disclosed herein are biofuels and other chemical compound or intermediate produced by any one or more of the cell-free processes described herein.

## BRIEF DESCRIPTION OF DRAWINGS

*Figure 1* shows a diagram of an exemplary cell-free multiphase reaction system of the invention designed to deliver methane and oxygen into the aqueous phase at a high rate.

*Figure 2* shows a diagram of an exemplary cell-free transformation platform of the invention.

*Figure 3* shows a diagram of an exemplary cell-free transformation platform of the invention.

*Figure 4* shows a diagram of the isobutanol pathway.

## CLAIMS

What is claimed is:

1. A cell-free method for conversion of methane to isobutanol, comprising:

combining, in a bioreactor at a pressure of at least 1 bar

(a) methane,

(b) oxygen, and

(c) cell lysates containing a methane monooxygenase, a methanol dehydrogenase, and each of the following enzymes that catalyze the conversion of formaldehyde to isobutanol: a hexulose-6-phosphate synthase, a 6-phospho-3-hexuloisomerase, a 6-phosphofructokinase, a fructose bisphosphate aldolase, a triose phosphate isomerase, a transketolase, a ribose-5-phosphate isomerase or a ribulose-5-phosphate 3-epimerase, a glyceraldehyde 3-phosphate dehydrogenase, a phosphoglycerate kinase, a phosphoglycerate mutase, an enolase, a pyruvate kinase, an acetolactate synthase, a acetohydroxy acid isomeroreductase, a dihydroxy acid dehydratase, an alpha-ketoisovalerate decarboxylase, and an isobutanol dehydrogenase, to form a cell-free reaction mixture; and

incubating under suitable conditions the cell-free reaction to convert methane to isobutanol.

2. The cell-free method of claim 1, wherein the methane monooxygenase is obtained from a methanotroph.

3. The cell-free method of claim 1 or 2, wherein the methanol dehydrogenase is a NAD-linked methanol dehydrogenase.

4. The cell-free method of any one of claims 1-3, wherein the enzymes that catalyze the conversion of formaldehyde to isobutanol are derived from recombinant bacteria.

5. The cell-free method of claim 4, wherein the recombinant bacteria are recombinant *Escherichia coli*.

6. The cell-free method of any one of claims 1 to 5, wherein the pressure is at least 2 bar.

7. The cell-free method of claim 6, wherein the pressure is at least 5 bar.

8. The cell-free method of any one of claims 1-7, wherein the isobutanol is produced at a production rate of at least 1 g/L-h.

9. The cell-free method of claim 8, wherein the isobutanol is produced at a production rate of at least 10 g/L-h.

10. The cell-free method of claim 9, wherein the isobutanol is produced at a production rate of at least 25 g/L-h.

11. The cell-free method of any one of claims 1-10, wherein the bioreactor comprises a gas phase and an aqueous phase.

12. The cell-free method of claim 11, wherein the bioreactor further comprises an organic solvent.

13. The cell-free method of claim 12, wherein the organic solvent is an alkane.

14. The cell-free method of claim 13, wherein the alkane is selected from the group consisting of pentane, hexane, heptane, octane, nonane, and decane.

15. A cell-free method for conversion of methane to pyruvate, comprising:

combining, in a bioreactor at a pressure of at least one bar

(a) methane,

(b) oxygen, and

(c) cell lysates containing a methane monooxygenase, a methanol dehydrogenase, and each of the following enzymes that catalyze the conversion of formaldehyde to pyruvate: a hexulose-6-phosphate synthase, a 6-phospho-3-hexuloisomerase, a 6-phosphofructokinase, a fructose bisphosphate aldolase, a triose phosphate isomerase, a transketolase, a ribose-5-phosphate isomerase or a ribulose-5-phosphate 3-epimerase, a glyceraldehyde 3-phosphate dehydrogenase, a phosphoglycerate kinase, a phosphoglycerate mutase, an enolase, and a pyruvate kinase, to form a cell-free reaction mixture; and

incubating under suitable conditions the cell-free reaction to convert methane to pyruvate.

16. The cell-free method of claim 15, wherein the methane monooxygenase is obtained from a methanotroph.

17. The cell-free method of claim 15 or 16, wherein the methanol dehydrogenase is a NAD-linked methanol dehydrogenase.

18. The cell-free method of any one of claims 15-17, wherein the enzymes that catalyze the conversion of formaldehyde to isobutanol are derived from recombinant bacteria.

19. The cell-free method of claim 18, wherein the recombinant bacteria are recombinant *Escherichia coli*.

20. The cell-free method of any one of claims 15 to 19, wherein the pressure is at least 2 bar.

21. The cell-free method of claim 20, wherein the pressure is at least 5 bar.

22. The cell-free method of any one of claims 15-21, wherein the bioreactor comprises a gas phase and an aqueous phase.

23. The cell-free method of claim 22, wherein the bioreactor further comprises an organic solvent.

24. The cell-free method of claim 23, wherein the organic solvent is an alkane.

25. The cell-free method of claim 24, wherein the alkane is selected from the group consisting of pentane, hexane, heptane, octane, nonane, and decane.

26. A cell-free system for conversion of methane to isobutanol, the system comprising a bioreactor that comprises:

a gas phase comprising methane and oxygen; and

an aqueous phase comprising a cell lysate containing (a) a methane monooxygenase, (b) a methanol dehydrogenase, and (c) each of the following enzymes that catalyze the conversion of formaldehyde to isobutanol: a hexulose-6-phosphate synthase, a 6-phospho-3-hexuloisomerase, a 6-phosphofructokinase, a fructose bisphosphate aldolase, a triose phosphate isomerase, a transketolase, a ribose-5-phosphate isomerase or a ribulose-5-phosphate 3-epimerase, a glyceraldehyde 3-phosphate dehydrogenase, a phosphoglycerate kinase, a phosphoglycerate mutase, an enolase, a pyruvate kinase, an acetolactate synthase, a acetohydroxy acid isomeroreductase, a dihydroxy acid dehydratase, an alpha-ketoisovalerate decarboxylase, and an isobutanol dehydrogenase.

27. The cell-free system of claim 26, wherein the methane monooxygenase is obtained from a methanotroph.

28. The cell-free system of claim 26 or 27, wherein the methanol dehydrogenase is a NAD-linked methanol dehydrogenase.

29. The cell-free system of any one of claims 26-28, wherein the enzymes that catalyze the conversion of formaldehyde to isobutanol are derived from recombinant bacteria.

30. The cell-free system of claim 29, wherein the recombinant bacteria are recombinant *Escherichia coli*.

31. The cell-free system of any one of claims 26-30, wherein the bioreactor further comprises an organic solvent.

32. The cell-free system of claim 31, wherein the organic solvent is an alkane.

33. The cell-free system of claim 32, wherein the alkane is selected from the group consisting of pentane, hexane, heptane, octane, nonane, and decane.

34. A cell-free system for conversion of methane to pyruvate, the system comprising a bioreactor that comprises:

a gas phase comprising methane and oxygen; and

an aqueous phase comprising a cell lysate containing (a) a methane monooxygenase, (b) a methanol dehydrogenase, and (c) each of the following enzymes that catalyze the conversion of formaldehyde to pyruvate: a hexulose-6-phosphate synthase, a 6-phospho-3-hexuloisomerase, a 6-phosphofructokinase, a fructose bisphosphate aldolase, a triose phosphate isomerase, a transketolase, a ribose-5-phosphate isomerase or a ribulose-5-phosphate 3-epimerase, a glyceraldehyde 3-phosphate dehydrogenase, a phosphoglycerate kinase, a phosphoglycerate mutase, an enolase, and a pyruvate kinase.

35. The cell-free system of claim 34, wherein the methane monooxygenase is obtained from a methanotroph.

36. The cell-free system of claim 34 or 35, wherein the methanol dehydrogenase is a NAD-linked methanol dehydrogenase.

37. The cell-free system of any one of claims 34-36, wherein the enzymes that catalyze the conversion of formaldehyde to isobutanol are derived from recombinant bacteria.

38. The cell-free system of claim 37, wherein the recombinant bacteria are recombinant *Escherichia coli*.

39. The cell-free system of any one of claims 34-38, wherein the bioreactor further comprises an organic solvent.

40. The cell-free system of claim 39, wherein the organic solvent is an alkane.

41. The cell-free system of claim 40, wherein the alkane is selected from the group consisting of pentane, hexane, heptane, octane, nonane, and decane.

42 A cell lysate of any one of claims 1-41.

## CLAIMS

What is claimed is:

1. A cell-free method for large-scale conversion of methane to isobutanol, comprising:

combining, in a bioreactor at elevated pressure, methane, oxygen, and cell lysates containing methane monooxygenase, methanol dehydrogenase, and enzymes that catalyze the conversion of formaldehyde to isobutanol, to form a cell-free reaction mixture; and

incubating under suitable conditions the cell-free reaction to convert methane to isobutanol.

2. The cell-free method of claim 1, wherein the methane monooxygenase is obtained from a methanotroph.

3. The cell-free method of claim 1 or 2, wherein the methanol dehydrogenase is a heterologous NAD-linked methanol dehydrogenase.

4. The cell-free method of any one of claims 1-3, wherein the enzymes that catalyze the conversion of formaldehyde to isobutanol are derived from recombinant bacteria.

5. The cell-free method of claim 4, wherein the recombinant bacteria are recombinant *Escherichia coli*.

6. The cell-free method of any one of claims 1-5, wherein the enzymes that catalyze the conversion of formaldehyde to isobutanol comprise one or more of hexulose-6-phosphate synthase, 6-phospho-3-hexuloisomerase, 6-phosphofructokinase, fructose biphosphate aldolase, triose phosphate isomerase, transketolase, ribose-5-phosphate isomerase, ribose-5-phosphate 3-epimerase, glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, enolase, pyruvate kinase, acetolactate synthase, acetohydroxy acid

isomeroreductase, dihydroxy acid dehydratase, alpha-ketoisovalerate decarboxylase, and isobutanol dehydrogenase.

7. The cell-free method of any one of claims 1-6, wherein the pressure is at least 1 bar.

8. The cell-free method of claim 7, wherein the pressure is at least 2 bar.

9. The cell-free method of claim 8, wherein the pressure is at least 5 bar.

10. The cell-free method of any one of claims 1-9, wherein the isobutanol is produced at a production rate of at least 1 g/L-h.

11. The cell-free method of claim 10, wherein the isobutanol is produced at a production rate of at least 10 g/L-h.

12. The cell-free method of claim 11, wherein the isobutanol is produced at a production rate of at least 25 g/L-h.

13. The cell-free method of any one of claims 1-12, wherein the bioreactor comprises a gas phase and an aqueous phase.

14. The cell-free method of claim 13, wherein the bioreactor further comprises an organic solvent.

15. The cell-free method of claim 14, wherein the organic solvent is an alkane.

16. The cell-free method of claim 15, wherein the alkane is selected from the group consisting of pentane, hexane, heptane, octane, nonane, and decane.

17. A cell-free method for large-scale conversion of methane to pyruvate, comprising:

combining, in a bioreactor at elevated pressure, methane, oxygen, and cell lysates containing methane monooxygenase, methanol dehydrogenase, and enzymes that catalyze the conversion of formaldehyde to pyruvate, to form a cell-free reaction mixture; and

incubating under suitable conditions the cell-free reaction to convert methane to pyruvate.

18. The cell-free method of claim 17, wherein the methane monooxygenase is obtained from a methanotroph.

19. The cell-free method of claim 17 or 18, wherein the methanol dehydrogenase is a heterologous NAD-linked methanol dehydrogenase.

20. The cell-free method of any one of claims 17-19, wherein the enzymes that catalyze the conversion of formaldehyde to isobutanol are derived from recombinant bacteria.

21. The cell-free method of claim 20, wherein the recombinant bacteria are recombinant *Escherichia coli*.

22. The cell-free method of any one of claims 17-21, wherein the enzymes that catalyze the conversion of formaldehyde to pyruvate comprise one or more of hexulose-6-phosphate synthase, 6-phospho-3-hexuloisomerase, 6-phosphofructokinase, fructose biphosphate aldolase, triose phosphate isomerase, transketolase, ribose-5-phosphate isomerase, ribose-5-phosphate 3-epimerase, glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, enolase, and pyruvate kinase.

23. The cell-free method of any one of claims 17-22, wherein the pressure is at least 1 bar.

24. The cell-free method of claim 23, wherein the pressure is at least 2 bar.

25. The cell-free method of claim 24, wherein the pressure is at least 5 bar.

26. The cell-free method of any one of claims 17-25, wherein the isobutanol is produced at a production rate of at least 1 g/L-h.

27. The cell-free method of claim 26, wherein the isobutanol is produced at a production rate of at least 10 g/L-h.

28. The cell-free method of claim 27, wherein the isobutanol is produced at a production rate of at least 25 g/L-h.

29. The cell-free method of any one of claims 17-28, wherein the bioreactor comprises a gas phase and an aqueous phase.

30. The cell-free method of claim 29, wherein the bioreactor further comprises an organic solvent.

31. The cell-free method of claim 30, wherein the organic solvent is an alkane.

32. The cell-free method of claim 31, wherein the alkane is selected from the group consisting of pentane, hexane, heptane, octane, nonane, and decane.

33. A cell-free method for large-scale conversion of methane to a biofuel or other chemical compound, comprising:

combining, in a bioreactor at elevated pressure, methane, oxygen, and cell lysates containing methane monooxygenase, methanol dehydrogenase, enzymes that catalyze the conversion of formaldehyde to pyruvate, and enzymes that catalyze the conversion of pyruvate to a biofuel or other chemical compound, to form a cell-free reaction mixture; and

incubating under suitable conditions the cell-free reaction to convert methane to the biofuel or other chemical compound.

34. The cell-free method of claim 33, wherein the methane monooxygenase is obtained from a methanotroph.

35. The method of claim 33 or 34, wherein the methanol dehydrogenase is a heterologous NAD-linked methanol dehydrogenase.

36. The cell-free method of any one of claims 33-35, wherein the enzymes that catalyze the conversion of formaldehyde to pyruvate and/or pyruvate to the biofuel or other compound are derived from recombinant bacteria.

37. The cell-free method of claim 36, wherein the recombinant bacteria are recombinant *Escherichia coli*.

38. The cell-free method of any one of claims 33-37, wherein the enzymes that catalyze the conversion of formaldehyde to pyruvate comprise one or more of hexulose-6-phosphate synthase, 6-phospho-3-hexuloisomerase, 6-phosphofructokinase, fructose biphosphate aldolase, triose phosphate isomerase, transketolase, ribose-5-phosphate isomerase, ribose-5-

phosphate 3-epimerase, glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, enolase, and pyruvate kinase.

39. The cell-free method of any one of claims 33-38, wherein the pressure is at least 1 bar.

40. The cell-free method of claim 39, wherein the pressure is at least 2 bar.

41. The cell-free method of claim 40, wherein the pressure is at least 5 bar.

42. The cell-free method of any one of claims 33-41, wherein the isobutanol is produced at a production rate of at least 1 g/L-h.

43. The cell-free method of claim 42, wherein the isobutanol is produced at a production rate of at least 10 g/L-h.

44. The cell-free method of claim 43, wherein the isobutanol is produced at a production rate of at least 25 g/L-h.

45. The cell-free method of any one of claims 33-44, wherein the bioreactor comprises a gas phase and an aqueous phase.

46. The cell-free method of claim 45, wherein the bioreactor further comprises an organic solvent.

47. The cell-free method of claim 46, wherein the organic solvent is an alkane.

48. The cell-free method of claim 47, wherein the alkane is selected from the group consisting of pentane, hexane, heptane, octane, nonane, and decane.

49. A cell-free system for large-scale conversion of methane to isobutanol, the system comprising a bioreactor that comprises:

a gas phase comprising methane and oxygen; and

an aqueous phase comprising a cell lysate containing methane monooxygenase, methanol dehydrogenase, and enzymes that catalyze the conversion of formaldehyde to isobutanol.

50. The cell-free system of claim 49, wherein the methane monooxygenase is obtained from a methanotroph.

51. The cell-free system of claim 49 or 50, wherein the methanol dehydrogenase is a heterologous NAD-linked methanol dehydrogenase.

52. The cell-free system of any one of claims 49-51, wherein the enzymes that catalyze the conversion of formaldehyde to isobutanol are derived from recombinant bacteria.

53. The cell-free system of claim 52, wherein the recombinant bacteria are recombinant *Escherichia coli*.

54. The cell-free system of any one of claims 49-53, wherein the enzymes that catalyze the conversion of formaldehyde to isobutanol comprise one or more of hexulose-6-phosphate synthase, 6-phospho-3-hexuloisomerase, 6-phosphofructokinase, fructose biphosphate aldolase, triose phosphate isomerase, transketolase, ribose-5-phosphate isomerase, ribose-5-phosphate 3-epimerase, glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, enolase, pyruvate kinase, acetolactate synthase, acetohydroxy acid

isomeroreductase, dihydroxy acid dehydratase, alpha-ketoisovalerate decarboxylase, and isobutanol dehydrogenase.

55. The cell-free system of any one of claims 49-54, wherein the bioreactor further comprises an organic solvent.

56. The cell-free system of claim 55, wherein the organic solvent is an alkane.

57. The cell-free system of claim 56, wherein the alkane is selected from the group consisting of pentane, hexane, heptane, octane, nonane, and decane.

58. A cell-free system for large-scale conversion of methane to pyruvate, the system comprising a bioreactor that comprises:

a gas phase comprising methane and oxygen; and

an aqueous phase comprising a cell lysate containing methane monooxygenase, methanol dehydrogenase, and enzymes that catalyze the conversion of formaldehyde to pyruvate.

59. The cell-free system of claim 58, wherein the methane monooxygenase is obtained from a methanotroph.

60. The cell-free system of claim 58 or 59, wherein the methanol dehydrogenase is a heterologous NAD-linked methanol dehydrogenase.

61. The cell-free system of any one of claims 58-60, wherein the enzymes that catalyze the conversion of formaldehyde to isobutanol are derived from recombinant bacteria.

62. The cell-free system of claim 61, wherein the recombinant bacteria are recombinant *Escherichia coli*.

63. The cell-free system of any one of claims 58-62, wherein the enzymes that catalyze the conversion of formaldehyde to pyruvate comprise one or more of hexulose-6-phosphate synthase, 6-phospho-3-hexuloisomerase, 6-phosphofructokinase, fructose biphosphate aldolase, triose phosphate isomerase, transketolase, ribose-5-phosphate isomerase, ribose-5-phosphate 3-epimerase, glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, enolase, and pyruvate kinase.

64. The cell-free system of any one of claims 58-63, wherein the bioreactor further comprises an organic solvent.

65. The cell-free system of claim 64, wherein the organic solvent is an alkane.

66. The cell-free system of claim 65, wherein the alkane is selected from the group consisting of pentane, hexane, heptane, octane, nonane, and decane.

67. A cell-free system for large-scale conversion of methane to pyruvate, the system comprising a bioreactor that comprises:

a gas phase comprising methane and oxygen; and

an aqueous phase comprising a cell lysate containing methane monooxygenase, methanol dehydrogenase, enzymes that catalyze the conversion of formaldehyde to pyruvate, and enzymes that catalyze the conversion of pyruvate to a biofuel or other chemical compound, to form a cell-free reaction mixture.

68. The cell-free system of claim 67, wherein the methane monooxygenase is obtained from a methanotroph.

69. The cell-free system of claim 67 or 68, wherein the methanol dehydrogenase is a heterologous NAD-linked methanol dehydrogenase.

70. The cell-free system of any one of claims 67-69, wherein the enzymes that catalyze the conversion of formaldehyde to isobutanol are derived from recombinant bacteria.

71. The cell-free system of claim 70, wherein the recombinant bacteria are recombinant *Escherichia coli.*

72. The cell-free system of any one of claims 67-71, wherein the enzymes that catalyze the conversion of formaldehyde to pyruvate comprise one or more of hexulose-6-phosphate synthase, 6-phospho-3-hexuloisomerase, 6-phosphofructokinase, fructose biphosphate aldolase, triose phosphate isomerase, transketolase, ribose-5-phosphate isomerase, ribose-5-phosphate 3-epimerase, glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, enolase, and pyruvate kinase.

73. The cell-free system of any one of claims 67-71, wherein the bioreactor further comprises an organic solvent.

74. The cell-free system of claim 73, wherein the organic solvent is an alkane.

75. The cell-free system of claim 74, wherein the alkane is selected from the group consisting of pentane, hexane, heptane, octane, nonane, and decane.

76. A cell lysate of any one of claims 1-75.