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APPLICATION NUMBER: 62/045,083
FILING DATE: September 03, 2014

THE COUNTRY CODE AND NUMBER OF YOUR PRIORITY APPLICATION, TO BE USED FOR FILING ABROAD UNDER THE PARIS CONVENTION, IS US62/045,083

By Authority of the
Under Secretary of Commerce for Intellectual Property
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**Title of Invention:**
Genetically engineered microorganisms and process for converting gaseous C1-carbon sources and/or gaseous C1-carbon sources and electrons into alkenes.

| **First Named Inventor/Applicant Name:** | Bernhard Guentner |
| **Customer Number:**                   | 121985 |
| **Filer:**                             | Bernhard Guentner |
| **Filer Authorized By:**               |          |
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National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.
**PROVISIONAL APPLICATION FOR PATENT COVER SHEET – Page 1 of 2**

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

**INVENTOR(S)**

Given Name (first and middle [if any]) | Family Name or Surname | Residence (City and either State or Foreign Country)
--- | --- | ---
Bernhard | Guentner | Vaals, Netherlands

Additional inventors are being named on the separately numbered sheets attached hereto.

**TITLE OF THE INVENTION (500 characters max):**

Genetically engineered microorganisms and process for converting gaseous C1-carbon sources and/or gaseous C1-carbon sources and electrons into alkenes.

**ENCLOSED APPLICATION PARTS (check all that apply)**

- ✔ Application Data Sheet. See 37 CFR 1.76.
- ✔ Drawing(s) Number of Sheets 10
- ✔ Specification (e.g., description of the invention) Number of Pages: 48

**Fees Due:** Filing Fee of $260 ($130 for small entity) ($65 for micro entity). If the specification and drawings exceed 100 sheets of paper, an application size fee is also due, which is $400 ($200 for small entity) ($100 for micro entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(g).

**METHOD OF PAYMENT OF THE FILING FEE AND APPLICATION SIZE FEE FOR THIS PROVISIONAL APPLICATION FOR PATENT**

- ✔ Applicant asserts small entity status. See 37 CFR 1.27.
- ✔ Applicant certifies micro entity status. See 37 CFR 1.29.
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☐ Yes, the invention was made under a contract with an agency of the U.S. Government. The name of the U.S. Government agency and Government contract number are:

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SIGNATURE /Bernhard Guentner/ DATE 09/03/2014

TYPED OR PRINTED NAME Bernhard Guentner REGISTRATION NO.  
(If appropriate)

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8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.
Provisional Patent Application:
Genetically engineered microorganisms and process for converting gaseous C1-carbon sources and/or gaseous C1-carbon sources and electrons into alkenes.

Inventor:
Bernhard Guentner
Akenrstraat 25A
6291BA Vaals
The Netherlands

Abstract:
I have invented genetically engineered microorganisms and their use in a process for converting gaseous C1-carbon sources and/or C1-carbon sources and electrons into the short chain alkenes ethene (ethylene), propene (propylene), 1-butene (1-butylene), isobutene (isobutylene) and 1-pentene (1-pentylene). The genetically engineered, anaerobic bacterial cells comprise one or more heterologous nucleic acids encoding one or more artificial short chain alkene pathways and are capable of producing ethene, propene, 1-butene, isobutene and 1-pentene from carbon monoxide and/or from carbon dioxide as well as from carbon dioxide and electrons and/or from carbon monoxide and electrons.

Objects:
The Invention provides a much-needed economical, nonpetrochemical route to produce unlimited amounts of ethene, propene, 1-butene, isobutene and 1-pentene in a sustainable manner by C1 gas fermentation and/or microbial electro synthesis. Because CO2 and/or CO can be used alone or together with electrons as a substrate, the carbon footprint is extremely low and environmental benefits are substantial while production costs are significantly lowered.
Relevant Prior art includes:

US Patent US20110269204
US Patent US20110091952
US Patent US20120329119
US Patent US20120288898
US Patent US20120259026
US Patent US20120064622
US Patent US20130210104
US Patent US20130130341
US Patent US20130210941
US Patent US20130224808
US Patent US20130274356
US Patent US20130123454
US Patent US20130316425
US Patent US20140039143
US Patent US20140134687
Patent WO 2012058606
Patent WO2012174271
Patent WO 2013148348
Patent WO 2013067833
Patent WO 2013181647
Patent WO 2013180584
Patent WO 2014047209
Patent WO 2014001517
Patent EP 2679568A1
Patent WO2014106122


Direct biosynthesis of ethylene.


D.R. Lovley, Powering microbes with electricity: direct electron transfer from electrodes to microbes, Env Microbilo Rep., 3 (2011) 27-35.


"Divergence of Function in the Hot Dog Fold Enzyme Superfamily: The Bacterial Thioesterase YciA” Zhihao Zhuang, Feng Song, Hong Zhao, Ling Li, Jian Cao, Edward Eisenstein, Osnat Herzberg, and Debra Dunaway-Mariano Biochemistry 2008 47 (9), 2789-2796

“A Synthetic Biology Approach to Engineer a Functional Reversal of the β-Oxidation Cycle” James M. Clombaugh, Jacob E. Vick, Matthew D. Blankschien, María Rodríguez-Moyá, and Ramon Gonzalez ACS Synthetic Biology 2012 1 (11), 541-554

“A Novel Paradigm of Fatty Acid β-Oxidation Examplified by the Thioesterase-Dependent Partial Degradation of Conjugated Linoleic Acid That Fully Supports Growth of Escherichia
coli” Lina Nie, Ying Ren, Anuradha Janakiraman, Stuart Smith, and Horst Schulz; Biochemistry 2008 47 (36), 9618-9626


Genomic Analysis of Carbon Monoxide Utilization and Butanol Production by Clostridium carboxidivorans Strain P7T. PLoS ONE 5(9): e13033. doi: 10.1371/journal.pone.0013033


“Genetically Modified Strains of Ralstonia eutropha H16 with β-Ketothiolase Gene Deletions for Production of Copolyesters with Defined 3-Hydroxyvaleric Acid Contents.” Nicole Lindenkamp, Elena Volodina, and Alexander Steinbüchel; Appl. Environ. Microbiol. August 2012 78:15 5375-5383

Belitsky BR, Sonenshein AL (2002). "GabR, a member of a novel protein family, regulates the utilization of gamma-aminobutyrate in Bacillus subtilis." Mol Microbiol 45(2);569-83. PMID: 12123465

Bessman SP, Rossen J, Layne EC (1953). "Gamma-Aminobutyric acid-glutamic acid transamination in brain." J Biol Chem 201(1);385-91. PMID: 13044808


Lengeler, JW (editor), Drews, G (editor), Schlegel, HG (editor) "Biology of the Prokaryotes." 1999, Blackwell Science, Malden, MA.


"Divergence of Function in the Hot Dog Fold Enzyme Superfamily: The Bacterial Thioesterase YciA." Zhihao Zhuang, Feng Song, Hong Zhao, Ling Li, Jian Cao, Edward Eisenstein, Osnat Herzberg, and Debra Dunaway-Mariano. Biochemistry 2008 47 (9), 2789-2796


Fall RR, Hector ML (1977). "Acyl-coenzyme A carboxylases. Homologous 3-methylcrotonyl-CoA and geranyl-CoA carboxylases from Pseudomonas citronellolis." Biochemistry 16(18);4000-5. PMID: 911753


Hoschle B, Gnau V, Jendrossek D (2005). "Methylcrotonyl-CoA and geranyl-CoA carboxylases are involved in leucine/isovalerate utilization (Liu) and acyclic terpene utilization (Atu), and are encoded by liuB/liuD and atuC/atuF, in Pseudomonas aeruginosa." Microbiology 151(Pt 11):3649-56. PMID: 16272386


Advantages:

The main advantages of the invention are:

- Economical nonpetrochemical route for short chain alkenes i.e. ethene, propene, 1-butene, isobutene and 1-pentene.
- One step process for ethene, propene, 1-butene, isobutene and 1-pentene production.
- Ethene, propene, 1-butene, isobutene and 1-pentene can produced simultaneously with other products or separately.
- Increase in cost effectiveness (valueless, waste gases CO and/or CO2 are used as substrates).
- Increased purity of ethene, propene, 1-butene, isobutene and 1-pentene compared to chemical routes e.g. cracking.
- When bacterial cells are grown on CO and/or, CO2 with supplied electrons growth and product formation is promoted.
• Provides a means to store excess electricity in high valuable chemical compound.
• It overcomes microbial growth limitations of prior art systems (shortage of reducing equivalents).
• Greatly facilitates the generation of unlimited supply of ethene, propene, 1-butene, isobutene and 1-pentene.
• Suitable for a robust industrial process.
• Extremely low carbon footprint.
• High environmental benefits.
• Process can be carried out at room temperature.

Introduction:

Increased demand for bulk chemicals by the global economy has placed increasing pressure on the cost of short chain alkenes. Many industries, including the chemical and plastics industry rely heavily on the availability of fossil hydrocarbon sources as feedstock for their products. Propene, 1-butene, isobutene and 1-pentene are short chain hydrocarbons which belong to the group of alkenes. These alkenes are of outstanding industrial importance, since they are the most important bulk chemicals of the chemical industry and serve as building blocks for plastics, poylesters, solvents, fuels, adhesives, paints and many more. Propene (propylene) for instance is a terminal olefin which is used to manufacture polyethylene, polypropylene, alpha olefins, styrene, polyesters, acrylics, ethylene glycol antifreeze, polyvinyl chloride (PVC), propylene oxide, oxo alcohols, and isopropanol. Propene is traditionally derived from fractional distillation from hydrocarbon mixtures obtained from cracking and other refining processes. Ethene is manufactured likewise and serves also as a raw chemical for use as a monomer for the production of polyurethanes, rubber products, laboratory chemicals, industrial fluids, and anti-freeze. It is also used to make fuel and fuel additives. 1-butene is used in the manufacture of a variety of other chemical products. It fills an important role in the production of materials such as linear low density polyethylene (LLDPE). The co-polymerisation of ethylene and 1-butene produces a form of polyethylene that is more flexible and more resilient. 1-butene can also help to create a more versatile range of polypropylene resins. It is also used in the production of polybutene, butylene oxide
and in the C4 solvents secondary butyl alcohol (SBA) and methyl ethyl ketone (MEK). Isobutene is used as an intermediate in the production of a variety of products. It is reacted with methanol and ethanol in the manufacture of the gasoline oxygenates methyl tert-butyl ether (MTBE) and ethyl tert-butyl ether (ETBE), respectively. Alkylaion with butane produces isooctane, another fuel additive. Isobutene is also used in the production of methacrolein. Polymerization of isobutene produces butyl rubber (polyisobutylene). Antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are produced by Friedel-Crafts alkylaion of phenols using isobutene. 1-Pentene is made as a byproduct of catalytic or thermal cracking of petroleum, or during production of ethene and propene via thermal cracking of hydrocarbon fractions. It is rarely isolated as a separate compound. Instead, it is most often blended into gasoline or, in a mixture with other hydrocarbons, alkylaied with isobutane to make gasoline. The only commercial manufacturer of 1-pentene is Sasol Ltd, where it is separated from crude made by the Fischer-Tropsch process. However, short chain alkene production by engineered host cells represents a significant alternative to traditional methods of production or fischer-tropsch synthesis. There are several patents which describe biological pathways to produce alkenes directly or indirectly (US 2011/0269204, US 2011/0091952, US 2011/0269204, WO 2012/058606, US 2012/0329119, US 2013/0224808, US 2013/0316425, WO 2013/148348, US 2014/0039143, WO 2014/047209, WO 2014/001517). But they are either not economical or not suitable for a large scale industrial process and have many other limitations. This invention employs new artificial, engineered pathways in a whole cell catalysis approach to produce propene, 1-butene, isobutene or 1-pentene from gaseous C1-carbon sources and/or gaseous C1-carbon sources and electrons and has several advantages compared to previously existing techniques.

Figures:

Fig. 1 shows the pathways and their key enzymes for the generation of propene from carbon monoxide and/or from carbon dioxide as well as from carbon dioxide and electrons and/or from carbon monoxide and electrons.

Fig. 2 shows the pathways and their key enzymes for the generation of 1-butene from carbon monoxide and/or from carbon dioxide as well as from carbon dioxide and electrons and/or from carbon monoxide and electrons.
Fig. 3 shows the pathways and their key enzymes for the generation of isobutene from carbon monoxide and/or from carbon dioxide as well as from carbon dioxide and electrons and/or from carbon monoxide and electrons.

Fig. 4 shows the pathways and their key enzymes for the generation of 1-pentene from carbon monoxide and/or from carbon dioxide as well as from carbon dioxide and electrons and/or from carbon monoxide and electrons.

Fig. 5 shows the pathways and their key enzymes for the generation of ethene from carbon monoxide and/or from carbon dioxide as well as from carbon dioxide and electrons and/or from carbon monoxide and electrons.

Fig. 6 shows plasmid SG455 comprising genes and pathways for propene biosynthesis.

Fig. 7 shows plasmid SG479 comprising genes and pathways for propene biosynthesis.

Fig. 8 shows plasmid SG539 comprising genes and pathways for 1-butene biosynthesis.

Fig. 9 shows plasmid SG523 comprising genes and pathways for 1-butene biosynthesis.

Fig. 10 shows plasmid SG582 comprising genes and pathways for isobutene biosynthesis.

Fig. 11 shows plasmid SG601 comprising genes and pathways for isobutene biosynthesis.

Fig. 12 shows plasmid SG498 comprising genes and pathways for 1-pentene biosynthesis.

Fig. 13 shows plasmid SG513 comprising genes and pathways for 1-pentene biosynthesis.

Fig. 14 shows plasmid SG557 comprising genes and pathways for ethene biosynthesis.

Fig. 15 shows plasmid SG479 comprising genes and pathways for ethene biosynthesis.

Figures – Reference Numerals:

30 – Acetoacetyl-Coenzyme A synthase thiolase (EC 2.3.1.9) = AtoB
32 – 3-hydroxybutyryl-CoA dehydrogenase (EC 1.1.1.157) = hbd
34 – 3-hydroxybutyryl-CoA dehydratase (EC 4.2.1.55) = crt
36 – acyl-CoA thioesterase (EC 3.1.2.-) = YciA, Ydil
38 – phenyl acrylic acid decarboxylase (EC 4.1.1.-) = PAD1, PADC
42 – acetyl-CoA carboxylase (EC 6.4.1.2) = AccABCD
44 – acetoacetyl-CoA synthase (EC 2.3.1.194) = nphT7
46 – acetoacetyl-CoA reductase (EC 1.1.1.36) = phaB1
48 – (R)-specific enoyl-CoA hydratase (EC 4.2.1.119) = phaJ
50 – 4-aminobutyrate aminotransferase (EC 2.6.1.19) = gabT
52 – glutamate dehydrogenase (EC 1.4.1.2) = gdh
54 – 2-hydroxyglutarate dehydrogenase (EC 1.1.99.2) = ygaF
56 – gluconate CoA-transferase (EC 2.8.3.12) = gctA
58 – 2-hydroxyglutaryl-CoA dehydratase (EC 4.2.1.-) = hdgAB
60 – glutaconyl-CoA decarboxylase (EC 4.1.1.70) = gcdABCD
62 – 4-hydroxybutyrate dehydrogenase (EC 1.1.1.61) = 4hbD
64 – 4-hydroxybutyrate CoA-transferase (EC 2.8.3.-) = abfT
66 – vinylacetyl-CoA-Delta-isomerase (EC 4.2.1.120) = abfD
68 – 4-hydroxybutyryl-CoA dehydratase (EC 5.3.3.3) = abfD
70 – Acetoacetyl-Coenzyme A synthase thiolase (EC 2.3.1.9) = Bktb
72 – 2-oxoglutarate/butyrate ferredoxin oxidoreductase (EC 1.2.7.3) = KorAB
74 – pyruvate/2-oxobutyrate ferredoxin oxidoreductase (EC 1.2.7.1) = PFOR
76 – methylmalonyl-CoA mutase (EC 5.4.99.2) = scpA
78 – methylmalonyl-CoA decarboxylase (EC 4.1.1.41) = scpB
80 – 3-hydroxy-3-methylglutaryl-CoA synthase (EC 2.3.3.10) = mvaS
82 – 3-methylglutaconyl-CoA hydratase (EC 4.2.1.18) = liuC
84 – 3-methylcrotonyl-CoA carboxylase (EC 6.4.1.4) = liuBD
86 – trans-2-enoyl-CoA reductase (EC 1.1.1.36) = ter
88 – acetyl-CoA acetyltransferase (EC 2.3.1.9) = Bktb
90 – 3-hydroxybutyryl-CoA dehydrogenase (EC 1.1.1.157) = pAAH1
92 – acyl-CoA hydrolase (EC 3.1.2.-) = mBACH
100 – lactate CoA-transferase (EC 2.8.3.-) = pct
102 – lactoyl-CoA dehydratase (EC 4.2.1.54) = lcaAB
104 – malonyl CoA reductase (EC 1.2.1.75) = mcr
106 – malonyl CoA reductase (EC 1.1.1.298) = mcr
108 – propionyl-CoA synthase (EC 6.2.1.36) = pcr
110 – propionyl-CoA synthase/acryloyl-CoA synthase (EC 4.2.1.116) = pcr, msed12
112 = propionyl-CoA synthase (EC 1.3.1.84) = pcr
pMB1 = origin of replication (E. coli)
repH = origin of replication (Clostridium)
catp = chloramphenicol/thiampenicol resistance marker
lacI = lac repressor

Description:

The microorganisms of the invention are prepared from a parental microorganism and one or more exogenous nucleic acids (plasmids) using genetic transformation by an electroporation procedure. Furthermore the invention provides a method and process for the production of propene, 1-butene, isobutene and 1-pentene by anaerobic fermentation using said genetically engineered cells and a bioreactor supplied with the cells and carbon monoxide and/or carbon dioxide and/or carbon dioxide and electrons and/or from carbon monoxide and electrons.

Preferred Embodiment – Example 1 (propene production):

Fig. 6 shows the plasmid SG455 (SEQ ID No 1) which comprises an engineered propene pathway (for genes see also reference numerals). This plasmid was electroporated into *Clostridium ljungdahlii DSM 13528* cells by the following protocol:

- Genetic Transformation of *Clostridium ljungdahlii DSM 13528* cells
  Preparation of electrocompetent *C. ljungdahlii DSM 13528* cells: The procedure for making *C. ljungdahlii DSM 13528* electro competent cells was modified from a protocol reported previously (Köpke, Held et al. 2010). All manipulations except centrifugation were carried out on ice in an anaerobic chamber. All buffers and centrifuge tubes were made ice cold and anoxic before use. All plastic ware was placed in the anaerobic chamber at least 24 h before use in order to eliminate any residual oxygen. Competent cells were maintained in SPM buffer (270 mM sucrose, 1 mM MgCl₂, 7 mM sodium phosphate, pH 6) with 10% DMSO at -80 °C until use. Electrocompetent *C. ljungdahlii DSM 13528* cells were prepared from cultures that were freshly inoculated from frozen stocks and then transferred twice in YTF liquid medium. About 15 to 16 h before preparation of competent cells, a mid- to late-log-phase culture was transferred into two serum bottles containing 100 mL fresh YTF medium supplemented with 40 mM DL-threonine (final optical density at 600 nm [OD₆₀₀] = 0.004). After overnight growth at 37 °C, early-log phase cells (OD₆₀₀ = 0.2 to 0.3; 200 mL) were harvested by centrifugation at 10,000 rpm for 10 min at 4 °C. The cells were washed twice with 200 mL of SPM wash buffer and resuspended in the same buffer at a final concentration of 10¹⁰ to 10¹¹ cells/mL. Antifreezing buffer (60% DMSO – 40% SMP, pH 6) was added to the competent cells a tone-fifth of the final resuspension volume to achieve a final concentration of 10% DMSO. The resulting competent cells (25 μL/tube) were stored at -80 °C for future use. The competence of these frozen competent cells remained stable for about 1 month.

  - Electrotransformation procedures for *C. ljungdahlii DSM 13528*: All procedures were carried out in an anaerobic chamber. Electrocompetent *C. ljungdahlii DSM 13528* cells (25 μL) were quickly transferred on ice from a -80 °C freezer to an anaerobic chamber. After thawing on ice (about 1 min), the cells were mixed with...
1 to 5 μg DNA and transferred to a prechilled, 0.1-cm-gap cuvette. Cells were pulsed at 0.625 kV with resistance at 600 Ohm and a capacitance of 25 μF by using a microbial electroporation system. Immediately after the pulse, cells were recovered with 0.5 mL of fresh YTF medium, transferred to a pressure tube containing 10 mL of YTF medium supplemented with 5 mM L-cysteine (pH 7), and incubated at 37 °C. The electroporated cells were allowed to recover at 37 °C until their cell densities were higher than immediately after the electroporation (about 9 to 12 h). Five-milliliter volumes of the outgrowth cultures or appropriately diluted cultures were mixed with 20 mL of YTF molten agar (1.5%) containing an appropriate antibiotic and poured into a petri dish. After the agar mixtures were solidified, plates were incubated upside down. After 2-3 days colonies become visible and can be inoculated in liquid culture.

- YTF medium:
  10 g/L yeast extract
  16 g/L Bacto tryptone
  4 g/L sodium chloride
  5 g/L fructose
  Supplemented with 2 mM L-cysteine
  Final pH is 6.

Propene production from CO, CO₂ and H₂ (syngas) by gas fermentation was performed using said genetically engineered C. ljungdahlii DSM 13528 cells harboring the plasmid SG455. The materials and methods used are described in the following:

- Used medium for growth with gas:

\[
\begin{align*}
&\text{NH}_4\text{Cl} & 1.0 \text{ g} \\
&\text{KCl} & 0.1 \text{ g} \\
&\text{MgSO}_4 \times 7\text{H}_2\text{O} & 0.2 \text{ g} \\
&\text{NaCl} & 0.8 \text{ g} \\
&\text{KH}_2\text{PO}_4 & 0.1 \text{ g} \\
&\text{CaCl}_2 \times 2\text{H}_2\text{O} & 20.0 \text{ mg}
\end{align*}
\]
Trace Elements (see below).................10.0 mL
Wolfe's Vitamin Solution (see below)....10.0 mL
Reducing Agent (see below)..............10.0 mL
Distilled water............................980.0 mL

Final pH is 6.

Trace Elements:
Nitrilotriacetic acid......................2.0 g
MnSO₄ x H₂O ..................................1.0 g
Fe(SO₄)₂(NH₄)₂ x 6H₂O ...................0.8 g
CoCl₂ x 6H₂O ..................................0.2 g
ZnSO₄ x 7H₂O .................................0.2 mg
CuCl₂ x 2H₂O .................................20.0 mg
NiCl₂ x 6H₂O ..................................20.0 mg
Na₂MoO₄ x 2H₂O ....................20.0 mg
Na₂SeO₄ ........................................20.0 mg
Na₂WO₄ ........................................20.0 mg
Distilled water .........................1.0 L

Add nitrilotriacetic acid to water and adjust to pH 6.0 with KOH. Add remainder of ingredients.

Wolfe's Vitamin Solution:
Biotin..............................................2.0 mg
Folic acid.......................................2.0 mg
Pyridoxine hydrochloride...........10.0 mg
Thiamine x HCl...............................5.0 mg
Riboflavin......................................5.0 mg
Nicotinic acid.................................5.0 mg
Calcium D-(+)-pantothenate ....5.0 mg
Vitamin B12.....................................0.1 mg
p-Aminobenzoic acid.................5.0 mg
Thiocytic acid..........................5.0 mg
Distilled water........................1.0 L

Reducing Agent:
L-Cysteine (free base)..................4.0 g
Distilled water........................100.0 mL

- Used gas: 60% CO, 10% CO₂, 30% H₂

The transformed cells were cultivated in the medium above supplied the 10 µg/mL thiamphenicol (antibiotic) at 37 °C in CSTR bioreactor provided with syngas (60% CO, 10% CO₂, 30% H₂) at 1 bar pressure. Gene expression was induced by 10 mM IPTG when a cell density of 0.3 OD₆₀₀ was reached. Product analysis was performed by GC-MS (Shimadzu GC-MS QP5050A). Headspace gas sampling was conducted using a 500 µL Hamilton gastight syringe. Propene production could be detected by GC-MS.

Preferred Embodiment – Example 2 (1-butene production):

Fig. 8 shows the plasmid SG539 (SEQ ID No 3) which comprises an engineered 1-butene pathway (for genes see also reference numerals). This plasmid was electroporated into Clostridium autoethanogenum DSM 10061 cells by the following protocol:

- Genetic Transformation of Clostridium autoethanogenum DSM 10061 cells
  Preparation of electrocompetent C. autoethanogenum DSM 10061 cells: The procedure for making C. autoethanogenum DSM 10061 electro competent cells was modified from a protocol for C. ljungdahlii DSM 13528 reported previously (Köpke, Held et al. 2010). All manipulations except centrifugation were carried out on ice in an anaerobic chamber. All buffers and centrifuge tubes were made ice cold and anoxic before use. All plastic ware was placed in the anaerobic chamber at least 24 h before use in order to eliminate any residual oxygen. Competent cells were maintained in SMP buffer (270 mM sucrose, 1
mM MgCl₂, 7 mM sodium phosphate, pH 6) with 10% DMSO at -80 °C until use. Electrocompetent *C. autoethanogenum DSM 10061* cells were prepared from cultures that were freshly inoculated from frozen stocks and then transferred twice in YTF liquid medium. About 15 to 16 h before preparation of competent cells, a mid- to late-log-phase culture was transferred into two serum bottles containing 100 mL fresh YTF medium supplemented with 40 mM DL-threonine (final optical density at 600 nm [OD₆₀₀] = 0.004). After overnight growth at 37 °C, early-log phase cells (OD₆₀₀ = 0.2 to 0.3; 200 mL) were harvested by centrifugation at 10,000 rpm for 10 min at 4 °C. The cells were washed twice with 200 mL of SMP wash buffer and resuspended in the same buffer at a final concentration of 10¹⁰ to 10¹¹ cells/mL. Antifreezing buffer (60% DMSO – 40% SMP, pH 6) was added to the competent cells a tone-fifth of the final resuspension volume to achieve a final concentration of 10% DMSO. The resulting competent cells (25 µL/tube) were stored at -80 °C for future use. The competence of these frozen competent cells remained stable for about 1 month.

- Electrotransformation procedures for *C. autoethanogenum DSM 10061*: All procedures were carried out in an anaerobic chamber. Electrocompetent *C. autoethanogenum DSM 10061* cells (25 µL) were quickly transferred on ice from a -80 °C freezer to an anaerobic chamber. After thawing on ice (about 1 min), the cells were mixed with 1 to 5 µg DNA and transferred to a prechilled, 0.1-cm-gap cuvette. Cells were pulsed at 0.625 kV with resistance at 600 Ohm and a capacitance of 25 µF by using a microbial electroporation system. Immediately after the pulse, cells were recovered with 0.5 mL of fresh YTF medium, transferred to a pressure tube containing 10 mL of YTF medium supplemented with 5 mM L-cysteine (pH 7), and incubated at 37 °C. The electroporated cells were allowed to recover at 37 °C until their cell densities were higher than immediately after the electroporation (about 9 to 12 h). Five-milliliter volumes of the outgrowth cultures or appropriately diluted cultures were mixed with 20 mL of YTF molten agar (1.5%) containing an appropriate antibiotic and poured into a petri dish. After the agar mixtures were solidified, plates were incubated upside down. After 2-3 days colonies become visible and can be inoculated in liquid culture.
YTF medium:
10 g/L yeast extract
16 g/L Bacto tryptone
4 g/L sodium chloride
5 g/L fructose
Supplemented with 2 mM L-cysteine
Final pH is 6.

1,3-butadiene production from CO, CO₂ and H₂ (syngas) by gas fermentation with electron supply by electrode was performed using said genetically engineered \textit{C. autoethanogenum DSM 10061} cells harboring the plasmid SG539. The materials and methods used are described in the following:

- Used medium for growth with gas:

\begin{itemize}
  \item NH₄Cl ..............................................1.0 g
  \item KCl ..............................................0.1 g
  \item MgSO₄ x 7H₂O ....................................0.2 g
  \item NaCl ............................................0.8 g
  \item KH₂PO₄ ............................................0.1 g
  \item CaCl₂ x 2H₂O ....................................20.0 mg
  \item Trace Elements (see below)........................10.0 mL
  \item Wolfe's Vitamin Solution (see below)........10.0 mL
  \item Reducing Agent (see below) .....................10.0 mL
  \item Distilled water ......................................980.0 mL
\end{itemize}

Final pH is 6.

Trace Elements:
- Nitrilotriacetic acid .................................2.0 g
- MnSO₄ x H₂O ..........................................1.0 g
- Fe(\textit{SO₄})₃(\textit{NH₄})₂ x 6H₂O ......................0.8 g
CoCl₂ x 6H₂O .........................0.2 g
ZnSO₄ x 7H₂O .........................0.2 mg
CuCl₂ x 2H₂O .........................20.0 mg
NiCl₂ x 6H₂O .........................20.0 mg
Na₂MoO₄ x 2H₂O .......................20.0 mg
Na₂SeO₄ ..............................20.0 mg
Na₂WO₄ ...............................20.0 mg
Distilled water ........................1.0 L

Add nitrilotriacetic acid to water and adjust to pH 6.0 with KOH. Add remainder of ingredients.

Wolfe's Vitamin Solution:
Biotin ......................................2.0 mg
Folic acid ...............................2.0 mg
Pyridoxine hydrochloride .............10.0 mg
Thiamine x HCl .........................5.0 mg
Riboflavin ..............................5.0 mg
Nicotinic acid ..........................5.0 mg
Calcium D- (+)-pantothenate .........5.0 mg
Vitamin B12 ............................0.1 mg
p-Aminobenzoic acid .................5.0 mg
Thioglycolic acid ........................5.0 mg
Distilled water ........................1.0 L

Reducing Agent:
L-Cysteine (free base) ...............4.0 g
Distilled water .........................100.0 mL

- Used gas: 60% CO, 10% CO₂, 30% H₂
The transformed cells were cultivated in the medium above supplied the 10 µg/mL thiamphenicol (antibiotic) at 37 °C in CSTR bioreactor provided with syngas (60% CO, 10% CO₂, 30% H₂) at 1 bar pressure and an electrode with power supply for electricity supply. Gene expression was induced by 10 mM IPTG when a cell density of 0.3 OD₆₀₀ was reached. Product analysis was performed by GC-MS (Shimadzu GC-MS QP5050A). Headspace gas sampling was conducted using a 500 µL Hamilton gastight syringe. 1-Butene production could be detected by GC-MS.

Preferred Embodiment – Example 3 (isobutene production):

Fig. 11 shows the plasmid SG601 (SEQ ID No 6) which comprises an engineered isobutene pathway (for genes see also reference numerals). This plasmid was electroporated into *Clostridium ljungdahlii DSM 13528* cells by the following protocol:

- Genetic Transformation of *Clostridium ljungdahlii DSM 13528* cells

  Preparation of electrocompetent *C. ljungdahlii DSM 13528* cells: The procedure for making *C. ljungdahlii DSM 13528* electro competent cells was modified from a protocol reported previously (Köpke, Held et al. 2010). All manipulations except centrifugation were carried out on ice in an anaerobic chamber. All buffers and centrifuge tubes were made ice cold and anoxic before use. All plastic ware was placed in the anaerobic chamber at least 24 h before use in order to eliminate any residual oxygen. Competent cells were maintained in SMP buffer (270 mM sucrose, 1 mM MgCl₂, 7 mM sodium phosphate, pH 6) with 10% DMSO at -80 °C until use. Electrocompetent *C. ljungdahlii DSM 13528* cells were prepared from cultures that were freshly inoculated from frozen stocks and then transferred twice in YTF liquid medium. About 15 to 16 h before preparation of competent cells, a mid- to late-log-phase culture was transferred into two serum bottles containing 100 mL fresh YTF medium supplemented with 40 mM DL-threonine (final optical density at 600 nm [OD₆₀₀] = 0.004). After overnight growth at 37 °C, early-log phase cells (OD₆₀₀ = 0.2 to 0.3; 200 mL) were harvested by centrifugation at 10,000 rpm for 10 min at 4 °C. The cells were washed twice with 200 ml of SMP wash buffer and resuspended in the same buffer at a final concentration of 10¹⁰ to 10¹¹
cells/mL. Antifreezing buffer (60% DMSO – 40% SMP, pH 6) was added to the competent cells a tone-fifth of the final resuspension volume to achieve a final concentration of 10% DMSO. The resulting competent cells (25 μL/tube) were stored at -80 °C for future use. The competence of these frozen competent cells remained stable for about 1 month.

- Electrotransformation procedures for C. ljungdahlii DSM 13528: All procedures were carried out in an anaerobic chamber. Electrocompetent C. ljungdahlii DSM 13528 cells (25 μL) were quickly transferred on ice from a -80 °C freezer to an anaerobic chamber. After thawing on ice (about 1 min), the cells were mixed with 1 to 5 μg DNA and transferred to a prechilled, 0.1-cm-gap cuvette. Cells were pulsed at 0.625 kV with resistance at 600 Ohm and a capacitance of 25 μF by using a microbial electroporation system. Immediately after the pulse, cells were recovered with 0.5 mL of fresh YTF medium, transferred to a pressure tube containing 10 mL of YTF medium supplemented with 5 mM L-cysteine (pH 7), and incubated at 37 °C. The electroporated cells were allowed to recover at 37 °C until their cell densities were higher than immediately after the electroporation (about 9 to 12 h). Five-milliliter volumes of the outgrowth cultures or appropriately diluted cultures were mixed with 20 mL of YTF molten agar (1.5%) containing an appropriate antibiotic and poured into a petri dish. After the agar mixtures were solidified, plates were incubated upside down. After 2-3 days colonies become visible and can be inoculated in liquid culture.

- YTF medium:
  10 g/L yeast extract
  16 g/L Bacto tryptone
  4 g/L sodium chloride
  5 g/L fructose
  Supplemented with 2 mM L-cysteine
  Final pH is 6.

Isobutene production from CO, CO₂ and H₂ (syngas) by gas fermentation was performed using said genetically engineered C. ljungdahlii DSM 13528 cells harboring the plasmid SG601. The materials and methods used are described in the following:
• Used medium for growth with gas:

NH₄Cl .........................................................1.0 g
KCl ............................................................0.1 g
MgSO₄ x 7H₂O ..............................................0.2 g
NaCl ............................................................0.8 g
KH₂PO₄ .......................................................0.1 g
CaCl₂ x 2H₂O ...............................................20.0 mg
Trace Elements (see below) ..................10.0 mL
Wolfe's Vitamin Solution (see below) ....10.0 mL
Reducing Agent (see below) ..............10.0 mL
Distilled water ..................980.0 mL

Final pH is 6.

Trace Elements:
Nitrilotriacetic acid .......................2.0 g
MnSO₄ x H₂O ..................................1.0 g
Fe(SO₄)₂(NH₄)₂ x 6H₂O ....................0.8 g
CoCl₂ x 6H₂O ......................................0.2 g
ZnSO₄ x 7H₂O ......................................0.2 mg
CuCl₂ x 2H₂O ..................................20.0 mg
NiCl₂ x 6H₂O ..................................20.0 mg
Na₂MoO₄ x 2H₂O ......................20.0 mg
Na₂SeO₄ ..................................20.0 mg
Na₂WO₄ ..................................20.0 mg
Distilled water ..................1.0 L

Add nitrilotriacetic acid to water and adjust to pH 6.0 with KOH. Add remainder of ingredients.

Wolfe's Vitamin Solution:
Biotin.................................2.0 mg  
Folic acid.............................2.0 mg  
Pyridoxine hydrochloride.............10.0 mg  
Thiamine x HCl.........................5.0 mg  
Riboflavin................................5.0 mg  
Nicotinic acid.........................5.0 mg  
Calcium D- (+)-pantothenate.............5.0 mg  
Vitamin B12............................0.1 mg  
p-Aminobenzoic acid....................5.0 mg  
Thiostic acid..........................5.0 mg  
Distilled water........................1.0 L  

Reducing Agent:  
L-Cysteine (free base)..................4.0 g  
Distilled water........................100.0 mL  

- Used gas: 30\% CO$_2$ and 60\% H$_2$

- The transformed cells were cultivated in the medium above supplied the 10 \mu g/mL thiamphenicol (antibiotic) at 37 °C in CSTR bioreactor provided with gas (30\% CO$_2$ and 60\% H$_2$) at 1 bar pressure. Gene expression was induced by 10 mM IPTG when a cell density of 0.3 OD$_{600}$ was reached. Product analysis was performed by GC-MS (Shimadzu GC-MS QP5050A). Headspace gas sampling was conducted using a 500 \mu L Hamilton gastight syringe. Isobutene production could be detected by GC-MS.

Preferred Embodiment – Example 4 (1-pentene production):  

Fig. 12 shows the plasmid SG498 (SEQ ID No 7) which comprises a second engineered 1-pentene pathway (for genes see also reference numerals). This plasmid was electroporated into *Clostridium autoethanogenum DSM 10061* cells by the following protocol:
Genetic Transformation of *Clostridium autoethanogenum DSM 10061* cells

Preparation of electrocompetent *C. autoethanogenum DSM 10061* cells: The procedure for making *C. autoethanogenum DSM 10061* electro competent cells was modified from a protocol for *C. ljungdahlii DSM 13528* reported previously (Köpke, Held et al. 2010). All manipulations except centrifugation were carried out on ice in an anaerobic chamber. All buffers and centrifuge tubes were made ice cold and anoxic before use. All plastic ware was placed in the anaerobic chamber at least 24 h before use in order to eliminate any residual oxygen. Competent cells were maintained in SMP buffer (270 mM sucrose, 1 mM MgCl₂, 7 mM sodium phosphate, pH 6) with 10% DMSO at -80 °C until use.

Electrocompetent *C. autoethanogenum DSM 10061* cells were prepared from cultures that were freshly inoculated from frozen stocks and then transferred twice in YTF liquid medium. About 15 to 16 h before preparation of competent cells, a mid- to late-log-phase culture was transferred into two serum bottles containing 100 mL fresh YTF medium supplemented with 40 mM DL-threonine (final optical density at 600 nm [OD₆₀₀] = 0.004). After overnight growth at 37 °C, early-log phase cells (OD₆₀₀ = 0.2 to 0.3; 200 mL) were harvested by centrifugation at 10,000 rpm for 10 min at 4 °C. The cells were washed twice with 200 mL of SMP wash buffer and resuspended in the same buffer at a final concentration of 10¹⁰ to 10¹¹ cells/mL. Antifreezing buffer (60% DMSO – 40% SMP, pH 6) was added to the competent cells a tone-fifth of the final resuspension volume to achieve a final concentration of 10% DMSO. The resulting competent cells (25 µL/tube) were stored at -80 °C for future use. The competence of these frozen competent cells remained stable for about 1 month.

- Electrotransformation procedures for *C. autoethanogenum DSM 10061*: All procedures were carried out in an anaerobic chamber. Electrocompetent *C. autoethanogenum DSM 10061* cells (25 µL) were quickly transferred on ice from a -80 °C freezer to an anaerobic chamber. After thawing on ice (about 1 min), the cells were mixed with 1 to 5 µg DNA and transferred to a prechilled, 0.1-cm-gap cuvette. Cells were pulsed at 0.625 kV with resistance at 600 Ohm and a capacitance of 25 µF by using a microbial electroporation system. Immediately after the pulse, cells were recovered with 0.5 mL of fresh YTF medium, transferred to a pressure tube containing 10 mL of YTF medium supplemented...
with 5 mM L-cysteine (pH 7), and incubated at 37 °C. The electroporated cells were allowed to recover at 37 °C until their cell densities were higher than immediately after the electroporation (about 9 to 12 h). Five-milliliter volumes of the outgrowth cultures or appropriately diluted cultures were mixed with 20 mL of YTF molten agar (1.5%) containing an appropriate antibiotic and poured into a petri dish. After the agar mixtures were solidified, plates were incubated upside down. After 2-3 days colonies become visible and can be inoculated in liquid culture.

- YTF medium:
  - 10 g/L yeast extract
  - 16 g/L Bacto tryptone
  - 4 g/L sodium chloride
  - 5 g/L fructose
  - Supplemented with 2 mM L-cysteine
  - Final pH is 6.

1-Pentene production from CO, CO₂ and H₂ (syngas) by gas fermentation with electron supply by electrode was performed using said genetically engineered *C. autoethanogenum DSM 10061* cells harboring the plasmid SG498. The materials and methods used are described in the following:

- Used medium for growth with gas:

  - NH₄Cl ..............................................................1.0 g
  - KCl ..............................................................0.1 g
  - MgSO₄ x 7H₂O ..............................................0.2 g
  - NaCl ..........................................................0.8 g
  - KH₂PO₄ ......................................................0.1 g
  - CaCl₂ x 2H₂O ..............................................20.0 mg
  - Trace Elements (see below) ..................10.0 mL
  - Wolfe's Vitamin Solution (see below) ....10.0 mL
  - Reducing Agent (see below) ...................10.0 mL

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Distilled water.............................980.0 mL

Final pH is 6.

Trace Elements:
Nitrilotriacetic acid......................2.0 g
MnSO₄ x H₂O .............................1.0 g
Fe(SO₄)₂(NH₄)₂ x 6H₂O ..............0.8 g
CoCl₂ x 6H₂O .............................0.2 g
ZnSO₄ x 7H₂O .............................0.2 mg
CuCl₂ x 2H₂O ............................20.0 mg
NiCl₂ x 6H₂O .............................20.0 mg
Na₂MoO₄ x 2H₂O .........................20.0 mg
Na₂SeO₄ ..................................20.0 mg
Na₂WO₄ ..................................20.0 mg
Distilled water ..........................1.0 L

Add nitrilotriacetic acid to water and adjust to pH 6.0 with KOH. Add remainder of ingredients.

Wolfe's Vitamin Solution:
Biotin.......................................2.0 mg
Folic acid..................................2.0 mg
Pyridoxine hydrochloride..............10.0 mg
Thiamine x HCl............................5.0 mg
Riboflavin..................................5.0 mg
Nicotinic acid............................5.0 mg
Calcium D-(+)-pantothenate............5.0 mg
Vitamin B12................................0.1 mg
p-Aminobenzoic acid....................5.0 mg
Thiolic acid..............................5.0 mg
Distilled water ..........................1.0 L
Reducing Agent:
L-Cysteine (free base)......4.0 g
Distilled water..............100.0 mL

- Used gas: 100% CO

The transformed cells were cultivated in the medium above supplied the 10 μg/mL thiamphenicol (antibiotic) at 37 °C in CSTR bioreactor provided with gas (100% CO) at 1 bar pressure and an electrode with power supply for electricity supply. Gene expression was induced by 10 mM IPTG when a cell density of 0.3 OD₆₀₀ was reached. Product analysis was performed by GC-MS (Shimadzu GC-MS QP5050A). Headspace gas sampling was conducted using a 500 μL Hamilton gastight syringe. 1-Pentene production could be detected by GC-MS.

Preferred Embodiment – Example 5 (ethene production):

Fig. 14 shows the plasmid SG557 (SEQ ID No 9) which comprises an engineered ethene pathway (for genes see also reference numerals). This plasmid was electroporated into Clostridium ljungdahlii DSM 13528 cells by the following protocol:

- Genetic Transformation of Clostridium ljungdahlii DSM 13528 cells

Preparation of electrocompetent C. ljungdahlii DSM 13528 cells: The procedure for making C. ljungdahlii DSM 13528 electro competent cells was modified from a protocol reported previously (Köpke, Held et al. 2010). All manipulations except centrifugation were carried out on ice in an anaerobic chamber. All buffers and centrifuge tubes were made ice cold and anoxic before use. All plastic ware was placed in the anaerobic chamber at least 24 h before use in order to eliminate any residual oxygen. Competent cells were maintained in SMP buffer (270 mM sucrose, 1 mM MgCl₂, 7 mM sodium phosphate, pH 6) with 10% DMSO at -80 °C until use. Electrocompetent C. ljungdahlii
**DSM 13528** cells were prepared from cultures that were freshly inoculated from frozen stocks and then transferred twice in YTF liquid medium. About 15 to 16 h before preparation of competent cells, a mid- to late-log-phase culture was transferred into two serum bottles containing 100 mL fresh YTF medium supplemented with 40 mM DL-threonine (final optical density at 600 nm [OD$_{600}$] = 0.004). After overnight growth at 37 °C, early-log phase cells (OD$_{600}$ = 0.2 to 0.3; 200 mL) were harvested by centrifugation at 10,000 rpm for 10 min at 4 °C. The cells were washed twice with 200 mL of SMP wash buffer and resuspended in the same buffer at a final concentration of $10^{10}$ to $10^{11}$ cells/mL. Antifreezing buffer (60% DMSO – 40% SMP, pH 6) was added to the competent cells a tone-fifth of the final resuspension volume to achieve a final concentration of 10% DMSO. The resulting competent cells (25 µL/tube) were stored at -80 °C for future use. The competence of these frozen competent cells remained stable for about 1 month.

- **Electrotransformation procedures for C. ljungdahlii DSM 13528**: All procedures were carried out in an anaerobic chamber. Electroporantent C. ljungdahlii DSM 13528 cells (25 µL) were quickly transferred on ice from a -80 °C freezer to an anaerobic chamber. After thawing on ice (about 1 min), the cells were mixed with 1 to 5 µg DNA and transferred to a prechilled, 0.1-cm-gap cuvette. Cells were pulsed at 0.625 kV with resistance at 600 Ohm and a capacitance of 25 µF by using a microbial electroporation system. Immediately after the pulse, cells were recovered with 0.5 mL of fresh YTF medium, transferred to a pressure tube containing 10 mL of YTF medium supplemented with 5 mM L-cysteine (pH 7), and incubated at 37 °C. The electroporated cells were allowed to recover at 37 °C until their cell densities were higher than immediately after the electroporation (about 9 to 12 h). Five-milliliter volumes of the outgrowth cultures or appropriately diluted cultures were mixed with 20 mL of YTF molten agar (1.5%) containing an appropriate antibiotic and poured into a petri dish. After the agar mixtures were solidified, plates were incubated upside down. After 2-3 days colonies become visible and can be inoculated in liquid culture.

- **YTF medium**:
  10 g/L yeast extract
16 g/L Bacto tryptone
4 g/L sodium chloride
5 g/L fructose
Supplemented with 2 mM L-cysteine
Final pH is 6.

Ethene production from CO, CO₂ and H₂ (syngas) by gas fermentation was performed using said genetically engineered C. ljungdahlii DSM 13528 cells harboring the plasmid SG557. The materials and methods used are described in the following:

- Used medium for growth with gas:

  NH₄Cl .................................................1.0 g
  KCl .................................................0.1 g
  MgSO₄ x 7H₂O ......................................0.2 g
  NaCl .................................................0.8 g
  KH₂PO₄ ..............................................0.1 g
  CaCl₂ x 2H₂O ......................................20.0 mg
  Trace Elements (see below) ......................10.0 mL
  Wolfe's Vitamin Solution (see below) ..........10.0 mL
  Reducing Agent (see below) ......................10.0 mL
  Distilled water ....................................980.0 mL

  Final pH is 6.

  Trace Elements:
  Nitrilotriacetic acid .........................2.0 g
  MnSO₄ x H₂O ......................................1.0 g
  Fe(SO₄)₂(NH₄)₂ x 6H₂O .........................0.8 g
  CoCl₂ x 6H₂O ......................................0.2 g
  ZnSO₄ x 7H₂O ......................................0.2 mg
  CuCl₂ x 2H₂O ......................................20.0 mg
  NiCl₂ x 6H₂O ......................................20.0 mg
Na₂MoO₄ x 2H₂O .................. 20.0 mg
Na₂SeO₄ .................................. 20.0 mg
Na₂WO₄ .................................. 20.0 mg
Distilled water ......................... 1.0 L

Add nitrilotriacetic acid to water and adjust to pH 6.0 with KOH. Add remainder of ingredients.

Wolfe's Vitamin Solution:
Biotin ..................................... 2.0 mg
Folic acid .................................. 2.0 mg
Pyridoxine hydrochloride ............. 10.0 mg
Thiamine x HCl .......................... 5.0 mg
Riboflavin .................................. 5.0 mg
Nicotinic acid ................................ 5.0 mg
Calcium D-(+)-pantothenate ........... 5.0 mg
Vitamin B12 .............................. 0.1 mg
p-Aminobenzoic acid .................... 5.0 mg
Thiotoic acid ............................. 5.0 mg
Distilled water ......................... 1.0 L

Reducing Agent:
L-Cysteine (free base) ................. 4.0 g
Distilled water ......................... 100.0 mL

- Used gas: 30% CO₂ and 60% H₂

- The transformed cells were cultivated in the medium above supplied the 10 μg/mL thiamphenicol (antibiotic) at 37 °C in CSTR bioreactor provided with gas (30% CO₂ and 60% H₂) at 1 bar pressure. Gene expression was induced by 10 mM IPTG when a cell density of 0.3 OD₆₀₀ was reached. Product analysis was performed by GC-MS (Shimadzu
GC-MS QP5050A). Headspace gas sampling was conducted using a 500 µL Hamilton gastight syringe. Ethene production could be detected by GC-MS.

Alternative Embodiments:

Plasmid SG479 (Fig. 7 and SEQ listing No 2), Plasmid SG523 (Fig. 9 and SEQ listing No 4), Plasmid SG582 (Fig. 10 and SEQ listing No 5), Plasmid SG513 (Fig. 13 and SEQ listing No 8), Plasmid SG598 (Fig. 15 and SEQ listing No 10) were also used to engineer Clostridium ljungdahlii DSM 13528, Clostridium autoethanogenum DSM 10061 and Clostridium aceticum cells to produce propene, 1-butene, isobutene, 1-pentene and ethene from various syngas-like gases. The same protocol and process like described in the preferred embodiments was performed. Additionally CO and a mixture of CO₂ and CO, as well as CO and electrons and/or CO₂ and electrons were also successfully used as substrates.

- All used substrates:
  - 60% CO, 10% CO₂, 30% H₂ (syngas)
  - 100% CO
  - 30% CO₂ and 60% H₂
  - 60% CO, 10% CO₂, 30% H₂ and electrons
  - 100% CO and electrons
  - 100% CO₂ and electrons
  - 30% CO₂ and 60% H₂ and electrons

Sequence Listing:

Seqlisting.txt contains all sequences:

No. 1 - nucleotide sequence of plasmid SG455
No. 2 - nucleotide sequence of plasmid SG479
No. 3 - nucleotide sequence of plasmid SG539
No. 4 - nucleotide sequence of plasmid SG523
No. 5 - nucleotide sequence of plasmid SG582
No. 6 - nucleotide sequence of plasmid SG601
No. 7 - nucleotide sequence of plasmid SG498
No. 8 - nucleotide sequence of plasmid SG513
No. 9 - nucleotide sequence of plasmid SG557
No. 10 - nucleotide sequence of plasmid SG598

Claims:

I claim:

1. Isolated, genetically engineered, anaerobic bacterial cells capable of producing ethene, propene, 1-butene, isobutene and 1-pentene from carbon monoxide and/or from carbon dioxide; said cells comprising one or more heterologous nucleic acids encoding one or more enzymes in one or more artificial alkene pathways, whereby the bacteria express the one or more enzymes, said enzymes selected from the group consisting of:

   a) Acetoacetyl-Coenzyme A synthase thiolase (EC 2.3.1.9)
   b) 3-hydroxybutyryl-CoA dehydrogenase (EC 1.1.1.157)
   c) 3-hydroxybutyryl-CoA dehydratase (EC 4.2.1.55)
   d) Acyl-CoA thioesterase [EC 3.1.2.-]
   e) Aldehyde/alcohol dehydrogenase (EC 1.1.1.- and EC 1.2.1.-)
   f) propionyl-CoA synthase (EC 1.3.1.84)
   g) Acetyl-CoA carboxylase (EC 6.4.1.2)
   h) Acetoacetyl-CoA synthase (EC 2.3.1.194)
   i) Acetoacetyl-CoA reductase (EC 1.1.1.36)
   j) phenyl acrylic acid decarboxylase (EC 4.1.1.-)
   k) (R)-specific enoyl-CoA hydratase (EC 4.2.1.119)
   l) 4-aminobutyrate aminotransferase (EC 2.6.1.19)
   m) glutamate dehydrogenase (EC 1.4.1.2)
n) 2-hydroxyglutarate dehydrogenase (EC 1.1.99.2)
o) glutaconate CoA-transferase (EC 2.8.3.12)
p) 2-hydroxyglutaryl-CoA dehydratase (EC 4.2.1.-)
q) glutaconyl-CoA decarboxylase (EC 4.1.1.70)
r) 4-hydroxybutyrate dehydrogenase (EC 1.1.1.61)
s) 4-hydroxybutyrate CoA-transferase (EC 2.8.3.-)
t) vinylacetyl-CoA-Delta-isomerase (EC 4.2.1.120)
u) 4-hydroxybutyryl-CoA dehydratase (EC 5.3.3.3)

v) 2-oxoglutarate/butyrate ferredoxin oxidoreductase (EC 1.2.7.3)
w) pyruvate/2-oxobutyrate ferredoxin oxidoreductase (EC 1.2.7.1)
x) methylmalonyl-CoA mutase (EC 5.4.99.2)
y) methylmalonyl-CoA decarboxylase (EC 4.1.1.41)
z) 3-hydroxy-3-methylglutaryl-CoA synthase (EC 2.3.3.10)
a2) 3-methylglutaconyl-CoA hydratase (EC 4.2.1.18)
b2) 3-methylcrotonyl-CoA carboxylase (EC 6.4.1.4)
c2) trans-2-enoyl-CoA reductase (EC1.1.1.36)
d2) 3-hydroxybutyryl-CoA dehydrogenase (EC 1.1.1.157)
e2) lactate CoA-transferase (EC 2.8.3.-)
f2) lactoyl-CoA dehydratase (EC 4.2.1.54)
g2) malonyl CoA reductase (EC 1.2.1.75)
h2) malonyl CoA reductase (EC 1.1.1.298)
i2) propionyl-CoA synthase (EC 6.2.1.36)
j2) acryloyl-CoA synthase (EC 4.2.1.116)

k2) lactate CoA-transferase (EC 2.8.3.-)

l2) propionyl-CoA synthase (EC 1.3.1.84)

2. The bacteria of claim 1 wherein in the absence of said nucleic acids, the bacteria do not express the corresponding polypeptides.

3. A process for converting carbon monoxide and/or carbon dioxide into ethene, propene, 1-butene, isobutene and 1-pentene, the process comprising: passing a gaseous carbon monoxide and/or carbon dioxide to a bioreactor containing a culture of genetically engineered anaerobic bacteria in a culture medium and providing electrons by an electrode such that the bacteria convert the carbon monoxide and/or carbon dioxide to propene, 1-butene, isobutene and 1-pentene, and recovering the ethene, propene, 1-butene, isobutene and 1-pentene from the bioreactor, wherein the anaerobic bacteria are genetically engineered to express one or more enzymes of one or more artificial alkene pathways.

4. The process of claim 3, wherein the process further comprises recovering the ethene, propene, 1-butene, isobutene and 1-pentene.

5. The process of claim 3 and 4, wherein the ethene, propene, 1-butene, isobutene and 1-pentene is recovered by absorption stripping, a cooling trap or a flash drum.

6. The isolated, genetically engineered, anaerobic bacterial cells of claim 1 which possess a Wood-Ljungdahl pathway.

7. The isolated, genetically engineered, anaerobic bacterial cells of claim 1 which are selected from the group consisting of Clostridium ljungdahlii DSM 13528, Clostridium autoethanogenum DSM 10061, Clostridium carboxidivorans, Clostridium aceticum, Clostridium drakei, Clostridium scatologenes, Clostridium ragsdalei, Clostridium formicoaceticum, Clostridium magnus, Butyribacterium methylotrophicum, Acetobacterium woodii, Alkalibaculum bacchii, Aecetoariaerobium riotera, Butyribacterium methylotrophicum, Blautia producta, Eubacterium limosum, Desulfotbacterium hafhierise, Moorella thermoacetica, Moorella thermautotrophica, Peptostreptococcus productus,
Rhodospirillum rubrum, Sporomusa ovata, Sporomusa silvacetica, Sporomusa sphaeroides, Thermoanaerobacter kiiui, Oxobacter pfennigii, Acetobacterium fimetarium, Acetohalobium arabaticum, Blautia wexlerae, Carbophilus carboxidus, Cloacibacillus evryensis, Hydrogenophaga pseudoalvea, Rhodopseudomonas palustris, Pseudomonas gazotropha, Ralstonia eutropha.

8. Isolated, genetically engineered, anaerobic bacterial cells capable of producing ethene, propene, 1-butene, isobutene and 1-pentene from carbon dioxide and electrons and/or from carbon monoxide and electrons; said cells comprising one or more heterologous nucleic acids encoding one or more enzymes in one or more artificial alkene pathways, whereby the bacteria express the one or more enzymes, said enzymes selected from the group consisting of:

   a) Acetoacetyl-Coenzyme A synthase thiolase (EC 2.3.1.9)
   b) 3-hydroxybutyryl-CoA dehydrogenase (EC 1.1.1.157)
   c) 3-hydroxybutyryl-CoA dehydratase (EC 4.2.1.55)
   d) Acyl-CoA thioesterase [EC 3.1.2.-]
   e) Aldehyde/alcohol dehydrogenase (EC 1.1.1.- and EC 1.2.1.-)
   f) propionyl-CoA synthase (EC 1.3.1.84)
   g) Acetyl-CoA carboxylase (EC 6.4.1.2)
   h) Acetoacetyl-CoA synthase (EC 2.3.1.194)
   i) Acetoacetyl-CoA reductase (EC 1.1.1.36)
   j) phenyl acrylic acid decarboxylase (EC 4.1.1.-)
   k) (R)-specific enoyl-CoA hydratase (EC 4.2.1.119)
   l) 4-aminobutyrate aminotransferase (EC 2.6.1.19)
   m) glutamate dehydrogenase (EC 1.4.1.2)
   n) 2-hydroxyglutarate dehydrogenase (EC 1.1.99.2)
o) glutaconate CoA-transferase (EC 2.8.3.12)

p) 2-hydroxyglutaryl-CoA dehydratase (EC 4.2.1.-)

q) glutaconyl-CoA decarboxylase (EC 4.1.1.70)

r) 4-hydroxybutyrate dehydrogenase (EC 1.1.1.61)

s) 4-hydroxybutyrate CoA-transferase (EC 2.8.3.-)

t) vinlyaceteryl-CoA-Delta-isomerase (EC 4.2.1.120)

u) 4-hydroxybutyryl-CoA dehydratase (EC 5.3.3.3)

v) 2-oxoglutarate/butyrate ferredoxin oxidoreductase (EC 1.2.7.3)

w) pyruvate/2-oxobutyrate ferredoxin oxidoreductase (EC 1.2.7.1)

x) methylmalonyl-CoA mutase (EC 5.4.99.2)

y) methylmalonyl-CoA decarboxylase (EC 4.1.1.41)

z) 3-hydroxy-3-methylglutaryl-CoA synthase (EC 2.3.3.10)

a2) 3-methylglutaconyl-CoA hydratase (EC 4.2.1.18)

b2) 3-methylcrotonyl-CoA carboxylase (EC 6.4.1.4)

c2) trans-2-enoyl-CoA reductase (EC1.1.1.36)

d2) 3-hydroxybutyryl-CoA dehydrogenase (EC 1.1.1.157)

e2) lactate CoA-transferase (EC 2.8.3.-)

f2) lactoyl-CoA dehydratase (EC 4.2.1.54)

g2) malonyl CoA reductase (EC 1.2.1.75)

h2) malonyl CoA reductase (EC 1.1.1.298)

i2) propionyl-CoA synthase (EC 6.2.1.36)

j2) acryloyl-CoA synthase (EC 4.2.1.116)
k2) lactate CoA-transferase (EC 2.8.3.-)

9. The bacteria of claim 1 wherein in the absence of said nucleic acids, the bacteria do not express the corresponding polypeptides.

10. A process for converting carbon dioxide and electrons and/or carbon monoxide and electrons into ethene, propene, 1-butene, isobutene or 1-pentene, the process comprising: passing a gaseous carbon monoxide and/or carbon dioxide to a bioreactor containing a culture of genetically engineered anaerobic bacteria in a culture medium and providing electrons by an electrode such that the bacteria convert the carbon dioxide and electrons and/or carbon monoxide and electrons to ethene, propene, 1-butene, isobutene or 1-pentene, and recovering the ethene, propene, 1-butene, isobutene and 1-pentene from the bioreactor, wherein the anaerobic bacteria are genetically engineered to express one or more enzymes of one or more artificial alkene pathways.

11. The process of claim 10, wherein the process further comprises recovering the ethene, propene, 1-butene, isobutene and 1-pentene.

12. The process of claim 10 and 11, wherein the ethene, propene, 1-butene, isobutene and 1-pentene is recovered by absorption stripping, a cooling trap or a flash drum.

13. The isolated, genetically engineered, anaerobic bacterial cells of claim 1 which possess a Wood-Ljungdahl pathway.

14. The isolated, genetically engineered, anaerobic bacterial cells of claim 1 which are selected from the group consisting of Clostridium ljungdahlii DSM 13528, Clostridium autoethanogenum DSM 10061, Clostridium carboxidivorans, Clostridium aceticum, Clostridium drakei, Clostridium scatologenes, Clostridium ragsdalei, Clostridium formicoaceticum, Clostridium magnum, Butyribacterium methylophilum, Acetobacterium woodii, Alkalibaculum bacchii, Aecetohalobium rietera, Butyribacterium methylophilum, Blautia producta, Eubacterium limosum, Desulfotuctbacterium haftherise, Moorella thermoacetica, Mooreella thermaotrophica, Peptostreptococcus productus, Rhodospirillum rubrum, Sporomusa ovata, Sporomusa silvatica, Sporomusa sphaeroides, Thermoanaerobacter kiiuvi, Oxobacter pfennigii, Acetobacterium fimetarium, Acetohalobium
arabaticu, Blautia wexlerae, Carbophilus carboxidus, Cloacibacillus evryensis, Hydrogenophaga pseudoflava, Rhodopseudomonas palustris, Pseudomonas gazotropha, Ralstonia eutropha.
Fig. 1

CO, CO₂ and/or CO, CO₂ and electrons

Wood-Ljungdahl pathway

glutamate ↔ 4-aminobutanoate ↔ acetyl-CoA → malonyl-CoA

52
54
56
58
Glutaconyl-CoA → crotonyl-CoA

60
62
64
66
68

3-butenoyl-CoA

34
32
30
50
50

3-butenyl-CoA

48
44
46

crotonate

36
38

propene
Fig. 2

\[ \text{CO}_2, \text{CO}_2 \text{ and/or} \]
\[ \text{CO}_2 \text{ and electrons} \]
\[ \text{Wood-Ljungdahl pathway} \]
\[ \text{lactate} \leftrightarrow 2\text{-oxobutyrate} \leftrightarrow \text{acetyl-CoA} \rightarrow \text{malonyl-CoA} \rightarrow \text{succinyl-CoA} \]
\[ 100 \quad 72, 74 \]
\[ \text{acryloyl-CoA} \rightarrow \text{propionyl-CoA} \rightarrow \text{ketovaleryl-CoA} \]
\[ 112 \quad 110 \quad 112 \quad 110 \quad 112 \quad 110 \quad 112 \quad 110 \]
\[ \downarrow \quad \downarrow \quad \downarrow \quad \downarrow \quad \downarrow \quad \downarrow \quad \downarrow \quad \downarrow \]
\[ + \text{acetyl-CoA} \rightarrow \text{(R)-methylmalonyl-CoA} \rightarrow \text{(R)-3-hydroxyvaleryl-CoA} \rightarrow \text{(S)-3-hydroxyvaleryl-CoA} \rightarrow \text{trans-2-pentenoyl-CoA} \rightarrow \text{trans-2-pentenoate} \rightarrow \text{1-butene} \]
\[ 70 \quad 76 \quad 78 \quad 70 \quad 70 \]
\[ 46 \quad 48 \quad 36 \quad 38 \]
\[ 32 \quad 34 \quad 1 \]
Fig. 3

\[
\text{CO, CO}_2 \quad \text{and/or} \quad \text{CO, CO}_2 \text{ and electrons} \\
\downarrow \\
\text{Wood-Ljungdahl pathway} \\
\downarrow \\
cis-geranyl-CoA \leftrightarrow \text{L-leucine} \leftrightarrow \text{acetyl-CoA} \rightarrow \text{acetoacetyl-CoA} \\
\downarrow^{30} \\
\text{HMG-CoA} \\
\downarrow^{82} \\
\text{3-methylcrotonyl-CoA} \leftrightarrow \text{3-methylglutaconyl-CoA} \\
\downarrow^{84} \\
\text{3-methylcrotonate} \\
\downarrow^{36} \\
isobutene \\
\downarrow^{38}
\]
Fig. 4

CO₂, CO₂ and/or CO₂ and electrons

↓

Wood-Ljungdahl pathway

↓

4-aminobutanoate ← ↔ acetyl-CoA → malonyl-CoA

50

50

60

62

64

66

Glutaconyl-CoA ← Glutamate

52

54

56

58

3-butenoyl-CoA

68

3-ketoheptanoyl-CoA

68

3-hydroxyheptanoyl-CoA

48

86

86

88

1-pentene

38

trans-2-hexenoate

36

trans-2-hexenoyl-CoA

34

3-hydroxyhexanoyl-CoA

90
Fig. 5

CO, CO₂
and/or
CO, CO₂ and electrons
↓
↓
↓
Wood-Ljungdahl pathway

lactate ↔ acetyl-CoA → malonyl-CoA

↓
100
lactoyl-CoA → acryloyl-CoA ↔
102
110
108
36
38
acrylate
ethylene
102
104
106
Fig. 6

SG455
15272 bps

Fig. 7

SG479
20891 bps