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APPLICATION NUMBER: 62/047,827
FILING DATE: September 09, 2014

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**INVENTOR(S)**

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<th>Family Name or Surname</th>
<th>Residence (City and either State or Foreign Country)</th>
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<tr>
<td>Bernhard</td>
<td>Guentner</td>
<td>Vaals, Netherlands</td>
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Additional inventors are being named on 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 1,3-butadiene.

**DIRECT ALL CORRESPONDENCE TO:**

**CORRESPONDENCE ADDRESS**

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

- [ ] Application Data Sheet. See 37 CFR 1.76.
- ✔ Drawing(s) Number of Sheets 3
- [ ] Specification (e.g., description of the invention) Number of Pages 40
- [ ] Other (specify) Sequence Listing

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PROVISIONAL APPLICATION FOR PATENT COVER SHEET – Page 2 of 2

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SIGNATURE /Bernhard Guentner/ DATE 09/09/2014

TYPED OR PRINTED NAME Bernhard Guentner REGISTRATION NO. __________________________

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Provisional Patent Application:
Genetically engineered microorganisms and process for converting gaseous C1-carbon sources and/or gaseous C1-carbon sources and electrons into 1,3-butadiene.

Inventor:
Bernhard Guentner
Akenerstraat 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 1,3-butadiene. The genetically engineered, anaerobic bacterial cells comprise one or more heterologous nucleic acids encoding 1,3-butadiene pathways and are capable of producing 1,3-butadiene 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 1,3-butadiene 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 US20110300597
US Patent US20120225466


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


S. Foss, J. Harder, Thauera linaloolentis sp. nov. and Thauera terpenica sp. nov., Isolated on Oxygen-containing Monoterpenes (Linalool, Menthol, and Eucalyptol and Nitrate, Systematic and Applied Microbiology, Volume 21, Issue 3, August 1998, Pages 365-373, ISSN 0723-2020,

Sabine Foß, Jens Harder, Microbial transformation of a tertiary allylalcohol: regioselective isomerisation of linalool to geraniol without nerol formation, FEMS Microbiology Letters, Volume 149, Issue 1, 1 April 1997, Pages 71-75, ISSN 0378-1097

"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. Clouberg, Jacob E. Vick, Matthew D. Blankschien, Maria Rodriguez-Moyá, and Ramon Gonzalez ACS Synthetic Biology 2012 1 (11), 541-554

"A Novel Paradigm of Fatty Acid β-Oxidation Exemplified 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


page 10


PMCID: PMC3126405 "Driving Forces Enable High-Titer Anaerobic 1-Butanol Synthesis in Escherichia coli" Claire R. Shen, Ethan I. Lan, Yasumasa Dekishima, Antonino Baez, Kwang Myung Cho and James C. Liao


„Genetische und biochemische Charakterisierung von Enzymen des anaeroben Monoterpen-Abbaus in Castellaniella defragrans.“ Dissertation Frauke Lüddeke


“Genetically Modified Strains of Ralstonia eutropha H16 with β-Ketothiolase Gene Deletions for Production of Copolysteres 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


Hector ML, Fall RR (1976). "Multiple acyl-coenzyme A carboxylases in Pseudomonas citronellolis." Biochemistry 15(16);3455-72. PMID: 8091

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


Wong BJ, Gerlt JA (2004). "Evolution of function in the crotonase superfamily: (3S)-methylglutaconyl-CoA hydratase from Pseudomonas putida." Biochemistry 43(16);4646-54. PMID: 15096032


Advantages:

The main advantages of the invention are:

- Economical nonpetrochemical route for 1,3-butadiene.
• One step process for 1,3-butadiene production.
• 1,3-butadiene 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 1,3-butadiene 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 1,3-butadiene.
• Suitable for a robust industrial process.
• Extremely low carbon footprint.
• Hugh environmental benefits.
• Process can be carried out at room temperature.

Introduction:

Butadiene, also known as 1,3-butadiene, is a colorless gas that condenses to a liquid at minus 4.5 degrees centigrade. Butadiene is conventionally derived, using an extractive distillation process, from the crude C4 stream, one of the cracker by-products of ethylene and propylene production. The largest single use for butadiene is in the production of styrene-butadiene rubber (SBR) which, in turn, is principally used in the manufacture of automobile tires. SBR is also used in adhesives, sealants, coatings and in rubber articles like shoe soles. Polybutadiene is also used in tires and can be used as an intermediate in the production of acrylonitrile-butadiene-styrene (ABS). ABS is widely used in items such as telephones, computer casings and other appliances. Other polymers made from butadiene include styrene-butadiene latex, used for example in carpet backings and adhesives; nitrile rubber, used in hoses, fuel lines, gasket seals, gloves and footwear; and styrene-butadiene block copolymers which are used in many end-uses ranging from asphalt modifiers (road and roofing construction applications), to adhesives, footwear and toys. Chemical intermediates
made from butadiene include adiponitrile and chloroprene which are used, respectively, in
the manufacture of nylon and neoprene. Approximately 10 million tons of butadiene are
currently produced annually. The sales of 1,3-butadiene exceed a value of over 10 billion US
dollars worldwide. The ability to manufacture butadiene from alternative and/or renewable
feedstocks represents a major advance in the quest for more sustainable chemical
production processes. Butadiene can be produced renewably by fermentation of sugars or
other feedstocks to produce diols, such as 1,4- butanediol or 1,3-butanediol, which are
separated, purified, and then dehydrated to butadiene in a second step involving metal-
based catalysis. However, direct fermentative production of butadiene from renewable
feedstocks obviates the need for dehydration steps since butadiene gas (boiling point, or bp,
is -4.4°C) could be continuously emitted from the fermenter and readily collected, e.g. by
condensation. The direct fermentative production process eliminates the need for fossil-
based butadiene and would allow substantial savings in cost, energy, and harmful waste and
emissions relative to petrochemically-derived butadiene. Improved microbial organisms and
methods for effectively producing butadiene from cheap renewable feedstocks such as
molasses, sugar cane juice, and sugars derived from biomass sources, including agricultural
and wood waste, especially C1 feedstocks such as syngas carbon monoxide and carbon
dioxide, as well as electrons are needed.

Figures:
Fig. 1 shows the two pathways and their key enzymes for the generation of 1,3-butadiene
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 plasmid SG387 comprising a 1,3-butadiene pathway type 1 (crotonyl-Coa
synthesis is derived from Clostridium acetobutylicum).

Fig. 3 shows plasmid SG411 comprising a 1,3-butadiene pathway type 2 (crotonyl-Coa
synthesis via malonyl-Coa is derived from Escherichia coli and other bacteria).

Fig. 4 shows plasmid SG661 comprising a 1,3-butadiene pathway type 3 (crotonyl-Coa
synthesis via via malonyl-Coa is derived from Escherichia coli and other bacteria and the
classical pathway from Clostridium acetobutylicum).
Fig. 5 shows plasmid SG411 comprising a 1,3-butadiene pathway type 4 (crotonyl-CoA synthesis via glutamate and 4-aminobutanoate).

**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 – aldehyde/alcohol dehydrogenase (EC 1.1.1.- and EC 1.2.1.-) = adhE2
40 – geraniol isomerase/linalool dehydratase (EC 5.4.4.4 and EC 4.2.1.127) = GIM, GIT
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 – glutaconate 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

pMB1 = origin of replication (E. coli)
repH = origin of replication (Clostridium)
catp = chloramphenicol/thiamphenicol resistance marker
lacI = lac repressor

page 19
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 1,3-butadiene 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:

Fig. 2 shows the plasmid SG384 (SEQ ID No 1) which comprises an engineered 1,3-butadiene pathway (for genes see also reference numerals). This plasmid was electroporated into Clostridium ljungdahlii cells by the following protocol:
• Genetic Transformation of *Clostridium ljungdahlii* cells

Preparation of electrocompetent *C. ljungdahlii* cells: The procedure for making *C. ljungdahlii* 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* 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 10⁹ 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*: All procedures were carried out in an anaerobic chamber. Electrocompetent *C. ljungdahlii* 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 was performed
using said genetically engineered C. ljungdahlii cells harboring the plasmid SG387. 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
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 ℃ 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. 1,3-butadiene production could be detected by GC-MS.

Preferred Embodiment – Example 2:

Fig. 3 shows the plasmid SG411 (SEQ ID No 2) which comprises a second engineered 1,3-butadiene pathway (for genes see also reference numerals). This plasmid was electroporated into *Clostridium autoethanogenum* cells by the following protocol:

- Genetic Transformation of *Clostridium autoethanogenum* cells
  Preparation of electrocompetent *C. autoethanogenum* cells: The procedure for making *C. autoethanogenum* electro competent cells was modified from a protocol for *C. ljungdahlii* 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 ℃ until use. Electrocompetent *C. autoethanogenum* 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 (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. autoethanogenum*: All procedures were carried out in an anaerobic chamber. Electrocompetent *C. autoethanogenum* 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 *C. autoethanogenum* cells harboring the plasmid SG411. 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
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. 1,3-butadiene production could be detected by GC-MS.
Preferred Embodiment – Example 3:

Fig. 4 shows the plasmid SG661 (SEQ ID No 3) which comprises an engineered 1,3-butadiene pathway (for genes see also reference numerals). This plasmid was electroporated into *Clostridium ljungdahlii* cells by the following protocol:

- Genetic Transformation of *Clostridium ljungdahlii* cells

  Preparation of electrocompetent *C. ljungdahlii* cells: The procedure for making *C. ljungdahlii* 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* 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 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*: All procedures were carried out in an anaerobic chamber. Electrocompetent *C. ljungdahlii* 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 by gas fermentation was performed using said genetically engineered C. ljungdahlii cells harboring the plasmid SG661. The materials and methods used are described in the following:

- Used medium for growth with gas:

  \[
  \begin{align*}
  \text{NH}_4\text{Cl} & \quad \text{1.0 g} \\
  \text{KCl} & \quad \text{0.1 g} \\
  \text{MgSO}_4 \cdot 7\text{H}_2\text{O} & \quad \text{0.2 g} \\
  \text{NaCl} & \quad \text{0.8 g} \\
  \text{KH}_2\text{PO}_4 & \quad \text{0.1 g} \\
  \text{CaCl}_2 \cdot 2\text{H}_2\text{O} & \quad \text{20.0 mg}
  \end{align*}
  \]
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
Thioctic 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 100% CO at 1 bar pressure. Gene expression was induced by 10 mM IPTG when a cell density of 0.3 OD<sub>600</sub> 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. 1,3-butadiene production could be detected by GC-MS.

Preferred Embodiment – Example 4:

Fig. 5 shows the plasmid SG696 (SEQ ID No 4) which comprises a second engineered 1,3-butadiene pathway (for genes see also reference numerals). This plasmid was electroporated into Clostridium autoethanogenum cells by the following protocol:

- Genetic Transformation of Clostridium autoethanogenum cells
  Preparation of electrocompetent C. autoethanogenum cells: The procedure for making C. autoethanogenum electro competent cells was modified from a protocol for C. ljungdahllii 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 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: All procedures were carried out in an anaerobic chamber. Electrocompetent C. autoethanogenum 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.
1,3-butadiene production from CO₂ and H₂ by gas fermentation with electron supply by electrode was performed using said genetically engineered *C. autoethanogenum* cells harboring the plasmid SG696. 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
ZnSO$_4 \times$ 7H$_2$O .................. 0.2 mg
CuCl$_2 \times$ 2H$_2$O .................. 20.0 mg
NiCl$_2 \times$ 6H$_2$O .................. 20.0 mg
Na$_2$MoO$_4 \times$ 2H$_2$O .......... 20.0 mg
Na$_2$SeO$_4$ ...................... 20.0 mg
Na$_2$WO$_4$ ......................... 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
Thioctic 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$_2$, 60% H$_2$
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, 60% 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. 1,3-butadiene production could be detected by GC-MS.

Alternative Embodiments:

Plasmid SG387 (Fig. 2 and SEQ listing No 1), Plasmid SG411 (Fig. 3 and SEQ listing No 2), Plasmid SG661 (Fig. 4 and SEQ listing No 3) and Plasmid SG696 (Fig. 5 and SEQ listing No 4) were also used to engineer Clostridium ljungdahlii and Clostridium autoethanogenum cells to produce 1,3-butadiene. 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 SG387
No. 2 - nucleotide sequence of plasmid SG411
No. 3 - nucleotide sequence of plasmid SG661
No. 4 - nucleotide sequence of plasmid SG696

Claims:

I claim:

1. Isolated, genetically engineered, anaerobic bacterial cells capable of producing 1,3-butadiene 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 1,3-butadiene 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) Geraniol isomerase/linalool dehydratase (EC 5.4.4.4 and EC 4.2.1.127)
   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) (R)-specific enoyl-CoA hydratase (EC 4.2.1.119)
   k) 4-aminobutyrate aminotransferase (EC 2.6.1.19)
   l) glutamate dehydrogenase (EC 1.4.1.2)
   m) 2-hydroxyglutarate dehydrogenase (EC 1.1.99.2)
   n) glutaconate CoA-transferase (EC 2.8.3.12)
o) 2-hydroxyglutaryl-CoA dehydratase (EC 4.2.1.-)

p) glutaconyl-CoA decarboxylase (EC 4.1.1.70)

q) 4-hydroxybutyrate dehydrogenase (EC 1.1.1.61)

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

s) vinylacetyl-CoA-Delta-isomerase (EC 4.2.1.120)

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

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 1,3-butadiene, 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 1,3-butadiene, and recovering the 1,3-butadiene from the bioreactor, wherein the anaerobic bacteria are genetically engineered to express one or more enzymes of one or more 1,3-butadiene pathways.

4. The process of claim 3, wherein the process further comprises recovering the 1,3-butadiene.

5. The process of claim 3 and 4, wherein the 1,3-butadiene 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, Clostridium autoethanogenum, Clostridium carboxidivorans, Clostridium aceticum, Clostridium drakei, Clostridium scatologenes, Clostridium ragsdalei, Clostridium formicoaceticum, Clostridium magnum, Butyribacterium methylophysicum, Acetobacterium woodii, Alkalibaculum bacillii,
Acetoarriaerobium riotera, Butyribacterium methylotrophicum, Blautia producta, Eubacterium limosum, Desulfitibacterium hafhierise, Moorella thermoacetica, Moorella thermautotrophica, Peptostreptococcus productus, Rhodospirillum rubrum, Sporomusa ovata, Sporomusa silvacetica, Sporomusa sphaeroides, Thermoanaerobacter kiiuvi, Oxobacter pfennigii, Acetobacterium fimetarium, Acetohalobium arabaticu, Blautia wexlerae, Carbophilus carboxidus, Cloacibacillus evyensis, Hydrogenophaga pseudoflava, Rhodopseudomonas palustris, Pseudomonas gazotropha, Ralstonia eutropha.

8. Isolated, genetically engineered, anaerobic bacterial cells capable of producing 1,3-butadiene 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 1,3-butadiene 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) Geraniol isomerase/linalool dehydratase (EC 5.4.4.4 and EC 4.2.1.127)

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) (R)-specific enoyl-CoA hydratase (EC 4.2.1.119)

k) 4-aminobutyrate aminotransferase (EC 2.6.1.19)

l) glutamate dehydrogenase (EC 1.4.1.2)

m) 2-hydroxyglutarate dehydrogenase (EC 1.1.99.2)
n) glutaconate CoA-transferase (EC 2.8.3.12)

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

p) glutaconyl-CoA decarboxylase (EC 4.1.1.70)

q) 4-hydroxybutyrate dehydrogenase (EC 1.1.1.61)

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

s) vinylacetyl-CoA-Delta-isomerase (EC 4.2.1.120)

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

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 1,3-butadiene, 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 1,3-butadiene, and recovering the 1,3-butadiene from the bioreactor, wherein the anaerobic bacteria are genetically engineered to express one or more enzymes of one or more 1,3-butadiene pathways.

11. The process of claim 10, wherein the process further comprises recovering the 1,3-butadiene.

12. The process of claim 10 and 11, wherein the 1,3-butadiene 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, Clostridium autoethanogenum,
Clostridium carboxidivorans, Clostridium aceticum, Clostridium drakei, Clostridium scatologenes, Clostridium ragsdalei, Clostridium formicoaceticum, Clostridium magnum, Butyribacterium methylotrophicum, Acetobacterium woodii, Alkalibaculum bacchii, Aecetoariaerobicium riotera, Butyribacterium methylotrophicum, Blautia producta, Eubacterium limosum, Desulfitbacterium hafnitrise, Moorella thermoacetica, Moorella thermautotrophica, Peptostreptococcus productus, Rhodospirillum rubrum, Sporomusa ovata, Sporomusa silvacetica, Sporomusa sphaeroides, Thermoanaerobacter kiiwi, Oxobacter pfennigii, Acetobacterium fimetarum, Acetohalobium arabaticu, Blautia wexlerae, Carbophilus carboxidus, Cloacibacillus evryensis, Hydrogenophaga pseudoflava, Rhodopseudomonas palustris, Pseudomonas gazotropha, Ralstonia eutropha.
Fig. 2

SG387
12647 bps

Fig. 3

SG411
15638 bps
Fig. 4

SG661
19082 bps

Fig. 5

SG696
24921 bps
Title of Invention: Genetically engineered microorganisms and process for converting gaseous C1-carbon sources and/or gaseous C1-carbon sources and electrons into 1,3-butadiene.

The application data sheet is part of the provisional or nonprovisional application for which it is being submitted. The following form contains the bibliographic data arranged in a format specified by the United States Patent and Trademark Office as outlined in 37 CFR 1.76. This document may be completed electronically and submitted to the Office in electronic format using the Electronic Filing System (EFS) or the document may be printed and included in a paper filed application.

Secrecy Order 37 CFR 5.2
☐ Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2 (Paper filers only. Applications that fall under Secrecy Order may not be filed electronically.)

Inventor Information:

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<tr>
<td>Bernhard</td>
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<tr>
<td>Residence Information (Select One)</td>
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Mailing Address of Inventor:

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All Inventors Must Be Listed - Additional Inventor Information blocks may be generated within this form by selecting the Add button.

Correspondence Information:

Enter either Customer Number or complete the Correspondence Information section below. For further information see 37 CFR 1.33(a).

☐ An Address is being provided for the correspondence Information of this application.

Customer Number: 121985
Email Address: Add Email Remove Email

Application Information:

<table>
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<th>Title of the Invention</th>
<th>Genetically engineered microorganisms and process for converting gaseous C1-carbon sources and/or gaseous C1-carbon sources and electrons into 1,3-butadiene.</th>
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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 1,3-butadiene.

Filing By Reference

Only complete this section when filing an application by reference under 35 U.S.C. 111(c) and 37 CFR 1.57(a). Do not complete this section if application papers including a specification and any drawings are being filed. Any domestic benefit or foreign priority information must be provided in the appropriate section(s) below (i.e., "Domestic Benefit/National Stage Information" and "Foreign Priority Information").

For the purposes of a filing date under 37 CFR 1.53(b), the description and any drawings of the present application are replaced by this reference to the previously filed application, subject to conditions and requirements of 37 CFR 1.57(a).

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Publication Information:

☐ Request Early Publication (Fee required at time of Request 37 CFR 1.219)

☐ Request Not to Publish. I hereby request that the attached application not be published under 35 U.S.C. 122(b) and certify that the invention disclosed in the attached application has not and will not be the subject of an application filed in another country, or under a multilateral international agreement, that requires publication at eighteen months after filing.

Representative Information:

Representative information should be provided for all practitioners having a power of attorney in the application. Providing this information in the Application Data Sheet does not constitute a power of attorney in the application (see 37 CFR 1.32). Either enter Customer Number or complete the Representative Name section below. If both sections are completed the customer Number will be used for the Representative Information during processing.

Please Select One: ☐ Customer Number ☐ US Patent Practitioner ☐ Limited Recognition (37 CFR 11.9)

Customer Number

Additional Representative Information blocks may be generated within this form by selecting the Add button.

Domestic Benefit/National Stage Information:

This section allows for the applicant to either claim benefit under 35 U.S.C. 119(e), 120, 121, or 365(c) or indicate National Stage entry from a PCT application. Providing this information in the application data sheet constitutes the specific reference required by 35 U.S.C. 119(e) or 120, and 37 CFR 1.78.

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Additional Domestic Benefit/National Stage Data may be generated within this form by selecting the Add button.
Title of Invention: Genetically engineered microorganisms and process for converting gaseous C1-carbon sources and/or gaseous C1-carbon sources and electrons into 1,3-butadiene.

Foreign Priority Information:

This section allows for the applicant to claim priority to a foreign application. Providing this information in the application data sheet constitutes the claim for priority as required by 35 U.S.C. 119(b) and 37 CFR 1.55(d). When priority is claimed to a foreign application that is eligible for retrieval under the priority document exchange program (PDX) the information will be used by the Office to automatically attempt retrieval pursuant to 37 CFR 1.55(h)(1) and (2). Under the PDX program, applicant bears the ultimate responsibility for ensuring that a copy of the foreign application is received by the Office from the participating foreign intellectual property office, or a certified copy of the foreign priority application is filed, within the time period specified in 37 CFR 1.55(g)(1).

Additional Foreign Priority Data may be generated within this form by selecting the Add button.

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications

This application (1) claims priority to or the benefit of an application filed before March 16, 2013 and (2) also contains, or contained at any time, a claim to a claimed invention that has an effective filing date on or after March 16, 2013.

NOTE: By providing this statement under 37 CFR 1.55 or 1.78, this application, with a filing date on or after March 16, 2013, will be examined under the first inventor to file provisions of the AIA.

Authorization to Permit Access:

Authorization to Permit Access to the Instant Application by the Participating Offices
## Application Data Sheet 37 CFR 1.76

| Title of Invention | Genetically engineered microorganisms and process for converting gaseous C1-carbon sources and/or gaseous C1-carbon sources and electrons into 1,3-butadiene. |

If checked, the undersigned hereby grants the USPTO authority to provide the European Patent Office (EPO), the Japan Patent Office (JPO), the Korean Intellectual Property Office (KIPO), the World Intellectual Property Office (WIPO), and any other intellectual property offices in which a foreign application claiming priority to the instant patent application is filed access to the instant patent application. See 37 CFR 1.14(c) and (h). This box should not be checked if the applicant does not wish the EPO, JPO, KIPO, WIPO, or other intellectual property office in which a foreign application claiming priority to the instant patent application is filed to have access to the instant patent application.

In accordance with 37 CFR 1.14(h)(3), access will be provided to a copy of the instant patent application with respect to: 1) the instant patent application-as-filed; 2) any foreign application to which the instant patent application claims priority under 35 U.S.C. 119(a)-(d) if a copy of the foreign application that satisfies the certified copy requirement of 37 CFR 1.55 has been filed in the instant patent application; and 3) any U.S. application-as-filed from which benefit is sought in the instant patent application.

In accordance with 37 CFR 1.14(c), access may be provided to information concerning the date of filing this Authorization.

## Applicant Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

### Applicant 1

If the applicant is the inventor (or the remaining joint inventor or inventors under 37 CFR 1.45), this section should not be completed. The information to be provided in this section is the name and address of the legal representative who is the applicant under 37 CFR 1.43; or the name and address of the assignee, person to whom the inventor is under an obligation to assign the invention, or person who otherwise shows sufficient proprietary interest in the matter who is the applicant under 37 CFR 1.46. If the applicant is an assignee under 37 CFR 1.46 (assignee, person to whom the inventor is obligated to assign, or person who otherwise shows sufficient proprietary interest) together with one or more joint inventors, then the joint inventor or inventors who are also the applicant should be identified in this section.

- [ ] Assignee
- [ ] Legal Representative under 35 U.S.C. 117
- [ ] Joint Inventor
- [ ] Person to whom the inventor is obligated to assign.
- [ ] Person who shows sufficient proprietary interest

If applicant is the legal representative, indicate the authority to file the patent application, the inventor is:

Name of the Deceased or Legally Incapacitated Inventor:

If the Applicant is an Organization check here.

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Additional Applicant Data may be generated within this form by selecting the Add button.

Assignee Information including Non-Applicant Assignee Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

Assignee 1

Complete this section if assignee information, including non-applicant assignee information, is desired to be included on the patent application publication. An assignee-applicant identified in the "Assignee Information" section will appear on the patent application publication as an assignee-applicant. For an assignee-applicant, complete this section only if identification as an assignee is also desired on the patent application publication.

If the Assignee or Non-Applicant Assignee is an Organization check here

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Mailing Address Information For Assignee including Non-Applicant Assignee:

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### Application Data Sheet 37 CFR 1.76

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### Signature:

**NOTE:** This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications.

<table>
<thead>
<tr>
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<th>/Bernhard Guentner/</th>
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<tbody>
<tr>
<td>First Name</td>
<td>Bernhard</td>
</tr>
<tr>
<td>Last Name</td>
<td>Guentner</td>
</tr>
<tr>
<td>Registration Number</td>
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