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(71) Applicant: **THE REGENTS OF THE UNIVERSITY OF CALIFORNIA** [US/US]; 1111 Franklin Street, 5th Floor, Oakland, California 94607-5200 (US).

(72) Inventors: **RIBBE, Markus Walter**; 5171 California Ave., Suite 150, University of California, Irvine, Irvine, California 92697-7700 (US). **HU, Yilin**; 5171 California Ave., Suite 150, University of California, Irvine, Irvine, California 92697-7700 (US). **REBELEIN, Johannes**; 5171 California Ave., Suite 150, University of California, Irvine, Irvine, California 92697-7700 (US).

(74) Agents: **EINHORN, Gregory P.** et al.; Greer Burns & Crain Ltd., 300 S. Wacker Drive #2500, Chicago, Illinois 60606 (US).

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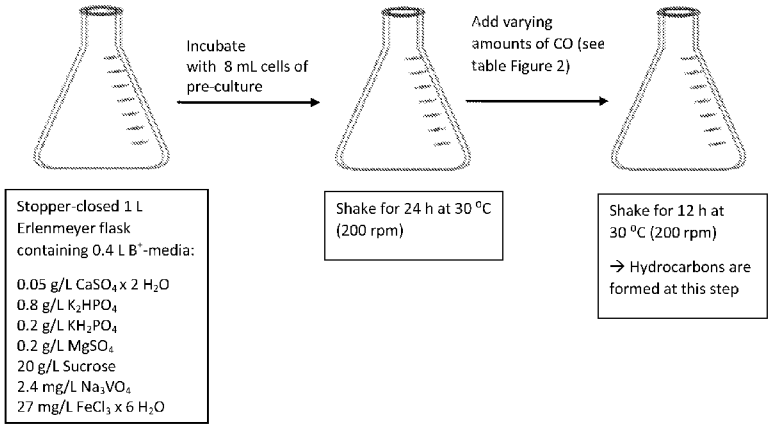
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(54) **Title**: RECOMBINANTLY ENGINEERED DIAZOTROPHS FOR WHOLE CELL HYDROCARBON PRODUCTION AND METHODS FOR MAKING AND USING THEM

Fig. 1



(57) **Abstract**: In alternative embodiments, provided are genetically or recombinantly engineered nitrogen-fixing, nitrogenase expressing bacteria capable of enzymatically synthesizing hydrocarbons, and methods for making and using them. In alternative embodiments, provided are genetically or recombinantly engineered nitrogen-fixing, nitrogenase expressing bacteria including nitrogen-fixing diazotrophs such as nitrogen-fixing bacteria of the family *Pseudomonadaceae*, or the genus *Azotobacter*, for the whole cell synthesis of hydrocarbons and carbon-carbon bonds. In alternative embodiments, nitrogen-fixing, nitrogenase-expressing bacteria used to practice the invention are genetically or recombinantly engineered to express an exogenous nitrogenase express more endogenous nitrogenase or have increased nitrogenase, activity. In alternative embodiments, nitrogen-fixing, nitrogenase-expressing bacteria used to practice the invention are genetically or recombinantly engineered to lack or have decreased molybdenum transporter activity. In alternative embodiments, provided are culture systems, fermenters and bioreactors using nitrogen-fixing, nitrogenase-expressing bacteria for enzymatically synthesizing hydrocarbons.

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RECOMBINANTLY ENGINEERED DIAZOTROPHS FOR WHOLE CELL HYDROCARBON PRODUCTION AND METHODS FOR MAKING AND USING THEM

RELATED APPLICATIONS

This application claims the benefit of priority to U.S. Provisional Patent Applications Serial No. (USSN) 62/093,955, filed December 18, 2014. The aforementioned application is expressly incorporated herein by reference in its entirety and for all purposes.

TECHNICAL FIELD

This invention generally relates to bacterial molecular and cellular biology, and biofuels. In alternative embodiments, provided are genetically or recombinantly engineered nitrogen-fixing, nitrogenase-expressing bacteria capable of enzymatically synthesizing hydrocarbons, and methods for making and using them. In alternative embodiments, provided are genetically or recombinantly engineered nitrogen-fixing, nitrogenase expressing bacteria including nitrogen-fixing diazotrophs such as nitrogen-fixing bacteria of the family *Pseudomonadaceae*, or the genus *Azotobacter*, including *Azotobacter vinelandii*, for the whole cell synthesis of hydrocarbons and carbon-carbon bonds. In alternative embodiments, nitrogen-fixing, nitrogenase-expressing (such as vanadium nitrogenase-expressing) bacteria used to practice embodiments as provided herein are genetically or recombinantly engineered to express an exogenous nitrogenase, e.g., vanadium nitrogenase, express more endogenous nitrogenase, e.g., vanadium nitrogenase, or have increased nitrogenase, e.g., vanadium nitrogenase, activity. In alternative embodiments, nitrogen-fixing, nitrogenase-expressing bacteria used to practice embodiments as provided herein are genetically or recombinantly engineered to lack or have decreased molybdenum transporter activity, for example, by deletion of molybdenum transporter genes or inhibition of molybdenum transporter expression. In alternative embodiments, provided are culture systems and bioreactors using nitrogen-fixing, nitrogenase-expressing bacteria provided herein for enzymatically synthesizing hydrocarbons.

BACKGROUND

Hydrocarbons such as propane, butane, and other alkanes and alkenes are in widespread use, both as fuels and as the precursors for many vital and necessary chemical compounds such as plastics, detergents, pharmaceuticals, etc. Currently the primary sources

of these hydrocarbons are fossil fuels, such as natural gas, from which they can be isolated. Such natural sources are, however, only available in limited supply, and retrieval and processing can have undesirable environmental impacts. In addition, the availability and pricing of such fossil fuels is greatly impacted by unpredictable political and social events.

Alternatives sources of such hydrocarbons have been developed, including the so-called Fischer-Tropsch synthesis. One version of this synthesis involves a series of reactions that uses hydrogen gas (H_2) to reduce carbon monoxide (CO) in the presence of a metal catalyst (such as cobalt-, nickel-, iron-, or ruthenium-based catalysts) to produce carbon-carbon bond containing alkanes ($C_nH_{(2n+2)}$) and water (H_2O). CO can be derived from a number of sources, including waste products of combustion of fossil fuels, natural gas, coal, and biomass. However, the efficiency of this synthesis is relatively low- generally only 25% to 50%. In addition, the process requires elevated temperatures and pressures in order to produce the desired range of alkane products, and careful control of reaction conditions and contaminants in the raw materials is necessary to avoid deactivation of the metal catalyst. As a result, while the Fischer-Tropsch synthesis has been developed for large scale production, it is not in widespread use, due in part to the high costs of reactor construction, operation, and maintenance.

In some instances it has been possible to replace such processes with the use of naturally occurring catalysts (e.g., enzymes) that can catalyze a wide variety of complex reactions at ambient temperatures and pressures, often at high efficiency. For example, monooxygenase enzymes have been used to catalyze oxidation of various hydrocarbons, such as ethylene, in the presence of oxygen to produce, for example, ethanol or ethylene oxide.

In another approach chemoautotrophic microorganisms, which are able to utilize inorganic carbon, are grown in a bioreactor using carbon dioxide (CO_2) as a carbon source. Growth of these bacteria provides a biomass that may then be dried and harvested for useful components, for instance lipids and fats can be extracted from dried biomass using solvents and after additional processing may subsequently be used as fuels. Reactor designs are, however, complex in order to accommodate the environmental requirements for chemoautotrophic bacteria. In addition, while this approach does provide reduction of inorganic carbon under relatively mild conditions the resulting product is a highly complex mixture of biomolecules that requires extensive processing in order to isolate useful compounds.

Thus, there is a need for a system and method that can provide reduction of inorganic carbon, such as CO and CO_2, to generate hydrocarbons such as alkanes and alkenes under mild conditions.

SUMMARY

In alternative embodiments, provided are nitrogen-fixing, nitrogenase-expressing diazotroph bacteria capable of enzymatically synthesizing hydrocarbons genetically or recombinantly engineered:

to lack or have decreased molybdenum transporter activity,

wherein optionally the lack or decreased molybdenum transporter activity is achieved by deletion of one or more, or all, molybdenum transporter genes in the bacteria, or inhibition of molybdenum transporter message (transcript) generation or expression, or inhibition of or decrease in molybdenum transporter protein expression,

wherein optionally the molybdenum transporter gene comprises an *Azotobacter vinelandii* modA1, or a molybdenum transporter-periplasmic molybdate-binding protein encoding gene, or a molybdenum ATP-Binding Cassette (ABC) transporter encoding gene,

and optionally the molybdenum transporter polypeptide comprises a molybdenum ATP-Binding Cassette (ABC) transporter protein,

and optionally the nitrogen-fixing, nitrogenase-expressing diazotroph bacteria comprise bacteria of the family *Pseudomonadaceae*, or the genus *Azotobacter*, or *Azotobacter vinelandii*,

and optionally the nitrogenase comprises a vanadium nitrogenase, or an *Azotobacter* vanadium nitrogenase or an *Azotobacter vinelandii* vanadium nitrogenase.

In alternative embodiments, the bacteria are genetically or recombinantly engineered to express an exogenous nitrogenase, to express more endogenous nitrogenase, or to have increased nitrogenase activity, or any combination thereof, and optionally the exogenous or endogenous nitrogenase comprises a vanadium nitrogenase, or an *Azotobacter* vanadium nitrogenase or an *Azotobacter vinelandii* vanadium nitrogenase, and optionally the exogenous nitrogenase and/or endogenous nitrogenase is modified (e.g., by site-directed or oligonucleotide-directed mutagenesis (including cassette mutagenesis, PCR site-directed mutagenesis, Cre-Lox recombination and whole plasmid mutagenesis), site-specific recombination, or mutagenesis *in vivo* or *in vitro*): to have increased enzymatic activity or velocity (Vmax); to be resistant to inhibition by ammonia; to have an enlarged active site to

allow the formation of larger hydrocarbons or to allow the formation of larger hydrocarbons at higher efficiency; or, any combination thereof,

and optionally the exogenous nitrogenase comprises an affinity tag, wherein optionally the affinity tag comprises: a CREB binding protein (CBP); an avidin or a streptavidin, a biotin or a desthiobiotin, a maltose binding protein (MBP); a glutathione-S-transferase (GST); a poly(His) tag; polyanionic amino acids; a FLAG-tag; or, any combination thereof.

In alternative embodiments, the nitrogen-fixing, nitrogenase-expressing diazotroph bacteria as provided herein can further comprise, or be constructed or engineered to have, deletion of or decreasing the activity of a gene or genes involved in the inhibition of nitrogenase by ammonia, wherein optionally the gene whose activity is deleted or decreased is Nitrogen fixation-L (NifL) (the gene *nifL* encodes the negative regulator NifL that is expressed in the presence of ammonia; NifL expression can lead to the inhibition of nitrogenase expression), and optionally further comprising overexpressing *nifA* (enhancement of NifA-mediated transcription by either elimination of *nifL* or overexpression of *nifA* can result in ammonium release, correlating with enhanced levels of *nifH* mRNA, and raised levels of nitrogenase).

In alternative embodiments, provided are methods for making a nitrogen-fixing, nitrogenase-expressing diazotroph bacteria capable of enzymatically synthesizing hydrocarbons, comprising genetically or recombinantly engineering the bacteria to lack or have decreased molybdenum transporter activity,

wherein optionally the lack or decreased molybdenum transporter activity is achieved by deletion of one or more, or all, molybdenum transporter genes in the bacteria, or inhibition of molybdenum transporter message (transcript) generation or expression, or inhibition of or decrease in molybdenum transporter protein expression,

wherein optionally the molybdenum transporter gene comprises an *Azotobacter vinelandii* modA1, or a molybdenum transporter-periplasmic molybdate-binding protein encoding gene, or a molybdenum ATP-Binding Cassette (ABC) transporter encoding gene,

and optionally the molybdenum transporter polypeptide comprises a molybdenum ATP-Binding Cassette (ABC) transporter protein,

and optionally the nitrogen-fixing, nitrogenase-expressing diazotroph bacteria comprise bacteria of the family *Pseudomonadaceae*, or the genus *Azotobacter*, or *Azotobacter vinelandii*,

and optionally the nitrogenase comprises a vanadium nitrogenase, or an *Azotobacter* vanadium nitrogenase or an *Azotobacter vinelandii* vanadium nitrogenase.

In alternative embodiments of methods as provided herein, the bacteria are genetically or recombinantly engineered to express an exogenous nitrogenase, to express more endogenous nitrogenase, or to have increased nitrogenase activity, or any combination thereof,

wherein optionally the exogenous or endogenous nitrogenase comprises a vanadium nitrogenase, or an *Azotobacter* vanadium nitrogenase or an *Azotobacter vinelandii* vanadium nitrogenase,

and optionally the exogenous nitrogenase and/or endogenous nitrogenase is modified (e.g., by site-directed or oligonucleotide-directed mutagenesis (including cassette mutagenesis, PCR site-directed mutagenesis, Cre-Lox recombination and whole plasmid mutagenesis), site-specific recombination, or mutagenesis *in vivo* or *in vitro*): to have increased enzymatic activity or velocity (Vmax); to be resistant to inhibition by ammonia; to have an enlarged active site to allow the formation of larger hydrocarbons or to allow the formation of larger hydrocarbons at higher efficiency; or, any combination thereof,

and optionally the exogenous nitrogenase comprises an affinity tag, wherein optionally the affinity tag comprises: a CREB binding protein (CBP); an avidin or a streptavidin, a biotin or a desthiobiotin, a maltose binding protein (MBP); a glutathione-S-transferase (GST); a poly(His) tag; polyanionic amino acids; a FLAG-tag; or, any combination thereof.

In alternative embodiments, methods as provided herein further comprise deletion of or decreasing the activity of a gene or genes involved in the inhibition of nitrogenase by ammonia, wherein optionally the gene whose activity is deleted or decreased is Nitrogen fixation-L (NifL), and optionally further comprising overexpressing *nifA*.

In alternative embodiments, provided are whole cell methods or processes for enzymatically synthesizing hydrocarbons, comprising contacting a nitrogen-fixing, nitrogenase-expressing diazotroph bacteria as provided herein, with a carbon-containing compound under conditions suitable to enzymatically form a carbon-carbon bond-comprising product compound, or a plurality of carbon-carbon bond-comprising product compounds, in the bacteria. In alternative embodiments, the carbon-containing compound comprises: a carbon monoxide (CO); a carbon dioxide (CO_2); or a C_2, C_3, C_4, or C_5 –comprising compound, and optionally the carbon-containing compound is input, or "fed" to the nitrogen-

fixing, nitrogenase-expressing diazotroph bacteria as a gas; or, the carbon-carbon bond-comprising product or products comprises or is a C_2, C_3, C_4, or C_5 product, or the carbon-carbon bond-comprising product comprises or contains at least one double bond, or the carbon-carbon bond-comprising product or products comprise or are methane, ethane, ethene, propane or butane.

In alternative embodiments of the methods, the contacting comprises conditions comprising high oxygen, low ammonia, or high oxygen and low ammonia. In alternative embodiments of the methods, the nitrogen-fixing, nitrogenase-expressing diazotroph bacteria are cultured, or in a cultured media, or in a bioreactor, and optionally the carbon-containing compound is input or "fed" to the culture, culture media or bioreactor as a gas.

In alternative embodiments, methods as provided herein further comprise harvesting, purifying and/ or isolating the carbon-carbon bond-comprising product compound.

In alternative embodiments, provided are bioreactors or fermenters comprising: a culture or a liquid system comprising a nitrogen-fixing, nitrogenase-expressing diazotroph bacteria as provided herein. In alternative embodiments, the bioreactors or fermenters as provided herein further comprise an inlet configured to provide a carbon-containing compound to the culture or liquid system in an amount effective to allow a nitrogenase in the nitrogen-fixing, nitrogenase-expressing diazotroph bacteria produce the carbon-carbon bond-comprising product compound, and optionally further comprising an outlet configured to remove the carbon-carbon bond-comprising product compound.

The details of one or more exemplary embodiments are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims.

All publications, patents, patent applications, GenBank sequences and ATCC deposits, cited herein are hereby expressly incorporated by reference for all purposes.

BRIEF DESCRIPTION OF THE DRAWINGS

The drawings set forth herein are illustrative of exemplary embodiments and are not meant to limit the scope of the invention as encompassed by the claims.

Fig. 1 schematically illustrates and exemplary process as provided herein.

Fig. 2 illustrates in graphic (Fig. 2B) and table form (Fig. 2A) a product analysis of an exemplary method as provided herein by GC-MS analysis. The experiments were performed with naturally-abundant ^{12}CO (upper panels) and with isotope-labeled ^{13}CO (lower panels)

and the masses of products in these experiments were subsequently analyzed by GC-MS. The GC-MS analysis shows that the carbon atoms in the hydrocarbon products originate from the carbon atoms of CO molecules, thereby demonstrating hydrocarbon formation from CO using exemplary genetically modified bacterial cells provided herein, i.e., genetically modified whole cells of *A. vinelandii* that express vanadium nitrogenase.

Like reference symbols in the various drawings indicate like elements.

Reference will now be made in detail to various exemplary embodiments as provided herein, examples of which are illustrated in the accompanying drawings. The following detailed description is provided to give the reader a better understanding of certain details of aspects and embodiments as provided herein, and should not be interpreted as a limitation on the scope of the invention.

DETAILED DESCRIPTION

In alternative embodiments, provided are genetically or recombinantly engineered nitrogen-fixing bacteria capable of enzymatically synthesizing hydrocarbons, and methods for making and using them. In alternative embodiments, provided are genetically or recombinantly engineered nitrogen-fixing bacteria including nitrogen-fixing diazotrophs such as nitrogen-fixing bacteria of the family *Pseudomonadaceae*, or the genus *Azotobacter*, including *Azotobacter vinelandii*, for the whole cell synthesis of hydrocarbons and carbon-carbon bonds. In alternative embodiments, provided are culture systems and bioreactors using nitrogen-fixing, nitrogenase-expressing bacteria as provided herein for enzymatically synthesizing hydrocarbons.

In alternative embodiments, nitrogen-fixing, nitrogenase-expressing diazotroph bacteria used to practice embodiments as provided herein are genetically or recombinantly engineered to express an exogenous nitrogenase, to express more endogenous nitrogenase, or to have increased nitrogenase activity, or any combination thereof. In alternative embodiments, the exogenous or endogenous nitrogenase comprises a vanadium nitrogenase, or an *Azotobacter* vanadium nitrogenase or an *Azotobacter vinelandii* vanadium nitrogenase. In alternative embodiments, enzymatically active fragments of nitrogenases, e.g., vanadium nitrogenases, are used, e.g., as exogenous sources of nitrogenase enzyme activity. In alternative embodiments, methods and devices (e.g., bioreactors or fermenters) as provided herein employ use or genetically or recombinantly engineered vanadium nitrogenases, vanadium nitrogenase fragments, vanadium nitrogenase isoforms, genetically modified

vanadium nitrogenase, and/or metal complexes derived from vanadium nitrogenase alone or in combination to produce hydrocarbons and/or to form carbon-carbon bonds.

Nitrogenases

In alternative embodiments, provided are nitrogen-fixing, nitrogenase-expressing diazotroph bacteria capable of enzymatically synthesizing hydrocarbons genetically or recombinantly engineered to lack or have decreased molybdenum transporter activity. In alternative embodiments, exogenous nitrogenases are added to a bacterial cell to supplement endogenous nitrogenase activity or the provide nitrogenase activity. In alternative embodiments, endogenous and/or exogenous nitrogenases are genetically or recombinantly engineered: to have increased enzymatic activity or velocity (Vmax); to be resistant to inhibition by ammonia; to have an enlarged active site to allow the formation of larger hydrocarbons or to allow the formation of larger hydrocarbons at higher efficiency; or, any combination thereof.

Any nitrogenases can be used to embodiments as provided herein, including vanadium-containing nitrogenases and molybdenum-containing nitrogenases, and nitrogenases not containing these or any metals. For example, in alternative embodiments, nitrogenases used to practice embodiments as provided herein comprise use of an Fe protein, which hydrolyzes ATP to drive the reaction, and a VFe protein, where reaction with a substrate molecule (which in nature is N_2) takes place. Nitrogenases used to practice embodiments as provided herein also can contain a number of metal complexes or metal clusters; and the Fe protein can contain an Fe_4S_4 iron-sulfur complex, while the VFe protein can contain both an iron-sulfur P cluster near the area of interaction with the Fe protein and a vanadium-iron (VFe) cofactor complex at the active site. Molybdenum containing nitrogenases also can be used to practice embodiments as provided herein, and they have a similar structure but have a molybdenum-iron (MoFe) cofactor complex in place of a VFe cofactor. Nitrogenases containing neither vanadium or molybdenum and utilizing iron-iron (FeFe) cofactors can be used to practice embodiments as provided herein.

In alternative embodiments, mutated forms of nitrogenases used to practice embodiments as provided herein have different forms of these metal complexes and may lack a functional cofactor at the active site. While N_2 is the best known substrate for these enzymes, some nitrogenases used to practice embodiments as provided herein exhibit catalytic activities other than reduction of nitrogen. For example, nitrogenases used to practice embodiments as provided herein also comprise enzymes able to convert acetylene

into ethylene, carbonyl sulfide into CO and H_2S, CO_2 into CO and H_2O, and H^+ into H_2. In some embodiments, H_2 is a competitive inhibitor, CO is a non-competitive inhibitor, for an exemplary Mo-containing nitrogenase.

Antisense inhibitory molecules

In alternative embodiments, provided are nitrogen-fixing, nitrogenase-expressing diazotroph bacteria capable of enzymatically synthesizing hydrocarbons that are genetically or recombinantly engineered to lack or have decreased molybdenum transporter activity.

In alternative embodiments, to decrease molybdenum transporter activity, provided are methods for using antisense inhibitory molecules comprising a sequence used to target either a sense or an antisense strand of a molybdenum transporter activity protein-expressing genes, e.g., such as *Azotobacter vinelandii* modA1, or a molybdenum transporter-periplasmic molybdate-binding protein encoding gene, or a molybdenum ATP-Binding Cassette (ABC) transporter encoding genes or transcripts. Naturally occurring or synthetic nucleic acids can be used as antisense oligonucleotides. The antisense oligonucleotides can be of any length; for example, in alternative aspects, the antisense oligonucleotides are between about 5 to 100, about 10 to 80, about 15 to 60, about 18 to 40. The optimal length can be determined by routine screening. The antisense oligonucleotides can be present at any concentration. The optimal concentration can be determined by routine screening. A wide variety of synthetic, non-naturally occurring nucleotide and nucleic acid analogues are known which can address this potential problem. For example, peptide nucleic acids (PNAs) containing non-ionic backbones, such as N-(2-aminoethyl) glycine units can be used. Antisense oligonucleotides having phosphorothioate linkages can also be used, as described in WO 97/03211; WO 96/39154; Mata (1997) Toxicol Appl Pharmacol 144:189-197; Antisense Therapeutics, ed. Agrawal (Humana Press, Totowa, NJ., 1996). Antisense oligonucleotides having synthetic DNA backbone analogues can also include phosphoro-dithioate, methylphosphonate, phosphoramidate, alkyl phosphotriester, sulfamate, 3'-thioacetal, methylene(methylimino), 3'-N-carbamate, and morpholino carbamate nucleic acids, as described above.

RNA interference (RNAi)

In one aspect, an RNA inhibitory molecule, a so-called "RNAi" molecule, comprising a sequence is used to practice embodiments as provided herein, e.g., a sequence inhibitory to molybdenum transporter activity protein-expressing genes or transcripts. In alternative embodiments, the RNAi molecule comprises a double-stranded RNA (dsRNA) molecule. The RNAi molecule can comprise a double-stranded RNA (dsRNA) molecule, e.g., siRNA,

miRNA (microRNA), an artificial micro RNA, and/or short hairpin RNA (shRNA) molecules. The RNAi molecule, e.g., siRNA (small inhibitory RNA), miRNA, or an artificial micro RNA, can inhibit expression of a molybdenum transporter activity protein-expressing gene or transcript.

In alternative aspects, the RNAi is about 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or more duplex nucleotides in length. While the invention is not limited by any particular mechanism of action, the RNAi can enter a cell and cause the degradation of a single-stranded RNA (ssRNA) of similar or identical sequences, including endogenous mRNAs. When a cell is exposed to double-stranded RNA (dsRNA), mRNA from the homologous gene is selectively degraded by a process called RNA interference (RNAi). A possible basic mechanism behind RNAi, e.g., siRNA for inhibiting transcription and/or miRNA to inhibit translation, is the breaking of a double-stranded RNA (dsRNA) matching a specific gene sequence into short pieces called short interfering RNA, which trigger the degradation of mRNA that matches its sequence. In one aspect, the RNAi 's are used in gene-silencing therapeutics, see, e.g., Shuey (2002) Drug Discov. Today 7:1040-1046. In one aspect, provided are methods to selectively degrade RNA using the RNAi 's. The process may be practiced in vitro, ex vivo or in vivo. In one aspect, the RNAi molecules used to practice embodiments as provided herein generate a loss-of-function mutation (of a molybdenum transporter activity protein-expressing gene or protein) in the bacteria.

In alternative embodiments, intracellular introduction of the RNAi (e.g., miRNA, artificial micro RNA or siRNA) is by internalization of a target cell specific ligand bonded to an RNA binding protein comprising an RNAi (e.g., microRNA) is adsorbed. The ligand is specific to a unique target cell surface antigen. The ligand can be spontaneously internalized after binding to the cell surface antigen. If the unique cell surface antigen is not naturally internalized after binding to its ligand, internalization can be promoted by the incorporation of an arginine-rich peptide, or other membrane permeable peptide, into the structure of the ligand or RNA binding protein or attachment of such a peptide to the ligand or RNA binding protein. See, e.g., U.S. Patent App. Pub. Nos. 20060030003; 20060025361; 20060019286; 20060019258. In one aspect, provided are lipid-based formulations for delivering, e.g., introducing nucleic acids as nucleic acid-lipid particles comprising an RNAi molecule to a cell, see e.g., U.S. Patent App. Pub. No. 20060008910.

In alternative embodiments, methods for making and using RNAi molecules, e.g., siRNA, artificial micro RNA and/or miRNA, for selectively degrade RNA include, e.g., U.S. Patent No. 6,506,559; 6,511,824; 6,515,109; 6,489,127.

In alternative embodiments, known and routine methods for making expression constructs, e.g., vectors or plasmids, from which an inhibitory polynucleotide (e.g., a duplex siRNA) is transcribed are used. A regulatory region (e.g., promoter, enhancer, silencer, splice donor, acceptor, etc.) can be used to transcribe an RNA strand or RNA strands of an inhibitory polynucleotide from an expression construct. When making a duplex siRNA (e.g., to molybdenum transporter activity protein-expressing gene) inhibitory molecule, the sense and antisense strands of the targeted portion of the targeted IRES can be transcribed as two separate RNA strands that will anneal together, or as a single RNA strand that will form a hairpin loop and anneal with itself.

For example, in alternative embodiments, a construct targeting a portion of molybdenum transporter activity protein-expressing gene is inserted between two promoters (e.g., two plant, viral, bacteriophage T7 or other promoters) such that transcription occurs bidirectionally and will result in complementary RNA strands that may subsequently anneal to form an inhibitory siRNA. Alternatively, a targeted portion of molybdenum transporter activity protein-expressing gene can be designed as a first and second coding region together on a single expression vector, wherein the first coding region of the targeted gene is in sense orientation relative to its controlling promoter, and wherein the second coding region of the gene is in antisense orientation relative to its controlling promoter. If transcription of the sense and antisense coding regions of the targeted portion of the targeted gene occurs from two separate promoters, the result may be two separate RNA strands that may subsequently anneal to form a gene or inhibitory siRNA, e.g., molybdenum transporter activity protein-expressing gene-inhibitory siRNA used to practice embodiments as provided herein.

In alternative embodiments, transcription of the sense and antisense targeted portion of the targeted nucleic acid, e.g., molybdenum transporter activity protein-expressing gene, is controlled by a single promoter, and the resulting transcript can be a single hairpin RNA strand that is self-complementary, e.g., forms a duplex by folding back on itself to create a (e.g., molybdenum transporter activity protein-expressing gene) -inhibitory siRNA molecule. In this configuration, a spacer, e.g., of nucleotides, between the sense and antisense coding regions of the targeted portion of the targeted (e.g., molybdenum transporter activity protein-expressing) gene can improve the ability of the single strand RNA to form a hairpin loop,

wherein the hairpin loop comprises the spacer. In one embodiment, the spacer comprises a length of nucleotides of between about 5 to 50 nucleotides. In one aspect, the sense and antisense coding regions of the siRNA can each be on a separate expression vector and under the control of its own promoter.

Inhibitory Ribozymes

In alternative embodiments, provided are ribozymes capable of binding molybdenum transporter activity protein-expressing genes, coding sequences or messages. These ribozymes can inhibit gene activity by, e.g., targeting mRNA.

Strategies for designing ribozymes and selecting the gene specific antisense sequence for targeting are well described in the scientific and patent literature, and the skilled artisan can design such ribozymes using the reagents and sequences used to practice embodiments as provided herein.

Ribozymes act by binding to a target RNA through the target RNA binding portion of a ribozyme which is held in close proximity to an enzymatic portion of the RNA that cleaves the target RNA. Thus, the ribozyme recognizes and binds a target RNA through complementary base-pairing, and once bound to the correct site, acts enzymatically to cleave and inactivate the target RNA. Cleavage of a target RNA in such a manner will destroy its ability to direct synthesis of an encoded protein if the cleavage occurs in the coding sequence. After a ribozyme has bound and cleaved its RNA target, it can be released from that RNA to bind and cleave new targets repeatedly.

Bioreactors, Culture Systems, and Fermenters

In alternative embodiments, provided are culture systems, bioreactors and fermenters comprising and comprising use of nitrogen-fixing, nitrogenase-expressing diazotroph bacteria as provided herein, for e.g., enzymatically synthesizing hydrocarbons.

In alternative embodiments, the various devices used to practice embodiments as provided herein (e.g., culture systems, bioreactors and fermenters) comprise an inlet configured to provide a carbon-containing compound to the culture or liquid system in an amount effective to allow a nitrogenase in the nitrogen-fixing, nitrogenase-expressing diazotroph bacteria produce the carbon-carbon bond-comprising product compound. In alternative embodiments, the various devices as provided herein further comprise an outlet configured to remove the carbon-carbon bond-comprising product compound.

In alternative embodiments, the various devices provided herein are manufactured or configured to comprise, culture and/or hold a liquid (e.g., a culture media) with exemplary nitrogen-fixing, nitrogenase-expressing diazotroph bacteria provided herein.

In alternative embodiments, the various devices provided herein are manufactured or configured to comprise an inlet that permits a carbon-containing compound to be introduced into the liquid in at a rate and/or amount that is effective in providing the nitrogenase with sufficient starting material for the formation of a hydrocarbon, i.e., the carbon-carbon bond-comprising product compound. In alternative embodiments, the various devices provided herein are manufactured or configured to comprise an outlet that permits removal of the hydrocarbon product, e.g., the carbon-carbon bond-comprising product compound.

In alternative embodiments, the various devices provided herein are manufactured or configured such the nitrogen-fixing, nitrogenase-expressing diazotroph bacteria provided herein are immobilized on a surface, e.g., a semi-solid or a solid surface, which may be conductive.

In alternative embodiments, devices provided herein, or devices used to practice embodiments of methods provided herein, including exemplary culture systems, bioreactors and fermenters, can be designed based on or comprise components of or can be practiced or used as described by, e.g., U.S. Patent Nos. 8,895,291 (describing e.g., closed cell expansion systems); 8,889,400 (describing e.g., bioreactor systems using gaseous exhausts comprising e.g., carbon monoxide); 8,865,460 (describing e.g., multi-chambered cell co-culture systems); 8,852,933 (describing e.g., flexible, deformable, chambers suitable for seeding and growing cells); 8,852,925 (describing e.g., bioreactors and fermenters comprising three-dimensional matrices, e.g., made of a hydrogel material); 8,852,923 (describing e.g., tissue conditioning bioreactor modules); 8,835,159 (describing e.g., static solid state bioreactors); 8,828,692 (describing e.g., membrane supported bioreactors for conversion of syngas components such as carbon monoxide to liquid products); 8,518,691 (describing e.g., horizontal array bioreactors for conversion of syngas components to liquid products), and 8,222,026 (describing e.g., stacked array bioreactors for conversion of syngas components to liquid products).

A number of exemplary embodiments have been described. Nevertheless, it can be understood that various modifications may be made without departing from the spirit and scope of the invention. Accordingly, other embodiments are within the scope of the following claims.

WHAT IS CLAIMED IS:

1. A nitrogen-fixing, nitrogenase-expressing diazotroph bacteria capable of enzymatically synthesizing hydrocarbons genetically or recombinantly engineered:

to lack or have decreased molybdenum transporter activity,

wherein optionally the lack or decreased molybdenum transporter activity is achieved by deletion of one or more, or all, molybdenum transporter genes in the bacteria, or inhibition of molybdenum transporter message (transcript) generation or expression, or inhibition of or decrease in molybdenum transporter protein expression,

wherein optionally the molybdenum transporter gene comprises an *Azotobacter vinelandii* modA1, or a molybdenum transporter-periplasmic molybdate-binding protein encoding gene, or a molybdenum ATP-Binding Cassette (ABC) transporter encoding gene,

and optionally the molybdenum transporter polypeptide comprises a molybdenum ATP-Binding Cassette (ABC) transporter protein,

and optionally the nitrogen-fixing, nitrogenase-expressing diazotroph bacteria comprise bacteria of the family *Pseudomonadaceae*, or the genus *Azotobacter*, or *Azotobacter vinelandii*,

and optionally the nitrogenase comprises a vanadium nitrogenase, or an *Azotobacter* vanadium nitrogenase or an *Azotobacter vinelandii* vanadium nitrogenase.

2. The nitrogen-fixing, nitrogenase-expressing diazotroph bacteria of claim 1, wherein the bacteria are genetically or recombinantly engineered to express an exogenous nitrogenase, to express more endogenous nitrogenase, or to have increased nitrogenase activity, or any combination thereof,

wherein optionally the exogenous or endogenous nitrogenase comprises a vanadium nitrogenase, or an *Azotobacter* vanadium nitrogenase or an *Azotobacter vinelandii* vanadium nitrogenase,

and optionally the exogenous nitrogenase and/or endogenous nitrogenase is modified (e.g., by site-directed or oligonucleotide-directed mutagenesis (including cassette mutagenesis, PCR site-directed mutagenesis, Cre-Lox recombination and whole plasmid mutagenesis), site-specific recombination, or mutagenesis *in vivo* or *in vitro*): to have increased enzymatic activity or velocity (Vmax); to be resistant to inhibition by ammonia; to have an enlarged active site to allow the formation of larger hydrocarbons or to allow the formation of larger hydrocarbons at higher efficiency; or, any combination thereof,

and optionally the exogenous nitrogenase comprises an affinity tag, wherein optionally the affinity tag comprises: a CREB binding protein (CBP); an avidin or a streptavidin, a biotin or a desthiobiotin, a maltose binding protein (MBP); a glutathione-S-transferase (GST); a poly(His) tag; polyanionic amino acids; a FLAG-tag; or, any combination thereof.

3. The nitrogen-fixing, nitrogenase-expressing diazotroph bacteria of claim 1, further comprising deletion of or decreasing the activity of a gene or genes involved in the inhibition of nitrogenase by ammonia, wherein optionally the gene whose activity is deleted or decreased is Nitrogen fixation-L (NifL),

and optionally further comprising overexpressing *nifA*.

4. A method for making a nitrogen-fixing, nitrogenase-expressing diazotroph bacteria capable of enzymatically synthesizing hydrocarbons, comprising genetically or recombinantly engineering the bacteria to lack or have decreased molybdenum transporter activity,

wherein optionally the lack or decreased molybdenum transporter activity is achieved by deletion of one or more, or all, molybdenum transporter genes in the bacteria, or inhibition of molybdenum transporter message (transcript) generation or expression, or inhibition of or decrease in molybdenum transporter protein expression,

wherein optionally the molybdenum transporter gene comprises an *Azotobacter vinelandii* modA1, or a molybdenum transporter-periplasmic molybdate-binding protein encoding gene, or a molybdenum ATP-Binding Cassette (ABC) transporter encoding gene,

and optionally the molybdenum transporter polypeptide comprises a molybdenum ATP-Binding Cassette (ABC) transporter protein,

and optionally the nitrogen-fixing, nitrogenase-expressing diazotroph bacteria comprise bacteria of the family *Pseudomonadaceae*, or the genus *Azotobacter*, or *Azotobacter vinelandii*,

and optionally the nitrogenase comprises a vanadium nitrogenase, or an *Azotobacter* vanadium nitrogenase or an *Azotobacter vinelandii* vanadium nitrogenase.

5. The method of claim 4, wherein the bacteria are genetically or recombinantly engineered to express an exogenous nitrogenase, to express more endogenous nitrogenase, or to have increased nitrogenase activity, or any combination thereof,

wherein optionally the exogenous or endogenous nitrogenase comprises a vanadium nitrogenase, or an *Azotobacter* vanadium nitrogenase or an *Azotobacter vinelandii* vanadium nitrogenase,

and optionally the exogenous nitrogenase and/or endogenous nitrogenase is modified (e.g., by site-directed or oligonucleotide-directed mutagenesis (including cassette mutagenesis, PCR site-directed mutagenesis, Cre-Lox recombination and whole plasmid mutagenesis), site-specific recombination, or mutagenesis *in vivo* or *in vitro*): to have increased enzymatic activity or velocity (Vmax); to be resistant to inhibition by ammonia; to have an enlarged active site to allow the formation of larger hydrocarbons or to allow the formation of larger hydrocarbons at higher efficiency; or, any combination thereof,

and optionally the exogenous nitrogenase comprises an affinity tag, wherein optionally the affinity tag comprises: a CREB binding protein (CBP); an avidin or a streptavidin, a biotin or a desthiobiotin, a maltose binding protein (MBP); a glutathione-S-transferase (GST); a poly(His) tag; polyanionic amino acids; a FLAG-tag; or, any combination thereof.

6. The method of claim 4, further comprising deletion of or decreasing the activity of a gene or genes involved in the inhibition of nitrogenase by ammonia, wherein optionally the gene whose activity is deleted or decreased is Nitrogen fixation-L (NifL),

and optionally further comprising overexpressing *nifA*.

7. A whole cell method for enzymatically synthesizing hydrocarbons, comprising contacting a nitrogen-fixing, nitrogenase-expressing diazotroph bacteria of any of claims 1 to 4, with a carbon-containing compound under conditions suitable to enzymatically form a carbon-carbon bond-comprising product compound, or a plurality of carbon-carbon bond-comprising product compounds, in the bacteria.

8. The whole cell method of claim 7, wherein the carbon-containing compound comprises: a carbon monoxide (CO); a carbon dioxide (CO_2); or a C_2, C_3, C_4, or C_5 –

comprising compound, and optionally the carbon-containing compound is input, or "fed" to the nitrogen-fixing, nitrogenase-expressing diazotroph bacteria as a gas.

9. The whole cell method of claim 7, wherein the carbon-carbon bond-comprising product or products comprises or is a C_2, C_3, C_4, or C_5 product, or the carbon-carbon bond-comprising product comprises or contains at least one double bond, or the carbon-carbon bond-comprising product or products comprise or are methane, ethane, ethene, propane or butane.

10. The whole cell method of any of claims 7 to 9, wherein the contacting comprises conditions comprising high oxygen, low ammonia, or high oxygen and low ammonia.

11. The whole cell method of any of claims 7 to 10, wherein the nitrogen-fixing, nitrogenase-expressing diazotroph bacteria are cultured, or in a cultured media, or in a bioreactor, and optionally the carbon-containing compound is input or "fed" to the culture, culture media or bioreactor as a gas.

12. The whole cell method of any of claims 7 to 11, further comprising harvesting, purifying and/ or isolating the carbon-carbon bond-comprising product compound.

13. A bioreactor or fermenter comprising: a culture or a liquid system comprising a nitrogen-fixing, nitrogenase-expressing diazotroph bacteria of any of claims 1 to 4.

14. The bioreactor or fermenter of claim 13, further comprising an inlet configured to provide a carbon-containing compound to the culture or liquid system in an amount effective to allow a nitrogenase in the nitrogen-fixing, nitrogenase-expressing diazotroph bacteria produce the carbon-carbon bond-comprising product compound, and optionally further comprising an outlet configured to remove the carbon-carbon bond-comprising product compound.

Fig. 1

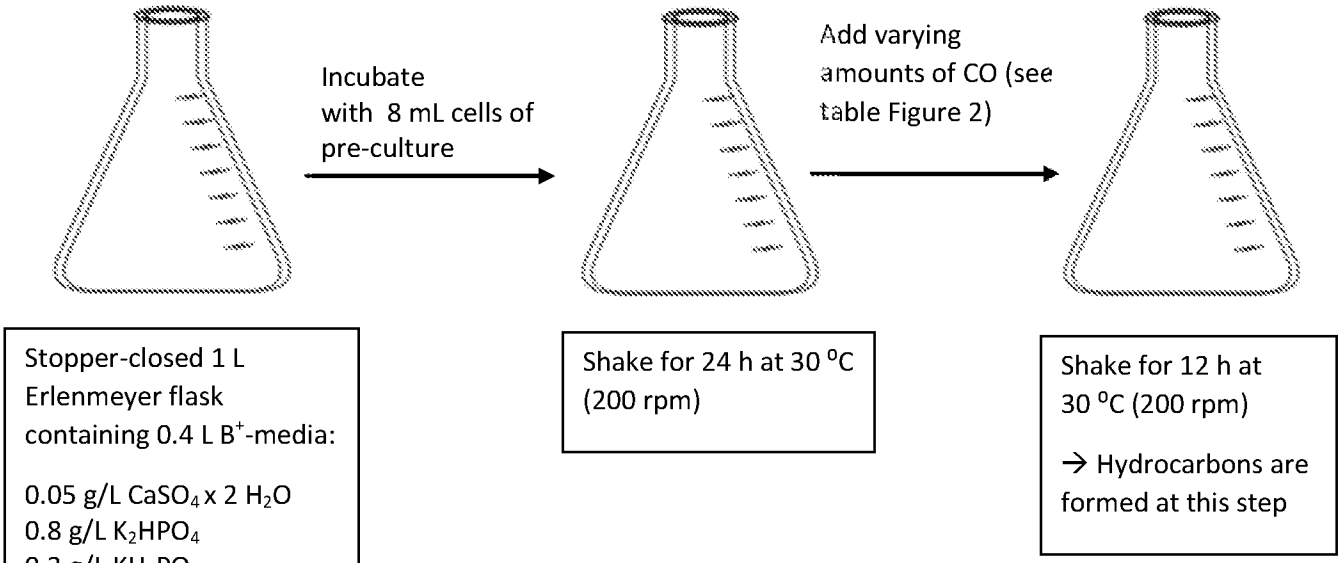
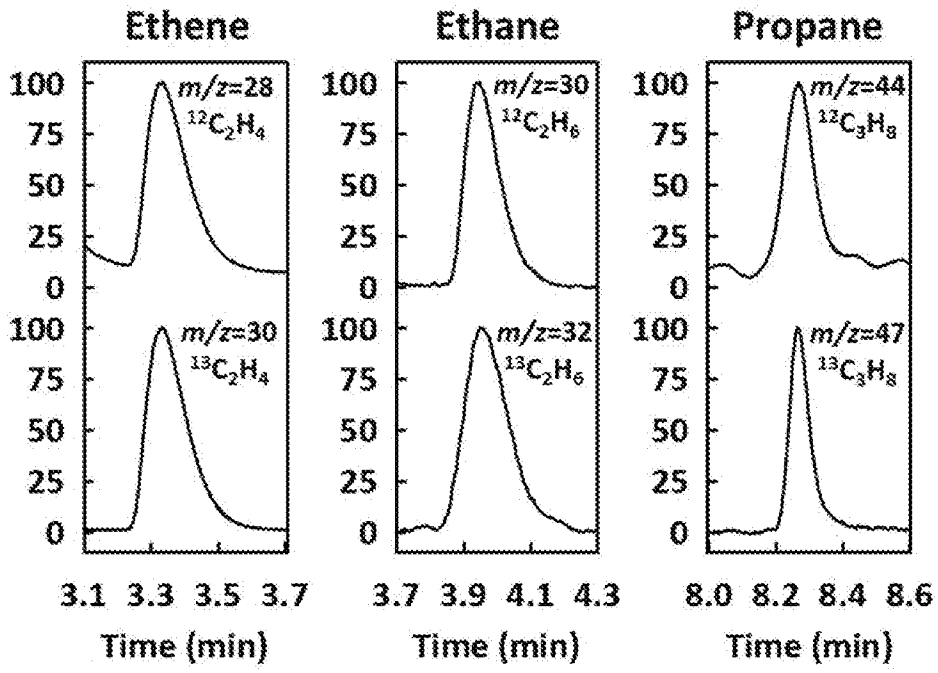


Fig. 2A

Product	Methane (CH_4)	Ethene (C_2H_4)	Ethane (C_2H_6)	Propane (C_3H_8)	Total
Amount of CO added (%)	(mol CH_4/ mol enzyme)	(mol C_2H_4/ mol enzyme)	(mol C_2H_6/ mol enzyme)	(mol C_3H_8/ mol enzyme)	Estimated total turnover per enzyme
0	0	0	0	0	0
2	9.84	15.204	0.72	0	25.77
5	**16.88**	**217.77**	**8.56**	**2.626**	**245.83**
7.5	**21.03**	**224.50**	**8.58**	**2.415**	**256.53**
10	**17.81**	**226.96**	**8.67**	**1.892**	**255.35**
20	**24.10**	**171.61**	**7.03**	**1.933**	**204.68**
30	**29.25**	**113.72**	**4.71**	**2.086**	**149.78**
40	**23.66**	**60.74**	**2.70**	**0.059**	**87.17**
50	21.05	18.26	1.10	0.431	40.85

Fig. 2B


Ethene
Ethane
Propane
m/z=28
12C2H4
m/z=30
13C2H4
m/z=30
12C2H6
m/z=32
13C2H6
m/z=44
12C3H8
m/z=47
13C3H8
100
75
50
25
0
3.1 3.3 3.5 3.7
3.7 3.9 4.1 4.3
8.0 8.2 8.4 8.6
Time (min)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/066481

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12Q 1/04 (2016.01)
CPC - C12Q 1/04 (2016.02)
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - A01G 7/00; C07K 14/195; C12N 1/02, 1/20, 1/21, 15/52, 15/74, 15/90; C12P 3/00; C12Q 1/02, 1/04 (2016.01)
CPC - C07K 14/195; C12N 15/52, 15/743, 15/90; C12Q 1/04 (2016.02)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC- 435/29, 168, 252.1 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Orbit, Google Patents, Google Scholar
Search terms used: Azotobacter vinelandii vanadium nitrogenase diazotroph hydrocarbon production "nitrogen fixation" "carbon fixation" nifA nifL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	EP 0339830 A2 (BIOTECHNICA INTERNATIONAL, INC.) 02 November 1989 (02.11.1989) entire document	1-3, 7, 13 --- 5, 6, 8, 9, 14
X --- Y	MOUNCEY et al. "Mutational analysis of genes of the mod locus involved in molybdenum transport, homeostasis, and processing in Azotobacter vinelandii". Journal of bacteriology. 1995 Sep 1;177(18):5294-302 (01.09.1995) entire document	4 --- 5, 6
Y	US 5,096,819 A (PAGE et al) 17 March 1992 (17.03.1992) entire document	8, 9, 14
A	US 2009/0042236 A1 (BISHOP et al) 12 February 2009 (12.02.2009) entire document	1-9, 13, 14
A	BOTHE et al. "Nitrogen fixation and hydrogen metabolism in cyanobacteria". Microbiology and Molecular Biology Reviews. 2010 Dec 1;74(4):529-51 (01.12.2010) entire document	1-9, 13, 14
A	FISHER et al. "Azotobacter vinelandii vanadium nitrogenase: formaldehyde is a product of catalyzed HCN reduction, and excess ammonia arises directly from catalyzed azide reduction". Biochemistry. 2006 Apr 4;45(13):4190-8 (04.04.2006) entire document	1-9, 13, 14
A	HERNANDEZ et al. "Molybdenum Trafficking for Nitrogen Fixation". Biochemistry. 2009 Oct 20;48(41):9711. (20.10.2009) entire document	1-9, 13, 14

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 February 2016	04 MAR 2016
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Authorized officer Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/066481

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 10-12
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.