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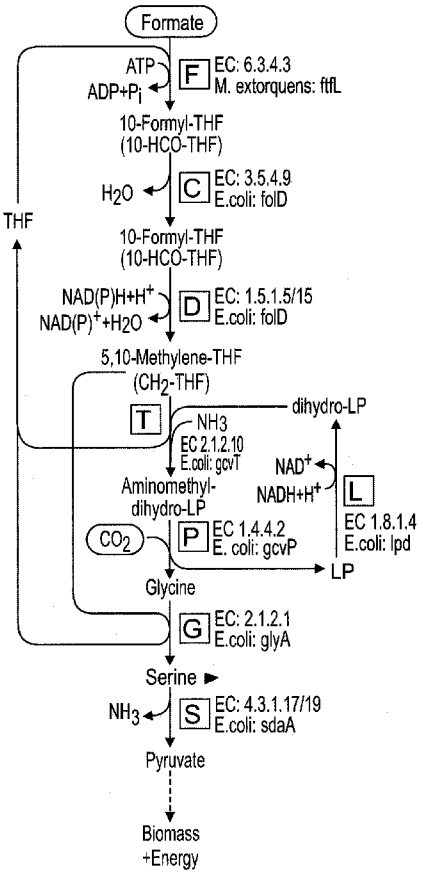
(54) Title: USE OF THE REDUCTIVE GLYCINE PATHWAY FOR GENERATING FORMATOTROPHIC AND AUTOTROPHIC MICROORGANISMS

FIG. 1



(57) Abstract: An isolated microorganism that expresses enzymes of the reductive glycine pathway is disclosed. The microorganism is capable of converting formate to pyruvate or glycerate via the formation of glycine and serine. Methods of generating same are further described.

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USE OF THE REDUCTIVE GLYCINE PATHWAY FOR GENERATING FORMATOTROPHIC AND AUTOTROPHIC MICROORGANISMS

FIELD AND BACKGROUND OF THE INVENTION

The present invention, in some embodiments thereof, relates to the use of the reductive glycine pathway for the generation of formatotrophic and autotrophic microorganisms.

The concept of biorefineries has become a wide spread notion in the last decade. It relies on the premise that living organisms can and should be used to supply the increasing demand by humanity for specialized chemicals, including fuels, solvents, plastics, pharmaceuticals, etc. Today, most of these chemicals are derived, directly or indirectly, from fissile carbons. However, with the imminent depletion of these fossil carbons and the increase in atmospheric CO_2 it has become essential to find alternative sources for these important materials.

The suggested feedstocks for most of the proposed biorefineries are simple sugars, starch, or lingocellulosic biomass. While the latter alternative has an apparent advantage over the former by not-competing with human consumption needs, it still presents numerous difficulties, including a problematic fermentation technology and feedstock availability and transportation. A fascinating alternative feedstock would be electric current. Electrons can be shuttled from an electrode to living cells, providing the necessary reducing equivalents and energy to support autotrophic growth and electrosynthesis of desired commodities (1-5). Since electricity is widely available, microbial electrosynthesis can be spatially and temporally decoupled from energy production and can take place at any convenient location and time.

Microbial electrosynthesis can be especially useful for the renewable energy market. One major drawback of most renewable energy sources, including solar, wind, hydro and nuclear, is that they are hard to store in a convenient way. Microbial electrosynthesis of fuels can thus serve to address this problem efficiently, converting electrical energy to kinetically stable chemical bonds.

Several methods of transferring reducing equivalents from an electrode to living cells were suggested and applied (reviewed in 1-5). Molecular hydrogen is one of the earliest electron carriers used in this manner since water electrolysis is a relatively

mature technology that can support efficient hydrogen production at high current density. However, the use of hydrogen suffers from numerous problems including its low solubility and the risk of explosion. Moreover, the hydrogenase enzymes that transfer hydrogen's electrons to the cellular carriers are generally complex, oxygen sensitive proteins, which are hard to recombinantly express and consume a significant fraction of the cell resources. As an alternative to molecular hydrogen, several inorganic compounds, such as ferric ion or nitrate, can serve as electron shuttles, supporting electricity-dependent cultivation (5). However, since the reduction potentials of these compounds are considerably higher than that of NAD(P)H, reverse electron flow must take place during growth on these substrates, limiting electrosynthesis to specific organisms which are less suitable to industrial use. A further option is direct electron transfer from the cathode to the microbes. While several advantages of this option were proposed (reviewed in 2-5) this approach is limited to a small group of organisms or requires complex adaption of others.

As an alternative to all of the above methods, CO_2 can be directly reduced at the cathode (the electrons are derived from water splitting at the anode) (6), providing organic compounds that can be used by living cells as a source of reducing equivalents, energy and even carbon. A diverse group of compounds can be produced in this manner (6-9). The production of simple alcohols, such as methanol, ethanol and propanol, hydrocarbons, such as methane and ethylene, or acids with more than one carbon, such as acetic acid and oxalic acid, has the advantage of supplying microbes with compounds relatively simple to metabolize and/or being rich in reducing equivalents. However, the electrocatalytic production of all of these compounds is generally inefficient (not specific to a single product and/or requiring high overpotential), requiring costly catalysts and/or supporting low current density (reviewed in 6, 7). In contrast, there are two compounds that can be produced by direct reduction of CO_2 at relatively high efficiency (although lower than that of molecular hydrogen) and an acceptable current density: carbon monoxide and formic acid (6-13). Since carbon monoxide is a toxic and flammable gas with low solubility, formic acid, being readily soluble and of low toxicity, is a preferred mediator of electrons. In fact, a formate-based economy was recently proposed as an alternative to the hydrogen-based economy or methanol-based economy concepts (14-17).

Various methylotrophic organisms can grow on formate as a sole carbon, electron and energy source (18-21). Such organisms can be used for formate-dependent microbial electrosynthesis (19, 22). However, as compared to model organisms extensively used in the bioindustry, such as *S. cerevisiae* or *E. coli*, the metabolism of these microbes is far less understood, their bulk cultivation is limited and their genetic manipulation is considerably less optimized. As a consequence, biotechnological usage of these natural methylotrophs is usually limited to the production of simple products.

SUMMARY OF THE INVENTION

According to an aspect of some embodiments of the present invention there is provided an isolated microorganism that expresses enzymes of the reductive glycine pathway, wherein the microorganism is capable of converting formate to pyruvate or glycerate via the formation of glycine and serine.

According to an aspect of some embodiments of the present invention there is provided an isolated microorganism that expresses enzymes of the reductive glycine pathway, wherein the microorganism is capable of converting formate to a metabolite of central metabolism via the formation of glycine and without the formation of serine, the metabolite being selected from the group consisting of acetyl CoA, oxaloacetate and glycerate 2/3-phosphate.

According to an aspect of some embodiments of the present invention there is provided a method of generating a microorganism comprising expressing in the microorganism at least one enzyme of the reductive glycine pathway, such that the microorganism is capable of converting formate to pyruvate or glycerate via the formation of glycine and serine.

According to an aspect of some embodiments of the present invention there is provided a system for culturing the microorganism described herein and an electrode for providing electrons to generate formate.

According to an aspect of some embodiments of the present invention there is provided a method of selecting the microorganism described herein comprising:

growing a microorganism on formate; and

analyzing for the production of metabolites of the reductive glycine pathway and/or activity of the enzymes of the reductive glycine pathway in the microorganism,

wherein a production of the metabolites of the reductive glycine pathway and an activity of the enzymes of the reductive glycine pathway above a predetermined level is indicative of a formatotrophic microorganism.

According to an aspect of some embodiments of the present invention there is provided a method of generating a biofuel comprising culturing the microorganism described herein under conditions that allow for biofuel formation, thereby generating the biofuel.

According to an aspect of some embodiments of the present invention there is provided a method of generating a human polypeptide comprising culturing the microorganism described herein under conditions that allow for expression of the human polypeptide, thereby generating the human polypeptide.

According to some embodiments of the invention, the isolated microorganism is genetically modified.

According to some embodiments of the invention, the microorganism is genetically modified to express at least one of the enzymes of the reductive glycine pathway.

According to some embodiments of the invention, the microorganism is formatotrophic.

According to some embodiments of the invention, the microorganism further expresses a formate dehydrogenase which is capable of reducing carbon dioxide to formic acid.

According to some embodiments of the invention, the microorganism is autotrophic.

According to some embodiments of the invention, the microorganism is phototrophic.

According to some embodiments of the invention, the microorganism is chemotrophic.

According to some embodiments of the invention, the microorganism is aerobic.

According to some embodiments of the invention, the microorganism does not express EC 2.1.2.1.

According to some embodiments of the invention, the microorganism is anaerobic.

According to some embodiments of the invention, the microorganism is a bacteria.

According to some embodiments of the invention, the microorganism is a gram positive bacteria.

According to some embodiments of the invention, the microorganism is a gram negative bacteria.

According to some embodiments of the invention, the bacteria is selected from the group consisting of Agrobacterium, Alicyclobacillus, Anabaena, Anacystis, Arthrobacter, Azobacter, Bacillus, Brevibacterium, Chromatium, Clostridium, Corynebacterium, Enterobacter, Erwinia, Escherichia, Lactobacillus, Lactococcus, Mesorhizobium, Methylobacterium, Microbacterium, Phormidium, Pseudomonas, Rhodobacter, Rhodopseudomonas, Rhodospirillum, Rhodococcus, Salmonella, Scenedesmun, Serratia, Shigella, Staphlococcus, Strepromyces, Synnecoccus, and Zymomonas.

According to some embodiments of the invention, the bacteria comprises Escherichia.

According to some embodiments of the invention, the Escherichia are genetically modified to express a first enzyme NAD-dependent formate dehydrogenase which is capable of oxidizing formate to carbon dioxide and a second enzyme formate-tetrahydrofolate ligase.

According to some embodiments of the invention, the Escherichia are genetically modified to further express bifunctional methenyltetrahydrofolate-cyclohydrolase-NAD-dependent-methylenetetrahydrofolate-dehydrogenase.

According to some embodiments of the invention, the Escherichia are genetically modified to further express at least one glycine cleavage system enzyme.

According to some embodiments of the invention, the Escherichia are genetically modified to further express serine hydroxymethyltransferase and/or serine deaminase.

According to some embodiments of the invention, the microorganism is a yeast.

According to some embodiments of the invention, the yeast comprises *S.cervavisciae*.

According to some embodiments of the invention, the microorganism is a fungi.

According to some embodiments of the invention, the fungi is selected from Aspergillus, Candida, Chlamydomonas, Chrysosporium, Cryotococcus, Fusarium, Kluyveromyces, Neotyphodium, Neurospora, Penicillium, Pichia, Saccharomyces, Trichoderma and Xanthophyllomyces.

According to some embodiments of the invention, the microorganism is an algae.

According to some embodiments of the invention, the formatotrophic microorganism is genetically modified to express a human polypeptide.

According to some embodiments of the invention, the human polypeptide is selected from the group consisting of an antibody, insulin, interferon, growth hormone, erythropoietin, growth hormone, follicle stimulating hormone, factor VIII, low density lipoprotein receptor (LDLR) alpha galactosidase A and glucocerebrosidase.

According to some embodiments of the invention, the microorganism is capable of producing a biofuel.

According to some embodiments of the invention, the biofuel is selected from the group consisting of ethanol, propanol, isobutanol and n-butanol.

According to some embodiments of the invention, the biofuel is selected from the group consisting of an alcohol, an alkene, an alkane, a lipid or a polysaccharide.

According to some embodiments of the invention, the microorganism is formatotrophic.

According to some embodiments of the invention, the microorganism is autotrophic.

According to some embodiments of the invention, the method further comprises selecting the microorganism that is formatotrophic by growing the microorganism on formate following the expressing.

According to some embodiments of the invention, the method further comprises selecting the microorganism that is autotrophic by growing the microorganism on carbon dioxide in the presence of an external electron source following the expressing.

According to some embodiments of the invention, the method further comprises analyzing for the production of metabolites of the reductive glycine pathway and/or activity of the enzymes of the reductive glycine pathway in the microorganism.

According to some embodiments of the invention, the method further comprises culturing the microorganism following the generating.

According to some embodiments of the invention, the culturing is effected in a presence of an electrical current so as to generate the formate.

According to some embodiments of the invention, when the analyzing is for the production of metabolites, the method is effected by performing a pulse chase experiment.

According to some embodiments of the invention, the method further comprises analyzing for an expression of the enzymes of the reductive glycine pathway.

According to some embodiments of the invention, the method further comprises collecting the biofuel.

According to some embodiments of the invention, the method further comprises isolating the human polypeptide.

Unless otherwise defined, all technical and/or scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of embodiments of the invention, exemplary methods and/or materials are described below. In case of conflict, the patent specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and are not intended to be necessarily limiting.

BRIEF DESCRIPTION OF THE DRAWINGS

Some embodiments of the invention are herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of embodiments of the invention. In this regard, the description taken with the drawings makes apparent to those skilled in the art how embodiments of the invention may be practiced.

In the drawings:

FIG. 1 is a diagram illustrating the reductive glycine pathway, producing pyruvate.

FIG. 2 is a diagram illustrating the reductive glycine pathway, producing glycerate.

FIG. 3 is a diagram illustrating the reductive glycine pathway, in which glycine – and not serine – is assimilated into central metabolism

DESCRIPTION OF SPECIFIC EMBODIMENTS OF THE INVENTION

The present invention, in some embodiments thereof, relates to the use of the reductive glycine pathway for the generation of microorganisms and, more particularly, but not exclusively, to formatotrophic and autotrophic microorganisms.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not necessarily limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways.

Electrosynthesis has recently received much attention for being a promising approach for use of a renewable energy for the production of commodities by living cells. Several techniques were proposed to mediate the transfer of electrons from the cathode to living cells. Of these, the electroproduction of formate as a mediator seems to be especially interesting: formate is readily soluble, of low toxicity and can be produced at high efficiency and at reasonable current density.

There are numerous metabolic pathways that, once expressed in a microorganism, can potentially support formatotrophic growth. The present inventors applied diverse computational methods to analyze and compare these pathways according to various criteria including biomass yield, thermodynamic favorability, chemical motive force, kinetics and expression challenges and found that the reductive glycine pathway, composed of the tetrahydrofolate system, the glycine cleavage system, serine hydroxymethyltransferase and serine deaminase, displays superior characteristics and is the most promising candidate to mediate electrosynthesis using bacteria, and more specifically, *E. coli* as a host.

While organisms that are capable of formatotrophic growth, i.e. growth on formate, exist naturally, they are generally less suitable for metabolic engineering and bulk cultivation. Further, there is no indication that any organism uses this pathway to support methylotrophic or autotrophic growth. Microorganisms that do use the

reductive glycine pathway as shown in Figure 1, use it to generate an electron sink, recycling reduced electron carriers that are generated during the fermentation of purines and amino acids (23).

Thus, according to one aspect of the present invention there is provided an isolated microorganism that expresses enzymes of the reductive glycine pathway, wherein the microorganism is capable of converting formate to pyruvate or glycerate via the formation of glycine and serine.

As used herein, the term "microorganism" refers to any organism of microscopic size. Non-limiting examples of microorganisms as the term is used herein include both prokaryotic and eukaryotic microorganisms, such as bacteria, protozoan, fungi, molds, yeasts, algae etc. The microorganism may be aerobic or anaerobic.

The term "isolated" as used herein refers to a microorganism that is at least partially separated from the natural environment e.g., from other microorganisms that are not capable of using formate as a carbon, reducing power and energy source (e.g. purified or semi-purified). Contemplated populations of microorganisms are ones which are enriched for the microorganism described herein, e.g. wherein at least 30 % thereof comprise the microorganism of the present invention, at least 40 % thereof comprise the microorganism of the present invention, at least 50 % thereof comprise the microorganism of the present invention, at least 60 % thereof comprise the microorganism of the present invention, at least 70 % thereof comprise the microorganism of the present invention, at least 80 % thereof comprise the microorganism of the present invention, at least 90 % thereof comprise the microorganism of the present invention, at least 95 % thereof comprise the microorganism of the present invention.

The organisms can be fermentative organisms. Exemplary microorganisms include, for example, Clostridium (e.g., C. acetobutylicum, C. Beijerinckii, C. saccharoperbutylacetonicum, C. saccharobutylicum, C. aurantibutyricum, C. tetanomorphum), Zymomonas, Escherichia (e.g., E. coli), Salmonella, Rhodococcus, Pseudomonas, Bacillus, Lactobacillus, Enterococcus, Alcaligenes, Klebsiella, Paenibacillus, Arthrobacter, Corynebacterium, Brevibacterium, Pichia, Candida, Hansenula, Zymomonas and Saccharomyces, e.g., Saccharomyces cerevisiae, Saccharomyces carlsbergensis, Kluyveromyces lactis, Saccharomyces lactis.

Bacteria may be gram positive or gram negative. Examples of bacteria which are contemplated by the present invention include, but are not limited to Agrobacterium, Alicyclobacillus, Anabaena, Anacystis, Arthrobacter, Azobacter, Bacillus, Brevibacterium, Chromatium, Clostridium, Corynebacterium, Enterobacter, Erwinia, Escherichia, Lactobacillus, Lactococcus, Mesorhizobium, Methylobacterium, Microbacterium, Phormidium, Pseudomonas, Rhodobacter, Rhodopseudomonas, Rhodospirillum, Rhodococcus, Salmonella, Scenedesmun, Serratia, Shigella, Staphlococcus, Strepromyces, Synnecoccus, and Zymomonas.

Examples of fungi contemplated by the present invention include, but are not limited to Aspergillus, Candida, Chlamydomonas, Chrysosporium, Cryotococcus, Fusarium, Kluyveromyces, Neotyphodium, Neurospora, Penicillium (e.g. *P. chrysogenum*), Pichia, Saccharomyces, Trichoderma and Xanthophyllomyces.

Examples of algae contemplated by the present invention include, but are not limited to a diatom or a cyanobacterium.

The diatom may be a microalgae of the class Coscinodiscophyceae, Fragilariophyceae or Bacillariophyceae.

The cyanobacterium can include, for example, Botryococcus braunii, Chlorella, Dunaliella tertiolecta, Gracilaria, Pleurochrysis carterae, Sargassum or Ulva.

The microorganism may be methylotrophic - i.e. is capable of growing on organic C1 compounds as their sole carbon, reducing power and energy source.

According to a particular embodiment, the methylotrophic microorganism is formatotrophic – i.e. uses formate as a carbon, reducing power and energy source. It will be appreciated that the formatotrophic organisms of the present invention may also be capable of growing on additional carbon sources – such as glucose, glycerol cellulose, acetate, butyrate, lactate, propionate, or valerate.

According to a particular embodiment, the formatotrophic uses formate as its sole carbon source.

According to another embodiment, the microorganism is autotrophic. One type of autotrophic microorganism is a phototrophic organism (one which requires light to get the reducing power and energy for carbon dioxide fixation). The electron source for photosynthesis may be for example, water, hydrogen sulfide, elemental sulfur or ferrous ion (Fe^{2+}).

Another type of autotrophic microorganism is a chemotrophic microorganism (one which requires an external electron source [e.g. molecular hydrogen, carbon monoxide (CO), hydrogen sulfide (H_2S), elemental sulfur (S), sulfite (SO_3^{2-}), phosphite (PO_3^{2-}), ammonia (NH_4^+), nitrite (NO_2^{2-}), ammonium hydroxide (NH_2OH), ferrous ion (Fe^{2+}), Mn^{2+} ion] to get the reducing power and energy for carbon dioxide fixation). Possible terminal electron acceptors include molecular oxygen, carbon dioxide (CO_2), sulfate (SO_4^{2-}), elemental sulfur (S), nitrate (NO_3^{2-}), ferric ion (Fe^{3+}).

As mentioned the microorganisms of the present invention express, either naturally or are genetically engineered so as to express, enzymes of the reductive glycine pathway such that the microorganism is capable of converting formate to pyruvate via the formation of glycine and serine (Figure 1) or is capable of converting formate to glycerate via the formation of glycine and serine (Figure 2). A further alternative is using the enzymes of the reductive glycine pathway such that the microorganism is capable of converting formate to acetyl-CoA, glycerate or oxaloacetate via the formation of glycine, but without the formation of serine (Figure 3).

The microorganism of embodiments of the present invention may use the enzyme serine deaminase for the conversion of the serine (generated via the reductive glycine pathway, Figure 1) to pyruvate. Alternatively, the microorganism of embodiments of the present invention may use a serine transaminase enzyme (or serine dehydrogenase) and hydroxypyruvate reductase (or hydroxypyruvate isomerase and tartronate semialdehyde reductase) for the conversion of the serine (generated via the reductive glycine pathway) to glycerate (Figure 2). Alternatively, the microorganism of embodiments of the present invention may directly assimilate glycine into central metabolism without producing serine, as shown in Figure 3.

Figure 1 illustrates the enzymes of the reductive glycine pathway in which the product is pyruvate. The pathway is composed of the tetrahydrofolate system, the glycine cleavage system, serine hydroxymethyltransferase and serine deaminase.

Thus, the microorganisms of the present invention may express formate tetrahydrofolate ligase (EC 6.3.4.3, *THFS*); methenyltetrahydrofolate cyclohydrolase (EC 3.5.4.9, *folD*); methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5/15, *folD*); aminomethyltransferase (EC 2.1.2.10, *gcvT*); dihydrolipoyl dehydrogenase (EC1.8.1.4, *lpd*); glycine dehydrogenase (decarboxylating; EC 1.4.4.2, *gcvP*); serine

hydroxymethyltransferase (EC2.1.2.1, *glyA*); and L-serine deaminase (EC 4.3.1.17, *sdaA*).

According to a particular embodiment the microorganism is capable of converting formate to pyruvate via an activity of serine deaminase or the microorganism is capable of converting formate to glycerate via an activity of a serine deaminase enzyme (*e.g.* EC 2.6.1.45, 2.6.1.51) and hydroxypyruvate reductase (EC 1.1.1.82 or 1.1.1.29).

The methenyltetrahydrofolate cyclohydrolase and methylenetetrahydrofolate dehydrogenase enzymes can be replaced by a bifunctional enzyme methenyltetrahydrofolate cyclohydrolase/methylenetetrahydrofolate dehydrogenase. Further, the enzyme formate tetrahydrofolate ligase and the bifunctional enzyme methenyltetrahydrofolate cyclohydrolase/methylenetetrahydrofolate dehydrogenase can be replaced by a trifunctional enzyme that carry formyl-THF synthethase, methenyltetrahydrofolate cyclohydrolase and methylenetetrahydrofolate dehydrogenase activities.

It will be appreciated that the enzyme formate tetrahydrofolate ligase can be bypassed by operating enzymes whose net reaction is identical to that of formate tetrahydrofolate ligase (for example, the sequential operation of *purT* (formate-dependent glycinamide ribonucleotide transformylase) in the forward direction and *PurN* (formyl-THF-dependent glycinamide ribonucleotide transformylase) in the reverse direction; another example is the sequential operation of *purP* (formate-dependent aminoimidazole carboxamide ribonucleotide transformylase) in the forward direction and *PurH* (formyl-THF-dependent aminoimidazole carboxamide ribonucleotide transformylase) in the reverse direction).

According to a particular embodiment the H-protein of the glycine cleavage system may also be expressed. In addition, the enzymes converting octanoic acid to lipoic acid attached to the H-protein (*e.g.* LipA, LipB and/or LplA) may also be expressed.

It will be appreciated that for the generation of formatotrophic microorganisms, expression of a formate deyhydrogenase (EC 1.2.1.2 or 1.2.1.43; for example GenBank CAB54834.1 – SEQ ID NO: 12) which is capable of oxidizing formate to obtain carbon dioxide is also necessary. The electrons which are released serve as the reducing power

required for the operation of the reductive glycine pathway and in order to generate ATP (*e.g.* via oxidative phosphorylation).

For the generation of autotrophic microorganisms, expression of a formate dehydrogenase which is capable of reducing carbon dioxide to formic acid is also necessary (EC 1.2.1.2, 1.2.1.43 or 1.1.99.33; for example GenBank AAB18330.2 (SEQ ID NO: 13) and AAB18329.1 (SEQ ID NO: 14).

Additional enzymes may also be expressed in the autotrophic microorganisms depending on the external electron source. Thus, for example if the electron source is hydrogen, the present invention contemplates expression of a hydrogenase enzyme. Further details on expression of hydrogenase in E. coli cells can be found in [24, 25].

Figure 2 illustrates the enzymes of the reductive glycine pathway in which the product is glycerate.

Thus, the microorganisms of this aspect of the present invention may express formate tetrahydrofolate ligase (EC 6.3.4.3, *THFS*); methenyltetrahydrofolate cyclohydrolase (EC 3.5.4.9, *folD*); methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5/15, *folD*); aminomethyltransferase (EC 2.1.2.10, *gcvT*); dihydrolipoyl dehydrogenase (EC 1.8.1.4, *lpd*); serine hydroxymethyltransferase (EC2.1.2.1, *glyA*); serine dehydrogenase (EC2.6.1.45) or serine-glyoxylate transaminase (EC 2.6.1.51) or any other transaminase enzyme (EC 2.6.1X); and hydroxypyruvate reductase (EC 1.1.1.26/19/79/81) or hydroxypyruvate isomerase (EC 5.3.1.22) + tartronate semialdehyde reductase (EC 1.1.1.60).

Figure 3 illustrates the enzymes of the reductive glycine pathway in which the products are metabolites of central metabolism. In these microorganisms, the enzyme serine hydroxymethyltransferase (EC2.1.2.1, *glyA*) is not expressed.

Thus, the microorganisms of this aspect of the present invention may express formate tetrahydrofolate ligase (EC 6.3.4.3, *THFS*); methenyltetrahydrofolate cyclohydrolase (EC 3.5.4.9, *folD*); methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5/15, *folD*); aminomethyltransferase (EC 2.1.2.10, *gcvT*); dihydrolipoyl dehydrogenase (EC1.8.1.4, *lpd*). When acetyl-CoA is produced as the central metabolite, the microorganism expresses the enzyme glycine reductase (EC 1.4.1.10) and phosphate acetyltransferase EC 2.3.1.8. Alternatively, glycine may be converted to glyoxylate and before being assimilated into central metabolism (as shown in Figure 3).

This requires the expression of glycine dehydrogenase (EC 1.4.1.10) or a transaminase (EC 2.6.1.51). When oxaloacetate is produced as the metabolite, the microorganism expresses enzymes EC 4.1.3.14 and 4.3.1.20. Alternatively, glycine may be converted to oxaloacetate via the reductive TCA cycle and glyoxylate shunt (as shown in Figure 3). When glycerate 2-phosphate or glycerate 3-phosphate are produced, enzymes EC4.1.1.47, EC1.1.1.60 and EC 2.7.1.31/165 are expressed. As shown in Figure 3, instead of EC1.1.1.60, the enzymes EC 5.3.1.22 and EC 1.1.1.26/29/79/81 may be expressed.

The term "enzyme" as used herein refers to a "catalytically functional biomolecule," which includes both whole native (or native-size) molecules and derivatives (e.g. genetic modifications) thereof.

Thus an enzyme of the present invention also refers to homologs and other modifications including additions or deletions of specific amino acids to the sequence (e.g., polypeptides which are at least 50 %, at least 55 %, at least 60 %, at least 65 %, at least 70 %, at least 75 %, at least 80 %, at least 85 %, at least 87 %, at least 89 %, at least 91 %, at least 93 %, at least 95 % or more say 100 % homologous to the lysomal amino acid sequences listed in Table 1, herein below as determined using BlastP software of the National Center of Biotechnology Information (NCBI) using default parameters). The homolog may also refer to an ortholog, a deletion, insertion, or substitution variant, including an amino acid substitution, thereof and biologically active polypeptide fragments thereof.

Table 1

Enzyme	***Examples of protein GenBank accession***
formate tetrahydrofolate ligase (EC 6.3.4.3);	ABY28836.1 - SEQ ID NO: 1
Bifunctional methenyltetrahydrofolate cyclohydrolase/methylenetetrahydrofolate dehydrogenase (EC 3.5.4.9 and EC 1.5.1.5/15, *folD*)	NP_006627.2 - SEQ ID NO: 2
aminomethyltransferase (EC	AAN81933.1 - SEQ ID NO: 3

2.1.2.10, *gcvT*); and.	
dihydrolipoyl dehydrogenase (EC 1.8.1.4, *lpd*)	ZP_03035757.1 - SEQ ID NO: 4
glycine dehydrogenase (decarboxylating; EC 1.4.4.2, *gcvP*)	YP_005276541.1 - SEQ ID NO: 5
serine hydroxymethyltransferase (EC 2.1.2.1, *glyA*)	ZP_03034169.1 - SEQ ID NO: 6
L-serine deaminase; EC 4.3.1.17, *sdaA*)	AFH11608.1 - SEQ ID NO: 7
serine-pyruvate transaminase (EC 2.6.1.51)	YP_004171157.1 - SEQ ID NO: 8
hydroxypyruvate reductase (EC 1.1.1.81, ghrA)	ZP_04001524.1 - SEQ ID NO: 9
serine-glyoxylate transaminase (EC 2.6.1.45)	YP_004370315.1 - SEQ ID NO: 10
serine dehydrogenase (EC 1.4.1.7)	BAB07807.1 - SEQ ID NO: 11

Nucleic acid sequences encoding the enzymes of some embodiments of the invention may be optimized for expression for a particular microorganism. Examples of such sequence modifications include, but are not limited to, an altered G/C content to more closely approach that typically found in the microorganism species of interest, and the removal of codons atypically found in the microorganism species commonly referred to as codon optimization.

The phrase "codon optimization" refers to the selection of appropriate DNA nucleotides for use within a structural gene or fragment thereof that approaches codon usage within the microorganism of interest. Therefore, an optimized gene or nucleic acid sequence refers to a gene in which the nucleotide sequence of a native or naturally occurring gene has been modified in order to utilize statistically-preferred or statistically-favored codons within the microorganism. The nucleotide sequence typically is examined at the DNA level and the coding region optimized for expression in the microorganism species determined using any suitable procedure, for example as

described in Sardana *et al.* (1996, Plant Cell Reports 15:677-681). In this method, the standard deviation of codon usage, a measure of codon usage bias, may be calculated by first finding the squared proportional deviation of usage of each codon of the native gene relative to that of highly expressed genes, followed by a calculation of the average squared deviation. The formula used is: 1 SDCU = n = 1 N [(Xn - Yn) / Yn] 2 / N, where Xn refers to the frequency of usage of codon n in highly expressed genes, where Yn to the frequency of usage of codon n in the gene of interest and N refers to the total number of codons in the gene of interest.

One method of optimizing the nucleic acid sequence in accordance with the preferred codon usage for a particular cell type is based on the direct use, without performing any extra statistical calculations, of codon optimization tables such as those provided on-line at the Codon Usage Database through the NIAS (National Institute of Agrobiological Sciences) DNA bank in Japan (worldwidewebdotkazusadotordotjp/codon/). The Codon Usage Database contains codon usage tables for a number of different species, with each codon usage table having been statistically determined based on the data present in Genbank.

By using the above tables to determine the most preferred or most favored codons for each amino acid in a particular species (for example, E. coli), a naturally-occurring nucleotide sequence encoding a protein of interest can be codon optimized for that particular species. This is effected by replacing codons that may have a low statistical incidence in the particular species genome with corresponding codons, in regard to an amino acid, that are statistically more favored. However, one or more less-favored codons may be selected to delete existing restriction sites, to create new ones at potentially useful junctions (5' and 3' ends to add signal peptide or termination cassettes, internal sites that might be used to cut and splice segments together to produce a correct full-length sequence), or to eliminate nucleotide sequences that may negatively effect mRNA stability or expression.

The naturally-occurring encoding nucleotide sequence may already, in advance of any modification, contain a number of codons that correspond to a statistically-favored codon in a particular species. Therefore, codon optimization of the native nucleotide sequence may comprise determining which codons, within the native nucleotide sequence, are not statistically-favored with regards to a particular plant, and

modifying these codons in accordance with a codon usage table of the particular species to produce a codon optimized derivative. A modified nucleotide sequence may be fully or partially optimized for microorganism codon usage provided that the protein encoded by the modified nucleotide sequence is produced at a level higher than the protein encoded by the corresponding naturally occurring or native gene. Construction of synthetic genes by altering the codon usage is described in for example PCT Patent Application 93/07278.

To express the enzymes of the present invention using recombinant technology, polynucleotides encoding the enzymes may be ligated into a nucleic acid expression vector, under the transcriptional control of a cis-regulatory sequence (e.g., promoter sequence) suitable for directing constitutive or inducible transcription of the enzymes in the microorganism.

Thus, the present invention contemplates isolated polynucleotides encoding the enzymes of the present invention.

The phrase “an isolated polynucleotide” refers to a single or double stranded nucleic acid sequence which is isolated and provided in the form of an RNA sequence, a complementary polynucleotide sequence (cDNA), a genomic polynucleotide sequence and/or a composite polynucleotide sequences (e.g., a combination of the above).

As used herein the phrase "complementary polynucleotide sequence" refers to a sequence, which results from reverse transcription of messenger RNA using a reverse transcriptase or any other RNA dependent DNA polymerase. Such a sequence can be subsequently amplified *in vivo* or *in vitro* using a DNA dependent DNA polymerase.

As used herein the phrase "genomic polynucleotide sequence" refers to a sequence derived (isolated) from a chromosome and thus it represents a contiguous portion of a chromosome.

As used herein the phrase "composite polynucleotide sequence" refers to a sequence, which is at least partially complementary and at least partially genomic. A composite sequence can include some exon sequences required to encode the polypeptide of the present invention, as well as some intronic sequences interposing therebetween. The intronic sequences can be of any source, including of other genes, and typically will include conserved splicing signal sequences. Such intronic sequences may further include cis acting expression regulatory elements.

The expression vector of the present invention includes additional sequences which render this vector suitable for replication and integration in prokaryotes, eukaryotes, or preferably both (e.g., shuttle vectors). Typical cloning vectors contain transcription and translation initiation sequences (e.g., promoters, enhancers) and transcription and translation terminators (e.g., polyadenylation signals).

Various methods can be used to introduce the expression vector of the present invention into the host cell system. Such methods are generally described in Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Springs Harbor Laboratory, New York (1989, 1992), in Ausubel et al., Current Protocols in Molecular Biology, John Wiley and Sons, Baltimore, Md. (1989), Chang et al., Somatic Gene Therapy, CRC Press, Ann Arbor, Mich. (1995), Vega et al., Gene Targeting, CRC Press, Ann Arbor Mich. (1995), Vectors: A Survey of Molecular Cloning Vectors and Their Uses, Butterworths, Boston Mass. (1988) and Gilboa et at. [Biotechniques 4 (6): 504-512, 1986] and include, for example, stable or transient transfection, lipofection, electroporation and infection with recombinant viral vectors. In addition, see U.S. Pat. Nos. 5,464,764 and 5,487,992 for positive-negative selection methods.

Exemplary bacterial based expression systems are disclosed in Baneyx et al., Current Opinion in Biotechnology, 1999; 10, 411-421 and Macrides et al, Microbiol Rev 1996, 60: 512-538, incorporated herein by reference.

The microorganisms may be transformed stably or transiently with the nucleic acid constructs of the present invention. In stable transformation, the nucleic acid molecule of the present invention is integrated into the microorganism genome and as such it represents a stable and inherited trait. In transient transformation, the nucleic acid molecule is expressed by the cell transformed but it is not integrated into the genome and as such it represents a transient trait.

The present invention contemplates using polynucleotide sequences which encode the enzymes of the reductive glycine pathway from any organism – e.g. human sequences, plant sequences, bacterial sequences, fungal sequences, yeast sequences.

It will be appreciated that the number of additional enzymes which have to be exogenously expressed in a particular microorganism will depend on the enzymes which are naturally expressed in that cell type and on the sub-cellular location thereof.

Thus, for example, in the case of E. coli, it is proposed that recombinant means are used to express:

1. NAD-dependent formate dehydrogenase which is capable of oxidizing formate to carbon dioxide; and
2. formate-tetrahydrofolate ligase.

In addition, expression of bifunctional methenyltetrahydrofolate-cyclohydrolase-NAD-dependent-methylenetetrahydrofolate-dehydrogenase may also be beneficial. Although endogenous to E. coli, overexpression of enzymes of the glycine cleavage system, serine hydroxymethyltransferase and serine deaminase are also contemplated.

Thus, for example, in the case of S. cervavisciae, it is proposed that recombinant means are used to express recombinant expression of NAD-dependent formate dehydrogenase, bifunctional methenyltetrahydrofolate-cyclohydrolase-NAD-dependent-methylenetetrahydrofolate-dehydrogenase and expression of an independent formate tetrahydrofolate ligase may also be beneficial. Enzymes of the glycine cleavage system should be expressed in the cytoplasm, since endogenously they are mitochondrial. Overexpression of cytoplasmatic serine hydroxymethyltransferase and serine deaminase is further contemplated.

Thus, for example, in the case of B. subtilitis, it is proposed that recombinant means are used to express formate-tetrahydrofolate ligase and serine deaminase. The recombinant expression of NAD dependent formate dehydrogenase and bifunctional methenyltetrahydrofolate-cyclohydrolase-NAD-dependent-methylenetetrahydrofolate-dehydrogenase may also be beneficial. Overexpression of enzymes of the glycine cleavage system and serine hydroxymethyltransferase is also contemplated.

Thus, for example, in the case of Corynobacterium glutamicum, it is proposed that recombinant means are used to express formate-tetrahydrofolate ligase, the glycine cleavage system enzymes (proteins H, P, T and H), and the enzymes converting octanoic acid to lipoic acid attached to the H-protein is required. The recombinant expression of NAD-dependent formate dehydrogenase and bifunctional methenyltetrahydrofolate-cyclohydrolase-NAD-dependent-

methylenetetrahydrofolate-dehydrogenase may also be beneficial. Overexpression of serine hydroxymethyltransferase and serine deaminase is also contemplated.

Thus, for example, in the case of Streptomyces spp., recombinant expression of formate-tetrahydrofolate ligase is required. The recombinant expression of NAD-dependent formate dehydrogenase and bifunctional methenyltetrahydrofolate-cyclohydrolase-NAD-dependent-methylenetetrahydrofolate-dehydrogenase is further contemplated. Overexpression of enzymes of the glycine cleavage system, serine hydroxymethyltransferase and serine deaminase is also contemplated.

Thus, for example in the case of Lactococcus lactis, recombinant expression of the glycine cleavage system enzymes (proteins H, P, T and H), the enzymes converting octanoic acid to lipoic acid attached to the H-protein of the glycine cleavage system is required. The recombinant expression of NAD-dependent formate dehydrogenase and bifunctional methenyltetrahydrofolate-cyclohydrolase-NAD-dependent-methylenetetrahydrofolate-dehydrogenase is further contemplated. Over-expression of formate-tetrahydrofolate ligase, serine hydroxymethyltransferase and serine deaminase is also contemplated.

Transformed cells are cultured under effective conditions, which allow for the expression of high amounts of the recombinant enzymes. Effective culture conditions include, but are not limited to, effective media, bioreactor, temperature, pH and oxygen conditions that permit protein production. An effective medium refers to any medium in which a cell is cultured to produce the recombinant polypeptide of the present invention. Such a medium typically includes an aqueous solution having assimilable carbon, nitrogen and phosphate sources, and appropriate salts, minerals, metals and other nutrients, such as vitamins. Cells of the present invention can be cultured in conventional fermentation bioreactors, shake flasks, test tubes, microtiter dishes and petri plates. Culturing can be carried out at a temperature, pH and oxygen content appropriate for a recombinant cell. Such culturing conditions are within the expertise of one of ordinary skill in the art.

It will be appreciated that until the microorganism is transformed from being a heterotrophic microorganism to a formatotrophic microorganism or autotrophic microorganism, the microorganism is cultured in a culture medium comprising other carbon sources such as glucose or glycerol.

Following generation of the microorganisms as described herein, preferably they are selected by growing (i.e. culturing) on a particular substrate. Preferably, the microorganisms are grown for at least one day, at least two days, at least three days, at least one week, at least one month, at least three months wherein any viable cells remaining after such time are the selected microorganism.

Thus, for example in the case of generating a formatotrophic microorganism, the microorganism should be cultivated in a culture medium comprising formate as the carbon source. In the case of an autotrophic microorganism, the microorganism should be cultivated in a culture medium comprising carbon dioxide as the carbon source and an external electron source as further described herein above.

The formate which is used may come from any source – e.g. sodium formate, potassium formate, formic acid or formic acid anhydride etc.

Alternatively, and/or additionally, the formate may be generated using electricity. CO_2 can be directly reduced at the cathode (the electrons are derived from water splitting at the anode) to generate formate at relatively high efficiency.

In order to generate the formate for use by the microorganism, the microorganism is placed in a bioreactor in a fluid (e.g. water). The cathode may optionally be placed inside the bioreactor in contact with the microorganism. Alternatively, the cathode may be placed in a separate container to the bioreactor and the formate may be channeled to the chamber comprising the microorganism. The fluid may contain other elements required by the microorganism for growth including for example salts, minerals, metals and other nutrients, such as vitamins.

Examples of such bioreactors and further methods are provided in Li et al. Science, 2012, Vol 335, page 1596, Rabaey et al, Current Opinion in Biotechnology, 2011, 22: 371-377; Lovley et al., Current Opinion in Biotechnology, 2011, 22: 441-448; Lovley D.R., Environmental microbiology reports, 2011, 3(1), 27–35; Nevin et al., Microbiology, May/June 2010 Volume 1 Issue 2; Rabaey et al., Applied and Industrial Microbiology, Nature Reviews, October 2010, Volume 8, page 706-716; each of which are incorporated herein by reference.

The electrodes may be fabricated from such conductive polymers and metallic materials including indium tin oxide (ITO), graphite, platinum and silver.

Thus, a system is contemplated for the microorganism described herein and an electrode for providing electrons to generate formate. The system may further comprise mechanism(s) for separating, collecting, and/or recovering the biofuel which is generated by the microorganism (as further detailed below).

In order to confirm that the formatotrophic or autotrophic microorganism is using the reductive glycine pathway for generation of pyruvate or glycerate, the present invention further contemplates analysis of the metabolites of the reductive glycine pathway.

Such metabolites include 10-formyl-tetrahydrofolate, 5,10-methenyl-tetrahydrofolate, 5,10-methylene- tetrahydrofolate, aminomethyl-dihydrolipoylprotein, dihydrolypoylprotein, lypoylprotein, glycine, serine and pyruvate (or hydroxypyruvate and glycerate).

Preferably, the analysis comprises ^{13}C- or ^{14}C-labeling analysis for the time taken to produce each labeled metabolite from label formate, such that it is evident that production of 10-formyl-tetrahydrofolate precedes that of 5,10-methylene-tetrahydrofolate, such that the production of 5,10-methylene- tetrahydrofolate precedes that of aminomethyl-dihydrolipoyl, such that the production of aminomethyl-dihydrolipoyl precedes that of glycine, such that of production of glycine precedes that of serine and the production of serine precedes that of pyruvate.

Preferably, formation of each of the metabolites precedes the next one in the chain by at least one second, at least 10 seconds and more preferably at least 20 seconds.

An exemplary method for analyzing the timing of the production of metabolites is via pulse chase analysis. A pulse-chase analysis is a method for examining a cellular process occurring over time by successively exposing the cells to a labeled compound (pulse) and then to the same compound in an unlabeled form (chase). Radioactivity is a commonly used label.

In one exemplifying method, the microorganisms of embodiments of the invention are first exposed to labeled formate (the pulse). The labeled formate then goes through the metabolic pathways and is used in the synthesis of pyruvate. Shortly after introduction of the labeled formate (usually about 5 minutes), excess of the same, but unlabeled, formate (the chase) is introduced into the environment. The production of

pyruvate would continue, but it would no longer contain the radioactive marker from the formate introduced in the pulse phase and would not be visible using radioactive detection methods.

In another exemplifying method, the compounds specified above can be analyzed to find which of their carbon atoms is labeled. If indeed they are produced via the reductive glycine pathway an indicative carbon-labeling pattern is expected.

The amount and/or activity of the enzymes of the pathway may be analyzed on the RNA or protein level using methods known in the art. Thus, on the RNA level, methods including RT-PCR, Northern blot analysis, oligonucleotide microarray. On the protein level, methods including ELISA, Western blot, radioimmunoassay, in situ activity assay, in vitro activity assay and immunohistochemical analysis are all contemplated. An indication for the pathway operation is if all pathway enzymes show activity sufficient to support cellular growth at the rate it has been shown to grow.

Further methods for analyzing production of metabolites of the reductive glycine pathway are found in Hugler et al, 2005, Methods In Enzymology, Vol. 397, p. 212-221; Berg et al., *Science* 318, 1782 (2007); Strauss et al., Eur. J. Biochem. 205, 853-866 (1992); Hertner et al., Journal Of Bacteriology, Vol 183, No. 14, July 2001, p. 4305–4316; Jahn et al., Journal Of Bacteriology, June 2007, p. 4108–4119 Vol. 189, No. 11; and Huber et al., PNAS June 3, 2008, vol. 105 , no. 22, 7851–7856, the contents of which are incorporated herein by reference.

According to one embodiment, the microorganism is one that produces an industrially important product – e.g. a biofuel. Alternatively, or additionally the microorganism expresses enzymes such that it is capable of producing an industrially important product - e.g. a biofuel. It will be appreciated that the precise choice of enzymes are selected according to the particular microorganism being used. Alternatively, or additionally the microorganism expresses an industrially important product - e.g. a recombinant protein. Additional industrial important products include antibiotics or other pharmaceutical, solvents, pigments, food additives, monomers for the plastic industry and industrially valuable polymers.

Biofuels include for example, an alcohol (e.g., methanol, ethanol, propanol, isobutanol, and n-butanol etc.), a hydrocarbon (e.g., an alkane such as methane, ethane, propane, butane, an alkene such as ethylene, propylene, isoprenes, an alkyne such as

acetylene etc.) hydrogen, a biodiesel (long-chain alkyl (methyl, propyl or ethyl) esters), an aldehyde or ketones (e.g. acetone, formaldehyde, 1-propanal, etc.). The biofuel can be a solid, a liquid or a gas.

Industrially useful microorganisms include the production of ethanol by Saccharomyces and the production of butanol by Clostridium.

The recombinant protein may be any protein – e.g. a human protein used for medicinal purposes. Examples of such proteins include an antibody, insulin, interferon, growth hormone, erythropoietin, growth hormone, follicle stimulating hormone, factor VIII, low density lipoprotein receptor (LDLR) alpha galactosidase A and glucocerebrosidase.

As mentioned, in order to express recombinant proteins in the microorganism, polynucleotide sequences encoding same are inserted into expression vectors as described herein above.

It will be appreciated that other than containing the necessary elements for the transcription and translation of the inserted coding sequence (encoding the industrially useful polypeptide), the expression construct for expression of the industrially useful polypeptide can also include sequences engineered to optimize stability, production, purification, yield or activity of the expressed polypeptide.

Depending on the vector and host system used for production, resultant polypeptides of the present invention may either remain within the recombinant cell, secreted into the fermentation medium, secreted into a space between two cellular membranes, such as the periplasmic space in *E. coli*; or retained on the outer surface of a cell or viral membrane.

Following a predetermined time in culture, recovery of the recombinant polypeptide is effected.

The phrase "recovering the recombinant polypeptide" used herein refers to collecting the whole fermentation medium containing the polypeptide and need not imply additional steps of separation or purification.

Thus, polypeptides of the present invention can be purified using a variety of standard protein purification techniques, such as, but not limited to, affinity chromatography, ion exchange chromatography, filtration, electrophoresis, hydrophobic interaction chromatography, gel filtration chromatography, reverse phase

chromatography, concanavalin A chromatography, chromatofocusing and differential solubilization.

To facilitate recovery, the expressed coding sequence can be engineered to encode the polypeptide of the present invention and fused cleavable moiety. Such a fusion protein can be designed so that the polypeptide can be readily isolated by affinity chromatography; e.g., by immobilization on a column specific for the cleavable moiety. Where a cleavage site is engineered between the polypeptide and the cleavable moiety, the polypeptide can be released from the chromatographic column by treatment with an appropriate enzyme or agent that specifically cleaves the fusion protein at this site (*e.g.* 26, 27).

Recovery of biofuels may be recovered according to methods known in the art. Alcohols such as ethanol, methanol, and/or butanol may be recovered from liquid material by molecular sieves, distillation, and/or other separation techniques. For example, ethanol can be concentrated by fractional distillation to about 90% or about 95% by weight. There are several methods available to further purify ethanol beyond the limits of distillation, and these include drying (e.g., with calcium oxide or rocksalt), the addition of small quantities of benzene or cyclohexane, molecular sieve, membrane, or by pressure reduction.

Product gas, for example, as produced by anaerobic metabolism or photosynthesis, may be processed to separate the methane and/or hydrogen components. Methane, hydrogen, or biogas may be drawn off from the system as pipeline gas.

In accordance with the invention, methane and/or hydrogen may be recovered as a biofuel product. Methane may be recovered and/or purified from biogas by known methods and systems which are commercially available, including membrane systems known for separating gases on the basis of different permeabilities. See, for example, U.S. Pat. No. 6,601,543, which is hereby incorporated by reference. Alternatively, various methods of adsorption may be used for separating methane and hydrogen.

Other ways of collecting biofuel products including centrifugation, temperature fractionalization, chromatographic methods and electrophoretic methods.

In certain embodiments, the biofuel recovery/purification components may be integrated into the microorganism culturing system (e.g. bioreactor), for example, by

connecting the respective device or apparatus to the gas or liquid effluents from the bioreactors. The purified biofuels and bioenergy products may be stoked in a separate container(s).

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination or as suitable in any other described embodiment of the invention. Certain features described in the context of various embodiments are not to be considered essential features of those embodiments, unless the embodiment is inoperative without those elements.

Various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below find calculated support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions illustrate some embodiments of the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel et al., "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson et al., "Recombinant DNA", Scientific American Books, New York; Birren et al. (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Culture of Animal Cells - A Manual of Basic Technique" by Freshney, Wiley-Liss, N. Y. (1994), Third Edition; "Current Protocols in Immunology" Volumes I-III

Coligan J. E., ed. (1994); Stites et al. (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); "Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and Translation" Hames, B. D., and Higgins S. J., eds. (1984); "Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984) and "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990); Marshak et al., "Strategies for Protein Purification and Characterization - A Laboratory Course Manual" CSHL Press (1996); all of which are incorporated by reference as if fully set forth herein. Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

EXAMPLE 1

Selecting an optimum pathway to support growth on formate

To choose which of the pathways described above is most suitable to support growth on formate they were compared according to several criteria (28). First, the expected biomass yield on formate was calculated for each of the different pathway (21, 29, 30).

Two quantitative methods were used to estimate the biomass yield on formate of each of the pathways. In the "carbon-source-conversion" method (21), the present inventors first calculated the yield of converting formate into a designated benchmark metabolite, $Y_{formate->metabolite}$, in units of mol/mol. Taking the experimentally measured biomass yield on that metabolite, $Y_{metabolite->biomass}$, in units of gCDW/mol (CDW being cellular dry weight), one can then estimate the biomass yield on formate, in units of gCDW/mol formate, as $Y_{formate->biomass} = Y_{formate->metabolite} \cdot Y_{metabolite->biomass}$.

For example, the present inventors calculated the number of formate molecules needed to be invested to generate one molecule of pyruvate. The reciprocal of this number is the pyruvate yield on formate in units of mol pyruvate /mol formate. Multiplying pyruvate yield on formate with biomass yield on pyruvate – 14.7 gCDW/mol, as measured experimentally (31) – provided the inventors with an estimation for biomass yield on formate in units of gCDW/mol formate. The same process was then repeated for glucose, taking the experimentally measured biomass yield on glucose, 70.8 gCDW/mole (31). In both cases it was assumed that ATP is produced via NADH and oxidative phosphorylation and that the P/O ratio (measuring how many ATP molecules are produced per one oxygen atom being reduced) is 1.5, as is relevant for *E. coli* (32). The second and third column of Table 2 herein below displays the estimated biomass yield calculated by choosing either pyruvate or glucose as the product of the carbon fixation and formate-assimilating pathways.

Table 2

Pathway	Biomass Yield, gCDW/mole-Formate *				Thermodynamics **	Number of Foreign Enzymes ***
	Carbon-Source-Conversion		Flux-Balance-Analysis			
	Formate conversion to pyruvate	Formate conversion to glucose	ATP maintenance as for growth on glucose	With no ATP maintenance		
Carbon fixation pathways						
reductive pentose phosphate	1.5	3.0	2.5	5.6	High CMF ‡	3
reductive TCA	2.3	3.8	2.9	6.7	Unfavorable	4
dicarboxylate-4-hydroxypropionate	1.8	3.1	2.4	5.3	Low CMF	5
3-hydroxypropionate-4-hydroxybutyrate	1.3	2.5	1.8	4.2	Low CMF	>5
3-hydroxypropionate	1.5	2.8	1.9	4.4	High CMF	>5
MOG (28)	1.6	3.1	2	4.5	High CMF	>5
Formate assimilating pathways						
reductive acetyl-CoA	2.6	4.1	2.7	6.2	Unfavorable (High CMF) †	4
serine	1.6	3.0	2.4	5.5	High CMF	5
ribulose monophosphate	2.1	3.8	2.8	6.4	Low CMF	4

xylulose 5-phosphate	1.9	3.5	2.6	5.9	High CMF	3
Reductive glycine	2.3	3.8	2.8	6.3	High CMF (High CMF) ††	2

* see main text for the difference between the methods of calculating biomass yield on fromate.

** Pathways marked with 'Unfavorable' contain a thermodynamically unfavorable reaction sequence; pathways marked with 'Low CMF' (i.e. low chemical motive force) are thermodynamically favorable but there is no metabolite concentration set, within the physiological range, that can support $\Delta G_r' < -3$ kJ/mol for all of its reactions; pathways marked with 'High CMF' are those for which such metabolite concentration set does exist.

*** Number of foreign enzymes that need to be expressed in *E. coli* to establish an active pathway.

‡ CMF stands for chemical motive force.

† The sign outside the parentheses refers to the case in which the reactions within the co-dehydrogenase-acetyl-coa-synthase complex are not coupled while the sign in the parentheses refers to the case they are coupled.

†† The sign outside the parentheses refers to the case in which the reactions within the glycine-cleavage-system complex are not coupled while the sign in the parentheses refers to the case they are coupled.

The results shown in Table 2 herein suggest that formate-assimilating pathways are generally more efficient than carbon fixation ones, with the exception of those carbon fixation pathways that bypass ATP-coupled carboxylation steps. Yet, these results also suggest that this type of analysis is problematic since the choice of the specific metabolite to serve as the product of the pathways can influence the estimation greatly: assuming glucose as the pathways' product gave much higher estimation for biomass yield on formate (~1.5 fold difference on average) just because the biomass yield of *E. coli* on glucose is higher than that on pyruvate.

An alternate approach to estimate biomass yield is to use flux balance analysis metrics (33,34) as implemented by (30). The advantage of this approach is that it is not biased by the choice of metabolite to which formate is converted to and which is then used as a carbon source, but rather treat the entire cellular biomass as the product of carbon fixation or formate assimilation (30). The core *E. coli* metabolic model was

selected (35) over the full metabolic model since the present inventors wanted to keep the analysis more general and less *E. coli* specific and since they wanted to avoid regulation complexities that can result in unsound solutions using the full metabolic model. Flux balance analysis was used to calculate growth yield rather than growth rate. Hence, formate input units were in mmol and biomass yield was given in gDW. Since the ATP maintenance cannot be estimated *a priori* for growth on formate the biomass yields were calculated in two different ways, once removing ATP maintenance altogether and once by assuming identical ATP maintenance to that of glucose. The fourth and fifth columns in Table 2 display the results of this analysis.

Importantly, while the methods of calculating biomass yield on formate differ substantially and resulted in up to 6 fold difference in estimated biomass yield (Table 2), they suggested very similar relative biomass yields of the pathways. In fact, the correlation between the biomass yields calculated by assuming pyruvate or glucose as the pathways' product and those calculated using flux balance analysis is $R^2 > 0.6$. Considering all the methods used, the reductive TCA cycle, reductive acetyl-coA pathway, ribulose monophosphate pathway and the reductive glycine pathway are the pathways supporting the highest biomass yield on formate.

EXAMPLE 2

Thermodynamic favorability

Not all metabolic pathways which operate in one organism are thermodynamically favorable in others, in which the cellular conditions (pH, ionic strength, etc.) might differ considerably (28, 36, 37). The present inventors therefore checked whether all the pathways discussed above are thermodynamically favorable within *E. coli* (pH ~ 7.5, I ~ 0.2 M). They tested not only the favorability of the pathway net reaction (28, 38) but also analyzed distributed thermodynamic bottlenecks composed of a subset of reactions within the pathways (28, 39). Notably, the CO-dehydrogenase-acetyl-CoA-synthase and the glycine-cleavage-system are complexes of several enzymes. Hence, the reactions that occur within these complexes are probably coupled to each other, overcoming any internal thermodynamic barrier (37). For pathways that contain these complexes both scenarios were considered - internal reactions within the complex are coupled or are uncoupled.

Two pathways were found that are predicted to be thermodynamically unfavorable since they contain reaction sets that cannot proceed in the forward direction at the cellular conditions of *E. coli* and under physiological reactant concentrations (the range 1μM-10mM for non co-factor metabolites was used) (37,40-42). These are the reductive TCA cycle and the reductive acetyl-CoA pathway in which CO_2 reduction to CO and acetyl-CoA synthesis are not coupled. Both pathways are marked as 'unfavorable' in the sixth column of Table 2.

The sequential reductive operation of 2-ketoglutarate synthase and isocitrate dehydrogenase (part of the reductive TCA cycle), catalyzing the overall reaction succinyl-CoA + 2 ferredoxin^{red} + NADPH + 2 CO_2 <=> isocitrate + CoA + 2 ferredoxin^{ox} + $NADP^+$, seems to be unfavorable in *E. coli*. Even if it is assumed that the concentration of dissolved CO_2 is kept at 1mM (~100 fold higher then ambient), [NADPH]=10·[$NADP^+$], [ferredoxin^{red}]~[ferredoxin^{ox}], [CoA]=1mM (the lowest cellular concentration of this cofactor (40) and the other metabolites are at their possible extreme values, [succinyl-CoA]=10mM and [isocitrate]=1μM, the change in Gibbs energy (Δ_rG') during the overall reaction is still positive at pH 7.5 and I= 0.2 M. This reasoning probably rules out the reductive TCA cycle from serving as a carbon fixation pathway in *E. coli*.

The energetic barrier of the reductive acetyl-CoA pathway is the result of the highly unfavorable reduction of CO_2 to CO with $\Delta_rG'^{o}$ = +46 kJ/mol when ferredxoin serve as electron donor (43). However, this huge energetic barrier can be flattened altogether if the reaction is indeed coupled to the very favorable reaction that follows it within the same complex, acetyl-CoA synthase (37). In such a case the overall reaction, methyl-THF + CO_2 + 2 ferredoxin^{red} + CoA <=> acetyl-CoA + 2 ferredoxin^{ox} + THF, will have $\Delta_rG'^{o}$ ~ -25 kJ/mol (43), making the entire pathway favorable.

EXAMPLE 3

Chemical motive force

Being thermodynamically favorable is not enough. The energy dissipated during a reaction (Δ_rG') can have a substantial effect on its kinetics. In fact, $\Delta G_r'$ dictates what fraction of the enzymatic machinery catalyzes the forward reaction (38,44-46): $\Delta G_r' = -RT\ln(J^+/J^-)$, where J^+ is the forward flux, J^- is the backward flux, R is the gas constant

and T is the temperature in Kelvin. Hence, a low (negative) ΔG_r' value, corresponding to a high chemical motive force, indicates that most of the enzymatic machinery is catalyzing the forward reaction and hence a high metabolic rate can be achieved. Assuming substrate saturation and similar kinetics in the forward and backward direction, ΔG_r' of -7.5 kJ/mol corresponds to a reaction that proceeds at 90% of its maximal velocity: 95% of the enzymes catalyze the forward reaction while 5% catalyze the backward reaction.

The present inventors asked which of the pathways analyzed can, in principle, support high flux in terms of the chemical motive force sustained by each of its reactions. For each pathway a linear optimization tool was used to ask whether a metabolite concentration set exists, within the physiological range, such that each reaction of the pathway is not only favorable but also operate at $\Delta G_r' < -3$ kJ/mol, corresponding to at least 55% of its maximal rate (assuming substrate saturation and similar kinetics in the forward and backward direction). The sixth column in Table 2 displays the results. Pathways marked as 'low CMF' (low chemical motive force) are thermodynamically favorable but kinetically poorer since there is no metabolite concentration set, within the physiological range, that can support $\Delta G_r' < -3$ kJ/mol for all of its reactions. Pathways marked as 'high CMF' are those for which such metabolite concentration set does exist and hence, in potential, can sustain a high chemical motive force through the pathway.

EXAMPLE 4

Pathway kinetics

The chemical motive force is not the only parameter determining the reaction flux. The kinetic parameters, i.e. maximal velocity (V_{MAX}) and affinities toward the substrates (Michaelis constants, K_M), play a role no less important.

The present inventors estimated what is the maximal growth rate for a bacterium utilizing formate only as an electron source and rely on carbon fixation pathways for carbon. They consider the growth rate limit imposed by the rate of formate dehydrogenase. Since formate dehydrogenase serves only auxiliary role in bacteria employing one of the formate-assimilating pathways (increasing cellular availability of reducing power and ATP), the analysis presented below do not hold in these cases.

Suppose that the cells express a formate dehydrogenase with a specific activity of 10 μmol/min/mg (maximal velocity for the simple enzyme variants, see above) at 20 % of its total protein (more than that is expected to be deleterious). Assuming that ~ 50% of the cellular dry weight is proteins (47), it is calculated that 10% of the cellular dry weight is formate dehydrogenase and hence the specific activity is ~1 μmol/min/mgCDW. If the reductive pentose phosphate pathway serves as the carbon fixation pathway, 12 NAD(P)H and 18 ATP molecules are required to fix six CO_2 molecules to glucose. Assuming a P/O ratio of 1.5 (32), 12+18/1.5 = 24 molecules of formate should be oxidized to support the formation of one glucose molecule. Hence, the rate of glucose formation will be 1/24 ~ 0.042 μmol-Glucose/min/mg CDW. Since the experimentally measured biomass yield on glucose is 70.8 gDW/mole (31) it is calculated that this rate to equal $0.042 \cdot 70.8 \cdot 10^{-6} \sim 3 \cdot 10^{-6}$ gCDW/min/mgCDW or 0.003 mgCDW/min/mgCDW. Hence, the growth rate equals 0.0031/min and the doubling time is ln(2)/0.003 ~ 230 min ~ 4 hours.

This calculation suggests that the doubling time of an autotrophic bacterium metabolizing formate using a formate dehydrogenase of the 'simple' type cannot be lower than 4 hours. Of course, this is only a lower limit and the doubling time might very well be limited by other factors, such as the rate of carbon fixation. In addition, even the rough limit of 4 hours can change when considering carbon fixation pathways with different ATP requirements or if restricting the expression of formate dehydrogenase to lower than 20% of total protein.

A similar kind of analysis can produce more conclusive results. For example, one can ask whether a bacterium can use the ribulose monophosphate or the xylulose 5-phosphate pathways for formate assimilation when limited by the rate of spontaneous cleavage of methylene-THF to formaldehyde and THF. According to the equilibrium and kinetic constants reported in (48) and assuming a high [methylene-THF]~10 mM, the maximal rate of methylene-THF cleavage can be calculated to be 0.027 mM/sec. Since the cellular volume of slowly growing *E. coli* is ~1 μm^3 (49) this rate equals $2.7 \cdot 10^{-17}$ mmol/sec/cell or $1.6 \cdot 10^{-12}$ μmol/min/cell. Since six such reactions are required for the production of one glucose molecule the rate of glucose production is limited to $1.6 \cdot 10^{-12}/6 = 2.7 \cdot 10^{-13}$ μmol-Glucose/min/cell. The dry weight of an *E. coli* cell with a volume of ~1 μm^3 is ~200 fg (50) and hence the former rate equals 0.0014 μmol-

Glucose/min/mgCDW. According to the above biomass yield this rate corresponds to $9.6 \cdot 10^{-8}$ gCDW/min/mgCDW which is $9.6 \cdot 10^{-5}$ mgCDW/min/mgCDW. The growth rate therefore equals $9.6 \cdot 10^{-5}$ 1/min and the doubling time is $\ln(2)/(9.6 \cdot 10^{-5}) > 7200$ min = 120 hours = 5 days.

Therefore, this calculation suggests that, by considering only a single reaction, two formate-assimilating pathways are kinetically infeasible and cannot sustain even a minimally acceptable growth rate. Indeed, a previous study has demonstrated that the reverse reaction – the condensation of THF with formaldehyde – is too slow to have any metabolic significance *in vivo* (51).

EXAMPLE 5

Pathway expression challenges

Finally, the present inventors asked how challenging the expression of an active pathway within *E. coli* is expected to be. One aspect that affects the expression difficulty is the number of foreign enzymes that should be expressed to enable pathway activity. The seventh column in Table 2 displays this number for each of the metabolic alternatives. Notably, all pathways require, or are strongly benefiting from, the expression of formate dehydrogenase to supply the cell with reducing power and energy.

The reductive pentose phosphate pathway and the xylulose 5-phosphate pathway seem to impose a small expression barrier, necessitating only three foreign enzymes. The reductive glycine pathway presents the smallest expression barrier: only foreign formate dehydrogenase and formate-tetrahydrofolate ligase are needed for pathway operation.

The oxygen sensitivity of some of the enzymes operating in some of the pathways (38, 52) was also contemplated. Specifically, the reductive TCA cycle and the dicarboxylate-4-hydroxypropionate pathways employ several oxygen sensitive enzymes (38-52). Also, the reductive acetyl-CoA pathway operates some of the most oxygen sensitive enzymes known (38, 52, 53). These pathways are therefore not suitable if the bacterium is to be cultivated under aerobic conditions.

Concluding remarks

Reviewing all the criteria suggests that one pathway stands out over the others. The reductive glycine pathway requires the expression of only two foreign enzymes, contains no oxygen sensitive enzyme, supports high biomass yield, is able to sustain high chemical motive force through the entire reaction set and is not severely kinetically restricted by any reaction (Table 1). This pathway seems to be the most promising route to establish a formatotrophic *E. coli*.

Some of the pathways analyzed by the present inventors have several variants, each with its own characteristics, advantages and drawbacks. For example, the reductive TCA cycle is probably not a good candidate since it contains a huge thermodynamic barrier at *E. coli*'s cellular conditions. However, *Hydrogenobacter thermophilus* has evolved an ATP-dependent mechanism to push the pathway in the reductive direction: the enzyme 2-ketoglutarate carboxylase catalyzes the ATP-dependent carboxylation of 2-ketoglutarate to oxalosuccinate in a biotin-dependent mechanism, while oxalosuccinate is further reduced to isocitrate by a non-carboxylating isocitrate dehydrogenase (54, 55). This pathway variant is thermodynamically favorable and even supports high chemical motive force of each of its enzymatic components. However, the soluble intermediate oxalosuccinate is unstable and readily undergoes decarboxylation, hence creating a futile cycle that reduces the overall efficiency of carbon fixation (54).

Finally, enzyme evolution can provide numerous metabolic solutions to the challenges raised by the present inventors. For example, the enzyme catalyzing the reversible condensation of formaldehyde and tetrahydromethanopterin to methylene-tetrahydromethanopterin (56) can be evolved to accept THF instead of tetrahydromethanopterin, thereby lifting the kinetic barrier imposed by the spontaneous cleavage of methylene-THF. Of special importance is the design of an enzyme that can condense two formate molecules to glyoxylate which can then be directly assimilated into central metabolism. While such an enzyme was previously suggested to operate in the chloroplast of greening potato tubers (57-59), the energetics of this unactivated condensation is extremely unfavorable, indicating that the report is probably erroneous(38). Any attempt to design an enzyme that catalyzes this condensation must therefore first activate the formate (with a phosphate group, for example).

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Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims.

All publications, patents and patent applications mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention. To the extent that section headings are used, they should not be construed as necessarily limiting.

WHAT IS CLAIMED IS:

1. An isolated microorganism that expresses enzymes of the reductive glycine pathway, wherein the microorganism is capable of converting formate to pyruvate or glycerate via the formation of glycine and serine.

2. An isolated microorganism that expresses enzymes of the reductive glycine pathway, wherein the microorganism is capable of converting formate to a metabolite of central metabolism via the formation of glycine and without the formation of serine, said metabolite being selected from the group consisting of acetyl CoA, oxaloacetate, glycerate 2-phosphate and glycerate 3-phosphate.

3. The microorganism of claim 2, not expressing EC 2.1.2.1.

4. The isolated microorganism of claims 1 or 2, being genetically modified.

5. The microorganism of claim 4, being genetically modified to express at least one of said enzymes of said reductive glycine pathway.

6. The microorganism of claims 1 or 2, being formatotrophic.

7. The microorganism of claim 1, further expressing a formate dehydrogenase which is capable of reducing carbon dioxide to formic acid.

8. The microorganism of claim 7 being autotrophic.

9. The microorganism of claim 8, being phototrophic.

10. The microorganism of claim 8, being chemotrophic.

11. The microorganism of claims 1 or 2, being aerobic.

12. The microorganism of claims 1 or 2, being anaerobic.

13. The microorganism of claims 1 or 2, being a bacteria.

14. The microorganism of claim 13, being a gram positive bacteria.

15. The microorganism of claim 13, being a gram negative bacteria.

16. The microorganism of claim 13, wherein said bacteria is selected from the group consisting of Agrobacterium, Alicyclobacillus, Anabaena, Anacystis, Arthrobacter, Azobacter, Bacillus, Brevibacterium, Chromatium, Clostridium, Corynebacterium, Enterobacter, Erwinia, Escherichia, Lactobacillus, Lactococcus, Mesorhizobium, Methylobacterium, Microbacterium, Phormidium, Pseudomonas, Rhodobacter, Rhodopseudomonas, Rhodospirillum, Rhodococcus, Salmonella, Scenedesmun, Serratia, Shigella, Staphlococcus, Strepromyces, Synnecoccus, and Zymomonas.

17. The microorganism of claim 16, wherein said bacteria comprises Escherichia.

18. The microorganism of claim 17, wherein said Escherichia are genetically modified to express a first enzyme NAD-dependent formate dehydrogenase which is capable of oxidizing formate to carbon dioxide and a second enzyme formate-tetrahydrofolate ligase.

19. The microorganism of claim 18, wherein said Escherichia are genetically modified to further express bifunctional methenyltetrahydrofolate-cyclohydrolase-NAD-dependent-methylenetetrahydrofolate-dehydrogenase.

20. The microorganism of claims 18 or 19, wherein said Escherichia are genetically modified to further express at least one glycine cleavage system enzyme.

21. The microorganism of any one of claims 18-20, wherein said Escherichia are genetically modified to further express serine hydroxymethyltransferase and/or serine deaminase.

22. The microorganism of claim 1 or 2, being a yeast.

23. The microorganism of claim 22, wherein said yeast comprises *S.cervavisciae*.

24. The microorganism of claim 1 or 2, being a fungi.

25. The microorganism of claim 24, wherein said fungi is selected from Aspergillus, Candida, Chlamydomonas, Chrysosporium, Cryotococcus, Fusarium, Kluyveromyces, Neotyphodium, Neurospora, Penicillium, Pichia, Saccharomyces, Trichoderma and Xanthophyllomyces.

26. The microorganism of claim 1 or 2, being an algae.

27. The microorganism of claim 1 or 2, being genetically modified to express a human polypeptide.

28. The microorganism of claim 27, wherein said human polypeptide is selected from the group consisting of an antibody, insulin, interferon, growth hormone, erythropoietin, growth hormone, follicle stimulating hormone, factor VIII, low density lipoprotein receptor (LDLR) alpha galactosidase A and glucocerebrosidase.

29. The microorganism of claim 1, capable of producing a biofuel.

30. The microorganism of claim 29, wherein the biofuel is selected from the group consisting of ethanol, propanol, isobutanol and n-butanol.

31. The microorganism of claim 29, wherein the biofuel is selected from the group consisting of an alcohol, an alkene, an alkane, a lipid or a polysaccharide.

32. A method of generating a microorganism comprising expressing in the microorganism at least one enzyme of the reductive glycine pathway, such that the microorganism is capable of converting formate to pyruvate or glycerate via the formation of glycine and serine.

33. The method of claim 32, wherein said microorganism is formatotrophic.

34. The method of claim 32, wherein said microorganism is autotrophic.

35. The method of claim 33, further comprising selecting the microorganism that is formatotrophic by growing the microorganism on formate following said expressing.

36. The method of claim 34, further comprising selecting the microorganism that is autotrophic by growing the microorganism on carbon dioxide in the presence of an external electron source following said expressing.

37. The method of claim 35 or 36, further comprising analyzing for the production of metabolites of said reductive glycine pathway and/or activity of said enzymes of said reductive glycine pathway in said microorganism.

38. The method of claim 32, further comprising culturing said microorganism following said generating.

39. The method of claim 38, wherein said culturing is effected in a presence of an electrical current so as to generate said formate.

40. A system for culturing the microorganism of claim 6 and an electrode for providing electrons to generate formate.

41. A method of selecting the microorganism of claim 6 comprising:

growing a microorganism on formate; and

analyzing for the production of metabolites of said reductive glycine pathway and/or activity of said enzymes of said reductive glycine pathway in said microorganism, wherein a production of said metabolites of said reductive glycine pathway and an activity of said enzymes of said reductive glycine pathway above a predetermined level is indicative of a formatotrophic microorganism.

42. The method of claim 41, wherein when said analyzing is for the production of metabolites, the method is effected by performing a pulse chase experiment.

43. The method of claim 41, further comprising analyzing for an expression of said enzymes of said reductive glycine pathway.

44. A method of generating a biofuel comprising culturing the microorganism of claims 29-31 under conditions that allow for biofuel formation, thereby generating the biofuel.

45. The method of claim 44, further comprising collecting the biofuel.

46. A method of generating a human polypeptide comprising culturing the microorganism of claims 29-31 under conditions that allow for expression of the human polypeptide, thereby generating the human polypeptide.

47. The method of claim 44, further comprising isolating the human polypeptide.

FIG. 1

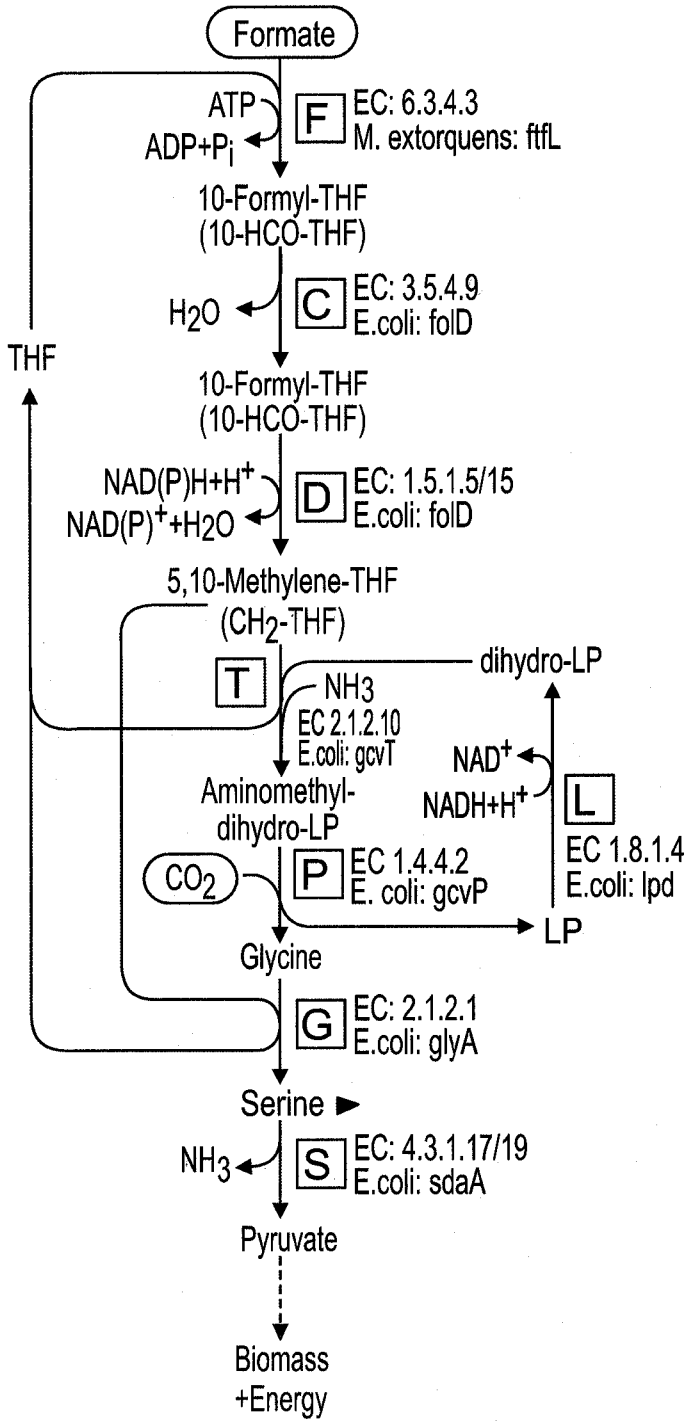
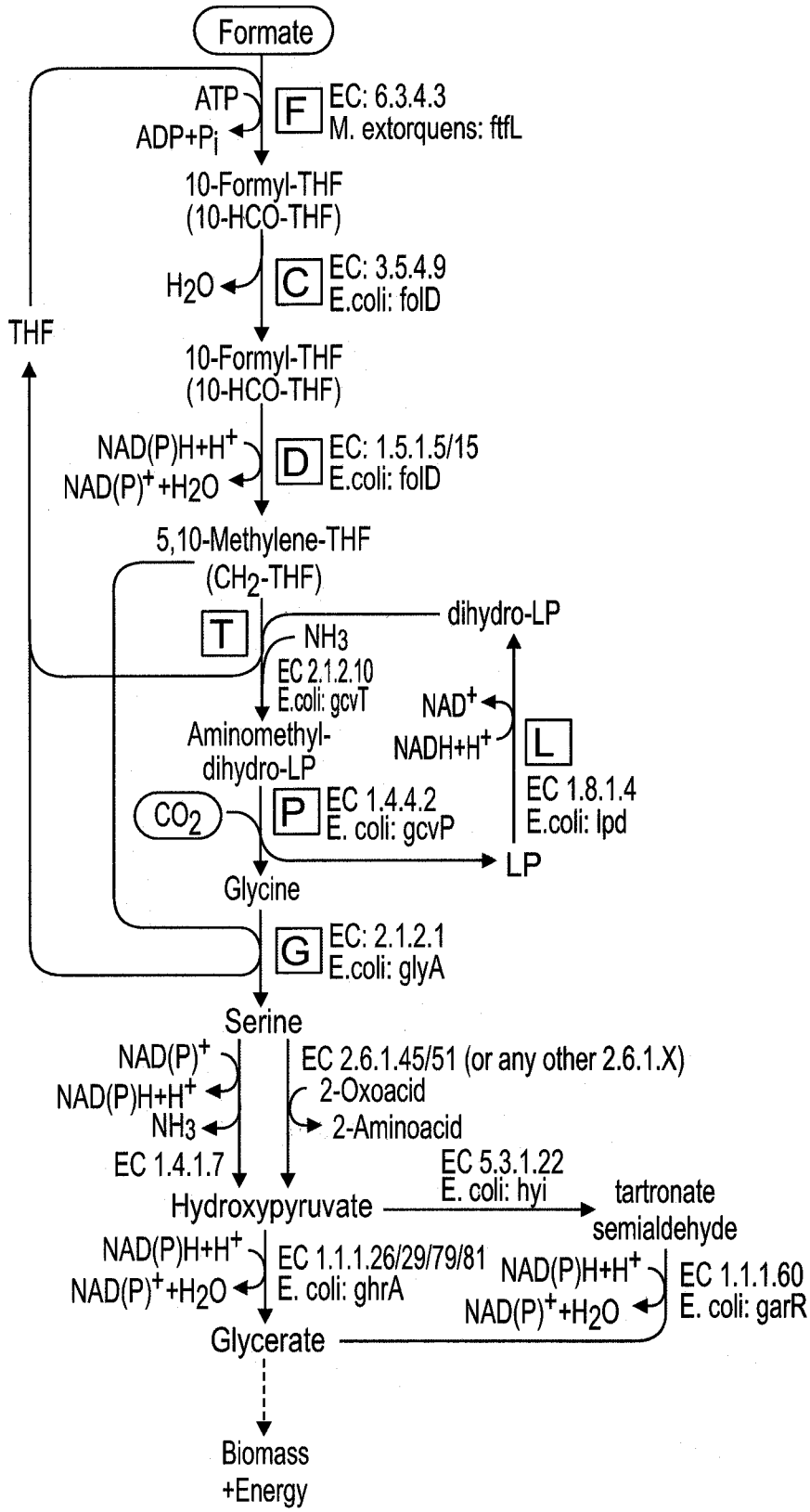

Formate
ATP
ADP+Pi
F
EC: 6.3.4.3
M. extorquens: ftfL
10-Formyl-THF
(10-HCO-THF)
H2O
C
EC: 3.5.4.9
E.coli: folD
THF
10-Formyl-THF
(10-HCO-THF)
NAD(P)H+H+
NAD(P)+ +H2O
D
EC: 1.5.1.5/15
E.coli: folD
5,10-Methylene-THF
(CH2-THF)
T
NH3
EC 2.1.2.10
E.coli: gcvT
dihydro-LP
NAD+
NADH+H+
L
EC 1.8.1.4
E.coli: lpd
Aminomethyl-
dihydro-LP
CO2
P
EC 1.4.4.2
E. coli: gcvP
LP
Glycine
G
EC: 2.1.2.1
E.coli: glyA
Serine
NH3
S
EC: 4.3.1.17/19
E.coli: sdaA
Pyruvate
Biomass
+Energy

FIG. 2


Formate
ATP
ADP+Pi
F
EC: 6.3.4.3
M. extorquens: ftfL
10-Formyl-THF
(10-HCO-THF)
H2O
C
EC: 3.5.4.9
E.coli: folD
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10-Formyl-THF
(10-HCO-THF)
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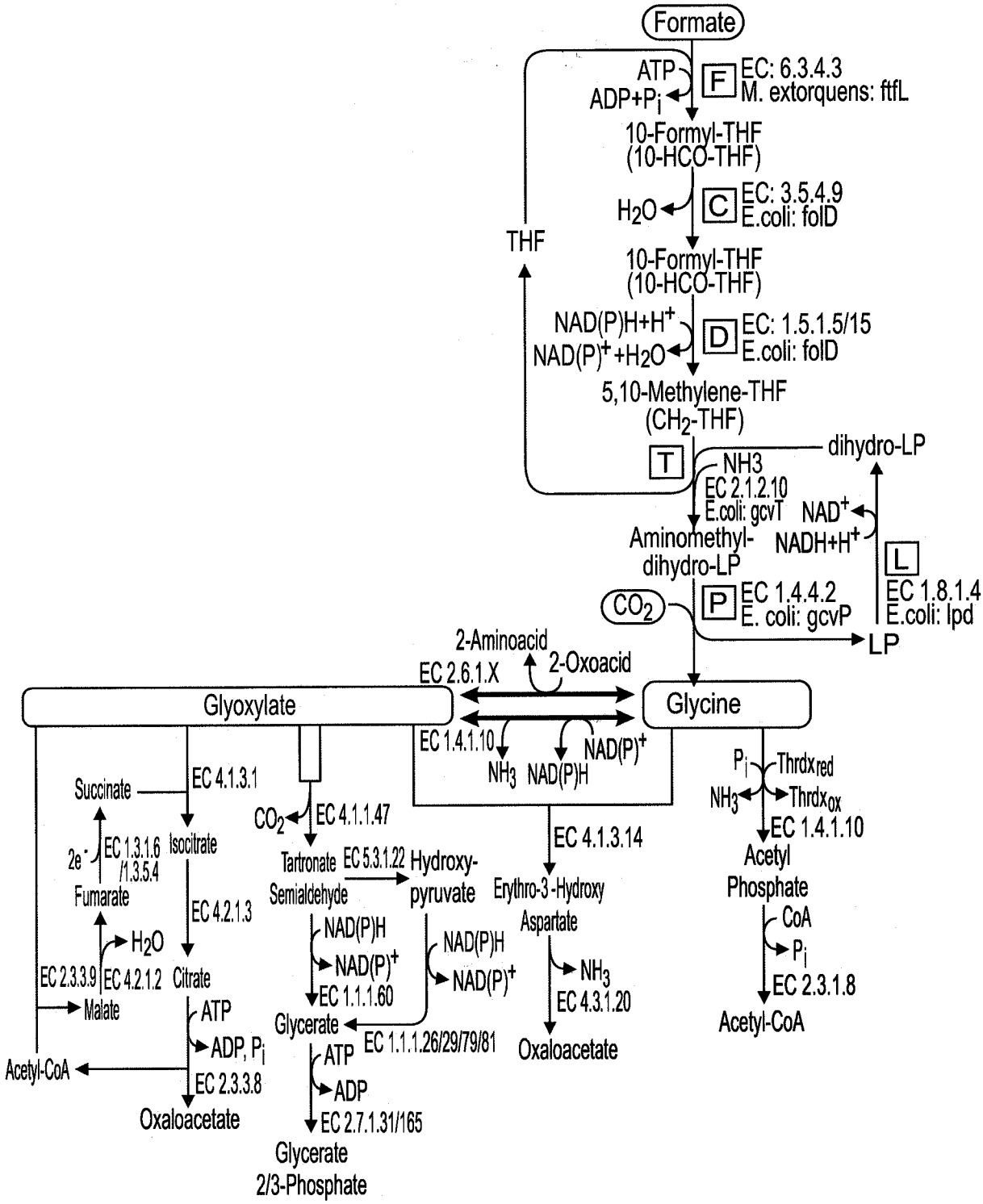
EC: 1.5.1.5/15
E.coli: folD
5,10-Methylene-THF
(CH2-THF)
T
NH3
EC 2.1.2.10
E.coli: gcvT
dihydro-LP
NAD+
NADH+H+
L
EC 1.8.1.4
E.coli: lpd
Aminomethyl-
dihydro-LP
CO2
P
EC 1.4.4.2
E. coli: gcvP
LP
Glycine
G
EC: 2.1.2.1
E.coli: glyA
Serine
NAD(P)+
NAD(P)H+H+
NH3
EC 1.4.1.7
EC 2.6.1.45/51 (or any other 2.6.1.X)
2-Oxoacid
2-Aminoacid
Hydroxypyruvate
EC 5.3.1.22
E. coli: hyi
tartronate
semialdehyde
NAD(P)H+H+
NAD(P)+ +H2O
EC 1.1.1.26/29/79/81
E. coli: ghrA
NAD(P)H+H+
NAD(P)+ +H2O
EC 1.1.1.60
E. coli: garR
Glycerate
Biomass
+Energy

FIG. 3


Formate
ATP
ADP+Pi
F
EC: 6.3.4.3
M. extorquens: ftfL
10-Formyl-THF
(10-HCO-THF)
H2O
C
EC: 3.5.4.9
E.coli: folD
THF
10-Formyl-THF
(10-HCO-THF)
NAD(P)H+H+
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EC: 1.5.1.5/15
E.coli: folD
5,10-Methylene-THF
(CH2-THF)
T
NH3
dihydro-LP
EC 2.1.2.10
E.coli: gcvT
NAD+
NADH+H+
Aminomethyl-
dihydro-LP
L
EC 1.8.1.4
E.coli: lpd
CO2
P
EC 1.4.4.2
E. coli: gcvP
LP
2-Aminoacid
2-Oxoacid
EC 2.6.1.X
Glyoxylate
Glycine
EC 1.4.1.10
NH3
NAD(P)H
NAD(P)+
Pi
NH3
Thrdxred
Thrdxox
EC 1.4.1.10
Acetyl
Phosphate
CoA
Pi
EC 2.3.1.8
Acetyl-CoA
Succinate
EC 4.1.3.1
CO2
EC 4.1.1.47
EC 4.1.3.14
2e-
EC 1.3.1.6
/1.3.5.4
Isocitrate
Tartronate
Semialdehyde
EC 5.3.1.22
Hydroxy-
pyruvate
Erythro-3-Hydroxy
Aspartate
Fumarate
EC 4.2.1.3
H2O
NAD(P)H
NAD(P)+
EC 1.1.1.60
NAD(P)H
NAD(P)+
NH3
EC 4.3.1.20
EC 2.3.3.9
EC 4.2.1.2
Citrate
Malate
ATP
ADP, Pi
Glycerate
EC 1.1.1.26/29/79/81
Oxaloacetate
Acetyl-CoA
EC 2.3.3.8
ATP
ADP
EC 2.7.1.31/165
Oxaloacetate
Glycerate
2/3-Phosphate

INTERNATIONAL SEARCH REPORT

International application No
PCT/IL2013/050643

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N9/02 C12N9/00 C12N15/52 C12P7/00 C12P7/40
ADD.

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)
C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ROGIER BRAAKMAN ET AL: "The Emergence and Early Evolution of Biological Carbon-Fixation", PLOS COMPUTATIONAL BIOLOGY, vol. 8, no. 4, April 2012 (2012-04), page e1002455, XP055090400, DOI: 10.1371/journal.pcbi.1002455 page 5, column 2, last paragraph - page 6, column 2; figure 2; table 1 -& ROGIER BRAAKMAN ET AL: "Text S1 Supporting Methods", , April 2012 (2012-04), XP055090478, Retrieved from the Internet: URL:http://files.figshare.com/335059/Text_S1.pdf [retrieved on 2013-11-27] table S1 ----- -/--	1-16,22, 24-26, 29-31, 41-43

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"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

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 December 2013	10/12/2013

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Dumont, Elisabeth

INTERNATIONAL SEARCH REPORT

International application No
PCT/IL2013/050643

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LI HAN ET AL: "Integrated Electromicrobial Conversion of CO2 to Higher Alcohols", SCIENCE (WASHINGTON D C), vol. 335, no. 6076, March 2012 (2012-03), page 1596, XP055090136, ISSN: 0036-8075 the whole document in particular Fig. 1A	40
X	US 2012/064622 A1 (FISCHER CURT R [US] ET AL) 15 March 2012 (2012-03-15) abstract paragraph [0130] - paragraph [0132] claims 1-20	1-47
A	BAR-EVEN ARREN ET AL: "Design and analysis of synthetic carbon fixation pathways", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES - PNAS, NATIONAL ACADEMY OF SCIENCES, US, vol. 107, no. 19, 11 May 2010 (2010-05-11) , pages 8889-8894, XP002638327, ISSN: 0027-8424, DOI: 10.1073/PNAS.0907176107 [retrieved on 2010-04-21] the whole document	1-47
X,P	ARREN BAR-EVEN ET AL: "Design and analysis of metabolic pathways supporting formatotrophic growth for electricity-dependent cultivation of microbes", BIOCHIMICA ET BIOPHYSICA ACTA (BBA) - BIOENERGETICS, vol. 1827, no. 8-9, 30 October 2012 (2012-10-30), pages 1039-1047, XP55090015, ISSN: 0005-2728, DOI: 10.1016/j.bbabio.2012.10.013 the whole document	1-47

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IL2013/050643

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2012064622 A1	15-03-2012	US 2012064622 A1	15-03-2012
		WO 2013066848 A1	10-05-2013