

(43) International Publication Date
24 December 2014 (24.12.2014)(10) International Publication Number
WO 2014/202695 A1(51) International Patent Classification:
CUP 7/40 (2006.01)(21) International Application Number:
PCT/EP2014/062892(22) International Filing Date:
18 June 2014 (18.06.2014)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
13 17241 1.4 18 June 2013 (18.06.2013) EP

(71) Applicant: JOHANN WOLFGANG GOETHE-UNIVERSITÄT [DE/DE]; Griineburgplatz 1, 60323 Frankfurt am Main (DE).

(72) Inventors: MÜLLER, Volker; Im Klausenstück 4, 60438 Frankfurt (DE). SCHUCHMANN, Kai; Außenring 12, 64331 Weiterstadt (DE).

(74) Agent: KRAUSS, Jan, B.; Boehmert & Boehmert Anwaltspartnerschaft mbB Patentanwälte Rechtsanwälte, Pettenkoferstrasse 20 - 22, 80336 Munich (DE).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY,

BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

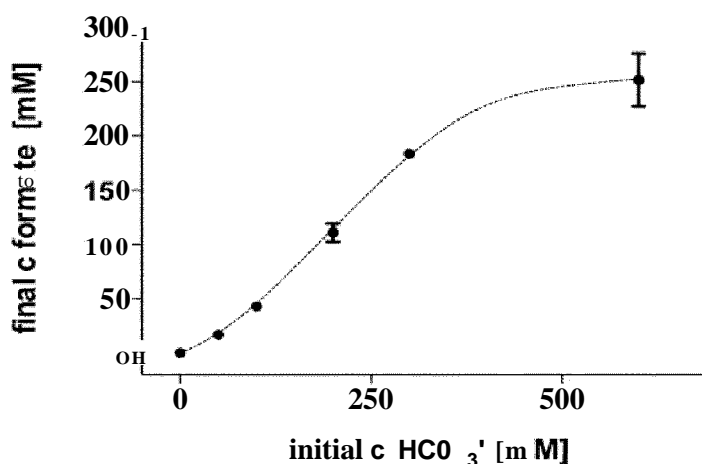
(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

- with international search report (Art. 21(3))
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))
- with sequence listing part of description (Rule 5.2(a))

(54) Title: METHOD FOR STORING GASEOUS HYDROGEN THROUGH PRODUCING METHANOATE (FORMATE)

Figure 3



(57) Abstract: The present invention relates to a method for storing gaseous hydrogen, comprising the steps of producing methanoate (formate) through contacting gaseous hydrogen with carbon dioxide in the presence of a hydrogen dependent carbondioxide reductase (HDCR), and thereby storing of said gaseous hydrogen. The HDCR and/or its complex is preferably derived from Acetobacterium woodii.

Method for storing gaseous hydrogen through producing methanoate (formate)

The present invention relates to a method for storing gaseous hydrogen, comprising the steps of producing methanoate (formate) through contacting gaseous hydrogen with carbon dioxide in the presence of a hydrogen dependent carbon dioxide reductase (HDCR), and thereby storing of said gaseous hydrogen. The HDCR and/or its complex is preferably derived from *Acetobacterium woodii*.

Background of the invention

One promising alternative to fossil fuels is hydrogen. Through its reaction with oxygen, hydrogen releases energy explosively in heat engines or quietly in fuel cells to produce water as its only byproduct. Hydrogen is abundant and generously distributed throughout the world without regard for national boundaries. Storing hydrogen in a high-energy-density form that flexibly links its production and eventual use is a key element of the hydrogen economy.

Boddien et al. (in: CO₂- "Neutral" Hydrogen Storage Based on Bicarbonates and Formates. Angew. Chem. Int. Ed., 2011 50: 6411-6414) describe a ruthenium catalyst generated in situ that facilitates the selective hydrogenation of bicarbonates and carbonates, as well as CO₂ and base, to give formates and also the selective dehydrogenation of formates back to bicarbonates. The two reactions can be coupled, leading to a reversible hydrogen-storage system.

KR 2004/0009875 describes an electrochemical preparation method of formic acid using carbon dioxide, thereby simultaneously carrying out reduction of carbon dioxide and conversion of carbon dioxide into useful organic matters. The method comprises electrochemical reduction of carbon dioxide using formic acid dehydrogenase or formic acid dehydrogenase-producing anaerobic bacteria and an electron carrier in which reversible oxidation/reduction is occurred at electric potential of -400 to -600 mV, wherein the concentration of the electron carrier is 5 to 15 mM; the anaerobic bacteria are selected from *Clostridium thermoaceticum*, *Clostridium thermoautotrophicum*, *Acetobacterium woodii*, *Acetogenium kivui*, *Clostridium aceticum*, *Clostridium ljungdahlii*, *Eubacterium limosum* or a mixture thereof; the electron carrier is selected from methylviologen, N,N-diethyl-4,4-bipyridyl, N,N-diisopropyl-4,4-

bipyridyl, 4,4-bipyridyl or a mixture thereof; the reduction temperature is 20 to 70°C; and the reduction pH is 6.0 to 7.0.

WO 2011/087380 describes methods for improving the efficiency of carbon capture in microbial fermentation of a gaseous substrate comprising CO and/or H₂; said method comprising applying an electrical potential across the fermentation. It further relates to improving the efficiency of carbon capture in the microbial fermentation of gaseous substrate comprising CO and/or H₂ to produce alcohol(s) and/or acid (s).

Catalytic processes may be used to convert gases consisting primarily of CO and/or CO and hydrogen (H₂) into a variety of fuels and chemicals. Microorganisms may also be used to convert these gases into fuels and chemicals. These biological processes, although generally slower than chemical reactions, have several advantages over catalytic processes, including higher specificity, higher yields, lower energy costs and greater resistance to poisoning.

The ability of microorganisms to grow on CO as a sole carbon source was first discovered in 1903. This was later determined to be a property of organisms that use the acetyl coenzyme A (acetyl CoA) biochemical pathway of autotrophic growth (also known as the Wood-Ljungdahl pathway and the carbon monoxide dehydrogenase / acetyl CoA synthase (CODH/ACS) pathway). A large number of anaerobic organisms including carboxydophilic, photosynthetic, methanogenic and acetogenic organisms have been shown to metabolize CO to various end products, namely CO₂, H₂, methane, n-butanol, acetate and ethanol. While using CO as the sole carbon source, all such organisms produce at least two of these end products. The Wood-Ljungdahl pathway of anaerobic CO₂ fixation with hydrogen as reductant is considered a candidate for the first life-sustaining pathway on earth because it combines carbon dioxide fixation with the synthesis of ATP via a chemiosmotic mechanism.

Schuchmann et al. (A bacterial electron-bifurcating hydrogenase. J Biol Chem. 2012 Sep 7; 287(37):31165-71) describe a multimeric [FeFe]-hydrogenase from *A. woodii* containing four subunits (HydABCD) catalyzing hydrogen-based ferredoxin reduction. Apparently, the multimeric hydrogenase of *A. woodii* is a soluble energy-converting hydrogenase that uses electron bifurcation to drive the endergonic ferredoxin reduction by coupling it to the exergonic NAD⁺ reduction.

Schiel-Bengelsdorf and Diirre (in: Pathway engineering and synthetic biology using acetogens, FEBS Letters, 2012, 586, 15, 2191) describe acetogenic anaerobic bacteria that synthesize acetyl-CoA from CO_2 or CO. Their autotrophic mode of metabolism offers the biotechnological chance to combine use of abundantly available substrates with reduction of greenhouse gases. Several companies have already established pilot and demonstration plants for converting waste gases into ethanol, an important biofuel and a natural product of many acetogens. Recombinant DNA approaches now opened the door to construct acetogens, synthesizing important industrial bulk chemicals and biofuels such as acetone and butanol. Thus, novel microbial production platforms are available that no longer compete with nutritional feedstocks.

WO 2011/028137 describes a bioreactor system for fermentation of a gaseous substrate comprising CO and optionally H_2 , or CO_2 and H_2 , to one or more products, including acid(s) and/or alcohol(s).

US 7,803,589 describes an *Escherichia coli* microorganism, comprising a genetic modification, wherein said genetic modification comprises transformation of said microorganism with exogenous bacterial nucleic acid molecules encoding the proteins cobalamide corrinoid/iron-sulfur protein, methyltransferase, carbon monoxide dehydrogenase, acetyl-CoA synthase, acetyl-CoA synthase disulfide reductase and hydrogenase, whereby expression of said proteins increases the efficiency of producing acetyl-CoA from CO_2 , CO or H_2 , or a combination thereof.

Poehlein et al. (An ancient pathway combining carbon dioxide fixation with the generation and utilization of a sodium ion gradient for ATP synthesis. PLoS One. 2012;7(3): e33439. doi:10.1371/journal.pone.0033439) describes that the synthesis of acetate from carbon dioxide and molecular hydrogen is considered to be the first carbon assimilation pathway on earth. It combines carbon dioxide fixation into acetyl-CoA with the production of ATP via an energized cell membrane. How the pathway is coupled with the net synthesis of ATP has been an enigma. The anaerobic, acetogenic bacterium *Acetobacterium woodii* uses an ancient version of this pathway without cytochromes and quinones. It generates a sodium ion potential across the cell membrane by the sodium-motive ferredoxin:NAD oxidoreductase (Rnf). The genome sequence of *A. woodii* solves the enigma: it uncovers Rnf as the only ion-motive enzyme cou-

pled to the pathway and unravels a metabolism designed to produce reduced ferredoxin and overcome energetic barriers by virtue of electron-bifurcating, soluble enzymes.

As mentioned above, hydrogen is one of the most discussed future energy sources. Methods for producing hydrogen are well known, but storage and transport of the gas is an unsolved problem. It is therefore an object of the present invention, to provide new and effective methods in order to provide for new ways to store hydrogen, in particular directly from the gaseous phase. Other objects and advantages will become apparent to the person of skill upon studying the following description and the examples of the invention.

According to a first aspect thereof, the object of the present invention is solved by providing a method for storing gaseous hydrogen, comprising the steps of producing methanoate (formate) through contacting gaseous hydrogen with carbon dioxide in the presence of a hydrogen dependent carbon dioxide reductase (HDCR), and thereby storing of said gaseous hydrogen.

The present invention is based on the surprising finding that the HDCR enzyme, and preferably a respective enzyme complex, has been found to convert gaseous $H_2 + CO_2$ directly into formate in the reaction $H_2 + CO_2 \rightarrow HCOOH$. The present biological system functions at normal, such as ambient, pressure and temperature, preferably at standard ambient temperature and pressure or at between about 20°C to about 40°C and normal pressure. The method furthermore has a high conversion rate, compared with known chemical catalysts. Also, preferably no additional energy has to be provided.

Since the reaction takes place closely to the thermodynamic equilibrium, in the reverse reaction, hydrogen can be readily released from the formate.

In contrast to the H_2 to be converted, the CO_2 can be provided in the method both in gaseous and/or solid form. Preferred is a method of the present invention, wherein the CO_2 is provided in the form of hydrogen carbonate (HCO_3^-) (see also Figure 3).

Preferred is a method according to the present invention, wherein the method does not involve electrochemical reduction, in particular of carbon dioxide. No electric energy has to be pro-

vided, and in particular no means for providing an electrical potential to a bioreactor as involved.

Preferred is a method according to the present invention, wherein said HDCR is selected from a bacterial enzyme, such as, for example FdhF1 (Acc. No. YP_005268500, SEQ ID No.1) or FdhF2 (Acc. No. YP_005268502, SEQ ID No.2) of *Acetobacterium woodii*. Also preferred are formate dehydrogenase enzymes that are at least 65% identical to the FdhF1 and/or FdhF2 enzyme, more preferably at least 70%, even more preferred at least 80%, and most preferred at least 90% identical to the FdhF1 and/or FdhF2 enzyme on the amino acid level. Preferred examples are selected from the formate dehydrogenase-H of *Clostridium difficile* 630 (Acc. No. YP_001089834.2), the formate dehydrogenase h of *Clostridium difficile* CD196 (Acc. No. YP_003216147.1), the formate dehydrogenase of *Clostridium* sp. DL-VIII (Acc. No. WP_009172363.1), the formate dehydrogenase of *Clostridium arbusti* (Acc. No. WP_010238540.1), the formate dehydrogenase of *Clostridium ragsdalei* (Acc. No. gb|AEI90724.1), the formate dehydrogenase H of *Paenibacillus polymyxa* E681 (Acc. No. YP_003871035.1), the formate dehydrogenase-H of *Clostridium difficile* 630 (Acc. No. YP_001089834.2), the formate dehydrogenase h of *Clostridium difficile* CD196 (Acc. No. YP_003216147.1), the formate dehydrogenase H of *Treponema primitia* ZAS-2 (Acc. No. ADJ1961.1.1), the formate dehydrogenase H of *Clostridium carboxidivorans* P7 (Acc. No. ADO12080.1), and the formate dehydrogenase I of *Clostridium ragsdalei* (Acc. No. gb|AEI90722.1), and mixtures thereof. All these proteins shall be understood as „homologs" of the proteins of *Acetobacterium woodii* as described herein.

Further preferred is a method according to the present invention, wherein said HDCR is part of an enzyme complex, for example with a formate dehydrogenase accessory protein, such as, for example, FdhD of *Acetobacterium woodii*, an electron transfer protein, such as, for example, HycB1 or HycB2 of *Acetobacterium woodii*, and a subunit harboring the active site characteristic of an [FeFe]-hydrogenase, such as, for example, HydA2 of *Acetobacterium woodii*. Also preferred are formate dehydrogenase accessory proteins and/or electron transfer proteins and/or [FeFe]-hydrogenase proteins, that are at least 65% identical to the HydA2, FdhD, HycB1 and/or HycB2 enzyme, more preferably at least 70%, even more preferred at least 80%, and most preferred at least 90% identical to the HydA2, FdhD, HycB1 and/or HycB2 enzyme on the amino acid level, and show an electron transfer activity, formate dehydrogen-

ase accessory protein activity, or [FeFe]-hydrogenase activity. Also these proteins shall be understood as "homologs" of the proteins of *Acetobacterium woodii* as described herein.

Particularly preferred is a method according to the present invention, wherein said HDCR is part of the enzyme complex comprising FdhF1/2, HycB1/2/3, and HydA2. Thereby, FdhF reduces CO_2 to formate, the electrons are provided by HydA2, the subunit of the H_2 -oxidation. More preferably the HDCR is a protein complex composed of the subunits FdhF1/FdhF2, HycB1/HycB2, HydA2 and HycB3. Most preferably the HDCR is selected from one of the complexes comprising FdhF1, HycB1, HydA2 and HycB3, or the complex comprising FdhF2, HycB2, HydA2 and HycB3

Further preferred is a method according to the present invention, wherein said method further comprises an inhibition of the cellular metabolism to further metabolize formate, such as an Na depletion, for example using sodium ionophores. When the metabolism of the cell is inhibited (and/or impaired), the formate as produced can no longer react further, and is advantageously produced as the final product. For the inhibition of the energy metabolism, all substances can be used that are known to the person of skill, and examples are selected from all ATPase inhibitors, such as DCCD (dicyclohexylcarbodiimide), heavy metals such as silver ions, copper ions, etc., all decoupling agents of the membrane potential, such as protonophores such as TCS (3,3',4',5-tetrachlorosalicylanilide), K-ionophores, such as valinomycin, propyl iodide as inhibitor of cobalt dependent reactions, phosphate starvation, which slows down ATP-synthesis, and tensides or substances that destroy the integrity of the membrane of the cell. It is important, that an enzyme and/or step of the energy metabolism is blocked, since this leads to an accumulation of the intermediate product. Since the HDCR is independent from the energy metabolism and does not require external electron carriers or energy, the process of formate formation can continue. This phenomenon can of course be applied both to reactions in whole cells, as well as in in vitro-reactions. The inventors have furthermore surprisingly found that the synthesis of acetyl-CoA can be stopped at formate, if Na is depleted. The system (for example bacteria) then nearly exclusively produces formate, which is used for hydrogen storage. Depletion can be achieved by using sodium-free buffers and/or media, and/or by using sodium-ionophores, such as, for example, Monensin, Gramicidin A, or the commercially available ETH 2120 (N,N,N',N'-Tetracyclohexyl-1,2-phenylenedioxydiacetamide, Selectophore™), or the like.

In another aspect of the present invention the present invention thus is based on the surprising finding that the inhibition of the cellular metabolism to further metabolize formate, such as by Na depletion (for example using sodium ionophores) can be advantageously used to produce formate. In this embodiment, the Na depletion leads to an accumulation of formate based on the effective blocking of the production of downstream products from the formate. The present invention thus further relates to a method for producing methanoate (formate) comprising contacting carbon dioxide in the presence of a hydrogen dependent carbon dioxide reductase (HDCR) under conditions that inhibit the cellular metabolism to further metabolize formate, such as, for example, under Na depletion, at an electric potential of -300 to -600 mV (e.g. using electrodes) and/or in the presence of an electron carrier. The concentration of the electron carrier can be from 5 to 15 mM, and the electron carrier can be selected from methylviologen, N,N,-diethyl-4,4-bipyridyl, N,N-diisopropyl-4,4-bipyridyl, 4,4-bipyridyl or a mixture thereof. Preferably, said HDCR is selected from a bacterial enzyme, such as, for example FdhF1 or FdhF2 of *Acetobacterium woodii*. More preferably the HDCR is a protein complex composed of at least one of the subunits FdhF1/FdhF2, HycB1/HycB2, HydA2 and HycB3. Most preferably the HDCR is selected from one of the complexes comprising FdhF1, HycB1, HydA2 and HycB3, or the complex comprising FdhF2, HycB2, HydA2 and HycB3. Further preferably, said method is performed under standard ambient temperature and pressure, or at between about 20°C to about 40°C and normal pressure. Other preferred embodiments of this method are analogous as described herein for the first aspect of the present invention.

Yet another aspect of the present invention then relates to a method according to the present invention, further comprising the step of converting carbon monoxide into carbon dioxide using a CO dehydrogenase, such as, for example a bacterial CO dehydrogenase, such as, for example AcsA of *Acetobacterium woodii*, and a ferredoxin. Also preferred are CO dehydrogenase enzymes that are at least 65% identical to the AcsA enzyme, more preferably at least 70%, even more preferred at least 80%, and most preferred at least 90% identical to the AcsA enzyme on the amino acid level, and show a CO dehydrogenase activity. All these proteins shall be understood as "homologs" of the proteins of *Acetobacterium woodii* as described herein.

In this aspect of the present invention, it was furthermore surprisingly found that the enzyme hydrogen dependent carbon dioxide reductase (HDCR) can also use carbon monoxide (via ferredoxin) as electron donor for the CO₂-reduction to formate. Thus, this enables the advan-

tageous use of synthesis gas (for example as feed-stock) for the method. Of course, this aspect of the invention also can be performed under the conditions and using the complex and enzymes as described above for the "direct" CO₂-use. Furthermore, this aspect of the method of the invention can be used to remove CO from gaseous phases, and thus can constitute a method for decontaminating CO-contaminated (or polluted) gases.

Yet another aspect of the present invention thus relates to a method for decontaminating CO-contaminated or polluted gases, comprising performing a method according to the invention as above using said CO-contaminated or polluted gas as a substrate. Preferably, said CO-contaminated or polluted gas is synthesis gas.

In the methods according to the present invention, both purified (or partially purified) enzyme(s) as well as bacterial cells can be used. Thus, methods according to the present invention can be performed *in vitro*, *in vivo* and/or in culture, for example in imidazole buffer (see below).

Most preferred is a method according to the present invention, which is performed in a bioreactor in a continuous operation or in batches. Respective methods and devices are known to the person of skill and described (for example, in Demler and Weuster-Botz; Reaction engineering analysis of hydrogenotrophic production of acetic acid by *Acetobacterium woodii*. Biotechnol Bioeng. 2011 Feb; 108(2):470-4).

Yet another aspect of the present invention thus relates to a recombinant bacterial organism comprising a genetic modification, wherein said genetic modification comprises transformation of said microorganism with exogenous bacterial nucleic acid molecules encoding the proteins FdhF1 and/or FdhF2, FdhD, HycB1 and/or HycB2, HydA2, and optionally HycB3 or AcsA of *Acetobacterium woodii*, or homologs thereof as described herein, whereby expression of said proteins increases the efficiency of producing format from CO₂, and/or CO and H₂. More preferably the nucleic acids encode at least one of the HDCR subunits FdhF1/FdhF2, HycB1/HycB2, HydA2 and HycB3. Most preferably the nucleic acids encode the proteins FdhF1, HycB1, HydA2 and HycB3, or the proteins FdhF2, HycB2, HydA2 and HycB3.

Further preferred is a method according to the present invention, further comprising the (re-combinant) expression of the genes of hydrogenase maturation as well as for a cofactor biosynthesis of the formate-dehydrogenase (e.g. described in Kuchenreuther JM, Grady-Smith CS, Bingham AS, George SJ, Cramer SP, et al. (2010) High-Yield Expression of Heterologous [FeFe] Hydrogenases in *Escherichia coli*. PLoS ONE 5(11): e15491. doi:10.1371/journal.pone.0015491).

Preferred is the use of the recombinant bacterial organism according to the present invention in a method according to the present invention as described herein.

Yet another aspect of the present invention relates to the use of a hydrogen dependent carbon dioxide reductase (HDCR), for example a bacterial enzyme, such as, for example FdhF1 or FdhF2 of *Acetobacterium woodii* or homologs thereof in a method according to the present invention as described herein. Preferred is a use, wherein said HDCR is part of an enzyme complex, for example with a formate dehydrogenase accessory protein, such as, for example, FdhD of *Acetobacterium woodii*, an electron transfer protein, such as, for example, HycB1 or HycB2 of *Acetobacterium woodii*, and a subunit harboring the active site characteristic of an [FeFe]-hydrogenase, such as, for example, HydA2 of *Acetobacterium woodii*, or homologs thereof. Further preferred is a use according to the present invention, wherein said complex further comprises a CO dehydrogenase, such as, for example a bacterial CO dehydrogenase, such as, for example AcsA of *Acetobacterium woodii*, and a ferredoxin, or homologs thereof. More preferably the HDCR is a protein complex composed of at least one of the subunits FdhF1/FdhF2, HycB1/HycB2, HydA2 and HycB3. Most preferably the HDCR is selected from one of the complexes comprising FdhF1, HycB1, HydA2 and HycB3, or the complex comprising FdhF2, HycB2, HydA2 and HycB3.

The following figures, sequences, and examples merely serve to illustrate the invention and should not be construed to restrict the scope of the invention to the particular embodiments of the invention described in the examples. For the purposes of the present invention, all references as cited in the text are hereby incorporated in their entireties.

Figure 1 shows the productions of formate using whole cell catalysis. Cell suspensions of *A. woodii* (1 mg/ml) were incubated using a gas phase of 0.8×10^5 Pa H_2 and 0.2×10^5 Pa CO_2

(A). Adding the Na^+ ionophore ETH2120 (30 μM) gives rise to the production of up to 8 mM formate and the production of acetate stopped (B).

Figure 2 shows the production of formate using HCO_3^- or CO_2 as substrate. Cell suspensions of *A. woodii* (1 mg/ml) were incubated using a gas phase of 0.8×10^5 Pa H_2 and 0.2×10^5 Pa CO_2 or 1×10^5 Pa H_2 with 300 mM KHCO_3 .

Figure 3 shows the relationship of final formate concentration to initial HCO_3^- . Cell suspensions of *A. woodii* (1 mg/ml) were incubated with increasing amounts of initial HCO_3^- and a gas phase of 1×10^5 Pa H_2 .

Sequence ID NOs. 1 to 8 show the amino acid sequences of the enzymes FdhFl, HycBl, FdhF2, HycB2, FdhD, HycB3, HydA2, and AcsA of *A. woodii*, respectively.

Examples

Measurements with the isolated HCDR

For the purification of HCDR *A. woodii* (DSM 1030) was grown at 30°C under anaerobic conditions in 20-l-liter flasks using 20 mM fructose to an OD_{600} of -2.5. All buffers used for preparation of cell extracts and purification contained 2 mM DTE and 4 μM resazurin. All purification steps were performed under strictly anaerobic conditions at room temperature in an anaerobic chamber filled with 100% N_2 and 2-5% H_2 . The cell free extract was prepared as described previously (Schuchmann et al, J Biol Chem. 2012 Sep 7; 287(37):3 1165-71). Membranes were removed by centrifugation at 130000 g for 40 minutes. Part of the supernatant containing the cytoplasmic fraction with approximately 1600 mg protein was used for the further purification. Ammonium sulfate (0.4 M) was added to the cytoplasmic fraction. Half of this sample was loaded onto a Phenyl-Sepharose high performance column (1.6 cm x 10 cm) equilibrated with buffer A (25 mM Tris/HCl, 20 mM MgSO_4 , 0.4 M $(\text{NH}_4)_2\text{SO}_4$, 20% glycerol, pH 7.5). Methylviologen-dependent formate dehydrogenase activity eluted around 0.33 M $(\text{NH}_4)_2\text{SO}_4$ in a linear gradient of 120 ml from 0.4 M to 0 M $(\text{NH}_4)_2\text{SO}_4$. This step was repeated with the second half of the sample in a separate run to gain more protein since otherwise large amounts of the activity eluted in the flowthrough. The pooled fractions of both runs were diluted to a conductivity of below 10 mS/cm with buffer C (25 mM Tris/HCl, 20 mM MgSO_4 , 20% glycerol, pH 7.5) and applied to a Q-Sepharose high performance column

(2.6 cm x 5 cm) equilibrated with buffer C. Protein was eluted with a linear gradient of 160 ml from 150 mM to 500 mM NaCl. Formate dehydrogenase eluted at around 360 mM NaCl. Pooled fractions were concentrated using ultrafiltration in 100-kDa VIASPIN tubes and applied to a Superose 6 10/300 GL preppacked column equilibrated with buffer C and eluted at a flow rate of 0.5 ml/min. Formate dehydrogenase activity eluted as a single peak. Pooled fractions were stored at 4°C.

Measurements of HCDR activity were performed at 30°C with in buffer 1 (100 mM HEPES/NaOH, 2 mM DTE, pH 7.0) in 1.8 ml anaerobic cuvettes sealed by rubber stoppers, containing 1 ml buffer and a gas phase of 0.8×10^5 Pa H_2 and 0.2×10^5 CO_2 . Production of formate was measured using Formate dehydrogenase of *Candida boidinii* with 2 mM NAD in the assay and production of NADH was followed.

For measurements with ferredoxin as electron carrier Ferredoxin was purified from *Clostridium pasteurianum*. For reduction of ferredoxin CO dehydrogenase of *A. woodii* was purified and in these experiments the gas phase of the cuvettes was changed to 100 % CO (1.1×10^5 Pa).

Whole cells

For experiments with whole cells, the inventors used cell suspensions of *A. woodii* for the conversion of H_2 and CO_2 to formate. The energy metabolism of *A. woodii* is strictly sodium ion dependent, and the ATP synthase uses Na^+ as the coupling ion. Thus, by omitting sodium ions in the buffer or by adding sodium ionophores (the inventors used the ionophore ETH2120 in this study), it is possible to switch off the energy metabolism specifically. Cells suspended in imidazole buffer (50 mM imidazole, 20 mM $MgSO_4$, 20 mM KCl, 4 mM DTE, pH 7.0) containing 20 mM NaCl converted $H_2 + CO_2$ to acetate, and only small amounts of formate were produced from a gas phase of 0.8×10^5 Pa H_2 and 0.2×10^5 CO_2 . By adding ETH2120 (30 μ M), acetate production ceased almost completely and formate was produced with an initial rate of 2 μ mol/min x mg cell protein (Figure 1). In agreement with the results obtained from the purified enzyme, formate production was also observed when hydrogen was absent and the electron donor was CO but with lower rates compared to hydrogen as electron donor.

The results in Figure 1 demonstrate that a maximal amount of around 8 mM of formate was produced in this experiment. The inventors next tested, if the final formate concentration is proportional to the initial gas pressure. From 0.5 to 2×10^5 Pa $H_2 + CO_2$ the final formate

concentration did not increase. Since the inventors observed a pH drop during the experiment, the inventors examined if the lower pH is the limiting factor. Increasing the buffer concentration from 50 to 200 mM resulted in a final formate concentration of around 14 ± 3 mM. If CO_2 was exchanged with KHCO_3 the effect was even more dramatic. By using the base HCO_3^- the overall process is almost pH neutral compared to the production of formic acid from CO_2 . The genome of *A. woodii* encodes for a carboanhydrase that allows the rapid interconversion of CO_2 and HCO_3^- . Figure 2 shows the production of formate from initially 300 mM KHCO_3 (with 1×10^5 Pa H_2) compared to CO_2 as substrate. Finally up to 184 ± 5 mM formate were produced with KHCO_3 .

The relationship of the final formate concentration to the initial concentration of HCO_3^- is shown in Figure 3. Up to 300 mM HCO_3^- , the final formate concentration increases with increasing substrate concentration. Furthermore the final formate concentration fits well to the theoretic thermodynamic limit of the reaction underlining the independence of the carboxylation of $\text{CO}_2/\text{HCO}_3^-$ from other cellular processes. At 1×10^5 Pa H_2 , the thermodynamic equilibrium is approximately $[\text{HCO}_3^-] = [\text{HCOOH}]$, so equimolar concentrations of substrate and product. At concentrations of HCO_3^- above 300 mM, this relationship does not exist anymore and the final amount of formate produced ceased around 300 mM.

Claims

1. A method for storing gaseous hydrogen, comprising the steps of producing methanoate (formate) through contacting gaseous hydrogen with carbon dioxide in the presence of a hydrogen dependent carbon dioxide reductase (HDCR), and thereby storing of said gaseous hydrogen.
2. The method according to claim 1, wherein said HDCR is selected from a bacterial enzyme, such as, for example FdhF1 or FdhF2 of *Acetobacterium woodii*.
3. The method according to claim 1 or 2, wherein said HDCR is part of an enzyme complex, for example with a formate dehydrogenase accessory protein, such as, for example, FdhD of *Acetobacterium woodii*, an electron transfer protein, such as, for example, HycB1 or HycB2 of *Acetobacterium woodii*, and a subunit harboring the active site characteristic of an [FeFe]-hydrogenase, such as, for example, HydA2 of *Acetobacterium woodii*.
4. The method according to any of claims 1 to 3, wherein said method is performed under standard ambient temperature and pressure or at between about 20°C to about 40°C and normal pressure.
5. The method according to any of claims 1 to 4, wherein said method further comprises an inhibition of the cellular metabolism to further metabolize formate, such as a Na depletion, for example using sodium ionophores.
6. The method according to any of claims 1 to 5, further comprising the step of converting carbon monoxide into carbon dioxide using a CO dehydrogenase, such as, for example a bacterial CO dehydrogenase, such as, for example AcsA of *Acetobacterium woodii*, and a ferredoxin.
7. The method according to any of claims 1 to 6, wherein said method is performed *in vitro*, *in vivo* and/or in culture.

8. The method according to any of claims 1 to 7, further comprising the release of hydrogen from the methanoate as produced.
9. A method for decontaminating CO-contaminated or polluted gases, comprising performing a method according to any of claims 5 to 7 using said CO-contaminated or polluted gas as a substrate.
10. The method according to claim 9, wherein said CO-contaminated or polluted gas is synthesis gas.
11. A recombinant bacterial organism comprising a genetic modification, wherein said genetic modification comprises transformation of said microorganism with exogenous bacterial nucleic acid molecules encoding the proteins FdhF1 and/or FdhF2, FdhD, HycB1 and/or HycB2, and HydA2, HycB3, and optionally AcsA of *Acetobacterium woodii*, or homologs thereof, whereby expression of said proteins increases the efficiency of producing format from CO₂, and/or CO and H₂.
12. Use of the recombinant bacterial organism according to claim 11 in a method according to any of claims 1 to 10.
13. Use of a hydrogen dependent carbon dioxide reductase (HDCR), for example a bacterial enzyme, such as, for example FdhF1 or FdhF2 of *Acetobacterium woodii* or homologs thereof in a method according to any of claims 1 to 10.
14. The use according to claim 13, wherein said HDCR is part of an enzyme complex, for example with a formate dehydrogenase accessory protein, such as, for example, FdhD of *Acetobacterium woodii*, an electron transfer protein, such as, for example, HycB1 or HycB2 of *Acetobacterium woodii*, and a subunit harboring the active site characteristic of an [FeFe]-hydrogenase, such as, for example, HydA2 of *Acetobacterium woodii*, or homologs thereof.
15. The use according to claim 14, wherein said complex further comprises a CO dehydrogenase, such as, for example a bacterial CO dehydrogenase, such as, for example AcsA of *Acetobacterium woodii*, and a ferredoxin, or homologs thereof.

Figure 1

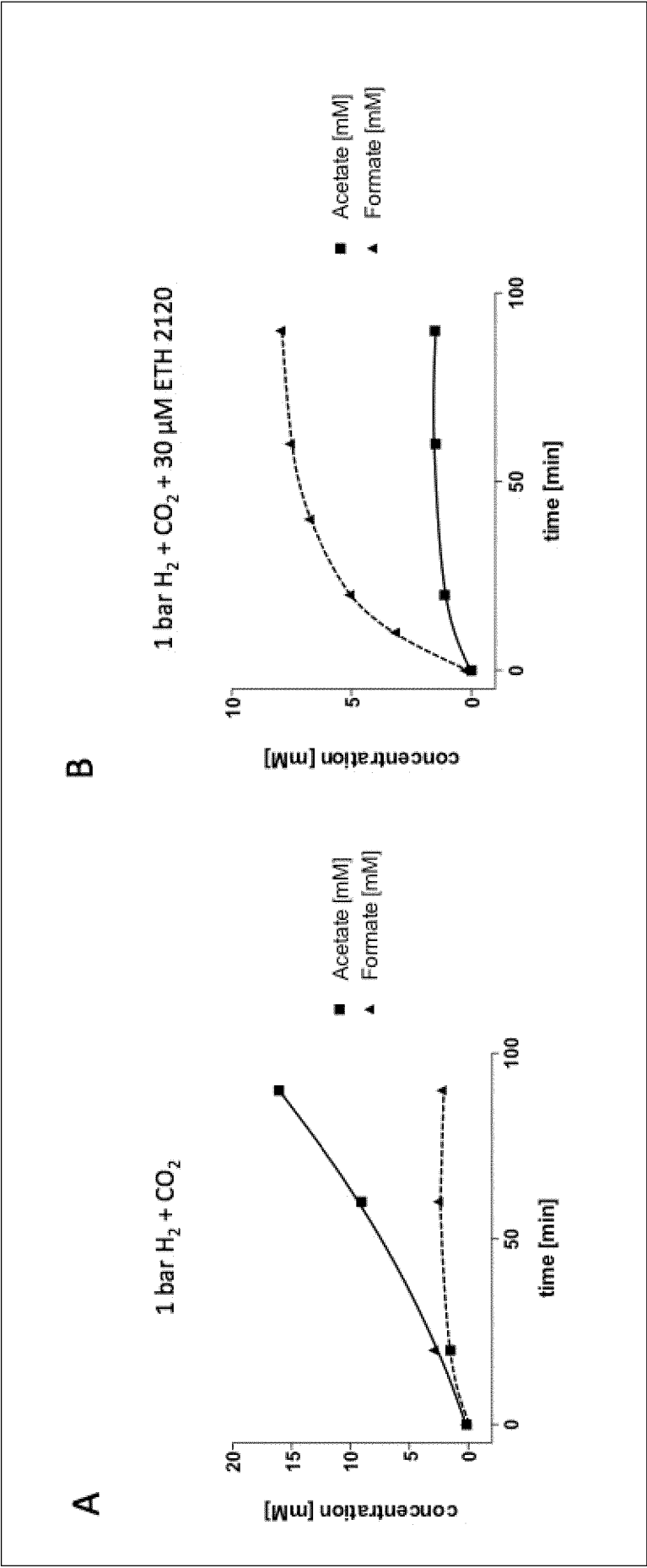


Figure 2

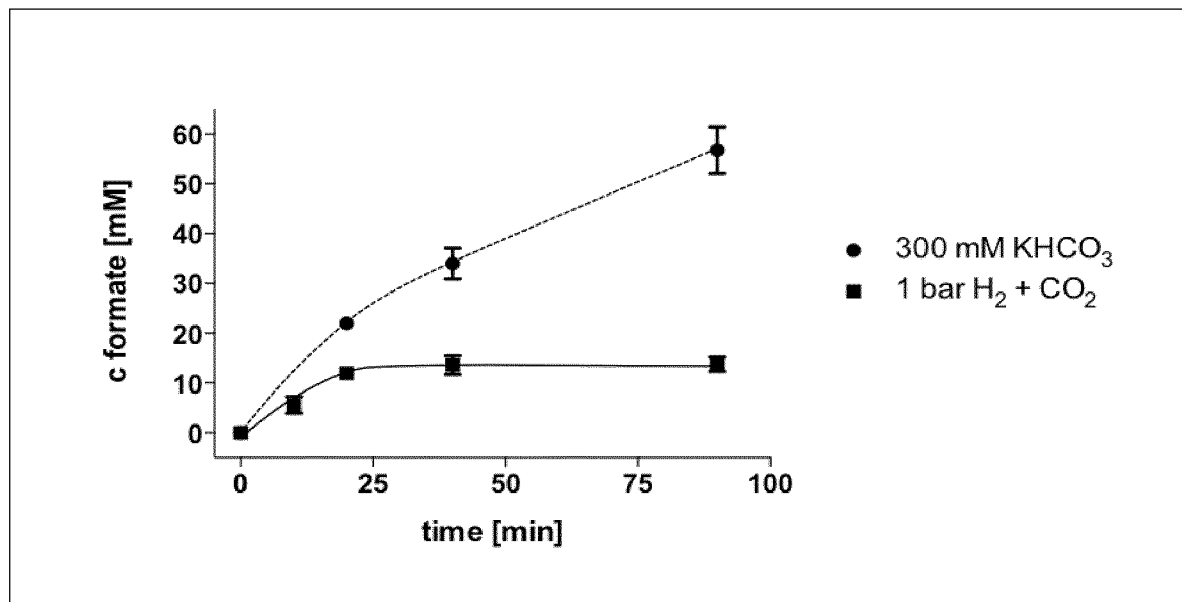
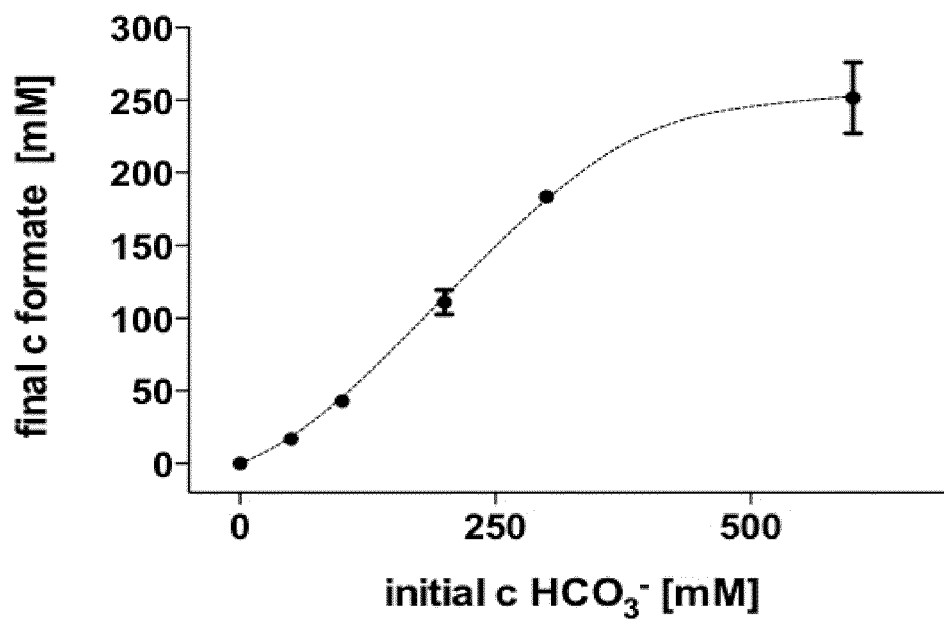


Figure 3



INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2014/062892

A. CLASSIFICATION OF SUBJECT MATTER

INV. 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)

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 , FSTA, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HULL ET AL: "Reversible hydrogen storage using CO ₂ and a proton-switchable iridium catalyst in aqueous media under mild temperatures and pressures" , NATURE CHEMISTRY, vol . 4, 2012, pages 383-388, XP002730516, * See pages 383-384 (Abstract and Introduction) *	1-15
Y	BODDIEN ET AL: "CO ₂ - "neutral " hydrogen storage based on bicarbonates and formates", ANGEWANDTE CHEMIE INTERNATIONAL EDITION , vol . 50, 2011, pages 6411-6414, XP055001843 , cited in the application * See page 6411 (and reference 8) * ----- -/-	1-15



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

2 October 2014

Date of mailing of the international search report

14/10/2014

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

Korsner, Sven-Erik

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2014/062892

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>LAURENCZY: "Hydrogen storage and delivery: The carbon dioxide - formic acid couple", CHIMIA, vol . 65, 2011, pages 663-666, XP008163293, * See page 663 (Abstract) *</p> <p>-----</p>	1-15
Y	<p>PETERS ET AL: "Transient production of formate during chemolithotrophic growth of anaerobic microorganisms on hydrogen", CURRENT MICROBIOLOGY, vol . 38, 1999, pages 285-289, XP002715917, * See page 285 (Abstract) *</p> <p>-----</p>	1-15
Y	<p>STRAUB: "Fermentative Acetatprodukte von durch Homoacetat-Gärung bzw. Acetatbildung", Dissertation / Thesis; Universität Ulm, 2012, page 86, 102, XP002715918, Ulm, Germany Retrieved from the Internet: URL: http://vts.uni-ulm.de/docs/2012/8114/vts_8114_11834.pdf [retrieved on 2013-11-05] * See esp. Figure 27(c); only these two pages are cited here *</p> <p>-----</p>	1-15
Y	<p>POEHLEIN ET AL: "An ancient pathway combining carbon dioxide fixation with the generation and utilization of a sodium ion gradient for ATP synthesis", PLOS ONE, vol . 7, E33439, 2012, pages 1-8, XP002715892, cited in the application * See page 2 (Figure 1) *</p> <p>-----</p>	1-15
Y	<p>SCHUCHMANN ET AL: "A bacterial electron-bifurcating hydrogenase", THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol . 287, 2012, pages 31165-31171, XP002715893, cited in the application * See pages 31165-31166 (Introduction) *</p> <p>-----</p>	1-15
X, P	<p>SCHUCHMANN ET AL: "Direct and reversible hydrogenation of CO₂ to formate by a bacterial carbon dioxide reductase", SCIENCE, vol . 342, 13 December 2013 (2013-12-13), pages 1382-1385, XP002730518, * Publication of the invention *</p> <p>-----</p> <p style="text-align: center;">-/--</p>	1-15

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2014/062892

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	<p>PEREIRA: "An enzymatic route to H₂ storage" , SCIENCE, vol . 342 , 13 December 2013 (2013-12-13) , pages 1329-1330, XP002730517 , * See page 1329 (Figure) *</p> <p>-----</p>	1-15
Y, P	<p>BELLER ET AL: "CO₂ fixation through hydrogenation by chemical or enzymatic methods" , ANGEWANDTE CHEMIE, INTERNATIONAL EDITION , vol . 53 , 6 April 2014 (2014-04-06) , pages 4527-4528, XP002730519 , * See both pages ; early online publication *</p> <p>-----</p>	1-15
Y, P	<p>ALISSANDRATOS ET AL: "Formate production through biocatalysis" , BIOENGINEERED, vol . 4 , 21 June 2014 (2014-06-21) , pages 348-350, XP002730520, * See page 348 (Abstract) *</p> <p>-----</p>	1-15
L	<p>SCHUCHMANN: "An electron bifurcating hydrogenase in the acetogenic bacterium Acetobacterium woodii" , Gordon Research Conferences ; Lewiston , Maine, U.S.A. Molecular basis of microbial one-carbon metabolism, 2012 , pages 1-5 , XP002730521 , Retrieved from the Internet: URL: http://www.grc.org/programs.aspx?year=2012&program=mol basis [retrieved on 2014-09-30] * The full disclosure at the conference may possibly be of relevance (?) *</p> <p>-----</p>	