Biofuels: Microbially Generated Methane and Hydrogen

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Article Contents

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The production of methane (CH_4) or hydrogen (H_2) from renewable biomass by microorganisms growing anaerobically has the potential for contribution to independence from fossil fuels. Anaerobes function in Nature by converting biomass to CH_4 through food chains comprised of fermentative and acetogenic species, which decompose the complex biomass to H_2, formate and acetate that are further metabolised to CH_4 by methanogens. Methanogens reduce the concentration of products to levels that permit the initial decomposition of biomass by fermentative and acetogenic species. Current H_2 production relies extensively on energy-intensive fossil fuel sources. Photosynthetic and fermentative species offer more efficient routes for H_2 production. Although fermentatives have significantly higher production rates, they have lower yields of H_2 but may be a source of other valuable compounds that are synthesised along with H_2. Further research must be conducted on obtaining H_2 from reductive pools of NAD(P)H to increase yields and increase economic competitiveness.

Introduction

Microbial production of hydrogen (H_2) and methane (CH_4) from renewable biomass has the potential to contribute to reducing dependence on fossil fuels. H_2 is a major intermediate, and CH_4 a final product, of the microbial decomposition of plant biomass in O_2-free (anaerobic) environments of the Earth's biosphere, an essential link in the global carbon cycle. **See also**: Methanogenesis: Ecology

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The cycle begins with CO_2 fixed into biomass via photosynthesis (step 1) (**Figure 1**). Aerobic (O_2-requiring) microbes, living in oxygenated environments, completely oxidise the biomass to CO_2 (step 2). However, a significant portion of the biomass enters anaerobic environments (step 3) such as termite hind guts, wetlands, rice paddy soils and the rumen of livestock, where anaerobic microbes (anaerobes) digest the biomass to CO_2 and CH_4 (steps 4, 5 and 6). The process in freshwater environments involves a food chain comprised of a minimum of three metabolic groups of anaerobes from the domains *Bacteria* and *Archaea*. The primary group (fermentative species) decomposes the biomass primarily to butyrate, propionate, acetate, formate and H_2 plus CO_2 (step 4). The secondary group (acetogens) converts butyrate and propionate to acetate, CO_2 and H_2 or formate for growth (step 5). However, the change in free energy of these conversions prohibits growth under standard conditions of equimolar reactants and

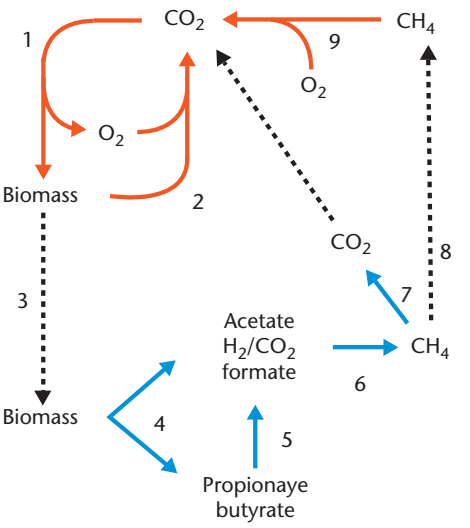

Figure 1 The global carbon cycle. Aerobic O_2-requiring conversions are shown in solid red arrows and anaerobic conversions in solid blue arrows. Black dotted arrows symbolise diffusion of substrates and products across the interface of aerobic and anaerobic zones.

Table 1 Reactions involved in the syntrophic metabolism of H_2-producing acetogens and methanogens

Reactions	ΔG° (kJ/mol)
$Propionate^- + 3H_2O \rightarrow Acetate^- + HCO_3^- + H^+ + 3H_2$	+76.1
$Butyrate^- + 2H_2O \rightarrow 2\ Acetate^- + H^+ + 2H_2$	+48.6
$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$	−135.6
$4\ Formate^- + H^+ + H_2O \rightarrow 3HCO_3^- + CH_4$	−130.4
$Acetate^- \rightarrow HCO_3^- + H^+ + CH_4$	−36.0

products (**Table 1**) which requires the tertiary CH_4-producing group (methanogens) to metabolise the products of the acetogens down to levels permitting their growth (step 6). The methanogens are incapable of metabolising complex substrates and rely on the primary and secondary metabolic groups to supply their growth substrates. The methanogens produce CH_4 by one of the two major pathways (**Table 1**). In the CO_2 reduction pathway, formate or H_2 is oxidised to provide the electrons for reduction of CO_2 to CH_4. In the aceticlastic pathway, acetate is cleaved with the carbonyl group oxidised to CO_2, providing the electron pair needed to reduce the methyl group to CH_4. The aceticlastic group is responsible for approximately two-thirds of the total CH_4 produced in most anaerobic environments, with the remaining one-third produced by the reduction of CO_2 with H_2 or formate. Some of the CH_4 is oxidised to CO_2 (step 7) by a consortium of anaerobes that reduces either sulfate, nitrate, manganese or iron (Thauer, 2010). The remaining CH_4 diffuses into aerobic environments (step 8), where O_2-requiring methanotrophic microbes oxidise it to CO_2, closing the carbon cycle (step 9).

Methane

The complete conversion of biomass via anaerobic microbial food chains produces a combustible 1:1 ratio of CH_4:CO_2 called biogas, a promising process for large-scale biogas production from renewable plant biomass that reduces dependence on fossil fuels. On a weight-for-weight basis, the energy content of CH_4 is approximately 3-fold greater than that of H_2, and CH_4 is stored and transported in a more efficient and safe manner. Improvement of the rate and reliability of the process is largely dependent on understanding the physiology of methanogens that function syntrophically with other members of anaerobic microbial food chains.

Reactions common to both the CO_2-reducing and aceticlastic pathways

Both pathways share reactions 10–12 producing CH_4 by reducing the methyl group from either methyl-tetrahydrosarcinapterin (CH_3-H_4SPT) in the aceticlastic pathway or methyl-tetrahydromethanopterin (CH_3-H_4MPT) in the CO_2 reduction pathway (**Figure 2**). The cofactors H_4SPT and H_4MPT are functionally equivalent analogues of tetrahydrofolate. Reaction 10 is catalysed by CH_3-H_4M(S)PT:coenzyme M (HS-CoM) methyltransferase (Mtr), a membrane-bound complex that couples the exergonic methyl transfer to translocation of sodium outside the cytoplasmic membrane, generating an electrochemical gradient that drives adenosine triphosphate (ATP) synthesis. Methyl-coenzyme M reductase (Mcr) catalyses reaction 11 (**Figure 2**), wherein the methyl group of CH_3-S-CoM is reduced to CH_4 with HS-CoB generating CoM-S-S-CoB that is reduced to HS-CoB and HS-CoM in reaction 12 (**Figure 2**) catalysed by heterodisulfide reductase (Hdr). Electrons for this reduction are derived from ferredoxin in the aceticlastic pathway and from H_2 or formate in the CO_2 reduction pathway. **See also**: Methanogenesis Biochemistry

Reactions unique to the aceticlastic pathway

Methyl-H_4SPT is synthesised by reactions 1–4 (**Figure 2**) in the aceticlastic pathway of *Methanosarcina* species. Homologues of enzymes catalysing these reactions play important roles in the fermentative and acetogenic groups. For example, acetate kinase and phosphotransacetylase, which catalyse the reverse of reactions 1 and 2 (**Figure 1**), are the primary energy-conserving enzymes of the fermentative and acetogenic groups (**Figure 1**) converting acetyl-CoA to ATP and acetate. Reaction 3 is central to the aceticlastic pathway of methanogenesis catalysed by the CO dehydrogenase/acetyl-CoA synthase complex that cleaves the C–C and C–S bonds of acetyl-CoA, transferring the methyl group to H_4SPT and oxidising the carbonyl group to CO_2 with transfer of electrons to ferredoxin. The conversion of acetate to CH_4 and CO_2 provides only a marginal amount of energy available for growth ($\Delta G^{\circ\prime} = -36$ kJ/CH_4). Thus, it is postulated that a carbonic anhydrase is located outside the cell membrane, where it hydrates CO_2 to membrane-impermeable HCO_3^- (**Figure 2**, reaction 4) facilitating removal of CO_2 from the cytoplasm that enhances the available energy (Zimmerman *et al.*, 2010). *Methanosaeta* (f. *Methanothrix*) is the only genus other than *Methanosarcina* utilising acetate for growth and methanogenesis. Reactions leading from acetate to steps 10–12 (**Figure 2**) are similar for both genera with the exception that acetate thiokinase catalyses a one-step conversion of acetate to acetyl-CoA in *Methanosaeta* species (Smith and Ingram-Smith, 2007).

All acetotrophic methanogens obtain energy for growth by coupling electron transfer from ferredoxin to CoM-S-S-CoB with generation of a proton gradient that drives ATP

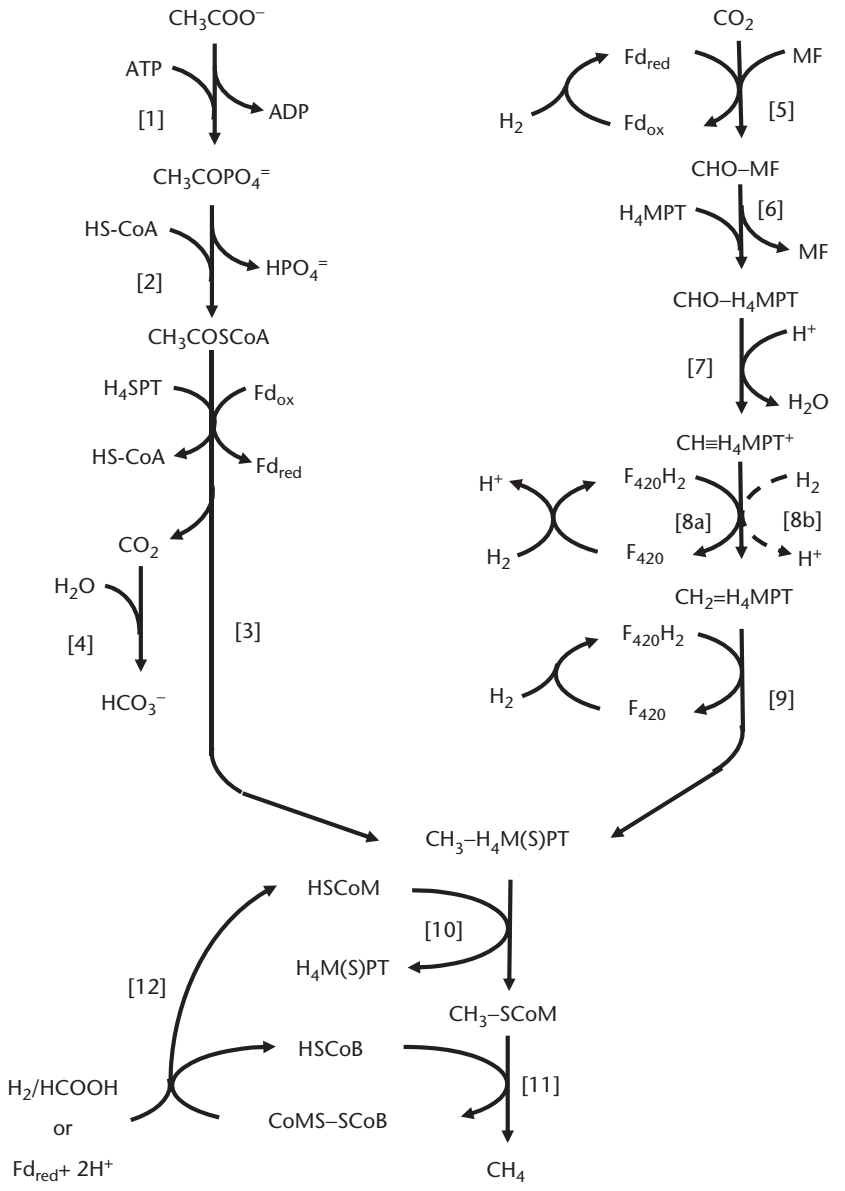

Figure 2 Composite of CO_2 reduction and aceticlastic methane-producing pathways. The left arm leading to CH_3-H_4M(S)PT shows reactions (1–4) unique to the aceticlastic pathway and the right arm leading to CH_3-H_4M(S)PT shows reactions (5–9) unique to the CO_2 reduction pathway. Both pathways have in common reactions (10, 11 and 12) leading to the formation of CH_4 from the methyl groups of CH_3-H_4M(S)PT. *Abbreviations*: ATP, adenosine triphosphate; H_4SPT, tetrahydrosarcinapterin; H_4MPT, tetrahydromethanopterin; Fd, ferredoxin; CoA, coenzyme A; CoM, coenzyme M; CoB, coenzyme B; MF, methanofuran; F_{420}, coenzyme F_{420}.

synthesis (Wang *et al*., 2011). In freshwater *Methanosarcina* species (**Figure 3a**), ferredoxin donates electrons to a membrane-bound hydrogenase (Ech) that evolves H_2 and generates a proton gradient driving ATP synthesis. It is proposed that a membrane-bound F_{420}-nonreducing hydrogenase (Vho) reoxidises H_2 and donates electrons to a quinone-like electron carrier methanophenazine (MP) that mediates electron transfer to CoM-S-S-CoB while translocating protons and contributing further to the proton gradient. Most acetate-utilising *Methanosarcina* species do not metabolise H_2; rather, it appears that these species contain an electron transfer complex (Rnf) first described in *Rhodobacter capsulatus* from the domain *Bacteria* (**Figure 3b**). Thus, it is anticipated that the Rnf complex is an acceptor of electrons from ferredoxin and donor to MP accompanied by translocation of either protons or sodium ions contributing to the gradient driving ATP synthesis. However, *Methanosaeta* species do not encode an Rnf complex, and are incapable of metabolising H_2, indicating an alternative pathway for transfer of

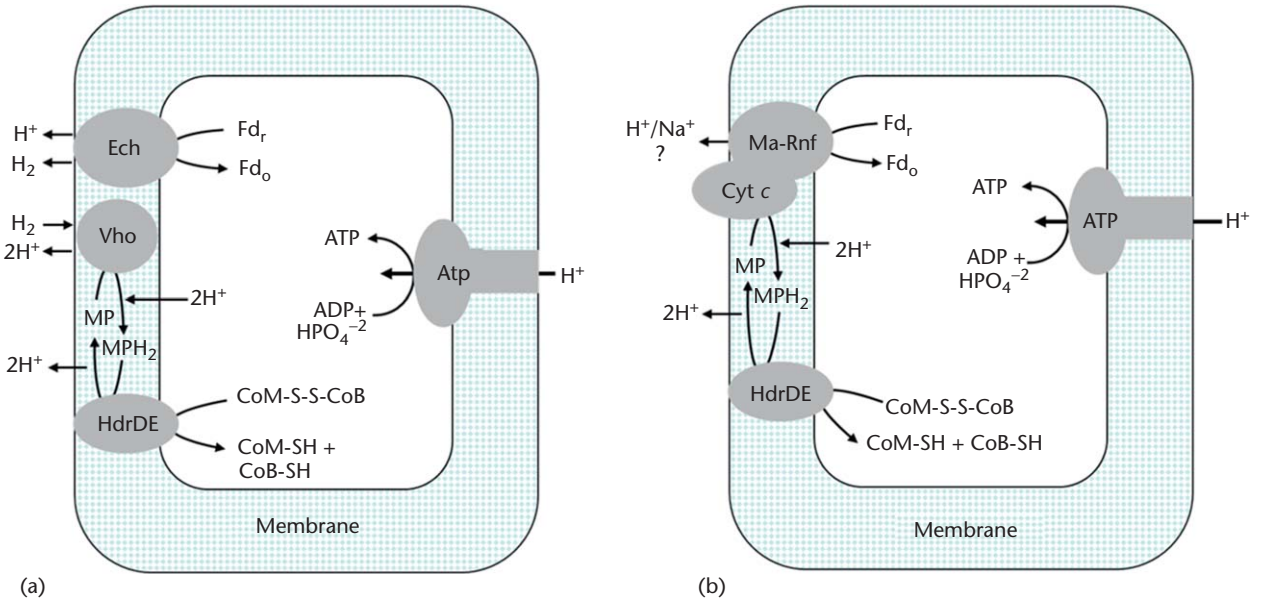

Figure 3 Comparison of electron transport pathways in acetotrophic methanogens. (a) H_2-dependent. (b) H_2-independent. *Abbreviations*: Ech, Ech hydrogenase; Fd_r, ferredoxin reduced; Fd_o, ferredoxin oxidised; Vho, Vho hydrogenase; MP, methanophenazine; HdrDE, heterodisulfide reductase; CoM-SH, coenzyme M; CoB-SH, coenzyme B; Atp, ATP synthase; Cyt *c*, cytochrome *c*; MaRnf, Rnf complex from *Methanosarcina acetivorans*; Mrp, putative sodium/proton antiporter.

electrons from the carbonyl carbon of acetyl-CoA to CoM-S-S-CoB, generating ion gradients that drive ATP synthesis (Smith and Ingram-Smith, 2007).

Reactions unique to the CO_2 reduction pathway

The CO_2-reducing methanogens are further divided into two metabolic groups: obligate CO_2-reducing species that only reduce CO_2 to CH_4 with either H_2 or formate and a few CO_2-reducing *Methanosarcina* species that reduce CO_2 to CH_4 with either H_2 or CO but also grow and produce CH_4 from acetate. Both groups reduce CO_2 via reactions 5–9 (**Figure 2**) to a methyl group bound either to H_4MPT in obligate CO_2 reducers or H_4SPT in *Methanosarcina* species. The three electron pairs required for reactions 5, 8 and 9 originate from oxidation of H_2, CO or formate with reduction of ferredoxin or coenzyme F_{420} serving as electron carriers. In the few *Methanosarcina* species that are able to metabolise H_2, CO is first oxidised to H_2 and CO_2, whereas in most *Methanosarcina* species the oxidation of CO is coupled to reduction of ferredoxin and F_{420}, avoiding H_2 as an intermediate (Lessner *et al.*, 2006). The utilisation of formate is limited to obligate CO_2-reducing species. Although enzyme systems are known that convert formate to H_2 and CO_2, the role of H_2 as an intermediate during growth with formate remains uncertain (Lupa *et al.*, 2008; Hendrickson and Leigh, 2008).

Reaction 5 (**Figure 2**) catalysed by formyl-methanofuran (MF) dehydrogenase is endergonic in the environment where partial pressures of H_2 are 1–10 Pa and therefore requires energy input for the forward reaction (Thauer *et al.*, 2008). The Ech hydrogenase of *Methanosarcina* species reduces ferredoxin driven by a proton gradient (high outside) generated by the membrane-bound electron transport chain originating with oxidation of H_2 and ending with reduction of CoM-S-S-CoB (reaction 12, **Figure 2**) (Deppenmeier and Muller, 2008). However, obligate CO_2 reducers do not contain a membrane-bound electron transport chain; instead, the exergonic H_2-dependent reduction of CoM-S-S-CoB is mechanistically coupled to the endergonic reduction of ferredoxin, which drives reaction 5 (Thauer, 2012). Supporting this hypothesis is the report of a protein complex from *Methanococcus maripaludis* that contains Hdr, formyl-MF dehydrogenase and a hydrogenase (Costa *et al.*, 2010).

In the next steps (reactions 6–9, **Figure 2**), the formyl group of formyl-MF is transferred to H_4M(S)PT, followed by two reduction steps culminating with CH_3-H_4M(S)PT. The obligate two-electron donor F_{420} is reduced with a Ni-containing hydrogenase (Thauer *et al.*, 2010). Under Ni-limiting conditions, involvement of the F_{420}-reducing hydrogenase is bypassed by a novel iron-only hydrogenase that directly oxidises H_2 and reduces $CH{\equiv}H_4MPT^+$ (reaction 8b) (Thauer *et al.*, 2010).

Electron transfer from H_2 to CoM-S-S-CoB (reaction 12, **Figure 2**) is significantly different in the CO_2-reducing pathways of *Methanosarcina* and obligate CO_2-reducing species. In *Methanosarcina*, the H_2:CoM-S-S-CoB oxidoreductase system is identical to the proton-pumping

segment of electron transport in the aceticlastic pathway involving the F_{420}-nonreducing hydrogenase, MP and Hdr. However, the membrane-bound electron transport chain of *Methanosarcina* species is absent in obligate CO_2-reducing species with no apparent mechanism for generating an ion gradient. Instead, the H_2:CoM-S-S-CoB oxidoreductase system is comprised of the cytoplasmic F_{420}-nonreducing hydrogenase tightly bound to Hdr with no experimentally determined mechanism for generating an ion gradient. The only remaining possibility for ATP synthesis is the sodium gradient generated by the membrane-bound methyl-H_4MPT:coenzyme M Mtr complex (**Figure 2**, reaction 10) driving the sodium translocating ATP synthase (Thauer *et al.*, 2008).

Biotechnological considerations

The small-scale conversion of biological waste and renewable plant material to CH_4, as a means of disposal and a source of biofuel, has been in use for decades. However, the fragile interactions of multispecies food chains are easily disrupted, a major impediment to efficient and reliable large-scale applications. An engineered pathway has been described derived from the domains *Bacteria* and *Archaea* that utilises the methyl esters of acetate and propionate for growth and methanogenesis (Lessner *et al.*, 2010). The pathway expands the exceptionally narrow range of substrates utilised by methanogens and exemplifies state-of-the-art approaches to simplify food chains leading to a more efficient and reliable process.

Hydrogen

Although H_2 has been proposed as a possible alternative to fossil fuels, current H_2 production amounts in industry rely heavily on fossil fuel sources itself. Global statistics reveal that the sources used for H_2 production are broken down as: 48% from natural gas, 30% from petroleum, 18% from coal and 4% from water electrolysis (Konieczny *et al.*, 2008). Efforts are underway to shift H_2 production to include renewable plant biomass to decrease dependence on nonrenewable fossil fuels that will eventually become depleted. Fermentative (**Figure 1**) and photosynthetic anaerobes offer possible routes for biological H_2 production.

Current H_2 production methods

Methods to obtain H_2 from fossil fuels include steam reformation of methane, partial oxidation, gasification and water hydrolysis (Konieczny *et al.*, 2008). In all these methods except for water hydrolysis, hydrocarbons are reacted with steam and/or oxygen at high temperatures. Water hydrolysis simply converts electrical energy, storing it as H_2 formed from water (Ursua *et al.*, 2012).

The most widely used system employed in industry for producing H_2, steam reforming of hydrocarbons, relies on an endothermic reaction (Wheeler *et al.*, 2004). In the case where methane (the main component of natural gas) is the substrate, the reaction is ($\Delta H = 205.8$ kJ/mol) (Wheeler *et al.*, 2004):

$$CH_4 + H_2O \leftrightarrow CO + 3H_2$$

Conventional steam reforming processes use a reaction temperature of approximately 800°C, and because the reaction is endothermic, large amounts of external heat must be provided (Wheeler *et al.*, 2004). Nonetheless, H_2 yields from this process are higher than from any other process, explaining why it is the most widely used form of H_2 production.

Partial oxidation, however, involves the incomplete reaction of hydrocarbons with oxygen to produce both carbon monoxide and H_2. Because this process is exothermic, it can be maintained autothermally (Wheeler *et al.*, 2004). Gasification involves reacting coal or another solid hydrocarbon with both steam and oxygen under high temperatures and pressures. Comparatively speaking, steam reformation achieves an energy yield of 0.74–0.81 J H_2/J natural gas (Jin *et al.*, 2008), whereas coal gasification achieves an yield of 0.59–0.65 J H_2/J coal (Jin *et al.*, 2008).

For steam reforming, partial oxidation and gasification, a water gas shift reaction is employed to convert CO and H_2O to CO_2 and H_2, respectively, to increase yield. Because it is exothermic, higher temperatures lead to the thermodynamic equilibrium favouring reactants (da Costa *et al.*, 2009). Thus, the reaction is carried out first at high temperature of 350–400°C, then the products are cooled down to 250–300°C before going through a second water gas shift reactor. The product gas mixture must be cooled down further to less than 50°C for conventional purification processes. The subsequent cooling processes inevitably lead to lower gas pressures, so they need to be recompressed to greater than 100 atm for transportation. Overall, large amounts of energy are wasted to cool large volumes of product gases and for recompression. Biological means for producing H_2 are carried out at milder conditions and do not require the consumption of energy for cooling and heating processes.

H_2 infrastructure

It is important to consider the changes in infrastructure needed for any alternative energy to be deemed economically competitive. If H_2 were to be used as a main energy source, pipelines would be the best option in the long term, as H_2 liquefaction processes to compress it for transportation in sizeable scales on trucks are energy intensive and inefficient (Balat, 2008). Pure H_2 gas cannot be transported using existing infrastructure available for natural gas because it embrittles steel and diffuses through materials easily (Haeseldonckx and D'haeseleer, 2007). However, it can be transported through the same pipelines as a mix of up to 17% with natural gas (Haeseldonckx and D'haeseleer, 2007). Capital costs for installing pipelines for the transportation of pure H_2 would be $200 000 to

$1 000 000 per mile (Balat, 2008), making a switch in infrastructure expensive. In addition, H_2 is difficult to compress and store in manageable volumes for vehicles.

In situ H_2 production provides an alternative to completely switching over pipeline transportation infrastructure. If small H_2 production systems can be designed, H_2 can be made wherever it is used. The raw materials consumed for H_2 will have to be transported instead, which presents considerably less risk. For example, using solid starch instead of H_2 would solve the transportation and storage problems associated with H_2 (Zhang *et al*., 2007).

Biological H_2 production

H_2 production can be accomplished by the use of either a nitrogenase or a hydrogenase, both of which are sensitive to oxygen. Nitrogenases are mostly used by phototrophic organisms and carry out the reaction (Dixon and Kahn, 2004):

$$N_2 + 8H^+ + 8e^- + 16\,ATP \rightarrow 2NH_3 + H_2 + 16\,ADP + 16\,P_i$$

Nitrogenases accept electrons from ferredoxins, although consuming large amounts of ATP to generate small amounts of ammonia and H_2 as a by-product. Very high yields of H_2 may be obtained with organisms harbouring nitrogenases, as best demonstrated by *Rhodopseudomonas palustris*, which produces H_2 at 6.69 mol H_2 per mol glycerol; this represents 96% of the theoretical yield of 7 mol H_2 per mol glycerol (not accounting for light energy used) (Ghosh *et al*., 2012). But because of their high metabolic energy demand, nitrogenase activity for H_2 production is only applicable in photosynthetic organisms, which tend to exhibit much slower H_2 production rates (as much as a thousand-fold) compared with non-photosynthetic H_2 batch cultures (Hallenbeck *et al*., 2012). The need for sunlight complicates photosynthetic fermentations further. As such, nitrogenases are not efficient enough to be considered for H_2 production.

Hydrogenases, however, carry out a much simpler reaction:

$$2H^+ + 2e^- \rightarrow 2H_2$$

Certain hydrogenases may fuse with other enzymes to form different complexes, as is the case in *Escherichia coli*. The hydrogenase 3 of *E. coli* fuses with formate dehydrogenase to form the formate H_2 lyase system, allowing H_2 production to be coupled with the breakdown of formate into cxide. Some hydrogenases accept electrons from other sources, with the physiological electron donors being ferredoxin or NAD(P)H (Vignais *et al*., 2001).

In a fermentative pathway using glucose as the sole carbon substrate, one mol of glucose is broken down into two moles of pyruvate via glycolysis, also yielding two mol of ATP and two moles of nicotinamide adenine dinucleotide (NADH) (**Figure 4**). This pyruvate is converted to acetyl-coA for entry into the tricarboxylic acid cycle (**Figure 4**). Pyruvate formate lyase can perform this step to yield formate as a by-product, or pyruvate ferredoxin oxidoreductase will yield cxide along with the reduction of ferredoxin. So, when a formate H_2 lyase pathway is employed, the theoretical yield is 2 mol H_2 per mol glucose. With hydrogenases able to accept electrons from ferredoxin or NAD(P)H, a greater pool of reductants are unlocked to use for H_2 production, giving a higher theoretical yield of 4 mol H_2 per mol glucose in fermentative metabolism. Certain bacteria, such as *Enterobacter aerogenes* (Zhang *et al*., 2011) and *Ruminococcus albus* (Miller and Wolin, 1979), use a combination of these routes for generating H_2.

In vitro versus *in vivo* Systems

Research into biological H_2 production has focused on using engineered or wild-type whole cells of organisms (*in vivo* systems) as well as using systems of purified enzymes (*in vitro* systems) essentially as catalysts for producing H_2 (Woodward *et al*., 2000a). The main rationale behind using *in vitro* systems instead is that microbes will use up some of the substrate for their own biomass production, reducing yields (Zhang *et al*., 2007). Thus, *in vitro* systems have higher H_2 yields than theoretical yields from *in vivo* fermentations (Zhang *et al*., 2007). However, *in vitro* systems have severe drawbacks when it comes to scaling up processes for use in industry. Enzyme purification can be costly, especially when multiple sets of enzymes are needed for an *in vitro* system. Also, a proper buffer must be developed to satisfy all the enzymes in the system as well. In addition, it is well-known that enzymes inevitably inactivate over time, meaning that the enzymes must be replaced even in immobilised systems (Yamane *et al*., 1987). The need for cofactors (i.e. NADPH for H_2 production) can present further complications, as proper regeneration systems must be used and the cofactors decompose into noncatalytic substances as well (Wong and Whitesides, 1981; Woodward and Orr, 1998). In an *in vivo* system, however, cells maintain the enzymes and produce the necessary cofactors, so these severe drawbacks are averted.

H_2 from NADPH

Although much research has been focused on gaining high H_2 production yields from NAD(P)H, no credible studies have successfully shown this *in vivo*. A preliminary report (Angenent *et al*., 2004) indicated that under standard conditions, H_2 production from NAD(P)H should be infeasible at partial pressures of more than 60 Pa.

This pathway and the thermodynamics behind a heterologously expressed route for electron transfer from NADPH to H_2 in *E. coli* has been explored (Veit *et al*., 2008). However, their studies encountered heavy thermodynamic limitations with the pathway in batch cultures, as the H_2 yield was strongly influenced by the headspace to liquid

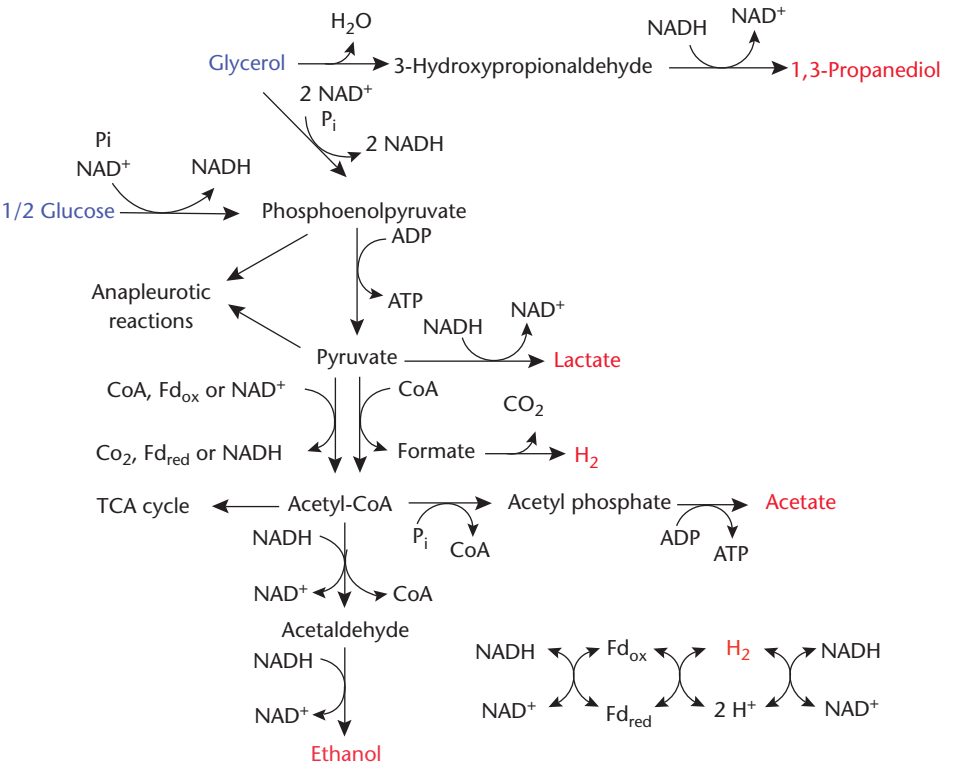

Figure 4 Fermentative pathways leading to production of H_2 and other important products. Metabolites shown in blue indicate carbon substrates, whereas those in red indicate final fermentation products. *Abbreviations*: Fd_{red}, reduced ferredoxin; Fd_{ox}, oxidised ferredoxin; P_i, orthophosphate.

ratio, and at most, 40 mmol H_2 per mol glucose could be obtained. Furthermore, the yield could be increased to just less than 0.2 mol H_2 per mol glucose by altering the NADPH:$NADP^+$ ratio with overexpression of *E. coli* glucose 6-phosphate dehydrogenase, which increases flux through the pentose phosphate pathway, altering the NADPH:$NADP^+$ ratio. Other studies have tried to increase H_2 production in *E. coli* by manipulating the NADPH:-$NADP^+$ ratio further (Kim *et al.*, 2011), or expressing a hydrogenase that accepts electrons directly from NADPH (Wells *et al.*, 2011), but the molar yields are low.

Credible reports of high H_2 yields from NADPH have been indicated *in vitro*. These systems coupled the breakdown of glucose 6-phosphate to H_2 production, using enzymes of the pentose phosphate pathway, as well as ferredoxin, NADPH:ferredoxin reductase and hydrogenase. Woodward *et al.* (2000a), Woodward and Orr (1998) and Woodward *et al.* (2000b) completed the first *in vitro* H_2 pathways, with the substrate being sucrose with a theoretical yield of 2 mol H_2 per mol sucrose or glucose 6-phosphate. Zhang *et al.* (2007) extended the substrate to starch by including glycogen phosphorylase, phosphoglucomutase and phosphate with the system. However, a [NiFe] hydrogenase was used, so Smith *et al.* (2012) improved on this *in vitro* pathway by using an [FeFe] hydrogenase instead, showing higher yields and catalytic rates. The concentrations of different enzymes in use were also experimented with, indicating that the main bottleneck was the transfer of electrons from NADPH to ferredoxin.

Why these pathways do not work so well as heterologously expressed systems *in vivo* is most likely due to a variety of causes. Synthetically high NADPH:$NADP^+$ ratios could have been attained in the *in vitro* studies, allowing for greater concentrations of H_2 to accumulate in the headspace before allowing the reaction to become thermodynamically infeasible. Most importantly, the transfer of electrons from NAD(P)H to a more negative redox couple such as that of the reduced/oxidised ferredoxin or H_2/proton presents a major bottleneck. Besides the latest report on an *in vitro* system, prior systems used constant sparging of the reaction medium with an inert gas such as helium to lower H_2 partial pressure, favouring the thermodynamics behind H_2 production (Woodward *et al.*, 2000b). Furthermore, the heterologous ferredoxins, NADPH:ferredoxin oxidoreductases and hydrogenases used in the *E. coli* studies may not have coupled well enough to provide for considerable H_2 production amounts.

Further thermodynamic analyses must be done to indicate which metabolic H_2 pathways are thermodynamically feasible because the only computational prediction of this was calculated using standard conditions. Thermodynamic-coupled flux balance analysis methods based on physiologically relevant ranges of metabolic

concentrations have been developed to predict the reversibility of metabolic reactions on a genome-scale level (Henry *et al*., 2006). This may be applied to overall metabolism to determine whether a pathway yielding H_2 is feasible and could be further developed to find the equilibrium value (or range) of dissolved H_2 concentration.

Producing H_2 along with coproducts

During dark fermentation processes, other fermentation end products are produced along with H_2. Although fermentation end products may eventually accumulate to toxic levels, inhibiting growth and H_2 production, they may be valuable products as well. Hence, it may be more economically feasible to utilise systems that produce H_2 along with coproducts such as ethanol or 1,3-propanediol, other potential biofuels.

Fermentative microbes can be metabolically engineered to modify ratios of H_2 coproducts or even change them to different products, potentially more valuable ones. For example, Zhu *et al*. (2011) reported replacing metabolic pathways for fermentative end products in *E. coli* (*adhE*, *ackA-pta*, *ldhA* and *frdC*) with an acetaldehyde dehydrogenase to successfully produce acetaldehyde as a coproduct.

In addition, many studies have analysed H_2 and coproducts from glycerol fermentation. Glycerol is more reduced than glucose, but the overall conventional metabolic pathway for glycerol catabolism is essentially the same (**Figure 4**). Glycerol is either used to remove reducing equivalents of NADH to form 1,3-propanediol or converted into glyceraldehyde 3-phosphate that enters glycolysis, yielding stoichiometric amounts of reduced NADH, although consuming ATP (Temudo *et al*., 2008). This extra NADH generated must be reoxidised to satisfy cellular redox balance and the cell must do that by forming more fermentation end products. Corroborating this approach, a strain of *E. coli* obtained by adaptive evolution and chemical mutagenesis that consumes glycerol at a rate five times as fast as the wild-type also produced both H_2 (via the formate hydrogen lyase) and ethanol (consuming acetyl-coA and NADH at stoichiometric amounts) at rates nearly five times as fast as the wild-type (Hu and Wood, 2010).

Conclusions

Methanogens are key players in methanogenic food chains by maintaining H_2, formate and acetate concentrations at levels favourable for the metabolism of fermentative and acetogenic groups at the front of the food chain. Thus, a fundamental understanding of methanogenic pathways is paramount to the identification of factors that optimise the rate and reliability of methanogenesis from biomass to be economically competitive with fossil fuels. Although considerable progress has been made in understanding pathway enzymes, additional research is necessary to understand other factors such as the stress response and regulatory mechanisms, particularly of aceticlastic methanogens. Finally, a fundamental understanding of pathways combined with recent advances in genetics provides the platform for a synthetic genomics approach to engineer methanogens with properties superior to native species.

Metabolic pathways for hydrogen production, however, are well-known. Nonetheless, current biological H_2 production occurs at rates or yields too low or is difficult and expensive to scale-up in order to compete with conventional fossil fuel-based H_2 production. For dark H_2 production, further research must be done on obtaining H_2 from reductive pools of intracellular NADPH to boost H_2 yields. It may also be necessary to focus on producing H_2 along with value-added coproducts such as ethanol and 1,3-propanediol to enhance its economic competitiveness. Then, dark H_2 production will likely become a major contributor to H_2 production in industry.

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