MINI-REVIEW

Clostridial whole cell and enzyme systems for hydrogen production: current state and perspectives

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Abstract
Strictly anaerobic bacteria of the Clostridium genus have attracted great interest as potential cell factories for molecular hydrogen production purposes. In addition to being a useful approach to this process, dark fermentation has the advantage of using the degradation of cheap agricultural residues and industrial wastes for molecular hydrogen production. However, many improvements are still required before large-scale hydrogen production from clostridial metabolism is possible. Here we review the literature on the basic biological processes involved in clostridial hydrogen production, and present the main advances obtained so far in order to enhance the hydrogen productivity, as well as suggesting some possible future prospects.

Keywords Clostridia · Fermentation · Hydrogen production · Hydrogenase · Metabolic engineering

Introduction
The use of molecular hydrogen (H₂) as a possible alternative to the dwindling supplies of fossil fuels available is one of the most promising strategies being investigated today. In addition to providing a rich source of energy, H₂ can be produced enzymatically, which means that it is a clean fuel which should be extremely useful in a large range of energy sectors (Das and Veziroglu 2008). Biological H₂ production by some microorganisms involves two classes of enzymes: hydrogenases (H₂ases) and nitrogenases. An overview of the progress achieved and the main challenges to be met in this field is available (Gupta et al. 2013). H₂ases are the most efficient enzymes known for biological H₂ production. They are structurally diverse enzymes which catalyze the reversible oxidation of H₂ into protons and electrons using various electron acceptors and donors. In heterotrophic microorganisms, they are involved in the anaerobic conversion of organic substances. This process, known as dark fermentation, has attracted considerable interest in recent years since it can be associated to the recycling of organic wastes. Among the various bacteria able to produce H₂ which have been identified so far (including Enterobacter sp., Bacillus sp., Klebsiella sp.), Clostridia are particularly promising candidates because of their comparatively high H₂-production efficiency. In addition to H₂, these obligate anaerobes produce several other substances of industrial interest, such as lactate, butyrate, acetate, ethanol, and butanol. Several aspects of H₂ production by Clostridia have been intensively studied during the past two decades, and some particularly noteworthy findings have been obtained as regards the structure of H₂ases and the mechanisms in which they are involved (reviewed in Calusinska et al. (2010)). Here we present a short review of H₂ases, focusing in particular on those which are involved in H₂ fermentative production, and discuss the current state of the art as regards H₂ production using Clostridia in the form of whole cells or enzymes. The contribution of metabolic engineering to enhance fermentative H₂-production methods is discussed and some possibilities for future improvements are suggested.

Clostridial hydrogenases involved in dark fermentation

H₂ases can be classified in three classes, depending on the nature of their active-site metal center: Fe, NiFe, or FeFe hydrogenases, which are phylogenetically unrelated (Vignais and Billoud 2007; Vignais et al. 2001). NiFe and FeFe
families have in common (i) the presence of cyanide (CN−) and carbon monoxide (CO) ligands coordinating the iron ions at the active site, (ii) FeS clusters forming an electron transfer chain between the active site and electron donors or acceptors at the surface of the enzyme, and (iii) gas transfer channels allowing gases (H2 as well as inhibitors O2 and CO) to diffuse towards or from the buried active site (Cohen et al. 2005; Fontecilla-Camps et al. 2007; Leroux et al. 2008; Montet et al. 1997; Nicolet et al. 1999; Peters et al. 2015). The maturation of NiFe H2ases is a complex process in which the biosynthesis and insertion of the NiFe catalytic center require the products of six hyp genes (Lacasse and Zamble 2016). In comparison, the maturation of [FeFe] H2ases is more simple; it involves only three accessory proteins for the assembly of the 2Fe subcluster of the active site: an FeS cluster-binding GTP-ase HydF, and two radical SAM (S-adenosyl-l-methionine) enzymes, HydE and HydG (Broderick et al. 2014; Peters et al. 2015; Shepard et al. 2014).

Most of the studies performed so far on fermentative H2 production by Clostridia have focused on members of the genus Clostridium and on the monomeric FeFe H2ase. The prototypes of this enzyme are CpI from C. pasteurianum and Cal from C. acetobutylicum. The H2ases in Clostridia are not restricted to this class, since the sequencing of the genome of many strains has shown the presence of genes putatively encoding a wide range of NiFe- and FeFe H2ases (Calusinska et al. 2010; Greening et al. 2016; Poudel et al. 2016). The NiFe H2ases produced by members of the genus Clostridium belong to groups 1 and 4 of NiFe H2ases in the system of classification by Vignais et al. in 2001 (Calusinska et al. 2010; Vignais et al. 2001). The physiological role of the group 1 enzymes in Clostridium has not yet been elucidated, nor has that of the group 4 enzymes, which include membrane-associated H2-evolving respiratory NiFe enzymes (Calusinska et al. 2010; Vignais et al. 2001).

FeFe H2ases occur in anaerobic bacteria such as Firmicutes and Thermotogae and some lower eukaryotes (Greening et al. 2016; Poudel et al. 2016). They are highly efficient H2-producing enzymes and show higher catalytic activity for H2 evolution than NiFe enzymes (Adams 1990; Frey 2002). Their catalytic site, which is called the H-cluster, consists of a single [4Fe4S] cluster ligated by four cysteine residues linked to a unique organometallic diiron subcluster (Nicolet et al. 1999; Peters et al. 1998). In addition to the catalytic domain containing three evolutionarily conserved binding motifs for the H-cluster (Vignais et al. 2001), some additional domains with accessory clusters can be present, conferring a modular organization on FeFe H2ases (Calusinska et al. 2010; Fontecilla-Camps et al. 2007; Poudel et al. 2016; Schwartz et al. 2013). The most thoroughly studied H2ase from Clostridia is the monomeric FeFe enzyme which occurs in some species of the genus Clostridium. The monomeric cytoplasmic CpI enzyme from C. pasteurianum was the first H2ase to be characterized, and the first of which the three-dimensional structure has been solved (Peters et al. 1998). CpI is involved in the recycling of the reduced ferredoxin produced during the dark fermentation process (see below) by producing H2 via a proton reduction step (Therien et al. 2017). Homologs of CpI are present in some Clostridium species (Calusinska et al. 2010) such as C. acetobutylicum, in which Cal produces H2 from reduced ferredoxin or flavodoxin (Demuez et al. 2007).

The model enzymes Cal and CpI have been studied at the molecular level with a view to improving our knowledge of the catalytic and structural properties of FeFe H2ases. In addition to the H-cluster, these enzymes contain a Y-shaped electron transfer chain consisting of four accessory FeS clusters, three 4Fe4S clusters, and one 2Fe2S cluster (Peters et al. 1998). Although it has been established that these accessory clusters contribute importantly to the activity of the enzyme, their exact role has not yet been determined (Artz et al. 2017; Gauquelin et al. 2018). FeFe H2ases are highly O2-sensitive enzymes, which considerably limits their potential for use in biotechnological systems for H2-production purposes. Cal and CpI, along with the FeFe H2ase HydA from Chlamydomonas reinhardtii, are the most suitable model enzymes for studying the mechanism involved in O2 inhibition (Koo et al. 2016; Kubas et al. 2017; Noth et al. 2015; Orain et al. 2015). In particular, the molecular mechanism of O2 diffusion within the enzyme and its reactions at the active site have been studied by combining several approaches such as electrochemistry, site-directed mutagenesis, and molecular dynamics approaches (Kubas et al. 2017; Orain et al. 2015). A better understanding of this mechanism should make it possible to design recombinant enzymes which are resistant to O2. In addition to CpI, two other genes of C. pasteurianum encode monomeric FeFe hydrogenases, CpII and CpIII (Rotta et al. 2015; Therien et al. 2017). CpIII still remains to be characterized, whereas CpII has been analyzed biochemically. This enzyme shows a lower H2-production rate than CpI but oxidizes H2 (Adams 1990). It was recently established that CpII is an uptake H2ase that captures electrons from the H2 produced during the nitrogen fixation process (Therien et al. 2017).

In addition to the monomeric FeFe enzymes, multimeric FeFe H2ases have been identified in Clostridia. These trimeric or tetrameric flavoenzymes have been found to bifurcate electrons. The flavin-based electron-bifurcation process, which was first discovered in 2008 (Li et al. 2008), was proposed to be a third mode of energy conservation in microorganisms in addition to electron transport phosphorylation and substrate level phosphorylation (Buckel and Thauer 2013). Electron-bifurcating enzymes couple an exergonic redox reaction to an endergonic one, and the simultaneous reduction or oxidation of two electron acceptors or donors occurs (Buckel and Thauer 2018a; Buckel and Thauer 2018b). Five bifurcating H2ases from strictly anaerobic bacteria have been described up to now (Kpebe et al. 2018; Schuchmann and Muller 2012; Schut and Adams 2009; Wang et al. 2013b;
Zheng et al. 2014). Three of them belong to the Clostridia (Acetobacterium woodii, Moorella thermoacetica, and Ruminococcus albus). Electron-bifurcating H$_2$ases, which have a similar conserved H$_2$ activation subunit to that of the Cpl of C. pasteurianum, are unique in that they catalyze the oxidation of H$_2$ by reducing ferredoxin and NAD$^+$ simultaneously. They are also electron-confurcating enzymes, since they can catalyze the coupled reduction of protons to H$_2$ via the oxidation of reduced ferredoxin and NADH. In C. autoethanogenum, an NADP+-specific electron-bifurcating FeFe H$_2$ase forms a complex with a formate dehydrogenase (Wang et al. 2013a).

Fermentative H$_2$ production in Clostridia

At least 33 clostridial species have been tested so far as possible means of H$_2$ production with a large range of substrates and growth techniques (Rittmann and Herwig 2012). In the dark fermentation process, Clostridia produce pyruvate, ATP, and NADH by oxidizing organic molecules, mainly those consisting of sugars, via a process of glycolysis (Fig. 1). Pyruvate is subsequently converted by pyruvate:ferredoxin (Fd) oxidoreductase (PFOR) into acetyl-CoA and CO$_2$, yielding reduced Fd. Under special conditions, the NADH thus formed can be oxidized and the Fd reduced by NADH:ferredoxin oxidoreductase (NFOR) (Vardar-Schara et al. 2008). This reduced Fd is used by electron-bifurcating or monomeric Fd-dependent FeFe H$_2$ases to reduce protons, yielding H$_2$. The acetyl-CoA formed follows several metabolic pathways during fermentation, mainly yielding acetate or butyrate. The butyrate pathway competes with H$_2$ production since it involves the use of NADH. Since the regeneration of NAD$^+$ is required for glycolysis to occur, several other fermentation products such as ethanol and butanol can be formed, and the H$_2$-production yield can vary depending on the pathway taken. Theoretically, 12 mol of H$_2$ can be produced per mole of glucose (Fig. 1). However, this theoretical yield is never actually reached in any fermentative organisms. The maximum yield possible with Clostridia (and strict anaerobes in general) is 4 mol of H$_2$ per mole of glucose when acetone or acetate are produced; this maximum, which is called the Thauer limit (Thauer et al. 1977), is never reached in practice since the thermodynamic conditions for the conversion of NADH into H$_2$ are not favorable. To enhance the H$_2$ productivity, the challenge is therefore to find means of circumventing all the latter obstacles (H$_2$ consumption, competitive pathways for reductant supply, and the theoretical limits of the pathway).

A large panel of physicochemical dark fermentation conditions has been tested with Clostridium species, with a view to increasing the H$_2$-production yield (some examples are provided in Table 1). Clostridium species are able to use a wide range of substrates, including pure carbohydrates (i.e., glucose/sucrose) and carbohydrate-enriched substrates, which have been the most widely tested. Abundant lignocellulosic biomass, cellulose, and hemicelluloses from organic waste and carbohydrate-rich waste from the agro-industry are also suitable substrates for H$_2$ production. This process therefore decreases the environmental impact of waste by harnessing it to biofuel production. To ensure the efficient use of these complex substrates, two steps have to be optimized, namely converting the substrate into sugar units that can be metabolized, and inducing the uptake of these forms through the membrane (Fig. 1). It has been established that in several Clostridium species such as C. acetobutylicum, a phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS) mechanism is responsible for the uptake of sugars (Tangney and Mitchell 2007).

Several Clostridium species and other anaerobic microorganisms comprise large extracellular enzymatic entities called cellulosomes which are required for the degradation of complex polysaccharides (Bayer et al. 2004). In these multi-enzyme complexes, several degradative enzymes such as cellulases and hemicellulases (Sabath et al. 2002) degrade cellulosic substrates. Some of the Clostridium species, such as C. acetobutylicum, with cellulolytic activity tested do not hydrolyze crystalline cellulose and are not able to grow on this substrate as the sole source of carbon (Lee et al. 1985). However, Clostridium thermocellum, and other thermophilic species, are able to use cellulose as carbon source and offer a high potential to produce H$_2$ by cellulosic material such as delignified wood fibers (Levin et al. 2006). A bioinformatics analysis of C. thermocellum 27,405 genome sequence has suggested that H$_2$ synthesis in this bacterium would involve either a ferredoxin (Fd)-dependent NiFe H$_2$ase, or a NAD(P)H-dependent Fe H$_2$ase (Carere et al. 2008). Further efforts are required in this field to tackle limiting obstacles to the effective use of Clostridium for H$_2$ production from lignocellulosic waste biomass.

In addition to the type of substrate, physical parameters (such as pH, agitation, temperature), the operating mode (the batch, continuous or semi-continuous process), and reactor design are other essential factors contributing to H$_2$ production, as reviewed in Kothari et al. (2017) and Show et al. (2008). It is necessary, for example, to maintain a low partial H$_2$ pressure to prevent inhibition by the product (Mizuno et al. 2000). The use of immobilized microorganisms (by adsorption, encapsulation, or entrapment in a matrix), contrary to cell suspensions, has been found to improve H$_2$ production by maintaining a high biomass concentration, and thus ensuring the stability of the system (Banu et al. 2018; Kumar et al. 2016). Using an immobilized Thermotoga, the H$_2$-production rate was found to reach 3.3 mol H$_2$/mol glucose, which is near the expected Thauer limit of these organisms. Immobilized Clostridium species have also been found to yield higher H$_2$-production rates than free suspensions (2.91 mol H$_2$/mol glucose versus 1.97 mol H$_2$/mol glucose) (Nomura et al. 2014; Seeert et al. 2015; Zhao et al. 2011) (Table 1).
Although *Clostridium* species are relatively high H₂ producers, mixed cultures can give better results with the fermentative procedure. One example of these mixed cultures is the sludge originating from various sources, which, in addition to strictly anaerobic microorganisms, also contains facultative anaerobes consuming O₂. This oxygen consumption benefits to *Clostridium* whose growth would otherwise be inhibited due to its O₂ sensitivity. A combination of *C. butyricum* and *Enterobacter aerogenes* produced a yield of 2 mol H₂/mol glucose using an industrial starch waste substrate from potato and corn (Yokoi et al. 2001). In addition, the interactions between microorganisms in mixed cultures can result in beneficial improvement of waste and agricultural residue degradation and sugar uptake. Improvement of the biochemical and structural features of H₂ases is indicated by blue dots. Yellow dots point to the possibility of inhibiting competitive pathways in order to rewire larger amounts of reductants to H₂ production. PTS, phosphotransferase sugar transport system.

### Table 1  Examples of H₂ yields obtained by dark fermentation using *Clostridia*

| Microorganism               | Feeding substrate and process                  | Yield of H₂ production (mol/mol glucose) | Ref.       |
|----------------------------|------------------------------------------------|------------------------------------------|------------|
| *Clostridium beijerinckii*  | Glucose                                        | 1.97                                     | Zhao et al. 2011 |
| *Clostridium* sp.           | Glucose                                        | 2.91                                     | Nomura et al. 2014 |
| *Clostridium beijerinckii*  | Glucose                                        | 2.1                                      | Seelert et al. 2015 |
| *Clostridium butyricum*     | Potato starch waste medium/peptone              | 2.4                                      | Yokoi et al. 2001 |
| Co-culture with *Enterobacter aerogenes* | Free suspension                                  | 3.4                                      | Benomar et al. 2015 |
| *Clostridium acetobutylicum* | Glucose                                        | 5.81                                     | Lo et al. 2010 |
| Co-culture with *Desulfovibrio vulgaris* | Sucrose                                        | 6.4                                      | Hitit et al. 2017 |
| *Clostridium butyricum*     | Potato juice/glucose                            | 7.2                                      | Yokoi et al. 2001 |
| Sequential culture with *Rhodospseudomonas palustris* | Free suspension                                  |                                         |            |
| *Clostridium butyricum*     | Potato starch waste medium/peptone              |                                         |            |
| Co-culture with *Rhodospseudomonas palustris* | Free suspension                                  |                                         |            |
physical interactions and a distribution of nutrients improving the metabolic fluxes in the cells, as observed, for example, in mixed cultures of \textit{C. acetobutylicum} and \textit{Desulfovibrio vulgaris}
Hildenborough, a sulfate reducing bacterium, which considerably increased the H$_2$-production rate (Benomar et al. 2015).

One particularly interesting mixed culture process which can be used for H$_2$ production, based on a combination between dark and photofermentation methods, can theoretically give a maximum yield of 12 mol H$_2$ per mol of glucose in two steps: the first dark fermentation step yields H$_2$, organic compounds, and CO$_2$, and these organic compounds are used in the second step by photofermenting bacteria, using captured light to enhance the levels of H$_2$ and CO$_2$ produced. In another fermentative process, a co-culture of \textit{C. butyricum} and the photosynthetic bacterium \textit{Rhodopseudomonas palustris} yielded up to 6.4 mol H$_2$/mol hexose (Hittit et al. 2017; Lo et al. 2010). Combining all three types of metabolism can be another advantageous strategy. A co-culture of \textit{C. butyricum} and \textit{E. aerogenes}, which was performed in a sequential fermentative process with \textit{Rhodobacter} sp., yielded 7.2 mol H$_2$/mol glucose (Yokoi et al. 2001) (Table 1).

Although the use of \textit{Clostridium} species for the production of H$_2$ in dark fermentation is very promising, other species, specifically hyperthermophiles, have shown higher yields in the conversion of substrates in comparison to many mesophilic \textit{Clostridium} species. However, the volumetric productivity in hyperthermophiles is lower (Rittmann and Herwig 2012). One of the factors that explain the high H$_2$-production yield of these organisms is related to the thermodynamic of enzymatic reactions; the change in Gibbs free energy of the overall reaction from glucose to acetate and H$_2$ makes the reaction more favorable (Hallenbeck 2005). High temperature also provides other advantages to the H$_2$-production process such as lower risk of contamination and lower viscosity. In addition, specific metabolism characteristics of the hyperthermophiles favor H$_2$ production (Verhaart et al. 2010). High yields of H$_2$ production (up to 3.8 mol H$_2$/mol hexose) have been obtained with the thermophile \textit{Thermoanaerobacterium} spp., and the hyperthermophiles \textit{Thermotoga} spp. and \textit{Pyrococcus furiosus} (Verhaart et al. 2010). Mixed cultures of these hyperthermophiles microorganisms with \textit{Rhodobacter} species also can produce high yields. A yield of 6.85 mol H$_2$/mol hexose was obtained in a mixed culture containing the hyperthermophile \textit{Caldicellulosiruptor saccharolyticus} and \textit{Rhodobacter capsulatus} (Ozgur et al. 2010).

### Metabolic engineering of clostridial strains to promote H$_2$ production

In the context of H$_2$ production using dark fermentation, metabolic engineering methods consisting in modifying enzymes or metabolic pathways have been used to overcome limiting factors in order to enhance the H$_2$-production yield. Since clostridial genomes are rather difficult to modify, relatively few studies have been conducted on these lines in comparison with those on other heterotrophic H$_2$-producing organisms. However, recent progress in the genetic engineering of \textit{Clostridia} has significantly improved this situation during the past decade. The genetic and synthetic biology toolbox available for use with \textit{Clostridia} has been summarized in (Joseph et al. 2018). Recent efforts to improve the H$_2$ yield have focused on the two main factors limiting the production pathway: (i) increasing and broaden the variety of carbohydrates that can be incorporated and used as carbon source for the growth (red dots in Fig. 1); (ii) and enhancing the yield and/or the rate of H$_2$ production from pyruvate. The first line of investigation, which focuses mainly on the growth on lignocellulosic substrates, involves the overexpression of hemicellulases, cellulases, and lignases as means of enhancing substrate digestion and glucose availability. This strategy is not specific to H$_2$, but enhances the amounts of all the fermentation products released, including ethanol, butanol, and acetate; the latest progress made on these lines has been summarized in Chandel et al. (2012), Olson et al. (2012), and Thomas et al. (2014)). The transport system for sugars in \textit{Clostridium} could also be object of manipulation as a strategy to optimize the uptake of substrates (Mitchell 2016). In a recent study, Jiang et al. (Jiang et al. 2017) reported that the overexpression of the inulinase gene from \textit{Paenibacillus polymyxa} in \textit{C. tyrobutyricum} resulted in relatively high levels of H$_2$ production from the fermentation of the inulin-rich Jerusalem artichoke. Another improvement strategy consists in broadening the panel of substrates used for the bacterial growth. In this context, the cloning and expression of xylose-utilization genes of \textit{Thermoanaerobacter ethanolicus} in \textit{Clostridium thermocellum} DSM 1313 allowed the recombinant strain obtained to co-ferment sugars that can be derived from cellulose and hemicellulose. The production of H$_2$ was twice higher when bacteria grew on cellulose and xylose as compared to cellulose alone (Xiong et al. 2018). This study represents an encouraging step towards the use of plant biomass for the synthesis of valuable products. All in all, the studies cited above confirm that combining metabolic engineering and use of alternative substrates is a promising strategy.

Once the sugar sources have been transformed into pyruvate, H$_2$-enhancement strategies are applied, consisting of either inhibiting competitive pathways or improving the H$_2$-producing branch of the fermentative metabolism (yellow and blue dots, respectively, in Fig. 1). In \textit{C. tyrobutyricum}, inhibiting the acetate pathway by generating a deletion mutant of the acetate kinase gene (ack) resulted in a 2-fold increase in the H$_2$-production level on glucose (Liu et al. 2006). Disrupting the ethanol pathway by inactivating the gene encoding the bifunctional aldehyde-alcohol-dehydrogenase was also tested with a view to directing larger amounts of NADH to H$_2$ases, but the recombinant strain did not show...
an enhanced level of H₂ production as expected, probably because the mutation favored the lactate pathway. Adding sodium acetate to stimulate lactate utilization induced a 20% increase in the H₂ production in this genetic background (Cai et al. 2013). Previous results suggested that inhibiting the butyrate pathway was not a useful approach for H₂-enhancement in this strain, although positive results were expected in view of the theoretical models, since this strategy may also increase the amount of NADH available for the H₂ pathway (Cai et al. 2011). As regards the strategies that have specifically addressed the H₂ pathway, an RNA antisense approach to down-regulating the expression of the uptake hydrogenase resulted in a 3.1-fold increase in the amount of H₂ accumulated by C. saccharoperbutylacetonicum (Nakayama et al. 2008). Homologous overexpression of the FeFe H₂ase encoding gene hydA has been found to enhance H₂ productivity in C. paraputrificum and C. tyrobutyricum 1.7- and 1.5-fold, respectively (Hyé et al. 2010; Morimoto et al. 2005). Interestingly, this approach did not affect the H₂ productivity in C. acetobutylicum, and the authors concluded that the Cal enzyme concentration must not be a limiting factor in this strain (Klein et al. 2010). All in all, the data obtained in these studies suggest that the overall amount of H₂ produced depends largely on the genetic background used, and that the predicted patterns are not always conclusive.

Use of clostridial hydrogenases in heterologous systems

In addition to investigating H₂ production from Clostridia cultures, the heterologous expression of clostridial H₂-ases has been investigated in both heterotrophic and autotrophic microorganisms. Using Escherichia coli as a host for the production and purification of H₂-ases has largely contributed to our knowledge on structure and function of these enzymes and on the mechanism of their maturation. Here we will focus on studies on the heterologous expression of clostridial genes as a means of enhancing H₂ production from E. coli. The powerful effects of the genetic engineering of E. coli have been illustrated in a study in which the authors succeeded in building a metabolic pathway yielding significant amounts of H₂ from pyruvate fermentation. The authors of the latter study co-expressed six genes encoding the following proteins: the pyruvate:ferredoxin oxidoreductase from E. coli, the C. pasteurianum [4Fe4S] ferredoxin, and the C. acetobutylicum monomeric FeFe H₂-ase Cal and the three enzymes involved in its maturation (HydE, HydF, and HydG). In addition, the deletion of the iscR gene encoding the repressor of the [FeS] cluster biogenesis operon resulted in 2-fold increase in the H₂-production yield (Akhtar and Jones 2009). Although the use of heterotrophic hosts growing on glucose is not economically sustainable for H₂ production, synthetic pathways such as those described in this study can pave the way to their use in microorganisms which are able to degrade and metabolize complex polysaccharides. Rewiring solar energy to activate H₂-ase is another attractive H₂-bioproduction strategy (Dubini and Ghirardi 2015; Khanna and Lindblad 2015; Martin and Frymier 2017). In this framework, the sensitivity of these enzymes to O₂ is one of the main obstacles to be overcome. Cal H₂-ase from C. acetobutylicum has been produced in the unicellular cyanobacterium Synechococcus elongatus and the recombinant strain produced 500-fold more H₂ than the parental strain under conditions where the activity of the O₂-producing photosystem was inhibited (Duca et al. 2011). We recently succeeded in producing the same enzyme as in the latter study in the heterocysts of the filamentous nitrogen-fixing cyanobacterium Nostoc PCC 7120. Contrary to what was expected to occur, these micro-oxic cells naturally hosting the O₂-sensitive nitrogenase turned out not to be appropriate for producing Cal. However, increasing the anaerobiosis in the heterocyst by overexpressing a cyanoglobin was found to be an effective strategy, since H₂ production could be achieved under conditions where the process of photosynthesis was active (Avilan et al. 2018). In addition to looking for the most biotechnologically suitable host for clostridial H₂-ases, direct evolution approaches can be expected in the future to enhance the yield of H₂ produced by the recombinant strains. As a proof of concept, a selected chimeric FeFe H₂-ase which evolved from two clostridial sequences produced and purified from E. coli showed a 400%-fold increase in the H₂-ase in vitro activity in comparison with the parental sequence (Plummer et al. 2016). In a recent study, a directed mutagenesis of the FeFe H₂-ase Cpl from Clostridium pasteurianum has showed that a single Cysteine substitution near the proximal delivery center significantly increased the tolerance to O₂ without lowering the amount of H₂ produced (Koo et al. 2016). Moreover, the O₂-tolerant enzymes obtained have been shown to be active in an in vitro system using light and a cyanobacterial photosystem I (Koo et al. 2016). This result suggests that these engineered O₂-tolerant H₂-ases can be implemented in photosynthetic organisms to take advantage of solar energy for H₂ production.

Conclusion and perspectives

Fermentative H₂-production processes have several advantages over other processes. The growth rate of fermentative organisms is relatively fast and a large range of carbon sources such as organic compounds, wastes, and cellulosic compounds can be used. They therefore have considerable potential for use in industrial applications. However, before this aim can be achieved, further research is required to deal with all the limiting factors reducing H₂ productivity. Future efforts will have to focus on improving the ability of the strains to degrade waste and complex polysaccharide substrates, and sugar uptake is another point which needs to be investigated more closely. The latest progress made in the genetic engineering of Clostridia should lead to the development of more successful means of shutting down
competitive pathways that rely on the same reductants than H₂. Gene shuffling and direct evolutionary approaches need to be further developed in order to enhance the activity of H₂ases and/or limit their sensitivity to O₂. These approaches are of great potential interest not only from the point of view of fermentative H₂ production but also as regards the use of clostridial enzymes in photosynthetic processes.

One of the more negative aspects of dark fermentation is the production of CO₂, a greenhouse gas. The possibility of coupling H₂ production from *Clostridia* to CO₂-consuming processes using algae or cyanobacteria for either the photosynthetic production of H₂ or other industrial applications would be worth investigating. Generally speaking, it would certainly be of great interest to find ways of using the metabolic and genetic interactions liable to occur in microbial communities: screening natural microbial communities or engineering specific microbiyal ecosystems should yield useful methods of boosting H₂ production by fermentative organisms.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

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**References**

Adams MW (1990) The structure and mechanism of iron-hydrogenases. Biochim Biophys Acta 1020(2):115–145

Akhbar MK, Jones PR (2009) Construction of a synthetic Ydbk-dependent pyruvate-H₂ pathway in *Escherichia coli* BL21(DE3). Metab Eng 11(3):139–147. https://doi.org/10.1016/j.menb.2009.01.002

Artz JH, Mulder DW, Rateloff MW, Lubner CE, Zadovornyy OA, LeVan AX, Williams SG, Adams MWW, Jones AK, King PW, Peters JW (2017) Reduction potentials of [FeFec]-hydrogenase accessory iron-sulfur clusters provide insights into the energetics of proton reduction catalysis. J Am Chem Soc 139(28):9544–9550. https://doi.org/10.1021/jacs.7b02099

Avilan L, Roumezi B, Risoul V, Bernard CS, Kpebe A, Belhadjhassine M, Rouset M, Brugna M, Latifi A (2018) Phototrophic hydrogen production from a clostridial [FeFe]-hydrogenase expressed in the heterocytes of the cyanobacterium *Nostoc PCC 7120*. Appl Microbiol Biotechnol 102(13):5775–5783. https://doi.org/10.1007/s00773-018-3889-2

Banu JR, Kannah RY, Kumar MD, Gunasekaran M, Sivagurunathan P, Park JH, Kumar G (2018) Recent advances on biogranules formation in dark hydrogen fermentation system: mechanism of formation and microbial characteristics. Bioresour Technol 268:787–796. https://doi.org/10.1016/j.biortech.2018.07.034

Bayer EA, Belaich JP, Shoham Y, Lamed R (2004) The cellulasesomes: multienzyme machines for degradation of plant cell wall polysaccharides. Annu Rev Microbiol 58:521–554. https://doi.org/10.1146/annurev.micro.57.030502.091022

Benomar S, Ranava D, Cardenas ML, Trably E, Rafrafi Y, Ducret A, Hamelin J, Lojou E, Steyer JP, Giudici-Orciconi MT (2015) Nutritional stress induces exchange of cell material and energetic coupling between bacterial species. Nat Commun 6:6283. https://doi.org/10.1038/ncomms7283

Broderick JB, Byer AS, Duschenke KS, Dufuiss BR, Betz JN, Shepard EM, Peters JW (2014) H-cluster assembly during maturation of the [FeFec]-hydrogenase. J Biol Inorg Chem 19(6):747–757. https://doi.org/10.1007/s00775-014-1168-8

Buckel W, Thauer RK (2013) Energy conservation via electron bifurcating ferredoxin reduction and proton/Na⁺ translocating ferredoxin oxidation. Biochim Biophys Acta 1827(2):94–113. https://doi.org/10.1016/j.bbobi.2012.07.002

Buckel W, Thauer RK (2018a) Flavin-based electron bifurcation, a new mechanism of biological energy coupling. Chem Rev 118(7):3862–3886. https://doi.org/10.1021/acs.chemrev.7b00707

Buckel W, Thauer RK (2018b) Flavin-based Electron bifurcation, ferredoxin, flavodoxin, and anaerobic respiration with protons (Ech) or NAD⁺ (Rnf) as electron acceptors: a historical review. Front Microbiol 9:401. https://doi.org/10.3389/fmicb.2018.00401

Cai G, Jin B, Monis P, Saint C (2013) A genetic and metabolic approach to redirection of biochemical pathways of *Clostridium butyricum* for enhancing hydrogen production. Biotechnol Bioeng 110(1):338–342. https://doi.org/10.1002/bit.24596

Cai G, Jin B, Saint C, Monis P (2011) Genetic manipulation of butyrate formation pathways in *Clostridium butyricum*. J Biotechnol 155(3):269–274. https://doi.org/10.1016/j.jbiotec.2011.07.004

Calusinska M, Happe T, Joris B, Wilmotte A (2010) The surprising diversity of clostridial hydrogenases: a comparative genomic perspective. Microbiology 156( Pt 6):1575–1588. https://doi.org/10.1099/mic.0.032771-0

Carere CR, Kalia V, Sparling R, Cicek N, Levin DB (2008) Pyruvate catabolism and hydrogen synthesis pathway genes of *Clostridium thermocellum* ATCC 27405. Indian J Microbiol 48(2):252–266. https://doi.org/10.1007/s12089-008-0036-z

Chandrasekhar G, Silva MB, Silverio da Silva S (2012) The realm of cellulases in biorefinery development. Crit Rev Biotechnol 32(3):187–202. https://doi.org/10.3109/01440410.2011.595385

Cohen J, Kim K, King P, Seibert M, Schulten K (2005) Finding gas diffusion pathways in proteins: application to O₂ and H₂ transport in Cpl [FeFec]-hydrogenase and the role of packing defects. Structure 13(9):1321–1329. https://doi.org/10.1016/j.str.2005.05.013

Das D, Vezziroglu TN (2008) Advances in biological hydrogen production processes. Int J Hydrog Energy 33:6046–6054

Demuzere M, Courmec L, Guerrini O, Soucaille P, Girbal L (2007) Complete activity profile of *Clostridium acetobutylicum* [FeFec]-hydrogenase and kinetic parameters for endogenous redox partners. FEMS Microbiol Lett 275(1):113–121. https://doi.org/10.1111/j.1574-6968.2007.00686.x

Dubini A, Ghirardi ML (2015) Engineering photosynthetic organisms for the production of biohydrogen. Photosynth Res 123(3):241–253. https://doi.org/10.1007/s11120-014-9991-x

Ducret DC, Sachdeva G, Silver PA (2011) Rewiring hydrogenase-dependent redox circuits in cyanobacteria. Proc Natl Acad Sci U S A 108(10):3941–3946. https://doi.org/10.1073/pnas.100626108

Fontecilla-Camps JC, Volbeda A, Cavazza C, Nicolet Y (2007) Structure/function relationships of [NiFe]- and [FeFec]-hydrogenases. Chem Rev 107(10):4273–4303. https://doi.org/10.1021/cr050195z

Frey M (2002) Hydrogenases: hydrogen-activating enzymes. ChemBiochem 3(2–3):153–160

Gauquelin C, Baffert C, Richaud P, Kamionka E, Etienne E, Guyeysse D, Girbal L, Fourmond V, Andre I, Guigliarelli B, Leger C, Soucaille P, Meyrial-Salles I (2018) Roles of the F-domain in [FeFec] hydrogenase. Biochim Biophys Acta 1859(2):69–77. https://doi.org/10.1016/j.bbabbio.2017.08.010
Plummer SM, Plummer MA, Merkel PA, Hagen M, Biddle JF, Waidner LA (2016) Using directed evolution to improve hydrogen production in chimeric hydrogenases from Clostridia species. Enzyme Microb Technol 93-94:132–141. https://doi.org/10.1016/j.enzmitec.2016.07.011

Poudel S, Tokmina-Lukaszewska M, Colman DR, Refai M, Schut GJ, King PW, Maness PC, Adams MW, Peters JW, Bothner B, Boyd ES (2016) Unification of [FeFe]-hydrogenases into three structural and functional groups. Biochim Biophys Acta 1860(9):1910–1921. https://doi.org/10.1016/j.bbagen.2016.05.034

Rittmann S, Herwig C (2012) A comprehensive and quantitative review of dark fermentative biohydrogen production. Microb Cell Factories 11:115. https://doi.org/10.1186/1475-2859-11-115

Rotta C, Poehlein A, Schwarz K, McClure P, Daniel R, Minton NP (2015) Xylanase and cellulase functional groups. Biochim Biophys Acta 1860(9):1910

Rotta C, Poehlein A, Schwarz K, McClure P, Daniel R, Minton NP (2015) Xylanase and cellulase functional groups. Biochim Biophys Acta 1860(9):1910

Sabathe F, Belaich A, Soucaille P (2002) Characterization of the cellulosytic complex (cellulosome) of Clostridium acetobutylicum. FEMS Microbiol Lett 217(1):15–22

Schuchmann K, Muller V (2012) A bacterial electron-bifurcating hydrogenase. J Biol Chem 287(37):31165–31171. https://doi.org/10.1074/jbc.M112.395038

Schut GJ, Adams MW (2009) The iron-hydrogenase of Thermotoga maritima utilizes ferredoxin and NADH synergistically: a new perspective on anaerobic hydrogen production. J Bacteriol 191(13):4451–4457. https://doi.org/10.1128/JB.01582-08

Schwartz E, Fritsch J, Friedrich B (2013) H2-metabolizing prokaryotes. In: Rosenberg E, DeLong EF, Lory S, Stackebrandt E, Thompson F (eds) The prokaryotes: prokaryotic physiology and biochemistry, 4th edn. Springer, Berlin, pp 119–199

Seelert T, Ghosh D, Yargeau V (2015) Improving biohydrogen production using Clostridium beijerinckii immobilized with magnetite nanoparticles. Appl Microbiol and Biotechnol 99(9):4107–4116. https://doi.org/10.1007/s00253-015-6484-6

Shepard EM, Mus F, Betz JN, Byer AS, Duffus BR, Peters JW, Broderick JB (2014) [FeFe]-hydrogenase maturation. Biochemistry 53(25):4090–4104. https://doi.org/10.1021/bi500210x

Show KY, Zhang ZP, Lee DJ (2008) Design of bioreactors for biohydrogen production. J Sci Ind Res 67:941–949

Tangney M, Mitchell WJ (2007) Characterisation of a glucose phosphotransferase system in Clostridium acetobutylicum ATCC 824. Appl Microbiol Biotechnol 74(2):398–405. https://doi.org/10.1007/s00253-006-0679-9

Thauer RK, Jungemann K, Decker K (1977) Energy conservation in chemotrophic anaerobic bacteria. Bacteriol Rev 41(1):100–180

Therien JB, Arzt JH, Poudel S, Hamilton TL, Liu Z, Noone SM, Adams MWW, King PW, Bryant DA, Boyd ES, Peters JW (2017) The physiological functions and structural determinants of catalytic bias in the [FeFe]-hydrogenases Cpi and CpiII of Clostridium pasteurianum strain W5. Front Microbiol 8:1305. https://doi.org/10.3389/fmicb.2017.01305

Thomas L, Joseph A, Gottumukkala LD (2014) Xylanase and cellulase systems of Clostridium sp.: an insight on molecular approaches for strain improvement. Bioresour Technol 158:343–350. https://doi.org/10.1016/j.biotech.2014.01.140

Vardar-Schara G, Maeda T, Wood TK (2008) Metabolically engineered bacteria for producing hydrogen via fermentation. Microb Biotechnol 1(2):107–125. https://doi.org/10.1111/j.1751-7915.2007.00009.x

Verhaert MR, Bielen AA, van der Oost J, Stams AJ, Kengen SW (2010) Hydrogen production by hyperthermophilic and extremely thermophilic bacteria and archaea: mechanisms for reductant disposal. Environ Technol 31(8–9):993–1003

Vignais PM, Billoud B (2007) Occurrence, classification, and biological function of hydrogenases: an overview. Chem Rev 107(10):4206–4272. https://doi.org/10.1021/cr050196r

Vignais PM, Billoud B, Meyer J (2001) Classification and phylogeny of hydrogenases. FEMS Microbiol Rev 25(4):455–501

Wang S, Huang H, Kahnt J, Mueller AP, Koprke M, Thauer RK (2013a) NADP-specific electron-bifurcating [FeFe]-hydrogenase in a functional complex with formate dehydrogenase in Clostridium autoethanogenum grown on CO. J Bacteriol 195(19):4373–4386. https://doi.org/10.1128/JB.00678-13

Wang S, Huang H, Kahnt J, Thauer RK (2013b) A reversible electron-bifurcating ferredoxin- and NAD-dependent [FeFe]-hydrogenase (HydABC) in Moorella thermoacetica. J Bacteriol 195(6):1267–1275. https://doi.org/10.1128/JB.02158-12

Xiong W, Reyes LH, Michener WE, Maness PC, Chou KJ (2018) Engineering cellulolytic bacterium Clostridium thermocellum to co-ferment cellulose- and hemicellulose-derived sugars simultaneously. Biotechnol Bioeng 115(7):1755–1763. https://doi.org/10.1002/bit.26590

Yokoi H, Saito A, Uchida H, Hirose J, Hayashi S, Takasaki Y (2001) Microbial hydrogen production from sweet potato starch residue. J Biosci Bioeng 91(1):58–63

Zhang F, Xu D, Fu N, Liu B, Ren N (2011) Hydrogen production by the newly isolated Clostridium beijerinckii RZF-1108. Bioresour Technol 102(18):8432–8436. https://doi.org/10.1016/j.biortech.2011.02.086

Zheng Y, Kahnt J, Mackie RI, Thauer RK (2014) Hydrogen formation and its regulation in Ruminococcus albus: involvement of an electron-bifurcating [FeFe]-hydrogenase, of a non-electron-bifurcating [FeFe]-hydrogenase, and of a putative hydrogen-sensing [FeFe]-hydrogenase. J Bacteriol 196(22):3840–3852. https://doi.org/10.1128/JB.02070-14