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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.

Key words

Clostridia, Fermentation, Hydrogen production, Hydrogenase, Metabolic engineering.

Introduction

The use of molecular hydrogen (H_2) 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, H_2 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 H_2 production by some microorganisms involves two classes of enzymes: hydrogenases (H_2 ases) and nitrogenases. An overview of the progress achieved and the main challenges to be met in this field is available (Gupta et al. 2013). H_2 ases are the most efficient enzymes known for biological H_2 production. They are structurally diverse enzymes which catalyze the reversible oxidation of H_2 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
57 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 6 *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 CaI 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 CaI 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. CaI 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 flavo-enzymes 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 H₂ases, which have a similar conserved H₂ activation subunit to that of the CpI of *C. pasteurianum*, are unique in that they catalyze the oxidation of H₂ by reducing ferredoxin and NAD⁺ simultaneously. They are also electron confurcating enzymes, since they can catalyze the coupled reduction of protons to H₂ via the oxidation of reduced ferredoxin and NADH. In *C. autoethanogenum*, an NADP-specific electron-bifurcating FeFe-H₂ase forms a complex with a formate dehydrogenase (Wang et al. 2013a).

Fermentative H₂ production in *Clostridia*

At least 33 clostridial species have been tested so far as possible means of H₂ 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 (Figure 1). Pyruvate is subsequently converted by pyruvate:ferredoxin (Fd) oxidoreductase (PFOR) into acetyl-CoA and CO₂, 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-H₂ases to reduce protons, yielding H₂. The acetyl-CoA formed follows several metabolic pathways during fermentation, mainly yielding acetate or butyrate. The butyrate pathway competes with H₂ 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 H₂ production yield can vary depending on the pathway taken. Theoretically, 12 moles of H₂ can be produced per mole of glucose (Figure 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 moles of H₂ 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 H₂ are not favorable. To enhance the H₂ productivity, the challenge is therefore to find means of circumventing all the latter obstacles (H₂ consumption, competitive pathways for reductant supply, and the theoretical limits of the pathway).

A large panel of physicochemical dark fermentation conditions have been tested with *Clostridium* species, with a view to increasing the H₂-production yield (some examples are provided in Table I). *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 H₂ 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 (Figure 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 (Sabathe 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 H₂ by cellulosic material such as delignified wood fibers (Levin et al. 2006). A bioinformatics analysis of *C. thermocellum* 27405 genome sequence has suggested that H₂ synthesis in this bacterium would involve either a Ferredoxin (Fd)-dependent NiFe H₂ase, or a NAD(P)H-dependent Fe-H₂ase (Carere et al. 2008). Further efforts are required in this field to tackle limiting obstacles to the effective use of *Clostridia* for H₂ 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 H₂ production, as reviewed in (Kothari et al. 2017; Show et al. 2008). It is necessary, for example, to maintain a low partial H₂ 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 H₂ 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 H₂ production rate was found to reach 3.3 mol H₂/mol glucose, which is near the expected Thauer limit of these organisms. Immobilized *Clostridium* species have also been found to yield higher H₂ production rates than free suspensions (2.91 mol H₂/mol glucose versus 1.97 mol H₂/mol glucose) (Nomura et al. 2014; Seelert 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 physical interactions and a distribution of nutrients improving the metabolic fluxes in the cells, as observed, for example, in mixed cultures of *C. acetobutylicum* and *Desulfovibrio vulgaris* Hildenborough, a sulfate reducing bacterium, which considerably increased the H₂ production rate (Benomar et al. 2015).

One particularly interesting mixed culture process which can be used for H₂ production, based on a combination between dark and photofermentation methods, can theoretically give a maximum yield of 12 mol H₂ per mol of glucose in two steps: the first dark fermentation step yields H₂, organic compounds and CO₂, and these organic compounds are used in the second step by photofermenting bacteria, using captured light to enhance the levels of H₂ and CO₂ produced. In another fermentative process, a co-culture of *C. butyricum* and the photosynthetic bacterium *Rhodospseudomonas palustris* yielded up to 6.4 mol H₂/mol hexose (Hitit et al. 2017; Lo et al. 2010). Combining all three types of metabolism can be another advantageous strategy. A co-culture of *C. butyricum* and *E. aerogenes*, which was performed in a sequential fermentative process with *Rhodobacter* sp. yielded 7.2 mol H₂/mol glucose (Yokoi et al. 2001) (Table 1).

Although the use of *Clostridium* species for the production of H₂ in dark fermentation is very promising, other species, specifically hyperthermophiles, have shown higher yields in the conversion of substrates in comparison to many mesophilic *Clostridium* species. However, the volumetric productivity in hyperthermophiles is lower (Rittmann and Herwig 2012). One of the factors that explain the high H₂ 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 H₂ makes the reaction more favorable (Hallenbeck 2005). High temperature also provides other advantages to the H₂ production process such as lower risk of contamination and lower viscosity. In addition, specific metabolism characteristics of the hyperthermophiles favor H₂ production (Verhaart et al. 2010). High yields of H₂ production (up to 3.8 mol H₂/mol hexose) have been obtained with the thermophile *Thermoanaerobacterium* spp., and the hyperthermophiles *Thermotoga* spp. and *Pyrococcus furiosus* (Verhaart et al. 2010). Mixed cultures of these hyperthermophiles microorganisms with *Rhodobacter* species also can produce high yields. A yield of 6.85 mol

H₂/mol hexose was obtained in a mixed culture containing the hyperthermophile *Caldicellulosiruptor saccharolyticus* and *Rhodobacter capsulatus* (Ozgun et al. 2010).

Metabolic engineering of clostridial strains to promote H₂ production

In the context of H₂ 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 H₂ 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 H₂ producing organisms. However, recent progress in the genetic engineering of *Clostridia* has significantly improved this situation during the past decade. The genetic and synthetic biology toolbox available for use with *Clostridia* has been summarized in (Joseph et al. 2018). Recent efforts to improve the H₂ 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 Figure 1); (ii) and enhancing the yield and/or the rate of H₂ 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 H₂, 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; Thomas et al. 2014). The transport system for sugars in *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 over-expression of the inulinase gene from *Paenibacilluspolymyxa* in *C. tyrobutyricum* resulted in relatively high levels of H₂ 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 *Thermoanaerobacter ethanolicus* in *Clostridium thermocellum* DSM 1313 allowed the recombinant strain obtained to co-ferment sugars that can be derived from cellulose and hemicellulose. The production of H₂ 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, H₂-enhancement strategies are applied, consisting either of inhibiting competitive pathways or improving the H₂-producing branch of the fermentative metabolism (yellow and blue dots, respectively, in Figure 1). In *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 H₂ 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 H₂ases, 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 downregulating 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 (Hye 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 CaI 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 H₂ases has been investigated in both heterotrophic and autotrophic micro-organisms. Using *Escherichia coli* as a host for the production and purification of H₂ases 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-H₂ase CaI 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 that 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 H₂ase 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. CaI H₂ase 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 (Ducat 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 CaI. 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 H₂ases, 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-H₂ase which evolved from two clostridial sequences produced and purified from *E. coli* showed a 400%-fold increase in the H₂ase *in vitro* activity in comparison with the parental sequence (Plummer et al. 2016). In a recent study, a directed mutagenesis of the FeFe H₂ase 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 1 (Koo et al. 2016). This result

suggests that these engineered O₂-tolerant H₂ases 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

microbial ecosystems should yield useful methods of boosting H₂ production by fermentative organisms.

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The authors declare that they have no conflict of interest.

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608 **Tables 1: examples of H₂-yields obtained by dark fermentation using *Clostridia***

Microorganism	Feeding substrate and process	Yield of H ₂ production (mol/mol glucose)	Ref
<i>Clostridium beijerinckii</i>	Glucose Free suspension	1.97	Zhao et al. 2011
<i>Clostridium sp.</i>	Glucose Cells immobilized in polyethylene glycol- <i>b</i> -polypropylene glycol	2.91	Nomura et al. 2014
<i>Clostridium beijerinckii</i>	Glucose Cells immobilized in Alginate–chitosan–magnetite nanoparticle	2.1	Seelert et al. 2015
<i>Clostridium butyricum</i> Co-culture with <i>Enterobacter aerogenes</i>	Potato starch waste medium/peptone Free suspension	2.4	Yokoi et al. 2001
<i>Clostridium acetobutylicum</i> Co-culture with <i>Desulfovibrio vulgaris</i>	Glucose Free suspension	3.4	Benomar et al. 2015
<i>Clostridium butyricum</i> Sequential culture with <i>Rhodopseudomonas palustris</i>	Sucrose Free suspension	5.81	Lo et al. 2010
<i>Clostridium butyricum</i> Co-culture with <i>Rhodopseudomonas palustris</i>	Potato juice/glucose Free suspension	6.4	Hitit et al. 2017
<i>Clostridium butyricum</i> Co-culture with <i>Enterobacter aerogenes</i> Sequential culture with <i>Rhodobacter sp.</i>	Potato starch waste medium/peptone Free suspension	7.2	Yokoi et al. 2001

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611 **Legend to Figure 1:** Schematic representation of dark fermentation in *Clostridia*.

612 H₂ is produced by a monomeric FeFe-H₂ase using ferredoxin (Fd) or by a bifurcating H₂ase

613 using both Fd and NADH. Electron transfer from NADH to Fd is indicated in dashed arrow.

614 Competitive pathways are indicated.

615 Possible lines of future research on H₂-production enhancement are indicated by colored dots.

616 Red dots stand for the improvement of waste and agricultural residue degradation and sugar

617 uptake. Improvement of the biochemical and structural features of H₂ases is indicated by blue

618 dots. Yellow dots point to the possibility of inhibiting competitive pathways in order to rewire

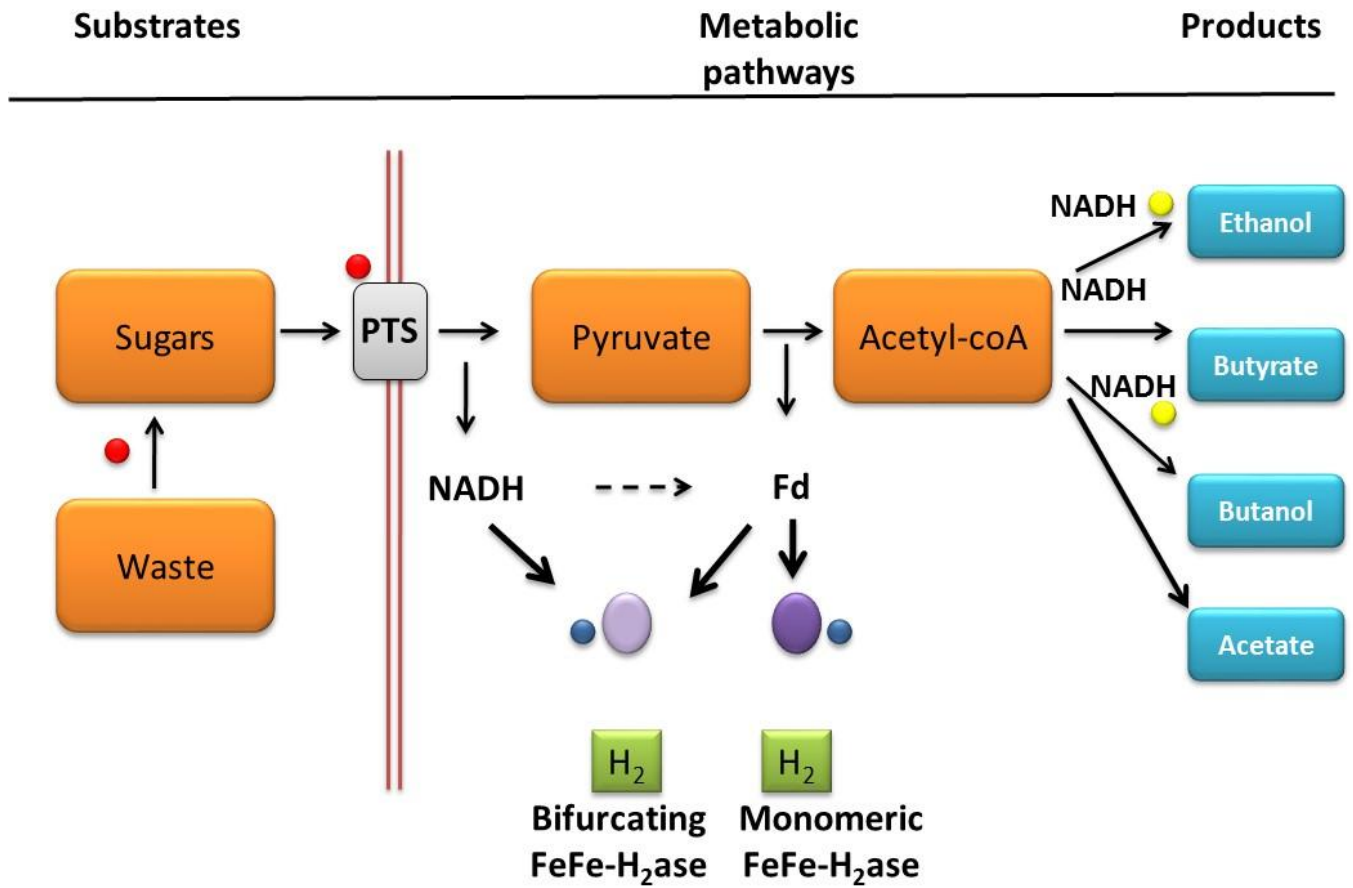
619 larger amounts of reductants to H₂ production. PTS: phosphotransferase sugar transport
620 system.

621

622 **Figure 1**

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Schematic representation of dark fermentation in *Clostridia*



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