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## REVIEW ARTICLE

# Microbial ecology of fermentative hydrogen producing bioprocesses: useful insights for driving the ecosystem function

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**One sentence summary:** This review establishes basic knowledge about the taxonomic and metabolic diversity of hydrogen producers in fermentation bioprocesses, and emphasizes the crucial role of interactions between effective producers, negative effectors inhibiting or outcompeting hydrogen production, and positive contributors facilitating the ecosystem function, with the final objective to propose bioengineering strategies and microbial resource management to enhance bioprocess function.

**Editor:** Marie-Therese Giudici-Orticoni

## ABSTRACT

One of the most important biotechnological challenges is to develop environment friendly technologies to produce new sources of energy. Microbial production of biohydrogen through dark fermentation, by conversion of residual biomass, is an attractive solution for short-term development of bioH<sub>2</sub> producing processes. Efficient biohydrogen production relies on complex mixed communities working in tight interaction. Species composition and functional traits are of crucial importance to maintain the ecosystem service. The analysis of microbial community revealed a wide phylogenetic diversity that contributes in different—and still mostly unclear—ways to hydrogen production. Bridging this gap of knowledge between microbial ecology features and ecosystem functionality is essential to optimize the bioprocess and develop strategies toward a maximization of the efficiency and stability of substrate conversion. The aim of this review is to provide a comprehensive overview of the most up-to-date biodata available and discuss the main microbial community features of biohydrogen engineered ecosystems, with a special emphasis on the crucial role of interactions and the relationships between species composition and ecosystem service. The elucidation of intricate relationships between community structure and ecosystem function would make possible to drive ecosystems toward an improved functionality on the basis of microbial ecology principles.

**Keywords:** Biohydrogen; dark fermentation; microbial community; eco-engineering; facultative anaerobic bacteria; microbial interactions

## ABBREVIATIONS

ASBR: Anaerobic sequencing batch reactor  
 CSTR: Continuous stirred tank reactor  
 EPS: Exopolysaccharides  
 FBA: Flux balance analysis  
 Fd<sub>red</sub>: Reduced ferredoxin  
 FHL: Formate hydrogen lyase  
 HPB: Hydrogen producing bacteria  
 HRT: Hydraulic retention time  
 LAB: Lactic acid bacteria  
 NAD<sup>+</sup>/NADH: Nicotinamide adenine dinucleotide an oxidized and reduced form, respectively  
 OLR: Organic loading rate  
 PFL: Pyruvate-formate-lyase  
 SRB: Sulfate reducing bacteria  
 UASB: Upflow anaerobic sludge blanket  
 VFAs: Volatile fatty acids

## INTRODUCTION

Nowadays, alternative energetic vectors are requested not only to reduce the global dependence on fossil fuels, but also to mitigate climate change originated by human activities, and redirect the current production processes (open cycles) toward natural closed ecological cycles. One of the most promising energetic scenario concerns the emergence of a hydrogen (H<sub>2</sub>) market, where H<sub>2</sub> is produced from renewable resources to be further used as primary energy carrier. Indeed, H<sub>2</sub> is an interesting energy vector as its combustion produces only water vapor instead of greenhouse gases, with a combustion yield 2.75 times higher (122 kJ/g) than hydrocarbons, and can be easily converted into electricity in fuel cells. Although H<sub>2</sub> can be produced by water electrolysis, one of the most important current challenges is to reduce the energy required for hydrogen production, by generating it from abundant and low cost renewable sources with environmental friendly technologies, through biological conversion of biomass (Hay et al. 2013). Among the diverse H<sub>2</sub>-producing biotechnologies, dark fermentation processes operated with mixed cultures have gained recently a lot of attention because they can convert many different types of organic matter—and more particularly waste streams—to hydrogen, with high productivity (Kleerebezem and van Loosdrecht 2007). Biohydrogen production by dark fermentation of ‘negative value’ organic waste streams combines the objectives of sustainable waste management with pollution control and with the generation of a valuable clean energy product (Guo et al. 2010; Hay et al. 2013).

Over the past decade, much of the research effort has focused on enhancing H<sub>2</sub> production rate by optimizing the operation parameters, modifying the reactor design, pretreating the substrate to increase bioavailability (Guo et al. 2010; Monlau et al. 2013b), especially focusing on pure and defined co-cultures (Lee, Show and Sud 2011; Rittmann and Herwig 2012; Elsharnouby et al. 2013). When working with mixed cultures, such process optimization targets the indirect, and often empiric, selection of more adapted microbial communities carrying the function of hydrogen production. During fermentation, microorganisms derive energy from a chain of redox reactions during which a part of the substrate carbon is oxidized and another part is reduced. Electrons derived from this process are used for the reduction of protons to molecular H<sub>2</sub>, in order to balance the cell redox

potential. H<sub>2</sub> evolution efficiently dissipates excess reductant as a diffusible gas during microbial fermentation (Schwartz, Fritsch and Friedrich 2013). Therefore, H<sub>2</sub> is a by-product of many bacterial metabolic pathways. Its role as a vector of the energetic chain in most bacterial metabolisms makes it ubiquitous with a wide taxonomic distribution (Greening et al. 2015a,b). Thus, there is currently no generally accepted selection criterion for the most favorable fermentative hydrogen production route in mixed cultures, which is one of the main limitations of the fermentative hydrogen production process (Kleerebezem and van Loosdrecht 2007). This limitation is partly due to the limited knowledge, to date, of the microbial ecology involved in hydrogen production in mixed cultures.

Biofermenters used for hydrogen production from mixed cultures can be considered as model ecosystems where environmental and operating parameters are reliably monitored and controlled, and can therefore be useful to elucidate the links between microbial community features and ecosystem services such as organic matter degradation and H<sub>2</sub> production. Microbial ecology examines how different levels of biodiversity (taxonomic and functional) affect the overall functionality of an ecosystem. Species composition and specific functional traits, as well as interactions between species, are often more important than the species richness itself in maintaining ecosystem processes and related services. Indeed, the efficient conversion of complex organic matter to hydrogen requires the participation of different microbial populations where ecological and metabolic interactions between microorganisms are of prime importance. Keystone species can be identified, which are critical within the complex network of community interactions and play a determining role in the global function (even though not dominant): their gain or loss can have amplifying effects at the ecosystem level (Rafrafi et al. 2013).

With the development of molecular tools, several studies have explored the microbial ecology of hydrogen-producing consortia, and revealed a wide phylogenetic diversity that contributes in different—and still mostly unclear—ways to hydrogen production in mixed cultures during fermentation of complex substrates. However, a global overview of the ecological complexities of these communities, including the fundamentals of microbial interactions, in relation with their function, is missing. Bridging this gap of knowledge is essential to further optimize bioprocesses toward maximization of the efficiency and stability of substrate conversion into biohydrogen.

The aim of this review is to provide an overview of microbial ecology in mesophilic dark fermentative hydrogen-producing ecosystems with an evaluation of the most up-to-date molecular biodata reported in literature. A first chapter will establish basic knowledge about the diversity of hydrogen-producing bacteria (HPB) (either spore-forming obligate anaerobe or no spore-forming obligate and facultative anaerobes) reported in hydrogen fermenters, with special attention to their functional performance and to the operating conditions favoring their emergence. The second section will emphasize the crucial role of microbial diversity not necessarily involved in primary hydrogen production but tightly interacting (either positively or negatively) with HPB, resulting in variable functional outcomes. Finally, based on the insights brought by microbial ecology studies, this review will evidence some of the bioengineering strategies and microbial resource management approaches proposed to date to enhance bioprocess function. A graphical outline of the manuscript structure is shown in Fig. 1.

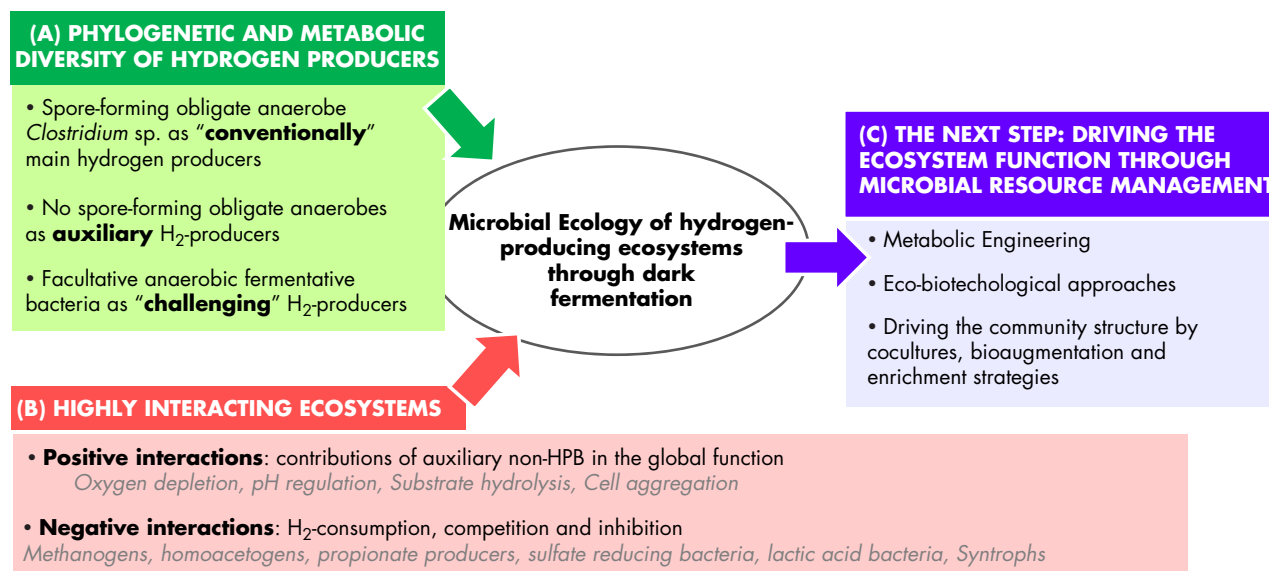


Figure 1. Outline of the review structure (HPB).

## MICROBIAL KEY PLAYERS IN FERMENTATIVE HYDROGEN-PRODUCING BIOPROCESSES

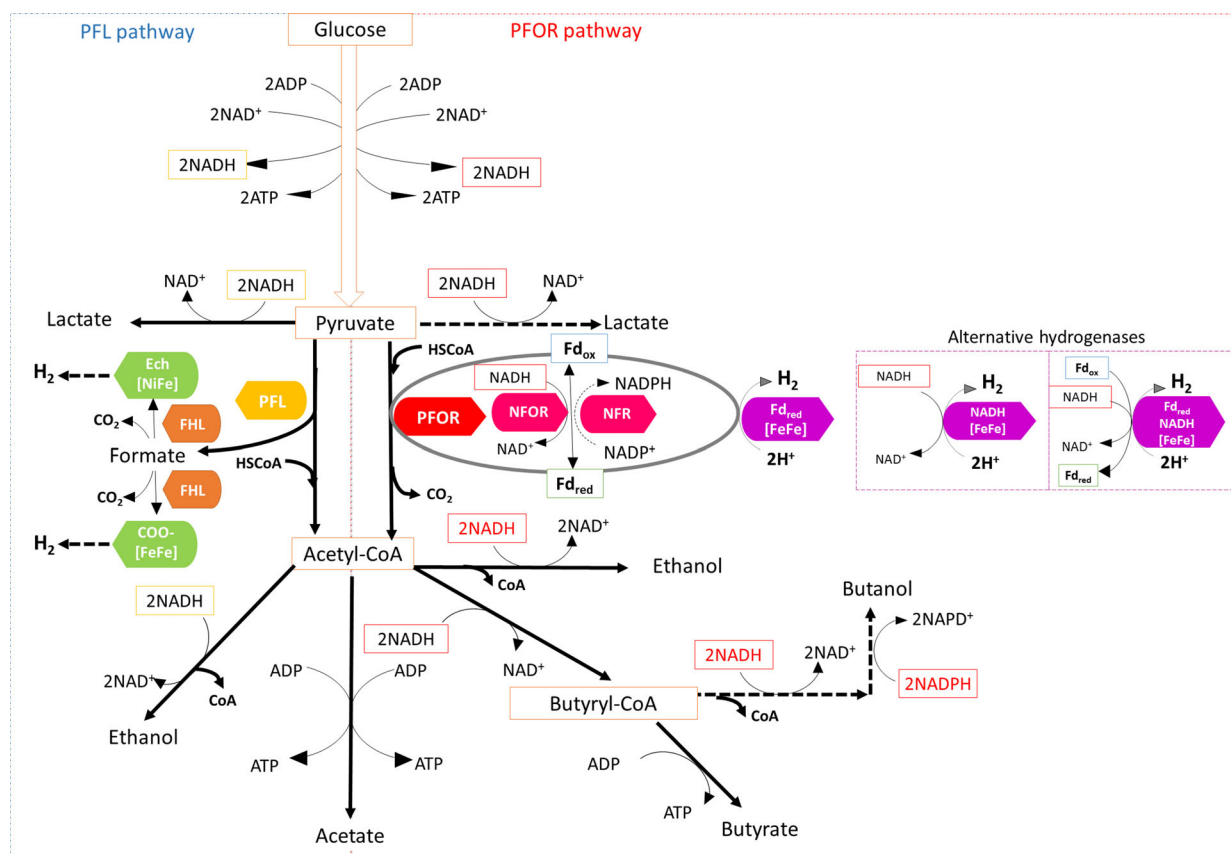
### Principles of dark hydrogen fermentation

Microorganisms are capable of different types of fermentation thanks to their high metabolic versatility, both among species and within the same species or strain. Fermentation is an anaerobic redox process, in which the substrate oxidation is partial and the final electron acceptor is an organic molecule (usually the same substrate itself or an intermediate from substrate oxidation). Molecular hydrogen production during microbial fermentation is a way to efficiently dissipate excess reductant (mainly by regenerating NAD<sup>+</sup> from NADH) as a diffusible gas (Schwartz, Fritsch and Friedrich 2013). Microorganisms produce H<sub>2</sub> using specialized metalloenzymes called hydrogenases (Vignais and Colbeau 2003; Schwartz, Fritsch and Friedrich 2013). Hydrogenases are classified into multiple groups and show a wide diversity that supports H<sub>2</sub>-based respiration, fermentation and carbon fixation processes in both oxic and anoxic environments, in addition to various H<sub>2</sub>-sensing, electron-bifurcation and energy-conversion mechanisms (Greening et al. 2015a,b). Several recent studies demonstrated that microbial H<sub>2</sub> metabolism is incredibly diverse and widespread at the taxonomic, community and ecosystem levels (Greening et al. 2015a,b). Typical substrates for fermentation include sugars and aminoacids, conventionally glucose is considered as the model substrate. In all thermodynamically feasible dark fermentation processes carried out by known microorganisms, H<sub>2</sub> is not produced as the single reduced compound but in combination with volatile fatty acids (VFA) and/or alcohols. H<sub>2</sub> evolution per se does not confer any advantage to microbes; however, it is obligatory for the elimination of excess electrons for some members of the microbial community.

Molecular hydrogen formation generally follows two routes in the presence of specific coenzymes, either through the reoxidation of nicotinamide adenine dinucleotide (NADH) pathway or through the pyruvate-formate-lyase (PFL) pathway (Nandi and Sengupta 1998; Hallenbeck and Benemann 2002; Hallenbeck 2005). In both cases, glucose is first converted to pyruvate, which then gives acetyl-CoA and either reduced ferredoxin

or formate (Fig. 2). In the first pathway, hydrogen production occurs through the oxidation of reduced ferredoxin (Fd<sub>red</sub>) with a ferredoxin-dependent hydrogenase (Fd-[FeFe]). This pathway is mainly used by obligate anaerobic microorganisms, such as Clostridia, which, under special conditions, are able to re-oxidate directly the NADH generated during glycolysis to produce additional hydrogen molecules through two other hydrogenases, i.e., NADH-dependent (NADH-[FeFe]) and bifurcating NADH-Fd<sub>red</sub>-dependent hydrogenase (NADH-Fd<sub>red</sub>-[FeFe]) (Tapia-Venegas et al. 2015). Therefore, if all the NADH are re-oxidated a total of 4 molecules of H<sub>2</sub> could be obtained from the fermentation of one molecule of glucose. In the second, the PFL pathway, formate is split into H<sub>2</sub> and CO<sub>2</sub> by a formate hydrogen lyase (FHL) complex, which contains a nickel-iron [NiFe] hydrogenase. This pathway is used by facultative anaerobes, such as Enterobacteria (Cai et al. 2011). Depending on the microorganism involved, this reaction can occur through [NiFe] hydrogenase (Ech hydrogenase) or formate-dependent [FeFe] hydrogenase (Tapia-Venegas et al. 2015). Organisms that only have the PFL pathway cannot access NADH for hydrogen production and thus theoretically are limited to 2 mol of hydrogen per mole of glucose (Hallenbeck, Abo-Hashesh and Ghosh 2012).

Acetyl-CoA is finally converted into acetate, butyrate and ethanol, depending on the microorganisms and the environmental conditions (Fig. 2). The final hydrogen yield depends on the main metabolite pathway orientation (Logan et al. 2002): acetate pathway provides the maximal yield of 4 mol<sub>H<sub>2</sub></sub>/mol<sub>glucose</sub>, while the butyrate and ethanol pathways are limited to 2 mol<sub>H<sub>2</sub></sub>/mol<sub>glucose</sub> (Gaudy and Gaudy 1980; Nandi and Sengupta 1998; Hwang et al. 2004). However maximum H<sub>2</sub> yield from fermentation can be achieved when only VFAs are produced and no microbial growth occurs (Angenent et al. 2004). Actual H<sub>2</sub> yields are usually lower than the theoretical ones (Kalia and Purohit 2008), being in the range of 1.2–2.3 mol H<sub>2</sub>/mol glucose (Benemann 1996; Angenent et al. 2004; Logan 2004). Apart from the energy involved in biomass production, there are several reasons for the low actual H<sub>2</sub> yield: glucose degradation toward non-H<sub>2</sub>-producing pathways; a stoichiometric yield is achievable only under near equilibrium condition, which implies a slow production rate and a low H<sub>2</sub> partial pressure (Woodward et al. 2000;



**Figure 2.** Fermentation pathways for hydrogen production from glucose, under anaerobic conditions. Pyruvate can be converted to either formate, through the PFL pathway which is most common in facultative anaerobes or to reduced ferredoxin and  $\text{CO}_2$ , through the pyruvate ferredoxin oxydoreductase (PFOR) pathway which is most common in strict anaerobes. In the PFL pathway, formate can be converted to hydrogen and  $\text{CO}_2$  by the FHL pathway, associated to either a [NiFe] (Ech) hydrogenase, or to a formate-dependent [FeFe] hydrogenase, depending on the microorganism. NADH generated during glycolysis is oxidized through the production of various reduced carbon compounds, such as lactate and ethanol. In the PFOR pathway, a ferredoxin-dependent (Fd-[FeFe]) hydrogenase enables to reoxidize ferredoxin and produce hydrogen. Moreover, NADH can also be used in hydrogen production, either by reducing ferredoxin (NFOR), by directly reducing hydrogenase (NADH-[FeFe]), or as a cosubstrate with reduced ferredoxin (Fd-NADH-[FeFe]). Excess NADH is used to produce other reduced fermentation products, such as butyrate. Adapted from Ramírez-Morales et al. (2015).

Hallenbeck and Benemann 2002);  $\text{H}_2$  consumption for other by-products (Vavilin, Rytow and Lokshina 1995). Hydrogen production capacity has also been reported in thermophilic and hyperthermophilic microorganisms from various extreme environments (e.g. *Thermotoga*, *Caldicellulosiruptor*, *Thermoanaerobacter*) with high production yields from a broad variety of substrates, using highly specific enzymatic metabolisms differing from the mesophilic ones (Adams 1990; Verhaart et al. 2010): they have been described elsewhere (especially in pure cultures) and are not in the scope of this review (Rittmann and Herwig 2012; Pawar and van Niel 2013; Cappelletti et al. 2014).

### Diversity of hydrogen producers

The ability to produce  $\text{H}_2$  gas in anaerobic mesophilic fermentative ecosystems was previously thought to be restricted to *Clostridium* species. With the emergence of molecular characterization techniques (which enabled to overpass the culture limitations but also have their own biases, as presented in Text Box 1), several works reported genomic evidence of the presence, diversity and activity of other HPB in hydrogen-producing communities. The most relevant mesophilic hydrogen producers will be presented in the next section. By convention, we divided HPB into three main key players: (i) spore-forming obligate anaero-

bic bacteria as 'conventionally' main hydrogen producers; (ii) no spore-forming obligate anaerobic bacteria as  $\text{H}_2$ -producers auxiliary contributing to the function; and (iii) facultative anaerobes as attractive challenging producers. The overview of the references used for this classification of phylogenetic and metabolic HPB diversity is proposed in Table S1 and Fig. S1 (Supporting Information), together with relevant information about the operating conditions prevailing in the reactors, performance indicators and main metabolites.

#### Text Box 1. Methodological and analytical considerations: biases of the 16S approach

To date, the most commonly used molecular tools to characterize complex microbial communities target highly variable fragments of the 16S ribosomal RNA, providing valuable phylogenetic identification of present and/or active species, thus overpassing some limits of the cultivation techniques. However, this approach is still limited by some methodological biases specific to the hydrogen communities: (i) low resolution of the 16S approach to distinguish between closely related species, due to the high similarity within *Clostridium* genus (Hung et al. 2008), and the high conservation degree in the Enterobacteriaceae family (Paradis et al. 2005); (ii) over-

estimation of the H<sub>2</sub> producers diversity, due to multiple 16S rRNA gene copies in many *Clostridium* sp. (Hung et al. 2008; Mariakakis et al. 2011); (iii) limited sensibility to encompass minor species which can be crucial for the process (Rafrafi et al. 2013); (iv) lack of functional inference due to the wide taxonomic diversity and functional redundancy within H<sub>2</sub>-producing communities.

To overpass the 16S limitations, functional approaches have been developed for strict anaerobic bacteria, targeting the *hydA* gene encoding for the [Fe-Fe]-hydrogenase which contributes to the unidirectional production of hydrogen through the reoxydation of the cellular-reducing elements (NADH and ferredoxine). However, as discussed in section 'Principles of dark hydrogen fermentation', H<sub>2</sub> could be produced by different metabolic pathways (i.e. involving the PFL and FHL complexes). It is therefore of prime importance to develop molecular tools targeting the genetic determinants of H<sub>2</sub> production in those systems. In this objective, primers targeting the Fe-hydrogenase of H<sub>2</sub>-producing *Enterobacter cloacae* (which have no homology with *Clostridium acetobutylicum*) have been proposed (Mishra et al. 2002).

Monitoring the *hydA* gene and its transcript enables to detect the presence and activity of functional (even if minority) populations directly involved in H<sub>2</sub> production within a mixed culture (Fang, Zhang, Li 2006; Chang et al. 2008b; Xing, Ren and Rittmann 2008), with improved sensitivity compared to the 16S approach (Quéméneur et al. 2010, 2011b). A possible bias when targeting the functional diversity through *hydA* gene is that the analysis is restricted to previously known species. Finally, some authors suggested to target the [FeFe]- and [NiFe]-hydrogenase genes separately, for a better and faster evaluation of the dynamics of both producers and consumers of hydrogen (Tolvanen et al. 2008a). Therefore, results have to be interpreted cautiously and future studies will need more integration of functional and phylogenetical approaches based on metatranscriptomics and metaproteomics.

#### Spore-forming obligate anaerobe *Clostridium* sp. as 'conventionally' main hydrogen producers

Clostridia are commonly considered as the most abundant and most efficient HPB in hydrogen reactors since they are usually predominant during the periods of highest hydrogen production efficiency, providing to reach H<sub>2</sub> yields from 1.5 to 3 mol<sub>H<sub>2</sub></sub>/mol<sub>hexose</sub> (Maintinguer et al. 2008; Chu et al. 2009; Hung et al. 2011; Masset et al. 2012; Laothanachareon et al. 2014) (Table S1). In batch experiments treating wastewater from beer-brewing industry inoculated with heat-shocked denitrifying sludge, the increase of the biohydrogen-producing potential was strongly correlated to the microbial shift toward a *Clostridium* spp.-dominated community, as a response to the initial pH increase (Boboescu et al. 2014). Due to their high efficiency, *Clostridium* sp. are often used as pure culture to inoculate or bioaugment reactors. As they are spore-forming bacteria, their capacity to resist heat shock or other drastic pre-treatment enables to easily and efficiently select them from mixed inoculum, with long-term stability (Ravindran, Adav and Yang 2010; Park et al. 2014; Goud, Sarkar and Mohan 2014). Therefore, most of the research effort on H<sub>2</sub> production by dark fermentation has focused on Clostridia to date, and some relevant examples are provided below.

*Clostridium butyricum* was the dominant cultivable HPB isolated from an anaerobic semi-solid fermentation system treating brewery yeast waste (Jen et al. 2007). It provided the highest

H<sub>2</sub> yield from starch in batch fermentations, among different clostridial pure cultures and defined co-cultures (Masset et al. 2012). *Clostridium pasteurianum* was the dominant HPB isolated from hydrogen-producing fermenters operated with mixed cultures and treating condensed molasses (Hsiao et al. 2009). It had the highest H<sub>2</sub> production rate, 3 to 8 folds higher than those of the other *Clostridium* species isolated at the same substrate concentrations (Hsiao et al. 2009; Liang et al. 2010). *Clostridium pasteurianum* is also suspected to promote granule formation thanks to its capacity of exopolysaccharides (EPS) production and self-flocculation (Liang et al. 2010). In large pilot-scale reactors fed with synthetic and real wastewater, *C. pasteurianum* represented up to 90% of the microbial community when the system approached its maximum hydrogen production (Cheng et al. 2011). *Clostridium beijerinckii* was one of the dominant HPB isolated in hydrogen fermenters fed with brewery yeast waste (Chang et al. 2008a) and palm oil mill effluent (Noparat, Prasertsan and O-Thong 2011). It had the highest hydrogen production rate compared to other pure HPB strains isolated from H<sub>2</sub> reactors (including other *Clostridium* species) and mixed anaerobic cultures (Chang et al. 2008a; Jeong et al. 2008) and exhibited the highest H<sub>2</sub> yield from glucose in batch assays, up to two times higher than those of other pure *Clostridium* species (Lin et al. 2007).

The different *Clostridium* species exhibit differential metabolic patterns and their relative abundances vary depending on the substrate type, the operating conditions and process configuration (Table S1). For example, *C. acetobutylicum* can switch from acidogenesis (hydrogen production through the acetate/butyrate pathway) to solventogenesis metabolism (acetone and butanol production, detrimental for H<sub>2</sub> production) (Lütke-Eversloh and Bahl 2011). Solventogenesis in *C. acetobutylicum* occurred mainly under conditions of low growth rate, low pH and high concentrations of carbohydrates (Baghchehsaraee et al. 2008). However, under optimal conditions, *C. acetobutylicum* provided the highest H<sub>2</sub> production rate from starch-containing waste in pure cultures (Argun, Kargi and Kapdan 2009). In glucose-fed continuous stirred tank reactors (CSTRs), *C. histolyticum* was dominant during the periods of ethanol-type fermentation (pH 4.0–4.5), with highest H<sub>2</sub> yield, while *C. lituseburense* was dominant during the periods of butyrate-type fermentation (pH 6.0–6.5), with lower H<sub>2</sub> yield (Song et al. 2011). In a sucrose-fed hydrogen-producing batch reactor, the hydrogen production rate was consistent with the succession of dominant species, reaching a maximum when the community got simplified toward only one dominant *hydA* gene type from *C. perfringens* and then declining when emerging *Clostridium* species appeared (Huang et al. 2010).

Clostridial species highly efficient for H<sub>2</sub> production have also other metabolic capacities of interest, such as saccharolytic activity for *C. tyrobutyricum* (Jo et al. 2007) or cellulolytic activity for *C. celerecrescens* (Li et al. 2010; Liang et al. 2010) and *C. acetobutylicum* (Wang et al. 2008). Interestingly, in CSTR fed with a mix of glucose and glycerol, *Clostridium* sp. were minor community members during the progressive adaptation to increasing glycerol concentrations, but became dominant and putatively responsible for hydrogen production when glycerol was the only substrate (Tapia-Venegas et al. 2015).

Eventually, *Clostridium* species can be the main hydrogen producers even though not dominant in the bioreactor, as reported in packed bed reactors treating sugar beet molasses (Chojnacka et al. 2011) and in a H<sub>2</sub>-producing granulated, anaerobic sequencing batch reactor (ASBR), bioreactor treating palm oil mill effluent where *Clostridium* isolates represented only 20% of the cultured community (the dominant community members being *Streptococcus* and *Lactobacillus* species), despite heat

treatment of the inoculum (Badiei et al. 2012). Many works evaluated the possible correlations between H<sub>2</sub> production performance and *Clostridium* abundance through their *hydA* gene quantification, with contrasted results depending on specific reactor conditions, as presented in Text Box 2.

#### Text Box 2. Correlations between H<sub>2</sub> production performance and *Clostridium* abundance

Several authors reported that the hydrogen production yield was positively correlated to the density of viable and active *Clostridium* sp. cells in starch-, glucose- and galactose-fed H<sub>2</sub>-producing fermenters (Cheng et al. 2008; Tolvanen et al. 2008b; Chu et al. 2009; Park et al. 2014). Conversely, the decrease of H<sub>2</sub> production performance was also correlated to a decrease of *Clostridium* spp. abundance (Monlau et al. 2013a). However, a strict correlation between hydrogen production performances and *Clostridium* spp. abundance is not always observed. In sucrose-fed CSTR, hydrogen was produced at the beginning of the experiment in spite of a very low number of clostridial hydrogenase genes detected (Tolvanen, Santala and Karp 2010), suggesting the key role played by other (facultative anaerobic) HPB during reactor startup. The relationship between HPB abundance and H<sub>2</sub> production performance also depends on specific operating conditions, such as the substrate type. When agitated granular sludge bed reactors were fed with simple (sucrose) substrate, the hydrogen production rate increased with the cell count of *Clostridium* spp., and was consistently correlated to the expression level of *Clostridium pasteurianum*-like *hydA* (Cheng et al. 2011). However, when the system was fed with real wastewater (condensed molasses soluble), *C. butyricum* was still the main HPB in the system, but its abundance was not correlated to the hydrogen production rate because of the dominance of other community members such as LAB (Cheng et al. 2011).

The poor correlations between hydrogen production performances and HPB abundance can be due to (i) hydrogen production by other HPB undetected by the molecular tool used; (ii) community composition changes toward the emergence of less efficient hydrogen producers, or negative effectors and/or hydrogen consumers; (iii) discrepancy between HPB density and effective hydrogenase activity. It may also suggest that biohydrogen production does not only depend on HPB abundance but also on environmental conditions (Tolvanen, Santala and Karp 2010; Mohd Yasin et al. 2011). Moreover, HPB abundance alone does not reflect the importance of HPB diversity and its effect on H<sub>2</sub> production. As an example, in batch experiments fed with sucrose and operated at different initial pH, the communities with highest *hydA* diversity provided higher H<sub>2</sub> production rates, suggesting that species coexistence may have positive effects on H<sub>2</sub> production (Quéméneur et al. 2011b). Furthermore, cell growth and hydrogen production are usually uncoupled during fermentation (Wang, Olson and Chang 2008): the increase of the HPB abundance can precede ahead of the increase of *hydA* expression level (Tolvanen et al. 2008a; Huang et al. 2010). Monitoring the *hydA* expression level seems to be a better early indicator of bioprocess functioning, well correlated to H<sub>2</sub> production rate, as reported in many cases (Chang et al. 2006; Fang, Zhang, Li 2006; Wang, Olson and Chang 2008; Huang et al. 2010).

#### No spore-forming obligate anaerobes as auxiliary hydrogen producers

Non-spore formers have been reported to persist and even dominate despite drastic heat-shock pretreatment in many mixed-culture reactors when the operating conditions are favorable, in terms of inoculum source, selection strategy, substrate and operative parameters (Luo et al. 2008; Liu et al. 2009) (Table S1). Under specific operating conditions, *Clostridium* sp. might not be the most adequate H<sub>2</sub> producers and other obligate anaerobic non-spore forming bacteria, mostly belonging to the Firmicutes and Bacteroidetes phyla, have been identified as major HPB, with specific metabolisms which enable to maintain acceptable (even though suboptimal) H<sub>2</sub> performance when *Clostridium* are inactive.

- I. Within the Clostridiales order, *Ethanoligenens harbinense* and *Acetanaerobacterium elongatum* are obligate anaerobes, able to ferment glucose into ethanol, acetic acid, H<sub>2</sub> and CO<sub>2</sub> (Chen and Dong 2004; Xing et al. 2006; Guo et al. 2011). They were predominantly enriched from anaerobic sludge during ethanol-type fermentation in acidic CSTR (pH 4.5–4.7) treating molasses wastewater from beet sugar refinery, and described as putative ethanol-H<sub>2</sub>-coproducing microorganisms with high efficiency (Ren et al. 2007; Xing, Ren and Rittmann 2008). They provided the highest H<sub>2</sub> yield during ethanol-type fermentation, compared to mixed-acid type fermentation (at higher pH), and coexisted with *Clostridium* sp. which was not the main H<sub>2</sub> producer in that case (Ren et al. 2007). A relative of *A. elongatum* (the new genus *Hydrogenoanaerobacterium saccharovorans*, able to produce ethanol, acetate, H<sub>2</sub> and CO<sub>2</sub> as main end products of glucose fermentation) has been reported in H<sub>2</sub>-producing granules in upflow anaerobic sludge blanket (UASB), demonstrating that the diversity of known HPB is still increasing and new studies enable to discover new strains (Song and Dong 2009).
- II. Within the Firmicutes phylum, the non-spore forming obligate anaerobe *Megasphaera* (Veillonellaceae family) were often reported as putative HPB in mesophilic fermentative systems. For example, *Megasphaera elsdenii* was dominant and proposed as the main hydrogen producer in fermentation processes inoculated with leaf-litter cattle-waste compost without pretreatment, fed with garbage slurry, operated under non-strict anaerobic conditions, where *Clostridium* members were absent (Marounek, Fliegerova and Bartos 1989; Ohnishi et al. 2010, 2012). *Megasphaera elsdenii* was also identified as putative prominent ethanol-H<sub>2</sub>-coproducing microorganism in acidic CSTR treating molasses wastewater from a beet sugar refinery under an ethanol-type fermentation (together with the previously described *A. elongatum* and *E. harbinense*) (Xing, Ren and Rittmann 2008). In that study, a hydrogenase gene transcript sequence was identified and affiliated to *M. elsdenii*-like [Fe]-*hydA*, thus confirming the contribution of this species to H<sub>2</sub> production. In cheese whey fermenting reactors inoculated from acidogenic sludge without pretreatment, *Megasphaera* was one of the prevalent putative hydrogen producers. However, the exact role and contribution of *Megasphaera* to H<sub>2</sub> production in mixed cultures is not clear to date. In some cases, its abundance is negatively correlated to H<sub>2</sub> production efficiency (Ren et al. 2007; Tapia-Venegas et al. 2015) and *Megasphaera*-dominated HPB populations often provided lower hydrogen yield compared to values reported

for different communities in similar conditions (Castelló et al. 2009, Ohnishi et al. 2010).

- III. Also belonging to the same Selenomonadales order within the Firmicutes phylum, *Acidaminococcus* sp. has been identified as a dominant HPB in CSTR treating condensed molasses fermentation soluble containing glutamate. Its active role in H<sub>2</sub> production from this substrate was confirmed by the expression of *Acidaminococcus*-like *hydA* transcripts (Chang et al. 2008b). *Acidaminococcus* was already present in the substrate itself and it may have been further favored by its capacity to use glutamate as substrate. *Acidaminococcus* was present at all tested hydraulic retention times (HRTs) in coexistence with *Clostridium*, but it overcompeted *Clostridium* at the lowest HRT condition tested, being the only HPB then (Chang et al. 2008b). However, its H<sub>2</sub> production efficiency depends on the species and substrate considered, as evidenced for *A. fermentans*: low hydrogen production from glutamate fermentation (Rogosa 1969), while the fermentation of citrate leads to significant hydrogen production (Cook et al. 1994).
- IV. *Prevotella* sp., a non-spore forming, strict anaerobe, from the Bacteroidetes phylum has been reported in several hydrogen reactors, without much emphasis to date because its exact role is not well defined yet. It might act either as positive contributor (by breaking down complex substrates) or as negative contributor (by competing for the substrates) (Castelló et al. 2009; Hung et al. 2011), and may also have the capacity to agglutinate with other microorganisms to form granules (Li, Zhang and Fang 2006). *Prevotella* was specifically selected and enriched by progressive adaptation to increasing glycerol concentrations in a CSTR fed with a mix of glucose and glycerol. It was strongly dominant and putatively responsible for hydrogen production at the highest glycerol concentration, in presence of glucose, but decreased when glucose was removed from the substrate mixture (Tapia-Venegas et al. 2015). *Prevotella* was abundant (but not dominant) in a fermentation system inoculated with untreated compost (Ohnishi et al. 2010) and in a large-scale continuous mixed reactor fed with sucrose, during the periods of highest hydrogen production (Mariakakis et al. 2011). However, low hydrogen production capacity has been evidenced for some species (Wu et al. 1992).

#### Facultative anaerobic fermentative bacteria as 'challenging' biohydrogen producers

The presence of facultative anaerobes is often limited by the drastic pretreatments of substrate/inoculum. Despite their theoretically lower yields, facultative anaerobes can be attractive for several reasons linked to their lower sensitivity to oxygen, thus rapidly depleting the oxygen in the culture either during start up or after accidental oxygen damage. Moreover, they make unnecessary the drastic heat-shock pretreatment which is not economically viable at full scale and has the drawback to suppress many non-spore forming HPB. As expected, facultative anaerobes are abundant when the substrate and/or inoculum origin and history is aerobic but not only: they can be detected even after drastic pretreatments. Facultative anaerobes can be considered as 'challengers' of H<sub>2</sub> production since in practice, some of them could reach or even exceed the hydrogen productivity reported for *Clostridium* cultures, as shown in Table S1 (Oh et al. 2003; Patel et al. 2014). In the following sections, the most relevant facultative anaerobic HPB will be presented.

H<sub>2</sub>-producing *Enterobacteriaceae*. Members of *Enterobacteriaceae* family, no-spore forming Gammaproteobacteria, are the most relevant facultative anaerobes for H<sub>2</sub> production in dark fermentation systems. When considering the indigenous self-fermenting microbial community of vegetable waste, all of the HPB isolated strains were facultative anaerobes, most of them belonging to the *Enterobacteriaceae* family, suggesting the influence of the aerobic history of the substrate (Marone et al. 2014). In granular packed bed reactors treating sugar beet molasses and inoculated with untreated sludge from a eutrophic meromictic lake, members of the family *Enterobacteriaceae* were the most dominant species detected by 454 pyrosequencing in both the granules and the biofilm, leading to significantly better hydrogen production performance than the *Clostridiaceae*- and *Leuconostocaceae*-dominated community which developed in the biofilm only, in absence of granular sludge (Chojnacka et al. 2011). Among *Enterobacteriaceae*, *Citrobacter*, *Klebsiella* and *Enterobacter* are especially considered as the front-runner H<sub>2</sub> producers to date (Patel et al. 2014), as evidenced in the following examples.

High hydrogen yield (exceeding 2 mol<sub>H<sub>2</sub></sub>/mol<sub>hexose</sub>) is usually reported for *Enterobacter aerogenes* (Jayasinghachari et al. 2009), which exhibited the highest H<sub>2</sub> production performance within the *Enterobacteriaceae* family compared to *Citrobacter amalonaticus* and *Escherichia coli* (Seol et al. 2008), and also when comparing to bacterial isolates from diverse (aerobic and anaerobic) environmental sources (Porwal et al. 2008). H<sub>2</sub> production by *Enterobacter* species has even been reported to exceed the clostridial production in some cases, as evidenced from waste wheat powder (Argun, Kargi and Kapdan 2009) and domestic landfill (Shin et al. 2007). Interestingly, in batch reactors that produce hydrogen from sucrose at pH 5.5 and inoculated with heat-shocked anaerobic sludge, the emergence and dominance of *En. cloacae* was favored at low to medium sucrose concentrations and outcompeted *Clostridium* sp., providing no lag phase and higher H<sub>2</sub> yields than in the *Clostridium*-dominated culture (Maintinguer et al. 2008). Similarly, superior H<sub>2</sub> yields of *Enterobacteriaceae* were reported in continuous flow bioreactor inoculated with heat-treated agricultural topsoil and fed with glucose at low to medium OLR, when clostridia were outcompeted (Luo et al. 2008).

*Citrobacter* spp. was identified as one of the H<sub>2</sub> producers (the only one from the *Enterobacteriaceae* family, in coexistence with *Clostridia*) in CSTR producing hydrogen from molasses wastewaters, independently of different pH and fermentation types (ethanol vs ethanol butyrate), showing its tolerance to various environmental conditions (Ren et al. 2007). The H<sub>2</sub> production capacity of *Citrobacter* has been demonstrated in pure cultures (Kim et al. 2008).

*Klebsiella* is known to produce hydrogen and alcohols from a variety of substrates, as verified in pure cultures (Niu et al. 2010) and also has the capacity to agglutinate with other microorganisms to form granules (Li, Zhang and Fang 2006). *Klebsiella pneumoniae* was a dominant community member active in hydrogen production in a CSTR treating sugarcane juice (Patra et al. 2011). *Klebsiella* sp. and other *Enterobacteriaceae* members (*Escherichia*, *Shigella*) were also strongly dominant in an hydrogen-producing community able to efficiently convert glycerol into H<sub>2</sub> and ethanol, after a specific enrichment of aerobic activated sludge (Varrone et al. 2013). Interestingly, *Klebsiella* sp. was the most abundant genus in the class Gammaproteobacteria in batch fermenters fed with sucrose, inoculated with activated sludge and exposed to heavy metals, suggesting that it stimulated hydrogen production and was able



to resist to a wide range of heavy metal concentrations (Cho and Lee 2011).

Obviously, all Enterobacteriaceae are not equivalent in terms of H<sub>2</sub> production efficiency, as demonstrated in pure strains isolated from various sources (Kanso et al. 2011): their H<sub>2</sub> production depends on environmental conditions (Trchounian, Sargsyan and Trchounian 2014). Various Enterobacteriaceae, including the genus *Klebsiella*, have been identified during periods of poor hydrogen production in batch fermenters fed with sucrose (Cho and Lee 2011) or in large pilot-scale reactors fed with real and synthetic wastewater (Cheng et al. 2011), indicating that all Enterobacteriaceae do not always promote efficient hydrogen generation. In a mixed culture of *E. coli* and *Clostridium* in glucose-fed continuous flow bioreactor, *E. coli* may not be involved in hydrogen production, as suggested by the inconsistency of detected metabolite patterns (Tolvanen, et al. 2008b).

**H<sub>2</sub>-producing Bacillales.** Phylogenetically closer to *Clostridium* sp, some spore-forming, facultative anaerobic Bacillales (Firmicutes phylum) have been reported to produce H<sub>2</sub> through the FHL pathway, providing high yields around 2 mol<sub>H<sub>2</sub></sub>/mol<sub>hexose</sub> (Patel et al. 2012, Kumar et al. 2013). Compared to *Clostridium* and Enterobacteriaceae, *Bacillus* spp. present the combined advantages to be little sensitive to oxygen and to resist heat-shock treatment through sporulation. Moreover, their metabolic versatility allows them to use a wide range of substrates, including lignocellulosic biomass (Patel, Purohit and Kalia 2010; Kumar et al. 2013), bagasse and molasses biomass (Sinha and Pandey 2014) or proteins (Cai, Liu and Wei 2004), maybe due to their capacity to produce hydrolytic enzymes. Several reports also indicate their resistance to a broad range of pH and salt concentrations (Liu and Wang 2012; Song et al. 2013). H<sub>2</sub>-producing *Bacillus* can be found in a wide range of different environments such as sewage sludge anaerobic digester (Kotay and Das 2007), corn-stalk biohydrogen reactor (Song et al. 2013), marine intertidal sludge (Liu and Wang 2012) or cattle dung enriched for H<sub>2</sub> production (Kalia et al. 1994). Interestingly, H<sub>2</sub> production in *Bacillus* sp. is often coupled to the production of other biomolecules of interest such as polyhydroxybutyrate (Porwal et al. 2008; Patel et al. 2012) or biofloculant polymers (Liu, Chen and Wang 2015). *Paenibacillus polymyxa* from the Bacillales order was the most abundant and the most effective in H<sub>2</sub> production from glucose under pH-uncontrolled batch culture, among eutrophic lake sediment isolates (Lal et al. 2012). The hydrogen production yield of *P. polymyxa* increased when the substrate shifted from glucose to xylose, together with a metabolic shift from alcohol production to acetate accumulation (Marwoto et al. 2004). Hydrogenase gene sequences of *Paenibacillus* have been detected during hydrogen production in batch system fed with sucrose and inoculated with heat-shocked cattle dung compost, confirming it was involved in H<sub>2</sub> production (Huang et al. 2010).

**Other facultative anaerobic HPB.** As research on dark fermentation advances, the diversity of identified microorganisms capable of H<sub>2</sub> production increases. Facultative anaerobic Gammaproteobacteria, such as *Shewanella oneidensis* (Meshulam-Simon et al. 2007) and *Pseudomonas stutzeri* (commonly known as a denitrifying bacterium) (Shiyan and Krishnaveni 2012), were characterized for their ability to produce H<sub>2</sub> at high yields in pure culture. Nevertheless, there is little knowledge about their role in mixed cultures and their capacity to maintain at long term in continuous systems operated at high flow rates with real wastewater (Cheng et al. 2011). Genes encoding a periplasmic [Fe-Fe] hydrogenase were expressed under anaerobic conditions

in *S. oneidensis*, and H<sub>2</sub> production in this species evolved from multiple parallel pathways, involving either pyruvate or formate intermediates (Meshulam-Simon et al. 2007).

Moreover, very little research was done up to now to investigate H<sub>2</sub> production in particularly extreme habitats. For example, under halophilic conditions, the hydrogen-producing community from a salt factory wastewater lagoon shifted toward the emergence and dominance of Vibrionaceae (facultative anaerobes from the Gammaproteobacteria) as main HPB, with ethanol, formate and acetate as main metabolites, at the expense of the initially abundant Clostridiales and Enterobacteriales (Pierra et al. 2014). Interestingly, the operation at high NaCl concentration permitted to reach the highest hydrogen yield and to reduce hydrogen consumption rate. *Vibrio* spp. have also been isolated from UASB granules treating high-strength organic wastewater, and demonstrated satisfactory H<sub>2</sub> yields, as high as other aerobic and anaerobic isolates from the same source (Oh et al. 2003). A new HPB isolated from mangrove sludge and identified as *Pantoea agglomerans* (Enterobacteriaceae family) was described as salt tolerant and offered a biotechnological interest for biological treatment of mariculture wastewater and marine organic waste (Zhu et al. 2008).

#### Synergism between diverse hydrogen producers

While the previous sections aimed at describing the main HPB individually, it is important to note that in practice, many examples of natural coexistence of diverse HPB (including strict and facultative anaerobes) have been reported in bioreactors, usually beneficial to hydrogen production.

In glucose-fed CSTR, some strains of Enterobacteriaceae contributed to hydrogen production as non-dominant HPB, in association with dominant *Clostridium*, and were resistant to environmental conditions: they maintained at the same abundance independently of the pH and the fermentation type (ethanol vs butyrate) imposed by the different *Clostridium* species (Song et al. 2011). In hydrogen-producing granules in packed bed reactors, the dominant *Clostridium* spp. developed in close association with *Klebsiella* and *Prevotella* which have the capacity to agglutinate with other microorganisms (Li, Zhang and Fang 2006). When both strict and facultative anaerobes naturally coexist in mixed cultures, as reported in sucrose-fed CSTR, *Enterobacter* likely contributed to H<sub>2</sub> production although it was not the main producer (Tolvanen, Santala and Karp 2010). Especially, it was probably responsible for H<sub>2</sub> production at the beginning of the operation, when the clostridial hydrogenase genes were not detected yet, and then was maintaining in the continuously renewed ecosystem. The fluctuating abundance of *Enterobacter* might therefore be the reason why H<sub>2</sub> production efficiency varied in a higher extent than the number of clostridial hydrogenases in that case (Tolvanen, Santala and Karp 2010). In continuous flow bioreactor inoculated with heat-treated agricultural topsoil and fed with glucose across a range of OLRs, the most diverse microbial community (comprising *Selenomonas*, *Enterobacter* and *Clostridium* spp) resulted in the highest yield of hydrogen production, at low OLR, compared to the *Clostridium*-dominated community at higher OLRs (Luo et al. 2008). Other examples of coexisting consortia efficient for H<sub>2</sub> production include *P. polymyxa* and *Eubacterium multiforme* from alkaline pretreated sewage sludge substrate (Cai, Liu and Wei 2004), or *C. tyrobutyricum* and *E. harbinense* in a large lab-scale ethanol-H<sub>2</sub> coproducing CSTR fed with sucrose (Mariakakis et al. 2011). Based on these observations, several authors proposed bioengineering strategies to increase process performance through co-culture

inoculation and/or bioaugmentation as discussed in the section ‘The next step: driving the ecosystem function through microbial resource management’.

As a conclusion of this first section, it is noteworthy to point out the high diversity of hydrogen producers in mesophilic bioprocesses. Even though *Clostridium* sp. were considered to date as the most efficient H<sub>2</sub> producers thanks to their high theoretical yields, they can be complicated to use in practice due to their strict anaerobic requirements or in case of specific operating conditions such as drastic environmental conditions or recalcitrant substrates. In those cases, other HPB can be useful for engineering bioprocesses, such as non-spore-forming anaerobes from Firmicutes and Bacteroidetes phyla, with specific metabolisms which enable to sustain acceptable (even though suboptimal) H<sub>2</sub> production performance when *Clostridium* are inactive. Eventually facultative anaerobes can be considered as challengers H<sub>2</sub> producers, combining high yields (comparable or higher to the Clostridial ones), tolerance to oxygen and metabolic versatility for various substrates, thus offering more flexibility in engineered ecosystems. In practice, the association of diverse HPB working in synergy should be favored to overpass the limitations of pure cultures in face of complex biowaste variability. Moreover, the natural diversity of mixed communities, including many microorganisms not directly involved in H<sub>2</sub> production, also has a critical role to play in the global process, as developed in the next section.

## THE MICROBIAL NETWORK OF H<sub>2</sub>-PRODUCING BIOPROCESSES: HIGHLY INTERACTING ECOSYSTEMS

Hydrogen production bioprocesses are complex and diverse ecosystems relying on interacting microorganisms and interconnected reactions, where direct hydrogen producers are not the only microbial players participating in the ecosystem. Most of this microbial diversity is part of the indigenous microflora from non-sterile substrates or untreated inocula. A high number of rare operational taxonomic units (OTUs) can be detected at low abundance in H<sub>2</sub> bioprocesses, with unclear role in H<sub>2</sub> production, as reported in packed bed reactors treating sugar beet molasses where rare OTUs represented 95% of the OTUs but only 2% of the sequences (Chojnacka et al. 2011). Xing, Ren and Rittmann (2008) reported that the total bacterial diversity increased with time during the adaptation phase in CSTR treating molasse wastewater from a beet sugar refinery. After acclimation, only 6% of the OTUs from the 16S rRNA gene clone libraries were affiliated to putative HPB, during stable H<sub>2</sub> production phase. In glucose-fed continuous flow bioreactors inoculated with either a pure culture (*Ethanoligenens harbinense*) or an enriched culture (dominated by *Clostridium butyricum*) and operated under non-sterile conditions, the community evolved rapidly toward more diversity: the initially dominant strain kept dominant after some weeks, but new invading species emerged progressively during stable H<sub>2</sub> production phase, forming a stable community including known HPB as well as microorganisms with unclear function, and others clearly unable to produce H<sub>2</sub> from glucose (Tolvanen et al. 2008b, Xing et al. 2008).

In the following sections, the role of such subdominant populations is discussed through the different (positive and negative) interactions they establish with HPB, paying special attention to the environmental and/or operating conditions that favor their emergence.

## Positive interactions: contributions of auxiliary non-HPB in the global function

As reported in many cases, the presence of auxiliary bacteria which are not able—or less efficient—to produce H<sub>2</sub> can be nonetheless associated with improved conversion of substrate to hydrogen, suggesting that these microorganisms can contribute positively to the global ecosystem service and enhance H<sub>2</sub> production through different mechanisms, such as cometabolism, granulation, oxygen consumption or hydrolysis.

### Oxygen consumption

The first way in which non-H<sub>2</sub> producers (or poorly efficient producers) can contribute to the ecosystem service is by depleting oxygen traces in the reactor and creating anaerobic conditions suitable for the growth of strict anaerobic HPB such as *Clostridium* sp., thus favoring high-rate hydrogen-producing pathways.

This role has been often attributed to *Bacillus*. In a sucrose-fed reactor, the growth and dominance of various aerobic *Bacillus* species and other facultative anaerobes during the initial lag phase, i.e. when the oxidation reduction potential was still high, was efficient for oxygen exhaustion and creating strict anaerobic environment (Huang et al. 2010). Thanks to its ability to grow faster at the beginning of the culture in presence of oxygen, *Bacillus thermoamylovorans* was considered as a ‘starter’ for biomass conversion in co-cultures with *C. butyricum* (Jen et al. 2007) and *C. beijerinckii* (Chang et al. 2008a) in anaerobic semi-solid batch reactors treating brewery yeast waste. In both cases, *B. thermoamylovorans* shortened the lag phase, favored subsequent *Clostridium* growth and was detected during the optimal hydrogen-producing phase. Consistently, a statistical response surface analysis showed that *B. thermoamylovorans* stimulated the specific hydrogen production rate of *C. beijerinckii* and *C. butyricum* from yeast waste, and enabled to determine the optimal ratio of each partner in co-culture (Chou et al. 2011). The exact type of symbiosis is not clear to date. It can be based on mutual benefits for both partners (as it might be the case for *C. beijerinckii* and *B. thermoamylovorans*) or on commensalism where one partner benefits from the other without affecting it (as it might be the case for *C. butyricum* and *B. Thermoamylovorans*) (Chou et al. 2011).

Apart from *Bacillus*, the capacity to act as oxygen consumer has also been attributed to facultative anaerobes from the Enterobacteriaceae family in packed bed reactors treating sugar beet molasses (Chojnacka et al. 2011), and specifically to *Klebsiella* sp. in glucose- and sucrose-fed granular sludge bed bioreactors, even at low abundance (Hung et al. 2007). The ability of *Klebsiella* to consume significantly O<sub>2</sub> has been verified in pure culture experiments (Hung et al. 2011), and its presence in codominance with *Clostridium* increased H<sub>2</sub> production of from sugarcane juice (Patra et al. 2011).

### pH regulation

pH is a critical ecological factor shaping the microbial community composition in hydrogen-producing ecosystems, and especially the HPB fraction of the community (Mohd Yasin et al. 2011). By influencing the equilibrium between different chemical forms, resulting in more or less availability and/or toxicity of the compounds, pH directly influences the enzymatic activity (Li, Zhang and Fang 2007; Cai et al. 2010).

Since hydrogen production is optimal at acid pH, a second way to contribute positively to hydrogen production in mixed culture fermentations is by slightly acidifying the local environment of HPB in the reactor, which can be achieved by lactic acid

bacteria (LAB). In packed bed reactors treating sugar beet molasses, the hydrogen production efficiency was directly correlated to the abundance of LAB such as Leuconostocaceae and Streptococcaceae (from the Lactobacillales order), which were among the most abundant species detected by 454 pyrosequencing and may have played a significant acidifying role, although non-H<sub>2</sub> producers (Chojnacka et al. 2011). Nevertheless, the exact role and contribution of LAB in hydrogen production ecosystems has not been fully elucidated and is probably underestimated, as extensively discussed in the section 'Syntrophic metabolism'.

Since a drastic acidification could be prejudicial to hydrogen production, some bacteria are also thought to contribute positively to the ecosystem service by oxidizing short-chain fatty acids and preventing their accumulation, therefore buffering against a collapsing pH drop. This role has been proposed for *Megasphaera elsdenii* (able to consume excessive lactic acid), *Syntrophobacter fumaroxidans* and *Syntrophomonas wolfei* (known as propionate degraders) in different fermenting systems (Xing, Ren and Rittmann 2008; Ohnishi et al. 2010).

### Substrate hydrolysis

A third way to contribute positively to hydrogen production in mixed culture fermentations is by hydrolyzing large organic molecules from complex substrates into smaller molecules that the HPB can use to produce H<sub>2</sub>, thus generating a metabolic synergy between hydrolyzers and HPB. The activity of these 'process helpers' is critical since the use of complex biowaste is one of the future challenges to ensure the economic viability of H<sub>2</sub> production at large scale (Patel et al. 2012).

Diverse hydrolyzers have been detected in hydrogen-producing ecosystems operated with raw substrates, including LAB. The dominant clones retrieved from a starch-fed hydrogen fermenter, a starch-peptone fed CSTR and a kitchen waste fermenter at low HRT were affiliated to *Bifidobacterium* sp., *Lactobacillus plantarum* and *Olsenella genomosp* (similar to *Lactobacillus* sp.), respectively (Cheng et al. 2008; Li et al. 2010, 2011). These three facultative heterofermentative LAB can hydrolyze starch to produce mainly lactate and some trace of acetate, but no hydrogen, and were more abundant during the periods of highest carbohydrate removal efficiency and H<sub>2</sub> production, indicating their amylolytic activity (Cheng et al. 2008; Li et al. 2010, 2011). In sludge from cattle manure compost incubated with cellobiose substrate, the functional bacterial consortium that could effectively hydrolyze cellobiose and produce hydrogen was composed of a cellobiose-hydrolyzing bacteria (*Enterococcus saccharolyticus*, from the order Lactobacillales, which harbors hydrolytic activity for a wide range of substrates) associated with an HPB (*C. butyricum*) (Adav et al. 2009).

Apart from LAB, other aerobic or facultative anaerobic microorganisms with hydrolyzing capacities have been detected in hydrogen-producing systems. When comparing the hydrogenic and hydrolytic activities of 35 bacterial isolates from diverse environmental sources, Porwal et al. (2008) found that the strains with the highest lipase, protease and amylase activities differed from the strains with highest H<sub>2</sub> production capacity and were mainly affiliated to *Bacillus* sp. and *Pseudomonas* sp. For example, *B. thermoamylovorans*, isolated from anaerobic semi-solid batch fermenters treating brewery yeast waste, could not produce H<sub>2</sub> but had multiple extracellular enzyme activities including lipase, protease,  $\alpha$ -amylase, pectinase and cellulase activities, suggesting that it could act as good partner for presaccharification in co-cultures with clostridial HPB (Jen et al. 2007). The extracellular hydrolytic activities of *B. thermoamylovorans* have been verified experimentally in pure cultures for several

complex substrates (Chang et al. 2008a). A strain of *Bacillus* sp. capable of hydrolyzing starch by secreting amylase has been used in co-culture with an Alphaproteobacteria HPB (*Brevundimonas* sp), thus providing high rate hydrogen production from starch in batch reactors (Bao, Su and Tan 2012). *Paenibacillus polymyxa* has been reported in hydrogen-producing reactors fed with complex substrates, which could be explained by the wide range of extracellular hydrolytic activities exhibited by this stain, such as glucanase, cellulase, chitinase, protease and xylanase activities (Lal and Tabacchioni 2009).

The use of mixed consortia combining hydrolytic bacteria (especially *Bacillus* sp. and *Proteus mirabilis*) and hydrogen producers enabled to reach high H<sub>2</sub> yields from lignocellulosic biowaste such as pea-shells slurry, exceeding the yields obtained with H<sub>2</sub> producers alone (Patel et al. 2012). The strictly aerobic, non-spore-forming *Microbacterium phyllosphaerae* (Actinobacteria phylum), known as proteolytic-saccharolytic bacteria able to produce organic acids but no hydrogen, was detected in a continuous reactor treating food waste (Kim and Shin 2008). In ASBR inoculated with anaerobic consortium and bioaugmented with different pure strains, the highest chemical oxygen demand (COD) removal efficiency was observed with *Pseudomonas stutzeri* bioaugmentation, suggesting its role in complex organic matter degradation (Goud et al. 2014).

Hydrolytic bacteria can also be strict anaerobes, as reported for *Ruminococcus*, known for its cellulolytic and lignocellulolytic activity, found in symbiosis with HPB lacking this activity (Ueno et al. 2001; Motte et al. 2014). In some cases, *Clostridium* sp. themselves are not used for their hydrogen-production capacity, but for their hydrolytic capacity. For example, the hydrolytic cellulose-degrader *C. acetobutylicum* was used in co-culture with the HPB *E. harbinense*, which could not degrade cellulose but could efficiently consume the reduced sugars, for hydrogen production from microcrystalline cellulose (Wang et al. 2008).

### Cell aggregation

Finally, another indirect way to contribute to efficient hydrogen production in mixed cultures is by favoring cell aggregation, thus increasing cell concentration within the reactor, preventing biomass wash-out, and offering protective barriers against toxic or hostile environments for the HPB.

This role has been often attributed to LAB. Illustratively, the facultative anaerobic *Streptococcus* sp. (Lactobacillales order) was probably implied in cellular granulation in glucose- and sucrose-fed granular sludge bed bioreactors, where it represented 7%–26% of all bacteria (Hung et al. 2007, 2011). Especially, it may act as a seed for the formation of self-flocculated core granules, subsequently colonized and surrounded by the *Clostridium* sp. HPB, as demonstrated by fluorescence in-situ hybridization (FISH) pictures (Hung et al. 2007, 2011). *Streptococcus* sp. abundance increased when HRT decreased, i.e. when self-granulation occurred (Hung et al. 2007). This observation was confirmed in pure cultures where *Streptococcus* sp. was able to produce EPS complexes to strengthen sludge granulation (Hung et al. 2011). *Streptococcus* sp. was also dominant in H<sub>2</sub>-producing granular ASBR treating palm oil mill effluent, where microscopy observations confirmed that it was involved in biomass retention through granule formation (Badiei et al. 2012).

Other microorganisms, different from LAB, have been identified as putative responsible of favoring cell aggregation in H<sub>2</sub>-producing systems. In chemostats continuously fed with a glucose-based medium and dominated by *Clostridium* sp., some minor bacteria such as *B. racemilacticus* was found in the culture with the highest biomass concentration, and consequently

the highest H<sub>2</sub> productivity: the positive effect of *B. racemilacticus* on H<sub>2</sub> production could be explained by its capacity to produce EPS that favor cell aggregation and biofilm development (Rafrafi et al. 2013). This positive contribution has also been attributed to *P. polymyxa*, present in a variety of hydrogen-producing reactors fed with complex substrates (Lal and Tabacchioni 2009). The interesting property of autoaggregation of *E. harbinense* by forming quick flocs has been demonstrated in continuous CSTR fed with glucose, highlighting its biotechnological interest in bioaugmented reactors despite continuous operation at high dilution rate under non-sterile conditions (Xing et al. 2008). *Prevotella* sp. and *Klebsiella* sp., despite their moderate performance in H<sub>2</sub> production, played a significant role in granule formation through the production of filamentous polysaccharides which efficiently retained *Clostridium* sp. in the packed bed reactor, thus enhancing H<sub>2</sub> production (Li, Zhang and Fang 2006). Independently of their H<sub>2</sub> production performance, the capacity of EPS production and self-flocculation promoting granule formation has also been reported in clostridial species such as *C. pasteurianum* (Liang et al. 2010).

### Negative interactions: H<sub>2</sub> consumption, competition and inhibition

The microbial diversity present in H<sub>2</sub>-producing reactors does not always contribute positively to the function of interest and can even reduce H<sub>2</sub> yield, mainly because of the activity of (i) symbiotic bacteria that directly consume H<sub>2</sub>, (ii) competitors that outcompete HPB for their substrates or (iii) bacteria that inhibit HPB through their metabolites. Negative contributors can be phylogenetically very diverse.

#### Hydrogen consumption by methanogens

One of the main mechanisms responsible for hydrogen loss is the use of H<sub>2</sub> as the primary electron donor to reduce CO<sub>2</sub> for methane production, by hydrogenotrophic methanogens such as Methanobacteriales and Methanomicrobiales (Chaganti, Lalman and Heath 2012). Since pretreatment methods such as sonication, aeration and freeze/thaw cycles are not efficient to prevent methane generation (Dong et al. 2010), methanogens are usually inhibited by heat-shock pretreatment of the inoculum (Oh, Van Ginkel and Logan 2003; Goud, Sarkar and Mohan 2014). Moreover, methanogens are usually sensitive to low pH. However, heat-shock pretreatment and low pH operation are not always sufficient, especially when treating real unsterilized substrate. Indeed, Chu et al. (2009) reported the persistence of methanogens in glucose-fed CSTR despite heat-shock pretreatment of the inoculum and despite the low pH (i.e. 5.5). When using seed sludge from an acidogenic lab-scale reactor, without pretreatment, at HRT between 12 and 24 h, methane production was substantial during the first 220 days, despite low pH operation (4.7) (Castelló et al. 2009). Due to their low growing rate, the persistence of methanogens is favored at long HRTs, as reported in a continuous reactor fed with cheese whey permeate and inoculated with untreated anaerobic sludge (Yang et al. 2007). Nevertheless, methanogenic activity could not be completely inhibited in a large lab-scale complete mixed reactor, fed with sucrose and inoculated with untreated anaerobic sludge, despite operation at pH 5.5 and HRT 12 h (Mariakakis et al. 2011). An increase of the purge rate enabled to decrease methane production and increase H<sub>2</sub> production by reducing excess biomass, but was not sufficient to completely wash the methanogens out of the reactor (Castelló et al. 2009).

#### Hydrogen consumption by homoacetogens

Fermentative homoacetogens are strict anaerobes that use hydrogen as electron donor to reduce CO<sub>2</sub> to acetate, autotrophically, through the acetyl-CoA pathway. They are very versatile bacteria, which can also have a chemoheterotrophic metabolism, thus converting a variety of different substrates to acetate as the major end product (Diekert and Wohlfarth 1994). In hydrogen fermenters, homoacetogenic activity results in lower hydrogen production and acetate accumulation.

Homoacetogenic microorganisms may overlap between H<sub>2</sub> producers and H<sub>2</sub> consumers (Chaganti, Lalman and Heath 2012). Indeed, several clostridial species exhibit a reverse metabolism that can either evolve or uptake hydrogen through bidirectional hydrogenases (Das et al. 2006). In a sucrose-fed batch reactor, *C. lundense*, *C. peptidivorans* and *C. vincentii* began to appear when H<sub>2</sub> production had stopped and H<sub>2</sub> uptake was observed (Huang et al. 2010). Clostridial [Ni-Fe] hydrogenases have been detected by proteomic approach in cellobiose cultures which showed the highest H<sub>2</sub> consumption rates (Quéméneur et al. 2011a). *Clostridium acetium*, *C. thermoautotrophicum*, *C. thermoacetium* and *C. stercorarium* are well-known homoacetogenic bacteria utilizing H<sub>2</sub> and CO<sub>2</sub> (Ueno et al. 2006; Guo et al. 2010), as well as *C. ljungdahlii*, which was detected before the efficient H<sub>2</sub> production phase in a large lab-scale CSTR fed with sucrose, and disappeared during high H<sub>2</sub> production periods, after increasing the OLR and decreasing the HRT (Mariakakis et al. 2011). The reversibility of hydrogenase has also been reported in Enterobacteria such as *Citrobacter amalonaticus* (Kim et al. 2008).

The fraction of hydrogen converted to acetate via homoacetogenesis cannot be established experimentally because of the complex interactions between H<sub>2</sub> producers and consumers, but it can be estimated through a flux balance analysis (FBA) of the experimental data. When comparing granular and flocculated mixed anaerobic cultures at pH 5.0, inoculated from UASB sludge, fed with glucose and exposed to linoleic acid inhibitor, the FBA revealed H<sub>2</sub> consumption by homoacetogenic activity in the granular culture (dominated by *Bacteroides* sp.), while there was no significant homoacetogenic activity in the flocculated culture (dominated by *Clostridium* sp. and *Bacillus* spp.), where the H<sub>2</sub> yield was thus higher (Saady et al. 2012). In that case, the homoacetogenic activity within the granular culture was putatively attributed to *Eubacterium* sp., a non-spore former from the Clostridia class able to grow autotrophically on H<sub>2</sub>/CO<sub>2</sub> (Mechichi et al. 1998). By contrast, the absence of homoacetogenic activity in the flocculated culture was attributed to a major inhibition of H<sub>2</sub> consumers by linoleic acid, in absence of 'protection' by the granular structure (Saady et al. 2012).

Heat-shock treatment of the inoculum is considered more efficient than other pretreatments (acid, base, aeration and chloroform) to suppress homoacetogenic activity (Wang and Wan 2008; Goud, Sarkar and Mohan 2014), albeit not always sufficient since many homoacetogenic bacteria are spore forming (Oh, Van Ginkel and Logan 2003; Baghchehsaraee et al. 2008). Chloroform pretreatment of immobilized anaerobic granules was suggested to inhibit the synthesis of acetate from H<sub>2</sub>/CO<sub>2</sub> (Hu and Chen 2007).

#### Hydrogen consumption by propionate-producing bacteria

Propionate-type fermentation is a hydrogen-consuming pathway that produces mainly propionate, acetate and some valerate, without significant gas production, and seems to be favored by acid pH (Ren et al. 2007; Guo et al. 2010) and high HRT. Propionate production was positively correlated to HRT in CSTRs fed with glucose, wheat starch or sugarcane juice (Hussy et al.

2003; Zhang, Bruns and Logan 2006; Pattra et al. 2011). The propionate production was suppressed at low HRT, thanks to the wash-out of propionate-producing bacteria (Zhang, Bruns and Logan 2006).

Most studies report the role of Veillonellaceae members (Firmicutes phylum) as propionate producers through direct H<sub>2</sub> consumption and lactate or succinate degradation, including the genus *Propionispira* and the closely related *Schwartzia* and *Selenomonas*, specifically detected during the periods of poor to null hydrogen production and propionate accumulation in continuous reactors fed with glucose and sucrose (Cohen et al. 1985; Mariakakis et al. 2011). In a CSTR fed with starch and peptone, *Propionispira arboris* was suspected to be responsible for H<sub>2</sub> consumption and propionate production through a reverse hydrogenase activity (Li et al. 2010). When comparing the settling and non-settling fractions in an ASBR converting starch to hydrogen, high propionate concentrations were detected only in the settleable sludge, which had the lowest specific H<sub>2</sub> activity; it was attributed to the propionate producers *Selenomonas* sp. which were favored by high settling times (Arooj et al. 2007). In a fluidized bed bioreactor fed with glucose, the shift from acetate-butyrate to acetate-propionate production (resulting in reduced H<sub>2</sub> production) was explained by community structure changes rather than metabolic changes, through the enrichment of propionate producers (identified as *Schwartzia succinivorans*) due to their efficient adhesion on the carrier forming a biofilm, regardless of the HRT changes (Koskinen, Kaksonen and Puhakka 2007). In a CSTR treating real industrial wastewater from a beverage plant, propionic acid accumulation was attributed to *Selenomonas lacticifex* and *Bifidobacterium catenulatum*, which could be successfully eliminated by a temperature increase from 37°C to 45°C leading to improved hydrogen production performance (Sivagurunathan, Sen and Lin 2014).

Other propionate producers have been reported, still favored by high HRT and low pH. For example, during the adaptation of anaerobic granules for hydrogen production from glucose, propionate production was favored by the highest HRT (24 h) and attributed to *Propionibacterium acidipropionici* (in continuous rather than discontinuous feeding regimes) which are able to produce propionate by transcarboxylase enzymes (Hernández-Mendoza, Moreno-Andrade and Buitrón 2014). Similarly, when comparing different pretreatments in glucose-fed batch experiments, *Propionibacterium* appeared after acid pretreatment, resulting in the lowest H<sub>2</sub> productivity and highest propionate production (Ren et al. 2008).

#### Hydrogen consumption by sulfate-reducing bacteria

Lithotrophic sulfate-reducing bacteria (SRB) use hydrogen as electron donor to reduce sulfate to hydrogen sulfide. Thermodynamically, sulfate reduction is the most efficient H<sub>2</sub>-consuming reaction. The growth of highly competitive SRB is favored by high sulfate concentration in the substrate (as reported in waste from pulp/paper industry, sea-food processing, distilleries or swine manure among others) (Guo et al. 2010). Short HRTs as low as 2 h are not sufficient to suppress SRB under sulfate-rich conditions, but their activity is inhibited by pH values lower than 6.0 (Guo et al. 2010).

One of the most studied SRB, *Desulfovibrio* spp., was detected in wastewater treatment plant sludge used as hydrogen production inoculum (Chaganti, Lalman and Heath 2012) and has [NiFe]-hydrogenase genes encoding uptake hydrogen enzymes (Wawer and Muyzer 1995). During H<sub>2</sub> fermentation from glucose in a fluidized bed bioreactor, the SRB *Desulfovibrio desulfuricans* was detected in the attached and suspended growth phase

communities (Koskinen, Kaksonen and Puhakka 2007), but the authors suggested that it was not involved in H<sub>2</sub> consumption or production in that case, because (i) the reactor did not contain sulfate for sulfate reduction activity and (ii) the substrates and products of other fermenting activities in *D. desulfuricans* (choline and pyruvate fermentation) do not include hydrogen. In kitchen waste fermenter, *Desulfovibrio* sp. was detected and suspected to be involved in lactate degradation (Li et al. 2011) which involves hydrogen production (McInerney and Bryant 1981). Interestingly, the ability to efficiently produce or consume molecular hydrogen depending on the environmental conditions has been evidenced in *Desulfovibrio*: when sulfate concentrations are low, it can grow on lactate and evolve varying amounts of hydrogen, during the early phase of growth (Odom and Peck 1981).

#### Contrasting effect of LAB

Lactate is a key intermediate metabolite in hydrogen fermenters, produced by a zero-hydrogen balance pathway. Although many bacteria produce lactic acid as a primary or secondary end product of fermentation, the term LAB is conventionally reserved for genera in the order Lactobacillales, including *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus*, *Streptococcus*, etc. LAB are non-spore-forming aerotolerant anaerobes. Lactate production is generally considered as detrimental for H<sub>2</sub> production since it is a deviation of the acetyl-coA pathway. However, many reports suggest the contribution of LAB to the global hydrogen production, through different mechanisms. Therefore, the positive or negative effect of LAB metabolism within hydrogen-producing consortia is still debated (Sikora et al. 2013), as discussed in the following sections.

#### Competition and inhibition of hydrogen production by LAB

The negative effect of LAB (even as minor species, as reported by Rafrafi et al. 2013) on hydrogen production can be explained by both substrate competition (for pyruvate) and HPB inhibition. The antimicrobial activity of LAB can be due to (i) the pH drop caused by the synthesis of lactic, acetic and propionic acids, or (ii) to the production of toxic compounds (hydrogen peroxide, diacetyl, bacteriocins polypeptides) as demonstrated in co-cultures (Noike et al. 2002). Therefore, community shifts favoring LAB growth are usually associated with process failure (Kawagoshi et al. 2005; Jo et al. 2007), making necessary a pH control throughout the fermentation.

LAB growth can be promoted by the substrate, especially when treating dairy products (Yang et al. 2007; Castelló et al. 2009) and other fermented waste. For example, in a continuous reactor treating fermented food waste (kimchi), the bloom of *Lactobacillus* spp. (normally present in the substrate) correlated with periods of poor hydrogen production, through the inhibition of *Clostridium* activity, resulting in a shift from acetate-butyrate to ethanol-lactate metabolisms (Jo et al. 2007). In CSTR inoculated with a pure culture of *C. butyricum* and treating sugarcane juice under non-sterile operation, *L. harbinensis* was a dominant community member at all tested HRTs and may have been responsible for lower hydrogen production and higher lactate accumulation than normally reported in biohydrogen processes (Pattra et al. 2011). *Bifidobacterium* spp. became highly dominant in a pilot-scale agitated granular sludge bed reactor treating real wastewater (condensed molasses solubles), contrary to what happened in the same reactor fed with synthetic sucrose substrate, and led to lower H<sub>2</sub> productivity (Cheng et al. 2011).

The inoculum also plays a major role in the development of LAB and subsequent H<sub>2</sub> production decline, as demonstrated by

Kawagoshi et al. (2005) who compared different inoculum origins with different tendencies for LAB development. Similarly, in glucose-fed batch reactors inoculated with different seeds (heat-treated aerobic or anaerobic sludge from wastewater treatment plant), LAB were brought in by the activated sludge inoculum only, resulting in lactate production and process instability (Baghchehsaraee et al. 2010). Inoculum pretreatment methods aiming to suppress LAB were decisive to enhance subsequent H<sub>2</sub> production; their efficiency depended greatly on the inoculum considered, especially whether or not the inoculum showed lactic acid production before pretreatment (Kawagoshi et al. 2005).

Heat treatment of substrate between 50°C and 90°C usually enables to prevent LAB growth (Noike et al. 2002). Acid pretreatment of food waste was also efficient to select HPB *Clostridium* sp. (representing more than 70% of the community sequences) and to reach the highest hydrogen yield, compared to the untreated control in which LAB such as *Lactobacillus* and *Streptococcus* were dominant (>90% of the community sequences) (Kim et al. 2014). However, pretreatments of inocula and/or substrate are not always effective in preventing LAB growth. Combined heat and acid treatment of sunflower stalks hydrolyzate was not efficient to suppress LAB: *Sporolactobacillus* became dominant (~70% of the bacterial community), at the expense of *Clostridium* (Monlau et al. 2013a). These authors suggested that the secondary by-products of lignocellulosic degradation released by the acid treatment (such as acetate, formate, furfural and phenolic compounds) induced a community shift toward LAB and that the subsequent activity of LAB generated stressful conditions for the remaining *Clostridium* HPB, therefore shifting toward solventogenesis (Monlau et al. 2013a). In batch reactors enriched from marine intertidal sludge, the LAB *Enterococcus* sp. persisted and dominated after drastic heat-shock or freeze-thaw pretreatments, despite being non-spore-forming bacteria (Liu et al. 2009).

LAB development is influenced by operating conditions in the reactor and mainly depends on pH. For example, when comparing different pH control strategies in glucose-fed anaerobic fluidized bed reactors, the presence of *Sporolactobacillus laevolacticus* was only observed at pH 5.0–5.5 and explained the lower H<sub>2</sub> yield obtained in that case (with acetate, ethanol and lactate production), in contrast with the better H<sub>2</sub> production of the *Clostridium*-dominated community at pH 3.7–4.1 (with acetate and butyrate as main products) (Shida et al. 2012). In CSTRs fed with molasses wastewater at different influent pH, the ethanol- and lactate-producer *Lactococcus* developed under the pH condition 5.5–6.0 (contrary to what happened at slightly higher and lower pHs), and led to mixed butyrate-ethanol fermentation and lower H<sub>2</sub> production, despite its low abundance (Ren et al. 2007). Other operating parameters favor LAB emergence, such as the combination of inoculum, substrate and citrate buffer concentrations (Sreela-or et al. 2011), the lower moisture content (Motte et al. 2014) and the higher loading rate (Oh et al. 2004).

#### Beneficial contribution of LAB to hydrogen production

Lactate can promote hydrogen production through a beneficial symbiosis between LAB and HPB. Efficient H<sub>2</sub> production has been demonstrated by the association of several dominant *Clostridium* species with different minor LAB (Fang, Liu and Zhang 2002; Sikora et al. 2013). In an UASB treating food waste, *Clostridium* coexisted with *Streptococcus* and *Weissella* (from the substrate) during the period of highest H<sub>2</sub> content (Laothanachareon et al. 2014). *Sporolactobacillus* was dominant in glucose-fed CSTR inoculated with heat-shocked anaerobic sludge, and its presence did not affect the hydrogen production efficiency (Wongtanet et al. 2007). In granular sludge from packed

bed reactors fed with sugar beet molasses, the highest number of LAB in the community (mainly from the Leuconostocaceae family) corresponded to the highest hydrogen production yield and the complete consumption of lactic acid (Chojnacka et al. 2011).

Interestingly, in biohydrogen reactors fed with starch and inoculated with either activated or anaerobic sludge, Baghchehsaraee et al. (2010) suggested the existence of a threshold of lactic acid concentration (around 650 mg/L), below which lactic acid enhanced both the hydrogen yield and production rate, simultaneously with a metabolic shift from acetate-ethanol to butyrate production. Above this threshold, hydrogen yield sharply decreased. Kim et al. (2012) also reported that within a certain range of concentration, artificially added lactate could enhance hydrogen production yield, in batch reactors treating glucose-based wastewater, inoculated with acid-treated sludge. Above this concentration range, lactate was no more degraded, but it accumulated and became detrimental to H<sub>2</sub> production (Kim et al. 2012). To provide a positive effect on H<sub>2</sub> production, it seems that lactic acid should be mixed to another carbon source such as acetic acid. For example, the addition of lactic acid to a mixed culture grown on starch increased both hydrogen production and butyric acid formation, with a complete consumption of produced lactic acid (Baghchehsaraee et al. 2009). However, when lactic acid was the only carbon source, the level of hydrogen production was very low.

In mixed cultures, the positive effect of lactate can be explained by its role as electron donor for hydrogen production. Lactate conversion to butyrate with concomitant H<sub>2</sub> production has been observed in mixed culture systems (Hashsham et al. 2000; Lee et al. 2008; Marone et al. 2012). The capacity to convert lactate into butyrate, CO<sub>2</sub> and H<sub>2</sub> in the presence of acetate, has been evidenced for *C. diolis* (from fermentation of sweet potato post-distillation slurry; Matsumoto and Nishimura 2007), as well as other clostridia species such as *C. beijerinckii*, *C. acetobutylicum* and *C. tyrobutyricum* (Cheng et al. 2010; Mariakakis et al. 2011). This capacity to reconsume lactate for enhanced H<sub>2</sub> production has also been proposed in pure and defined co-cultures of *C. butyricum*, *C. beijerinckii*, *C. felsineum* and especially *C. pasteurianum* in starch fermentations where the highest H<sub>2</sub> yields were obtained when lactate was the main metabolite (Masset et al. 2012). In kitchen waste fermenter, the lactate produced by *Olsenella* LAB was efficiently degraded with concomitant H<sub>2</sub> production, but the putative lactate degraders could not be identified certainly (Li et al. 2011). In a fermenter fed with food waste garbage slurry, lactate (coming from the substrate and/or produced by the abundant and diverse LAB including *Bifidobacterium* and *Lactobacillus*) was suggested as one of the main substrate of H<sub>2</sub> production by lactate-utilizing HPB *M. elsdenii* (Ohnishi et al. 2010).

Eventually, even though LAB have not been described as hydrogen producers since the lactate pathway recycles the reduced cellular components under a strict stoichiometry, the capacity of H<sub>2</sub> production has been suggested in a *Lactobacillus* strain isolated from a continuous reactor fed with cheese whey permeate, where its abundance correlated to the H<sub>2</sub> production efficiency (Yang et al. 2007). Also highly abundant in cheese whey fermenter and palm oil mill effluent fermenter inoculated with heat-shocked anaerobic sludge, *Lactobacillus* species (probably coming from the unsterilized substrate itself) were proposed by some authors as putative H<sub>2</sub>-producer species, in association with the other consortium members, either *Clostridium* or not (Castelló et al. 2009; Badieli et al. 2012). Marone et al. (2014) found a *Lactococcus lactis* strain among the HPB isolated from vegetable waste, and characterized its H<sub>2</sub> production metabolism in axenic culture, confirming the hydrogen production capacity of

LAB species. The LAB *Olsenella* was proposed as putative HPB in anaerobic sludge treated with chloroform fed with glucose (Ning et al. 2012).

LAB may therefore be directly involved in hydrogen production, by providing extra substrate to HPB in the form of lactate, or by directly converting lactate to hydrogen. In addition, LAB can contribute indirectly to hydrogen production in mixed cultures, through different auxiliary mechanisms that were extensively presented in the section 'Positive interactions: contributions of auxiliary non-HPB in the global function'. Therefore, as postulated by Sikora et al. (2013), the stimulatory effects of LAB on hydrogen producers seem to exceed in some cases their potentially unbeneficial features.

### Syntrophic metabolism

Hydrogen production can be carried out by syntrophic microorganisms, which are able to metabolize fatty acids and produce high molar ratios of hydrogen from NADH in association with hydrogen/formate-using microorganisms. Model syntrophic microorganisms include the butyrate-degrading *Sy. wolfei* within the Clostridia class, as well as the benzoate-degrading *Syntrophus aciditrophicus* and the propionate-degrading *Syn. fumaroxidans* within the Deltaproteobacteria (Jackson et al. 1999; McInerney et al. 2008; Stams and Plugge 2009). Syntrophy is defined as a thermodynamically based interaction between microorganisms, where the degradation of a compound (such as a fatty acid) by a microorganism occurs only when its degradation products (usually hydrogen, formate and acetate) are maintained at very low concentrations by a second microorganism, usually a methanogen or sulfate reducer, making the reaction energetically favorable (Sieber et al. 2010). Even under optimal conditions, hydrogen production through syntrophic reactions is thermodynamically difficult, leading to low growth rates and growth yields. Syntrophic associations usually rely on interspecies transfer of hydrogen and formate by diffusion (Sieber, Le and McInerney 2014), therefore requiring close physical proximity between partners, organized within multicellular structures such as flocs or compact aggregates, as evidenced in anaerobic digester sludge (Conrad, Phelps and Zeikus 1985).

The observation that microorganisms have evolved biochemical mechanisms to overcome energetic barriers to use protons as electron acceptors offers interesting perspectives for biotechnological hydrogen production (Stams and Plugge 2009). *Syntrophomonas wolfei* and *Synt. aciditrophicus* contain multiple hydrogenase genes, but some of them are more highly expressed under syntrophic growth with a hydrogen consumer (Sieber, Le and McInerney 2014). Interestingly, *Synt. aciditrophicus* was able to ferment benzoate and produce H<sub>2</sub> in pure culture via a dismutation reaction in the absence of hydrogen-using microorganisms or terminal electron acceptors, but high hydrogen and acetate levels inhibited benzoate metabolism (Elshahed and McInerney 2001). Some syntrophic bacteria (*Sy. wolfei*, *Syn. fumaroxidans*) have been reported in ethanol-H<sub>2</sub>-coproducing bioreactors at pH 4.5, within a diverse microbial community (Xing, Ren and Rittmann 2008). They were carrying [Fe]-hydrogenase genes and thus identified as putative novel HPB in these systems, together with other well-known HPB such as *Clostridium* and *Ethanoligenens*. *Syntrophomonas wolfei* represented up to 20% of the *hydA* gene library. According to these authors, these syntrophs consume short-chain fatty acids, produce H<sub>2</sub> and play a role in buffering against a pH drop in this acidogenic sludge (Xing, Ren and Rittmann 2008). However, due

to the operating conditions of most hydrogen production systems, syntrophic bacteria usually do not maintain from the inoculum to the fermenter (Laothanachareon et al. 2014). Some strategies to release the product inhibition on syntrophic bacteria have been proposed in order to enhance H<sub>2</sub> production, such as venting and flushing with N<sub>2</sub> the headspace of batch reactors treating paper mill wastes (Valdez-Vazquez et al. 2005). However, hydrogen production through syntrophic reactions is not expected and desired in H<sub>2</sub> production bioreactors due to the strict metabolic requirements of syntrophic bacteria implying that H<sub>2</sub> concentration must remain extremely low.

### Engineered ecosystems for H<sub>2</sub> production: far from pure cultures

All the findings discussed above clearly illustrate that even though known efficient HPB are present in the community, the H<sub>2</sub> production efficiency will also depend on the composition of the whole community, including H<sub>2</sub> consumers and competitors or inhibitors, which counteract the HPB activity. On the other hand, many species within hydrogen reactors have been reported to have an essential auxiliary role thus providing a positive contribution to the global function. Therefore, the global ecosystem service can benefit from this diverse microflora, especially when the substrate is complex and variable, since the contribution of diverse bacteria in mixed inoculum results in a range of different metabolic capabilities to span a large substrate spectrum and yielding to high H<sub>2</sub> production rates. In batch fermentation of waste wheat powder, Argun, Kargi and Kapdan (2009) showed that the use of a complex (uncharacterized) mixed-culture inoculum provided better hydrogen yields and rates than the use of pure culture and co-culture inocula, including *C. acetobutylicum*, *C. butyricum* and *Enterobacter aerogenes* inocula. In ASBR treating palm oil mill effluent exposed to variable HRT conditions, the highest H<sub>2</sub> productivity was associated with the highest community diversity, where *Clostridium*, *Lactobacillus* and *Streptococcus* species coexisted (at HRT 72h) (Badiei et al. 2012).

Moreover, when the diversity is low and the community dominated by an overrepresented species, an unwanted diauxic growth can be observed, favoring the consumption of easily degradable substrates and leading to delayed consumption of complex substrates, as reported during fermentation of lignocellulosic residues where *C. butyricum* dominated (>90%) the community (Monlau et al. 2013c).

The contribution of subdominant bacteria to fermentative H<sub>2</sub> production has been highlighted by Rafrafi et al. (2013) in chemostats continuously fed with a glucose-based medium and inoculated with different biomass sources. *Clostridium pasteurianum* was dominant (representing 67% to 89% of the community) in most assays at steady state, and the differences between chemostats only came from persistent bacterial populations of low abundance. According to the competitive exclusion principle, these subdominant bacteria should have been washed out, unless they interacted with their environment. Therefore, the authors suggest that these subdominant bacteria impacted substantially the microbial metabolic network of the overall ecosystem, acting as keystone species despite their low abundance: while *Bacillus* spp. and *Lactobacillus* spp. lowered the H<sub>2</sub> yields by diverting a part of the H<sub>2</sub> potential to lactate production, the presence of *Escherichia coli* increased the H<sub>2</sub> yield by redirecting the metabolic network to acetate and butyrate hydrogen-producing pathways (Rafrafi et al. 2013).

On the basis of the insights brought by microbial ecology studies, the next section will evidence some of the bioengineering strategies and microbial resource management approaches proposed to date to enhance bioprocess function.

## THE NEXT STEP: DRIVING THE ECOSYSTEM FUNCTION THROUGH MICROBIAL RESOURCE MANAGEMENT

### Metabolic engineering of HPB

A better knowledge of metabolic and molecular outcomes of hydrogen production enabled to enhance H<sub>2</sub> production in HPB strains through metabolic engineering. Given the crucial role of hydrogenase gene abundance in hydrogen production, as evidenced by empirical correlations, several authors suggested that the enhancement of hydrogenase activity using genetic engineering would improve hydrogen production. Indeed, the overexpression of [Fe]-hydrogenase gene in *Clostridium paraputrificum* led to increased hydrogen productivity and acetic acid production, while it abolished lactic acid production (Morimoto et al. 2005), as also reported for the [Fe] hydrogenase of the facultative anaerobe *Enterobacter cloacae* (Mishra et al. 2004).

Since the hydrogen production rate is influenced by the global metabolic pathways, H<sub>2</sub> production efficiency can be improved by eliminating competing pathways through genetic engineering. Especially, butyrate production is recognized to reduce the H<sub>2</sub> yield because it consumes more NADH than other pathways. As an example, the disruption of the *hbd* gene encoding for the  $\beta$ -hydroxybutyryl-CoA dehydrogenase enzyme (involved in butyrate formation pathway) in *C. butyricum* led to increased H<sub>2</sub> production and simultaneous decrease of ethanol production, under low partial pressure of hydrogen (Cai et al. 2011). However, the success of the genetic manipulation depends on some operating conditions. For example, the same *hbd* disruption led to a drastic decrease of H<sub>2</sub> yield and simultaneous increase of ethanol production when the partial pressure of hydrogen was high (Cai et al. 2011). In *Escherichia coli*, the yield of hydrogen production by FHL could be enhanced by various metabolic engineering modifications such as deletion of a negative regulator of FHL, deletion of uptake hydrogenases, deletion of lactate dehydrogenase and fumarate reductase (Manish, Venkatesh and Banerjee 2007; Kim et al. 2009).

However, there are still many discrepancies in the H<sub>2</sub> production yield of genetically engineered strains, possibly resulting from incompatibility between reactor conditions and genetically modified cell requirements and/or the poor performance of the parental strains (Kim et al. 2009). Moreover, at large scale, the continuous operation of hydrogen production reactors fed with unsterile biowaste substrates and inoculated with genetically engineered strains is still hardly feasible to date, because of (i) possible contamination/outcompetition by substrate endogenous strains (loss of the genetically engineered strain) and (ii) possible dispersion of the genetically engineered strain in the environment through the release of the treated effluent.

### Controlling H<sub>2</sub> production by ecobiotechnological approach

As extensively discussed along the review, the most common strategy to select HPB up to now is based on drastic pretreatment of the inoculum. Many pretreatment methods have been proposed and compared so far (Kawagoshi et al. 2005; Ren et al. 2008; Wang and Wan 2008). However, the conventional drastic

pretreatments specifically enriching spore-forming HPB (namely *Clostridium* sp.) are not necessarily the most adequate in fermentation communities where the major hydrogen producers do not form spore in response to environmental stress and where non-spore formers play a crucial role in the ecosystem service (Ohnishi et al. 2010). After exploring the effect of different physicochemical pretreatments to maximize the hydrogen production from sewage sludge substrate, Kotay and Das (2009) concluded that pretreatment was essential to reduce the abundance of microbial competitors and to improve the sludge nutrient solubilization, but not sufficient to develop a suitable microbial consortium for H<sub>2</sub> production (without further inoculation/bioaugmentation of the sludge).

An alternative approach for the sustainable production of biohydrogen could be the application of ecobiotechnology. The principle of ecobiotechnology is based on natural selection and competition rather than on genetic or metabolic engineering: selective pressure for a desired metabolism is applied on a diverse community by choosing the substrate and operating conditions of the bioreactor in an appropriate way, that is, to engineer the ecosystem rather than the microorganisms (Johnson et al. 2009). In most cases, given operating conditions can impose a sufficient driving force to select and enrich specific members of the community, as demonstrated for the progressive increase of a recalcitrant substrate concentration (Tapia-Venegas et al. 2015), or adaptation to low pH (Boboescu et al. 2014) or high salt concentration (Pierra et al. 2014). Valdez-Vazquez and Poggi-Varaldo (2009) have extensively reviewed the different methods used to apply a biokinetic control (i.e. current methods applied to control H<sub>2</sub>-consuming organisms alternative to heat-shock pretreatments) to select fermentative consortia. In expanded granular sludge bed reactors fed with glucose, the 'washout' pretreatment of the inoculum resulted in higher H<sub>2</sub> production rate than the heat-shock pretreatment, probably because of the significant role played by Enterobacteriaceae in H<sub>2</sub> production, which outnumbered *Clostridium* in that case since they were not depleted by the drastic pretreatment (Cisneros-Perez et al. 2015).

Although controlling HPB population structure and abundance is of high importance for optimizing fermentative H<sub>2</sub> production, a better knowledge of the physiology of putative H<sub>2</sub> consumers would also help to find the optimal reactor conditions to avoid their growth and thus improve the overall H<sub>2</sub> production (Castelló et al. 2009). Among the negative symbiotic bacteria, some homoacetogens are related to *Clostridium* sp., such as *C. aceticum*, with similar growth properties as HPB. It is therefore difficult to remove these H<sub>2</sub> consumers from *Clostridium*-based ecosystems. Nevertheless, H<sub>2</sub> consumption by homoacetogens was found to begin only after H<sub>2</sub> accumulation close to saturation. Therefore, homoacetogenic activity can be avoided by a continuous release of H<sub>2</sub> gas or by maintaining low H<sub>2</sub> partial pressure (Chaganti, Lalman and Heath 2012). Reducing the H<sub>2</sub> partial pressure can also be favorable if the hydrogen production is driven by clostridial *hydA*-carrying species, since it would release the hydrogenase inhibition by their own product (H<sub>2</sub>), thus maintaining high activity for NADH direct oxidation and enhance H<sub>2</sub> yield (Ramírez-Morales et al. 2015). As competitors, LAB are presumably responsible for poor H<sub>2</sub> performances in several studies. Their identification enabled to implement appropriate corrective actions such as temperature control of the substrate to reduce LAB fermentation (Jen et al. 2007). However, as discussed in the sections 'Positive interactions: contributions of auxiliary non-HPB in the global function' and 'Contrasting effect of lactic acid bacteria', some bacteria which are not directly involved in primary H<sub>2</sub> production (or even which were



previously thought to be negative effectors, such as LAB) can also be useful helpers of H<sub>2</sub> production through different auxiliary activities. Therefore, the optimal community consortium depends on the specificity of each substrate and operating conditions, and high diversity in mixed cultures can be preferred to high dominance of HPB.

### Driving the microbial community structure for enhanced hydrogen production

#### Co-culture fermentation

The co-culture of different microorganisms appears to be advantageous over a single microorganism because of the potential synergy between the different metabolic pathways of all involved strains, resulting in increased yield, and the possibility of utilizing cheaper substrates (Bader et al. 2010).

First, these positive interactions can occur between different HPB belonging to the same *Clostridium* genus. As an example, a co-culture of the carbohydrate-degrading strain *C. pasteurianum* with the glutamate-utilizing strain *C. sporosphaeroides* efficiently enhanced hydrogen production by 12%–220% (compared to the pure cultures) depending on the substrate concentration in a batch fermentation of condensed molasses collected from a glutamate manufacturing factory (Hsiao et al. 2009). These two strains may possess different metabolic pathways and substrate utilization patterns, which might reduce their nutrient competition and thus allow an efficient symbiotic mixed-culture function (Hsiao et al. 2009). Similarly, Masset et al. (2012) reported that, in general, co-cultures of *C. butyricum*, *C. pasteurianum*, *C. beijerinckii* and *C. felsineum* produced H<sub>2</sub> at higher rates than the pure cultures during glucose and starch fermentation, and were able to completely hydrolyze starch without pretreatment. Moreover, the fact that the members of the co-cultures maintained stable at long term suggested that positive interactions between the community members were at least as important as the simple competition for nutrients (Masset et al. 2012).

However, in some cases the coexistence of several clostridial HPB can be detrimental to the process performance. For example, the hydrogen production efficiency of the co-culture *C. tyrobutyricum* F4 and *C. sporosphaeroides* F52 was lower than those of their pure cultures, probably because of substrate competition, differences of growth rates and lag-phase times, as well as possible H<sub>2</sub> consumption by one of the partners or modifications of the local environment (e.g. ammonium, alkalinity, pH) by one of the strain which does not fit the requirements of the other strain (Hsiao et al. 2009). A similar observation was reported in large lab-scale continuous mixed reactor fed with sucrose, where the lowest *Clostridium* diversity (one or two coexisting species) was associated with the periods of highest H<sub>2</sub> production, while the *Clostridium* diversity significantly increased during periods of poor to null H<sub>2</sub> production (Mariakakis et al. 2011).

In addition, the co-culture synergism also occurs between HPB from different types, such as strict and facultative anaerobic isolates, and was thus suggested as an efficient strategy to enhance hydrogen production (Elsharnouby et al. 2013). For example, the crucial synergistic role of *Bacillus cereus* enhancing the hydrogen production yield from glucose (up to 3 mol<sub>H<sub>2</sub></sub>/mol<sub>hexose</sub>) has been highlighted when added to *Enterobacter* culture (Patel et al. 2014). The positive interaction can be due to O<sub>2</sub> depletion or hydrolytic activity. Effective and economical production of hydrogen was also reported from nitrogen-

rich corn steep liquor and sweet potato starch residue, using a defined co-culture of *C. butyricum* and *En. aerogenes*, which permitted to remove O<sub>2</sub> without any reducing agents (Yokoi et al. 1998, 2002). A co-culture of *C. acetobutylicum* and *Ethanoligenens harbinense* improved cellulose hydrolysis and subsequent hydrogen production rates as compared with monoculture inoculation. The two strains had a synergistic cooperation since *E. harbinense* alone could not degrade cellulose but it could rapidly remove the reduced sugars released by the cellulose-degrader *C. acetobutylicum*, allowing to obtain the highest yield among those reported in the literature at that time, with pure or mixed cultures, for H<sub>2</sub> production from cellulose materials (Wang et al. 2008). Similarly, a strain of *Bacillus* sp. capable of hydrolyzing starch by secreting amylase has been used in co-culture with the Alphaproteobacteria HPB *Brevundimonas* sp. (efficiently fermenting glucose into hydrogen), thus providing high rate hydrogen production from starch in batch reactors (Bao, Su and Tan 2012).

Recently, Benomar et al. (2015) demonstrated the formation of an artificial consortium between two anaerobic bacteria, *C. acetobutylicum* (HPB) and *Desulfovibrio vulgaris* (SRB) in which physical interactions between the two partners induce emergent properties. The particular condition of nutrient starvation for *D. vulgaris* induced interspecies interaction, allowing exchange of cytoplasmic material. This physical interaction induced changes in the expression of two genes involved in pyruvate metabolism in *C. acetobutylicum*, with concomitant changes in the distribution of metabolic fluxes, leading to a substantial increase in hydrogen production compared to the pure culture (Benomar et al. 2015). These authors suggested that the understanding of microbial consortia interactions may offer a type of ecological engineering of microbial ecosystems that could provide new approaches for modifying or controlling metabolic pathways without requiring genetic engineering (Benomar et al. 2015).

#### Bioaugmentation strategies

Apart from 'natural' adaptation and selection of the microbial community to specific operating conditions, bioaugmentation strategies have been proposed to artificially increase the proportion of key hydrogen-producing species with high performance, in real substrates already containing a high endogenic microbial diversity. As exposed in details below, several authors reported that bioaugmentation with pure or defined co-cultures of specific HPB can be a better alternative than 'diluted' microbial consortia (from thermal pretreated sludge, soils or compost), because by using well-known species it is possible to drive the metabolic pathways toward desired products. Hypothetically, by enriching a complex waste with specific H<sub>2</sub>-producing bacteria previously isolated from it, metabolically well-adapted consortia might be obtained. This could be more effective than inoculating with a generic H<sub>2</sub>-producing pure culture which may be ill equipped for this specific waste. Moreover, the hydrogen production is usually improved when the augmented strain does not work alone but with the support of substrate endogenic microflora: the hydrogen production was favored when the applied HRT permitted the coexistence of the bioaugmented (strict anaerobe) *Clostridium* strain with the indigenous (facultative anaerobe) *Klebsiella pneumoniae* in CSTR treating sugarcane juice at different HRT (Pattra et al. 2011).

In a brewery yeast waste fermentation system inoculated with *C. butyricum*, hydrogen production rate and concentration were 2-fold higher than that of the same system inoculated with compost microflora, the lag phase was shortened and the hydrogen production period in batch lasted longer (Jen et al.

2007). The identification of key players in brewery yeast waste fermentation process, either those directly involved in H<sub>2</sub> production (*C. beijerinckii*) or those indirectly related (e.g. *B. thermoamylovorans* which creates anaerobic environment), led Chang et al. (2008a) to propose the modification of the original microflora toward a syntrophic bacterial co-culture containing both members, which resulted in increased hydrogen-producing potential and rate and reduced lag phase.

Kotay and Das (2009) reported that different physicochemical pretreatments were not sufficient to develop a suitable microbial consortium for maximal H<sub>2</sub> production from sewage sludge substrate without further inoculation/bioaugmentation of the sludge. In that case, bioaugmentation with a defined microbial consortium of *En. cloacae*, *Citrobacter freundii* and *B. coagulans*, isolated from the sewage sludge itself, was found to improve H<sub>2</sub> yield by 1.5–4 times with respect to self-fermentation of pretreated sewage sludge (Kotay and Das 2009). In that case, *B. coagulans* was enriched from the original (1:1:1) constructed consortium and became dominant, and contributed the most efficiently to H<sub>2</sub> production (Kotay and Das 2010). The effectiveness of bioaugmentation has also been demonstrated at large pilot scale (100 m<sup>3</sup>), in a reactor treating sugar cane distillery effluent, by using co-cultures of *Ci. freundii* and *En. aerogenes* (previously isolated from the effluent treatment plant), together with *Rhodospseudomonas palustris*, which resulted in successful scale-up of H<sub>2</sub> production (Vatsala, Raj and Manimaran 2008). Interestingly, the H<sub>2</sub> yield obtained with the co-culture of the three isolates exceeded the one of each strain individually.

Similarly, Marone et al. (2012) proposed a bioaugmentation strategy for hydrogen production from vegetable waste by re-inoculating, individually and in a constructed consortium, three facultative anaerobic HPB (*Buttiauxella*, *Rahnella* and *Raoultella*), previously isolated and enriched from the same types of vegetable waste. The increase of hydrogen production with the addition of each single strain compared to the endogenous vegetable waste microbial community demonstrated the effectiveness of the bioaugmentation strategy. Moreover, the triple co-culture, inoculated at 1:1:1 ratio, outperformed the pure cultures, suggesting some synergistic interaction (Marone et al. 2012). These results suggested that a further optimization of the artificial consortium composition, for example, by mirroring the natural proportions of the consortia, could also influence the functional efficiency of the bioaugmentation strategy. Goud et al. (2014) confirmed the improvement of process performance in ASBR fed with real field food wastewater at elevated organic load, by bioaugmentation of native acidogenic microflora with three different acidogenic bacterial isolates (*B. subtilis*, *Pseudomonas stutzeri* and *Lysinibacillus fusiformis*) previously isolated from long-term operated acidogenic bioreactors producing biohydrogen. Interestingly, FISH analysis confirmed the survivability and persistence of augmented strains at long term (Goud et al. 2014). The different augmented strains showed different performances: higher VFA production (especially acetate) with *B. subtilis*, higher COD removal with *Ps. stutzeri*. Moreover, bioelectrochemical analysis depicted specific changes in the metabolic activity after bioaugmentation which also facilitated enhanced electron transfer.

The association of facultative and strict anaerobes can be beneficial for H<sub>2</sub> production. The use of a mixed *Enterobacter* (48%)/*Clostridium* (25%) inoculum enriched from a coastal lake sediment enabled to produce hydrogen efficiently in an anaerobic glucose fed CSTR (Izzo et al. 2014), and it showed higher yield from complex substrates codigestion (cheese whey, crude glycerol, sorghum silage, buffalo manure and slurry) when used

for bioaugmentation compared with more specialized inocula such as *Enterobacteriaceae* alone (Marone et al. 2015). This high substrate versatility was probably due to the simultaneous presence of *Enterobacter* sp. and *Clostridium* sp. which can cooperate through mutualistic and/or commensalistic relationships, providing different metabolic pathways to degrade different carbon sources.

However, the efficiency of bioaugmentation was in some cases contrasted. For example, in sugar cane-fed CSTR bioaugmented with *C. butyricum*, the augmented strain could maintain at short HRT (4–12 h), but it decreased and even disappeared at longer HRT (36 h), outcompeted by contaminants from the substrate (*Klebsiella* sp. and *Lactobacillus* sp.) (Pattra et al. 2011). Also, face to the washout risk, some authors proposed strategies to retain the bioaugmented strains within the reactor, for example, by encapsulating them: the use of appropriate co-culture in carrier (activated carbon)-induced granules, comprising *Enterobacter cloacae* and *Ci. freundii* isolated from soil and sewage sludge, respectively, allowed to generate H<sub>2</sub> from sucrose in an anaerobic fluidized bed bioreactor at high volumetric rate (Thompson et al. 2008).

#### Strategies to enrich functional consortia

Inoculation and bioaugmentation strategies imply previous isolation and culture steps, which are time consuming and can be limited since only a small fraction of the microbial diversity from natural microflora can be cultivated *in vitro*. Moreover, some (uncultivable) key species of the H<sub>2</sub> process can be missed by this method. Several authors proposed different acclimatization strategies to obtain an enriched and simplified functional consortium exhibiting the target function from an original mixed community, without the need to isolate pure strains. Especially, successful acclimatization to specific low degradable substrates has been proposed in batch and continuous modes, through community shifts and adaptation. As an example, Ren et al. (2010) obtained a functional consortium (exempt of methanogens) able to produce H<sub>2</sub> while degrading cellulose, by enriching continuously cow dung compost in a defined medium containing cellulose. Varrone et al. (2013) obtained a stable functional consortium characterized by *Enterobacteriaceae* (*Klebsiella* and *Escherichia/Shigella*) and *Betaproteobacteria* (*Cupriavidus*), able to efficiently convert different crude glycerol types into H<sub>2</sub> and ethanol, after several months of adaptation of aerobic-activated sludge by a series of successive sequencing batches using a minimal medium, using glycerol as unique carbon source, without any nutrient supplements. With the same objective, Tapia-Venegas et al. (2015) proposed a different strategy consisting in the progressive adaptation of untreated anaerobic sludge community to stepwise-increasing glycerol concentrations, in CSTR fed with a mix of glucose and glycerol. Contrary to the previous case, here the final consortium responsible of efficient hydrogen production when glycerol was the only substrate was dominated by *Clostridium* sp., after passing through community shifts from *Veillonellaceae* (when glucose was the only substrate) to *Prevotella* (when glucose and glycerol were mixed in the feed) (Tapia-Venegas et al. 2015).

Wang et al. (2010) proposed a ‘dilution-to-extinction’ approach by serially diluting the original mixed community from rumen liquid, to select the simplest microbial consortium exhibiting the function of interest (in that case simultaneous cellulose degradation and H<sub>2</sub> production). In this example, the 10<sup>7</sup> dilution comprised only three strains (*Ruminococcus* sp., *Butyrivibrio* sp. and *Succinivibrio* sp.), while the loss of *Ruminococcus* sp. at dilution >10<sup>9</sup> induced the loss of cellulose degradation and

H<sub>2</sub> production capacities, suggesting the key role of this strain in interaction with the other two dominant ones. This approach is relatively easy to implement in practice; however, it requires that the functional strains were initially abundant enough to remain in the reactor after several dilutions; conversely, the non-functional strains should be sparse enough to be eliminated by the dilutions: this is not always the case, as reported in many previous examples. An alternative 'concentration-to-extinction' approach was applied by Adav et al. (2009) to select (from cattle manure compost) a simple functional consortium able to hydrolyze cellobiose and produce H<sub>2</sub>. Here, the authors increased the cellobiose concentration, resulting in a significant decrease of microbial diversity, together with an increase of hydrogen production and a metabolic shift from ethanol to acetate–butyrate pathways. However, this approach is beneficial only when the functional strains of interest (H<sub>2</sub> producing) in the original mixed community are more tolerant than the non-functional strains to the strong selective pressure applied.

## CONCLUSION

H<sub>2</sub> is a ubiquitous by-product of many bacterial metabolic pathways, with a wide taxonomic distribution. No specific criterion exists for selecting the most favorable fermentative hydrogen production route in mixed cultures, which is one of the main limitations for optimizing the hydrogen-producing bioprocesses. This limitation is also due to the limited knowledge of the microbial ecology involved in mixed cultures fermentation. Future proteomic approaches are needed to clarify the function of the diverse microbial community in hydrogen-producing reactors.

The analysis of microbial community composition revealed a wide phylogenetic diversity that contributes in different—and still mostly unclear—ways to hydrogen production in mixed cultures during mesophilic fermentation of complex substrates. Hydrogen production bioprocesses are diverse and interacting ecosystems, where direct hydrogen producers are not the only microbial players participating in the ecosystem service. Most of this microbial diversity is part of the indigenous microflora from non-sterile substrates or untreated inocula. Under strictly controlled operating conditions, high hydrogen production yields have been conventionally attributed to the dominance of *Clostridium* sp. in mesophilic ecosystems, especially in lab-scale reactors after heat-shock pretreatment. However, diverse communities including other obligate anaerobes (e.g. *Acetanaerobacterium*, *Ethanoligenens*, *Megasphaera*, *Acidaminococcus*, *Prevotella*) as well as facultative anaerobes (e.g. *Enterobacteriaceae*, *Bacillales*, *Shewanella*, *Pseudomonas*), alone or in combination, have been reported to be preferable under real variable and unsterile conditions, and enabled to reach similar or higher H<sub>2</sub> yields than the conventional *Clostridium* HPB, especially under very specific operating conditions such as recalcitrant substrate.

In most cases, the operating conditions can impose a sufficient driving force to select and enrich specific members of the community, leading to characteristic species succession. The result is a coexistence of different strongly interacting species, which determines the functionality of the ecosystem. Even though known efficient HPB are present in the community, the H<sub>2</sub> production efficiency will also depend on the composition of the whole community, including H<sub>2</sub> consumers and competitors (methanogens, homoacetogens, propionate producers, sulfate reducers) or inhibitors (LAB), which counteract the HPB activity. On the other hand, many species within hydrogen reactors

have been reported to have an essential auxiliary role (e.g. pH regulation, substrate hydrolysis, cell aggregation, oxygen consumption) thus providing a positive contribution to the global function.

Apart from, not always preferred, heat-shock pretreatment of inocula, different successful bioengineering strategies have been proposed for controlling the microbial consortium toward maximal H<sub>2</sub> production, such as synthetic *de novo* co-cultures, bioaugmentation with efficient HPB, selective pressure through specific inoculum pretreatment or operating conditions and metabolic engineering. Considering that the composition of the optimal community diverges according to the substrate and other environmental conditions, the optimal ecosystem driving approach has to be determined for each specific condition.

Apart from a basic interest and a better knowledge of the microbial mechanisms involved in biohydrogen production processes, the elucidation of intricate relationships between community structure and ecosystem function offers a practical interest for process monitoring and optimization. Such advanced knowledge should allow, by a tight control of the operating conditions, to orient the metabolism and activity of the community in order to achieve required ecosystem services. With a better knowledge of the microbial interactions, it would be possible to specifically select and enrich microorganisms of interest and therefore trigger the microbial community in a favorable way for achieving the function of interest.

## SUPPLEMENTARY DATA

Supplementary data are available at [FEMSRE](https://femsre.onlinelibrary.wiley.com/doi/10.1111/femsre.12158) online.

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