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Biological hydrogen production: Trends and perspectives

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Abstract

Biologically produced hydrogen (biohydrogen) is a valuable gas that is seen as a future energy carrier since its utilization via combustion or fuel cells produce pure water. Heterotrophic fermentations for biohydrogen production are driven by a wide variety of microorganisms such as strict anaerobes, facultative anaerobes and aerobes kept under anoxic conditions. Substrates such as simple sugars, starch, cellulose, as well as diverse organic waste materials can be used for biohydrogen production. Various bioreactor types have been used and operated under batch and continuous conditions; substantial increases in hydrogen yields are been achieved through optimum design of the bioreactor and fermentation conditions. This mini-review explores the research work carried out in

fermentative hydrogen production using biomass as substrates. The mini-review also presents the state of the art in novel molecular strategies to improve the hydrogen production.

Key words: Anaerobic conditions; biohydrogen; biomass; bioreactor; dark fermentation; gene manipulation; hydrogenases; hydrogen production; mixed culture

1. Introduction

A large proportion of the world energy needs are being covered by fossil fuels which have led to an accelerated consumption of these non-renewable resources. This has resulted in both, the increase in CO_2 concentration in the atmosphere and the rapid depletion of fossil resources. The former is considered the main cause of global warming and associated climate change, whereas the latter will lead to an energy crisis in the near future. For these reasons, large efforts are being conducted worldwide in order to explore new sustainable energy sources that could substitute fossil fuels. Processes which produce energy from biomass are a typical example of environmentally friendly technologies as biomass is included in the global carbon cycle of the biosphere. Large amounts of biomass are available in the form of organic residues such as solid municipal wastes, manure, forest and agricultural residues among others. Some of these residues can be used after minor steps of pre-treatment (usually dilution and maceration), while others may require extensive chemical transformations prior to being utilized as a raw material for biological energy production. Biological processes such as methane and hydrogen production under anaerobic conditions, and ethanol fermentation are future oriented technologies that will play a major role in the exploitation of biomass from energy.

By using some of the microbial mechanisms of anaerobic digestion, hydrogen (biohydrogen) can be the final by-product of the digestion process together with organic acids. The major advantage of energy from hydrogen is the lack of polluting emissions since the utilization of hydrogen, either via combustion or via fuel cells, results in pure water (Claassen et al. 1999).

This mini-review provides an overview of the state of the art and perspectives of biohydrogen production by microorganisms. The review focuses on heterotrophic

fermentation (dark hydrogen fermentation) revising the literature published mainly during the years 2005 and part of 2006. For a full view of previous works in this topic the reader is referred to excellent reviews published elsewhere (Nandi & Sengupta 1998; Claassen et al. 1999; Hallenbeck & Benemann 2002; Hawkes et al. 2002; Nath & Das 2004; Kapdan & Kargi 2006).

2. Biohydrogen producing microorganisms

Hydrogen can be produced by strict and facultative anaerobes (*Clostridia*, *Micrococci*, *Methanobacteria*, *Enterobacteria*, etc), aerobes (*Alcaligenes and Bacillus*) and also by photosynthetic bacteria (Nandi & Sengupta 1998). As can be seen from Table 1 and 2, some species used to produce H_2 belong to the genus *Clostridium* and in the majority of the cases mixed cultures were used. It also can be noticed that there are different sources of inocula (soil, sediment, compost, aerobic and anaerobic sludges, etc.) and all of them undergo some kind of conditioning before being used (heat, acid treatment). This is due to the need to select hydrogen producing microorganisms from the starting mixed culture. Fortunately hydrogen producing microorganisms are tolerant to harsher conditions. Various studies have been carried out to identify the microbial community present in mixed cultures used for H_2 production (Ueno et al. 2001; Fang et al. 2002; Ueno et al. 2004; Kawagoshi et al. 2005; Kim et al. 2006). Fang et al. (2002) identified the microbial species in a granular sludge used for H_2 production from sucrose. They found that 69.1% of microorganisms were *Clostridium* species and 13.5% were *Bacillius/Staphylococcus* species. Kawagoshi et al. (2005) studied the effect of both pH and heat conditioning on different inoculums. In their study they concluded that the highest hydrogen production was obtained with heat-conditioned anaerobic sludge. They also found DNA bands with high similarity (>95%) to

Clostridium tyrobutyricum, Lactobacillus ferintoshensis, L. paracasei, and *Coprothermobacter* spp. Kim et al. (2006) suggested that heat-treatment caused a change in the microbial community composition of a fresh culture used to produce H_2 from glucose in a membrane bioreactor. They reported that most of the species founded in the fresh sludge were affiliated to the *Lactobacillus sp.* and *Bifidobacterium sp.*; in contrast a *Clostridium perfringens* band was established in the heat-treated sludge. When mixed cultures are used as inocula the predominance of species in a bioreactor depends on operational conditions as temperature, pH, substrate, inoculum type, hydrogen partial pressure, etc. Kotay and Das (2006) showed the potential of a defined microbial consortium consisting of three facultative anaerobes, *Enterobacter cloacae*, *Citrobacter freundii* and *Bacillus coagulans* for H_2 production and with glucose and sewage sludge as substrates. They carried out experiments with the consortium (three species) and the species individually. *E. cloacae* produced higher yield than the other strains but similar to the consortium suggesting that *E. cloacae* dominated in the consortium. Some studies using pure strains have been also carried out for H_2 production. *Escherichia coli* (genetically modified strains), *Clostridium butyricum, C. saccharoperbutylacetonicum, C. thermolacticum,* and *C. acetobutylicum* are among the microorganisms used (Tables 1 and 2).

3 Substrates used for biohydrogen production

The main criteria for substrate selection are: availability, cost, carbohydrate content and biodegradability Kapdan & Kargi (2006). Glucose, sucrose and to a lesser extent starch and cellulose, have been extensively studied as carbon substrates for biohydrogen production (Tables 1 and 2). They have been used as *model* substrates for research purposes due to their easy biodegradability and because they can be present in different carbohydrate-rich

wastewaters and agricultural wastes. Other substrates suitable for biohydrogen production are protein- and fat-rich wastes. Although they are less available than carbohydrate-rich wastes, they represent potential feeds for the biological conversion of organic wastes to hydrogen (Svensson & Karlsson 2005).

A maximum theoretical yield of 12 mol of H_2 per mol of hexose is predicted from the complete conversion of glucose:

$$C_6H_{12}O_6 + 6\,H_2O \rightarrow 12\,H_2 + 6\,CO_2 \qquad (1)$$

It should be noticed that essentially no energy is obtained from this reaction to allow microbial growth (Hallenbeck 2005). Actual yields in metabolisms that lead to H_2 production are lower compared to the maximum theoretical yield. Recent works (Tables 1 and 2) show that even when substrate consumptions are high, hydrogen yields do not exceed 4 mol of H_2 per mol of monosaccharide or 8 mol of H_2 per mol of disaccharide. This so called *fermentation barrier* is maintained regardless of the fermentation system used for H_2 production e.g. batch, semi-continuous or continuous one step-processes (Logan 2004). Another important feature of hydrogen fermentation is volumetric H_2 production rate (VHPR). Levin et al. (2004) suggested to express VHPR in units that allow comparison between different hydrogen producing systems. For this reason, it was made an effort to report VHPR in the same units in Tables 1 and 2.

4. Biohydrogen production in batch, continuous and semi-continuous systems

Biohydrogen production by dark fermentation is highly dependent on the process conditions such as temperature, pH, mineral medium formulation, type of organic acids produced, hydraulic residence time (HRT), type of substrate and concentration, hydrogen partial pressure, and reactor configuration (Tables 1 and 2).

Temperature is an operational parameter that affects the growth rate and metabolic activity of microorganism. Fermentation reactions can be operated at mesophilic (25-40°C), thermophilic (40-65°C), extreme thermophilic (65-80°C), or hyperthermophilic (>80°C) temperatures. Most of the results presented on Tables 1 and 2 were obtained under mesophilic conditions and some under thermophilic conditions. Apparently, operation at thermophilic conditions is more favorable for mixed cultures. Oh et al. (2004) reported that thermophilic (60°C) conditions suppress lactate-forming bacteria and increase VHPR. These results can be explained thermodynamically by considering the Gibbs energy and standard enthalpy of the conversion of glucose to acetate and assuming a maximum theoretical yield of 4 mol H_2 per mol glucose (Vazquez-Duhalt 2002):

$C_6H_{12}O_6$	$+2H_2O$	$\rightarrow 2CH_3COOH$	$+4H_2$	$+2CO_2$	Gibbs energy and standard enthalpy, (KJ/mol)
-917.22	-237.17	-389.45	0.0	-394.38	$\Delta G° = -176.1$
-1274.45	-285.84	-484.21	0.0	-393.51	$\Delta H° = +90.69$

The Gibbs energy of the reaction indicates that the reaction can occur spontaneously. The van´t Hoff (Smith et al. 2000) equation explains the effect of the temperature on the equilibrium constant and in consequence on the yield coefficient:

(2)

If temperature increases the kinetic constant also increase because the reaction is endothermic ($\Delta H°$ has positive sign). Therefore, increasing the temperature in the fermentation of glucose enhances VHPR as is shown in Tables 1 and 2. Valdez-Vazquez et al. (2005) studied the semi continuous H_2 production at mesophilic and thermophilic conditions, they found that VHPR was 60% greater at thermophilic than mesophilic conditions. They suggested that this behavior is related with the optimal temperature for the enzyme hydrogenase (50 and 70°C) present in thermophilic Clostridia. Wu et al. (2005) showed that VHPR was greater at 40°C than at 30°C in batch tests using immobilized sludge in vinyl acetate copolymer. In addition, fermentation at high temperatures inhibited the activity of hydrogen consumers and destroyed pathogens present in some residues allowing the use of these residues as fertilizers for application on agricultural soil.

On the other hand, high temperatures can induce proteins thermal denaturation affecting the microorganism activity. Lee et al. (2006) studied the effect of temperature on hydrogen production in a CIGSB (Carrier induced granular sludge bed) bioreactor. They found that temperatures around 45°C affected the biomass growth of granular sludge (Table 2). Another potential disadvantage of thermophilic process is that they can increase energy costs.

In some of the studies presented in Table 1 and 2 the maximum VHPR was obtained between pH 5.0 and 6.0. However, in other studies the maximum VHPR was found around

pH 7.0 (Lee et al. 2006; Lin & Cheng 2006; Mu & Yu 2006). Various studies have recently pointed out that in order to inhibit methanogenesis, increase VHPR and enhance stability of continuous systems, moderate acid pH and high temperatures should be applied (Oh et al. 2004; Atif et al. 2005; Kotsopoulos et al. 2006). For the operation of batch systems an optimum initial pH of 5.5 has been reported (Fan et al. 2006; Fang et al. 2006; Mu et al. 2006b; Mu et al. 2006c). However, final pH in batch systems is around 4-5 regardless of initial pH. This is due to the production of organic acids which diminishes the buffering capacity of the medium resulting in low final pH. Mu et al. (2006c) found that VFA (volatile fatty acids) formation was pH dependant. When pH was decreased from 4.2 to a lower level or increased to a higher level, the fermentative pathway shifted from butyrate to caproate or ethanol. It is well documented that high VHPR is associated with butyrate and acetate production and inhibition of hydrogen production has been demonstrated with propionic acid formation (Oh et al. 2004; Wang et al. 2006). Therefore, control of pH at the optimum level is critical. Initial pH also influences the extent of lag phase in batch hydrogen production. Some studies reported that low initial pH in the range of 4 to 4.5 causes longer lag periods than high initial pH levels around 9 (Cai et al. 2004). However, the yield of hydrogen production decreased at high initial pH.

The mineral salt composition (MSC) also effects on hydrogen production. Lin and Lay (2005) found an optimal MSC by using the Taguchi fractional design method. The VHPR obtained with the optimal MSC was 66% greater than the value obtained with conventional acidogenic nutrient formulation. Also, they found that magnesium, sodium, zinc and iron were important trace metals affecting VHPR. Magnesium was the most important nutrient factor that produced a notorious effect on VHPR. Recently, other authors studied the effect of sulfate and ammonia concentrations on VHPR in CSTR systems with sucrose and

glucose as substrate (Lin & Chen 2006; Salerno et al. 2006). Increasing sulfate concentration from 0 to 3000 mg/L, at pH 6.7, reduced the activity of H_2 producing microorganism and shifted the metabolic pathway from butyrate to ethanol fermentation. However, increasing the with sulfate concentration to 3000 mg/L, at pH 6.7, raised the VHPR to 40% (Lin & Chen 2006). A decrease of 40% on VHPR and hydrogen yield was observed at ammonia concentrations of 7.8 g N-NH_4/L compared with the value obtained at 0.8 g N-NH_4/L which was the optimal ammonia concentration for hydrogen production (Salerno et al. 2006).

Hydrogen and VFA can be produced during exponential and stationary growth phases. However, various authors have shown that VFA and hydrogen production are maximal during the exponential growth phase, and decrease during the stationary phase due to alcohols production (Lay 2000; Levin et al. 2004). Hydrogen production in continuous and discontinuous systems is dependant on both biomass and substrate concentrations. Yoshida et al. (2005) studied the effect of biomass concentration on hydrogen production. They found that increasing cell density from 0.41 g/L to 74 g /L the specific hydrogen production rate (SHPR) increased 67 %.

The maximum hydrogen yield (HY) of 4 mol/mol has not been reached because in nature fermentation serves to produce biomass and not hydrogen. Also, hydrogen production by fermenting cells is considered as wasted energy by the bacteria, and therefore elaborated machineries exist to recycle the evolved hydrogen in these cells. Additionally, the HY is negatively affected by the partial pressure of the product. Theoretically, up to 33% of the electrons in hexose sugars can go to hydrogen when growth is neglected and at least 66% of the substrate electrons remain on VFA production.

The most appropriated parameter to analyze continuos systems is the mass loading rate (L) which is function of substrate concentration (S) and the hydraulic retention time (HRT):

(3)

VHPR increase when substrate concentration increase and HRT diminishes. However, at low HRT microbial washout might be greater than microbial growth. Thus, the low concentration of biomass in the reactor led to the decrease of VFA production and the increase of pH. High substrate concentration would result in the accumulation of VFA and a fall of pH in the reactor, and even inhibition of hydrogen producing bacteria. In addition, when substrate concentration increases in batch systems the partial pressure of hydrogen rises and the microorganism would switch to alcohol production, thus inhibiting hydrogen production (Fan et al. 2006). Park et al. (2005) showed that chemical scavenging of the CO_2 increased hydrogen production by 43% in batch glucose fermentation. It has been demonstrated that applying vacuum, gas sparging or CO_2 scavenging may all be effective methods of increase hydrogen production (Levin et al. 2004; Valdez-Vazquez et al. 2006).

For most of the results presented on Table 2, optimal HRT between 0.5 to 12 h and substrate concentrations around 20 g/L were reported. Chang & Lin (2004) studied the effect of HRT on HY, VHPR and SHPR in an up-flow anaerobic sludge blanket (UASB) reactor fed with sucrose. They found that HY was independent on HRT between 8-20 h and VHPR and SHPR were dependent on HRT. Oh et al. (2004) showed that decreasing HRT at 4 h and increasing substrate concentration from 6.86 to 20.6 g/L resulted in an increase of lactate concentration reducing VHPR.

The reactor configuration is another parameter that affects VHPR as is shown on Table 2. The VHPR varied from different reactor configurations, having the best performance with immobilized cell bioreactors. High cell densities are needed to maximize hydrogen production rates. Therefore, major improvements are expected in systems with biomass retention, e.g. by immobilized cells, under nutrient limitations operating in a continuous mode. Oh et al. (2004) studied hydrogen production in a trickling biofilter (TBR) with glucose as substrate and found a maximum VHPR of 37.5 mmol/L-h. TBR could maintain a high density of 18-24 g VSS/L which is higher than other immobilized systems and significantly higher than most of the cell suspended reactors like the continuous stirring tank reactor (CSTR). More else, packed bed reactors maintain a lower gas hold up since biogas is removed more efficiently. This alleviates both the inhibition by hydrogen and the severe channeling of liquid and gas flows in the reactor. Recently, fluidized bed reactor (FBR) and draft tube bed reactor (DTFBR) systems with effluent recycle and immobilized cells were studied for the production of hydrogen using sucrose (Lin et al. 2006a; Wu et al. 2006a). A VHPR of 95.23 mmol/L-h was obtained with DTFBR which was 50% greater than the one obtained with FBR. However, when using immobilized systems it could be important to consider the biogas accumulation and excessive gas hold up produced.

The maximum VHPR that has been obtained is 612.5 mmol/L-h by using a CSTR containing silicone immobilized sludge (10% v/v) and sucrose as substrate (Wu et al. 2006a). This VHPR is at least six times greater than any other VHPR reported (Table 2). This work demonstrated that an appropriate process design containing simultaneously granular, immobilized and freely suspended sludge had a major contribution on hydrogen production. Also, in that study HY was 3.86 mol/mol which is similar to the highest yield of 3.88 mol/mol obtained in a CIGSB (Carrier induced granular sludge) system for

fermentation of sucrose (Lee et al. 2006). Ren et al. (2006) reported an adequate performance of a pilot scale CSTR to produce hydrogen from molasses. However, CSTR problems could be present when high dilution rates are used and washout of the cells is experienced.

Gavala et al. (2006) obtained similar VHPR in CSTR and UASB reactors for glucose fermentation. But the HY attained in the CSTR was greater than the obtained with UASB. Overall, analogous VHPR are obtained by using UASB and CSTR systems (Table 2) (Chang & Lin 2004; Gavala et al. 2006; Lin & Chen 2006; Zhang et al. 2006).

Kim et al. (2006) showed that the use of membrane bioreactor (MBR) for hydrogen production allows advantages such as high cell density, high organic removal rates, and high quality effluent by the membrane and easy control of pH and temperature. They used a MBR system with glucose as substrate and found a maximum VHPR of 71.4 mmol/L-h. However, the use of MBR system has been limited at laboratory scale because high cost and this technology has not been demonstrated at full-scale. Although immobilized cells and MBR systems have shown the highest VHPR, it is not easy to compare various configurations of reactors to draw a conclusion regarding which configuration is better, even under a specific set of conditions. This is due to the fact that many factors such as VHPR, HY, long-term stability of the reactor, scale up, etc. have an impact on the economics of fermentative hydrogen production. In particular, the VHPR and HY change significantly depending on experimental conditions including temperature, pH, substrate concentration, type of substrate and HRT as was analyzed.

5. Molecular approach

Among few microorganisms genetically modified reported for biohydrogen production, *Escherichia coli* is the most used because its metabolic pathways as well as genomic sequence are known. Also, there are molecular tools for its manipulation. The metabolic pathway producing biohydrogen by enterobacteria is shown in Figure 1. Under anaerobic conditions, a fraction of pyruvate can be transformed to lactate by the lactate dehydrogenase (LDH), but most of it is hydrolyzed by the pyruvate formate liase (PFL) into acetyl-CoA and formate. PFL cleaves pyruvate only when cells grow fermentatively, while pyruvate dehydrogenase (PDH) decarboxylates pyruvate under aerobic conditions. Both enzymes are active under limiting oxygen conditions. The acetyl-CoA is partially converted in ethanol and acetate. Formate is the electron donor in anaerobic metabolism for nitrate reduction or can be transformed into hydrogen by the formate-hydrogen lyase complex (FHL). In *E. coli* there are three formate dehydrogenases (FDH) denominated O, N and H. The FDH-H (encoded by the *fdhF* gene) forms part of the FHL. The enzymes required for formate metabolism are encoded in the formate regulon.

The formate regulon includes genes *hycB-I, hypA-E, hycA* and *hypF*. Hyc proteins are the structural proteins forming the FHL and Hyp proteins are involved in the maturation of the FHL, whereas HycA is the negative transcriptional regulator for the formate regulon and FhlA (encoded by *fhl*A gene) is the positive transcriptional regulator for the expression of *fdhF* gene (Figure 2). Thus, HcyA mutants are hydrogen overproducing strains. A description of formate regulon has been published elsewhere (Sawers 2005).

The hydrogenases 1 and 2 and formate dehydrogenase N and O are located on the periplasmic space, and they must be transported by Twin arginine translocation (Tat) protein system to be active. Thus, Tat mutant do not uptake formate needed for hydrogen production, whereas hydrogenase 3 and FDH-H are located on cytoplasm and hence are not

to be transported. Penfold et al. (2006) reported that mutant strains defective of Tat transport (Δ*tatC* and Δ*tatA*-*E*) showed a hydrogen production comparable to *E. coli* strain carrying a Δ*hycA* allele. However, Δ*tatC* Δ*hycA* double mutant strain did not increase hydrogen production. Thus, it is possible that hydrogen production by *E. coli* could be increased by discarding activities of the uptake hydrogenases, which recycle a portion of hydrogen and the formate hydrogenases N and O which oxidize the formate without hydrogen production.

Penfold & Macaskie (2004) transformed *E. coli* HD701, a hydrogenase-upregulated strain and FTD701 (a derivative of HD701 which has a deletion of the *tatC* gene), with the plasmid pUR400 carrying the *scr* regulon to yield *E. coli* strains, which produce hydrogen from sucrose, as an alternative to coupling-in a upstream invertase. The parenteral strains did not produce hydrogen, whereas recombinant strains produced 1.27 and 1.38 ml H_2/mg dry weight-$L_{culture}$.

Mishra et al. (2004) overexpressed a [Fe]-hydrogenase from *Enterobacter cloacae* (obtained with degenerate primers designed from the conserved zone of *hydA* gene) in a non-hydrogen producing *E. coli* BL21. The resultant recombinant strain showed the ability to produce hydrogen. Yoshida et al. (2005) constructed an *E. coli* strain overexpressing FHL by combining *hycA* inactivation with *fhl*A overexpression. With these genetic modifications, the transcription of *fdhF* (large-subunit formate dehydrogenase) and *hycE* (large-subunit hydrogenase) increased 6.5 and 7-fold, respectively, and hydrogen production increased 2.8-fold compared with the wild-type strain. The effect of mutations in uptake hydrogenases, in lactate dehydrogenase gene (*ldhA*) and *fhlA* was studied by Bisaillon et al. (2006). They reported that each mutation contributed to a modest increase in hydrogen production and the effect was synergistic.

As expected, the amino acid sequence of FDH-H (E.C.1.2.1.2) from *E. coli* was highly homologous to the FDH-H sequences reported by NCBI for other Enterobacteria (Figure 3). FDH-H sequence identities of 98.5%, 98.3% and 79.4% were calculated for *Salmonella enterica* YP_153159, *Salmonella typhimurium*, and *Erwinia carotovora* YP_049356 respectively. The partial sequence available for *Enterobacter aerogenes* CAA38512 showed high homology as well. In addition, the identity with the FDH-H from the Archeas *Methanocaldococcus jannaschii* NP_248356 and *Thermoplasma acidophilum* NP_393524 were 58.1% and 54.9%, respectively and the FDH-H from *Photobacterium profundum* ZP_01218756 (belongs to *Vibrionaceae* family) was 59.8%. The high homology of FDH-H sequence among diverse bacteria suggests a high evolutive conservation of FDH-H.

The hydrogen production by Gram-positive bacteria such as *Clostridium* is shown in Figure 4. The pathway for hydrogen production uses two enzymes: ferredoxin-NAD reductase (FNR) and [Fe]-hydrogenase (FR). The overexpression of *hydA* gene encoding the FR has been used as a strategy to enhance hydrogen production. For instance, Morimoto et al. (2005) reported that hydrogen yield increased 1.7-times in *Clostridium paraputrificum* with respect to a wild-type bacteria. Harada et al. (2006) proposed the disruption of *nuoG* gene (encoding NuoG essential protein for the function NADH dehydrogenase I) and mutagenesis of the *thl* gene (encoding thiolase involved in the butyrate formation) from *Enterobacter aerogenes* and *Clostridium butyricum*, respectively, like novel molecular strategies to improve the hydrogen production. Nevertheless, at this time, results on hydrogen production are not available. Overexpression of FNR may improve hydrogen production. However, strains overexpressing FNR has not been reported.

6. Economics of biohydrogen production and perspectives

Even when there are many reports in the literature about biohydrogen production, only few economic analyses are available. In general, the molar yield of hydrogen and the cost of the feedstock are the two main barriers for fermentation technology. The main challenge to fermentative production of hydrogen is that only 15% of the energy from the organic source can typically be obtained in the form of hydrogen (Logan 2004). Consequently, it is not surprising that mayor efforts are directed to substantially increase the hydrogen yield. The U.S. DOE´s 2015 program goal for fermentation technology is to realize a yield of 6 mol hydrogen per mol of glucose and achieve six months of continuous operation (Sverdrup et al. 2006). Nevertheless, the remaining energy of the unused substrate can be recovered by photobiological systems producing hydrogen, by methane production or by microbial fuel cells producing electricity (Logan 2004).

de Vrije & Classen (2003) reported the cost of hydrogen production using a locally produced lignocellulosic feedstock. The plant was set at a production capacity of 425 Nm^3 H_2/h and consisted of a thermo-bioreactor (95 m^3) for hydrogen fermentation followed by a photo-bioreactor (300 m^3) for the conversion of acetic acid to hydrogen and CO_2. Economic analysis resulted in an estimated overall cost of € 2.74/kg H_2. This cost is based on acquisition of biomass at zero value, zero hydrolysis costs and excludes personnel costs and costs for civil works, all potential cost factors. Current estimation for hydrogen production cost is € 4/ kg H_2 or € 30/GJ H_2. The estimation is done on the basis of process parameters which seem presently feasible (http://www.biobasedproducts.nl/UK/5%20Projects/frame%205%20projecten.htm).

Regarding feedstock costs, commercially produced food products, such as corn and sugar are not economical for hydrogen production (Benemann 1996). However, byproducts from agricultural crops or industrial processes with no or low value represent a valuable resource

for energy production. Nevertheless, besides hydrogen biological production, other biofuels (bioethanol, biodiesel, biobutanol, etc.) processes are being under development (Reisch 2006) and, eventually, the demand of agricultural byproducts would increase its present low value. Wastewater has a great potential for economic production of hydrogen; only in the Unites States the organic content in wastewater produced annually by humans and animals is equivalent to 0.41 quadrillion British thermal units, or 119.8 terrawatt h (Logan 2004). Currently, biologically produced hydrogen is more expensive than other fuel options. There is no doubt that many technical and engineering challenges have to be solved before a sustainable hydrogen economy can be implemented.

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Legends to Figures

Figure 1. Metabolic routes of pyruvate and formiate in *E. coli*. Key reactions in the generation of hydrogen are shown in bold.

Figure 2. The formate regulon of *E. coli*: formate is generated by the *pfl* gene product. Genes or operons positively regulated by formate through the action of the transcriptional regulator FhlA are designated by + (Modified from Sawers 2005).

Figure 3. Multiple alignment of the FDH-H protein of *E. coli* with the FDH-H of two archeas (*M. jannaschii* and *T. acidophilum*), a vibrionales (*P. profundum*) and other enterobacteria. Sequence from last line represents the total conserved amino acids.

Figure 4. Metabolic routes of pyruvate in *Clostridium paraputrificum*. Key reactions in the generation of hydrogen are shown in bold.

1 Table 1. Hydrogen production rates and yield coefficients from pure and complex substrates under batch conditions.

2

Inoculum	**Substrate[a]**	**Volumetric hydrogen production rate (mmol $H_2/L_{culture}$-h)[f]**	**H_2 yield**	**Culture conditions[a] [pH, temperature (°C), %H_2 in biogas (%V/V)]**	**Reference**
Clostridium butyricum CGS5	Sucrose (20 g COD/L)	8.2	2.78 mol H_2/mol sucrose	5.5-6.0[c], 37, 64	Chen et al. 2005
Clostridium saccharoperbutylacetonicum ATCC 27021	Crude cheese whey (*ca*. 41.4 g lactose/L)	9.4	2.7 mol H_2/mol lactose	6.0[d], 30, NR[b]	Ferchichi et al. 2005
Escherichia coli strains	Glucose (4 g/L)	NR[b]	~2 mol H_2/mol glucose	7.0, 37, NR	Bisaillon et al. 2006

Escherichia coli strains	Formic acid (25 mM)	11795	1 mol H_2/mol formiate	6.5[d], 37, NR	Yoshida et al. 2005
Defined consortium (1:1:1, and separately tested): *Enterobacter cloacae* IIT-BT 08, *Citrobacter freundii* IIT-BT L139, *Bacillus coagulans* IIT-BT S1	Glucose (10 g/L)	NR	41.23 ml H_2/ g COD removed	6.0[d], 37, NR	Kotay & Das 2006
Mesophilic bacterium HN001	Starch (20 g/L)	59	2 mol H_2/mol glucose	6.0[c], 37, NR	Yasuda & Tanisho 2006
Aerobic and anaerobic sludges, soil and lake sediment (acid and heat conditioned)	Glucose (20 g/L)	NR	1.4 mol H_2/mol glucose	6.0[c], 35, NR	Kawagoshi et al. 2005
Aerobic sludge (heat conditioned)	Glucose (2 g/L)	NR	2.0 mol H_2/mol glucose	6.2[d], 30, 87.4	Park et al. 2005

Soil (heat conditioned)	Organic matter present in four carbohydrate-rich wastewaters.	6.2	100 ml H_2/g $COD_{removed}$	6.1[d], 23, 60	Van Ginkel et al. 2005
Anaerobic sludge (acid treatment and acclimated in a CSTR)	Sucrose (20 g COD/L)	96	1.74 mol H_2/mol sucrose	6.1[d], 40, 45	Wu et al. 2005
Anaerobic sludge (heat conditioned)	Glucose (10 g/L)	27.2 mmol/g_{VSS}-$L_{culture}$-h	1.75 mol H_2/mol glucose	6.0[d], 37, 40	Zheng & Yu 2005
Anaerobic sludge (acid treatment)	Glucose (~21.3 g/L)	4.9-8.6	0.8-1.0 mol H_2/mol hexose	5.7[c], 34.5, 59-66	Cheong & Hansen 2006
Microflora from a cow dung compost (heat treatment)	Wheat straw wastes (25 g/L)	2.7 mmol H_2/g $_{TVS}$ $L_{culture}$-h	2.7 mmol H_2/g $_{TVS}$	7.0[d], 36, 52	Fan et al. 2006
Anaerobic sludge (heat treated)	Sucrose (10 g/L)	8	1.9 mol H_2/mol sucrose	5.5[c], 35, NR	Mu et al. 2006a

Anaerobic sludge (heat treated)	Sucrose (24.8 g/L)	20	3.4 mol H_2/mol sucrose	5.5[c], 34.8, 64	Mu et al. 2006b
Anaerobic sludge (heat treated)	Glucose (3.76 g/L)	9	1.0 mol H_2/mol glucose	6.2[d], 30, 66	Salerno et al. 2006
Anaerobic sludge (heat treated)	Glucose (2.82 g/L)	NR	0.968 mol H_2/mol glucose	6.2[d], 25, 57-72	Oh et al. 2003
Microflora from soil (heat shocked)	Glucose, sucrose, molasses, lactate, potato starch, cellulose (each: 4 g COD/L)	NR	Glucose (0.92 mol H_2/mol glucose). Sucrose (1.8 mol H_2/mol sucrose). Potato starch (0.59 mol H_2/mol starch)[e] Lactate (0.01 mol H_2/mol lactate). Cellulose (0.003 mol H_2/mol cellulose)[e]	6.0[d], 26, 62	Logan et al. 2002

Notes: [a]When optimization trials were carried, optimum values are reported. [b]NR: Not reported. [c]Controlled value. [d]Initial, not controlled. [e]starch, celulose:[$(C_6H_{10}O_5)_n$]. [f] In some cases unit conversions were made according to the conditions reported by the authors.

1 Table 2. Hydrogen production rates and yield coefficients from pure and complex substrates under continuous and semi-continuous

2 bioreactors.

3

System	**Inoculum**	**Substrate**	**Volumetric H_2 production rate, (mmol $H_2/L_{culture}$ h)**	**H_2 yield**	**Culture conditions[a] [HRT (h), Load, pH, Temperature (°C), H_2 in biogas (% v/v)]**	**Reference**
Fed Batch	Mixed culture	OFMSW	14.7 mmol/$g_{VSdestroyed}$	NR[b]	504, 11 g_{VS}/Kgwmr d, 6.4, 55, 58	Valdez-Vazquez et al. 2005
	POME sludge	Palm oil mill effluent (2.5% w/v)	17.82	NR	24, NR, 5.5, 60, 66	Atif et al. 2005
	Windrow yard waste compost	Glucose (2 g/L)	7.44	1.75 mol H_2/mol Glucose	76, NR, 5.4, 55, NR	Calli et al. 2006

CSTR	Mixed culture	Sucrose (20 g COD/L)	17	3.5 mol H_2/mol sucrose	12, NR, 6.8, 35, 45.9	Lin et al. 2006b
	Mixed culture	Sucrose (40 g/L)	20	1.15 mol H_2/mol hexose	12, 80 g/L-d, 5.2, 35, 60	Kyazze et al. 2006
	Mixed culture immobilized in silicone gel	Sucrose (30 g COD/L)	612.5	3.86 mol H_2/mol sucrose	0.5, NR, 6.5, 40, 44	Wu et al. 2006a
	Mixed culture	Xylose (20 g COD/L)	5	1.1 mol H_2/mol	12, NR, 7.1, 35, 32	Lin & Cheng 2006
	Mixed culture	Broken kitchen wastes (10 Kg COD/m^3-d) and corn starch (10 Kg COD/m^3-d)	1.7	NR	96, NR, 5.3-5.6, 35, NR	Cheng et al. 2006

	Mixed culture	Glucose (15 g COD/L)	13.23	1.93 mol H_2/mol glucose	4.5, 80 g COD/L-d, 5.5, 37, 67 Hydrodynamic properties	Zhang et al. 2004
	Dewatered and thickened sludge	Glucose (4 g COD/L)	3.47	1.9 mol H_2/mol glucose	10, NR, 5.5, 35, 67 Ammonia effect	Salerno et al. 2006
	Mixed culture	Sucrose (20 g COD/L)	15.6	3.6 mol H_2/mol sucrose	12, NR, 5.5, 35, 50 Sulfate effect	Lin & Chen 2006
	Mixed culture	Organic waste water (4000 mg COD/L)	4.96	NR	12, NR, 4.4, 8 Kg_{COD}/m^3 d, 30, NR Effect of propionic acid	Wang et al. 2006
	See sludge	Sucrose (20 g COD/L)	52.6	3.43 molH_2/mol sucrose	12, NR, 6.8, 35, 50.9 Nutrient formulation effect	Lin & Lay 2005
	Mixed culture	Sucrose and sugarbeet	5.15	1.9 molH_2/mol hexose	15, 16 Kg sugar/m^3-d, 5.2, 32, NR	Hussy et al. 2005

	Mixed culture	Glucose (15 g/L)	0.115 g $_{H2\text{-}COD}$/g $_{Feed\ COD}$	1.38 mol H_2/mol hexose	10, NR, 5.5, 35, 45 Two phase system	Kraemer & Bagley 2005
	C. thermolacticum (DSM 2910)	Lactose(10 g/L)	2.58	2.1-3 mol H_2/mol lactose	17.2, NR, 7.0, 58, 55	Collet et al. 2004
	Seed sludge	Molasses (3000 mg COD/L)	26.13 molH_2/Kg COD removed	NR	11.4,27.98 Kg COD/m^3 reactor-d, 4.5, 35, 45	Ren et al. 2006
	Mixed culture	Glucose (10 g/L)	2.18	2.47 molH_2/mol glucose	26.7, NR, 4.8 – 5.5, 70, NR Metanogenic inhibitor	Kotsopoulos et al. 2006
UASB	Mixed culture	Sucrose rich waste water	5.93	1.61 molH_2/mol glucose	12, NR, 7, 39, NR	Mu & Yu 2006

	Mixed culture	Citric acid waste water (18 kg COD/L)	1.23	0.84 molH_2/mol hexose	12, 38.4 Kg COD/m^3-d, 7, 35, NR	Yang et al. 2006
	Mixed culture	Sucrose (20 g COD/L)	11.3	1.5 mmol H_2/mol sucrose	8, 175 mmol sucrose/L-d, 6.7, 35, 42.4	Chang & Lin 2004
	Mixed culture	Glucose (7.7 g/L) (1.3 g/L)	18.4 19	1.7 mol H_2/mol glucose 0.7 mol H_2/mol glucose	2, NR, 6.4, 55, 36.8 2, NR, 4.4, 35, 29.4	Gavala et al. 2006
CSTR and UASB	Mixed culture	Starch and xylose (10g /L starch and xylose 1:1 w/w)	4.5 4.76 2.54		32.9, NR, 7, 35, 68 6.7, NR, 7, 35, 68 20.5, NR, 7, 35, 68	Camilli & Pedroni 2005
CSTR UASB	Mixed culture	Glucose (6.86 g/L)	37.5	1.6 molH_2/mol glucose	12, NR, 5.5, 60, 48	Oh et al. 2004

UFBR						
TBR	Clostridium acetobutylicum (ATCC 824)	Glucose (10.5 g/L)	8.9	0.9 mol H_2/mol glucose	0.035, 8.3 g/L-h, 4.9, 30, 74	Zhang et al. 2006
	Mixed culture	Glucose (2 g/L)	NR	2.48 molH_2/mol glucose	0.5, 96 Kg/m^3-d, 7.7, 30, NR	Leite et al. 2006
PBR	Mixed culture	Sucrose (17.8 g/L)	0.298	3.88 mol H_2/mol sucrose)	0.5, NR, 6.7, 40, 42 Temperature effect	Lee et al. 2006
CIGSB	Cow dung	Palm oil mill effluent (5-60 g DQO/L)	0.42 L/g $_{\text{COD destroyed}}$	NR	3-7, NR, 5, NR, 53-56	Vijayaraghavan & Ahmad 2006
UACF	Cow dung	Jackfruit peel (22.5 g VS/L)	0.72 L biogas/g $_{\text{VS destroyed}}$	NR	288, NR, 5, NR, 56	Vijayaraghavan et al. 2006
	Mixed culture	Glucose (10 g/L)	71.4	1.1 mol H_2/mol glucose	0.79, NR, 5.5, 37, 70	Kim et al. 2006

MBR	Mixed culture	Sucrose (20 g COD/L)	50.27 95.23	2.10 mol H_2/mol sucrose 1.22 mol H_2/mol sucrose	2, NR, 6.9, 40, 40 0.5, NR, 7, 40, 35	Wu et al. 2006b

[a]When optimization trials were carried, optimum values are reported. [b] NR: Not reported. POME: Anaerobic pond of a palm oil mill effluent. COD: Chemical oxygen demand. CSTR: Continuous stirring tank reactor. TBR: Trickling biofilter. OFMSW: Organic fraction of municipal solid wastes. PBR: Packed bed reactor. MBR: Membrane bioreactor. FBR: Fluidized bed bioreactor. DTFBR: Draft tube bed reactor. UFBR: Up-flow fixed bed reactor. CIGSB: Carrier induced granular sludge bed. UASB: Upflow anaerobic sludge blanket. UACF: Up-flow anaerobic contact filter.