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REVIEW ARTICLE

Microbiological and engineering aspects of biohydrogen production

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Abstract Dramatically rising oil prices and increasing awareness of the dire environmental consequences of fossil fuel use, including startling effects of climate change, are refocusing attention worldwide on the search for alternative fuels. Hydrogen is poised to become an important future energy carrier. Renewable hydrogen production is pivotal in making it a truly sustainable replacement for fossil fuels, and for realizing its full potential in reducing greenhouse gas emissions. One attractive option is to produce hydrogen through microbial fermentation. This process would use readily available wastes as well as presently unutilized bioresources, including enormous supplies of agricultural and forestry wastes. These potential energy sources are currently not well exploited, and in addition, pose environmental problems. However, fuels are relatively low value products, placing severe constraints on any production process. Therefore, means must be sought to maximize yields and rates of hydrogen production while at the same time minimizing energy and capital inputs to the bioprocess. Here we review the various attributes of the characterized hydrogen producing bacteria as well as the preparation and properties of mixed microflora that have been shown to convert various substrates to hydrogen. Factors affecting yields and rates are highlighted and some avenues for increasing these parameters are explored. On the engineering side, we review the potential waste pre-treatment technologies and discuss the relevant bioprocess parameters, possible reactor configurations, including emerging technologies, and how engineering design-directed research might provide insight into the exploitation of the significant energy potential of biomass resources.

Keywords Biofuels · Biohydrogen · Fermentation · Bioreactors · Waste treatment

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Climate change and biofuels: the case for biohydrogen

Impending climate change and increased concern about dwindling fossil fuel reserves have focused the world's attention on a search for alternative energy sources. Although the magnitude of the near and long term effects due to global warming is somewhat uncertain, and whether or not we have reached "peak oil" is still being debated [1], there is a general consensus emerging that large environmental changes are imminent and that fossil fuels will be rapidly depleted at present, or even greater future [2], rates of consumption. Public attention, seen daily on the front pages of newspapers, has led to government action. Worldwide biofuel production has quickly ramped up, spurred on by government incentives; subsidies and alternative fuel mandates. In 2007 worldwide production of ethanol reached 50 billion liters, biodiesel stood at 9 billion liters. However, it has become obvious that first generation technologies producing biofuels from food crops are untenable in the long term [3–5]. In fact, it is apparent that greatly expanded biofuels production requires

thoughtful consideration of the many social, economic, and environmental impacts that might arise [6–9]. However, it is only somewhat belatedly that some of these issues are starting to be addressed [10]. Obviously these important policy issues will require intense public debate informed by science and critical thinking, but are beyond the scope of this review.

A large variety of biofuel options are possible for use as mobile energy carriers [11, 12], but it is not clear at present which one is to be preferred in the long term, or indeed perhaps a restricted variety of biofuels would more appropriately match locally available resources and needs. Nevertheless, biologically produced hydrogen would seem to have a number of advantages. First, it can be converted to useful power using fuel cells at about twice the efficiency of burning a biofuel in an internal combustion engine. Secondly, its use leads to near zero levels of pollution whereas the use of some other biofuels is predicted to lead to appreciable levels of air pollution; for example in the case of ethanol, ozone and peroxyacetyl nitrate (photochemical smog), and acetaldehyde and formaldehyde (carcinogens). Thirdly, other biofuels emit CO_2 when combusted whereas the CO_2 associated with biohydrogen is released at source during fermentation, thus more easily allowing its potential capture and sequestration which could even make biohydrogen carbon negative.

Microbial processes producing hydrogen

Diversity in microbial physiology and metabolism means that there are a variety of different ways in which microorganisms can produce hydrogen, each one with seeming advantages, as well as problematic issues [13]. From an engineering perspective, they all potentially offer the advantages of lower cost catalysts (microbial cells) and less energy intensive reactor operation (mesophilic) than the present industrial process for making hydrogen (steam reformation of methane). Four distinct approaches for biohydrogen production include: 1) biophotolysis of water using algae/cyanobacteria, 2) photodecomposition (photofermentation) of organic compounds using photosynthetic bacteria, 3) dark fermentative hydrogen production using anaerobic (or facultative anaerobic) bacteria and 4) bioelectrohydrogenesis.

Biophotolysis, the concerted action of the two photosystems of plant-type photosynthesis to split water with absorbed photons and generate reduced ferredoxin to drive the reduction of protons to hydrogen, is carried out by both some green algae and some cyanobacteria (Fig. 1). This is an inherently attractive process because it uses water, an abundant and easily obtainable substrate. On the other hand, its simultaneous production of oxygen and hydrogen poses a number of possibly severe problems; the generation of potentially explosive mixtures of these gases, and inhibition of hydrogenase (green algae), highly sensitive to even moderately low concentrations of O_2. Hydrogen production by cyanobacteria, where hydrogen is usually produced by nitrogenase in heterocysts, is much less sensitive to oxygen. However, this comes at a metabolic cost, both due to heterocyst biosynthesis and maintenance, and to the burdensome ATP requirement of nitrogenase. Additional problems arise because of the low solar energy conversion efficiencies obtained, effectively increasing dramatically the surface area requirement for the necessary transparent, hydrogen imperable, enclosed photobioreactors. Thus these problems have proved daunting, and presently reported rates of solar energy conversion with these systems are not much higher than they were 30 years ago [14, 15].

Another process that requires input of light energy is light driven hydrogen production from various substrates, in particular organic acids, by photosynthetic bacteria, a process that has been called photofermentation (Fig. 2). Indeed, photosynthetic bacteria have long been studied for their capacity to produce significant amounts of hydrogen due to their high substrate conversion efficiencies and ability to degrade a wide range of substrates. Although pure substrates have usually been used in model studies, some success in using industrial wastewater as substrate has been shown [16, 17]. However, pre-treatment may be needed prior to photosynthetic biohydrogen gas production due to either the toxic nature of the effluent, or its colour/opaqueness. For example, high biomass concentration is not desirable due to the reduction of light diffusion into

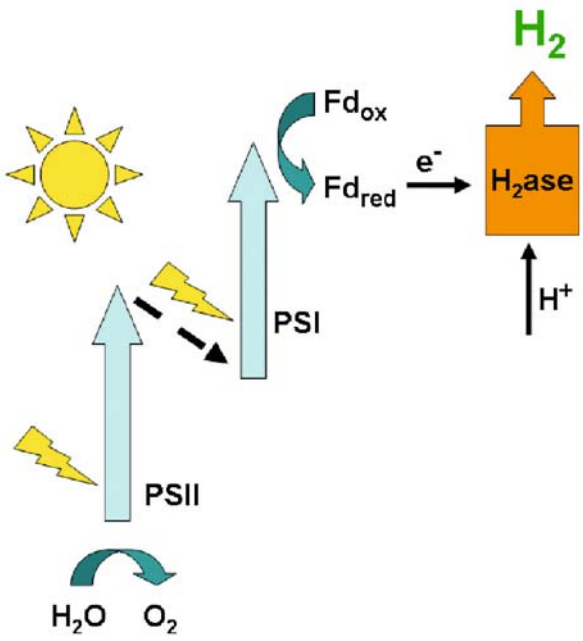

Fig. 1 Biophotolysis (green algae – cyanobacteria)

the bioreactor. Despite the successes of hydrogen generation via photosynthetic degradation of organic compounds, much work is still needed to create a large-scale, economically attractive process. Though the conversion of substrate is generally high, the production rate of H_2 is slow and hydrogen yields are still far from the theoretical maximum. As with any other light-based production process, light diffusion and intensity play a key role in maximizing product (hydrogen) yield. Increasing light intensity (to a certain threshold) increases the hydrogen yield and production rate, but has a negative effect on light conversion efficiency. Expensive equipment and the requirement for large reactor surface areas remain serious drawbacks. Though cyclic light process operation (i.e. light–dark cycles) has been shown to increase the amount of hydrogen evolved when compared to continuous illumination [18] and a number of other improvements could possibly be made (replace N_2ase with H_2ase, etc.), many questions remain about as to whether overall light conversion efficiencies could ever be high enough to warrant large-scale systems. Photosynthetic hydrogen production might have to be coupled with another process in order to make it economically viable.

A third method of the biological production of hydrogen is dark fermentation, where hydrogen production is inherently more stable since it takes place in the absence of oxygen. Indeed, anaerobic systems have an advantage over their photosynthetic counterparts in that they are simpler, less expensive, and produce hydrogen at much higher rates. The major drawback of course is that these bacteria are unable to overcome the inherent thermodynamic energy barrier to full substrate decomposition. Thus, in general fermentative systems suffer from low hydrogen yields [19]. The reason for this is that anaerobic metabolism is evolutionarily optimized for maximizing biomass and not hydrogen [13]. Typically, anaerobic species (ex. from the genus Clostridium) generate gas in the exponential growth phase, and then the metabolism shifts from H_2/acid production to solventogenesis when the culture reaches stationary growth phase [20, 21]. Poor hydrogen yields have also been linked to high hydrogen partial pressure, high substrate concentration, low iron concentration, and/or low pH [20, 22, 23, 24]. Current maximum hydrogen yields obtained do not make the fermentative process an attractive one from an economic point of view when compared to conventional reforming techniques. Ongoing research is attempting to address this issue and identify a set of parameters under which both yield and production rate can be maximized.

It has been argued that in order for hydrogen production by dark fermentation to be economically feasible and sustainable, a two-step/hybrid biological hydrogen production process would be necessary [25]. By combining the anaerobic and photosynthetic steps, as shown in Fig. 3, higher overall substrate conversion efficiency is possible as the photosynthetic microbes can degrade the soluble metabolites from the fermentative step using sunlight to overcome the energy barrier. VFAs are the main soluble breakdown products from the first step, and these are preferred substrates of photo-heterotrophic bacteria [26–28].

Theoretically, 12 moles of hydrogen can be produced from 1 mole of glucose in the two step process. It should be pointed out that some photosynthetic bacteria are theoretically capable of doing this in a single step since species are known which can use some sugars as substrate. Thus, the only real advantage of such a two-step process might be to decrease the time and volumes required for initial substrate

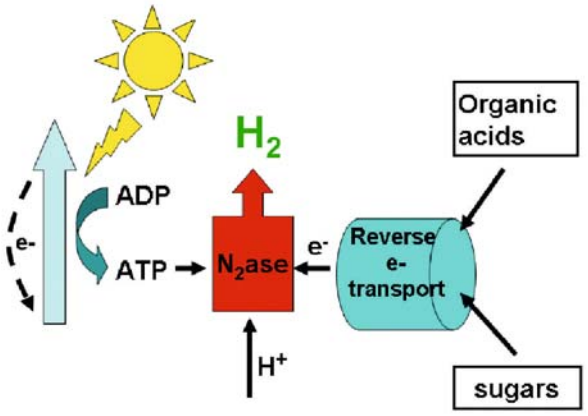

Fig. 2 Photofermentation (Photosynthetic bacteria)

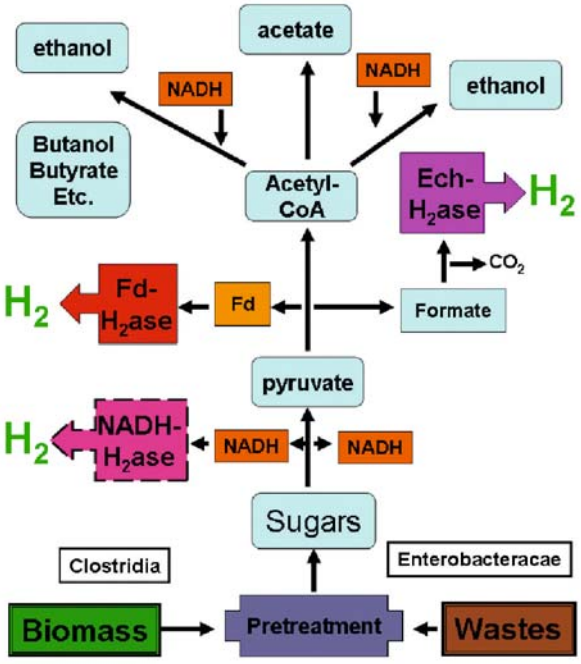

Fig. 3 Dark fermentation (Clostridia, Enterobacteraceae)

conversion. Indeed, complex substrates, i.e. most carbohydrate-containing wastes, would not be readily degraded by photosynthetic bacteria, and probably require the use of mixed consortia. There have been a number of recent reports on two-stage systems as shown in Fig. 4 [26-33]. One study successfully produced hydrogen using olive mill wastewater for the production of biohydrogen in a two-stage process, with a three fold increase in hydrogen production when compared to photo-fermentation alone, and a COD conversion efficiency of ~55% [29]. High COD concentrations may have had an inhibitory effect since COD removal could be increased by diluting the wastewater. In similar work, an almost 70% conversion efficiency of *Chlamydomonas reinhardtii* biomass (mainly glucose-starch) was achieved in a two-step process that utilized *Clostridium butyricum* and *Rhodobacter sphaeroides.*

There have been several studies in which co-cultures of fermentative and photosynthetic organisms were examined [26, 33]. Work with co-cultures of both *C. butyricum* and *R. sphaeroides* showed only a slight increase in the hydrogen yield when compared with production obtained from pure cultures separately [26]; even at high *Rhodobacter* ratios (~6:1) it appeared that *R. sphaeroides* was not able to compete with *Clostridium* for substrate (glucose). However, it is difficult to draw conclusions as to the efficacy of having both types of organism present in the same reaction vessel since molar yields, either alone or in co-culture, were very low, < 1 mol H_2/mole glucose, and large quantities of fermentation products, acetate and butyrate accumulated (i.e. were not used as a substrate for photofermentation). A co-immobilized two stage system using *Lactobacillus* and *R. sphaeroides* was much more successful with a maximum yield of 7 mol H_2/mole glucose [33]. However, it remains to be seen if the extra manipulation and costs involved, especially if the system is run in batch mode, can be justified for any practical application. Indeed it may be difficult to rationalize the use of any type of co-culture system. Thus, a two-step approach to two-stage fermentations, where the two species are separated, may show more promise in achieving economical hydrogen production yields in large-scale applications.

Finally, a new hybrid biological hydrogen production process has very recently been described and is under active study [34–41]. It is based on the concept and practice of a microbial fuel cell (MFC). In fact, the idea is to add a little electrical potential to that generated by a microbial fuel cell, thus reaching a sufficient force to reduce protons to hydrogen, in a process that can be called bioelectrohydrogenesis as shown in Fig. 5. Thus the cell could be called a microbial electrohydrogenesis cell (MEC). (Microbial electrolysis cell is an unacceptable term since it implies that the protons are derived from water splitting.)

The advantage of MFCs and MECs is that the energy available in waste streams can be directly recovered as electricity (MFC) or hydrogen (MEC). The metabolic pathways involved are not clear, and in fact thus far MEC studies have been carried out only with mixed cultures, often using those already enriched and active in microbial fuel cells (MFC).

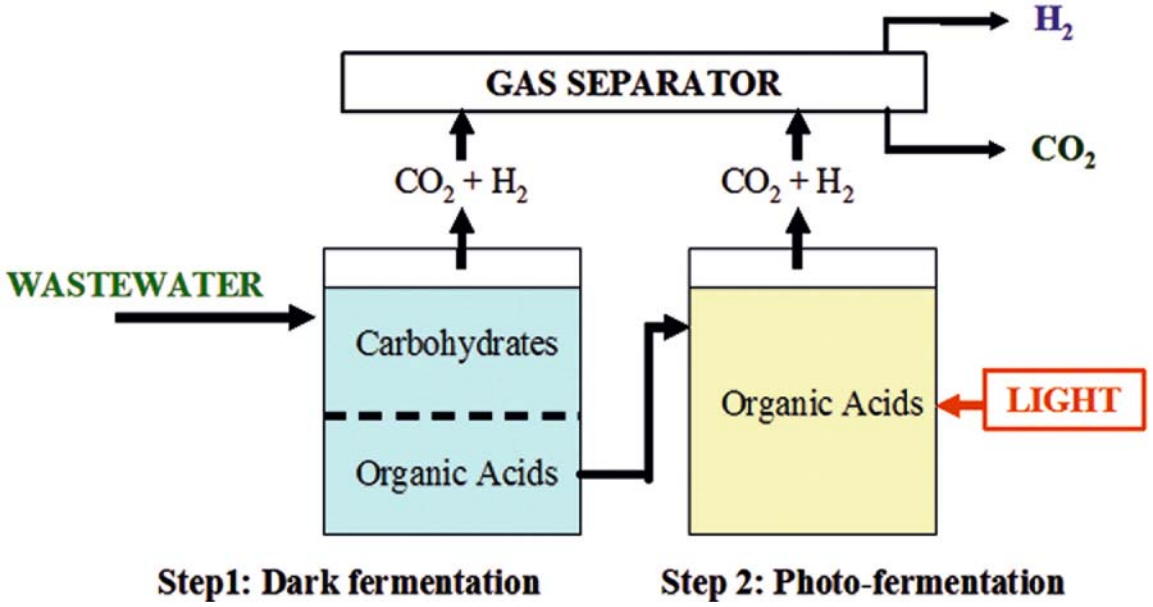

Fig. 4 A two-step approach for biohydrogen production

However, MFCs usually contain bacteria such as *Geobacter* and *Shewanella*, which are known to effectively couple their metabolism to electrode surfaces. These reactions are essentially anaerobic respirations where the external electron acceptor is an electrode instead of the more usual oxidized compound (nitrate, TMAO, fumurate, etc.). Thus bioelectrohydrogenesis utilizes electrochemically active micro-organisms which, with a small to moderate voltage input, convert dissolved organic matter into hydrogen inside an electrochemical cell/microbial fuel cell via coupled anode-cathode reactions. Thus, in principal, and in practice, sufficient energy can be added to allow the conversion of compounds such as acetate, products of dark fermentation, to hydrogen. Ordinarily of course, as discussed elsewhere, microbes such as these are unable to do this conversion on their own except in syntrophic association with a hydrogen-consuming organism capable of maintaining very low hydrogen partial pressures [42, 43]. Although an appealing concept, and obviously one with the potential to permit the complete conversion of simple substrates, sugars or acetate, or even wastewaters [36] to hydrogen, there are a number of serious challenges in several problem areas to overcome. Not surprisingly, many of these are also faced in the further development of microbial fuel cells [44]. Power densities at the electrode surface are low, which translates into low volumetric hydrogen production. However, a variety of manipulations involving electrode materials and cell construction [38–41] have increased volumetric hydrogen production by several orders of magnitude over the original reports, so that values near 1 m^3H_2/m^3 reactor liquid volume/day can now be obtained [37, 41]. However, this remains well below that obtainable in a standard dark fermentation. While yields of 50% or greater can be demonstrated, higher yields require increased voltage, adversely affecting energy efficiency. Other issues include the need for a noble metal catalyst in cathode fabrication, and decreased hydrogen production due to potential methanogenic reactions in both the anodic and cathodic chambers. In fact, it has been proposed that syntrophic reactions are the basis for the normal functioning of a MFC [45], which very well could apply to MECs. Much remains to be learned about the microbiology involved in these processes, and it is likely that novel organisms can be isolated from functioning MFCs and MECs [46, 47]. Obviously, much more research is needed to address key limitations. Nonetheless, bioelectrohydrogenesis appears to be a promising future approach to hydrogen generation from wastewater, especially for effluents with low organic content.

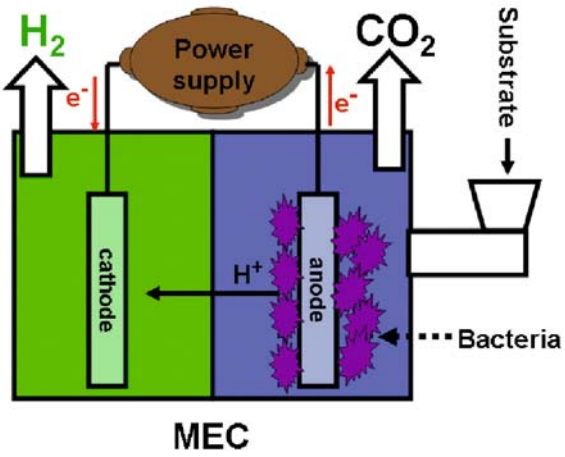

Fig. 5 Hybrid biological hydrogen production

Isolation and properties of novel hydrogen producers

Modern molecular techniques have revolutionized and expanded the scope of microbial systematics and physiology. We are now aware that only a very small fraction of what is out there has been isolated and studied in the laboratory. In the present perspective of hydrogen production technology, there is a no evidence that any naturally isolated microbe can produce more that 4 moles of H_2 per mole of glucose. Moreover, there is no clear contender for a robust, industrially capable microorganism that can be used as a platform for research to genetically alter its metabolic pathway to produce more than 4 moles of H_2 per mole of glucose equivalent. Therefore, data mining of genomic and metagenomic sequences might provide insight into potentially useful hydrogenases. As well, it is possible that continued isolation and cultivation efforts might yield novel isolates with some unique properties for waste decomposition and higher hydrogen yields and production rates.

An initial data mining study targeted genomes containing both a Ni-Fe hydrogenase and formate dehydrogenase, and found a number of interesting possibilities [48]. This screen would presumably find systems similar to the well known *Escherchia coli* Fhl system which consists of a formate dehydrogenase and hydrogenase 3, the paradigm of the Ni-Fe hydrogenase subclass known as Ech hydrogenases [49-51], although other possible combinations might be found as well. Although the Ech hydrogenases are now known to be wide spread, occurring in at least 56 different bacterial and 28 different archeal genomes [51], sequence homology alone can not assign physiological function and some of Echs are known to preferentially carry out

hydrogen oxidation. The recently described isolates that belong to the genera *Bacillus* and *Proteus* [52] may contain this enzyme complex. Reported hydrogen yields were quite low, but conditions were not optimized. More commonly, hydrogen fermenting bacteria are thought to contain a [FeFe] hydrogenase. However, their occurrence in the environment, at least in an initial metagenomic survey, appears to be quite limited [53], and this is borne out by a recent blast search of over one billion base pairs of non-redundant sequence where only 10 hits to a [FeFe] hydrogenase bait sequence were found [54]. A search of 371 fully sequenced microbial genomes only found 25, and not all these appeared to be bona fide [FeFe] hydrogenase sequences [54]. A large number of the sequences were in microbes belonging to the Class Clostridia. There were a number found in the δ-proteobacteria, but many of these, as well as the lone γ-proteobacterium, *Shewanella oneidensis*, are thought to function in hydrogen oxidation, not proton reduction. Thus, in this case too, caution is required in interpreting data mining results.

A number of somewhat novel fermentative hydrogen producing bacteria have been isolated recently. *Enterobacter asburiae* SNU-1 was isolated from a domestic landfill and gave a yield of 0.43 mol hydrogen/ mol formate. It showed rather high maximum and overall hydrogen production productivities (398 and 174 ml/l/h) with glucose. Unlike many hydrogen producers, this strain produced hydrogen in both the exponential and stationary phase [55]. A new thermophilic hydrogen producer *Thermoanaerobacterium thermosaccharolyticum* PSU-2 was found to carryout an ethanol-acetate type fermentation with inorganic nitrogen medium medium, whereas a butyrate-acetate type fermentation was found with a medium containing organic nitrogen. Maximum hydrogen yields were 2.53 mol H_2.mol^{-1} hexose and rates were 270 ml H_2.l^{-1}.h^{-1} . As is typical of most fermentations, hydrogen production slowed dramatically with time due to acidification [56]. *Citrobacter* sp. Y19, originally isolated for CO-dependent H_2 production can also ferment glucose over a wide range of temperatures (25–40°C) and pH (5–9) with a maximum H_2 yield of 2.49 mol H_2.mol^{-1} glucose and an H_2 production rate of 32.3 mmol H_2.g^{-1} cells. h^{-1} [57]. Other major metabolic end products are acetate and ethanol. Another bacterium, *Rhodopseudomonas palustris* P4, isolated by the same group for its CO-dependent H_2 production abilities was also studied for its capacity for fermentative H_2 production in batch mode. Maximum H_2 yields were 2.76 mol H_2. mol^{-1} glucose and H_2 product-ion rates were 29.9 mmol H_2. g^{-1} cell. h^{-1} with ethanol, acetate and CO_2 as the other major metabolites. As to be expected from the well-known effects of pH and high hydrogen partial pressures on fermentative hydrogen production [19], a high concentration of phosphate buffer or intermittent sparging with argon improved overall performance [58].

A novel extreme thermophile, *Caldicellulosiruptor saccharolyticus* (Class Clostridia), was found to produce hydrogen from a variety of substrates including: glucose, xylose, and an industrial waste stream, paper sludge, as a renewable cheap feedstock, with major metabolic end products acetate and lactate. As is typically found for extreme thermophiles [19], maximal volumetric H_2 production rates were quite low, 9–10 mmol H_2.l^{-1}.h^{-1} with simple sugars, and even lower rates, 5 to 6 mmol H_2.l^{-1}.h^{-1}, were found with the complex substrate indicating the possible presence of inhibiting components in paper sludge hydrolysate [59]. Two strains of the mesophilic anaerobic bacterium *Sporacetigenium mesophilum* (Class Clostridia) were isolated from an anaerobic sludge digester treating municipal waste. Unusually, optimal hydrogen production was detected at pH 8.8, with a moderate yield of 1.4 mol H_2.mol^{-1} glucose and the major metabolic end products were acetate, ethanol, and CO_2, typical for a clostridial-type fermentation [60]. As noted previously in various other studies [19], yields under thermophilic conditions tend to be higher. Thus, a thermophilic bacterium, *Thermotoga neapolitana,* gave a hydrogen yield of 2.4 ± 0.3 mol H_2 mol^{-1} glucose with acetic acid and lactic acid as additional metabolic end products [61]. For reasons that aren't clear, malonic acid addition increased H_2 yields to 3.5–3.8 mol H_2 mol^{-1}glucose. These authors were unable to reproduce the earlier surprising and even somewhat fantastic claims of microaerobic hydrogen production by this bacterium with yields greater than 4 mol H_2/mol glucose [62, 63].

Preparation and properties of mixed microbial consortia

Large scale, and ever increasing, industrialization and urbanization are creating massive organic waste disposal problems. Obviously, it would be desirable to turn these into a useful product, such as a biofuel, while at the same time carrying out effective waste treatment. Thus, they could be ideal inexpensive feed-stocks for biological hydrogen production. However, probably no single microorganism possesses the necessary various hydrolytic activities required to use complex wastes for biohydrogen production. Thus, efforts are being made to develop suitable mixed microbial consortia (also called mixed microflora) capable of decomposing various organic waste streams, and to improve the hydrogen yields and rates by these cultures. In this section we describe the development and some characteristics of

described hydrogen producing microbial consortia, afterwards we discuss specific substrates.

Typically, innocula for cultures are prepared by pretreatment of various sludges to remove methanogens, normally incubation at high temperatures or low pHs, or a combination of these. This is usually quite effective in enriching for hydrogen producers, not surprisingly since these treatments will favour the survival of spore-forming Clostridia. The resulting bacterial populations can be followed using techniques such as DGGE and phylogenetic assignments made through analysis of 16S RNA. One such consortium produced hydrogen (149.69 ml H_2.g^{-1} TVS) from cornstalk wastes, which are becoming a burning problem as an environmental pollutant. Morphological and physico-chemical characteristics, and comparative sequence analysis of 16S rDNA indicated that two the dominant strains belonged to *Clostridium* sp. and *Micrococcus* sp. Accordingly, conversion of cornstalks into hydrogen was accompanied by production of acetate, propionate, butyrate and ethanol [64]. In another study, hydrogen (319 ml H_2. g^{-1} COD) was produced from cattle waste water by an enriched consortium. In this case, screened waste was subjected to ultrasonic pre-treatment (at 100 KHz for 30 min) for the selective enrichment of hydrogen producers [65]. Of course, reactor configuration and mode of operation will affect the composition of the microbial consortia, as suggested by a recent study on hydrogen production from chemical wastewater in a biofilm configured reactor operated in periodic discontinuous batch mode [66]. Thus, microbial consortia have been shown to be capable of producing hydrogen, with various yields, from a variety of substrates, including; probiotic wastewater [67], dairy wastewater [68], and heat-treated cassava starch [69], to name only a few recent examples (more are given in the next section). Of course, hydrogen amounts are highly dependent upon the composition of the substrate and carbohydrate rich materials will give higher yields. It might be expected that the species composition of the consortium might vary with substrate, but this has not been examined in a systematic way. One substrate of potentially great interest is cellulose. Not surprisingly, growth on cellulose enriches for Clostridia [70, 71], known for their cellulytic capabilities. Another consideration is that the developed microbial consortium is likely to be quite complex, with not every organism present directly producing hydrogen. One way to determine which organisms that are present could potentially be responsible for the observed hydrogen production is to probe the population for metabolism specific genes. Thus, one recent study of a consortium, growing on molasses wastewater and producing hydrogen and ethanol, where the population was probed for its content in *hydA* (catalytic subunit of the [FeFe] hydrogenase) found 11 different phylotypes. All however, were in the Class Clostridia, and most closely related to *Ethanoligenens harbinense*, *Clostridium thermocellum*, and *Clostridium saccharoperbutylacetonicum* [72].

Potential substrates

Many studies have examined the hydrogen production potential of different carbon sources varying from simple sugars such as glucose to more complex substrates such as biomass. A brief summary of yields and rates of biohydrogen production (batch and continuous) on various simple substrates is shown in Table 1 and Table 2 (Note: reported rates and yields may not have occurred under same operating conditions). Glucose and sucrose are the most common pure substrate used in both batch and continuous processes, due to their relatively simple structures, ease of biodegradability and presence in several industrial effluents [73] and presence in polymers that can be obtained from agricultural and biomass wastes. In general, the maximum yield by mixed or pure inocula for both types of systems under atmospheric/near atmospheric operating pressures is in the range of 45–60% [74] (based on 4 moles of hydrogen per glucose).

Wastewater substrates

Biohydrogen production from simple sugars has been well researched, however, relatively few studies have dealt with using industrial/domestic wastewater as a potential feedstock. These are summarized in Table 3. (Note: reported rates and yields may not have occurred under same operating conditions). In a recent study, the ability of a mixed culture to produce hydrogen from composite chemical wastewater (CW) in conjunction with co-substrates was studied [84], The CW was an heterogenous mixture of pharmaceuticals, pesticides, wastes from numerous chemical processing units and synthetic wastewater (SW) containing glucose (2 g/L) and nutrients, domestic sewage wastewater (DSW) and glucose served as co-substrates. It was found that a 40%/60% mixture of CW/DSW gave the highest yield and highest relative H_2 production rate, followed by a 40%/60% mixture of CW/SW+1 g/L glucose. Interestingly, synthetic wastewater alone showed poor hydrogen evolution, as did increasing the glucose co-substrate concentration. Various studies have shown that addition of trace nutrients does not consistently increase hydrogen gas production [85, 86].

Of course with waste streams of complex composition, COD is a convenient measure of substrate potentially available for conversion to hydrogen while at the same

time it allows one to gauge the efficacy of treatment. For example, a recent study showed that dairy wastewater was suitable for biohydrogen production in a continuous set-up [88]. Thus, not only were significant amounts of hydrogen produced, but also high COD removal efficiencies (>60% in some cases) were achieved. Similar results have been obtained by others [see for examples; 85, 87]. Generally, such removal efficiencies are acceptable for the first stage of wastewater treatment. Some studies have examined the effect of wastewater substrate concentration (OLR-organic loading rate) on the rates and yields of hydrogen production. For example, one study showed that both hydrogen yield and production rate increased with increasing sucrose up to moderate OLR (5 to 20 g COD/l); however both values decreased when the substrate concentration reached 30 g COD/l [78]. Similar results have been observed in other studies, although inhibition occurred at different substrate concentrations [22, 83]. These results suggest that elevated substrate loadings have a toxic effect on the bacteria.

Biomass substrates

Plant biomass, agricultural wastes and industrial effluents from such sectors as the pulp/paper and food industries represent an abundant potential source of substrate for the production of biohydrogen. Production of hydrogen by dark fermentation of cellulosic material usually requires substrate pretreatment procedures, which significantly increase the hydrogen production cost. (Note that the same would be true for the production of bioethanol or most other biofuels). Different microorganisms have been studied in order to obtain higher efficiencies in microbial hydrolysis of a variety of cellulosic materials [90]. Significant amounts of hydrogen can be produced from cellulosic feedstocks (straw, wood chips, grass residues, paper waste, saw dust, etc) using natural consortia and conventional fermentors operated under conditions that favor H_2-producing bacteria able to degrade cellulose for example, *Clostridium thermocellum* [91]. The hydrogen production obtained is however variable and depends greatly on the bacterial consortium and culture medium. As an alternative, a two-stage hydrolysis-fermentation approach can be used with a comparable biohydrogen production efficiency to that reported in other studies using cellulose or hydrolyzed cellulose as the substrate [90]. Other types of waste such as municipal food waste and sewage sludge have also been studied and a combination of feedstocks in optimal ratios can significantly enhance the production of hydrogen as compared with the individual wastes [92].

Reactor configurations for dark fermentation

CSTRs are the most frequently studied reactor type for continuous fermentative H_2 production [93]. However, CSTRs do have some disadvantages. In general, a CSTR system is very sensitive to environmental conditions such as changes in pH and HRT. Additionally, operation at a high dilution rate (or short residence time) can lead to washout of biomass [94]. Upflow anaerobic sludge blanket (UASB) reactors are another popular option for continuous fermentative H_2 production due to their high treatment efficiency, short HRT and excellent process stability. For example, one study on hydrogen production from high-strength wastewater (rice winery wastewater) by a mixed anaerobic culture concluded that because of a higher hydrogen productivity of the biomass coupled with a higher concentration of biomass, the UASB reactor possessed much higher volumetric H_2 production rates than a CSTR [89]. Similar conclusions have been drawn by other studies. For example, a comparison of H_2 producing CSTR and UASB reactors operating on glucose showed that the UASB configuration was more stable and had a higher volumetric H_2 production rate, although in this case the molar H_2 yield was higher in the CSTR for all conditions tested [95]. One possible disadvantage of a UASB is that start-up might be time consuming, however this is more than likely compensated by the long period of stable operation that can be obtained [80]. A very closely related approach would be to use cell immobilization or granulation to increase biomass retention in reactors. Thus, one study evaluated the performance of a continuously stirred anaerobic bioreactor (CSABR) seeded with silicone (SC) – immobilized sludge [94]. Start-up of the reactor took 1–2 days before biogas with a H_2 content of over 40% was produced, and then the CSABR was able to produce H_2 from sucrose at a stable rate (1.15 l/h/l) for more than 300 days with a H_2 yield of 3.71 mol H2/mol sucrose. [94]

Relevant continuous bioprocess parameters

One decision that needs to be made is whether to use a pure or a mixed culture. The downside of using a pure strain is that sterile and anaerobic conditions should be maintained throughout the experiment, which may prove difficult on a larger industrial scale [93]. The drawback of using a mixed culture inoculum, on the other hand, is that a culture shift can occur after a period of continuous operation causing a drop in hydrogen yield and rate of production. With pure strains, it may be necessary to include an expensive reducing agent into the liquid. As a result, large scale hydrogen production using a pure culture may not prove to be economically feasible. In

a novel way to circumvent this problem, mixed cultures of a facultative anaerobe, *Enterobacter aerogenes* was used with *Clostridium butyricum* without the addition of a reducing agent, achieving a nearly 50% increase in hydrogen production when compared to *C. butyricum* with reducing agent alone [96]. Similar results were reported elsewhere [76]. Thus, the use of *E. aerogenes* in conjunction with *Clostridium* could simultaneously resolve the need for addition of expensive reducing agents and increase hydrogen production.

Hydraulic retention time (HRT) is another factor potentially affecting both hydrogen production rates and yields in different continuous reactor systems. For example, an upflow anaerobic reactor with rice winery wastewater as substrate was used to study the effect of varying HRTs on hydrogen production [89]. It was demonstrated that H_2 yield increased with HRT while the specific hydrogen production rate (SHPR) decreased. Additionally, HRT was shown to influence the product composition during fermentation of substrates that are more recalcitrant to biodegradation. Another group used an upflow anaerobic sludge blanket (UASB) reactor to examine hydrogen production rates and yields over a wide range of HRTs (4–24 h) [80]. The hydrogen production rate peaked at 8 h whereas yields were fairly constant (1.5 mol H_2/mol sucrose) in the range of HRTs from 8 to 20 h. In this case, both rates of hydrogen production and yields declined with increasing HRT below 8h. It was found that granule formation, which was HRT-dependent, could serve as an indicator of a successful USAB reactor operation; the largest granules were found at the same HRT, 8 h, at which hydrogen productivity was maximum. Of course, HRT is equally important in CSTRs as shown, for example, in one recent study using brewery waste as substrate [97]. Here 18 h was determined to be the optimum HRT based on the highest H_2 concentration obtained and the highest hydrogen production rate. Above 18h, both the hydrogen yield and production rate drastically decreased with increasing HRT. Volatile fatty acid (VFA) concentrations increased with increasing HRT, with ethanol being the major alcohol produced, with especially high concentrations at lower HRTs [97]. In reality, a wide range of optimal HRTs, ranging from 2 hours to 18 hours, have been observed in various studies. This suggests, not surprisingly, that optimal HRT is a specific characteristic of each system dependent upon a multitude of factors including; reactor configuration, substrate used, and the particular organism or microbial consortia.

Conclusion

Biological systems have significant potential as an environmentally friendly means of producing hydrogen, a widely touted possible fuel of the future. However, a number of obstacles must be overcome if this potential is to be realized on a practical scale. One attractive route that was discussed in detail in this review is the use of dark fermentation. In the initial stages of its use, it could be used to recover useable energy from various waste streams while at the same time effecting waste treatment. Here we have reviewed various approaches to this problem using either pure cultures or microbial consortia in a variety of reactor configurations with different substrates. Pure cultures and defined substrates have proven useful for probing limiting factors presented by different microbial physiologies whereas more engineering-type "black box" studies can highlight areas that need to be targeted in bioprocess development. Finally, searches for new strains, either by direct isolation from the environment, or guided by bioinformatics, may well yield novel strains with interesting and useful properties. Obviously, the horizon for practical application of biohydrogen is still in the distance. However, the search for alternative fuels is imperative and the advantages of biohydrogen suggest that is pursuit is a worthwhile endeavour.

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