Chapter 11
Fundamentals of Hydrogen Production via Biotechnology (Bio-H2)

Nuri Azbar

Abstract Hydrogen is considered to be the fuel of the future due to its promising properties in terms of sustainability. Among common hydrogen production methods processes like thermo-chemical, physico-chemical, electro-chemical and biological have been gaining more and more interest lately. A special term "biohydrogen" has been coined which refers to hydrogen production by living organisms. It is considered to be more environmentally friendly alternative since it neither requires high temperature nor high pressure during the production, moreover it provides eco-friendly solution to organic wastes via conversion of organics into a biofuel which emits only water vapor when combusted. Among different technologies of hydrogen production, bio-hydrogen production perhaps exhibits the greatest potential to replace fossil fuels. In this chapter, various hydrogen production methods such as anaerobic dark fermentation and light-driven photo-biological processes are discussed.

Keywords Biohydrogen · Anaerobic dark fermentation · Light · Driven fermentation · Hydrogen economy · Photo · Fermentation

1 Introduction

The main drivers of the hydrogen economy are; ever-growing consumption of energy and decarbonisation need due to the negative environmental effects of refinery based energy alternatives, which have negative environmental impacts related to climate change and global warming, both threatening our existence on the planet earth. It is estimated that over 38 Mt (5,000 petajoules), with a market value of $ 60 billion hydrogen is produced worldwide (Levin and Azbar 2012). Hydrogen has been most commonly used for the purpose of processing of oil in refineries and for the production of industrial chemicals such as ammonia and methanol. Other uses of hydrogen are in industrial processes, chemical manufacturing, and food preparation. The current use of hydrogen is expected to increase significantly in

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Table 11.1 Comparison of various conventional methods of hydrogen production

Method	Advantages	Disadvantages
Reformation of natural gas $CH_4 + H_2O \rightarrow CO + 3H_2$ $CO + H_2O \rightarrow CO_2 + H_2$	Most common (80% H_2 production) Well understood process Widespread infrastructure	Dependent on non-renewable natural gas High CO_2 (GHG) emissions
Gasification of coal $C + H_2O \rightarrow CO + H_2$ $CO + H_2O \rightarrow CO_2 + H_2$	Coal is abundant and inexpensive	Low yields High CO_2 (GHG) emissions High SOx and CO emissions
Electrolysis of water $H_2O \rightarrow O_2 + 2H_2$	Second most common method used Well understood Widespread infrastructure Potentially emission free, depending on source of electricity generation	Energy intensive High CO_2 (GHG) emissions if fossil fuels (coal, natural gas) used to generate electricity
Biomass reformation $C_6H_7O_2N + 2H_2O \rightarrow 4CO + CH_4 + 3/2H_2$ $CH_4 + H_2O \rightarrow CO + 3H_2$ $CO + H_2O \rightarrow CO_2 + H_2$	Potentially carbon neutral Inexpensive Can use organic waste streams	Not yet well understood
Biohydrogen production $H_2O + \text{light energy} \rightarrow O_2 + 2H_2$ $C_6H_{12}O_6 + H_2O \rightarrow CO_2 + H_2$ + Organic molecules	Carbon neutral Can use light or organic waste streams Low energy input	May have poor yields Not yet well understood

the near future in order to meet the demand for refining increasingly heavier, higher sulfur crude oils and oil sands and to meet more stringent regulations on the levels of sulfur in gasoline and diesel fuel. Hydrogen use will also increase up to 40 million t of hydrogen per year in order to meet the fuel need of transportation sector for 100 million fuel cell-powered cars after full market penetration.

Thermo-chemical and electro-chemical methods are the common hydrogen production methods using a diverse array of potential feedstock including fossil fuels, water, and organic matter (Table 11.1). Currently, over 80% of hydrogen production occurs via steam reformation of natural gas during which methane, the primary constituent of natural gas, is combined with high temperature steam (700–1000 °C) in the presence of a catalyst, breaking it apart into H_2 and CO. The CO produced further reacts with water at high temperatures to produce H_2 and CO_2 via a process known as the gas shift reaction. The main drawback of this process is that it is dependent on a limited reserve of natural gas and the carbon dioxide emissions. Similar to natural gas gasification, hydrogen can be produced via coal gasification, however this process produces even more CO_2 emissions and is more expensive (H_2 to CO_2 production ratios: 1:1 for coal gasification and 4:1 for natural gas reformation).

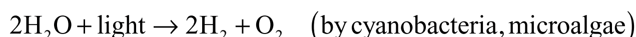
Alternatively; biomass gasification, a potentially carbon neutral method; can be used to break down biomass into H_2, CH_4, and CO, which can in turn be used for steam reformation and the gas shift reaction. Although net CO_2 production is observed during these processes, the overall process of biomass gasification can be considered carbon neutral since new biomass generated during the photosynthesis fixes CO_2. With this process, municipal and agricultural wastes could also be turned into valuable commodities. The electrolysis of water, which is one of the most common methods for H_2 production, is carried out by an electric current passing through water, splitting it up into H_2 and O_2. This is a very energy intensive process and could be potentially emission free if only clean, renewable sources such as wind, solar, hydro, or geothermal energy are used for the generation of electricity.

Hydrogen production via biotechnology (Bio-H_2), on the other hand, employs the use of either dark fermentative or light dependent hydrogen producing organisms. This method of hydrogen production is an attractive alternative to conventional thermo-chemical and electro-chemical methods since it is a potentially carbon neutral process which is carried out at lower temperatures and pressures, and is therefore less energy-intensive than thermo-chemical and electro-chemical processes. Furthermore, unlike thermo-chemical methods, which involve the conversion of nonrenewable fossil fuels into hydrogen, fermentative hydrogen production can utilize renewable carbohydrate-rich substrates such as waste biomass from municipal, agricultural, and forestry sectors, while light driven biological hydrogen production processes utilize light energy, water, and/or CO_2.

2 Biohydrogen Production

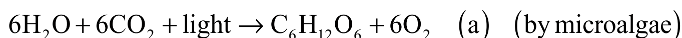
The term “biohydrogen” refers to hydrogen production by living organisms. Hydrogen production via biotechnology can be classified as follows:

2.1 *Direct Biophotolysis of Water*



$$2H_2O + \text{light} \rightarrow 2H_2 + O_2 \quad (\text{by cyanobacteria, microalgae})$$

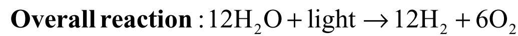
2.2 *Indirect Biophotolysis of Water*



$$6H_2O + 6CO_2 + \text{light} \rightarrow C_6H_{12}O_6 + 6O_2 \quad (a) \quad (\text{by microalgae})$$

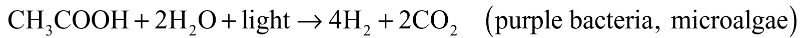
$$C_6H_{12}O_6 + 2H_2O \rightarrow 4H_2 + 2CH_3COOH + 6CO_2 \quad (b) \quad (\text{by cyanobacteria})$$

$$2CH_3COOH + 4H_2O + \text{light} \rightarrow 8H_2 + 4CO_2 \quad (c)$$



$$\textbf{Overall reaction}: 12H_2O + \text{light} \rightarrow 12H_2 + 6O_2$$

2.3 Photo-Fermentation



$$CH_3COOH + 2H_2O + \text{light} \rightarrow 4H_2 + 2CO_2 \quad (\text{purple bacteria, microalgae})$$

2.4 Water-Gas Shift Reaction

$$CO + H_2O \rightarrow H_2 + CO_2 \quad (\text{fermentative bacteria, photosynthetic bacteria})$$

2.5 Two-Phase Anaerobic Process

$$C_6H_{12}O_6 + 2H_2O \rightarrow 4H_2 + 2CH_3COOH + 2CO_2 \quad (\text{fermentative bacteria})$$

$$2CH_3COOH \rightarrow 2CH_4 + 2CO_2 \quad (\text{methanogenic bacteria})$$

2.6 Hybrid Hydrogen Production System (Dark Fermentation + Photo-Fermentation)

Biohydrogen production methods can also be grouped into light independent processes (dark anaerobic fermentation) and light dependent hydrogen production methods with or without oxygen evolution (bio-photolysis) in terms of the energy sources and electron donors used by microorganisms. Light-dependent hydrogen results from the process of photosynthesis. Among photosynthesizing microorganisms capable of evolving hydrogen most attention is paid to microalgae, heterocyst cyanobacteria, and purple non-sulfur bacteria. Microalgae and cyanobacteria possess two photo-systems and can decompose water to release oxygen. Bacteria with one photosystem (first of all purple and green sulfur and non-sulfur bacteria) are incapable of evolving oxygen, and need more reduced electron donors than water to affect photosynthesis. Biohydrogen processes can convert high carbohydrate content waste streams into useable renewable energy, while reducing waste disposal costs and negative environmental impacts. The main criteria for the selection of

organic wastes are availability, low cost, carbohydrate content, and biodegradability. In this chapter, each potential biohydrogen production method is discussed.

3 Light Independent Hydrogen Production—Dark Fermentation

Dark fermentation, which is a naturally occurring process for a variety of microbes, can convert organic material (especially carbohydrate rich ones) into H_2, CO_2, and organic acids. This is a promising alternative to light dependent processes, particularly when waste biomass is used as a feedstock for the generation of H_2. The main advantages of this method over light dependent biohydrogen method is that fermentation does not require a constant light supply, it can be run continuously using inexpensive and commercially available systems and H_2 production rates are much higher than photosynthesis-based systems (Levin et al. 2004). On the other hand, there are also some disadvantages as summarized in Table 11.1.

Although fermentative hydrogen production has many advantages as mentioned above, it is necessary to note that there are also some constraints such as thermodynamic limitations, product inhibition, the presence of branched catabolic pathways, media composition, and the nature of substrate, which all have an impact on hydrogen yields. These constraints are discussed below.

3.1 Basics of Dark Fermentative H_2 Production

Fermentative H_2 production yields only about 10–20 % of the hydrogen potentially available in the substrate (theoretical upper limit: 12 mol H_2 mol^{-1} hexose) (Hawkes et al. 2007; Kraemer and Bagley 2007; Hallenbeck and Ghosh 2009). Somewhat higher yields, up to 25 % (3 mol H_2 mol^{-1} hexose) can be achieved with thermophilic fermentations using either pure cultures or co-cultures at the expense of volumetric productivities (Panagiotopoulos 2010; Zeidan and van Niel 2009).

Fermentative hydrogen production, where hydrogenase enzymes are involved, is carried out via two metabolic types; facultative anaerobes, such as *Escherichia coli*, and strict anaerobes, like *Clostridia* as shown in Fig. 11.1.

Heterotrophic organisms (bacteria growing on organic substrates) have special problems with respect to the disposition of electrons from energy-yielding oxidation reaction during the anaerobic mode of growth. Various kinds of specific controls are required to regulate electron flow in the metabolism of strict and facultative anaerobes. The ability of "disposing off" excess electrons (e^-) in the form of H_2 through the activity of hydrogenase is among them.

As shown in Fig. 11.1, substrate is first broken down to pyruvate by Embden-Meyerhof-Parnas pathway (glycolysis) which results in ATP production and reduction of NAD to NADH. Oxidation of NADH is necessary for glycolysis to continue and this is achieved via the production of a variety of reduced products (e.g. ethanol

Table 11.2 Advantages and disadvantages of dark fermentation processes

No	The process	Organisms	Key enzyme	Advantages	Disadvantages
1	Dark anaerobic fermentation	Wide range of anaerobic bacteria	Hydrogenase	Substrates: wide range of organics including wastes; highest rates of the process	Low yield of the process (not more than 4 moles H_2 per 1 mol of glucose); organic acids, alcohols as by-products; high H_2 concentration inhibits the process
2	Dark aerobic or anaerobic	All chemotrophic diazotrophic bacteria during nitrogen fixation	Nitrogenase	All organic substrates available for aerobic or anaerobic decomposition by diazotrophic bacteria under nitrogen fixing conditions	Low efficiency of the bioconversion, especially under aerobic conditions
3	Dark anaerobic CO decomposition (water-shift reaction)	Some purple bacteria	Hydrogenase in conjunction with CO-dehydrogenase	The substrate: toxic gas which represents big part of syngas	High sensitivity to inactivation by oxygen; organic substrates and H_2 can inhibit the process

and acetate during facultative anaerobic pathway and ethanol, butyrate, butanol, acetone during strict anaerobic pathway).

Pyruvate, which is the key intermediate, is catabolized in two different ways. Facultative anaerobes degrade pyruvate to formate and acetyl-CoA through the action of pyruvate formate lyase (PFL). Strict anaerobes produce acetyl-CoA, CO_2, and reduced ferredoxin with the help of pyruvate ferredoxin oxidoreductase enzyme (PFOR) (Hallenbeck 2009). Thus, facultative anaerobes produce hydrogen from formate by the formate hydrogen lyase (*fhl)* complex which possesses an energy-converting Ni-Fe hydrogenase, a member of the Ech family of hydrogenases (Vignais and Billoud 2007; Vignais 2008) and in the other case, hydrogen production is derived by reduced ferredoxin by a Fe-Fe hydrogenase (Hallenbeck 2009; Hallenbeck and Ghosh 2009). Reducing ferredoxin can lead to additional hydrogen production by re-oxidizing NADH under very low hydrogen partial pressures which is not common in typical hydrogen fermentations. In both facultative and strict anaerobic hydrogen production pathways, some of the acetyl-CoA is used for the

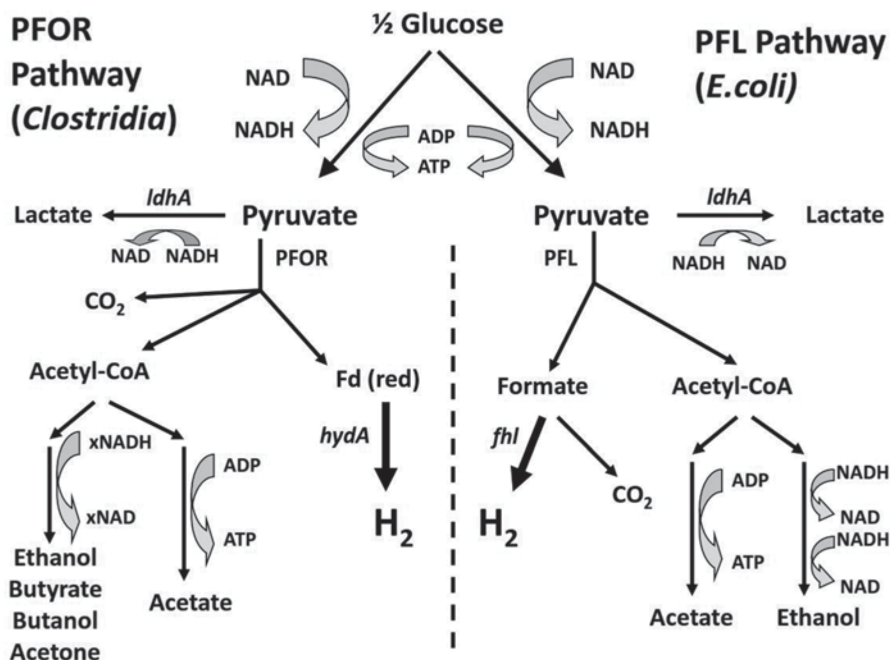


Fig. 11.1 Metabolic pathways in fermentative H_2 production. (Hallenbeck and Ghosh 2012)

production of reduced products and rest is required for ATP synthesis. Even though many organic compounds enable the production of hydrogen during dark fermentation, estimations of potential yields are mostly based on hexose conversions. The theoretical yield per mole of glucose is described as follows:

$$C_6H_{12}O_6 + 4H_2O \rightarrow 2CH_3COO^- + 2HCO_3 + 4H^+ + 4H_2 + \Delta G^\circ = -206\ \mathrm{kj\,mol^{-1}}$$

Theoretically, a maximum of 4 moles of H_2 per mole of glucose can be produced concurrently with the production of 206 kJ energy per mole of glucose in acetic acid fermentations. The remainder of the hydrogen in the hexose is conserved in the byproduct acetate, and under non ideal circumstances, more reduced products such as ethanol, lactate, or alanine (at high H_2 partial pressures). The production of more reduced organic acids and/or alcohols results in lower H_2 yield. For example, the conversion of 1 mol of glucose into butyrate is accompanied by the production of only 2 moles of H_2. Generally, a mixture of products is produced especially by *Clostridia* and the available H_2 from glucose is determined by the butyrate/acetate ratio. The complete oxidation of glucose to hydrogen and carbon dioxide yields 12 mol H_2 mol^{-1} of glucose without taking the metabolic energy needed. In practice, hydrogen yield is 2 mol H_2/mol glucose (2 mol H_2 mol^{-1} glucose only at $PH_2 < 0.1$ kPa) for both PFOR and PFL pathways. The overall yields in these metabolisms are relatively low. This is a natural consequence of the fact that

fermentations have been optimized by evolution to produce cell biomass and not hydrogen. Thus a portion of the substrate (pyruvate) is used in both cases to produce ATP giving a product "acetate" that is excreted. Also, in many organisms the actual yields of hydrogen are reduced by hydrogen recycling due to the presence of one or more uptake hydrogenases, which consume a portion of the hydrogen produced. It is unknown to what extent hydrogen production could be increased through metabolic engineering and manipulation of culture conditions.

3.1.1 Microbes Involved

Various kinds of microorganisms take part in the dark fermentative hydrogen generation. *Bacillus, Escherichia, Enterobacter, Ruminococcus, Citrobacter* and *Clostridia* are most common microbial genera capable of producing hydrogen via fermentation (Das 2009; Davila-Vazquez et al. 2008). Many anaerobes are capable of producing hydrogen from hexoses in acetic acid, butyric acid and acetone-butanol ethanol fermentations. *Clostridia* (*C. butyricum, C. welchii, C. pasteurianum, C. beijerincki*) and mixtures have been used in many studies dedicated to hydrogen production. The hyperthermophile *Pyrcoccus furious*, an archaebacterium, is also known to produce H_2, organic acids and CO_2 from carbohydrates (Fiala and Stetter 1986; Brown and Kelly 1989; Godfroy et al. 2000). There are other cellulotic thermophiles and extreme hyperthermophilic bacteria producing hydrogen such as *Anaerocellum, Caldicellulosiruptor, Clostridium, Dictyoglomus, Fervidobacterium, Spirocheta, Thermotoga and Thermoanaerobacter* (Schröder et al. 1994).

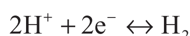
Rumen bacteria are other strict anaerobic bacteria, which are capable of H_2 production and other products such as acetate, ethanol, formate and CO_2 from carbohydrates. *Ruminococcus albus* is one of the most commonly known one (Innotti et al. 1973). Methanogens have hydrogenase which is usually involved in the oxidation of H_2 coupled to CH production and CO_2 reduction. On the other hand, it is well known that *Methanosarcina barkeri*, under the conditions of inhibition of CH_4 formation, is capable of carrying out so-called water-gas shift reaction (production of H_2 and CO_2 in stoichiometric amounts from CO and H_2O) (Bott et al. 1986). Strict anaerobes have no tolerance over oxygen and do not usually survive low oxygen concentrations, on the other hand, facultative anaerobes are resistant to oxygen. These bacteria are capable of rapidly consuming oxygen and restoring anaerobic conditions. Among these bacteria, *Enterobacter* and other members of *Enterobacteriaceae* are able to produce H_2 and are not inhibited by high H_2 pressures, but the H_2 yield on glucose is lower than that of *Clostridia* (Tanisho and Ishiwata 1994). Hydrogen production by *Escherichia coli* is mediated by the formate hydrogenlyase (*fhl*) complex as shown in Fig. 11.1. *E. coli* can perform a 'mixed-acid fermentation' in which glucose is metabolised to ethanol and various organic acids, including formate. This formate is further disproportionated to carbon dioxide and hydrogen by the formate hydrogenlyase (FHL) complex. Another facultative anaerobe, genus *Citrobacter* is considered under the family *Enterobacteriaceae*. They are gram-negative, non spore forming, facultative anaerobic and motile bacilli employing peritrichous flagella for locomotion and commonly utilizing citrate as their sole carbon source. *Citrobacter*

species, especially *C. freundii* have been shown to produce H_2 from CO and H_2O by the water-gas shift reaction under anaerobic conditions (Jung et al. 1999). Some aerobic bacteria like *Alcaligenes eutophus* and *Bacillus licheniformis* are known to produce H_2. *B. licheniformis* was reported to produce 0.5 mol H_2 per mole of glucose. Use of cell immobilization enhanced H_2 yield increasing it up to 1.5 mol $mole^{-1}$ glucose. *Alcaligenes eutophus*, which contains a soluble NAD-reducing hydrogenase, can grow heterotrophically on gluconate and fructose and produces H_2 when exposed to anaerobic conditions (Kalia et al. 1994; Kumar et al. 1995).

3.1.2 Enzymes Involved

Availability of a hydrogen-producing enzyme is the most crucial aspect of all biohydrogen processes. One should note that the catalytic activity of the various enzymes differs enormously and the quantity or inherent activity of these enzymes could limit the overall process. However, currently there is no evidence proving that the quantity of hydrogen-producing enzyme is the limiting factor in any known system. In contrast, potential catalytic activity far surpasses the amount of hydrogen produced which means that other metabolic factors might be limiting.

Hydrogen-producing enzymes catalyze the simplest chemical reaction:



$$2H^+ + 2e^- \leftrightarrow H_2$$

However, it is known that enzymes capable of hydrogen evolution contain complex metallo-clusters as active sites harboring Ni and Fe atoms. At present, three enzymes carrying out this reaction are known; nitrogenase, Fe-hydrogenase, and Ni-Fe hydrogenase. Like most metalloenyzmes, hydrogenases are quite sensitive to oxygen, high temperature and some other environmental factors. Protein matrix surrounding the metal centers allows hydrogenases to function properly, selectively and effectively (VoIbeda et al. 1995).

3.1.3 Feedstock for Dark Fermentative Hydrogen Production

There are two concerns regarding the feedstock that could be utilized i) the range of organic compounds and ii) quality of the feedstock. Carbohydrates are the preferred organic carbon source for H_2 production. Glucose or in principle its isomer hexoses or its polymers starch and cellulose, give maximum yield of $4H_2$ per glucose when acetic acid is the by-product

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COO^- + 2CO_2 + 4H_2$$

On the other hand, half of this yield per glucose is obtained with butyrate as the fermentation end product as follows:

$$C_6H_{12}O_6 + 2H_2O \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2$$

Table 11.3 Examples for various substrates used for Bio-H_2 production and H_2 yield

Substrate	Microorganism	H_2 yield mol H_2 mol^{-1} glucose equiv
Glucose	Mixed *Clostridium* sp.	1.43
Glucose	Acclimatized sludge	1.66
Glucose	Heat-treated sewage sludge	0.96
Sucrose	Mixed, undefined community	1.0–1.9
Pulped sugar beet	Mixed, undefined community	0.9–1.7
Soluble starch	Mixed, undefined community	2.14
Wheat starch	Mixed, undefined community	1.9
Mixed wastes	Heat-treated sludge compost	0.9–2.4
Mixed wastes	Heat-treated sludge compost wastewater	2.59
Glucose	*Clostridium butyricum*	1.4–2.3
Glucose	*Enerobacter aerogens* HU−101	1.17
Glucose	*Clostridium acidisoli*	1.0
Glucose	*Enerobacter cloacae IIT BT 08*	2.3
Glucose	*Enerobacter cloacae* DM 11	3.8
Glucose	*Caldicellulosiruptor saccharolyticus*	3.3–3.6
Sucrose	*Clostridium butyricum*	2.78
Molasses	*Enerobacter aerogens*	1.58
Cellobiose	*Clostridium thermocellum ATCC 27405*	1.5
Cellobiose	*Clostridium termitidis CT112*	0.5
Delignified wood fibers	*Clostridium thermocellum ATCC 27405*	1.6
α-cellulose	*Clostridium thermocellum ATCC 27405*	1.28–1.9
α-cellulose[a]	*Clostridium thermocellum ATCC 27405*	0.98–1.29
Dried distillers grains	*Clostridium thermocellum ATCC 27405*	1.27
Barley hulls	*Clostridium thermocellum ATCC 27405*	1.18–1.24
α-cellulose	*Clostridium termitidis CT112*	1.4

[a] Continuous fermentation over 3,000 h with a 24 h HRT

Table 11.3 summarizes a wide variety of organic materials employed for the hydrogen generation, using mixed, undefined microbial communities or defined pure cultures. Most common substrates used for biohydrogen production during dark fermentation studies include sugars, such as glucose, fructose, galactose and arabinose, sucrose, xylose, starch, and cellulose and various industrial waste streams that contain heterogeneous mixtures of sugars and starch (Das 2009; Davila et al. 2008).

It is obvious that the cost of raw material or substrate is crucial and plays a significant role for the overall economics of biohydrogen production, in view of this, finding inexpensive feedstock from agro-based wastes and industrial organic wastes is indispensable for the economical sustainability of biohydrogen production.

The majority of research has been directed towards expensive pure substrate or to a much lesser degree solid waste or wastewaters. However, more sustainable feedstock will be needed for a sustainable process. These could be achieved by sugar-containing crop such as sweet sorghum and sugar beet, starch based crops such as corn or wheat, or ligno-cellulosics such as fodder grass and *Miscanthus*. Ligno-cellulosic biomass is a complex of biopolymers that makes up the structural components of plant material. The approximate composition of lingo-cellulose found in most biomass feedstocks is roughly 45–60 % cellulose, 20–40 % hemicellulose, 25 % lignin, and 1–5 % pectin (Demain et al. 2005; Desvaux 2005; Lynd et al. 2005). Cellulose consists of linear, insoluble polymers consisting of up to 25,000 repeating β-1,4 linked β-D-glucopyranose units. Cellulose is a highly ordered molecule consisting of 15–45 crystalline microfibril chains, which in turn associate to form cellulose fibers. In nature, cellulose is found primarily in plant cell walls and is associated with varying degrees of other biopolymers, including: (i) hemi-cellulose, a random, amorphous hetero-polysaccharide composed of typically β-1,3 linked xylans, arabinoxylan, gluco-mannan, and galactomannan; (ii) lignin, a complex hydrophobic network of phenyl-propanoid units; (iii) pectins, composed of α-(1–4)-linked D-galacturonic acid; and (iv) proteins. Ligno-cellulosic biomass is renewable, inexpensive, constitutes a large fraction of waste biomass from municipal, agricultural, and forestry sectors, and thus offers excellent potential as a feedstock for renewable biofuels. Cellulose is, however, difficult to hydrolyze due to its crystalline structure. Current strategies that produce fuel ethanol from lingo-cellulosic biomass (or "second-generation" biofuels) use simultaneous saccharification and fermentation (SSF) or simultaneous saccharification and co-fermentation (SSCF). Both SSF and SSCF require extensive pre-treatment of the cellulosic feedstock by steam-explosion and/or acid treatment, followed by addition of exogenously produced cocktails of cellulolytic enzymes to hydrolyse cellulose chains and release the glucose monomers required for fermentation. These pre-treatments are costly, and some of the by-products generated, for example furfurals, can inhibit downstream processes. Steam-explosion of corn stover, with or without acid treatment, can be a suitable substrate for H_2 production. Hydrogen production from fermentable biomass has the advantage over ethanol production that the microflora is able to use a wider range of cellulosic substrates than the yeast.

Fermentative H_2 production using cellulose as the sole carbon source is under extensive investigation (Wang et al. 2008; Lo et al. 2008). The mesophilic, cellulolytic bacterium, *C. termitidis* strain CT1112 has displayed a cell generation time of 18.9 h when grown on 2 g L^{-1} α-cellulose (Ramachandran et al. 2008). The major soluble fermentation byproducts were acetate and ethanol. Maximum yields of acetate, ethanol, H_2, and formate on α-cellulose are 7.2, 3.1, 7.7 and 2.9 mmol L^{-1} culture, respectively. Although, the generation time was longer when cultured on α-cellulose than on the soluble cellulodextrin cellobiose, acetate and H_2 synthesis were favored over ethanol synthesis, indicating that carbon flow to ethanol and formate was restricted. During log phase, H_2 was produced at a specific rate of 2.79 mmoles h^{-1} g^{-1} dry weight- of cells on α-cellulose.

The thermophilic, cellulolytic bacterium *Clostridium thermocellum* strain 27405 produced greater amounts of H_2 when cultured (in Balch tubes) on cellulosic

substrates compared with the soluble cellulodextran cellobiose, with an average yield of 1.6 mol H_2 mol^{-1} glucose equivalent (Islam et al. 2009). The major soluble fermentation byproducts include ethanol, acetate, and formate, with lactate being produced when the pH drops below 6.3. Hydrogen production by *C. thermocellum* 27405 was also investigated using dried distillers grain (DDGS), barley hulls (BH), or *Fusarium* head blight contaminated barley hulls (CBH) as the carbon source in batch fermentation experiments (Magnusson et al. 2008). Overall, DDGS produced the highest concentration of H_2 gas at 1.27 mmol H_2 mol^{-1} glucose equivalent, while CBH and BH produced 1.18 and 1.24 mmol H_2 mol^{-1} glucose equivalent, respectively.

Hydrogen production in a continuous pure culture of *C. thermocellum* 27405 was established in a 5 L working volume fermentor, and growth experiments were maintained for over 3,000 h (Magnusson et al. 2009), substrate concentrations varied from 1 to 4 g L^{-1} and the feed was introduced with continuous N_2 gas sparging to prevent clogging of the feed-line; pH and temperature of the reactor were maintained at 7.0 and 60 °C, respectively, throughout the study. At concentrations above 4 g L^{-1}, the delivery of α-cellulose was impaired due to feed-line clogging and it became difficult to maintain a homogenous suspension. At a dilution rate of 0.042 h^{-1} and substrate concentration of 4 g L^{-1}, the H_2 production rate was 5.06 mmol L^{-1} h^{-1}. Acetate and ethanol were the major soluble end-products, while lactate and formate were greatly reduced compared to production in batch cultures. Concentrations of all metabolites increased with increasing substrate concentration, with the exception of lactate. Despite a number of short-term electrical and mechanical failures during the testing period, the system recovered quickly, exhibiting substantial robustness. A carbon balance indicated near 100 % carbon recovery. This study shows that long-term, stable H_2 production can be achieved during direct fermentation of an insoluble cellulosic substrate under continuous culture conditions.

4 Light—Driven Biohydrogen Production

Light energy is essential to hydrogen evolution by photosynthetic cells. Table 11.4 compares various light driven bio-hydrogen processes including enzymes involved. Photoautotrophic green microalgae and cyanobacteria use sunlight and CO_2 as the sole sources for energy and carbon. The reducing power for cellular photosynthesis and/or biophotolysis comes from water oxidation under light irradiation.

4.1 Bio-Photolysis Based Hydrogen Production

Bio-photolysis is classified in to two groups; **direct** and **indirect**. Direct photolysis refers to sustained hydrogen evolution under light irradiation. The light energy is absorbed by the pigments at PSII, or PSI or both, which raises the energy level of electrons from water oxidation when they are transferred from PSII via PSI to ferrodoxin. A portion of the light energy is directly stored in hydrogen gas.

Table 11.4 Various light driven bio-hydrogen production methods

No	The process	Organisms	Key enzyme	Advantages	Disadvantages
1	Anaerobic photosynthetic under N deficiency	Nitrogen fixing anoxygenic photosynthetic bacteria	Nitrogenase	High rates, especially by purple non-sulfur bacteria, might be activated by near-infrared light; low sensitivity to high H_2 pressure	Sensitivity to N sources (due to repression and inactivation of nitrogenase); needs in simple organics as electron donor for photosynthesis; narrow range of organics; inactivation by oxygen; low efficiency (needs in electrons and ATP)
2	Anaerobic photosynthetic under over reduced conditions	Anoxygenic photosynthetic bacteria and cyanobacteria under anaerobic conditions with reversible hydrogenase (*hox YH*)	Hydrogenase	Relatively high efficiency (does not need ATP)	At present knowledge appears only during short-term experiments under over-reduced conditions; high H_2 concentration inhibits the process; under mesophilic conditions a possibility for pathogenic bacteria development
3	Aerobic photosynthetic	Nitrogen fixing cyanobacteria	Nitrogenase	Converts light energy into H_2 fuel in stoichiometry near to $2H_2O<->2H_2+O_2$; low sensitivity to high H_2 concentrations; temporal or spatial separation of O_2 and H_2 production	Low rates of the process; low efficiency (needs in electrons and ATP)
4	Temporal separation of oxygenic photosynthesis and light-dependent H_2 production under sulfur deprivation	Microalgae with Fe-Fe hydrogenase; proved for several species including *Chlamydomonas* and *Chlorella*	Hydrogenase	Converts light energy into H_2 fuel; long term process; possibility to repeat the cycle "oxygenic photosynthesis-light-dependent H_2 production"	Low rates; high sensitivity to oxygen inactivation; still need experimental studies to determine efficiency of process
5	Aerobic photosynthetic	Microalgae with Fe-Fe hydrogenase	Hydrogenase	Converts light energy into H_2 fuel in stoichiometry near to $2H_2O<->2H_2+O_2$; high efficiency; high rates of the process	Appears only during short-term experiments at the start of anaerobic cultures illumination; under S-deprivation shows decreased rates but for longer period. By-products under S-deprivation are organic acids and ethanol

Table 11.4 (continued)

No	The process	Organisms	Key enzyme	Advantages	Disadvantages
6	Temporal separation of oxygenic photosynthesis with accumulation of polysaccharides and dark fermentative H_2 production	Proved for some cyanobacteria with reversible hydrogenase (*hoxYH*) and marine microalgae	Hydrogenase	Converts light energy into H_2 fuel; long term process; possibility to repeat the cycle "oxygenic photosynthesis-dark fermentative H_2 production"	Low rates; high sensitivity to oxygen inactivation; organic acids (especially acetate) as by-products

In photosynthesis, the reduced carbon is stored as endogenous carbohydrates, such as starch in microalgae and glycogen in cyanobacteria. Studies on the mechanisms involved in hydrogen evolution have found that the electrons or reducing equivalents of hydrogenase and nitrogenase do not always come from water, but may sometimes originate from the intracellular energy reserve including carbohydrates. The stored energy is released through fermentation of the endogenous carbohydrates in dark conditions, and the excess reducing power can be deposited by hydrogenase on protons (H^+) forming molecular hydrogen. Hydrogen evolution from endogenous carbon reserve under dark anaerobic conditions looks very similar to the conventional anaerobic hydrogen fermentation, but the endogenous carbon reserve is made in vivo during photosynthesis. In this sense, the electrons or reducing equivalents in indirect bio-photolysis are derived from water by photoautotrophic cells. This indirect bio-photolysis, therefore, consists of two stages in series: photosynthesis for carbohydrate accumulation, and dark fermentation of the carbon reserve for hydrogen production. This way the oxygen and hydrogen evolutions are temporally and/or spatially separated. This separation not only avoids the incompatibility of oxygen and hydrogen evolution (e.g., enzyme deactivation and the explosive property of the gas mixture), but also makes hydrogen purification relatively easy because CO_2 can be conveniently removed from the H_2/CO_2 mixture.

In cells of certain green algae (e.g. *Chlamydomonas reinhardtii, Chlorella fusca*) and blue-green algae (cyanobacteria), hydrogen production occurs as a result of light-driven splitting of water during photosynthesis. In direct bio-photolysis, the photosynthetic apparatus captures light and the recovered energy is used to couple water splitting to the generation of a low-potential reductant, which can be used to reduce a hydrogenase enzyme. This is an inherently attractive process since solar energy is used to convert a readily available substrate, water, to oxygen and hydrogen:

$$2H_2O + \text{light energy} \rightarrow 2H_2 + O_2$$

This reaction was first demonstrated with a cell free chloroplast-ferredoxin-hydrogenase system, although the existence of such a reaction in green algae had been suggested earlier (Spruit 1958). Anaerobic conditions are indispensable for this process to occur. A stream of electrons and protons originating from water is generated upon the light energy with wavelength lower than 680 nm and absorbed by photosystem II (PSII). On the other hand, photosystem I (PSI) is induced with light wavelength lower than 700 nm which allows the transportation of electrons from PSII to PSI via chain of reductors called cytochrome bf. Electrons from PSI system are transferred via ferrodoxine to hydrogenase (algae or cyanobacteria) or nitrogenase (cyanobacteria) and these enzymes reduce protons to molecular hydrogen. In direct biophotolysis, neither CO_2 nor liquid metabolites are observed. The constant removal of oxygen is required since oxygen inhibits hydrogenase activity irreversibly (Das and Veziroglu 2008).

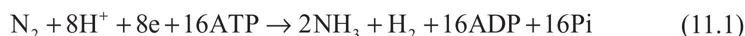
Photoautotrophic microorganisms, either prokaryotic cyanobacteria or eukaryotic green microalgae, possess chlorophyll *a* and other pigments to capture sunlight energy and use photosynthetic systems (PSII and PSI) to carry out oxygenic

photosynthesis. All oxygenic photosynthetic organisms extract electrons and protons from water and use them to reduce $NADP^+$ and plastoquinone for use as energy sources for metabolism such as the Calvin cycle (CO_2 fixation) and other pathways. However, oxygenic phototrophs, such as cyanobacteria and microalgae, can transiently produce H_2 under anaerobic conditions *via* proton reduction, catalyzed by a hydrogenase (or nitrogenase) in competition with other intracellular processes. In this case the electrons and protons ultimately produced by water oxidation are redirected at the level of ferredoxin/NADPH into hydrogenase (Kruse et al. 2005).

Hydrogen producing cyanobacteria may be either nitrogen-fixing or non-nitrogen-fixing. The examples of nitrogen-fixing, hydrogen producing cyanobacteria include non-marine *Anabaena* species, marine species of *Anabaena,* such as *Anabaena cylindrica, Anabaena variabilis, Anabaena variabilis* PK84, *Anabaena* AMC41, marine cyanobacteria in the genera *Calothrix, Oscillatori*a, *Gloebacter* PCC7421, and *Synechococcus* PCC602, and the marine species *Aphanocapsa montana.*

Some hydrogen producing species of *Synechococcus*, *Gloebacter,* and *Anabaena* are non-nitrogen-fixing and produce more hydrogen than nitrogen-fixing cyanobacteria. Heterocystous filamentous *Anabaena cylindrica* is a well-known hydrogen producing cyanobacterium, but *Anabaena variabilis* has received more attention in recent years, because of higher hydrogen yield (Liu et al. 2006).

Heterocysts provide an oxygen-free environment for the oxygen-sensitive nitrogenase enzyme that reduces molecular nitrogen into NH_3, and protons to H_2 (Eq. 11.1). In a N_2-containing atmosphere, nitrogen-fixation is the predominant reaction while H_2 is a minor byproduct. More H_2 can only be formed in the absence of molecular nitrogen according to the Eq. 11.2. The reducing power for H_2 evolution is derived from the energy-rich carbohydrate (CH_2O) stored in the heterocyst or transferred from neighbor cells. Because of the high energy demand (4 ATP per H_2), the energy conversion efficiency from light to H_2 by nitrogenase is quite low (<1 %) (Yoon et al. 2006).



$$N_2 + 8H^+ + 8e + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi \quad (11.1)$$

$$8H^+ + 8e + 16ATP \rightarrow 4H_2 + 16ADP + 16Pi \quad (11.2)$$

The maximum specific H_2 evolution rate per gram of cell mass or chlorophyll *a* (the pigment content accounts for 2–3 % of cell mass) for the representative nitrogen-fixing cyanobacteria varies between 0.21–3.06 mmol g^{-1} h^{-1}. Volumetric productivity of H_2, on the other hand, has been reported to be between 0.084–0.93 mmol H_2 L^{-1} h^{-1}. Surface area is one of the major cost factors for photobioreactors. Hydrogen production is also compared in terms of energy productivity, a general performance parameter for energy generation based on the energy output per volume per time. The energy productivity is calculated by multiplying the volumetric productivity (mmol H_2 L^{-1} h^{-1}) by the heat of combustion of hydrogen at 25 °C (ΔHc, $H_2 = -0.24$ kJ $mmol^{-1}$) (Weissmann and Benemann 1977).

Biological H_2 production is often conducted in sequential mode under different headspace conditions (argon is preferred during the hydrogen production stage): the

first stage for cell growth, followed by the second stage for H_2 evolution. The head-space gas component could be a significant cost factor in large-scale hydrogen production. A N_2-free gas phase is needed, and argon plus CO_2 is preferred, for higher H_2 evolution rate, since nitrogenase uses the reducing power for nitrogen reduction, rather than H_2 evolution when nitrogen is present in headspace. *A. variabilis* PK84, for instance, was reported to produce almost 15 times more H_2 in CO_2-enriched argon than in CO_2-enriched air. Therefore, a metabolic stress in the form of nitrogen starvation is often required at the end of the growth period to induce the activity of nitrogenase (Fernando et al. 2002). Light intensity also influences H_2 production by cyanobacteria. The most commonly applied light intensity varies between 20 and 100 w m^{-2} (Fernando et al. 2002).

It is clear that there is a need for a significant technological breakthrough for H_2 productivity by nitrogen-fixing cyanobacteria. Progress up to a certain extent has been made with the genetic engineering of nitrogen-fixing cyanobacteria. In wild type strains, uptake of hydrogenase enzymes to reoxidize H_2 to protons takes place, thus reducing H_2 evolution. A genetically modified *A. variabilis* PK84, in which the uptake of hydrogenase was mutated, displayed a four-fold greater (3.1 mmol g^{-1} dry wt h^{-1}) H_2 production rate compared with the wild type strain (Masukawa et al. 2002). There are reports in literature indicating that energy conversion efficiency of photosynthetically active radiation (PAR) could also be increased from 0.005 % of a wild type to above 1 % for strains with impaired uptake hydrogenases (Masukawa et al. 2002).

It is known that unicellular, non-nitrogen-fixing cyanobacteria, such as *Gloebacter* PCC7421, *Synechococcus* PCC602, and *Aphanocapsa montana* are also able to produce H_2. Hydrogen evolution values of these cyanobacteria are lower than the heterocystous nitrogen-fixing strains, and there is a need for a highly reducing gas to protect the hydrogenase from oxygen (O_2) inhibition.

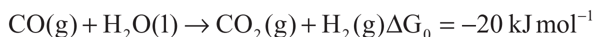
Hydrogen production is also possible with green microalgae. Among the green algal species studied (*Scenedesmus obliquus*, *Chlorococcum littorale* and *Platymonas subcordiformis*), *Chlorella vulgaris*, *Chlorococcum humicolum* and *Chlamydomonas reinhardtii* are among the best-known H_2 producing algae. The main problem with algal H_2 production is O_2 inhibition. Various methods to overcome the inhibitive effect of oxygen on hydrogenase have been investigated, with limited success including spatial separation of O_2 from H_2 production, oxygen scavenging, and gas purging. Two fundamental approaches were developed by Ghirardi et al. (2000) and Melis et al. (2000). One involves the temporal separation of the usually incompatible reactions of O_2 and H_2 production in green algae, and the second involves the use of classical genetics to increase the O_2 tolerance of the reversible hydrogenase enzyme (Ghirardi et al. 2000; Melis et al. 2000).

Algal H_2 production could be an ecologically acceptable process, since the raw material, water, comes from a sustainable and renewable source, and CO_2 consumption during the process provides advantages with respect to reduction of greenhouse gases, specifically CO_2. However, low H_2 evolution and strong inhibition due to the O_2 on hydrogenase enzyme are major limitations for algal H_2 production (Greenbaum 1982). Furthermore, algae are not able to utilize organic waste materials.

Therefore, other biohydrogen methods such as anaerobic dark and photo-fermentations are more advantageous, since simultaneous waste utilization and H_2 evolution are possible (Hallenbeck and Benemann 2002).

4.2 Water-Gas Shift Reaction

Photo-heterotrophic bacteria are also able to produce hydrogen via water-gas shift reaction. During this reaction H_2 is released while carbon monoxide (CO) is oxidized to carbon dioxide (CO_2) in the presence of anaerobic bacteria as follows:



$$CO(g) + H_2O(1) \rightarrow CO_2(g) + H_2(g) \Delta G_0 = -20\ \text{kJ mol}^{-1}$$

Both the gram-negative organisms, photo-heterotrophic bacteria (*Rhodospirillum rubrum* and *Rubrivax gelatinosus*) and gram-positive bacteria (*Carboxydothermus hydrogenoformans*) are able to utilize this reaction. Under anaerobic conditions, CO induces the synthesis of several proteins, including CO dehydrogenase, Fe-S protein, and CO-tolerant hydrogenase. Electrons produced from CO oxidation are conveyed via the Fe-S protein to the hydrogenase for hydrogen production.

4.3 Photo-Fermentation

Much work has been done on the capacity of photosynthetic bacteria to produce significant amounts of H_2. Nitrogen deficient conditions using light energy and reduced compounds are required for the photosynthetic bacteria to evolve molecular H_2 catalyzed by nitrogenase (Fig. 11.2). The overall reaction of hydrogen production is given as follows:

$$C_6H_{12}O_6 + 6H_2O + \text{Light energy} \rightarrow 12H_2 + 6CO_2$$

The main advantage of this reaction over biophotolysis is the fact that the lack of PSII in this organism automatically eliminates the O_2 inhibition of H_2 production. Furthermore, the ability of these organisms to utilize wide variety of organic compounds for hydrogen production is another additional advantage. Some of the photo-heterotrophic bacteria capable of hydrogen production under anaerobic conditions in the presence of light are *Rhodobacter spheroides*, *R. capsulatus*, *Rhodovulum sulfidophilum* W-1S and *Rhodopseudomonas palustris*. The highest conversion efficiency reported in the literature has been obtained when lactic acid was used as the sole carbon source. *Rhodospirillum rubrum* and *Rhodopseudomonas palustris* P4, which express a CO-dependent dehydrogenase (CODH) enzyme, have also been reported to be able to produce H_2 from CO or other organic acids (Hallenbeck and Benemann 2002). The optimum growth for the photosynthetic bacteria is reported to be in the range of 30–35 °C and at pH 7.0. Hydrogen production by these bacteria

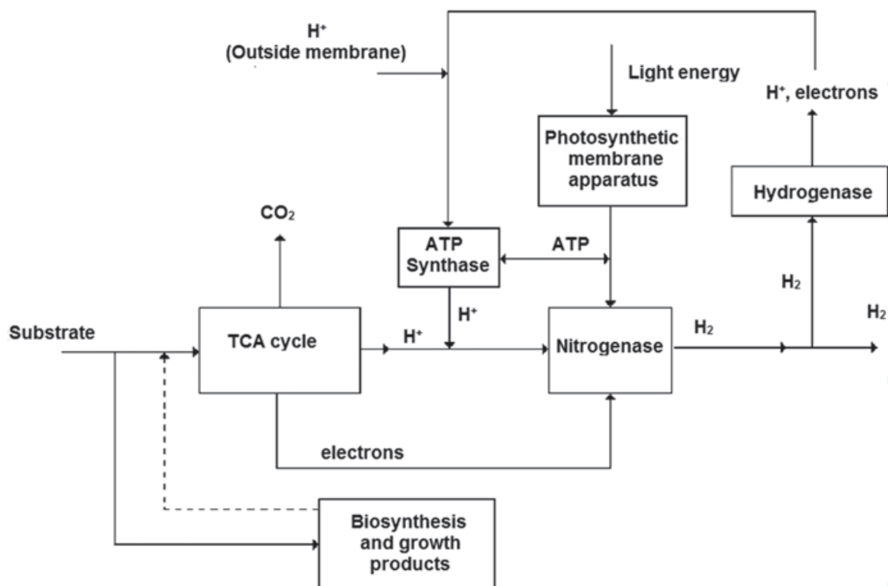


Fig. 11.2 Hydrogen generation via photofermentation. (Koku et al. 2002)

requires anaerobic conditions under illumination (Bolton 1996). Even though these organisms prefer organic acids as carbon source, other industrial effluents are amenable to H_2 production (Koku et al. 2002).

H_2 production rates by photo-heterotrophic bacteria are mainly affected by the factors such as light intensity, carbon source, enzymes involved and the type of microbial culture. Nitrogenase, however, is the key enzyme catalyzing H_2 production by these bacteria. The presence of oxygen, ammonia and high N/C ratios inhibit the activity of nitrogenase. For example, H_2 production by *R. sphaeroides* is completely inhibited at ammonia concentrations above 2 mM (Koku et al. 2002). Under the presence of high nitrogen, metabolic pathway shifts from hydrogen production to the utilization of organic substrates for cell growth which in return prevents the light penetration. For this reason, ammonium concentration in the reactor has to be limited and oxygen should be eliminated. For nitrogen source proteins such as albumin, glutamate, and yeast extract are generally preferred. Uptake of hydrogenase enzymes in photo-fermentative bacteria, oxidizes H_2 and are antagonistic to nitrogenase activity, therefore uptake hydrogenase activity should be eliminated for enhanced H_2 production. Two to three times more H_2 production has been achieved by using hydrogenase deficient mutant cultures of photo-fermentative bacteria (Kars et al. 2008).

Light intensity is another important parameter affecting the performance of photo-fermentations. Increasing light intensity has a stimulatory affect on H_2 yields and production rates, but has an adverse effect on the light conversion efficiencies. It was reported that the reduced antenna mutant of *R. sphaeroides* MTP4 produces

H_2 more efficiently under high light intensity as compared to the wild type strain. Reduced antenna mutants have been studied for many biotechnological applications, since the benefits deriving from a reduced absorption of light may affect a number of physiological pathways, in different microorganisms. Torzillo et al. (2009) has reported that a great benefit can be derived from the mutants of green algae. Light intensity also affects the consumption rates of organic acids. For example, butyrate consumption requires higher light intensities (4,000 lx) as compared to acetate and propionate. Exposure time to light also affects H_2 production. Alternating 14 h light/10 h dark cycles yielded slightly higher H_2 production rates and cell concentrations compared to continuous illumination. More frequent exposure to dark/light cycle has a better effect on H_2 production (Kapdan and Kargi 2006).

Industrial effluents that do not cause any inhibition on light penetration (colored wastewater) are also amenable for H_2 production by photosynthetic organisms. Ammonia content of industrial effluents may also inhibit the nitrogenase enzyme and reduce the H_2 productivity. Therefore, pretreatment to remove ammonia and toxic compounds (heavy metals, phenols, etc.) and dilution of high organic matter content (COD) in industrial effluents may be required before using such industrial wastewater during biohydrogen production.

An extensive summary of H_2 production studies from some food industry wastewaters has been given by Kapdan and Kargi (2006). Glucose, sucrose, starch, wheat starch, lactose, food waste, potato processing waste, apple, domestic sludge, molasses, rice winery, biosolids, filtrate, sweet potato starch residues, and organic fraction of municipal solid wastes have been used as substrates for H_2 production. Tofu wastewater, which is a carbohydrate and protein rich effluent, has also been used for H_2 production. Hydrogen yield from tofu wastewater (1.9 L H_2 L^{-1} wastewater at 30 °C) has been reported to be comparable to H_2 yield from glucose (3.6 L H_2 L^{-1} wastewater) using *R. sphaeroides* RV immobilized in agar gel (Zhu et al. 1999). No ammonia inhibition (2 mM) was observed and 41 % of total organic carbon (TOC) was removed. Similarly, the dilution of the wastewater at a ratio of 50 % resulted in an increase in H_2 yield of up to 4.32 L H_2 L^{-1} wastewater and 66 % TOC removal (Zhu et al. 1999).

Other agro-based waste materials such as potato starch, sugar cane, juice and whey have also been investigated in terms of H_2 production using *Rhodopseudomonas* sp. Sugar cane juice yielded the maximum level of H_2 production (45 mL [mg DW h^{-1} basis]) as compared to potato waste (30 mL [mg DW h^{-1} basis]) and whey (25 mL [mg DW h^{-1} basis]). There was no H_2 production by the photosynthetic bacterium, *Rhodobium marinum* using raw starch as the substrate (Singh et al. 1994).

Use of photo-bioreactors is also critical factor for efficient photobiological H_2 production. Most common photo-bioreactors configurations used for H_2 production in literature are tubular, flat panel, and bubble column reactors. One of the attempts to increase volumetric hydrogen production in suspended cell bioreactor systems is to keep high biomass inventory in the reactor, but it fails due to the exponential decay of light intensity with increasing density of the cell culture. Therefore, suspension layer thicknesses of 1–5 cm have to be provided in photo-bioreactors. Another attempt to provide higher biomass inventory in the reactor is by cell immobilization

on light transmitting matrices with high surface area/volume ratios. In this case, up to 12 g of cells (dry weight) can be immobilized in 1 L of matrix. Therewith, the rates of H_2 evolution per unit volume increase considerably. Thus, *R. sphaeroides* immobilized on a porous glass steadily evolves H_2 at a rate of 1.1 L L^{-1} h^{-1} for more than 1,000 h. The maximum volumetric H_2 rate attained is 3.8 L L^{-1} h^{-1}, with an 80% conversion of the organic acid substrate (Tsygankov et al. 1998). Use of mutant photosynthetic bacteria has also been considered by many researchers to enhance the light conversion efficiency, and hence H_2 production rate. Although an improvement has been observed by mutant type, the light conversion efficiency was around 6% (El-Shishtawy et al. 1997), which is still less than theoretical efficiency. The light penetration length is important for the hydrogen productivity. In relation to solar energy driven H_2 production, the light conversion efficiency has been reported to be less during mid-day because of high light intensity (1.0 kW m^{-2}). In addition, a delay of 2–4 h has been observed in maximum hydrogen production rate (3.4 L H_2 (m^2 h^{-1}) after the highest light intensity at noon with an average light conversion efficiency of 1.4% (El-Shishtawy et al. 1997). A 3.5% light conversion efficiency with an over 0.8 kW m^{-2} light intensity at midday has been obtained using a photo-bioreactor system with light shade bands, whereas photo-inhibition has been observed at 0.4 kW m^{-2} in photo-bioreactors without shade bands Miyake et al. 1999).

Mixing of reactor content is the other important factor affecting H_2 production. Some literature reports suggest gas injection using argon gas for mixing, although not cost-effective. On the other hand, it is also known that continuous argon sparging may inhibit the growth of *Rhodopseudomonas* in a pneumatically agitated photo-bioreactor. Re-circulation of reactor content was also a choice for mixing which provides better growth of the culture. A novel flat-panel airlift photo-bioreactor with baffles has provided a significant increase in the biomass productivity and therefore it could also be used for H_2 production (Wakayama et al. 2000).

Multi-tubular photo-bioreactors made up of parallel transparent tubes are another preferred reactor configuration, generally used for the cultivation of *Spirulina*. The system is inclined with a 10–30% slope to allow gas bubbles to rise. The hydrogen production rate from lactate using a modified tubular reactor reaches 2 L m^{-2} h^{-1} with light conversion efficiency of 2% in outdoor experiments (Modigell and Holle 1998).

4.4 Biohydrogen Production by Hybrid Systems

Although the theoretical maximum yield of H_2 from a single dark fermentation reaction is limited to 4 mol H_2 mol^{-1} glucose, yields higher than 4 mol H_2 mol^{-1} glucose can be achieved through hybrid systems. Hybrid hydrogen production system is composed of two sequential reactors in which dark fermentation is carried out in the first reactor and then a second photosynthetic reactor is integrated where hydrogen atoms sequestered in low molecular weight VFAs are converted to H_2 via photosynthetic organisms. This is required since hydrogen production is possible up to a certain extent in dark fermentation and significant amount of H_2 is sequestered

in the form of volatile fatty acids (VFAs) such as acetic acid, butyric acid, propionic acid etc. This type of hydrogen production system strategy includes dark fermentation followed by photo-fermentation or dark fermentation followed by a microbial electrohydrolysis cell (MEC), which is also referred to as 'electrohydrogenesis'. Thermodynamic constraints limit the release of the hydrogen atoms bound up in fermentation end-products by dark fermentation, so integration of dark fermentation with photosynthetic bacteria is needed for the maximization of H_2 yield.

The combined use of anaerobic bacteria and purple non-sulfur photosynthetic bacteria for efficient conversion of wastewater into H_2 using effluents from three different carbohydrate-fed reactors (CSTR, ASBR, and UASB) has been reported by Lee et al. (2002). The authors report that CSTR effluent is the most suitable for photohydrogen production. Azbar and Dokgoz (2010) have reported the use of a two-stage reactor to maximize the H_2 yield from cheese whey wastewater. For this purpose, effluent from a thermophilic anaerobic digester fed with cheese whey has been used in photo-fermentation reactors using *Rhodopseudomonas palustris* strain DSM 127. In this study, overall H_2 production yield (for dark + photo fermentation) has been found to vary between 2 and 10 mol H_2 mol^{-1} lactose. It is suggested that cheese whey effluent with a co-substrate containing L-malic acid, such as apple juice processing effluents could provide successful hydrogen production.

A hybrid hydrogen production system employing dark-fermentation process followed by a photo-fermentation process has been used by Lo et al. (2008) for hydrogen production from acid-hydrolyzed wheat starch. The effluent from dark fermentation reactor in which hydrolyzed starch was continuously converted to H_2 by *Clostridium butyricum* CGS2, was fed into photo H_2 production process inoculated with *Rhodopseudomonas palustris* WP3–5 (ToC = 35 °C, pH 7.0, light 100 W m^{-2} irradiation). Combining enzymatic hydrolysis, dark fermentation and photo fermentation has led to a marked improvement of overall H_2 yield, up to 16.1 mmol H_2 g^{-1} COD or 3.09 mol H_2 mol^{-1} glucose, and COD removal efficiency (ca. 54.3 %), suggesting the potential of using the proposed integrated process for efficient and high-yield bio-H_2 production from starch feedstock. Similar experiments have been conducted using *Enterobacter cloacae* DM11 in the first stage, followed by photo-fermentation by *Rhodobacter sphaeroides* strain OU001 (Nath et al. 2008). The yield of H_2 in the first stage has been approx. 3.3 mol H_2 mol^{-1} glucose (approx. 82 % of theoretical), while the yield of H_2 in the second stage is between 1.5–1.7 mol H_2 mol^{-1} acetic acid (37–43 % of theoretical). The combined yield of H_2 in the two-stage process is 4.8–5.0 mol H_2 mol^{-1} substrate, significantly higher than the 3.3 mol H_2 mol^{-1} glucose obtained in the dark fermentation alone.

5 Conclusions

H_2 fuel is clearly a promising solution for energy security as a sustainable alternative energy carrier and also a reliable choice against climate change. Biotechnology seems to provide much more environmentally friendly alternative H_2 production in

comparison to the conventional hydrogen production methods (thermo-chemical and electro-chemical), which are dependent on fossil fuels or highly energy intensive. Numerous reports show that each biohydrogen process has its advantages and disadvantages in terms of technology and productivity. In order to compete with aforementioned conventional hydrogen production methods, there is a need for intensive work on both enhancement of biohydrogen yield and energy efficiencies of the respective processes. It is particularly imperative to address several techno-economic challenges for cost-effective production as well as commercial application of biohydrogen.

References

Azbar N, Dokgoz F (2010) The effect of dilution and L-malic acid addition on biohydrogen production with *Rhodopseudomonas palustris* from effluent of an acidogenic anaerobic reactor. Int J Hydrog Energy 35:5028–5033

Bolton JR (1996) Solar photoproduction of hydrogen. Sol Energy 57:37–50

Bott M, Eikmanns B, Thauer RK (1986) Coupling of carbon monooxide to carbon dioxide and hydrogen with the phosphorylation of ADP in acetate grown *Methanosarcina barkeri*. Eur J Biochem 159:393–398

Brown SH, Kelly RM (1989) Cultivation techniques for hyperthermophilic archaebacteria: continuous culture of *Pyrococcus furiosus* at temperatures near 100 °C. Appl Environ Microbiol 55:2086–2088

Das D (2009) Advances in biohydrogen production processes: an approach towards commercialization. Int J Hydrog Energy 34:7349–7357

Das D, Veziroglu TN (2008) Advances in biological hydrogen production processes. Int J Hydrog Energy 33:6046–6057

Davila-Vazquez G, Arriaga S, Alatriste-Mondragón F, de León-Rodríguez A, Rosales-Colunga LM, Razo-Flores E (2008) Fermentative biohydrogen production: trends and perspectives. Rev Environ Sci Biotechnol 7:27–45

Demain AL, Newcomb M, Wu JHD (2005) Cellulase, *Clostridia*, and ethanol. Microbiol Mol Biol Rev 69:124–154

Desvaux M (2005) *Clostridium cellulolyticum*: model organism of mesophilic cellulolytic *Clostridia*. FEMS Microbiol Rev 29:741–764

El-Shishtawy RMA, Kawasaki S, Morimoto M (1997) Biological H_2 production using a novel light-induced and diffused photobioreactor. Biotechnol Tech 11:403–407

Fernando A, Pinto F, Troshina O, Lindblad P (2002) A brief look at three decades of research on cyanobacterial hydrogen evolution. Int J Hydrog Energy 27:1209–1215

Fiala G, Stetter KO (1986) *Pyrococcus furiosus* sp. nov. represents a novel genus of marine heterotrophic archaebacteria growing optimally at 100 °C. Arch Microbiol 145:56–61

Ghirardi LM, Zhang L, Lee JW, Flynn T, Seibert M, Greenbaum E, Melis A (2000) Microalgae: a green source of renewable H_2. Trends Biotechnol 18(12):506–511

Godfroy A, Raven NDH, Sharp RJ (2000) Physiology and continuous culture of the hyperthermophilic deep-sea vent archaeon *Pyrococcus abyssi* ST549. FEMS Microbiol Lett 186:127–132

Greenbaum E (1982) Photosynthetic hydrogen and oxygen production: kinetic studies. Science 196:879–880

Hallenbeck PC (2009) Fermentative hydrogen production: principles, progress and prognosis. Int J Hydrog Energy 34:7379–7389

Hallenbeck PC, Benemann JR (2002) Biological hydrogen production; fundamentals and limiting processes. Int J Hydrog Energy 27:1185–1193

Hallenbeck PC, Ghosh D (2009) Advances in fermentative biohydrogen production: the way forward? Trends Biotechnol 27:287–297

Hallenbeck PC, Ghosh D (2012) Improvements in fermentative biological hydrogen production through metabolic engineering. J Environ Manage 95:S360–S364

Hawkes FR, Hussy I, Kyazze G, Dinsdale R, Hawkes DL (2007) Continuous dark fermentative hydrogen production by mesophilic microflora: principles and progress. Int J Hydrog Energy 32:172–184

Innotti EL, Kafkawitz D, Wolin MJ, Bryant MP (1973) Glucose fermentation products of *Ruminococcus albus* grown in continuous culture with *Vibrio succinogenes*: changes caused by interspecies transfer of H_2. J Bacteriol 114:1231–1240

Islam R, Cicek N, Sparling R, Levin DB (2009) Influence of initial cellulose concentration on the carbon flow distribution during batch fermentation by *Clostridium thermocellum* ATCC 27405. Appl Microbiol Biotechnol 82:141–148

Jung GY, Kim JR, Jung HO, Park J-Y, Park S (1999) A new chemo-heterotrophic bacterium catalyzing water-gas shift reactions. Biotechnol Lett 21:869–873

Kalia VC, Jain SR, Kumar A, Joshi AP (1994) Fermentation of bio-waste to H_2 by *Bacillus licheniformis*. World J Microbiol Biotechnol 10:224–227

Kapdan IK, Kargi F (2006) Biohydrogen production from waste materials. Enzyme Microb Technol 38:569–582

Kars G, Gündüz U, Rakhely G, Yücel M, Eroglu I, Kovacs KL (2008) Improved hydrogen production by uptake hydrogenase deficient mutant strain of *Rhodobacter sphaeroides* O.U.001. Int J Hydrog Energy 33(12):3056–3060

Koku H, Eroglu I, Gündüz U, Yücel M, Türker L (2002) Aspects of the metabolism of hydrogen production by *Rhodobacter sphaeroides*. Int J Hydrog Energy 27:1315–1325

Kraemer JT, Bagley DM (2007) Improving the yield from fermentative hydrogen production. Biotechnol Lett 29:685–695

Kruse O, Rupprecht J, Mussgnug JH, Dismukes GC, Hankamer B (2005) Photosynthesis: a blueprint for energy capture and conversion technologies. Photochem Photobiol Sci 4:957–970

Kumar A, Jain SR, Sharma CB, Joshi AP, Kalia VC (1995) Increased H_2 production by immobilized microorganisms. World J Microbiol Biotechnol 11:156–159

Lee CM, Chen PC, Wang CC, Tung YC (2002) Photohydrogen production using purple nonsulfur bacteria with hydrogen fermentation reactor effluent. Int J Hydrog Energy 27:1309–1313

Levin D, Azbar N (2012). Introduction: biohydrogen in perspective. State of the art and progress in production of biohdyrogen, pp 3–7. Bentham eBooks, USA (eISBN: 978-1-60805-224-0, ISBN: 978-1-60805-411-4)

Levin DB, Pitt L, Love M (2004) Biohydrogen production: prospects and limitations to practical application. Int J Hydrog Energy 29:173–185

Liu J, Bukatin VE, Tsygankov AA (2006) Light energy conversion into H_2 by *Anabaena variabilis* mutant PK84 dense cultures exposed to nitrogen limitations. Int J Hydrog Energy 31:1591–1596

Lo Y-C, Chen S-D, Chen C-Y, Huang T-I, Lin C-Y, Chang J-S (2008) Combining enzymatic hydrolysis and dark-photo fermentation processes for hydrogen production from starch feedstock: a feasibility study. Int J Hydrog Energy 33:5224–5233

Lynd LR, Van Zyl WH, McBride JE, Laser M (2005) Consolidated bio-processing of cellulosic biomass: an update. Curr Opin Biotechnol 16:577–583

Magnusson L, Islam R, Sparling R, Levin D, Cicek N (2008) Direct hydrogen production from cellulosic waste materials with a single-step dark fermentation process. Int J Hydrog Energy 33:5398–5403

Magnusson L, Cicek N, Sparling R, Levin DB (2009) Continuous hydrogen production during fermentation of α-cellulose by the thermophilic bacterium *Clostridium thermocellum*. Biotechnol Bioeng 102:759–766

Masukawa H, Mochimaru M, Sakurai H (2002) Hydrogenases and photobiological hydrogen production utilizing nitrogenase system in cyanobacteria. Int J Hydrog Energy 27:1471–1474

Melis A, Zhang L, Forestier M, Ghirardi ML, Seibert M (2000) Sustained photobiological hydrogen gas production upon reversible inactivation of oxygen evolution in the green alga *Chlamydomonas reinhardtii*. Plant Physiol 122:127–136
Miyake J, Wakayama T, Schnackenberg JS, Arai T, Asada Y (1999) Simulation of daily sunlight illumination pattern for bacterial photo-hydrogen production. J Biosci Bioeng 88:659–663
Modigell M, Holle N (1998) Reactor development for a biosolar hydrogen production process. Renew Energy 14:421–426
Nath K, Muthukumar M, Kumar A, Das D (2008) Kinetics of two-stage fermentation process for the production of hydrogen. Int J Hydrog Energy 33:1195–1203
Panagiotopoulos IA (2010) Prospects of utilization of sugar beet carbohydrates for biological hydrogen production in the EU. J Clean Prod 18:S9–S14
Ramachandran U, Wrana N, Cicek N, Sparling R, Levin DB (2008) Hydrogen production and end-product synthesis patterns by *Clostridium termitidis* strain CT1112 in batch fermentation cultures with cellobiose or alpha-cellulose. Int J Hydrog Energy 33:7006–7012
Schröder C, Selig M, Schönheit P (1994) Glucose fermentation to acetate, CO_2 and H_2 in the anaerobic hyperthermophilic eubacterium *Thermotoga maritima*: involvement of the Embden-Meyerhof pathway. Arch Microbiol 161:460–470
Singh SP, Srivastava SC, Pandey KD (1994) Hydrogen-production by *Rhodopseudomonas* at the expense of vegetable starch, sugar juice and whey. Int J Hydrog Energy 19:437–440
Spruit CJP (1958) Simultaneous photoproduction of hydrogen and oxygen by *Chlorella*. Meded Landbouwhogesch Wagening 58:1–17
Tanisho S, Ishiwata Y (1994) Continuous hydrogen production from molasses by the bacterium *Enterobacter aerogenes*. Int J Hydrog Energy 19:807–812
Torzillo G, Scoma A, Faraloni C, Ena A, Johanningmeier U (2009) Increased hydrogen photoproduction by means of a sulfur-deprived *Chlamydomonas reinhardtii* D1 protein mutant. Int J Hydrog Energy 34:4529–4536
Tsygankov AA, Fedorov AS, Talipova IV, Laurinavichene TV, Miyaki D, Gogotov IN (1998) Use of immobilized phototrophic microorganisms for waste water treatment and simultanenous production of hydrogen. Appl Biochem Microbiol 34:362–366
Vignais PM (2008) Hydrogenases and H^+-reduction in primary energy conservation. Results Probl Cell Differ 45:223–252
Vignais PM, Billoud B (2007) Occurrence, classification, and biological function of hydrogenases: an overview. Chem Rev 107:4206–4272
VoIbeda A, Charon M-H, Piras C, Hatchikian EC, Frey M, Fontecilla-Camps HC (1995) Crystal structure of the nickel-iron hydrogenase from *Desurfovibrio gigus*. Nature 373:580–587
Wakayama T, Nakada E, Asada Y, Miyake J (2000) Effect of light/dark cycle on bacterial hydrogen production by *Rhodobacter sphaeroides* RV. Appl Biochem Biotechnol 84–86:431–440
Wang A, Ren N, Shi Y, Lee D-J (2008) Bio-augmented hydrogen production from microcrystalline cellulose using co-culture-*Clostridium acetobutylicum* X9 and *Ethanoigenens harbinense* B49. Int J Hydrog Energy 33:912–917
Weissman JC, Benemann JR (1977) Hydrogen production by nitrogen-starved cultures of *Anabaena cylindrica*. Appl Environ Microbiol 33:123–131
Yoon JH, Shin JH, Kim MS, Sim SJ, Park TH (2006) Evaluation of conversion efficiency of light to hydrogen energy by *Anabaena variabilis*. Int J Hydrog Energy 31:721–727
Zeidan AA, van Niel EWJ (2009) Developing a thermophilic hydrogen-producing co-culture for efficient utilization of mixed sugars. Int J Hydrog Energy 34:4524–4528
Zhu H, Suzuki T, Tsygankov AA, Asada Y, Miyake J (1999) Hydrogen production from tofu wastewater by *Rhodobacter sphaeroides* immobilized agar gels. Int J Hydrog Energy 24:305–310