Chapter 2
Biohydrogen Production via Lignocellulose and Organic Waste Fermentation

Chen-Yeon Chu and Bing-Shun Huang

Abstract Hydrogen is a promising energy carrier and a replacement for fossil fuels, since it is clean and has high energy and its application does not contribute to the greenhouse effect. Renewable resources, such as lignocellulosic materials and organic wastes, in particular, dark fermentative hydrogen methods, as the feedstock for hydrogen production have great potential for supplying hydrogen.

The development of novel and effective cellulase enzymes, the optimization and improvement of cellulase systems, and engineering approaches for cellulose pretreatment and saccharification to produce biohydrogen have high interest in the scientific community. This chapter gives an introduction to the feedstocks (lignocellulosic materials and organic wastes), primary technologies (physical, chemical, physicochemical, and biological), process of feedstock pretreatments, microorganisms, fermenter types (continuous stirred tank reactors, upflow anaerobic sludge blanket, anaerobic biofilm and granule reactor, membrane bioreactor, etc.), and operational conditions (substrate concentrations, nutrients, pH, temperature, hydraulic retention time, etc.) for producing biohydrogen.

Pretreatment and saccharification are at the heart of producing biological hydrogen from lignocellulosic feedstocks. Efficient production of biohydrogen via lignocellulosis and organic waste depends largely on the fermenter type. Selection of the pretreatment system and fermenter type is the key for economic success of the biohydrogen production plant. This chapter also aims to develop a fundamental understanding of key technologies and variables during biohydrogen production from lignocellulosic raw materials and organic feedstock.

Keywords Biohydrogen • Lignocellulosic materials • Organic wastes • Pretreatment • Fermenter type

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2.1 Introduction to Feedstocks

Biofuel sources can be classified into first-, second-, and third-generation feedstocks. The first-generation biofuel feedstocks are traditionally food related such as corn for ethanol, vegetable oil, and animal fats. Second-generation biofuel feedstocks are of the nonfood crop type, wastewaters, lignocellulosic agriculture, and forest residues. However, one issue is that the energy crops required for the biofuel source can compete with land use for agriculture [1]. The key challenge for developing the next generation of biofuels is acquiring an economically viable feedstock. Feedstock costs contribute 80–90 % to the final fuel price for many processes and therefore they are important to the economics of biofuels [2].

The feedstock is made up of organic compounds such as carbohydrates that can be converted to biohydrogen by biological metabolism [3]. Biological hydrogen is produced via dark fermentation of organic wastes [4]. Fermentative hydrogen production is carried out by anaerobic bacteria that ferment organic compounds to volatile fatty acids (VFAs), alcohols, carbon dioxide, and hydrogen (Fig. 2.1). Many different substrates can be fermented to produce hydrogen [5]. Dark fermentation has advantages, for example, high rate of bacterial growth, low energy requirement, no oxygen limitation problems, low cost [6, 7], and biohydrogen production without light, and various carbon sources can be utilized as the substrate [8].

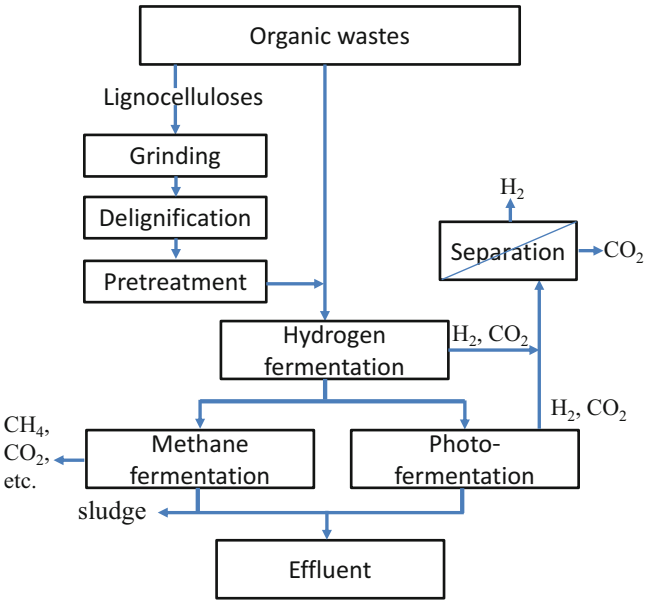

Fig. 2.1 Schematic diagram for biohydrogen production from organic wastes and agricultural wastes containing lignocelluloses

Waste materials that have been used as substrate for biohydrogen include palm oil mill effluent (POME) [6, 9–17], starch-based materials [18–28], food waste [29–33], and condensed molasses fermentation soluble (CMS) [34–38]. Sugary wastewater [39–42] is a more efficient source of carbohydrates than raw materials for biohydrogen production. Simple sugars, such as sucrose and glucose, can be converted at high temperatures into hydrogen at high conversion efficiencies [43]. Glucose, which is an easily biodegradable carbon source, is present in many industrial effluents and can be obtained abundantly from agricultural wastes [44]. Therefore, sugary wastewater can be considered to be most useful for industrial hydrogen production.

2.1.1 Organic Wastes

Various organic solid wastes or wastewaters have attracted considerable attention for biohydrogen production due to the advantages of high organic loading possibilities, low nutrient requirements, concurrent wastewater treatment, and positive net energy gain. Table 2.1 shows complex organic wastes that are considered as feedstock, such as kitchen, food processing, mixed, and municipal wastes for biohydrogen production. These organic wastes usually have high concentrations of protein and fat, making their hydrogen conversion efficiencies lower than that of the carbohydrate-based wastewaters. As a matter of fact, previous studies show that the hydrogen production prospective of carbohydrate-based wastes was higher than that of fat- and protein-based wastes by about 20 times [57]. This is partially due to the protein degradation that produces nitrogen that consumes the free hydrogen. In many kinds of wastes, the organic fraction of municipal solid waste (OFMSW) is considered to be quite favorable as a potential raw material for biohydrogen production, because it is able to represent up to 70 % of the total MSW produced, consisting of paper (up to 40 %), food wastes, garden residues, and wood [56]. It should be noted that the process will lead to an extra cost, because an initial selection/separation process would be necessary in order to obtain the suitable substrates. Starchy- and sugary-based biomass and wastes are readily fermented by microorganisms for hydrogen generation. Although lignocellulosic biomass is abundant in agricultural residues, it needs pretreatment to be useable.

2.1.2 Lignocelluloses

Lignocellulose is the most abundant renewable biomass in the world with an annual quantity of about 220 billion tons (dry weight) per year [58]. To use this feedstock, pretreatment is necessary to decompose the lignin structure and loosen the crystalline cellulose structure to promote enzyme accessibility. Pretreated lignocellulosic materials (e.g., sugarcane bagasse, corncob, wheat straw, cornstalks, grass, energy

Table 2.1 Fermentative hydrogen production from different types of waste and wastewaters

Type	Microorganism	Operation mode	H_2 production rate	Maximum H_2 yield	Ref.
Rice winery wastewater	Mixed culture	Continuous	380 mmol/g VSS/day 155.5 mmol/l·d	2.14 mol/mol hexoses	[42]
Food waste	Mixed thermophilic culture	Batch	11.8 mmol/g VSS/day	1.8 mol/mol hexoses	[31]
Food waste sewage sludge	Mixed mesophilic culture	Batch	109 mmol/g VSS/day	122.9 ml/g COD carbohydrate	[30]
Dairy wastewater	Mixed mesophilic culture	Continuous	1.59 mmol/l·d	–	[45]
Molasses	Mixed mesophilic culture	Continuous	8.1 mmol/l·d	–	[46]
Cheese whey	Mixed mesophilic culture	Batch	194 mmol/l·d	5.9 mol/mol lactose	[47]
Dairy wastewater	Mixed mesophilic culture	Batch	47.67 mmol/g VSS/day	17.2 mmol/g COD	[48]
Olive pulp	Mixed mesophilic culture	Continuous	10.6 mmol/l·d	0.19 mol/kg TS	[49]
Olive oil mill wastewater	Mixed mesophilic culture	Continuous	8.2 mmol/l·d	196.2 ml/g hexose	[50]
Coffee drink manufacturing wastewater	Mixed thermophilic culture	Continuous	4,153 mmol/l·d	2.57 mol H_2/mol hexoses	[51]
Sugar factory wastewater	Mixed mesophilic culture	Continuous	4.1 mmol·gML VSS/l·d	–	[52]
Starch wastewater	Mixed mesophilic culture	Sequencing batch	140.8 mmol/l·d	5.79 ± 0.41 mmol H_2/g COD_{added}	[53]
Textile wastewater	Mixed mesophilic culture	Intermittent-flow, stirred tank reactor (IFSTR)	408 mmol/l·d	0.97 mol H_2/mol hexoses	[54]
Enzymatic hydrolyzed food waste	Mixed mesophilic culture	Continuous mixed immobilized sludge reactor (CMISR)	346.7 mmol/l·d	–	[55]

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crops, oil palm trunk, and beer lees [59]) can be used for fermentative hydrogen production. Some studies have focused on biohydrogen and biomethane production from raw or pretreated solid wastes such as olive pulp, household solid waste, and potato waste [60–68]. The feedstock costs are high due to processing (shredding, densifying, pulverizing, and handling), collection, and transportation. Despite the challenges, these second-generation feedstock options are plentiful and produce great amounts of fuel. Lignocellulosic material basically consists of three different types of polymers, namely, cellulose, hemicellulose, and lignin. Agricultural and forestry residues are rich in carbohydrates and the cost of obtaining these residues is negligible. However, these do not contain readily accessible free sugars necessary for efficient fermentation. To convert cellulose, it is necessary to transform the carbohydrate polymers into fermentable sugars through the use of enzymes and change the structure of the cellulosic biomass. Therefore, biotransforming it into hydrogen is a difficult task in most cases. In Table 2.2 different types of residues used as feedstock for hydrogen production are presented, along with the achieved hydrogen yields and rates.

2.2 Pretreatment of Lignocellulosic Feedstock

Pretreatment is widely accepted to be an essential step for making lignocellulosic biomass accessible to enzymatic attack by breaking the lignin seal, removing hemicellulose, or disrupting the crystalline structure of cellulose [56]. An effective and economical pretreatment should meet the following requirements: (a) delignify feedstock for enzymatic attack, (b) avoid destruction of hemicelluloses and cellulose, (c) avoid formation of possible inhibitors, (d) minimize energy demand, (e) reduce cost for size reduction of feedstock, (f) reduce reactor costs, (g) produce low residues, and (h) decrease chemical costs [82]. Several methods have been introduced for pretreating lignocellulosic materials, namely, prior enzymatic hydrolysis or digestion. These methods are classified into physical, chemical, physicochemical, and biological pretreatments. The main principles of each pretreatment method are illustrated below.

2.2.1 Physical

The objective of the physical pretreatment is to reduce particle size, pore size, crystallinity of lignocellulosic material, and the degree of polymerization and increase the specific surface [83, 84]. Different types of physical processes such as milling (e.g., ball milling, two-roll milling, hammer milling, colloid milling, and vibro energy milling) and irradiation (e.g., by gamma rays, electron beam, or microwaves) can be used to improve the enzymatic hydrolysis or biodegradability of lignocellulosic waste materials [82].

Table 2.2 Fermentative hydrogen production from lignocellulosic residues

Lignocellulosic residue	Pretreatment	Microorganism	Operation mode	H_2 production rate (mmol /l·d)	Maximum H_2 yield (mol/mol consumption hexose)	Ref.
Rice straw hydrolysate	Concentrated acid 55 % + calcium hydroxide to remove the SO^{4-}	Mixed mesophilic cultures	Continuous	666.1	1.02	[4]
Rice straw hydrolysate	Concentrated acid 55 % + calcium hydroxide to remove the SO_4^{2-}	Mixed mesophilic cultures	Continuous	408.2	0.69	[69]
Wood fibers	Mechanical	*Clostridium thermocellum*	Batch	–	1.47	[70]
Corn stover	Steam explosion (90–220 °C, 3–5 min)	Mixed mesophilic cultures	Continuous	10.56	3	[71]
Sugarcane bagasse hydrolysate	Acid–thermal hydrolysis H_2SO_4 0.27–7(v/v), +121 °C, 60 min	*Clostridium butyricum*	Batch	–	1.73 mol/mol total sugar	[72]
Fodder maize juice	Mechanical	Mixed mesophilic cultures	Continuous	–	69.4 ml H_2/g dry mass	[73]
Sweet sorghum residues	Mechanical	*Ruminococcus albus*	Batch	–	2.59	[74]
Wheat straw	Mechanical	*Caldicellulosiruptor saccharolyticus*	Batch	–	3.8 (44.7 l/kg dry biomass)	[75]
Maize leaves	Mechanical	*Caldicellulosiruptor saccharolyticus*	Batch	–	3.6 (81.5 l/kg dry biomass)	[75]

Wheat straw	Pretreated with 7.4 % (w/w) $Ca(OH)_2$	Mixed mesophilic culture	Batch	–	58.78 ml/g-VS	[76]
Wheat straw	Pretreatment via steam explosion	*Clostridium* sp.	Batch	–	2.54 ± 0.2 mol H_2/mol reducing sugar	[77]
Bagasse	Alkali–thermal 0.2–4 g/l NaOH, 100 °C, 2 h	Mixed thermophilic cultures	Batch	0.28 mmol/h.g TVS	13.39 mmol H_2/g TVS	[78]
Corn stover	Acid–thermal hydrolysis H_2SO_4 0.25–4(v/v), +121 °C, 30–180 min	*Thermoanaerobacterium thermosaccharolyticum*	Batch	–	2.24	[79]
Disposable wooden chopsticks (DWC) waste	Alkaline pretreatment and enzymatic hydrolysis	Enriched hot spring culture	Batch	–	195 ml H_2/g total sugars consumed	[80]
Rice husk	Enzyme treated	*Clostridium beijerinckii*	Batch	9.7 mmol/l	2.93 mmol H_2/g of reducing sugar	[81]

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Milling can be employed to alter the inherent ultrastructure of lignocellulosic material and the degree of crystallinity and consequently make it more amenable to cellulase [85]. Milling and size reduction have been applied prior to enzymatic hydrolysis or even other pretreatment processes such as dilute acid, steam, or ammonia [85, 86]. Among the milling processes, the colloid mill, fibrillator, and dissolver are the only ones suitable for wet materials, e.g., wet paper from domestic waste separation or paper pulps. However, the extruder, roller mill, cryogenic mill, and hammer mill are usually used for dry materials.

Irradiation by gamma rays, electron beam, and microwaves can improve enzymatic hydrolysis of lignocellulosic materials as well. The combination of radiation and other methods such as acid treatment can further accelerate enzymatic hydrolysis [87]. Irradiation has enhanced enzymatic degradation of cellulose into glucose. However, pre-irradiation was found to be more effective in air than in an acid solution [88].

Ultrasound can be used for disintegration of waste-activated sludge and aquacultural effluents [89, 90] due to its advantage on the mechanical properties of sludge hydrolysis. In this method, the sludge is disintegrated and the bacterial cell walls are disrupted [91]. Several factors such as ultrasonic density and intensity, sludge pH, and sludge concentration have an impact on disintegration [92].

2.2.2 Chemical

Chemical pretreatment for lignocellulosic feedstocks employs different chemicals such as acids, alkalis, and oxidizing agents, e.g., peroxide and ozone [93]. Among these methods, dilute acid pretreatment using H_2SO_4 is the most widely used method. Depending on the type of chemical used, pretreatment can have different effects on lignocellulose structural components. Alkaline pretreatment, ozonolysis, and peroxide and wet oxidation pretreatments are more effective in lignin removal, whereas dilute acid pretreatment is more efficient in hemicellulose solubilization [94–96].

Alkali pretreatment refers to the application of alkaline solutions such as NaOH, $Ca(OH)_2$ (lime), or ammonia to remove lignin and a part of the hemicellulose. The purpose of alkali pretreatment is to (1) induce swelling of the biomass and lead to an increase of internal surface area, (2) separate cellulose from hemicellulose and lignin, (3) reduce crystallinity of cellulose, (4) disrupt lignin structure, (5) eliminate both hydrolysis and fermentation inhibitors, and (6) improve accessibility of cellulose and hemicellulose toward enzymatic hydrolysis [97, 98]. Alkaline peroxide is an effective method for the pretreatment of biomass. In this method, the lignocellulose is soaked in pH-adjusted water (e.g., to pH 11–12 using NaOH) containing H_2O_2 at room temperature for a period of time (e.g., 6–24 h). The process can improve the enzymatic hydrolysis after delignification.

Acid pretreatment has received considerable research attention over the years [99]. Dilute sulfuric acid has been added to cellulosic materials for some years to

commercially manufacture furfural [100]. Dilute sulfuric acid is mixed with a biomass to hydrolyze hemicellulose to xylose and other sugars and then continue to break xylose down to form furfural. The most widely used and tested approaches are based on dilute sulfuric acid. However, nitric acid, hydrochloric acid, and phosphoric acid have also been studied.

Processing of lignocellulosic biomass with ionic liquids (IL) and other solvents has gained importance in the last decade due to the tunability of the solvent chemistry and hence the ability to dissolve a wide variety of biomass types. Ionic liquids are salts, typically composed of a small anion and a large organic cation, which exist as liquids at room temperature and have very low vapor pressure [101].

2.2.3 Physicochemical

Pretreatments that combine both chemical and physical methods are referred to as physicochemical processes. Physicochemical pretreatment for lignocellulosic feedstock employs different methods such as steam explosion, steam explosion with addition of SO_2, ammonia fiber explosion (AFEX), liquid hot-water pretreatment, and microwave–chemical pretreatment [71, 102–105]. Steam-explosion pretreatment is one of the most commonly used pretreatment options, as it uses both chemical and physical techniques to break the structure of the lignocellulosic material. This hydrothermal pretreatment method subjects the material to high pressures and temperatures for a short duration of time after which it rapidly depressurizes the system, disrupting the structure of the fibrils. The disruption of the fibrils increases the accessibility of the cellulose to the enzymes during hydrolysis. Particle size is a major contributing factor on the effectiveness of the process, and it has been observed that relatively large particle sizes have been able to yield maximum sugar concentrations [106]. The steam-explosion pretreatment process is a proven technique for the pretreatment of different biomass feedstocks as it is able to generate complete sugar recovery while utilizing a low capital investment. Steam-explosion pretreatment also has a low environmental impact in regard to the chemicals being used and the way the process is implemented. Steam-explosion pretreatment method is highly efficient [106].

Acid catalysts have been used within the steam-explosion processes in dilute quantities to improve hemicellulose hydrolysis during the pretreatment stage and cellulose digestibility in later stages of the process. Dilute acids have the ability to decrease retention times and temperatures of the current operating systems and allow for the use of softwoods in this pretreatment technique, where it was originally thought to be uneconomical. By decreasing the retention time and temperature with the addition of this acid catalyst, a reduction of inhibitory compounds formed is observed, nearly all the hemicellulose is removed, and there is an increase rate of hydrolysis later on in the production [107].

Table 2.3 Effect of pretreatment methods on the chemical composition and chemical/physical structure of lignocellulosic biomass

Methods	Effects
Uncatalyzed steam explosion, liquid hot water, pH-controlled hot water, flow-through liquid hot water	Increases accessible surface area and removes hemicellulose, low effect on altering lignin structure
Dilute acid, flow-through acid, biological pretreatment	Increases accessible surface area, removes hemicellulose, and alters lignin structure
Thermal alkaline	Increases accessible surface area, removes hemicellulose, and alters lignin structure. Low effect on removing hemicellulose
AFEX (ammonia fiber expansion), ARP (ammonia recycled percolation)	Increases accessible surface area, decreases cellulose crystallinity, removes hemicellulose, and alters lignin structure. Low effect on removing hemicellulose
Lime	Increases accessible surface area, removes hemicellulose, and alters lignin structure. Low effect on removing hemicellulose
Ozonolysis	Removes lignin
Organosolv	Removes hemicellulose and removes lignin
PEF (pulsed electric field)	Increases accessible surface area and alters lignin structure
Ionic liquids	Increases accessible surface area, removes hemicellulose, lowers cellulose crystallinity, and removes hemicellulose

2.2.4 Biological

Biological hydrolysis of cellulose is carried out by cellulolytic microorganisms or by the cellulose enzyme complex [108–111]. In nature, cellulosic materials are degraded by microorganisms, of which brown-white and soft-rot fungi have proven to readily degrade lignin and hemicellulose in waste materials and are used in biological pretreatment processes [112]. A mixed culture [113, 114] comprising of cellulolytic bacterium and a noncellulolytic bacterium could degrade natural cellulosic materials aerobically or anaerobically without sterilization, thereby having a high degree of stability to degrade cellulosic material for long periods of time. The advantages of biological pretreatment include minimal cost, low energy requirement, and mild impact on the environment. However, utilizing these microorganisms and enzymes to process natural cellulosic materials without pretreatment and/or sterilization is difficult and the rate of hydrolysis is also low.

The main aim of pretreatment is to increase accessible surface area, to decrystallize cellulose, and to remove hemicellulose and lignin. The effects of different pretreatments are listed in Table 2.3. Several factors are mentioned to have a positive effect on the overall economy of the process. It is, for example, favorable to avoid the production of inhibitors [115], because the detoxification of the liquid fractions showed to be costly and/or ineffective [116, 117], leaving the

lignin with the substrate and removing it after the hydrolysis of the (hemi)cellulose will minimize the overall cost of the process [118], and the use of low concentrations of water, energy, and alkali/acid during pretreatment can be attractive for industrial applications [106].

2.2.5 Organosolv Pretreatment

It is known that organosolvent (organosolv) pretreatment can be applied with a large number of organic or aqueous–organic solvent systems with or without added catalysts in the temperature range of 100–250 °C [119], while organic acid pretreatment can be applied under mild conditions, even at room temperature [120, 121]. For most organosolv processes, there is no need for acid addition if the pretreatment is conducted at high temperatures (185–210 °C), as it is believed that organic acids act as catalysts for breaking the lignin–carbohydrate complex [122]. However, when acid catalysts are added, the rate of delignification is increased and high yields of xylose are obtained. Mineral acids (hydrochloric acid, sulfuric acid, and phosphoric acid) are good catalysts to accelerate delignification and xylan degradation, while some organic acids such as formic, oxalic, acetylsalicylic, and salicylic acid also can be used as catalysts [123, 124]. Most of the hemicellulose and lignin are solubilized, but the cellulose remains as solid. The organic solvents used in the process need to be recycled to reduce the cost. On the other hand, removal of solvents from the system is necessary because the solvent may be inhibitory to the growth of organisms, enzymatic hydrolysis, and fermentation. Organosolv pretreatment yields three separate fractions: dry lignin, an aqueous hemicellulose stream, and a relatively pure cellulose fraction [122].

Heterogeneous catalysis for lignocellulosic biomass conversion is gaining attention in the literature [125–130]. This type of acid catalyst is a good alternative to concentrated sulfuric acid for hydrolysis reaction. It has numerous advantages over sulfuric acid in terms of activity, selectivity, catalyst lifetime, and reusability. Moreover, the use of solid acid reduces liquid pollutants and cost of wastewater treatment and thus reduces the costs [131–135].

2.3 Fermentative Hydrogen Production

Biohydrogen production performance is directly determined by operational strategy and key process parameters such as microorganism, fermenter type, substrate concentration, pH, temperature, and hydraulic retention time (HRT).

2.3.1 Microorganisms

In a previous review paper, the major hydrogen-producing bacteria identified are related strictly to facultative anaerobic genera (*Escherichia coli*, *Enterobacter*, *Citrobacter*), to anaerobic genera (*clostridia*, *methylotrophs*, *rumen bacteria*, *methanogenic bacteria*, *archaea*), and to aerobic genera (*Alcaligenes*, *Bacillus*). Table 2.4 shows hydrogen-producing bacteria and their characteristics. Dark

Table 2.4 Hydrogen-producing bacteria and their characteristics

Organisms	Functions	Characteristics	References
Clostridium spp.	H_2 production	Obligate and mesophilic anaerobes	[136–139]
		The most popular H_2 producer	
		Ferment a wide range of carbohydrates and produce H_2	
		E.g., *Clostridium butyricum*, *C. acetobutylicum*, *C. tyrobutyricum*, *C. saccharolyticum*	
Thermoanaerobacterium spp.	H_2 production	Obligate and thermophilic anaerobes	[140]
		E.g., *Thermoanaerobacterium thermosaccharolyticum*	
Ethanoligenens spp.	H_2 production	Facultative anaerobes	[141]
		May possess important features such as salt tolerance	
		E.g., *Bacillus megaterium*	
Bacillus spp.	H_2 production	Facultative anaerobes	[142]
		May possess important features such as salt tolerance	
		E.g., *Bacillus megaterium*	
Enterobacter spp.	H_2 production	Facultative anaerobes	[141]
		Have better tolerance against oxidative stress	
		E.g., *Enterobacter aerogenes*	
Klebsiella spp.	H_2 production	Facultative anaerobes	[143]
		Have better tolerance against oxidative stress	
		E.g., *Klebsiella pneumonia*	
Methanogens	H_2 consumption	Obligate anaerobes	[144]
		Utilize H_2 for methane production	
		E.g., *Methanobacterium* spp., *Methanococcus* spp., etc.	
Other H_2-consuming bacteria	H_2 consumption	Obligate/facultative anaerobes	[145, 146]
		Utilize H_2 as electron donor and precursors for metabolic compounds	
		E.g., *Lactobacillus* spp. and *Bifidobacterium* spp.	

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fermentative hydrogen production is the most practical to be applied among the various biological hydrogen production methods [148, 149] due to its efficient processes to convert organic substrates to energy and electrons. Three types of metabolism to dark fermentative hydrogen production are as follows. The first type is for *Escherichia coli* and *Enterobacteriaceae* [150, 151], which has two major enzymes: (1) pyruvate formate lyase (PFL) and (2) formate hydrogen lyase (FHL). Pyruvate formed via the Embden–Meyerhof–Parnas (EMP) pathway is split into acetyl-CoA and formate by PFL under anaerobic conditions. H_2 and CO_2 are then generated from formate by FHL [152]. The second type typical for *Clostridium* species [153] includes pyruvate:ferredoxin oxidoreductase (PFOR) and Fd-dependent hydrogenase (HydA) [152]. Pyruvate:ferredoxin oxidoreductase catalyzes the oxidative decarboxylation of pyruvate to form acetyl-CoA and CO_2 under anaerobic conditions. The electrons are first transferred to Fd_{ox} with a highly negative potential (−420 mV) [154]. The electrons in Fd_{rd} are then transferred to protons to generate hydrogen by Hyd_A. The third type is reported to exist in many thermophilic bacteria and some *Clostridium* species for utilizing NAD(P)H to form hydrogen. This biochemical reaction is catalyzed by two major enzymes, NAD(P)H:ferredoxin oxidoreductase (NFOR) and Hyd_A [155]. NAD(P)H formed during carbon metabolism by Fd_{ox} reaction. This hydrogen-producing reaction is then processed by Fd_{rd} and Hyd_A.

2.3.2 Fermenter Types

2.3.2.1 CSTR

Continuous stirred tank reactors (CSTR) are commonly used for continuous biohydrogen production [7, 56, 156–158]. In a CSTR, hydrogen-producing microbes are completely mixed and suspended in the reactor liquor by the mixing pattern. Biomass is well suspended in the mixed liquor, which has the same biomass concentration in the effluent [159]. Under such hydrodynamics, good substrate–microbe contact and mass transfer can be accomplished. On the other hand, the CSTR is unable to maintain high levels of fermentative biomass because of the rapidly mixed operating pattern. Biomass washout may occur at short hydraulic retention times (HRTs) [160]; thus, the hydrogen production rates are considerably restricted [7]. To retain high biomass concentrations in reactors, various techniques have been developed for hydrogen fermentation, including sludge immobilization [35, 157, 158], utilization of the upflow reactor [42], and immobilization on a porous support such as loofah sponges, expanded clays, activated carbons [161], and membrane reactors [162, 163].

2.3.2.2 UASB

An upflow anaerobic sludge blanket (UASB) process is a widely applied anaerobic treatment system that has high treatment efficiency and a short hydraulic retention time (HRT). UASB hydrogen production systems have been used in granulation enhancement and granule microstructure [164–166]. Numerous works have dealt with hydrogen-producing UASBr, since hydrogen production granule (HPG) formation was first reported by Fang et al. [167], and as mentioned above, this reactor generally demonstrates a high and stable performance. However, for most studies in this field, synthetic wastewater is generally applied as a substrate. Chang and Lin [168] produced hydrogen from sucrose using a UASBr seeded with heat-pretreated sewage sludge. The highest HY (hydrogen yield) and HPR (hydrogen production rate) values were 0.75 mol H_2/mol hexose and 0.25 l H_2/l·h, respectively, at a HRT of 8 h. In an effort to decrease the start-up period in the UASBr, Jung et al. [169, 170] inoculated heat-treated sludge to a CSTR, and then the mixed liquor in the CSTR was transferred to the UASBr as a seeding source. As a result, hydrogen production granule with an average size of 1.9 mm was successfully formed in the UASBr after 45 days of operation using coffee drink manufacturing wastewater (CDMW), which was the first report on the formation of HPG from actual wastewater.

2.3.2.3 Anaerobic Biofilm and Granule Reactor

To overcome biomass washout problems, an addition of immobilized cells into the conventional CSTR for increasing the biomass retention in biohydrogen-producing fermenters has been previously attempted. Biohydrogen-producing fermenters, such as a carrier-induced granular sludge bed reactor (CIGSB) [160] and continuously stirred anaerobic bioreactor (CSABR) used with the silicone immobilized cells and agitated granular sludge bed reactor (AGSBR), have been investigated [35, 171–173]. Attempts to enhance biomass retention by immobilized cells exhibited a better hydrogen production performance than that of conventional CSTR, with HPR ranging from 6 to 360 l/l·d [160, 172]. Consequently, immobilized cells created by natural or synthetic matrices [174] were often used to allow better retention of hydrogen-producing bacterial cells for stable operations at high feeding rates. Cell immobilization by surface attachment [175] or self-flocculation [35, 173], [168, 176, 177] may have higher feasibility in practical environmental applications. Wu et al. [178] studied the hydrogen production from a sucrose-rich wastewater in a fluidized bed reactor by immobilized cell. Results showed that a stable yield of 182 ml H_2/g hexose and a hydrogen production rate of 22.3 l/l·d were obtained in the fluidized bed reactor.

2.3.2.4 Membrane Bioreactor

The membrane bioreactor (MBR) has emerged as an effective means of attaining performance improvement in wastewater treatment and has been applied to anaerobic processes due to its capability of increasing biomass retention via membrane separation [179, 180]. Attempts have been made to apply the MBR process to hydrogen production, but relatively little research has been carried out so far. Oh et al. [181] demonstrated that HPR increased by 25 %–0.32 l/l.h due to a 164 % increase in biomass concentration from 3.53 to 5.8 g/l with an increase of the slurry retention time (SRT) from 3.3 to 12 h using an external cross-flow membrane. Membrane fouling is a key process limitation and remains one of the most challenging issues with future MBR development [182].

2.3.3 Environmental Operational Conditions

2.3.3.1 Substrate Concentration

Many review reports [61, 65, 183, 184] have summarized the optimum values of substrate concentration, although most studies focus on lab-scale systems. The indexes for identifying high biogas production efficiency are biohydrogen or biomethane production yield (HY or MY, defined as the biohydrogen or biomethane production per unit weight of consumed substrate, mol H_2/g COD or mol CH_4/g COD) and biohydrogen/biomethane production rate (HPR or MPR, defined as the biohydrogen or biomethane production per unit working volume per day, l/l·d).

Finding the optimal substrate concentration in continuous operation mode is more meaningful and practical, since the batch mode does not take into consideration the hydrodynamic effect, steady state of the substrate concentration, and pH condition for bacterial growth. The best performance is found to be at 30 g sucrose COD/l with HY of 1.09 mol H_2/mol hexose using a CSTR [185]. At inlet substrate concentrations below 20 g COD/l, the HY decreases along with a significant decrease in the n-butyrate/acetate ratio. The appearance of hydrogen-consuming bacteria and decrease of substrate removal efficiency was observed at over 35 g COD/l [185].

High substrate concentration allows more energy-efficient operation but product inhibition is likely to set the upper limit. Certain levels of metabolic products in the dark fermentative hydrogen production reactor may inhibit the hydrogen-producing pathway as well as microbial activity. It is known that butyrate has the highest inhibiting effect on *Clostridium sp*., among various acids; thus many attempts have been made to alleviate butyrate inhibition, mostly by chemical extraction [186].

Table 2.5 shows the biohydrogen production performance data in continuous operation mode in 2013–2015. Most investigators try to use agriculture waste or the residue from biofuel production processes or food waste to extend the feedstock

Table 2.5 The biohydrogen production performance in continuous operation mode in 2013–2015

Substrate	Reactor type	Microorganism	Substrate concentration	pH	Temperature (°C)	HRT (h)	H_2 production rate (mmol/l·d)	Maximum H_2 yield	References
Synthetic substrate based on sucrose	UASB	Mixed Culture	2 g COD/l	–	55	2	80.7	1.73 mol H_2/mol sucrose	[187]
Palm oil mill effluent wastewater	UASB	PEG-immobilized *Clostridium* sp. LS2	60 g COD/l	5.5	37	12	360	0.35 l H_2/g $COD_{removed}$	[188]
Rice straw hydrolysate	Continuously stirred anaerobic bioreactor (CSABR)	Mixed culture	20 g total sugar/l	5.5	37	4	446	0.69 mol H_2/mol total sugar	[69]
Rice straw hydrolyzate	Continuously external circulating bioreactor (CECBR)	Mixed culture	20 g total sugar/l	5.5	37	4	728	1.02 mol H_2/mol hexoses	[4]
Crude glycerol from biodiesel industry	CSTR	*C. pasteurianum* CH_4	2 g glycerol/l	–	35	12	177.8	0.77 mol H_2/mol glycerol	[189]

Molasses with liquid swine manure	Anaerobic sequencing batch reactor (ASBR)	Mixed culture	10 g sugar/l	5.32	37	15.62	99.6	1.57 mol H_2/mol sugar	[190]
De-oiled jatropha waste (DJW)	ASBR	Mixed culture	200 g DJW/l	6.5	55	48	66	8.7 ml H_2/g volatile $solid_{added}$	[191]
Food waste	CSTR	Mixed culture	29.17 g COD/l	5.0	35	20	16.9	261 ml H_2/g VS_{added}	[32]
Food waste	Continuous mixed immobilized sludge reactor (CMISR)	*A. awamori* and *A. oryzae*	10 g food waste hydrolysate/l	4	55	6	379	85.6 ml H_2/g food waste	[55]
Textile wastewater (TW)	Intermittent-flow, stirred tank reactor (IFSTR)	Mixed culture	33.1 g hexoses/l	5.5	35	24	44.6	0.97 mol H_2/mol hexoses	[54]

resources for the biohydrogen production. The study trend for enhancement of hydrogen production rate seems to change the hydrodynamic properties by changing the fermenter type.

To recover more energy from the biomass substrate, researchers have begun to explore two-stage (biohydrogen+biomethane) [192–195] production technology system recently. Two-stage biohydrogen and biomethane production systems can really increase the energy gain from biomass resource by around 8–43 % energy compared with one-stage anaerobic digestion systems [192, 196].

2.3.3.2 Nutrients and Metals

Excluding the main substrate, carbohydrate materials, dark fermentative hydrogen production (DFHP) requires nutrients for bacterial activity like all biological treatment processes. The nutrients include nitrogen (N), phosphorous (P), ferrous (Fe), and some trace metals. Among the many kinds of nutrients, N is the most essential one for bacterial growth. Optimal C/N ratio is 47 according to Lin and Lay [197]. P and Fe concentrations affect the metabolic pathway of *Clostridium* sp., and hydrogen production potential decreases when their concentrations are limited.

The effect of iron has been investigated many times in DFHP, since it is an essential component of hydrogenase. Lin and Lay [198] studied the requirement of 11 trace metals in hydrogen fermentation. Magnesium, sodium, zinc, and iron were found to be the important trace metals with magnesium being the most significant one. Hydrogen production is enhanced by 30 % at optimal combined concentrations, 4.8 mg Mg^{2+}/l, 393 mg Na^{+}/l, 0.25 mg Zn^{2+}/l, and 1 mg Fe^{2+}/l.

The effect of metal ions on the fermentative hydrogen production has been widely studied such as Ni [199–201], Fe [199, 202, 203], Cu [201, 204–206], Cr [201, 204], Zn [201, 204, 206], Cd [201], and Pb [201] ions. Hydrogenase enzymes catalyze the reduction of proton to H_2. Hydrogenase enzymes are classified into [Ni–Fe] and [Fe–Fe] hydrogenases, according to the metal content at their active site [207]. [Ni–Fe] hydrogenases are extensively distributed among bacteria [208], and both nickel and iron have important effects on fermentative H_2 yields [199, 200, 202, 209].

In a biohydrogen production process, electrons are transported via an intramolecular electron transfer chain from the redox partner of the [Ni–Fe] hydrogenases to the active site, and then the protons are reduced by producing biohydrogen [210, 211]. Since nickel is a fundamental component making up the [Ni–Fe] hydrogenases, it plays an important role in fermentative hydrogen production.

Karadag and Puhakka [199] investigated the effect of Fe^{2+} and Ni^{2+} on continuous hydrogen production in anaerobic completely stirred tank reactor (ACSTR). They found that hydrogen production increased about by 71 % with the increasing of iron and nickel supplementation, and the highest yields were achieved at the concentrations of 50 mg Fe^{2+}/l and 25 mg Ni^{2+}/l. Wang and Wan [200] reviewed the effects of Fe^{2+} on anaerobic hydrogen production and reported some inconsistency on the optimal Fe^{2+} concentration. They also found that increasing Ni^{2+}

concentration up to 0.2 mg/l enhanced the hydrogen production by using batch experiments at 35 °C. Metabolic pathway shifted at different Ni^{2+} concentrations and higher Ni^{2+} concentration promoted the growth of hydrogen-producing bacteria. Lee et al. [202] investigated the effect of iron on the efficiency of continuous hydrogen production in a submerged membrane bioreactor system. They found $FeSO_4$ concentration is the key factor affecting the fermentation pathway for hydrogen production with the membrane bioreactor. Both increase in the hydrogen production rate and the hydrogen yield were obtained by adding $FeSO_4$. They indicated that iron sulfate increased hydrogenase activity and hydrogen production in a membrane bioreactor when $FeSO_4$ concentration closes to 10.9 mg/l.

Lin and Shei [204] investigated the effect of Cr, Cu, and Zn ions on biohydrogen production using anaerobic sewage sludge microflora. Cr, Cu, and Zn significantly affect hydrogen-producing microflora enriched from sewage sludge with Zn and Cr being the most and least toxic metals, respectively. The microflora's hydrogen production activity could be reduced by 50 % for a biomass in contact with 4.5 mg Zn/l, 6.5 mg Cu/l, and 60 mg Cr/l. However, low concentrations of 2 mg Cu/l and 15 mg Cr/l resulted in peak hydrogen production by 20 and 10 %, respectively. Zheng and Yu have reported that the specific hydrogen production rate was enhanced by the dosage of Cu at 50–100 mg/l, but was inhibited by Cu over 200 mg/l from glucose by enriched anaerobic culture [206]. Li and Fang found that Cu strongly inhibited the bioactivity of hydrogen-producing sludge [201]. Han et al. [205] investigated Cu^{2+} concentration effect in a sucrose-fed CSTR on fermentative hydrogen production by mixed cultures. The result shows that 6.4 mg/L Cu^{2+} is the optimal concentration for the CSTR at HRT 4 h. In addition, copper causes shift in the metabolic pathway.

Li and Fang [201] studied the inhibition of six heavy metals and found the bioactivity of hydrogen-producing sludge in the following order: Cu (most toxic) > Ni >Zn > Cr > Cd > Pb (least toxic). Hydrogen-producing sludge exhibited in general higher resistance to metal toxicity than methanogenic granular sludge. Furthermore, Han et al. [203] studied the effects of hematite nanoparticle concentration on hydrogen production in batch system. The optimum hematite nanoparticle concentration was 200 mg/l, with the maximum hydrogen yield of 3.21 mol H_2/mol sucrose which was 32.64 % higher than the blank test. The slow release of hematite nanoparticles had been verified by transmission electron microscopy (TEM). In addition, TEM analysis indicated that the hematite nanoparticles can increase the length and narrow the width of bacteria.

2.3.3.3 pH

The control of pH is crucial to fermentative hydrogen and methane production due to its effects on hydrogenase activity and metabolic pathways. When the pH of a fermentation medium is too low, hydrogenase activity and methanogens would be inhibited or there would be a switch in metabolic pathway resulting in cessation of hydrogen and methane generation. Anaerobic hydrogen production process is

typical during the exponential growth phase of *clostridia* [212]. The reactions shift from a hydrogen/acid production phase to a solvent production phase when the population reaches the stationary growth phase. The accumulation of volatile fatty acids such as butyric and propionic acids and hydrogen during the exponential growth phase prompts this shift. Some researchers claimed that this shift occurred when the pH dropped to 4.5 or below [213, 214], while others found that the shift occurred at pH levels above 5.7 due to enzyme synthesis or enzyme activation, which led to solvent production [215]. Thus, it is important to remove excess hydrogen from the system and control the pH at an optimal range to maintain hydrogen production. Otherwise, the biohydrogen production will stop due to the microbial population shift caused by pH uncontrolled in the desired range.

Khanal et al. [216] investigated the effect of pH on biological hydrogen production using sucrose and starch as organic substrates in batch system. Based on the evaluation of maximum hydrogen production rate, the optimum operational pH range was about 5.5–5.7. This result could be applied in continuous-flow processes to maintain a high rate of hydrogen production. Lin and Lay [217] found that phosphate acted as a better buffer source for hydrogen production than carbonate. Its addition enhanced hydrogen production by 1.9 times and decreased the lag period. Cavinato et al. [218] found that recirculation of anaerobic digested sludge after a mild solid separation to control the pH in an optimal hydrogen production range of 5–6 resulted in a stable hydrogen production output. The importance of pH control for continuous hydrogen production has been investigated extensively. The rapid pH depletion could cause a metabolic change of the microorganisms in the hydrogen production process, resulting in the shift of intermediate production pathway and a decrease in hydrogen production or system upset [216].

Reported optimal pH values for different systems or substrates differed substantially from 4.0 to 6.5, but for each specific situation, the optimal pH range was quite narrow within 0.5 [219]. Chu et al. [220] also reported a pH-phased two-stage fermentation process (combining thermophilic hydrogen production and mesophilic methane production) with recirculating digested sludge. These results show that a recirculation of precipitated digester sludge to a hydrogen reactor can maintain the hydrogen reactor pH at an optimal range without adding any reagents. Recently, most researches operated the hydrogen production fermenter at pH 5–5.5 as an optimal condition at mesophilic temperature [54, 69, 221–223].

2.3.3.4 Temperature

Fermentative hydrogen production via mixed cultures is conducted mostly under mesophilic (20–40 °C) and thermophilic (50–60 °C) conditions with only few studies being carried out under hyperthermophilic (65–75 °C) conditions. Biohydrogen production temperatures within 23–60 °C show that hydrogen production yield and hydrogen production rate increase along with the temperature increment [183].

Vatsala et al. [224] improved hydrogen production from a sugarcane distillery effluent using co-cultures at 100 m^3 reactor and found that unsteady hydrogen production was presumably due to temperature variation during daytime (32–39 °C) and nighttime (26–32 °C). Zhang et al. [225] reported biogas production from brown grease at mesophilic temperature (34.3–37.9 °C) in a pilot-scale high-rate anaerobic digester with a methane yield of 0.40–0.77 m^3 CH_4/kg-VS (higher than a typical range of other food wastes, 0.11–0.42 m^3 CH_4/kg-VS), a mean methane content of 75 %, and <200 ppm of hydrogen sulfide.

Cheong and Hansen [226] indicate that thermophilic acidogenesis enhances hydrogen production consistent with the biochemical pathway of butyrate fermentation. Under thermophilic temperatures (55 °C), the maximum hydrogen production potential of 134 ml with a specific hydrogen production rate of 25 ml H_2/h.g cell can be achieved. Throughout the thermophilic batch experiments, the main intermediate metabolites were acetate, n-butyrate, and ethanol. Propionate formation was suppressed completely during fermentation. Yields of produced hydrogen were correlated with increasing concentrations of n-butyrate, and quantities of ethanol present were significant in the batches producing lower yields of hydrogen.

Zhang et al. [19] studied conversion of starchy wastewater into hydrogen at thermophilic condition (55 °C) with batch experiments. The mixed liquor was composed mostly of acetate (40.2–53.4 %) and butyrate (26.0–40.9 %). Luo et al. [227] evaluated the pretreatment methods on mixed inoculum for both batch and continuous thermophilic biohydrogen production from cassava stillage. They found that butyrate was predominant and accounted for more than 75 % of the total amount of VFA/ethanol except the case of loading-shock pretreated sludge (56 % in this study). Butyrate concentration was observed to be correlated with the hydrogen production. Chen et al. [228] also found highest biohydrogen production was obtained when butyrate was predominate (70–85 % of the total VFA/ethanol).

Lin et al. [229] studied biohydrogen production with mesophilic conditions with a mixed microflora on a pilot scale. They found that the primary soluble microbial products (SMP) were butyrate (iso- and n-butyrate) accounting for 44.4–53.2 % of SMP. Acetate was also produced and accounted for 21.3–26.4 % of SMP. Productions of propionate and ethanol ranged from 7.2–10.6 % to 14.3–22.3 % of SMP, respectively. However, propionate and ethanol are unfavorable metabolites for hydrogen production [158, 172, 230]. The ratios of ethanol/acetate and acetate/butyrate have been used to indicate the performance of hydrogen production [231, 232]. Wu et al. [172] indicated that there might be an optimal acetate/butyrate ratio for hydrogen production, but the ratio is highly dependent on the anaerobic culture or the carbon substrate used.

2.3.3.5 HRT

Shortening hydraulic retention times (HRTs) is a well-used and effective operation strategy to enhance hydrogen production from organic wastewater and solid wastes because of its ability to exclude methanogens which have longer generation time.

The proper HRTs for hydrogen and methane production from organic fractions of municipal solid wastes (OFMSW) are 1–2 days and 10–15 days, respectively. A thermophilic hydrogen production reactor operating at HRT 1.3 days and a mesophilic methane production reactor operating at HRT 5.0 day have been combined to convert OFMSW into a pilot-scale two-phase fermentation system [220]. A pilot-scale two-phase hydrogen/methane fermentation system for food waste was operated at HRT 21 h with a peak hydrogen yield of 1.82 H_2 mol/mol glucose. Over 80 % of the methane was produced in the methane fermentation tank with acetic acid as the dominant organic acid. An economic evaluation shows that two-phase hydrogen/methane fermentation has greater potential for recovering energy than that of methane fermentation [233]. Cavinato et al. [218] operated pilot-scale hydrogen and methane fermenters by using HRTs of 3.3 and 12.6 days resulting in a specific hydrogen yield of 66.7 l/kg total volatile solids (TVS) and a specific biogas yield of 0.72 m^3/kg TVS respectively.

For most studies on continuously dark fermentative hydrogen production (DFHP), continuous systems are expected to operate at a low HRT 36–12 h [222, 234], very low of HRT 12–2 h [69, 229, 235–239], for obtaining a high biohydrogen production that can be operated at extremely low of HRT 2–0.5 h [35, 157, 240–243] with immobilized cell in the biohydrogen production fermenters. A mixture of food industry wastewater with rice straw hydrolyzate as substrate was conducted in a continuously stirred anaerobic bioreactor (CSABR) at HRT 4 h and found the hydrogen production of 10 l/l·d [69]. The rice straw hydrolyzate as sole substrate in continuously external circulating bioreactor (CECBR) prevented biomass washout by using a high volumetric flow rate with HRT of 4–2 h. It was found that the value of hydrogen production rate of 16.32 l/l·d at HRT 4 h was three times more than that at HRT 8 h [4].

2.4 Conclusions and Future Outlook

In recent years, the goal of biohydrogen systems has been to design economically viable hydrogen production processes. In this chapter, the potential feedstock, pretreatment processes, microorganisms, fermenter types, and operational conditions have been introduced. From this segment, readers will have attained a basic knowledge on biohydrogen production technologies from biomass waste. However, commercial biohydrogen production plants are not yet established because abundant and suitable feedstocks are not easily accessible yet. The economic feasibility of the dark fermentative hydrogen production process is dependent on the availability of cellulosic materials and organic wastes. Enhancements in the hydrogen production rate and yield from these feedstocks are important subjects for metabolic engineering. From an engineering point of view, the easily converted and abundant feedstock should be the first option to supply the biohydrogen production plants. Carbohydrate-rich organic wastes are a promising feedstock for anaerobic biohydrogen production. The sugary wastewater has a big potential for hydrogen

Fig. 2.2 Two-stage biohydrogen and biomethane production pilot plant in Feng Chia University campus with hydrogen fermenter of 0.4 m^3 and methane fermenter of 2 m^3

fermentation for future industrial applications. The potential economic gains from the internal rate of return (IRR) evaluation result in the motivation to apply this technology. From a global economic perspective, sugary wastewater could have higher profits with biogas energy used on-site for replacing natural gas and also could satisfy the energy requirements of some local areas. The adoption of fermentative hydrogen production from organic wastes will potentially lead to great advancements in energy and the environment. Finally, there are many pilot plants still at work in Spain [244], Taiwan (see Fig. 2.2) [229, 245], Italy [246], and the UK [247]. Toyota already has the first commercial fuel cell car (Mirai) [248] selling in Europe and North America right now. We believe that profitable and sustainable hydrogen production from biomass waste will be achieved in the near future.

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