Biohydrogen Production Potential of Different Biomass Sources

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Abstract

Hydrogen production from renewable energy sources has gained special attention in recent years. Especially biohydrogen production from biomass resources is accepted as an environmentally-friendly and sustainable approach. However, this process is slow due to the recalcitrant biomass structure hindering the liberation of readily fermentable sugars for fermentation. Therefore, biohydrogen production is usually integrated with a relevant biomass pretreatment process. This book chapter presents an overview of potential biomass resources, biomass pretreatment options, and fermentation processes used for biohydrogen gas production. The text focuses especially on separate and integrated dark and photofermentative hydrogen production processes by discussing principles and recent research outcomes from the literature.

2.1 Introduction

Hydrogen is a clean energy carrier with a high energy content and has a wide range of applications from transportation fuel to electricity generation. Currently, most hydrogen demand is supplied from fossil resources, such as natural gas and coal by steam reforming or gasification. However, these processes require high energy. Besides, CO_2 is produced as a by-product which is a main gas that causes greenhouse effect. In response to those problems, hydrogen production from renewable sources like biomass draws great attention.

Biomass is a general term which is called as organic material that is produced via photosynthesis by green plants including algae,

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trees, and crops (McKendry 2002). Utilization of biomass as feedstock for hydrogen production is not only cost-effective but also environmentally-friendly option, because the processes are carbon neutral (have net zero CO_2 emission) due to the fact that CO_2 is fixed in the atmosphere by plants during photosynthesis. Besides, agricultural crops (sugar and oilseed crops) and their waste by-products, lignocellulosic products such as wood and wood waste, aquatic plants like algae and water weeds, industrial or municipal solid wastes, and animal wastes are accepted as biomass sources (Nath and Das 2003; Caputo et al. 2005; Ni et al. 2006). Hydrogen can be produced from these biomass sources via thermochemical or biological processes. Over the last few decades, investigations on biological hydrogen production as a by-product of microorganism metabolism have accelerated for generating sustainable energy to meet increasing global energy demand (Gupta et al. 2013).

Dark fermentation and photofermentation are the major bioprocesses for hydrogen generation from carbohydrate-rich substrates. The most important criteria for raw material selection for biohydrogen production are its availability, carbohydrate content, fermentability, and cost (Kapdan and Kargi 2006). It is a fact that biomass is the most abundant biopolymer on Earth and an alternative resource to fossil fuels. Biomass sources for biohydrogen production have been categorized as first-generation biomasses (agricultural crops), second-generation biomasses (lignocellulosic wastes), and third-generation biomasses (algae). Second- and third-generation biomasses are preferred ones, since they are not food source. Recalcitrance of these biomasses is the limiting factor hindering microbial biomass degradation for hydrogen production. Therefore, fermentative hydrogen production is usually integrated with a relevant pretreatment process. Physical and chemical biomass pretreatments are mostly used processes prior to biohydrogen production. Enzymatic biomass pretreatments on the other hand are in minority due to slow conversion rates and practical difficulties. The complexity of pretreatment is directly related with the biomass content. Therefore, selection of a suitable biomass requiring less pretreatment steps is of great importance.

Production of clean energy source and utilization of biomasses make biological hydrogen production a novel and promising approach to meet the increasing energy needs as a substitute for fossil fuels. On the basis of these facts, this chapter focuses on biomass types, potential use of different biomasses as the raw material, biomass pretreatment options, biohydrogen production potentials from biomasses, bioprocessing strategies, and challenges on biohydrogen production.

2.2 Biomass Sources for Biohydrogen Production

First-generation biomasses are often edible agricultural crops which are grown for food and animal feed purposes (Sims et al. 2008; Lee and Lavoie 2013). Sugar-containing crops such as sugarcane and sugar beet; starch-containing ones such as wheat, barley, potato, and corn; and oily plants and seeds such as soybeans, sunflower, and palm are some of the examples for first-generation biomasses. A number of studies have been reported in the literature for biohydrogen production from first-generation biomasses, especially from starchy and sugar-rich biomasses due to easy fermentability attribute of these feedstocks by anaerobic organisms. Even though higher hydrogen yields are obtained from first-generation biomasses, the biggest obstacle when using these sources as feedstock is the utilization of arable land to produce energy crops instead of food production. This will lead both severe food shortages and overmuch usage of water and fertilizers (Dragone et al. 2010). For this reason, nowadays, biohydrogen production studies have been shifted from first-generation biomass to second-generation biomass.

Lignocellulosic biomasses including agricultural and forestry wastes and nonedible crop residues, as well as industrial and municipal organic wastes, wastes from food processing, and industrial effluents, constitute second-generation biomasses (Cheng et al. 2011; Singh et al. 2011). Beet molasses is a by-product of sugar industry and a

common biomass that is used in fermentation processes due to its high sucrose content. Besides carrot pulp (de Vrije et al. 2010), solid organic wastes such as carbohydrate-rich wastes (apples, carrots, Jerusalem artichoke roots, maize flour, oats, potatoes, and wheat flour), protein-rich wastes (soybean milk cake, chicken meat, cow manure with straw, fish residues, and meat waste from restaurants), agro-industrial wastes (including food waste from restaurants, rapeseed oil cakes, sunflower oil cakes, grape marc, fruit peels – orange peels and banana peels – and maize cob), agricultural residues (Jerusalem artichoke leaves and stalks, giant reed stalks and leaves, maize stalks, rice straw, and sorghum stalks) (Guo et al. 2014), palm oil mill effluent (POME) (Al-Shorgani et al. 2014), distillery wastewater (Sridevi et al. 2014), and waste papers (Ntaikou et al. 2009) are second-generation biomasses that were used as substrate for biohydrogen production.

Second-generation biomasses should be considered as organic wastes of agricultural or industrial activities, in general. Therefore, these biomasses are abundant and cheap and they do not compete with food production. Researchers have been taking a great interest in biohydrogen production from second-generation biomasses especially agricultural and forestry by-products due to their low cost. Qian (2014) stated that estimated annual production of lignocellulosic biomass is over 200 billion tons on Earth. The main component of plant biomass and primary building block of plant cell walls is lignocellulose. The structure of lignocellulose is complicated because it is composed of mainly 20–45 % cellulose, 16 % and 37 % hemicellulose, and 12–26 % lignin with wide range of carbon to nitrogen ratio between C/N = 118 and C/N = 10 (Sawatdeenarunat et al. 2015). Cellulose is a linear polymer of D-glucose which is linked to each other by β-(1,4)-glycosidic bonds. Hemicellulose is a branched heteropolymer consisted of pentoses (D-xylose, L-rhamnose, and L-arabinose), hexoses (D-glucose, D-mannose, and D-galactose), and uronic acid derivatives (e.g., D-glucuronic, D-galacturonic acids). Lignin is a complex, hydrophobic polymer and is made of cross-linked phenolic monomers. These biopolymers are difficult to be fermented by anaerobic microorganisms directly for biohydrogen production. Thus, a pretreatment step is required to remove lignin and to hydrolyze complex carbohydrates into their monomers. Biohydrogen production from second-generation biomass on a large scale is a challenge due to the requirement of high pretreatment costs.

Algae are third-generation biomasses and they have been in use as feedstock for biohydrogen production due to their rich carbohydrate content. They are unicellular or multicellular organisms which can be classified as prokaryotic, like cyanobacteria (blue-green algae), or eukaryotic such as green algae, red algae, and brown algae. Generally, algae are grouped as microalgae and macroalgae, according to their morphology and size. They may grow autotrophically (use atmospheric CO_2 via photosynthesis), heterotrophically (use organic carbon), or mixotrophically (use both inorganic and organic carbon depending on the condition). Algae can store carbon in the form of starch, cellulose, and lipids. The carbohydrate source in algae is mainly starch which is deposited in the cytoplasm and cellulose in the cell wall. The carbohydrate storage type can be different according to the types of algae. For instance, cyanobacteria have glycogen, green algae and red algae have starch, and brown algae have β-glucans as a storage carbohydrate (Mollers et al. 2014). The carbohydrate content of algae can vary between 30.7 and 48.2 % (Batista et al. 2014; Yun et al. 2014; Liu and Wang 2014; Nayak et al. 2014). Algae are common biomass in bioethanol and biodiesel production. But, researchers have focused on biohydrogen production from third-generation biomass due to many advantages such as high CO_2 capture rate, ease of cultivation, rapid biomass production, and high carbohydrate content. The other advantage of using algae as substrate in biohydrogen production is the absence of lignin and hemicellulose. The pretreatment requirement for the separation of lignin and hemicellulose as applied in second-generation biomass is omitted. Thus, cost of pretreatment is reduced and formation of some toxic end products as furfurals and 5-hydroxymethylfurfural (5-HMF) during pretreatment does not occur.

2.3 Pretreatment (Hydrolysis) of Biomasses for Biohydrogen Production

Biomass is an abundant renewable resource for biological hydrogen gas production and can be found in different forms in nature as presented in Sect. 2.2. However, in most cases biomass cannot directly be used as feedstock for fermentative hydrogen production due to the presence of hardly biodegradable compounds and unsuitable nitrogen levels. Hydrogen-producing microorganisms prefer fermentable sugar monomers and low nitrogen in the substrate. Therefore, biomass is usually subjected to a convenient pretreatment in order to provide the desired microbial milieu conditions. A general schematic diagram covering various pretreatment steps for biohydrogen production is shown in Fig. 2.1.

Biomass pretreatment is related with the use of several sources like energy, water, chemicals, equipment, and by-product formations like wastes and toxic compounds as shown in Fig. 2.1. Therefore pretreatment with little resource consumption requirement and waste production under simple operation conditions with high product formation yields should be preferred. An ideal pretreatment process is identified as a process that produces a disrupted, hydrated substrate that is easily hydrolyzed with no toxic by-product formation (Agbor et al. 2011).

Feedstock preparation from biomass involves a number of steps prior to fermentation as shown in Fig. 2.1. Therefore, the selection of a proper biomass is of great importance. Feedstock preparation requirements for hydrogen production from green or brown colored biomass, for example, are different. Green biomasses such as grass leaves or cabbage residues are rich in hemicellulose, nitrogen, and lignin (Ruggeri and Tommasi 2012). Therefore green biomass requires more pretreatment steps when compared to brown biomass (corn, wheat straw, corn stover, etc.) that is generally rich in cellulose but poor in nitrogen (CalRecycle 2015). Thus the decision on starting with a convenient biomass will significantly

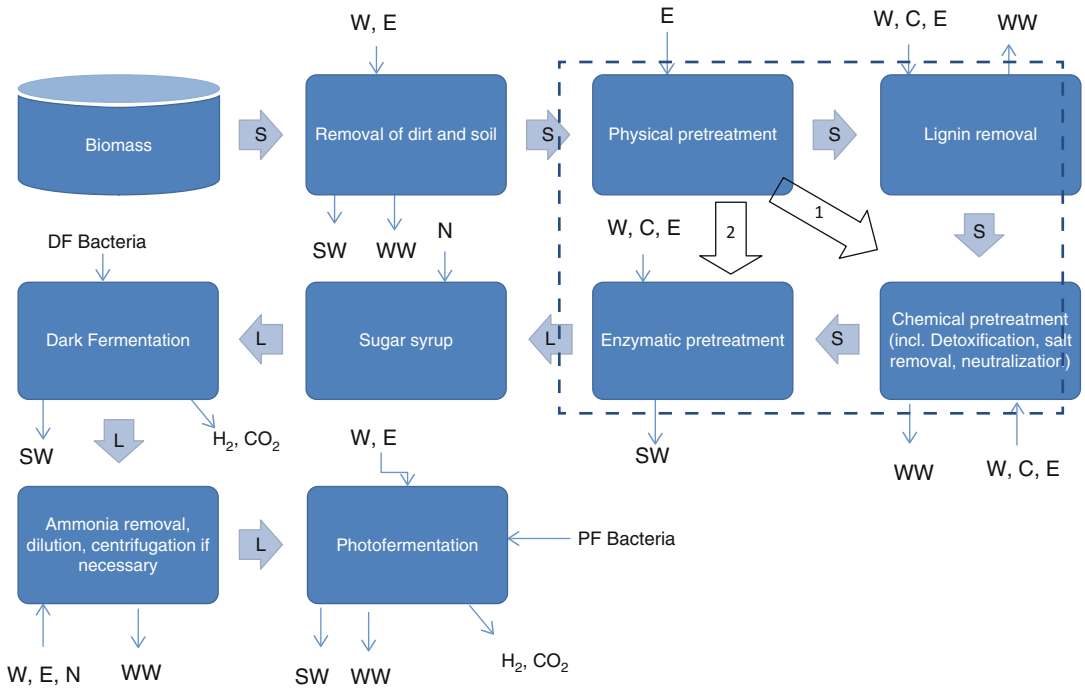

Fig. 2.1 Fermentative hydrogen gas production from biomass (*E* energy, *S* solid, *L* liquid, *C* chemicals, *W* water, *SW* solid waste, *WW* wastewater, *1* 1st alternative route, *2* 2nd alternative route, *area within dashed line* biomass pretreatment processes, *DF* dark fermentation, *PF* photofermentation, *N* nutrient)

affect required pretreatment stages and operation costs in hydrogen production.

After starting with a proper biomass, the next step is the removal of dirt and soil. Soil may contain hydrogen-consuming methanogens that may negatively affect hydrogen production performance. On the other hand, any coarse material within the biomass might cause damage during further operation phases. Dirt and soil removal can be accomplished by washing or by hand sorting. Usually washing is applied if there is dirt or soil on biomass. Wet biomass cannot be stored due to microbial attack and degradation of fungi. Therefore, it has to be dried by solar or oven heating if storage is considered (Velázquez-Martí et al. 2011). Drying at high temperature could cause collapse of cellulose pores which is an irreversible process limiting microbial degradation (Dowe and Nrel 2001).

If biomass is considered to be used as feedstock in fermentation, the lignins and hemicelluloses that hinder or block enzymatic attack and the crystallinity of cellulose have to be reduced by suitable pretreatment processes. Depending on the biomass structure, pretreatment can be applied in single or multisteps (Agbor et al. 2011; Alvira et al. 2010; Mosier et al. 2005). Biomass pretreatment processes are classified in general as physical, chemical, and biological processes (FitzPatrick et al. 2010). Physical pretreatment is related with size reduction or the contribution of a physical force to decompose the biomass structure to some extent. Chemical pretreatment is usually applied at high temperatures under severe acidic or alkaline conditions. Biological pretreatments, on the other hand, can be accomplished at ambient operation conditions with lower conversion rates and yield of complex carbohydrates to their monomers. The objectives in all pretreatment steps are to enable microbial access to fermentable sugars within the biomass. Factors limiting this access are the cellulose crystallinity, degree of polymerization, lignin and hemicellulose structure, available surface area for enzymatic attack, particle size, and porosity of biomass (Alvira et al. 2010). The effects of different pretreatment processes on biomass structure are summarized in Table 2.1, and the principles are briefly explained in the following sections.

2.3.1 Physical Pretreatment

Physical pretreatment involves the treatment of biomass by physical forces without using any chemicals or microorganisms (Zheng et al. 2014). Comminution, steam explosion, liquid hot water, extrusion, and irradiation are the most used physical pretreatment processes (Zheng et al. 2014). The main goal in physical pretreatment is to enhance the accessible biomass surface area by decreasing the particle size, cellulose crystallinity, and degree of polymerization (Alvira et al. 2010). Achieving these goals enables a more efficient chemical or microbial hydrolysis of the biomass matrix and decreases hydrolytic enzyme limitations (Chandra et al. 2007; Mansfield et al. 1999). Most of the studies in the literature relating to biohydrogen production from biomass usually report the application of physical treatment prior to fermentation, and mechanical size reduction is the most popular method applied.

Size reduction by mechanical comminution is an energy-intensive process and can be accomplished by different equipment such as ball or hammer millers, shredders, and grinders (Alvira et al. 2010). The energy consumption of this process increases depending on the biomass structure, the moisture, and the particle size that is to be obtained. For example, a particle size less than 2 mm is desired prior to delignification of different types of biomass by the alkaline peroxide pretreatment process (Sun et al. 2000; Gould 1985; Banerjee et al. 2011). Size reduction after chemical pretreatment was suggested as an option to decrease energy consumption and process costs. However, this is not applicable for all kind of biomass types (Zhu et al. 2010).

Physical pretreatment of biomass can also be accomplished without using mechanical size reduction. Steam explosion, for example, enables a direct pretreatment of biomass. This technique was reported to be one of the most cost-effective

Table 2.1 Effects of different pretreatment methods on biomass structure

	Pretreatment	Increase of accessible surface area	Decrystallization of cellulose	Solubilization of hemicellulose	Solubilization of lignin	Alteration of lignin structure	Formation of toxic products like furfural/hydroxymethylfurfural (HMF)
Physical	Mechanical	●	●				
	Irradiation	●	○	○			○
	Steam explosion	●		●	○	●	●
	Liquid hot water	●	ND	●	○	○	○
Chemical	Catalyzed steam explosion	●		●	●/○	●/○	●
	Acid	●		●	○	●	●
	Alkaline	●		○	●/○	●	○
	Oxidative	●	ND		●/○	●	○
	Ionic liquids	●	●	○			
	Thermal acid	●	ND	●			●
	Thermal alkaline	●	ND	○	●/○	●	○
	Thermal oxidative	●	ND	○	●/○	●	○
	Ammonia fiber explosion	●	●	○	●	●	○
Biological	Enzymatic	●	ND	●	●	●	

Adapted from Mosier et al. (2005) and Zheng et al. (2014)

options for agricultural biomass pretreatment (Prasad et al. 2007). During steam explosion, biomass is subjected to a quick pressure release under high temperature (Han et al. 2010), which causes cell wall disruption due to heating and shearing forces and the hydrolysis of glycosidic bonds by formed organic acids (Jacquet et al. 2011). However this process still suffers from problems such as toxic and inhibitory by-product (e.g., 5-hydroxymethylfurfural (5-HMF) and furfurals) formation (Cantarella et al. 2004) and incomplete removal of lignin (Shevchenko et al. 1999; Martín-Sampedro et al. 2012). Factors affecting the efficiency in steam explosion are particle size, biomass humidity, temperature, pressure, and residence time (Jeoh 1998). Liquid hot water pretreatment is a similar process with steam explosion, however, with higher retention times and no sudden pressure release. During liquid hot water treatment, biomass fractions are exposed to hot water resulting in cellulose hydrolysis, hemicellulose solubilization, and delignification to some extent due to penetration of hot water into the cell structure (Zheng et al. 2014). Different from steam explosion, liquid hot water treatment retention times vary from minutes up to hours (Zheng et al. 2014). Steam explosion and liquid hot water treatments can be preceded in temperature- and pressure-resistant reactors.

Another way of physical treatment is extrusion where the biomass is exposed to heating, mixing, and shearing while passing through an extruder (Agbor et al. 2011). In this process the biomass is forced to move through a barrel with the torque of single or twin screws (Zheng and Rehmann 2014). This movement produces compression and expansion within the zones of the barrel, disrupting the biomass structure into shorter fibers with broken-down cell walls (Karunanithy et al. 2012). Temperature, screw speed, retention time, and biomass moisture are critical parameters affecting the extrusion process (Karunanithy and Muthukumarappan 2012).

Biomass disruption by physical treatment could also be accomplished by irradiation methods like microwave, gamma ray, ultrasound, or electron beam applications (Zheng et al. 2014. When biomass is irradiated with microwaves, macromolecules and water within the cells absorb the wave energy resulting in enormous heat generation and consequently cell wall degradation in a short period of time (Chaturvedi and Verma 2013). Biomass irradiation by gamma rays is another option that causes glycosidic bond breakage and cell degradation (Orozco et al. 2012). When biomass is subjected to ultrasonication, a phenomenon called bubble collapse occurs by cavitation, resulting in high pressure and heat formation which disrupts the cell wall (Luo et al. 2014). Electron beam irradiation is a pretreatment process that induces a cleavage and depolymerization mechanism within the lignocellulosic biomass structure (Bak 2014). This process can proceed under normal pressure and temperature which is accepted as an advantage (Mehnert 1995).

2.3.2 Chemical Pretreatment

Chemical pretreatment (CPT) is the process where chemicals are used to depolymerize the biomass (Harmsen et al. 2010). The objective of CPT is to enable enzymatic access to fermentable sugars by breaking down the lignin and hemicellulose structures or the related chemical bonds (Badiei et al. 2014). Acid, alkaline, organosolv, ammonia fiber explosion (AFEX), ionic liquids (IL), oxidation, or any combination of those processes is the most applied CPT methods (Harmsen et al. 2010). There exist many studies in the literature regarding CPT of biomass for various biofuel productions and the mechanisms are explained as well (Harmsen et al. 2010; Ulhoff HET 2012). Therefore, this section will focus on some intensively used CPT technologies that were applied for biohydrogen production after a short description of each process (see Table 2.1).

Most of the studies on chemical biomass pretreatment in the literature report dilute acid hydrolysis (AH). Among several acids like HCl, HNO_3, H_3PO_4, and some volatile fatty acids, H_2SO_4 is preferred since it is less corrosive and relatively cheaper (Jeihanipour et al. 2011). The acid used in hydrolysis can be in liquid or solid form (Guo et al. 2012). Dilute AH can be accom-

plished at 100–250 °C, in 0.5–30 min with 0.5–3 % acid concentrations. Concentrated AH usually takes place at room temperature with acid concentrations above 30 % (Taherzadeh and Karimi 2007a). The main disadvantages in AH are toxic by-product (Harmsen et al. 2010) and excess salt formations (Liu et al. 2013). Also corrosion, temperature, and pressure-resistant reactors required are other factors to be considered for process simplicity (Taherzadeh and Karimi 2007a). Toxic compounds like 5-HMF and furfural are produced more during dilute AH due to dehydration of hexose (C_6) and pentose (C_5) sugars at high reaction temperatures (Mosier et al. 2005). Biomass dissolution can proceed faster in concentrated AH (Jung et al. 2013); however, the need of high alkaline dosages for neutralization results in more salt formation than that in dilute AH. During AH, biomass depolymerization occurs due to hemicellulose dissolution and breakage of glycosidic bonds (Taherzadeh and Karimi 2007a). Hydrogen can be produced from the obtained C_5 and C_6 sugar monomers after AH, though C_6 sugars like glucose are preferred by the majority of microorganisms (Lai et al. 2014). The efficiency of AH can be enhanced by optimizing pretreatment operation condition parameters like temperature, pressure, retention time, solid/liquid ratio, and acid dosage (Panagiotopoulos et al. 2011).

Biomass depolymerization can also be done by alkaline pretreatment where NaOH, KOH, and $Ca(OH)_2$ (lime) are used as chemicals (Chaturvedi and Verma 2013). High-temperature alkaline pretreatment, which is also known as the Kraft pulping process, has been in use for years in the pulp and paper industry (Perlack et al. 2005). Lignin and hemicellulose can effectively be removed at hot and alkaline conditions resulting in a recovery of the cellulose for paper production (Sixta and Rutkowska 2006). The recovered cellulose in this way has also been used for biofuel production (Jeihanipour and Taherzadeh 2009). The alkaline treatment is usually applied at high temperature where cellulose cleavage occurs due to scission and cleavage reactions (Knill and Kennedy 2002). This, however, may result in unfavorable toxic 5-HMF and furfural productions due to dehydration of C_5 and C_6 sugars at elevated temperatures (Yin et al. 2011) Those unfavorable conditions could be prevented by integrating the usage of hydrogen peroxide which is known as the alkaline peroxide (ALPER) process that can be operated at room temperature and does not result in toxic by-product formation (Karagöz et al. 2012; Gould and Freer 1984).

2.3.3 Enzymatic Pretreatment

It has been clearly stated that biomass can be converted into fermentable feedstock for biohydrogen production after a suitable physical or chemical pretreatment process. However, it should be realized that physical or chemical biomass pretreatments may require intensive energy, chemicals, and sometimes severe operation conditions resulting in wastewater and toxic by-product formations.

Therefore, selection of an environmentally-friendly and sustainable process is of great importance. In this context, enzymatic pretreatment could be considered as an alternative option to physical and chemical biomass pretreatments.

Microorganisms such as fungi (*Trichoderma reesei*, *Phanerochaete chrysosporium*) and some bacteria (*Clostridium thermocellum*, *Ruminococcus albus*) can produce special proteins that can degrade biomass to liberate fermentable sugars which is known as enzymatic biomass degradation (Rodrigo de Souza 2013). Enzymatic reactions can proceed under mild operation conditions without toxic by-product formation (Verardi et al. 2012). However, this process is slower and more expensive compared with physical and chemical pretreatment processes (Taherzadeh and Karimi 2007b). Enzymes that take part in biomass degradation are diverse. In general cellulose-, hemicellulose-, and lignin-degrading enzymes are cellulases, xylanases, and lignin peroxidases and laccases, respectively (Perez et al. 2002). Cellulases can degrade β-(1,4)-glycosidic bonds of cellulose resulting in glucose and cellobiose formation which can further be used as feedstock for biohydrogen gas production (Carere et al. 2008). Xylanases, on

the other hand, can degrade hemicelluloses into C_5 sugars like xylose, arabinose, or mannose (De Menezes et al. 2010). Mostly, enzymatic hydrolysis is used for C_6 production since the majority of microorganisms can metabolize C_6 sugars rather than C_5 sugars for hydrogen production (Chen et al. 2013). Enzymes can be used in pure form or could be generated by microorganisms spontaneously during the fermentation process. The latter is generally preferred in hydrogen production due to economic and practical considerations.

Table 2.2 summarizes different pretreatments applied to first-, second-, and third-generation biomass resources prior to fermentative hydrogen production. The biomass and hydrolysate content along with experimental pretreatment conditions are presented as well. As can be seen from Table 2.2, there is no single way to treat biomass for hydrogen production. Usually pretreatment starts with physical size reduction followed with diverse combinations of chemical and enzymatic treatments. Also detoxification is usually applied after chemical pretreatments in order to remove toxic by-products like 5-HMF, furfural, salts, and volatile fatty acids from the pretreatment effluents. Detoxification stage is not required in enzymatic pretreatment. Activated carbon adsorption, ion-exchange resin, and lime treatment are the most intensively used detoxification processes among several detoxification methods.

2.4 Biohydrogen Production from Biomass

Biological processes for hydrogen production from carbon-containing materials are achieved mainly by dark fermentation and photofermentation (light fermentation). The major substrates required in the dark fermentation are simple sugars like glucose and sucrose. The substrate for photofermentation is organic acids such as acetic, butyric, and lactic acids. Sequential dark fermentation and photofermentation or combined dark fermentation and photofermentation processes are two-stage fermentation approaches used in order to increase the yield of production. The simple sugars are converted to organic acids in dark fermentation, and then these products are used as substrate in photofermentation.

The main problem in biohydrogen production is the cost of the substrate when pure simple sugars are used for this purpose. In order to solve this problem, the recent trend is the utilization of carbon-rich waste materials or biomasses. Organic wastes must be somehow converted into environmentally acceptable form. The conventional approach to achieve this goal is applying one of the waste treatment or disposal technology in order to convert carbon content of these wastes into CO_2. However, these methods are cost intensive and they are not successful all the time for the complete conversion of their carbon content all the way to CO_2. Using biomass for energy production like hydrogen helps to overcome both substrate requirement of biohydrogen and intensive cost requirement of waste disposal.

Two main stages in biohydrogen production from biomass are hydrolysis and fermentation. The approaches for the realization of these stages are (i) separate hydrolysis and fermentation (SHF), (ii) simultaneous saccharification and fermentation (SSF), and (iii) direct fermentation (DF) or also known as consolidated bioprocessing (CBP).

Separate hydrolysis and fermentation is the process in which biomass is first hydrolyzed by pretreatment methods as mentioned in Sect. 2.3 to obtain fermentable sugars and to remove nonfermentable lignin in a separate unit. Then, the sugar solution is subjected to dark fermentation or photofermentation in a bioreactor for hydrogen production. SHF provides certain advantages. Hydrolysis of complex substrates is the rate-limiting step in most of the bioprocesses, and conducting hydrolysis in a separate process overcomes this problem. The operating conditions in pretreatment can be optimized to reach maximum biomass to sugar solution conversion yield. The desired hydrolysis products as lignin and toxic substance-free sugar solution can be obtained by using appropriate pretreatment methods. Finally, required or optimized sugar concentration with addition of other nutrients, which may be required

Table 2.2 Biomass pretreatment processes for fermentative hydrogen production

Biomass and its composition	Physical pretreatment	Chemical pretreatment	Enzymatic pretreatment	Detoxification	Pretreated product composition	Reference
Rice straw	Shredding and crushing 200 µm	Concentrated acid hydrolysis (55 % H_2SO_4, 40 °C, 2 h)	–	Sulfate removal and neutralization by $Ca(OH)_2$ treatment for 20 h	51.73 g total sugar/L	Liu et al. (2013)
Cornstalk	Milling <40 mesh size	– –	1st step: Lignin and holocellulose removal by *Phanerochaete chrysosporium* for 21 days 2nd step: Enzymatic saccharification by enzymes of *Trichoderma viride* for 4 days	– – –	6 g/L glucose 3.5 g/L xylose 1.1 g/L arabinose 54 mg/L acetic acid No toxic product formation	Zhao et al. (2014)
Water hyacinth (24.9 % cellulose, 23.2 % hemicellulose, 10.1 % lignin 20.4 % protein	Pulverization	Acid pretreatment by microwave heating (1 % H_2SO_4, 140 °C for 15 min)	Cellulase treatment for 120 h (0.15 g, P10 U/mg)	Detoxification by 1.67 g/L activated carbon (37 °C, 180 rpm, 20 min)	17.24 g/L glucose 1.56 g/L acetic acid 1.21 g/L furfural 0.51 g/L 5-HMF 0.076 g/L vanillin	Cheng et al. (2015)
Mixture of cassava starch and *Chlorella pyrenoidosa*	–	Steam heating with dilute acid (1 % H_2SO_4, 135 °C for 15 min)	–	–	13.7 g/L reducing sugar (glucose equivalent)	Xia et al. (2014)

Wheat straw	Hammer milling <0.5 mm	Alkaline pretreatment (7.4 % $Ca(OH)_2$, 20 °C, 48 h)	–	–	0.685 g total carbohydrates/g vs untreated straw	Reilly et al. (2014)
Corn stover 42.2 % glucan 25.7 % xylan 15.6 % lignin 11.0 % extractives, 1.9 % protein, and 3.6 % ash		1st step: acid pretreatment 1.2 % H_2SO_4, 2 h 2nd step: steam explosion (200 °C, 1 min)	– – –	Detoxification by 100 g/L activated charcoal treatment (30 °C, 200 rpm, 60 min) Detoxification by 100 g/L activated charcoal treatment (30 °C, 200 rpm, 60 min)	13.14 g/L glucose 32.32 g/L xylose 2.85 g/L-galactose 4.63 g/L-arabinose 1.97 g/L-mannose	Datar et al. (2007)
Wetland biomass mixture of water arum (*Phragmites* (sp.)) and green reed (*Thalia dealbata*) (37–43 % cellulose, 23–33 % hemicelluloses, and 13–14 % lignin)	Cutting and milling 60 mesh	1st step: steam explosion 2nd step: ionic liquid pretreatment with 1-butyl-3-methyl-imidazolium chloride as ionic liquid ([Bmim]Cl)	Cellulase treatment for 24 h (20 FPU/g cellulase/ substrate)	–	72.11–88.8 % glucose (%w/w biomass) 0.62–1.27 % xylose (%w/w biomass)	Peng et al (2014)
Red algae	Blender milling	Dilute acid pretreatment	–	Detoxification by 100 g/L activated carbon (35 °C, 150 rpm, 6 h)	15 g/L total carbohydrate by	Park et al. (2013)

(continued)

Table 2.2 (continued)

Biomass and its composition	Physical pretreatment	Chemical pretreatment	Enzymatic pretreatment	Detoxification	Pretreated product composition	Reference
(*Gelidium amansii*) (2.4 % agar, 14.9 % cellulose, 15.6 % protein, and 5.7 % ash)	<0.3 mm	1.30 % H_2SO_4 159 °C, 15 min			69.7 % hydrolysis efficiency	
Miscanthus 38.2 % cellulose 24.2 % hemicellulose 25 % lignin 1.3 % protein 4.2 % solvent extract 1.4 % hot water extract 2 % ash	1st step: chopping to 0.5–5 cm 2nd step: extrusion by twin extruder	Alkaline treatment During extrusion (12 % NaOH, 70 °C, 4 h)	Cellulase treatment for 72 h (10 FPU/g glucan)	–	10 g glucose 3.7 g xylose+ Arabinose	de Vrije et al. (2002)
Soybean straw 39.93 % cellulose 14.45 % hemicellulose 23.31 % lignin	Comminution to get 20 mesh	Alkaline peroxide treatment 16 % H_2O_2 and 0.5 % NaOH, 30 min	–	–	18 % total reducing sugar 33 % cellulose 5.5 % hemicellulose 8 % lignin	Han et al. (2012)

Corncob 32 % cellulose 41.8 % hemicellulose 8.6 % lignin and 0.9 % ash	Knife milling 2 mm	Acid steam explosion 0.5 % H_2SO_4, 121 °C, 20 min	Cellulase treatment for 72 h	–	5.1 g/L reducing sugar	Tang et al. (2013)
Wheat straw 35.1 % cellulose 24.8 % hemicellulose and 20.4 % lignin	Milling <2 mm	Ozonation	–	–	564 mg sugar/g dry straw	Wu et al. (2013)
Arthrospira platensis (*Spirulina*) (CYA-1)	–	Ultrasonication (200 W ultrasound, 15 min)	1st step: α-amylase (activity ≥ 50 U/mg) treatment for 2 h (60 °C, 120 rpm) 2nd step: glucoamylase (activity 70 U/mg) treatment for 12 h (60 °C, 120 rpm)	–	407 mg sugar/g dry weight	Cheng et al. (2012)
Cornstalk (37.1 % cellulose, 21.5 % hemicelluloses, and 18.5 % lignin)	Milling 0.5–2 mm	Lime treatment 0.1 g/g biomass, 23 ± 2 °C, 96 h	–	–	40.1 % cellulose 18.5 % hemicellulose 16.7 % lignin	Cao et al. (2012)
Cotton (85 % cellulose, 0 % lignin)	–	Concentrated acid hydrolysis (55 % H_2SO_4, 40 °C, 2 h)	–	Sulfate removal by ion-exchange resin	51.73 g reducing sugar/L	Chu et al. (2011)

for fermentation, can be controlled. The disadvantage of SHF is the cost of pretreatment used for hydrolysis. Selection of a pretreatment method with high yield of sugar formation and free of toxic substances, hydrogen production potentials depending on pretreatment methods, and biomass are the major interests in SHF

In simultaneous saccharification and fermentation, hydrolysis of biomass to sugar solution by corresponding enzymes (see Sect. 2.3.3) and fermentation of that sugar by hydrogen-producing organisms are realized in a single unit. The problem in this approach is the difference in the rate of hydrolysis and fermentation. Low rate of hydrolysis will cause low concentration of sugar formation which could then reduce the rate of fermentation and thus hydrogen formation. Another problem is adjusting the necessary conditions for maximum activity of enzyme in hydrolysis and growth or activity of microorganisms for biohydrogen production. In case raw biomass is used, there is a possibility of the occurrence or existence of some inhibitory substance that could lower the activity of enzyme. A physical or chemical pretreatment stage may be required prior to SSF to make the biomass suitable for hydrolysis reaction. The selection of enzyme with high activity and pretreatment methods are the major concerns in SSF studies.

Direct microbial conversion is achieved in a single unit by using single microorganism culture or consortium of microorganisms which are capable of hydrolyzing and then fermenting the biomass to hydrogen gas. The process is more cost-effective with respect to SSF and SHF since no chemical consumption and external enzyme additions are required for pretreatment and hydrolysis of biomass, respectively. The process would be ideal if only hydrolysis and fermentation are achieved by single organisms without a rate-limiting step. Nevertheless, the problem about the difference in the rate of hydrolysis and fermentation still exists in this approach. The optimal growth conditions could be different when the consortium of organisms as hydrolyzing and fermenting is used. Then, control of operating condition with respect to organism type could be a nuisance which may affect both hydrolysis and fermentation rates. Moreover, sugars obtained from microbial hydrolysis can be consumed by hydrolyzing organisms for growth instead of hydrogen-producing organisms with the result of decreasing hydrogen production potential. One of the strategies developed in order to divert sugar consumption to hydrogen production rather than growth of hydrolyzing culture was "temperature shift" (Lo et al. 2011). Hydrolysis was conducted at 35 °C to obtain sufficient amount of cellulase/xylanase production by *Cellulomonas uda* E3-01, and then temperature was raised to 45 °C to increase the rate of hydrolysis and to prevent the sugar consumption by the hydrolyzing organisms. But, results indicated that sugar was used for growth by hydrolyzing organism even though an effective temperature shift was achieved (Nasirian et al. 2011). Direct microbial conversion studies concentrate on isolation and selection of microbial cultures capable of producing high concentration of hydrolytic enzyme and hydrogen. Cocultures or consortium of either mesophilic or thermophilic organism has been identified and used for direct conversion of lignocellulosic biomass to biohydrogen. It has been stated that production by cocultures is higher than that of monocultures (Ho et al. 2012; Li and Liu 2012; Ren et al. 2008; Liu et al. 2008; Geng et al. 2010).

2.4.1 Dark Fermentative Hydrogen Production from Biomass

Hydrogen production by dark fermentation is realized under the acidogenic phase of the anaerobic process. Methanogenic phase is inhibited to prevent the consumption of hydrogen. In other words, substrate is converted to organic acids preferably acetic and butyric acid, and then no further conversion of these organic acids to methane or CO_2 is desired. Hydrogen production potential of dark fermentation process is evaluated through the yield of formation as mol H_2/mol glucose. Theoretically conversion of 1 mol of glucose yields 12 mol of hydrogen gas (H_2). According to the reaction stoichiometry, bioconversion of 1 mol of glucose into acetate yields 4 mol H_2/mol glucose, but only 2 mol H_2/mol glucose is formed when butyrate is the end product.

Ethanol and lactate are non-hydrogen-forming and propionic acid is a hydrogen-consuming end product of fermentation. The observed yields from glucose are in general around 2.0–2.5 mol H_2/mol glucose (Kapdan and Kargi 2006) which is lower than theoretical estimations. The reasons of low yields are the formation of butyric and acetic acid mixture which closes the yield around 2.5 mol H_2/mol glucose, utilization of substrate as an energy source for bacterial growth rather than desired organic acid generation and hence hydrogen formation, or generation of non-hydrogen-forming or hydrogen-consuming end products. The effects of environmental conditions, media compositions, and microbial culture type to reach the maximum theoretical yield of hydrogen formation from simple sugars have been evaluated in detail (Kapdan and Kargi 2006). The recent challenge in dark fermentative hydrogen production is to develop process technologies for biomass.

Hydrogen production can occur under mesophilic, thermophilic, and hyperthermophilic conditions. The well-known mesophilic hydrogen-producing microorganism species is *Clostridium* sp. which is a Gram-negative, spore-forming bacterium (Chong et al. 2009; Kapdan and Kargi 2006). Pure *Clostridium* sp. can be used in the production. However, anaerobic sludge is pretreated by acid, heat, or chemicals for the formation of *Clostridium* spore and elimination of methanogens. The most common method is the heat treatment in which anaerobic sludge is exposed to high temperature (90–100 °C) for a certain period of time (Ho et al. 2012; Panagiotopoulos et al. 2010; Saraphirom and Reungsang 2010; Fang et al. 2006; Argun et al. 2008a). Then, *Clostridium* spores are activated by adjusting the media composition and environmental conditions for hydrogen production. The recent trend is the utilization of thermophilic and hyperthermophilic cultures for hydrogen production. Some of those used for this purpose are *Caldicellulosiruptor* sp. (Pawar et al. 2013; Panagiotopoulos et al. 2010), *Thermoanaerobacter* sp. (Brynjarsdottir et al. 2013; Cao et al. 2009; Hniman et al. 2011; Phummala et al. 2014), and *Thermotoga* sp. (Nguyen et al. 2008; de Vrije et al. 2002).

2.4.1.1 Biohydrogen Production from First-Generation Biomass

First-generation biomasses are mainly starch and sugar-rich agricultural products such as sweet sorghum, sugar beet, potato, wheat, pumpkin, etc., and their residues after being processed (Ghimire et al. 2015; Argun et al. 2008a; Oztekin et al. 2008; Panagiotopoulos et al. 2010; Saraphirom and Reungsang 2010). Lignin and hemicellulose contents of those biomasses are lower compared with those of second-generation biomasses. Therefore, pretreatment of these biomasses is more effective in obtaining high concentration of fermentable sugar solution. Moreover, formation of fermentation inhibitory substances such as furfural and 5-HMF is not generally encountered.

Hydrogen production potentials of first-generation biomass have been widely studied. Sugar beet is supposed to be a promising energy crop because of its high sucrose and water content. Biohydrogen production by batch dark fermentation of sugar beet juice by *Caldicellulo-siruptor saccharolyticus* under thermophilic condition that resulted in 3 mol H_2/mol hexose was obtained which is comparable with theoretical yield of 4 mol H_2/mol glucose. It was mentioned that sugar beet juice contains some required nutrients such as amino acids (e.g., glutamine, glutamic acid, asparaginic acid, leucine, isoleucine, alanine), organic acids (mainly lactic acid), and inorganic acids (mainly phosphoric acid) for cell growth and hydrogen production (Panagiotopoulos et al. 2010). Another sugar-rich biomass that attracted attention for biohydrogen production is sweet sorghum. Batch dark fermentation of sweet sorghum syrup by anaerobic mixed cultures resulted in maximum hydrogen production potential of 6864 mL H_2/L medium and hydrogen yield of 2.22 mol H_2/mol hexose (Saraphirom and Reungsang 2010). Potato is a starch-rich first-generation biomass. Xie et al. (2008) used potato in two-phase anaerobic fermentation in which hydrogen was produced in the first stage, and methane was produced in the second stage. The effect of enzyme hydrolysis was investigated in this study. The maximum hydrogen yield of 271.2 mL/g TVS was achieved with α-amylase and glucoamylase

hydrolyzed potato feedstock. On the other hand, Mars et al. (2010) studied hydrogen production by the extreme thermophiles *Caldicellulosiruptor saccharolyticus* and *Thermotoga neapolitana* from potato steam peel (PSP) which is a coproduct of the potato processing industry. Untreated PSP and enzyme hydrolyzed PSP were used as substrate for batch fermentation. Amyloglucosidase-hydrolyzed PSP resulted in higher sugar concentration compared to that of α-amylase. The yields from amyloglucosidase-hydrolyzed PSP were 3.4 mol H_2/mol glucose by *C. saccharolyticus* and 3.3 mol H_2/mol glucose by *T. neapolitana*. However, they were 3.5 mol H_2/mol glucose by *C. saccharolyticus* and 3.8 mol H_2/mol glucose by *T. neapolitana* for untreated PSP. Similarly, hydrogen production yield from batch dark fermentation of untreated sweet potato was obtained as 1.24 mol H_2/mol hexose by mix cultures of *Ruminococcus schinkii* and *Lactovum miscens* (Lay et al. 2012). These results indicated that pre-hydrolysis stage is not essential for potato and its peels when hydrolyzing microbial cultures are available during fermentation.

Kargi and his research group investigated hydrogen production from acid-hydrolyzed and partially hydrolyzed wheat powder by boiling. The effects of factors such as substrate concentration (Argun et al. 2008a), media composition (Oztekin et al. 2008; Argun et al. 2008b), fermentation temperature (Cakır et al. 2010), inoculum type (Argun et al. 2009c), bioreactor operation mode in continuous processes (Kargi and Pamukoglu 2009; Sagnak et al. 2010) on biohydrogen yield, and production rate were evaluated. The cumulative biohydrogen formation, hydrogen yield, and formation rate were at maximum at the heat-treated WP concentration of 20 g/L. Higher WP concentrations resulted in lower fermentation performance probably due to substrate and product (VFA) inhibition (Argun et al. 2008a). Wheat powder was nitrogen and phosphorus limited for dark fermentative hydrogen production and required external nutrient addition. The optimal nutrient ratios for boiled wheat powder were determined as C/N = 200 and C/P = 1000 for the maximum yield of formation of 281 mL H_2/g starch (Argun et al. 2008b). When acid-hydrolyzed wheat powder solution was used, the optimum nutrient ratios reaching the maximum hydrogen yield (Y = 2.84 mol H_2 mol/glucose) were C/N = 0.02, P/C = 0.008, and Fe(II)/C = 0.015 (Oztekin et al. 2008). Dark fermentation of acid-hydrolyzed ground wheat under thermophilic conditions (55 °C) was proven to be more beneficial as compared to mesophilic or thermophilic fermentation of boiled wheat powder (Cakır et al. 2010). Another approach used for hydrogen production from wheat was to apply enzyme for hydrolysis (Han et al. 2015a, b). The hydrolysate was fermented at 37 °C by *Biohydrogenbacterium* R3 and anaerobic sludge. The yields of hydrogen formation were 2.34 mol H_2/mol glucose and 1.9 mol H_2/mol glucose, respectively.

2.4.1.2 Biohydrogen Production from Second-Generation Biomass

The hydrogen production potential from lignocellulosic materials depends on applied pretreatment method, microbial culture, and biomass type (Nissila et al. 2014). Pretreatment of lignocellulosic materials increases the porosity, removes lignin, and reduces the cellulose crystalline structure. As a result, cellulose becomes more accessible to be converted into simple sugars through enzymatic hydrolysis during fermentation (Nasirian et al. 2011). It is evident that applying pretreatment or hydrolysis stage enhances hydrogen production (Cui et al. 2009, 2010; Brynjarsdottir et al. 2013; Han et al. 2012; Cao et al. 2012; Sekoai et al. 2013). The yield of production with pretreatment application prior to fermentation could reach up to 3 mol/mol which is 75 % of the theoretical yield (Nissila et al. 2014).

Some of the lignocellulosic materials used for hydrogen production are bagasse (Lo et al. 2011), cornstalk (Cao et al. 2012; Sawatdeenarunat et al. 2015), rice straw (Liu et al. 2013, 2014a, b; He et al. 2014), wheat straw (Nasirian et al. 2011), hemp (*Cannabis sativa*), barley straw, Timothy grass (*P. pratense*) (Brynjarsdottir et al. 2013), soybean straw (Han et al. 2012), oil palm trunk (Hniman et al. 2011), and poplar leaves (Cui et al. 2010) and even mixture of agricultural

waste and organic fraction of municipal wastes (Sekoai et al. 2013). High cellulose and hemicellulose contents rather than lignin are required. Those carbohydrates are easily converted to fermentable sugars as glucose and xylose through hydrolysis. Since lignin is the non-fermentable part of the lignocellulosic materials, hydrogen is not generated from lignin (Ho et al. 2012; Klimiuk et al. 2010; Thomsen et al. 2008; Cheng et al. 2010). Hydrolysis of lignocellulosic materials will result in mixture of glucose and xylose after lignin separation. Although glucose is known as the most preferred carbon source by hydrogen-producing organisms, it was reported that xylose is the preferred sugar over glucose due to higher attainable hydrogen yields per substrate (Prakasham et al. 2009, 2010). The optimal ratio for maximum hydrogen production was suggested as glucose/xylose: two-third (Prakasham et al. 2009, 2010). High xylose utilization for hydrogen production was attributed to metabolic and biochemical nature of inoculum (buffalo dung) which may contain microbial consortia that are well adapted to digest lignocellulosic materials as food. Xylose utilization was improved by using cocultures of *C. beijerinckii* and *Geobacter metallireducens*. The hydrolysates with the lowest glucose/xylose ratio of 1:10 resulted in the highest xylose utilization, hydrogen production, and no accumulation of acetate which could be inhibitory to hydrogen production through metabolic shift from hydrogen generation to solvent generation as ethanol (Zhang et al. 2013). Patel et al. (2015) reported that xylose resulted in the highest hydrogen production and organic acid generation among other carbon sources as cellulose, cellobiose, starch, and sucrose. On the other hand, no significant difference was observed in hydrogen production when xylose, glucose, and xylose–glucose mixture was used as substrate under thermophilic fermentation condition (Hniman et al. 2011). The theoretical yield of hydrogen formation from xylose is 3.33 mol H_2/mol xylose. The observed yield of formations varies depending on the bacterial cultures used. Some of the observed yields from xylose were 0.45 mol H_2/mol xylose with *Bacillus firmus* (Sinha and Pandey 2014) and 0.56 mol/mol by the mix culture of *Caldoanaerobacter subterraneus* and *Caloramator fervidus* (Yokoyama et al. 2007). Better yields from xylose were obtained under thermophilic fermentation conditions such as 1.62 mol/mol by extreme thermophilic mixed culture (Kongjan et al. 2009), 2.09 mol/mol by *Thermoanaerobacterium thermosaccharolyticum* (Khamtib and Reungsang 2012), and 2.24 mol/mol by *Caldicellulosiruptor saccharolyticus* (Kádár et al. 2004).

Acid and alkaline pretreatments are the most common hydrolysis methods applied to second-generation biomass. The hydrolysate content and hydrogen production potential vary depending on acid or alkali pretreatment. Therefore, it is not possible to suggest one of these pretreatment methods superior to the other. The use of cornstalk as substrate for H_2 production has gained special attention due to its abundance. Cellulose, hemicellulose, and lignin content of fresh cornstalk are around 37 %, 17 %, and 20 %, respectively. Alkaline pretreatment with NaOH provided increase in cellulose content to 40 % and hemicellulose to 27 % with the decrease in lignin content to 10 % and the yield of hydrogen was 82.5 mL/g substrate (Amutha and Murugesan 2013). Lime pretreatment of cornstalk provided more hemicellulose and lignin removal compared with cellulose, and maximum yield of hydrogen formation was achieved as 170 mL/g TVS at 0.10 g lime/g raw biomass (Cao et al. 2012). Acid treatment of soybean straw was more effective in obtaining high sugar content with decreasing hemicellulose and lignin than alkaline treatment, and 11-fold increase in hydrogen production was achieved with acid-treated soybean straw compared to that of raw one (Han et al. 2012). Hydrogen production from acid hydrolysis of bagasse mainly by H_3PO_4 was higher than alkaline treatment (Lo et al. 2011). Acid treatment of beer lees was more effective on hydrolysis of hemicellulose than cellulose or lignin, and cumulative hydrogen yield was 17-fold more compared to that of raw beer lees (Cui et al. 2009). Similarly, the increase in the cumulative hydrogen yield of formation from acid-hydrolyzed wheat straw was 136-fold higher than that of raw

wheat straw (Fan et al. 2006). Higher hydrogen formation yields after acid or alkaline pretreatment is due to substantial decrease in mainly lignin content and increase in accessibility of organisms to cellulose for hydrogen production.

Enzymatic hydrolysis of chemically treated lignocellulosic biomass enhances hydrogen production. Nasirian et al. (2011) compared the hydrogen production from fresh wheat straw, with dilute acid pretreatment, by simultaneous saccharification and fermentation (SSF) and by separate hydrolysis and fermentation (SHF). No hydrogen was produced from fermentation of fresh wheat straw. The highest yields and volumes of production were obtained from enzymatic SSF and from fermentation of acid-hydrolyzed wheat straw. Similar results were observed when lime-treated wheat straw was subjected to enzymatic hydrolysis with celluloses, hemicelluloses, and beta-glucosidases in SSF (Reilly et al. 2014). Enzymatic SSF resulted in higher yield of formation (58.78 ± 4.02 mL H_2/g TVS) compared to single-stage alkaline treatment (43.28 ± 3.77 mL H_2/g TVS). The findings from hydrogen production from poplar leaves support these results. Cumulative hydrogen yield from enzymatic hydrolysis was threefold higher than that of raw poplar leaves and 1.34-fold higher than that of substrate pretreated with HCl (Cui et al. 2010).

Utilization of thermophilic organisms for hydrogen production from lignocellulosic materials has recently received more attention. It has been stated that the enzymatic activity is slow at mesophilic fermentation condition which causes slow rate of biomass hydrolysis and low yield of sugar formation. Thermophilic organisms have thermostable cellulose and xylanase enzymes which exert high activity and rate of hydrolysis at fermentation temperatures above 50 °C. On the other hand, low amount of enzyme production is the limitation in thermophilic fermentation (Bhalla et al. 2013). Therefore, pretreatment could be required before fermentation to provide at least partial hydrolysis of biomass. Dark fermentation of acid-pretreated corn stover hydrolysate by thermophilic organism, *T. thermosaccharolyticum*, resulted in 2.24 mol H_2/mol sugar hydrogen formation yield (Cao et al. 2009). Sequential alkali and enzymatic treatment of wooden chopsticks resulted in 195 mL H_2/g total sugar under extreme thermophilic conditions with *Thermoanaerobacterium* sp. (Phummala et al. 2014). Hydrogen production studies from hemp (*Cannabis sativa*), barley straw, and Timothy grass (*P. Pratense*) by *Thermoanaerobacter* sp. showed lower yields on biomass without chemical treatment as compared with acid or alkali-pretreated substrates. The yield obtained from barley straw was the highest and almost ten times more yield was achieved by alkali-treated straw (Brynjarsdottir et al. 2013). Pretreatment stage can be eliminated by using cocultures of thermophilic cellulolytic and hydrogen-producing organisms. These cultures form a cellulose–enzyme–microbe complex (Lu et al. 2006). Cellulolytic bacteria hydrolyze the cellulose to sugars for hydrogen-producing bacteria in the coculture. Some of the studies conducted on coculture hydrogen production revealed that hydrogen production was significantly improved. In most cases, pretreatment of biomass is not required. Direct fermentation of cornstalk powder without any pretreatment was achieved by using thermophilic coculture of *Clostridium thermocellum* and *Clostridium thermosaccharolyticum*. The yield of hydrogen formation with coculture was 94 % more than that of monoculture (Li and Liu 2012). Similarly, a twofold increase in the yield (1.8 mol H_2/mol glucose) was obtained when coculture of *Clostridium thermocellum* and *Thermoanaerobacterium thermosaccharolyticum* (Liu et al. 2008) was used. Cellobiose and glucose accumulation was observed during fermentation with monoculture of *C. thermocellum* indicating that culture cannot completely utilize cellobiose and glucose produced by the cellulose degradation. Utilization of coculture increased cellobiose and glucose consumption, cell division, and organic acid production. This result was supported by coculture of *Clostridium thermocellum* and *Clostridium thermopalmarium* which depend on cellulose and soluble sugar for growth, respectively. The soluble sugars produced by *C. thermocellum* from hydrolysis of cellulose were consumed by

C. thermopalmarium and twofold increase was obtained in hydrogen production compared to monoculture fermentation. However alkali treatment was required at high substrate concentrations (Geng et al. 2010). Ren et al. (2008) identified mesophilic cocultures of *Clostridium acetobutylicum* and *Ethanoigenens harbinense* in hydrogen production. Coculture provided 20 % more cellulose hydrolysis and 2.7 more hydrogen production than that of monoculture of *C. acetobutylicum*.

Particle size of the biomass could be a factor in hydrogen generation by dark fermentation. Hydrogen and organic acid concentrations increased with decreasing particle size of raw wheat straw from 10 to 1 mm (Yuan et al. 2011). On the other hand, Song et al. (2014) used cornstalk as biomass without pretreatment and investigated the effect of particle size on biohydrogen yield by using *Clostridium* sp. FS3 which was isolated from anaerobic acclimated sludge. It was concluded that particle size didn't substantially affect hydrogen yield. The maximum yield was 92.9 mL H_2/g cornstalk under optimized media composition.

Hydrolysis of lignocellulosic materials generates some by-products such as furfural, 5-HMF, phenols, formate, acetate, and other unknown ones (Monlau et al. 2013, 2015). Furfural, 5-HMF, and phenolic compounds are known as inhibitory on hydrogen-producing organisms. Severe alkaline and acid treatments could increase the amount of inhibitory phenolic compounds in the hydrolysate and thereby adversely affect the hydrogen production potential (Cui et al. 2009; Phummala et al. 2014). Detoxification of hydrolysate through resin adsorption could substantially improve the hydrogen production (Rai et al. 2014). Monlau et al. (2015) used with and without alkaline-pretreated sunflower stalks as feedstock in hydrogen production. The yield of formation from fresh stalk (7.1 mL H_2/g TVS) was higher than that of pretreated one (6 ml H_2/g TVS). Lower yield formation from alkaline-pretreated stalks was attributed to inhibition effect of phenolic compounds released from lignin degradation. Similar result was observed when dilute acid hydrolysis was applied to sunflower stalk (Monlau et al. 2013). Hydrogen production yield substantially decreased when hydrolysate volume in the media was 7.5 % at which by-product concentrations in the fermentation media reached to 61 mg/l acetate, 45 mg/L formate, 86.2 mg/L furfural, 9.5 mg/L 5-HMF, and 1.5 mg/L phenolic compounds. A shift of dominant microbial culture from *Clostridium* sp. to *Sporolactobacillus* sp. was observed which indicates specific inhibition of biohydrogen-producing bacteria by hydrolysate. Depending on this population shift, a metabolic shift from acetate/butyrate to non-hydrogen-generating ethanol/lactate was also observed. On the other hand, Liu et al. (2015) reported that although furfural inhibits hydrogen production, 5-HMF enhances. Forty percent higher hydrogen production was obtained from 100 mg/L HMF containing steam-exploded cornstalk hydrolysate in comparison to control experiment without addition of HMF. It was also observed that volatile fatty acid production increased, and 90 % HMF was degraded up to 1000 mg/L HMF concentration during hydrogen formation. Microbial community analysis indicated that HMF stimulated higher proportion of hydrogen-producing *Clostridium* and cellulose-hydrolyzing rumen bacteria *Ruminococcaceae*. It was stated that furfural inhibits hydrogen generation, VFA concentration is not affected by furfural concentration, and it is degraded by anaerobic culture. Similarly, no inhibition effects on hydrogen production up to 0.14 g/L HMF and 1.09 g/L furfural concentrations were observed in pretreated corncob (Nasr et al. 2014).

Most of the hydrogen production studies from lignocellulosic biomass were conducted in small-scale batch fermentation. A summary of hydrogen yields and rates obtained from batch fermentation of different second-generation biomasses with applied pretreatment method is given in Table 2.3. Recently, attentions were diverted to continuously operated bioprocesses to determine optimal operating conditions such as initial substrate concentration, hydraulic retention time (HRT), and organic loading rates for the used second-generation biomass and bioreactor. Hydrogen production potentials of continuously

Table 2.3 Hydrogen production yields and rates obtained from batch dark fermentation of different second-generation biomasses with applied pretreatment method

Biomass	Pretreatment	Culture	T[b] C	H_2 yield	H_2 rate	Reference
Corn stover	Acid	Heat-treated river sediment	37	4.17 mmol/g sugar	0.78 mmol/L/h	Zhang et al. (2014a, b)
Corn stover	Acid	*Thermoanaerobacterium thermosaccharolyticum*	60	2.24 mol/mol sugar		Cao et al. (2009)
Cornstalk	Alkaline	*Bacillus licheniformis* MSU AGM 2	37	80 ml/g substrate	0.14 ml/h	Amutha and Murugesan (2013)
Cornstalk	Wet stream explosion	Anaerobic digested sludge		12 ml/g TVS	–	Liu et al. (2014a, b)
Corncob	Acid	Anaerobic digested sludge	35	265 ml/g COD	–	Nasr et al. (2014)
Corncob	High-pressure autohydrolysis	Anaerobic digested sludge	35	31 ml/g COD	–	Nasr et al. (2014
Wheat straw	Acid and enzyme	Mixed culture	35	1 mol/mol glucose	–	Nasirian et al. (2011)
Wheat straw	Acid and stream explosion	*Clostridium* sp. IODB03	37	2.52 mol/mol sugar	153 mol/L/h	Patel et al. (2015)
Wheat straw	Acid, stream explosion, and enzyme	*Clostridium* sp. IODB03	37	2.62 mol/mol sugar	136 mol/L/h	Patel et al. (2015)
Wheat straw	Acid and microwave heating	Cow dung	36	64 ml/g TVS	–	Fan et al. (2006)
Wheat straw	Acid	Mixed culture	35	1.19 mol/mol glucose	–	Nasirian et al. (2011)
Wood chopsticks	Alkaline and enzyme	*Thermoanaerobacterium*	50	195 ml/g TVS	116 ml/d	Phummala et al. (2014)
Rice straw	Acid	Heat-treated sludge	37	0.44 mol/mol sugar	6.43 ml/h	Liu et al. (2013)

Soybean straw	Acid	*Clostridium buytricum*	35	47.65 ml/g substrate	2.26 ml/h	Han et al. (2012)
Potato and pumpkin	–	BESA-treated anaerobic sludge	35	171 ml/g TVS	–	Ghimire et al. (2015)
Oil palm trunk	Acid and alkaline	*Thermoanaerobacterium*, *Thermoanaerobacter*, *Caloramator*	60	301 ml/g sugar	–	Hniman et al. (2011)
Poplar leaves	Enzyme	*Clostridium pasteurianum*	35	44.9 ml/g poplar leaves	2.17 ml/h	Cui et al. (2010)
Sugarcane leaves	Acid	Anaerobic sludge	37	248.05 ml/g	–	Moodley and Kana (2015)
Waste paper	–	*Ruminococcus albus*	37	282 ml/g paper		Ntaikou et al. (2009)
Miscanthus	Hydrothermal	*Clostridium beijerinckii*, *Geobacter metallireducens*	30		2.43 mmol/L/h	Zhang X et al. (2013)
Beer lees	Acid	*Clostridium pasteurianum*	35	53 ml/g dry beer lees	6.68 ml/h	Cui et al (2009)
Raw cornstalk	–	*C. sartagoforme FZ11*	35	87.2 mL/g cornstalk	6.2 mL/g/h	Zhang et al. (2015)
Sugarcane bagasse	Acid	*Enterobacter aerogenes MTCC 2822*	30		8.3 mL/h	Rai et al. (2014)
Coffee mucilage	–	Swine manure		77.6 mL/g COD	7.6 Nl/L/d	Hernández et al. (2014)
Water hyacinth	Acid	Anaerobic digester sludge	37	134.9 mL/g TVS	90 mL/L/h	Cheng et al. (2015)

stirred tank reactor (CSTR) (Zhao et al. 2013; Liu et al. 2013; Pawar et al. 2013), anaerobic contact filter (Vijayaraghavan et al. 2006), continuously external circulating bioreactor (Liu et al. 2014a, b), upflow anaerobic sludge blanket (UASB) reactor, anaerobic filter (Kongjan and Angelidaki 2010), and anaerobic biotrickling filter (Arriaga et al. 2011; Vargas et al. 2014) from different second-generation biomasses have been investigated. Hydrogen production rates and yields at optimal operating conditions of these continuously operated bioreactors, used substrate, and applied pretreatment method were given in Table 2.4. HRT is one of the most significant operating parameters that should be optimized in a bioprocess to obtain the highest product yield. The optimal HRT of a bioprocess is the factor of substrate type, substrate concentration, environmental conditions, microorganism type, and certainly bioreactor type as immobilized and suspended growth systems. The advantage of CSTR is the presence of homogenous conditions which eliminate substrate limitation and provide easy control of environmental conditions within the reactor. On the other hand, HRTs for CSTR are generally longer than that of immobilized systems due to difficulty in holding slow-growing organism in bioreactor at short retention times and consequently washing out of organism. Accumulation of VFAs, decrease in pH, lower glucose and xylose utilization, more lactic acid production, and washout of organisms are the problems encountered in hydrogen production in CSTR at short HRTs (Zhao et al. 2013; Liu et al. 2014a, b; Kongjan and Angelidaki 2010). Liu et al. (2014a, b) developed continuously external circulating bioreactor (CECBR) with volumetric circulation rate of 9.6 L/min, and a substantial decrease in optimal HRT to 4 h was provided with external recirculation of effluent. Immobilized systems are more advantageous in terms of high biomass holding capacity and ability to operate at lower HRTs without washing out of organisms. There are limited studies on hydrogen production in immobilized systems. Kongjan and Angelidaki (2010) studied UASB and anaerobic filter (AF) system with hydrothermal-treated wheat straw. It was observed that hydrogen production in immobilized bioprocesses can easily be recovered when system was operated in optimal conditions after inappropriate operation period. Efficient toxic substance removal was obtained, a stable long-term acetic acid production was achieved under optimal operation conditions, but lactic acid accumulation occurred when the systems were operated at shorter HRT (Kongjan and Angelidaki 2010). Vargas et al. (2014) examined hydrogen production from acid and enzymatic hydrolysate of oat straw in trickling biofilter. Although there is efficient hydrogen production from enzymatic hydrolysate, production is totally suppressed when acid hydrolysate was used. The reason for the suppression was explained as sugar gap occurred between enzymatic and acid hydrolyses rather than the presence of 5-HMF or furfural in the hydrolysate. On the other hand, no hydrogen production suppression was observed for acid hydrolysate of oat straw in trickling biofilter (Arriaga et al. 2011). The difference in these two studies could be acid hydrolysis condition which results in different sugar contents. By considering the advantages of immobilized systems over CSTR, more studies are needed to evaluate the performance of immobilized systems in hydrogen production from lignocellulosic biomasses.

2.4.1.3 Biohydrogen Production from Third-Generation Biomass

Algae are the main source of third-generation biomass. Low-cost cultivation of algae is possible with no requirement for expensive substrate and energy input (Park et al. 2011). Utilization of algae as substrate for hydrogen production provides CO_2 reduction besides clean and sustainable fuel production. Algae are a CO_2-fixing organism and hydrogen production will require massive cultivation of this microorganism. Therefore, large-scale production of algae offers massive CO_2 reduction from the atmosphere.

Algae can fix CO_2 in the form of carbohydrate, such as glycogen, starch, or other cellular storage materials in cytoplasm and in the form of lipids in cell membrane (Nayak et al. 2014). Direct fermentation of algae biomass is possible (Shi et al. 2011). But, it was stated that biodegradation

Table 2.4 Hydrogen production yields and rates obtained from batch dark fermentation of third-generation biomasses

Biomass	Pretreatment	Culture	T[a] °C	H_2 yield	H_2 rate	References
Chlorella vulgaris	Acid	*Clostridium buytricum* CGS5	37	1.15 mol/mol reducing sugar	246 ml/L/h	Liu et al. (2012)
Chlorella vulgaris	–	Heat-treated anaerobic sludge	60	19 ± 2.94 ml/g TVS	0.123 ml/h	Wieczorek et al. (2014)
Chlorella vulgaris	Enzyme	Heat-treated anaerobic sludge	60	135 ± 3.11 ml/g TVS	3.14 ml/h	Wieczorek et al. (2014)
Chlorella vulgaris	–	TC60 from compost pile	60	4.2 mmol/g TVS	–	Carver et al. (2011)
Chlorella vulgaris FSP-E	Acid	*Clostridium butyricum* CGS5	35	1.42 mol/mol reducing sugar	176.9 mL/L/h	Chen et al. (2015)
Chlorella sp.	–	Heat-treated anaerobic digested sludge	35	7.13 ml/g TVS	0.417 ml/h	Sun et al. (2011)
Chlorella sorokiniana	Acid	Thermophilic mixed culture (*Thermoanaerobacterium thermosaccharolyticum*)	60	2.68 mol/mol hexose	330 ml/L/h	Roy et al. (2014)
Chlorella sorokiniana	Acid	*Enterobacter cloacae* IIT-BT 08	37	9 ± 2 mol/kg COD	148 ml/L/h	Kumar et al. (2013)
Gelidium amansii	Acid	Anaerobic digested sludge	35	1.07 mol/mol sugar added	420 ml/h	Park et al. (2011)
Scenedesmus obliquus	Grinding	Coculture of anaerobic sludge and *Clostridium butyricum*	37	2.74 mol/mol sugar added	154 ml/h	Ortigueira et al. (2015)
Scenedesmus obliquus	Acid	*C. butyricum* DSM 10702	37	2.9 mol/mol sugars	–	Ferreira et al. (2013)
Laminaria japonica	–	*Clostridium* sp.	35	0.92 mol/mol hexose (120 ml/g TVS)	–	Yan et al. (2011)
Anabaena PCC 7120	Enzyme	Thermophilic mixed culture	60	2.68 mol/mol hexose	–	Nayak et al. (2014)
Dunaliella tertiolecta	–	TC 60 from compost pile	60	2.1 mmol/g TVS	–	Carver et al. (2011)
Taihu blue algae	Alkaline	Heat-treated anaerobic granular sludge	35	105 mL/g TVS	–	Yan et al. (2010)

[a]Fermentation temperature

of intact macroalgae cell membranes and walls could be slow and prolong fermentative hydrogen production. For an efficient fermentation, the embedded carbohydrate within the cell has to be liberated properly (Liu and Wang 2014). Therefore, pretreatment of algae or third-generation biomass is the first step in hydrogen production as it was the case for hydrogen production from the first- and second-generation biomasses. Enzymatic, heat, sonication, alkaline, and acid–heat treatments are some of the pretreatment methods that have been applied to algae biomass for hydrolysis purpose (Nayak et al. 2014; Yun et al. 2014; Batista et al. 2014; Liu and Wang 2014; Park et al. 2011).

Hydrogen production from different algae biomasses has been presented in the literature. Nayak et al. (2014) studied thermophilic dark fermentative hydrogen production using pretreated *Anabaena* PCC 7120 as substrate by mixed microflora. *Anabaena* PCC 7120 is a cyanobacterium and the carbon content of it was found to be 48.2 % on dry-weight basis.

It was mentioned that the carbohydrate content of *Anabaena* sp. PCC 7120 was mostly composed of glycogen stored in cytoplasm and several polysaccharides associated with the cell envelope. No direct fermentation of this type of biomass has been accomplished yet. Enzymatic (α-amylase), autoclaving, sonication, and acid–heat pretreatments were applied to algae biomass, and the maximum yield was obtained as 2.68 mol H_2/mol of hexose (6.42 mmol H_2/g COD reduced), by α-amylase pretreatment. Another algae biomass used for hydrogen production is *Chlorella vulgaris*. It is a well-known microalgae and it was supposed to be a potential feedstock for hydrogen production due to its starch content in the cell wall which should be hydrolyzed to make fermentation feasible. A crude hydrolytic extracellular enzyme solution extracted from the H_2-fermented effluent of food waste was used instead of a commercial enzyme for a cost-effective hydrolysis. Carbohydrate content of microalgal biomass was 38.8 g/100 g of cell, and the reducing sugar concentration was ca.6 g/L after the hydrolysis of biomass with enzyme solution. The highest H_2 yield and production rate were 43.1 mL H_2/g dry cell weight and 21.8 mL H_2/L/h, respectively (Yun et al. 2014). Liu et al. (2012) reported 100 % recovery of glucose and xylose by acid hydrolysis of *C. vulgaris* with the maximum hydrogen yield of 1.15 mol H_2/mol sugar. But, alkaline treatment provided only breakdown of the cell wall, and enzymes were required for further hydrolysis of the components to fermentable sugars.

Laminaria japonica, which is a brown macroalgae, was used as substrate for dark fermentative hydrogen production by Liu and Wang (2014). The carbohydrate content of *L. japonica* was 47.6 % and the main carbohydrate types were laminarin (β-glucan) and alginate. Heat, acid, alkaline, and ultrasonication pretreatment methods were applied to *L. japonica*, and the highest cumulative hydrogen volume of 66.7 ml H_2/g was obtained by heat pretreatment, which was approximately sixfold greater than that of raw *L. japonica* (10.0 mL/g). *Gelidium amansii* is a red algae which mainly contains glucose and galactose as the main sugar monomers. Acid-hydrolyzed red algae yielded maximum hydrogen production of 2.8 mol H_2/mol galactose and 1.5 mol H_2/mol glucose (Park et al. 2011). Combined pretreatment of *Chlorella pyrenoidosa* by steam heating with diluted acid and by microwave heating with diluted acid enhanced the dark fermentative hydrogen yield about tenfold compared to single pretreatments like steam heating, microwave heating, and ultrasonication (Xia et al. 2013). *C. pyrenoidosa* was reported to be composed of 26.2 % carbohydrates, 35.2 % proteins, 16.3 % lipids, 9.2 % moisture, and 13.1 % ash, and its low C/N ratio was considered as a limiting factor in dark fermentative hydrogen production. Steam heating with dilute acid-pretreated *Chlorella pyrenoidosa* (CP) was mixed with cassava starch (CS) to increase the C/N ratio. The maximum dark fermentative hydrogen yield of 276.2 mL/g total volatile solids (TVS) from the mixed biomass at C/N molar ratio of 25.3 showed 3.7-fold and 1.8-fold increases, respectively, compared with those from only CP and only CS (Xia et al. 2014). Batista et al. (2014) compared batch fermentative hydrogen production by *Enterobacter aerogenes* ATCC 13048 and

Clostridium butyricum DSM 10702 from dried (5 % moisture) and wet (69 % moisture) *Scenedesmus obliquus*. The main carbohydrate storage of *S. obliquus* was determined as starch and its sugar content was around 30.7 % on dry-weight basis. H_2 yields obtained from batch fermentation by *C. butyricum* were considerably higher than those obtained by *E. aerogenes*, and the highest H_2 yield was 113.1 ml H_2/g TVS algae without any pretreatment of algae. Formation of high hydrogen yields from raw algae was attributed to the fact that *C. butyricum* produces amylases able to hydrolyze starch, which is not possible by *E. aerogenes*. No significant difference in hydrogen production potentials from wet and dried algae biomass was observed for both cultures. It was stated that algal hydrogen production could be cost-effective when the high-energy-demanding drying process could be eliminated. Table 2.4 summarizes applied pretreatment, hydrogen production yields, and rates obtained from batch fermentation of third-generation biomasses.

2.4.2 Hydrogen Production from Biomass by Photofermentation

Hydrogen-producing photofermentative microorganisms are purple nonsulfur bacteria (PNS). *Rhodobacter sphaeroides*, *Rhodobacter capsulatus*, *Rhodospirillum rubrum*, and *Rhodopseudomonas palustris* are some of those bacteria used for hydrogen production (Kars and Ceylan 2013; Kapdan et al. 2009; Argun et al. 2008c). The main substrates for hydrogen production in photofermentation are organic acids such as acetic, butyric, and lactic acids (Kars and Ceylan 2013; Keskin et al. 2011). Photofermentation requires strict environmental conditions and media compositions. Light intensity, light source type, and lighting regime are the major environmental conditions needed to be optimized for maximum hydrogen production by photofermentation (Argun and Kargi 2010a). Fe and Mo are essential elements for hydrogen production and they should be externally added into the fermentation media (Kars and Ceylan 2013). Although theoretically high yield of hydrogen can be obtained from conversion of organic acids to hydrogen, low light conversion efficiency and production volumes are reported obstacles in photofermentative hydrogen production (Azwar et al. 2014).

Photofermentation has a complementary role of dark fermentative hydrogen production despite its strict process requirements and low conversion hydrogen rates. As it was mentioned in Sect. 2.4.1.1, the theoretical yield for glucose conversion to hydrogen is 12 mol H_2/mol glucose. Dark fermentation stage can achieve only, e.g., 4 mol H_2/mol glucose which is one-third of theoretical yield if acetate is the end product. The theoretical yield of hydrogen formation from acetate is 4 mol H_2/mol acetate. Two moles of acetate obtained from dark fermentation of glucose are further converted to H_2 and CO_2 by photofermentation with a total yield of 8 mol H_2/mol. Therefore, photofermentative hydrogen production is considered to be the next stage of dark fermentation in order to enhance the yield for hydrogen formation. The two main bioprocess approaches for this purpose are sequential dark fermentation and photofermentation and combined dark fermentation and photofermentation. On the other hand, there are reports about photofermentation of simple sugars and organic acids obtained from hydrolysis of biomass for hydrogen production. In this case, single-stage photofermentation of biomass hydrolysate is another option in hydrogen production.

2.4.2.1 Single-Stage Photofermentation

Photofermentative organisms cannot use polysaccharides directly as substrate. Therefore, utilization of biomass for photofermentative hydrogen production requires a pretreatment step for the hydrolysis of polysaccharides into monosaccharides. Lignocellulosic biomass hydrolysate may contain organic acids such as acetic, lactic, formic, propionic, and citric acid as well as sugars (Kars and Ceylan 2013; Patel et al. 2015; Pattanamanee et al. 2012; Pattra et al. 2008).

Hydrogen production potential of single-stage photofermentation of biomass hydrolysate has not been studied in detail, yet. There are limited numbers of studies about direct photofermentation of biomass hydrolysate to hydrogen production in literature. In one of those, acid-hydrolyzed waste ground wheat was used as substrate for direct photofermentation by pure culture of *Rhodobacter sphaeroides* cultures. The maximum hydrogen yield of 1.23 mol H_2/mol glucose was obtained with *Rhodobacter sphaeroides* RV at a sugar concentration of 5 g/L (Kapdan et al. 2009). In another study waste barley was acid hydrolyzed and the hydrolysate was subjected to photofermentation directly by *Rhodobacter sphaeroides* O.U.001 without a dark fermentation stage.

The starch content of waste barley in that study was 37 % and other components were 2 % fat and 11 % protein. Acid hydrolysate of waste barley contained mainly acetic, lactic, formic, propionic, and citric acid besides sugar. The maximum volumetric hydrogen production was 0.4 L H_2/L culture. The ammonia concentration in the hydrolysate was lower than inhibitory concentration, but the addition of Fe and Mo was required for an effective fermentation (Kars and Ceylan 2013). Zhang et al. (2014a, b) reported hydrogen production from different types of pretreated milled agricultural residues like corn stover, corncobs, sorghum stover, soybean stalks, cotton stalks, and rice straw by photofermentation. After the enzymatic hydrolysis of feedstock with Solarbio cellulase, agricultural residue hydrolysates were used as substrate in photofermentation by a mix inoculum of *Rhodospirillum rubrum*, *Rhodobacter capsulatus*, and *Rhodopseudomonas palustris*. Maximum cumulative hydrogen yield of 229 mmol H_2/L culture and hydrogen production rate of 5.97 mmol H_2/L/h were obtained with corncob. Hydrolysis of oil palm with acid treatment resulted in a hydrolysate containing mainly glucose, xylose, and acetic acid. Hydrogen production from hydrolysate by direct photofermentation with *R. sphaeroides* S10 was highly affected by media composition such as Fe, Mo, and yeast extract. The maximum production rate obtained at optimized media composition was 22.4 ml H_2/L/h (Pattanamanee et al. 2012).

2.4.2.2 Sequential Dark and Photofermentation

Organic acids required for photofermentative hydrogen production can be generated by dark fermentation of mono- and polysaccharide containing biomass. The process may require a pretreatment like acid, alkaline, and enzymatic hydrolysis for polysaccharides to monosaccharides and lignin separation from biomass as the first step. The second step is dark fermentation of sugars for hydrogen organic acid production. This step is followed with the utilization of dark fermentation effluent as substrate for photofermentative hydrogen production.

The optimal growth and hydrogen production conditions for dark and photofermentative organisms are different. Running the process in two different reactors provides better control of corresponding optimal conditions. For example, optimal hydrogen production in photofermentation occurs at 30 °C. However, dark fermentation requires higher temperatures like 37 °C or 50 °C. A cooling stage of dark fermentation effluent is required especially in thermophilic dark fermentation. Initial organic acid requirement of photofermentative organisms is generally lower than the amount of organic acids produced in dark fermentation. High organic acid concentrations generated during dark fermentation could be inhibitory to photofermentative organisms (Oh et al. 2004; Rai et al. 2014; Ozkan et al. 2012). The effluent of dark fermentation can be diluted to the required level of organic acids for photofermentative organism to eliminate organic acid inhibition. Similarly, high ammonia concentration could inhibit photofermentative hydrogen production. Argun et al. (2008c) reported the optimum TVFA and NH_4–N concentrations in dark fermentation effluent of ground wheat as 2350 and 47 mg/L. Özgür and Peksel (2013) suggested the maximum acetic and ammonia concentrations as 30–40 mM and 2 mM, respectively. Ammonia concentration can be decreased to certain levels by applying ammonia separation techniques or by diluting the effluent before photofermentation.

Sequential dark fermentation and photofermentation of first- and second-generation biomasses have been widely studied. Pretreated

barley straw was first subjected to dark fermentation with hyperthermophilic bacteria *Caldicellulosiruptor saccharolyticus* and then photofermentation with *Rhodobacter capsulatus*. Dark fermentation effluent was composed of mainly acetate with relatively low concentrations of lactate and formate. It was reported that external additions of Fe and Mo are crucial in photofermentative hydrogen production (Özgür and Peksel 2013). Hydrogen production from beet molasses by sequential dark fermentation and photofermentation resulted in 13.7 mol of H_2/mol of sucrose production yield which corresponds to 57 % of the theoretical yield of 24 mol of H_2/mol of sucrose. The effluent of dark fermentation effluent was ammonia, Fe, and Mo deficient and external addition of those elements were required for photofermentation (Özgür et al. 2010). Laurinavichene et al. (2010) reported total yield of 5.6 mol H_2/mol glucose from potato homogenate without pretreatment where the contribution of dark fermentation and photofermentation was 0.7 mol H_2/mol glucose and 4.9 mol H_2/mol glucose, respectively. The effluent of dark fermentation was supplemented with Fe/Mg/phosphate nutrients and diluted (≤10 % in water) to decrease organic acid and nitrogen to non-inhibitory level for photofermentative organisms. Sugar beet was first subjected to thermophilic dark fermentation and then to photofermentation in an outdoor process. Dark fermentation effluent was cooled to 30 °C, diluted three times to adjust the acetate to non-inhibitory concentration of 40 mM, and supplemented with potassium phosphate, Fe, and Mo. The total yield obtained in the two-stage process was 77 % of the theoretical yield (Ozkan et al. 2012). Rai et al. (2014) studied biohydrogen production from sugarcane bagasse (SCB) by integrating dark fermentation and photofermentation. Acid-hydrolyzed SCB contained glucose, xylose, arabinose, and inhibitors such as furfural, 5-HMF, and ferulic compounds. Total volatile fatty acid-rich media obtained from dark fermentation of acid hydrolysate was used as substrate for photofermentation by *Rhodopseudomonas* BHU 01. The total hydrogen production potential was 1753 mL H_2/L.

2.4.2.3 Combined Dark Fermentation and Photofermentation

Hydrogen production by combined dark fermentation and photofermentation from biomass is realized in a single reactor which contains both dark fermentative and photofermentative organisms. The theoretical background of this approach can be explained as that organic acids produced in dark fermentation are simultaneously converted to hydrogen gas by photofermentative organisms. The process offers distinct advantages over single-stage dark fermentation or photofermentation due to the prevention of organic acid accumulation by photofermentative bacteria. However, the process has to be operated at convenient fermentation conditions both satisfying dark fermentative and photofermentative microorganisms. Some important parameters affecting hydrogen production performance in combined dark fermentation and photofermentation are the proper selection of microbial culture, dark fermentative/photofermentative biomass ratio, initial biomass and substrate concentration, light source, lighting regime, and light intensity. The effects of those parameters on hydrogen gas production from waste wheat powder, for example, have been reported in the literature. Among various combinations of dark fermentative and photofermentative microorganism, compositions using heat-treated anaerobic sludge with *Rhodobacter sphaeroides* NRLL-1727 reported the most convenient culture combination for hydrogen production from waste wheat hydrolysate by combined fermentation (Ozmihci and Kargi 2010). Argun et al. used a combination of heat-treated anaerobic sludge with four types of photofermentative bacteria *Rhodobacter sphaeroides* (NRLL-1727), *Rhodobacter sphaeroides* (DSMZ-158), *Rhodopseudomonas palustris* (DSMZ-127), and *Rhodobacter sphaeroides* (RV) for combined fermentation of wheat powder solution and reported that the optimum initial dark fermentative/photofermentative biomass ratio as 1:7 (Argun et al. 2009a). In another study, the same group investigated the effects of initial biomass and wheat powder concentrations on hydrogen formation yield and rate by using the same culture composition. Optimum initial

biomass and wheat powder concentrations resulting most convenient hydrogen yield and rate were determined as 5 g wheat powder/L and 1.1 g total biomass/L, respectively (Argun et al. 2009b). Same authors reported that using halogen lamp as light source with 670 w/m2 continuous light illumination was the most effective option compared with other light sources such as tungsten, incandescent, fluorescent, and infrared lamps (Argun and Kargi 2010a). Hydrogen gas production from waste wheat powder solution by combined fermentation was also produced in a continuously operated annular hybrid reactor where *Clostridium beijerinckii* DSM-791 and *Rhodobacter sphaeroides* RV were used as inoculum (Argun and Kargi 2010b). The reactor was continuously illuminated with halogen and fluorescent lamps at light intensity of 10 klux resulting in 85 mLH_2/g starch (Argun and Kargi 2010b). The highest hydrogen formation yields and rates from waste wheat powder by the abovementioned studies were 1.45 mol H_2/mol glucose and 50.26 ml H_2/g cells/h, respectively. However combined fermentation studies with yields up to 7.1 mol H_2/mol glucose have been reported in the literature (Asada et al. 2006).

2.4.3 Challenges in Biohydrogen Production from Biomass

Hydrogen is one of the best choices in terms of renewable energy production. The method used for hydrogen production should provide high productivity and high purity at an affordable cost. Physicochemical methods are highly efficient in productivity and in purity of hydrogen but not cost-effective due to high energy requirement in the production line. Biological methods for hydrogen production have received considerable attention in the last decades since bioprocesses operate under mild conditions (25–35 °C, 1 atm) which make the process cost-effective. The other factor that attracts attentions in biohydrogen production is the utilization of organic residues which need high technologies to handle safely or to convert into environmentally acceptable form. The limitations in this approach are somewhat lower rate and yields of production as compared to the other methods. Therefore, the challenges in biohydrogen production are to develop strategies in order to increase the yield and rate. The main obstacles to achieve high rates and yields have been summarized as partial pressure of hydrogen gas in the produced gas mixture, competing reactions, research needs to develop bioprocess technology, insufficient active hydrogenase enzyme, and efficient hydrogen-producing culture (Hallenbeck 2009). *Clostridium* sp. produces two moles of more hydrogen during glycolysis of glucose by reoxidizing NADH. Redox potential difference between hydrogen and NADH/NAD couple makes this reaction unfavorable. Therefore, one of the solutions is to reduce partial pressure of hydrogen appreciably to derive electrons form NADH (Hallenbeck 2009). The simplest method to decrease partial pressure is to sparge inert gas such as argon and nitrogen. However, more cost-effective or technological methods should be developed to overcome this problem.

The reactions that reduce hydrogen production are metabolic shift from acetic acid generation to solvent or hydrogen-consuming organic acid generation and consumption of hydrogen by uptake hydrogenase and homoacetogens (Hallenbeck 2009; Mathews and Wang 2009). Elimination of uptake hydrogenase has minor effect on hydrogen production. Therefore, studies should be devoted to the development of methods to eliminate homoacetogens and enhance acetic acid production by controlling process conditions or through metabolic engineering.

Commercial production of hydrogen from lignocellulosic biomasses can be achieved by the development of bioreactors. It is evident that immobilized systems are superior over suspended growth systems in hydrogen production (Azbar and Kapdan 2012). The studies on hydrogen production in immobilized system should be diverted to the selection of microbial support particle type or immobilization methods. Support particle characteristics should include high surface area, high porosity, high biomass holding capacity, no toxic component, and low cost. The main

immobilization techniques are entrapment in a porous material, attachment on a surface, containment, and self-aggregation. Both support particle type and immobilization technique should be suitable for effective substrate diffusion from liquid phase into biofilm and hydrogen separation from liquid phase to gas phase. Optimization of operating parameters specific to immobilization method or support particle and substrate type is essential. HRT, organic loading rate, and substrate concentration are the major operating parameters. Bioreactors should provide high yield and rate for hydrogen production with efficient substrate removal at short HRTs, high substrate concentrations, or organic loadings for the sake of process economy. Characteristic of lignocellulosic hydrolysate after applied pretreatment is another factor that affects hydrogen production potential of a bioreactor. The effect of glucose, xylose, arabinose, or toxic substance concentrations (5-HMF, furfural, or phenols) and glucose to xylose ratio in the hydrolysate on hydrogen production in studied bioreactor should be considered. Another factor is bioprocess development to convert substrate into hydrogen for target hydrogen yield of 12 mol/mol glucose. Two-stage biohydrogen production as dark fermentation and photofermentation is the first approach to achieve this yield. However, photofermentation needs extensive studies to overcome the problems that limit the expected performance of the process to reach its theoretical potential. Therefore, biohydrogen process needs development of an efficient and low-cost second-stage fermentation process to valorize dark fermentation effluent for hydrogen production and to obtain processed water which does not require further treatment before discharging to receiving media. Although it is desired to have a biological process as second stage, alternatively, methods such as electrohydrolysis can be applied to dark fermentation effluent to obtain high-purity and high-volume hydrogen (Tuna et al. 2009).

Isolation of hydrogen-producing microorganisms with high hydrogen production ability is the major challenge. Mesophilic cultures might be preferred due to lower heating requirement and hence consumption of less energy for the process. However hydrogen production ability of thermophilic organisms is the utilization of thermophilic hydrogen-producing cultures. Thermophilic organisms have thermostable cellulose and xylanase enzymes which exert high activity and rate of hydrolysis at fermentation temperatures above 50 °C. On the other hand, although the yield of thermophilic hydrogen production is higher than mesophilic ones, volumetric productivities are comparable. Therefore, more researches on thermophilic hydrogen production are needed to evaluate yields and rates if it is worth to spend energy for heating to obtain relatively higher yields by thermophilic fermentation.

Another problem in biohydrogen production is the purity of hydrogen in gas phase. Hydrogen purity varies between 30 % and 60 % according to available literature (see Table 2.5). Sparging the head space with an inert gas to reduce hydrogen partial pressure causes further dilution in hydrogen percentage. Simultaneous production and separation of hydrogen by using selective membranes could help both reducing hydrogen partial pressure and increasing purity of hydrogen (Neves et al. 2009).

Pretreatment of lignocellulosic biomasses is the major cost-intensive stage of biohydrogen production. Although there are different methods, each has its own merit and limits. Acid, enzyme, and hydrothermal pretreatments are still the most common methods used for lignocellulosic biomasses. Recent trend is the utilization of ionic liquids for pretreatment. Effective separation of lignin, obtaining pure cellulose and hemicellulose, no toxic substance generation, recovery, and repeated use of liquids without substantial decrease in the separation of cellulose and hemicellulose are the major advantages of ionic liquids. One of the disadvantages of ionic liquids is their cost due to small amount of production. Large-scale utilization of these chemicals, for example, for pretreatment of lignocellulosic biomass for hydrogen production will help to commercialize them and reduce their cost. Another approach is to eliminate pretreatment stage completely. Direct fermentation of lignocellulosic material by coculture and solid

Table 2.5 Hydrogen production yields and rates obtained from dark fermentation of different second-generation biomasses by continuously operated bioreactors

Biomass	Pretreatment	Bioprocess	Culture	T[b] C	Substrate conc.	HRT h	% H_2	H_2 rate	H_2 yield	Reference
Rice straw	Acid treatment	CECBR	Heat-treated anaerobic sludge	40	20 g TS/L	8		5.52 L/L/d	0.72 mol/mol hexose	Liu et al. (2014a, b)
Rice straw	Acid treatment	CECBR	Heat-treated anaerobic sludge	40	20 g TS/L	4		16.32 L/L/d	1.02 mol/mol hexose	Liu et al. (2014a, b)
Cornstalk	Fungal and enzymatic	CSTR	*T. thermosaccharolyticum* W16	60	6.9 g glucose/L 3 g xylose/L	50	60.6	8.4 mmol/L/h	1.9 mol/mol sugar	Zhao et al. (2013)
Wheat straw	Steam–acid pretreatment and enzymatic hydrolysis	CSTR	*C. saccharolyticus* DSM 8903	37	6.7 g glucose/L 3.7 g xylose/L 0.4 g arabinose/L	20		5.09 L/L/d	3.43 mol/mol sugar	Pawar et al. (2013)
Wheat straw[a]	Acid treatment	CSTR	Heat-treated anaerobic sludge	37	20 g TS/L	4	30–40	10 L/L/d	0.69 mol/mol hexose	
Wheat straw	Hydrothermal	CSTR	Mixed extreme thermophilic culture	70	3.9 g glucose/L	72	41	241 ml/L/d	188 ml/g sugar	
Wheat straw	Hydrothermal	UASB	Sterilized methanogenic granules	70	3.9 g glucose/L	24	43	821 ml/L/d	212 ml/g sugar	Kongjan and Angelidaki (2010)
Wheat straw	Hydrothermal	AF	Mixed extreme thermophilic culture	70	3.9 g/glucose/L	24		488 ml/L/d	126 ml/g sugar	
Oat straw	Acid	TBF	Heat-treated anaerobic granular sludge	28	1.2 g COD/L	24	45	81.4 mL/L/h	2.9 mol/mol hexose	Arriaga et al. (2011)
Oat straw	Enzyme	TBF	Heat-treated triticale silage	35	5 g COD/L	12		26 ml/L/h	2.3 mol/mol sugar	Vargas et al. (2014)
Oat straw	Acid						66	71 ml/L/h	0.59 mol/mol sugar	Vargas et al. (2013)
Oat straw	Enzymatic	ASBR	Heat-treated anaerobic granular sludge	35	4.5 g COD/L	8	50	29.6 ml/L/h	0.81 mol/mol sugar	
Oat straw	Acid and enzyme						58	27 ml/L/h	0.38 mol/mol sugar	

TS total sugar, *CECBR* continuously external circulating bioreactor, *CSTR* continuously stirred tank reactor, *UASB* upflow anaerobic sludge blanket reactor, *AF* anaerobic filter, *TBF* trickling biofilter, *ASBR* anaerobic sequencing batch reactor

[a]Wheat straw hydrolysate was mixed with food industry wastewater

[b]Fermentation temperature

state fermentation are two strategies for this purpose to reduce the cost of production. These approaches need detailed investigations to determine process control parameters and to select microorganism types.

2.5 Conclusions

Hydrogen is certainly accepted as an energy carrier and alternative to fossil fuels. The development of economical large-scale hydrogen production is required to replace hydrogen with fossil fuels as one of the main energy sources. It is evident that cost-effective hydrogen production cannot be achieved by energy-intensive processes and by using fossil fuels as raw material which are already limited and highly competitive for the generation of other energy sources. Sustainable, renewable, and green energy demands address biohydrogen production from biomasses. Generation of biohydrogen from, especially, second- and third-generation biomasses combines these three concepts along with providing economical process. Therefore, hydrogen production from biomass by fermentative processes is a viable alternative to existing physical and chemical methods. Process limitations or obstacles are well known, and intensive studies are conducted by researchers to overcome all these obstacles and to make biohydrogen competitive with the conventionally produced ones. Other areas that need extensive effort are commercialization and adaptation of hydrogen sector to biohydrogen. It is necessary to transfer research outcomes into practice. Interdisciplinary studies with participants from sectors could accelerate application of biohydrogen technology in large scale.

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