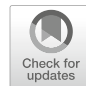
Chapter 5
Biohydrogen Production from Lignocellulosic Feedstocks Using Extremophiles



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What Will You Learn from This Chapter?

- Different pretreatment methods for hydrogen production from lignocellulosic biomass
- Microbial process for hydrogen production
- Thermophilic hydrogen production from untreated lignocellulosic biomass
- Mechanism of extremophilic microbial hydrogen production

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5.1 Introduction

Fossil fuels are the major energy provider for current economy and day-to-day life (Demirbas 2007). Maximum percentage of fossil fuel like petrol, diesel, compressed natural gas (CNG), and liquefied natural gas (LPG) are used in the transportation sector. Fossil fuels also emit the greenhouse gases (GHG: CO, CH_4, CO_2), which affect the environment adversely. Therefore, researchers around the world are focusing on finding the alternate energy resources, which are environmental friendly and renewable in nature (Singh et al. 2013a, b). Hydrogen (H_2) is one such attractive energy source, which has the highest energy yield (calorific value of 143 MJ/kg) among any known fuel. It can be easily transported through conventional means and has been accepted globally as environmentally safe energy resource (Das 2009). Due to high current global demand for H_2 (>45 million tons per annum), a vast array of physical, chemical, physiochemical, and biological processes is currently being employed for H_2 production (Rittmann and Herwig 2012). These include water electrolysis, steam reformation, catalytic steam gasification of biomass, biomass pyrolysis, supercritical water gasification, photolysis of water, and microbial fermentation.

To date, however, 96% of the current H_2 supply comes from fossil fuels (49%, natural gas; 29%, crude oil; and 18%, coal) through steam reforming, and 4% H_2 comes through electrolysis as shown in Fig. 5.1 (Evers 2008; Suresh et al. 2010). Fossil fuel reforming generates greenhouse gases and is not renewable. On the other hand, the biohydrogen ($BioH_2$) production process is eco-friendly (nonpolluting in nature), generates no GHG, and renewable as it can be produced from biomass. Therefore, generating H_2 from microbial origins can meet the requirements of a viable biofuel prospect, providing a cost-effective, pollution-free, and energy-saving alternative to current production practices. Several options for the biological production of H_2 are being investigated such as biophotolysis of water through microalgae and

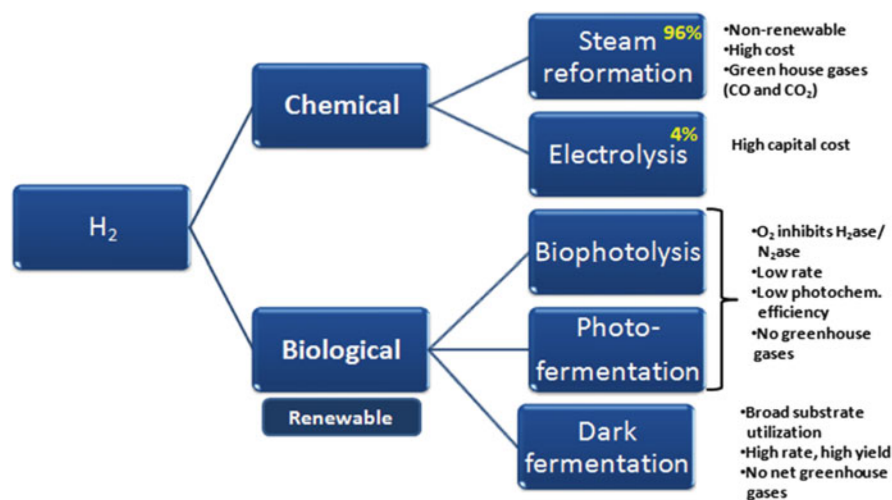


Fig. 5.1 Hydrogen production methods

cyanobacteria, the use of photosynthetic bacteria for the photo-fermentation of organic substances, and dark fermentation of organic substances by anaerobic organisms. The last approach, i.e., dark fermentation, is generally preferred because it does not rely on the availability of light sources. The major advantages of dark fermentation are (1) its simplicity of reactor design, (2) process operation, (3) its wide variety of feedstocks utilization, and (4) higher H_2 production rates compared to other biological methods of H_2 production (Kumar et al. 2015; Saripan and Reungsang 2013).

The utilization of biomass for energy, food, and chemical could solve waste disposal problems and help in finding the alternate route to meet the future energy demands by providing a convenient and renewable source of energy (Ragauskas et al. 2006). Therefore, it has been proposed that the use of inexpensive renewable resources such as lignocellulosic biomass (LCB) for $BioH_2$ production, especially by using extremophiles, can fulfill the huge demand of future energy supply (Lynd et al. 2008). These LCB must be first pretreated in order to remove lignin and hemicelluloses and to increase the surface area of material to enhance the release of sugars (Mosier et al. 2005).

Pretreatment methods improve the fermentability of LCB by overcoming recalcitrance of the lignocellulosic complex by altering its structure, which makes the cellulose and hemicelluloses accessible to the enzymes (Mosier et al. 2005). One of the methods of interest is pretreatment with an alkaline agent at relatively low temperatures (<100 °C). There are few reports on thermophilic bioprocessing (≥ 55 °C) of LCB, which results in higher $BioH_2$ yield as compared to mesophilic conditions (30–40 °C), due to favorable thermodynamical conditions at high temperatures and reduced variety in by-product formation (de Vrije and Claassen 2003; Hallenbeck 2005; Jones 2008). However, the rate of production of $BioH_2$ from complex substrates using thermophiles is too low to be commercially viable. Further, many thermophilic bacteria, including *Thermotoga neapolitana* and *Caldicellulosiruptor saccharolyticus*, can utilize different substrate ranging from simple sugars to complex carbohydrates (Blumer-Schuette et al. 2008). Several investigators have reported a $bioH_2$ yield, closer to theoretical yield 4.0 mol-H_2/mol-glucose by using extremophiles (Munro et al. 2009; d'Ippolito et al. 2010). The use of elevated temperature to produce $BioH_2$ offers several benefits including higher mass transfer rates leading to substrate solubility, enhanced hydrolysis of LCB, and lower risk of microbial contamination.

5.2 Lignocellulosic Biomass and its Preprocessing

Plant cell walls are the major source of renewable biomass with annual production of 150–170 $\times$ 10^9 tones (Pauly and Keegstra 2008). Few studies have been reported to produce $BioH_2$from the feedstocks such as agricultural wastes and other waste materials. Plant cell wall is the major source of LCB including solid materials such as wheat straw waste (Fan et al. 2006), delignified wood fibers (Levin et al. 2006), and other cellulose waste materials (Magnusson et al. 2008). Other biomass like corn stalks and corn stover have also been used for $BioH_2$ production followed by pretreatment and hydrolysis to obtain soluble biomass with mixture of sugars and oligosaccharides (Datar et al. 2007; Ren et al. 2008a, b).

Conversion of LCB includes hydrolysis of feedstock to produce reducing sugars and production of H_2 and higher valuable products via fermentation. Porosity of waste materials, crystallinity of cellulose fiber, and lignin and hemicelluloses content affect the hydrolysis of LCB (McMillan 1994). Two classes of processing strategies have been explored in a way so that the hemicellulose and cellulose fractions can be processed together or separated and processed individually. Due to the close association of cellulose and hemicellulose with lignin in the plant cell wall, pretreatment is necessary to expose the cellulose present in the plant biomass (Radeva et al. 2012; Behera et al. 2014). The goal of the pretreatment process is to break down the lignin structure and disrupt the crystalline structure of cellulose, so that the acids or enzymes can easily access to hydrolyze the cellulose (Mosier et al. 2005). The process can enhance the bio-digestibility of the wastes for $BioH_2$ and ethanol production and increase accessibility of the enzymes to the materials. It results in enrichment of the difficult biodegradable materials and improves the yield of $BioH_2$ from the wastes (Taherzadeh and Karimi 2008).

5.2.1 Physical Treatment

The mechanical disruption (such as grinding, milling, or chipping) of LCB is an environmentally friendly pretreatment process. The milling pretreatment has several advantages for lignocelluloses, including the higher accessible surface area, decreased crystallinity, and no loss of low molar mass components (Lin et al. 2010). In mechanical pretreatment, reduction of particle size and crystallinity of lignocellulosic feedstock should be achieved in order to increase the specific surface and reduce the degree of polymerization. It includes chipping, grinding, or milling depending on the final particle size of the material (10–30 mm after chipping and 0.2–2 mm after milling or grinding). Different types of milling processes such as ball milling, two-roll milling, hammer milling, colloid milling, and vibro-energy milling can be used to improve the biodegradability of LCB. Irradiation processes such as gamma and electron rays can also be used to break the lignocellulose structure by changing physical and chemical structure of cellulose (Shin and Sung 2008).

5.2.2 Physicochemical Treatment

Physicochemical methods include steam explosion, hot water treatment, ammonia fiber explosion, wet oxidation, and CO_2 explosion. Steam explosion method breaks the LCB by sudden release of applied pressure. However, it is necessary to maintain the reaction condition (temperature, moisture content and chip size, etc.) in order to reduce the inhibitor production. In the AFEX method, biomass is treated with liquid ammonia at high temperature and pressure, whereas CO_2 explosion method includes the use of supercritical fluid under high pressure, where lignin solubilizes effectively. In liquid hot water (LHW) treatment, hot water at a temperature of 160–240 °C is used to remove the

lignin and to hydrolyze the hemicelluloses. However, due to recondensation of soluble components, complete delignification is not possible (Cara et al. 2007). Okuda et al. (2008) used the liquid hot water method at 100–300 °C for 30 min to treat the green algae (*Monostroma nitidum*) and red algae (*Soleria pacifica*). Maximum glucose yield from cellulose using enzymatic hydrolysis were 79.9% and 87.8% for *M. nitidum* and *S. pacifica*, respectively. Mosier et al. (2005) reported the increase of enzymatic hydrolysis of corn stover with controlled pH by using liquid hot water pretreatment. Maximum glucose yield of 90% was obtained using 16% slurry of corn stover at 190 °C for 15 min. In addition, by using liquid hot water pretreatment (230–240 °C for 2–15 min) prior to enzymatic saccharification process, the glucose yield of the poplar (*Populus nigra*) biomass can be improved by 60% (Negro et al. 2003).

5.2.3 Chemical Treatment

This method involves the chemical reactions for disruption of the LCB structure, which includes acid treatment, alkali treatment, liquid hot water, ionic liquids, organosolv, and ozonolysis. In acid treatment, the LCB is hydrolyzed to fermentable sugar by using both dilute and concentrated acid. Sulfuric acid is the most used acid for the acid pretreatment process followed by nitric acid, hydrochloric acid, and phosphoric acid. In alkali treatment, lignin is removed from biomass along with acetyl and other uronic acid substitutions, which decreases the accessibility of enzyme to the cellulose surface (Chang and Holtzapple 2000). In ionic liquid (IL) methods, there is a formation of electron donor-electron acceptor complexes (EDA) which interacts with ionic liquids. ILs such as imidazolium salts like 1-n-butyl-3-methylimidazolium chloride (BMIMCl), N-methylmorpholine-N-oxide monohydrate (NMMO), 3-methyl-N-bytylpyridinium chloride (MBPCl), 1-allyl-3- methylimidazolium chloride (AMIMCl), and benzyldimethyl (tetradecyl) ammonium chloride (BDTACl) are used for fractionation of biomass (Lee et al. 2009; Li et al. 2009). In ozonolysis method, the lignin and hemicelluloses present in the feedstock like wheat straw, rice straw, etc. are degraded by using ozone. This method does not produce any toxic inhibitor and is carried out at room temperature and pressure. However, this method is expensive because large quantity of ozone is required for efficient pretreatment.

5.3 Microbial Routes for BioH_2 Production from Lignocellulosic Biomass

BioH_2 can be produced by biophotolysis of water by blue-green algae, dark fermentation in anaerobic conditions, and photo-fermentation by photo heterotrophic bacteria. BioH_2 production by microbial routes has been studied for the last few years using polysaccharide derived from plants. During the dark fermentation, the anaerobic culture utilizes the carbohydrates to produce bioH_2 in mesophilic (25–55 °C),

thermophilic (60–75 °C), or hyperthermophilic (75–90 °C) environment. Higher yields of H_2 have been obtained by using thermophilic and mixed hyperthermophilic microorganisms with different simple sugar as well as complex substrates (Table 5.1). Thermophiles or hyperthermophiles contain membrane-bound NADPH-dependent hydrogenase enzyme which is responsible for the thermodynamic feasibility of the process and higher yields of H_2. There are two methods available for the conversion of LCB into bioH_2 production including direct process and two stage process. In direct process, single microorganism is capable to hydrolyze the cellulose/hemicellulose and to produce bioH_2 in a single step; whereas in two stage process, the cellulose is hydrolyzed by pure or mixed cultures, and H_2 is produced using the different culture separately.

It has been reported that extremophiles can effectively act on pretreated biomass to produce H_2. In a study, thermophiles *C. saccharolyticus* and *T. neapolitana* were employed for H_2 production from lignocellulosic energy crop *Miscanthus*. The *Miscanthus* was pretreated using alkali pretreatment, and H_2 yield of 2.9–3.4 mole of H_2/mole of hexose was obtained which is about 74–85% of the theoretical yield (de Vrije et al. 2009).

One of the extreme thermophiles, *C. saccharolyticus* DSM 8903, has been reported for its efficiency to produce BioH_2 from untreated and dried biomass like sweet sorghum, sugarcane bagasse, maize leaves, wheat straw, silphium, and pine wood. Wheat straw produced maximum H_2 yield of 44.7 L/Kg of dry biomass which is equivalent to 3.8 mole H_2/mol of glucose (Ivanova et al. 2008, 2009). Further study was conducted using 0.5% (w/v) untreated pine wood biomass for up to 91 days to produce BioH_2 by the same strain DSM 8903. It was found that significant amount of H_2 was produced for up to 55 days (Ivanova et al. 2008). However, the total yield of H_2 was low, but this study provided possible use of DSM 8903 strain in future to enhance the H_2 production from untreated plant biomass. Further, the strain DSM 8903 produced H_2 from untreated switch grass (SWG) without any chemical or physiochemical treatments yielding 11.2 mmol H_2/g of SWG in a single step (Talluri et al. 2013).

5.4 BioH_2 Production from Untreated Lignocellulosic Biomass Using Thermophiles

Literature suggests that the use of thermophilic microorganisms such as *Caldicellulosiruptor saccharolyticus* or *Thermotoga maritima* has shown promising results for H_2 production (Talluri et al. 2013; Willquist et al. 2010; Ivanova et al. 2009). Thermophilic H_2 fermentations have higher H_2 yields than mesophilic ones due to the suppression of H_2-consuming bacteria such as methanogens and sulfate-reducing bacteria (Hallenbeck et al. 2012; Ren et al. 2010). For example, higher H_2 yield of H_2 (19.01 mmol H_2/g of sugar) was obtained at a temperature of 80 °C in CSTR by a mixed culture as compared to yield of 15.2 mmol H_2/g of sugar at 55 °C

Table 5.1 BioH_2 production by thermophilic and hyperthermophilic culture from simple and complex substrates

Organism	Substrate	Temperature (°C) and culturing type	End products	Yield (mol/mol)[a]	Reference
Thermotoga neapolitana	Xylose	75 °C, batch	Acetic acid, lactic acid	2.22	Ngo et al. (2012)
Thermotoga neapolitana DSM 4359	Glucose	80 °C, batch	Acetic acid, lactic acid	3.8	Eriksen et al. (2011)
Thermoanaerobacter mathranii A3N	Glucose	70 °C, batch	Acetic acid, lactic acid, butyric acid, ethanol	2.64	Jayasinghearachchi et al. (2012)
Pyrococcus furiosus DSM 3638	Cellobiose	70 °C, batch	Acetic acid, alanine, ethanol	3.8	Chou et al. (2008)
Thermobrachiumcelere, *Thermoanaerobacterium aotearoense*, *Clostridium thermopalmarium*	Xylose	70 °C, batch	Acetic acid, ethanol	1.84	Zhao et al. (2010)
Thermococcus kodakaraensis TSF100	Starch	85 °C, batch	Acetic acid, alanine	3.3	Kanai et al. (2005)
Thermoanaerobacterium, *Thermoanaerobacter*	Glucose	70 °C, batch	Acetate, butyrate, ethanol, propionate	2.38	Lu et al. (2012)
Mixed enriched culture	Glucose	70 °C, UASB	Acetate, butyrate	2.47	Kotsopoulos et al. (2006)
Thermococcus onnurineus	CO/Sodium formate/ Starch	72 °C, batch	Acetic acid, butyric acid. ethanol	3.13	Bae et al. (2012)
Thermotoga neapolitana	Rice straw	75 °C, batch	N/A	2.7 mmol of H_2/g rice straw	Nguyen et al. (2010)
Caldicellulosiruptor saccharolyticus	Miscanthus hydrolysate	72 °C, batch	Acetic acid, lactic acid	3.4	de Vrije et al. (2009)
Caldanaerobacter subterraneus, Thermoanaerobacter subterraneus, Thermoanaerobacterium thermosaccharolyticum	Wheat straw hydrolysate	70 °C, CSTR	Acetic acid, butyric acid, propionic acid, lactic acid, formic acid, ethanol	9.46 mmol H_2/g sugars	Kongjan and Angelidaki (2010)

(continued)

Table 5.1 (continued)

Organism	Substrate	Temperature (°C) and culturing type	End products	Yield (mol/mol)[a]	Reference
Thermotoga neapolitana	Miscanthus hydrolysate	80 °C, batch	Acetic acid, lactic acid	2.9	de Vrije et al. (2009)
Thermoanaerobacterium saccharolyticum, T. thermosulfurigenes, Bacillus sp., Geobacillus sp.,	Sago starch	60 °C, batch	Acetate, ethanol	19.72 mmol H_2/g Starch	Hasyim et al. (2011)
Anaerobic mixed microflora	Cellulose	60 °C, CSTR	Acetic acid, butyric acid	19.01 mmol H_2/g Cellulose	Gadow et al. (2012)
Thermotoga neapolitana	Potato stem Peels	75 °C, CSTR	Acetic acid	2.6–3.8	Mars et al. (2010)
Thermoanaerobacterium sp.	Sweet sor-ghum bagasse	70 °C, batch	Acetate, lactate	2.6	Panagiotopoulos et al. (2010)

[a]Mole of H_2 produced/mole of substrate consumed

and 0.6 mmol H_2/g of sugar at 37 °C (Gadow et al. 2012). In addition, several thermophiles can produce H_2 from both C_5 and C_6 sugars. The thermophile *C. saccharolyticus* produces H_2 from pentose sugars, while *Thermoanaerobacterium thermosaccharolyticum* W16 was shown to ferment a biomass hydrolysate containing a mixture of glucose and xylose to H_2 (Ren et al. 2008a, b). Microbial production of H_2 has been studied for the past few years, but it has not yet been developed to an economically viable status. As discussed above, most of the microorganisms including thermophiles produce higher yields of BioH_2 only when pretreated LCBs are used. This suggests that conversion of lignocellulose to H_2 will require pretreatment, removal of inhibitors released during pretreatment, hydrolysis, and fermentation. Pretreatment of LCBs, detoxification of hydrolysates, and the high current costs of lignocellulose-deconstructing commercial enzymes make dark fermentation more challenging. These factors warrant the development of a cost-effective H_2 production route. Using acid-treated sugarcane bagasse hydrolysate, *T. thermosaccharolyticum* KKU-ED1 produced only 1.12 mole-H_2/mol sugar. *C. saccharolyticus* has been shown to achieve the theoretical Thauer limit of 4 moles of H_2/mole of glucose used (Willquist et al. 2010); however, it generated only 11.2 mmol H_2/g switchgrass (Talluri et al. 2013).

The commercial H_2 production is hindered by high cost of pretreatment steps and formation of some inhibitory compounds like organic acids, aromatics, and hydroxyl methyl furfural (HMF) in acid hydrolysates. A viable option to lower the costs of feedstock and lignocellulolytic commercial enzymes costs is to screen for and identify H_2-producing microorganisms capable of utilizing cellulose and hemicellulose directly from the biomass without pretreatment at higher temperatures (≥60 °C). This would eliminate the need for the separate steps of pretreatment, lignocellulolytic enzyme production, and enzymatic hydrolysis of biomass and would improve the process sustainability and economics. However, information on such microorganisms which produce H_2 from untreated LCB is relatively scarce. Table 5.2 summarizes the recent studies of H_2 production from untreated LCB using thermophiles. All these reports mentioned in Table 5.2 have used different parameters for H_2 yield calculations. Therefore, it was not feasible to compare H_2 yields. Some of these reports did utilize pretreatments, e.g., physical pulverization, treatment with α-amylase (Chen et al. 2012; Mars et al. 2010).

5.5 Mechanism of H_2 Production by Extremophiles

Most of the extreme thermophilic bacteria from the phylum *Clostridia* follow the Embden-Meyerhof-Parnas pathway (EMP) to metabolize hexose sugars to pyruvate followed by formation of BioH_2via decarboxylation of pyruvate to acetyl CoA, and proton are reduced to H_2 by reduced ferredoxin (Fd_{red}) (Verhaart et al. 2010) as shown in Fig. 5.2. Pyruvate is formed at the end of glycolysis, and it can be reduced to lactate by lactate dehydrogenase but most of the anaerobic bacteria oxidize pyruvate into acetyl CoA by enzyme POR (pyruvate oxidoreductase), and the end

Table 5.2 $BioH_2$ gas production from untreated lignocellulosic feedstocks

Feedstock	Bacterial strain	Pretreatment	Temp (°C)	Reference
Switch grass and microcrystalline cellulose	*Caldicellulosiruptor saccharolyticus* DSM 8903	None	65	Talluri et al. (2013)
Rice straw	Heat treated sludge	Physical pulverization	55	Chen et al. (2012)
Potato stem peels	*Thermotoga neapolitana*	Treatment with α-amylase (Novozymes)	75	Mars et al. (2010)
Potato stem peels	*Caldicellulosiruptor saccharolyticus*	Treatment with α-amylase (Novozymes)	72	Mars et al. (2010)
Maize leaves	*Caldicellulosiruptor saccharolyticus*	None	70	Ivanova et al. (2009)
Poplar, switch grass, napier and bermuda grasses	*Anaerocellum thermophilum* DSM 6725	None	70	Yang et al. (2010)
Dried distillers grain, barley hulls, and Fusarium head blight contaminated barley hulls	*Clostridium thermocellum* ATCC 27405	None	60	Magnusson et al. (2008)

product formed is acetic acid/butyric acid depending upon the microbes involved and environmental conditions. Two pathways are available for bioH_2 production by strict anaerobes: first, from a NAD(P)H by GAPDH (glyceraldehyde-3-P dehydrogenase) and from pyruvate ferredoxin oxidoreductase (PFOR) (Jones 2008). BioH_2 production from either ferredoxin or NAD(P)H is thermodynamically unfavorable; therefore, the H_2 yield observed by mesophilic and thermophilic bacteria is low (Jones 2008; Hallenbeck 2009). The redox potential of Fd_{red}/Fe_{ox} couple depends on the microorganism and temperature involved. In nature, the activity of methanogens and sulfate-reducing bacteria reduces the partial pressures of H_2. This results in a low partial pressure of BioH_2 which is favorable for a complete oxidation of glucose to acetate and CO_2. Higher temperatures and partial pressure of BioH_2 do not affect the activity of key enzymes responsible for bioH_2 production. Hence, extremophilic bacteria are able to produce up to 4 moles of bioH_2 along with 2 moles of acetate in pure cultures and also for the fact that microorganisms growing at lower temperatures direct their end product formation to other reduced products.

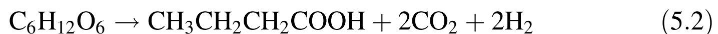
It has been estimated that conversion of 1 mole of glucose can yield 12 mol of BioH_2. The H_2 production with different end products using glucose as substrate is shown below:

If the end product is acetic acid, 1 mole of glucose gives 4 moles of H_2.

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (5.1)$$

Fig. 5.2 Embden-Meyerhof-Parnas pathway for $BioH_2$ production by extremophiles (adapted from Abreu et al. 2012)

If the end product is butyric acid and formic acid, 1 mole of glucose produces 2 and 1 moles of H_2, respectively.



$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \tag{5.2}$$

$$HCOOH \rightarrow CO_2 + H_2 \tag{5.3}$$

All the fermentative methods to produce H_2 depend on the activity of enzymes (nitrogenase and hydrogenase) whose properties are shown in Table 5.3. Hydrogenase is the main enzyme responsible for $BioH_2$ production and is of two types (Fe hydrogenase and Ni-Fe hydrogenase) depending upon their metal content. During the fermentative EMP pathway, the H_2 is produced during the conversion of

Table 5.3 Properties of nitrogenase and hydrogenase (Ni et al. 2006)

Property	Nitrogenase	Hydrogenase
Substrates	ATP, H^+ or nitrogen	H^+, hydrogen
Products	H_2, NH_4^+	ATP, H^+ or nitrogen
Number of proteins	2 (Mo-Fe and Fe)	1
Metal components	Mo, Fe	Ni, Fe, S
Optimal temperature	30 °C (*A. vinelandii*)	55 °C (*R. rubrum*) 70 °C (*R. capsulatus*)
Optimal pH	7.1–7.3 (*A. vinelandii*)	6.5–7.5 (*R. sulfidophilus*)
Inhibitors	N_2, NH_4^+, O_2, high N:C	CO, EDTA, O_2 and some organic compounds
Stimulators	Light	Absence of organic compounds

pyruvate to acetyl CoA. In this step, NADH is oxidized, and ferredoxin is reduced to produce molecular H_2. *C. saccharolyticus* anaerobe has been reported to produce BioH_2 by producing some cellulolytic enzymes, which degrade the LCB like switchgrass, sweet sorghum, paddy straw, etc. *C. saccharolyticus* and *T. tengcongensis* show a different and modified form of EMP (Kumar et al. 2015). *C. saccharolyticus* produces high yield of molecular H_2 as the level of lactic acid and ethanol produced is low. Further research on the genetic level of this microbe should explore its potential to produce BioH_2. In the pathway, glucose molecule is metabolized in to glyceraldehyde-3-phosphate (GAP). Electron carrier NADH is formed by further conversion of GAP into pyruvate. Pyruvate is further oxidized into acetyl CoA by enzyme POR (pyruvate oxidoreductase), and ferredoxin is reduced in this step. The acetyl CoA is then converted into acetic acid. However, *T. tengcongensis* does not grow on the LCB. The enzyme present in *T. tengcongensis* is NADH-dependent hydrogenase, which uses direct NADH to produce H_2 at low concentration of H_2 (Soboh et al. 2004). Studies have shown that both species of extremophiles can use NADH directly for H_2 production. On increasing the pressure, enzyme lactate dehydrogenase will utilize NADH to produce lactate instead of acetate and BioH_2 thereby decreasing the BioH_2 yield.

5.6 Future Scope

The laboratory scale production of BioH_2 from LCB includes several steps which ultimately increase the production cost. The combination of several steps will reduce the overall cost efficiency of the process. Therefore, an efficient and cost-effective single step process can degrade and ferment the untreated lignocelluloses. As a result, there will be no need of any additional step like pretreatment, lignocellulolytic enzyme production, enzymatic hydrolysis of biomass, and fermentation. One of such practice is presented with consolidated bioprocessing (CBP) process in which enzyme production, enzyme hydrolysis of the biomass, and fermentation step are combined in a single step. This single step CBP process can reduce the production

cost up to 50% by eliminating the cost associated with enzyme production as in other methods like simultaneous saccharification and fermentation (SSF) or co-fermentation (Xu et al. 2009). The use of extremophiles offers several advantages like increase mass transfer rates, favorable thermodynamic conditions, less chances of microbial contamination, etc., thereby, increasing the overall economics of the process.

Combining dark fermentation with photo-fermentation or combining dark fermentation with methanogenesis is also an alternative or holistic approach for BioH_2 production from LCB. The volatile acids such as acetic acid, butyric acid, etc. are present in the dark fermentative effluent, which can be used by methanogens to produce methane gas. As a result, the combined hybrid system will increase the overall energy recovery from biomass. In a different novel approach, the lignocellulose-based BioH_2 system can be designed by combining sequencial dark and photo-fermentation with microalgae photoautotrophic process, where microalagae will utilize all CO_2 produced in sequencial dark and photo-fermentation. Microbial biomass produced can be used further in biorefinery to produce value-added biofuels as shown in Fig. 5.3.

If the composition of substrate is complex, the use of microbial consortia to degrade the biomass might increase the BioH_2 yield as compared to pure culture. In vitro system can also be designed to build a modified BioH_2 production pathway. For example, glucose substrate can be converted into H_2 and glucuronic acid by combining glucose dehydrogenase from *Thermoplasma acidophilum* and NADPH-

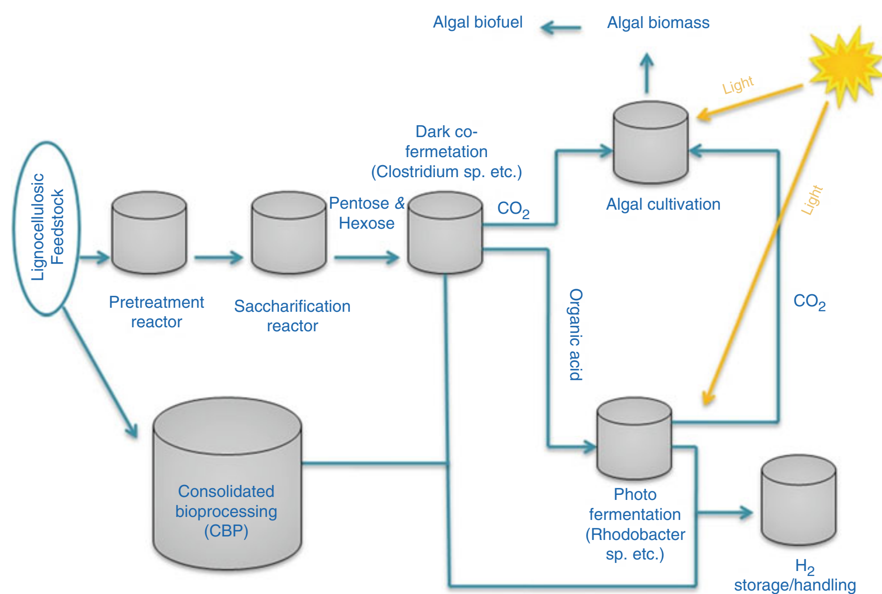


Fig. 5.3 Conceptual approach of sequential dark fermentation and photo-fermentation augmented with microalgal photoautotrophic process

dependent hydrogenase from *Pyrococcus furiosus*. Metabolic engineering has also been reported to increase the BioH_2 production rate. The modification in the native pathways depends on redirecting the metabolic flux which blocks those pathways which compete with H_2 production. However, the yield cannot be increased above the network potential. Therefore, nonnative pathways are employed which involves the expression of nonnative hydrogenase. For example, the nonnative hydrogenase from *E. cloacae* can be over expressed in non-H_2-producing *E. coli* to get the enhanced production rate of BioH_2 as compared to yield obtained from wild strain *E. cloacae*. Also, the significant progress has been achieved by using genetic tools, which improved our understanding of extremophilic microorganisms by manipulating the genetic characteristics for H_2 and carbon metabolism. Further, research and development in bioprocessing of extremophiles can lead to the commercialization of extremophile-based application in the near future.

5.7 Summary and Outlook

Conversion of LCB to BioH_2 shows an attractive pathway to meet the future demand of energy. These feedstocks are abundant and can be efficiently degraded by microorganisms to produce BioH_2. The methods involved in the production of BioH_2 from LCB are pretreatment, hydrolysis, removal of inhibitors, and fermentation. Use of extremophiles in producing renewable H_2 from LCB is gaining attention due to its advantages over other biological methods.

Existing processes of BioH_2 production from complex wastes, such as LCB, utilize several steps. The inclusion of several steps reduces the overall cost-efficiency of the process. An alternative to the use of various steps in H_2 production is the development of an efficient and cost-effective single-step process utilizing untreated lignocellulose-degrading and fermentative thermophiles (second-generation consolidated bioprocessing, CBP). Growth at high temperature favors the thermodynamics of stoichiometric H_2 yield and decreases the possibility of contamination by unwanted microorganisms that compete for the same substrates. The use of elevated temperatures also offer several potential advantages such as (1) improved hydrolysis of cellulosic substrates, (2) higher mass transfer rates leading to better substrate solubility, and (3) lowered risk of potential contamination, thus improving the overall economics of the process (Bhalla et al. 2013). The innovative CBP may impact current multiple-step conversion processes of complex wastes to biofuels by providing a safe, more efficient, sustainable, and economical process. However, this method needs further research and development to make this process feasible for commercial application. In a long term, progress in lignocelluloses breakdown and genetic tools to manipulate the H_2 production capabilities in thermophiles is expected to offer unique advantages to the design, construction, and application of an economically viable BioH_2 production system.

Take-Home Message

The use of second-generation feedstocks (lignocelluloses) to produce $BioH_2$ can be a promising and efficient method, which fulfill the future demand of energy. Recently $BioH_2$ production using extremophiles has gained high attention due to fast production rate without any preprocessing or mild processing of plant biomass. Extremophiles are reported to produce $BioH_2$ and other value-added products even from untreated lignocellulosic biomass. This chapter presented a review and in-depth analyses of extremophilic $BioH_2$ production from lignocellulosic biomass. The chapter also provided the knowledge on how to develop a more efficient and economical integrated processes for enhanced conversion of lignocellulosic feedstocks to $BioH_2$.

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