

Outlook of biohydrogen production from lignocellulosic feedstock using dark fermentation – a review

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Hydrogen becomes a promising alternative energy carrier to fossil fuels since it is clean, renewable, contains high energy content and does not contribute to greenhouse effect. Therefore, using cheap or renewable resources, such as lignocellulosic materials, as the feedstock for hydrogen production, in particular, dark fermentative hydrogen production has a great potential to give major contribution to future energy supply. The main challenges are the low hydrogen yield arising from poor efficiency on direct microbial assimilation of cellulosic materials. Considerable research efforts have been made to improve the pretreatment and hydrolysis of lignocellulosic materials. Development of novel and effective cellulase enzymes, optimization and improvement of cellulase system, as well as engineering approaches on cellulose pretreatment and saccharification are gaining increasing interest. Information from genomics and molecular genetics combined with improved genetic engineering offer a wide range of possibilities for enhancing performance of cellulose feedstock utilization and biohydrogen production. This study reviews key technologies and variables to be considered during biohydrogen production from lignocellulosic feedstock.

Keywords: Biofuels, Biohydrogen, Cellulase, Dark fermentation, Feedstock pretreatment, Lignocellulose, Saccharification

Introduction

Major energy provider (80%) for current economy and lifestyle are fossil fuels¹. Transport sector, a major consumer of petroleum fuels [diesel, gasoline, liquefied petroleum gas (LPG) and compressed natural gas (CNG)], is likely to suffer badly because oil reserves are decreasing, and therefore, there is a continuous rise of crude oil prices (Fig. 1)². Fossil fuels emit greenhouse gases (CO₂, CH₄ and CO) resulting in global warming and pollution. Intensive research is going on to generate clean and sustainable energy sources from renewable carbon resources³. Today's energy system is unsustainable because of equity issues as well as environmental, economic, and geopolitical concerns⁴. Lignocellulosic biomass (LB) is most abundant renewable biological resource^{5,6} continually replenished by photosynthetic reduction of carbon dioxide (CO₂) by sunlight energy⁷. LB constitutes a major portion of agricultural and forest wastes and industrial effluents

such as pulp/paper and food industry. On the earth, annual biosynthesis of cellulose by both land plants and marine algae occurs at a rate of (0.85×10^{11}) tonnes per annum equivalent to more than four times the world's yearly total energy consumption⁸⁻¹⁰. There is enormous worldwide interest in the development of new and cost-efficient processes for converting plant-derived biomass to bioenergy in view of fast depletion of oil reserves and food shortages¹¹⁻¹². Thus, biomass utilization for energy, food and chemicals could solve waste disposal problems and also help to displace growing dependence on fossil fuels by providing a convenient and renewable source of energy as glucose^{5,13-15}.

Biofuels represent ecofriendly, biodegradable, sustainable, cost competitive and promising alternative energy source for fossil fuels¹⁶. Among which, hydrogen (H₂) is a clean and high-energy fuel (122 MJ/kg), which is three times higher than hydrocarbon fuels¹⁷. Combustion of H₂ fuel produces water and hence does not contribute to greenhouse gas (GHG) effect. Heating value (61,100 Btu/lb) of H₂ is nearly three times that of methane (23,879 Btu/lb) (Table 1)¹⁸. Therefore, using cheap or renewable resources, such as lignocellulosic

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materials, as feedstock for biohydrogen production has a great potential to give major contribution to future energy supply. Thus, H₂ has been predicted to play a major role in energy supply by 2100¹⁹⁻²⁰.

This paper reviews methods for pretreatment and hydrolysis of lignocellulosic feedstock and technologies leading to generation of biohydrogen by using lignocellulosic feedstock involving microbial/enzymatic treatment of cellulose followed by anaerobic dark fermentation.

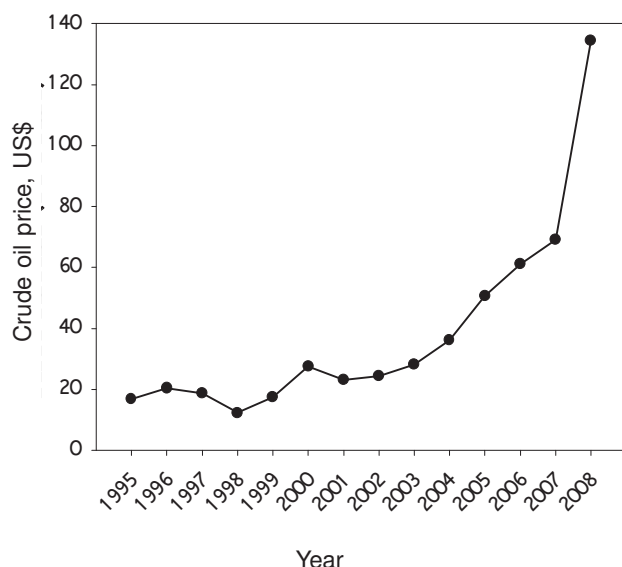


Fig. 1—Annual profile of crude oil price²

Cellulose Degradation and Cellulosic Waste Management

Conventional Cellulose Conversion Techniques and Pretreatment Methods

Conversion of LB includes hydrolysis of cellulosic materials to reducing sugars and production of H₂ and higher valuable products via fermentation. Factors affecting hydrolysis of cellulose include porosity of waste materials, crystallinity of cellulose fiber and lignin, and hemicellulose content²¹. Table 2²²⁻²³ presents LB constituents (cellulose, hemicellulose and lignin).

Pretreatment aims to get rid of lignin and hemicellulose, reduce crystallinity of cellulose and increase surface area of materials to improve formation of sugars (Fig. 2)²⁴. Pretreatment procedures should be economically feasible and could prevent formation of byproducts inhibitory to subsequent hydrolysis and fermentation processes²⁵. Also, pretreatment outcomes must be balanced against their impact on the cost of downstream processing steps and trade-off between operating, capital, and biomass costs²⁶⁻²⁸. Several methods have been used to treat cellulosic feedstock (polysaccharides to corresponding monomers) and each generates a different pretreatment product stream (Fig. 3)²⁹. Physical pretreatment (mechanical comminution and pyrolysis) found to be effective in breaking down cellulose crystallinity but requires more cost for power and gives all the three major compounds in one product stream³⁰. Chemical methods (ozonolysis, acid hydrolysis, alkaline hydrolysis, oxidative delignification, solvent extraction) are also effective pretreatment procedure, but require more energy and chemicals than biological processes and may cause secondary pollution problem³¹. Among physicochemical pretreatment procedures, steam

Table 1—Comparison of energy, carbon emissions and low heating value (LHV) of combustible fuels

Sr No.	Fuel type	Energy /unit mass, MJ/kg	Energy/Vol MJ/l	Carbon emission kg C/kg fuel	LHV MJ/kg
1	Hydrogen gas	120	2	0	120.1
2	Hydrogen liquid	120	8.5	0	120.1
3	Coal (anthracite)	15–19	—	0.5	33.3
4	Natural gas	33–50	9	0.46	38.1
5	Gasoline	42–45	38	0.84	42.5
6	Diesel	42.8	35	0.9	43.0
7	Petrol (naphtha)	40–43	31.5	0.86	44.9
8	Bio-diesel	37	33	0.5	—
9	Ethanol	21	23	0.5	27.0

Table 2-Contents of cellulose, hemicellulose, and lignin in common agricultural residues and wastes²²⁻²³

Sr No.	Lignocellulosic materials	Cellulose, %	Hemicellulose, %	Lignin, %
1	Hardwoods stems	40-55	24-40	18-25
2	Softwood stems	45-50	25-35	25-35
3	Nut shells	25-30	25-30	30-40
4	Corn cobs	45	35	15
5	Grasses	25-40	35-50	10-30
6	Paper	85-99	0	0-15
7	Wheat straw	33-38	26-32	17-19
8	Sorted refuse	60	20	20
9	Leaves	15-20	80-85	0
10	Cotton seed hairs	80-95	5-20	0
11	Newspaper	40-55	25-40	18-30
12	Waste papers from chemical pulps	60-70	10-20	5-10
13	Primary wastewater solids	8-15	NA ^a	24-29
14	Swine waste	6.0	28	NA ^a
15	Solid cattle manure	1.6-4.7	1.4-3.3	2.7-5.7
16	Coastal Bermuda grass	25	35.7	6.4
17	Switch grass	45	31.4	12.0
18	Sorghum stalk	27	25	11
19	Rice bran	35	25	17
20	Rice straw	32-47	19-27	5-24
21	Coconut fiber	36-43	1.5-2.5	41-45
22	Wheat bran	30	50	15
23	Barley bran	23	32	21.4
24	Barley straw	31-45	27-38	14-19

^aNA-not available

explosion is recognized as one of the most cost-effective pretreatment processes for hardwoods and agricultural residues, but having limitation due to incomplete disruption of lignin-carbohydrate matrix, and generates compounds inhibitory to microorganisms used in downstream processes³². Ammonia fiber explosion (AFEX) pretreatment shows better performance³³ but ammonia makes process expensive and also causes secondary pollution problems. Αλτηουγη αλλ τησε μεθοδος, ιν γενεραλ, ηαπε ποτεντιολ φορ χελλυλοσε ηιδρολυσισ, βυτυσολλιν ινπολπε χομπλιχαεδπροχεδυρεσ ορορε εχονομικαλ ινυφεασιβλε³⁴.

Biological Pretreatment

Biological hydrolysis of cellulose is carried out by cellulolytic microorganisms or catalyzed by cellulase enzyme complex. In nature, cellulosic materials are degraded by microorganisms, of which, brown-, white- and soft-rot fungi have more ability to degrade lignin and hemicellulose in waste materials and used in biological pretreatment processes³⁵. White rot fungi are most effective basidiomycetes for biological pretreatment of

LB⁷. *Pleurotus ostreatus* converted wheat straw into reducing sugar (35%) in 5 weeks, whereas *Phanerochaete sordida* 37 and *Pycnoporus cinnabarinus* 115 contributed similar conversion within 4 weeks³⁶. Some white rot fungi (*Ceriporiopsis subvermispora* and *Cyathus stercoreus*) were found effective in delignification of bermuda grass³⁷.

A mixed culture^{38,39} comprising a cellulolytic bacterium and a noncellulolytic bacterium could degrade natural cellulosic materials aerobically or anaerobically without sterilization, thereby having a high degree of stability to degrade cellulosic material for long time. Advantages of biological pretreatment include inexpensive, low energy requirement and mild environmental conditions. However, utilizing these microorganisms and enzymes to process natural cellulosic materials without pretreatment and/or sterilization is difficult and hydrolysis rate is also low.

Enzymatic Hydrolysis of Cellulose

Cellulose, a linear condensation polymer of glucose joined together by glycosidic bonds [degree of polymerization (DP), 100-20,000], is water insoluble and

Effect of Pretreatment

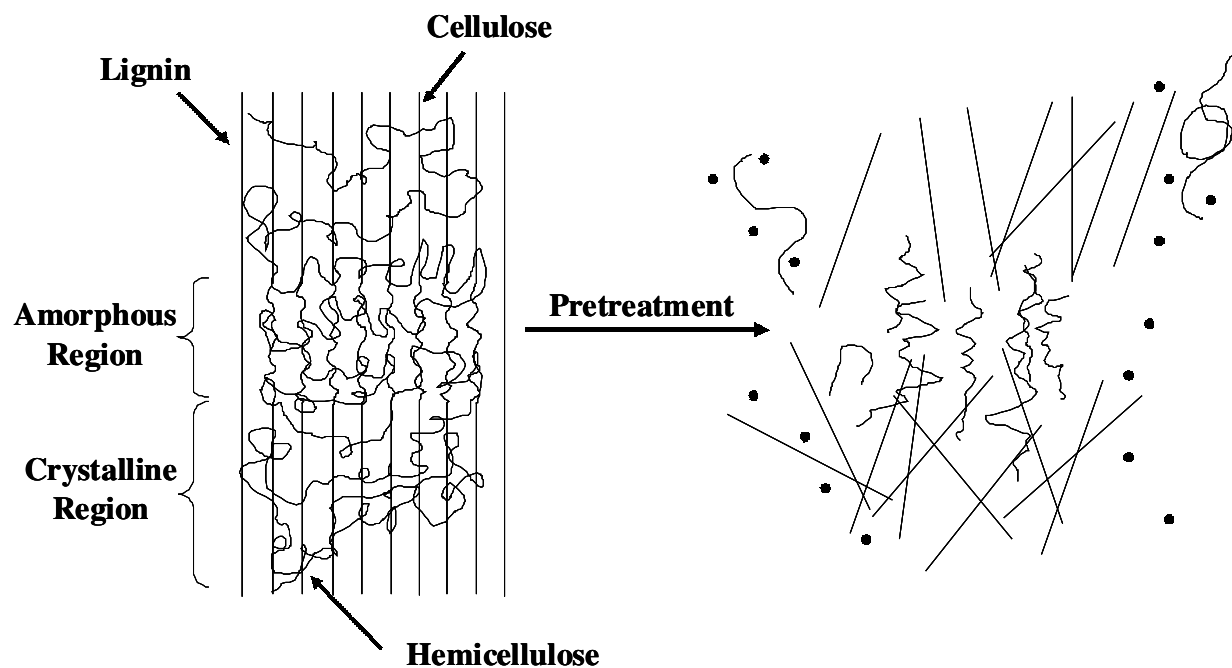


Fig. 2—Schematic description of pretreatment on lignocellulosic material²⁴

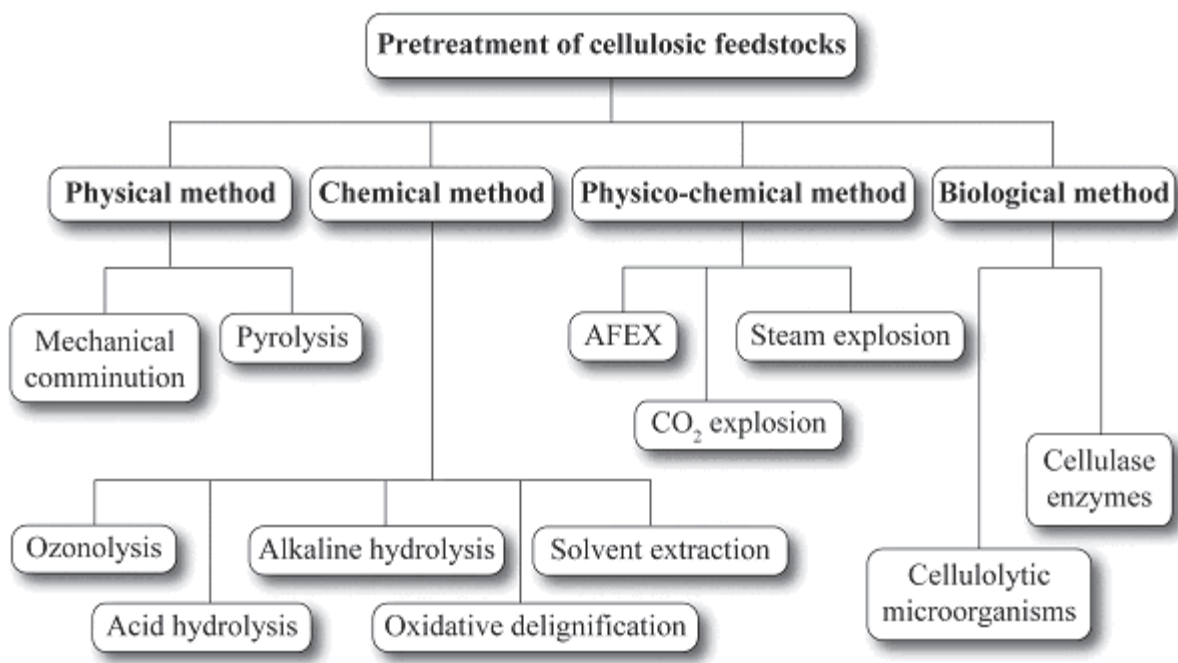


Fig. 3—Methods for pretreatment of cellulosic feedstock

recalcitrant to hydrolysis into its individual glucose subunit because of tightly packed, highly crystalline structure with straight, stable supra-molecular fibers of great tensile strength and low accessibility in its polymer form^{40,41}. Conversion of cellulosic mass to fermentable sugars through biocatalyst cellulase derived from cellulolytic organisms is economically feasible process and offers potential to reduce use of fossil fuels and reduce environmental pollution relative to physicochemical processes⁴². Formation of soluble sugars from cellulose in agricultural residues relies on sequential/coordinated action of individual components [β -endoglucanase (EC 3.2.1.4), β -exoglucanase (EC 3.2.1.91) and β -D-glucosidase (EC 3.2.1.21)] in cellulase enzymes⁴³⁻⁴⁴. Endoglucanases cleave intramolecular β -1,4-glucosidic linkages randomly and releases reducing sugars in reaction mixture; having more applications in textile and detergent industries. Exoglucanases acts on accessible ends of cellulose molecules to liberate glucose and cellobiose but cellobiohydrolase (CBH I & II) by *Trichoderma reesei* act on reducing and non-reducing cellulose chain ends⁴⁵. β -D-glucosidases hydrolyze soluble cellobiose and other cellooligosaccharides to produce glucose in aqueous phase⁴⁵. In addition to three major groups of cellulases, there are also a number of ancillary enzymes (glucuronidase, acetylsterase, xylanase, β -xylosidase, galactomannanase and glucomannanase) that attack hemicellulose⁴⁶.

Microorganisms and enzymes (cellulase, xylanase and peroxidase) that degrade cellulosic materials have been well studied and several microbial related applications have been developed for textile, food and paper-pulp processing^{47,48}. Cellulolytic bacteria (*Acetovibrio*, *Bacillus*, *Bacteriodes*, *Cellulomonas*, *Clostridium*, *Erwinia*, *Microbispora*, *Ruminococcus*, *Streptomyces*, and *Thermomonospora* genus) can produce cellulases effectively⁴⁹. Although many cellulolytic bacteria, particularly cellulolytic anaerobes (*Clostridium thermocellum* and *Bacteroides cellulosolvens*) can produce cellulases with high specific activity but low enzyme production rate due to slow growth profile. Hence, for commercial cellulase production, most research has focused on fungi⁴⁶ that include *Sclerotium rolfsii*, *Phanerochaete chrysosporium* and species of *Trichoderma*, *Aspergillus*, *Schizophyllum* and *Penicillium*^{7,46}. White-rot fungus especially *P. chrysosporium* produces lignin-degrading oxidizing enzymes extracellularly can degrade wood cell wall and lignin⁵⁰. *Trichoderma* and *Aspergillus* species⁵¹⁻⁵²

produce most commercial cellulases (including β -glucosidase).

Key Issues in Developing Effective Cellulase Complex

Cellulases are relatively costly enzymes, thereby significant cost reduction will be important for their commercial use in the preparation of cellulosic feedstock. There is a need to increase cellulase enzyme volumetric productivity by using cheaper substrates, with higher stability and specificity (substrates) for specific processes. Large market potential (US \$ 400 million/y)⁵³ and important role that cellulases play in bioenergy and bio-based products industries⁵¹ require to develop better cellulases for cellulose hydrolysis.

Factors affecting enzymatic hydrolysis of cellulose include substrates, cellulase activity, reaction conditions (temperature, pH, etc.) and end product inhibition (cellobiose and glucose). Higher substrate concentration can cause substrate inhibition, which substantially lowers hydrolysis rate, and extent of substrate inhibition depends on the ratio of total substrate to total enzyme⁵⁴. Lignin interferes with hydrolysis by blocking access of cellulases to cellulose and by irreversibly binding hydrolytic enzymes²¹. Enzymatic hydrolysis of cellulose consists adsorption of cellulase onto cellulose surface, biodegradation of cellulose to fermentable sugars and desorption of cellulase. Retardation of cellulase activity during hydrolysis may be because of irreversible adsorption of cellulase on cellulose⁵⁵. Addition of surfactants (Tween 20, Tween 80 etc.) during hydrolysis modifies cellulose surface property and minimizes irreversible binding of cellulase on cellulose. Increasing dosage of cellulases in the process, to a certain extent, can enhance yield and hydrolysis rate, but would significantly increase the process cost. Thus, improved cellulases must show higher catalytic efficiency on insoluble cellulosic substrates, increased stability at elevated temperature and at a certain pH and higher tolerance to end-product inhibition. For this purpose now a days, research has focused on three major directions: 1) Rational design for each cellulase, based on knowledge of cellulase structure and catalytic mechanism⁵⁶⁻⁵⁷; 2) Directed evolution for each cellulase, in which improved enzymes or ones with new properties were selected or screened after random mutagenesis and/or molecular recombination^{51,58-60}; and 3) Reconstitution of cellulase mixtures (cocktails) active on insoluble cellulosic substrates, yielding an improved hydrolysis rate or higher cellulose digestibility⁶¹⁻⁶⁴.

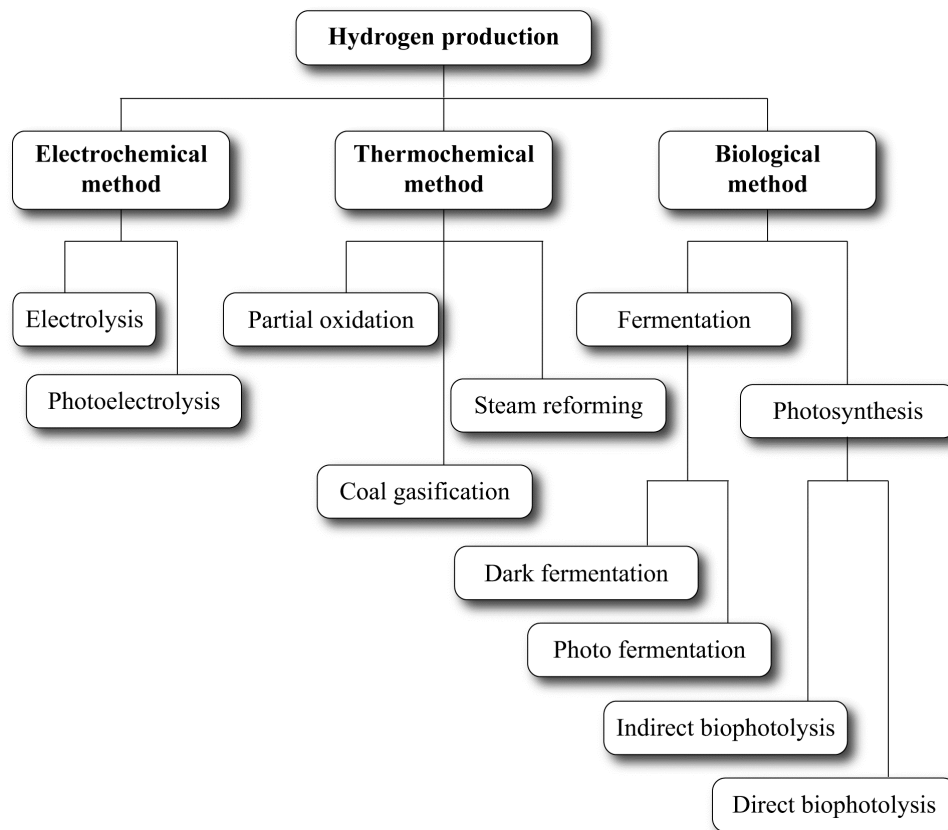


Fig. 4—Methods used for hydrogen production

Hydrogen Energy - Its Importance and Production Methods

Importance of Hydrogen Energy

H₂ is a promising alternative to fossil fuel with many social, economic and environmental benefits. Concept of H₂ economy has been proposed as a clean and efficient replacement for petroleum based economy and recognized by US Department of Energy (US-DOE), International Partnership for Hydrogen Economy (IPHE) and European Hydrogen Association (EHA)⁶⁵. H₂ has low emission, represents a cleaner and more sustainable energy system and could contribute substantially in the reduction of GHG emissions^{66,67}. H₂ acts as a versatile energy carrier with potential for extensive use in power generation and in many other applications. H₂ gas is a widely used feedstock for the production of chemicals (ammonia and methanol), in oil refineries for removal of impurities or for upgrading heavier oil fractions into lighter and more valuable products, production of electronic devices, processing steel, desulfurization and reformulation of gasoline in refineries and also used in

the world's space programmes (1%)⁶⁸. Vehicles can be powered with H₂ fuel cells, which are three-times more efficient than a gasoline powered engine. As on today, in all these areas H₂ utilization is equivalent to 3% of energy consumption, but it is expected to grow significantly in future⁶⁹. More than 50 million tonnes of H₂ are produced annually worldwide with a growth rate of nearly 10% per year⁷⁰. This amount of H₂ could produce 6.5 EJ of energy, equivalent to about 1.5% of world energy consumption. H₂ (99%) is produced from fossil fuels, primarily natural gas, with chemical production and renewable energy sources accounting for the rest⁷⁰⁻⁷¹. Based on the National Hydrogen Program of the United States, contribution of H₂ to total energy market will be 8-10 % by 2025⁷².

Hydrogen Production with Physicochemical Methods

Although H₂ is most abundant element in the Universe, it must be produced from other H₂-containing compounds such as fossil fuels, biomass, or water⁷³. Conventional physicochemical methods (Fig. 4) for H₂ production are based on steam reforming of natural gas

(methane and other hydrocarbons), partial oxidation of hydrocarbons heavier than naphtha, coal gasification, and pyrolysis or gasification of biomass, which produces a mixture of gases (H_2 , CH_4 , CO_2 , CO and N_2). All these processes require high temperatures ($>850^\circ C$), derived from combustion of fossil fuels, thereby being energy intensive and expensive. Among these methods, steam reforming process alone produces nearly 90% of H_2 but it requires more cost for power⁷⁴. Water can be used as renewable resources for H_2 gas production and methods are based on electrolysis, photolysis, thermochemical process, direct thermal decomposition or thermolysis⁷⁵. Electrolysis of water can be attractive and cleanest technology for H_2 gas production. However, electricity costs account for 80%. Moreover, to avoid deposits on electrode and corrosion problems, feeding water should be mineralized, which ultimately increase cost of the process⁷². Although all these methods, in general, have potential for effective H_2 production but require a source of energy, which derived from fossil fuels, usually involve complicated procedures, economically unfeasible and not always environmentally benign⁷⁶.

Biological Methods for Hydrogen Production and their Advantages

Biological H_2 production from renewable LB presumes paramount importance as an alternative and renewable bioenergy resource (Fig. 4). Methods adopted to produce H_2 from biological methods are based on biophotolysis of water by algae and cyanobacteria, photodecomposition of organic compounds by photosynthetic bacteria, dark-fermentative H_2 production during acidogenic phase of anaerobic digestion of organic matter, and hybrid systems using two stage dark/photo-fermentative production of H_2 ^{75,77-79}. Key advantages of biological H_2 production are: 1) Process catalyzed by microorganisms in an aqueous environment at ambient temperature and pressure; 2) Inexpensive; 3) Low energy requirement; and 4) Well suited for decentralized energy production in small-scale installations in locations where biomass or wastes are available, thus avoiding energy expenditure and costs for transport.

Role of Hydrogenase in Hydrogen Production

Hydrogenases, key enzymes of H_2 metabolism, are distributed in many microorganisms located at cytoplasm or periplasm and also involved in many biological processes where H_2 is involved. Hydrogenases oxidize H_2 to protons and electrons or reduce protons to release

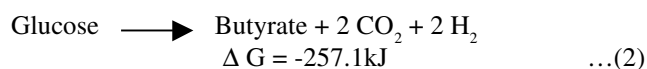
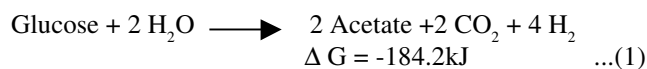
molecular H_2 ^{80,81}. In biosphere, mostly biological H_2 production is derived from microbial fermentation processes. These organisms decompose organic matter to H_2 , CO_2 and metabolites like volatile fatty acids (VFAs) and ethanol⁸². In natural habitat, H_2 bacteria can even grow autotrophically with H_2 gas as sole reducing power and energy substrate⁸³. In these bacteria, oxygen serves as a terminal electron acceptor leading to water as the end product. Around 200 million tonnes of H_2 are cycled within these ecosystems per year, atmosphere only harbors some 7.8×10^{-5} vol % H_2 ⁸⁴. Physiological role and biochemical characteristics of hydrogenases are variable for different microbial processes^{75,80,81,85-87}.

Hydrogen Gas Production by Dark Fermentation

A broad spectrum of biological H_2 -production processes has been investigated, including direct biophotolysis, indirect biophotolysis, photo-fermentations and dark fermentation⁸⁸. Mainly three kinds of microorganisms capable of H_2 production are cyanobacteria or green algae, photosynthetic bacteria and anaerobic bacteria. Cyanobacteria/green algae directly decompose water to H_2 and O_2 in presence of light energy by photosynthesis. Algal H_2 production could be considered as an economical and sustainable method in terms of water utilization as a renewable resource and CO_2 consumption as one of the air pollutants. However, natural-borne organisms of these species examined so far show rather low rates of H_2 production due to complicated reaction systems and inhibition of hydrogenase by oxygen. Another drawback encountered is the requirement of a carrier gas to collect evolved gas from culture. Ready separation of O_2 and H_2 is also an unsolved subject⁸⁹⁻⁹². Therefore, dark and photo-fermentations are considered to be more advantageous due to simultaneous waste treatment and H_2 gas production. Photosynthetic bacteria utilize organic substrates like organic acids instead of water as starting compound for H_2 production. Compared to algal hydrolysis, photosynthetic bacteria require less free energy (+8.5 kJ/mol H_2 for lactate) to produce H_2 and can completely degrade organic substances toward mineralization. However, this process requires high activation energy to drive nitrogenase, which is responsible for H_2 production in photosynthetic bacteria⁹³ and consequence is low solar conversion efficiencies, typically not higher than that for algal biophotolysis systems⁹⁴. In addition, phototrophic H_2 production with photosynthetic bacteria is extremely suspicious to

ammonia and oxygen contents, making it difficult in practical applications⁷⁹.

After mid 1990s, much attention has been paid to H₂ production by anaerobic dark fermentation system, which has the best potential for practical applications⁹⁵. Some basic advantages relative to other processes include process simplicity on technical grounds, low energy requirements, higher rates of H₂ production, economically feasible or better process economy, and ability to generate H₂ from a large number of carbohydrates (or other organic materials) frequently obtained as waste products⁹⁵⁻⁹⁷. A variety of microbes¹⁷ [anaerobic bacteria (*Clostridium* sp.), facultative anaerobes (*Enterobacter* and *Bacillus* sp.), as well as bacterial consortium from organic wastes, (anaerobic digester sludge, soil, animal feces etc.)] can be used for dark H₂ fermentation. Major soluble metabolites from dark fermentation include VFAs and alcohols and their further decomposition is not possible under anaerobic conditions^{88,98,99}. Anaerobic bacteria utilize organic substances as sole source of electrons and energy, converting them into H₂¹⁷. The reactions involved in H₂¹⁷ production (Eqs. 1 and 2) are rapid and these processes do not require solar radiation, making them useful for treating large quantities of organic waste by using an appropriate fermentor.



Thus, theoretically maximal H₂ yield from dark fermentation is 4 mol H₂/mol glucose. In addition, since dark fermentation is only an incomplete degradation of organic substrates, production of H₂ gas is accompanied by formation of acetate and/or butyrate with a stoichiometrical ratio of 2 mol H₂ per 1 mol acetate or butyrate. Production cost of biohydrogen production by dark fermentation is 340 times lower than photosynthetic process and thus is considered to be more commercially viable¹⁰⁰. However, H₂ yield could be further elevated by integration of dark and photo-fermentation processes, as theoretically highest H₂ production yield (12 mol H₂/mol glucose) could be expected¹⁰¹. Biotechnology Research Group at Iowa State University¹⁰² has developed a new fermentation process that converts negative-value organic waste streams into H₂-rich gas. Most recent studies on H₂ production used pure isolated anaerobic bacteria as H₂ producer^{103,104}. In some cases,

process employs using mixed microflora or acclimated sewage sludge for H₂ production^{105,106}.

Anaerobic H₂ fermentation processes from *Clostridium* species have been well studied^{107,108}. Mainly the obligate anaerobes and spore forming organisms such as *C. buytricum* (on sweet potato starch)¹⁰⁹, *C. thermolacticum* (on lactose)¹¹⁰, *C. pasteurianum* (on starch)¹¹¹ and *C. paraputrificum* M-21 (on chitinous waste)¹¹² and *C. bifermentans* (on wastewater sludge)¹¹³ show maximum H₂ production at exponential growth phase. Dominant and enriched culture of *Clostridia* can be easily obtained by thermal treatment of biological sludge as well as pH control and HRT control of treatment system¹¹⁴. Spores formed at high temperatures can be germinated when required environmental conditions are provided for H₂ gas production. A study of microbial diversity of mesophilic H₂ producing sludge shows the presence of *Clostridia* species (up to 64.6%), indicating that *Clostridia* species were dominant microbes for H₂ production¹¹⁴. H₂ production by *Thermotogales* sp. and *Bacillus* sp. were detected in mesophilic acidogenic cultures¹¹⁵. In anaerobic granular sludge along with *Clostridium* sp., some anaerobic cultures (*Actinomyces* sp., *Porphyromonas* sp.) show H₂ yield between 1 and 1.2 mmol/mol glucose when cultivated under anaerobic conditions¹¹⁶. Facultative anaerobes (*Enterobacter* sp) metabolize carbohydrates and produce gaseous (H₂ & CO₂), mixture of acids, ethanol and 2-3 butanediol as valuable products. Capacity of H₂ production of *Enterobacter aerogenes* using different substrates has been widely studied¹¹⁷⁻¹¹⁹. Enhancement of H₂ production (2.2 mol H₂/mol glucose) by using *E. cloacae* ITT-BY 08 have been reported¹²⁰. Some anaerobic thermophilic organisms (*Thermoanaerobacterium thermosaccharolyticum* and *Desulfotomaculum geothermicum*) produce H₂ gas in thermophilic acidogenic culture¹¹⁵. *Thermococcus kodakaraensis* KOD1 and *C. thermolacticum* strains produce H₂ at 85°C and 58°C¹²¹, respectively, whereas *Klebsiella oxytoca* HP1 produce maximal H₂ at 35°C¹²². Isolated *Klebsiella* sp. HE1 produce 2,3- butanediol, ethanol and H₂ using sucrose as a substrate under dark fermentation process¹²³.

For maximum H₂ yield, optimum pH is reported^{106,124-126} between 5.0-6.0, whereas some reported^{110-111,119,126} pH range between 6.8-8.0. During dark fermentation along with H₂ production, formation of organic acids deplete buffering capacity of the medium resulting in low final pH, which inhibits H₂ production

since pH affects activity of iron containing hydrogenase enzyme¹²⁷. Culture pH also affects H₂ production yield, biogas content, type of organic acids produced and specific H₂ production rate¹²⁸. Therefore, control of pH at optimum level may be useful for better yield. In addition, composition of substrate, medium composition, temperature, and type of microbial culture are also important parameters affecting duration of lag phase as well as efficiency of H₂ production. In anaerobic organisms, hydrogenase enzyme oxidizes reduced ferredoxin to produce molecular H₂ and external iron addition may shorten lag phase and also increases H₂ production. For example, an iron (conc. 10 mg/l) was found to be optimum in batch H₂ production by *C. pasteurianum* from starch¹¹¹. Nitrogen is also essential and effective factor for H₂ production by dark fermentation under anaerobic conditions. Polypepton, (NH₄)₂ HCO₃ and corn-steep liquor (waste of corn starch) were found to be good inducers for better H₂ yield^{109,129}. Lin¹³⁰ reported that C/N ratio affected H₂ productivity more than specific H₂ production rate. H₂ gas producing organisms requires strict anaerobic condition, thereby purging of reducing agents (argon, nitrogen, H₂ gas and l-cystine·HCl) might be essential to remove trace amounts of oxygen present in the medium. As a consequence, this additional engineering effort may make biohydrogen process less economically unfeasible for industrial production of H₂ gas. Yokoi *et al*¹⁰⁹ proposed application of co culture of *Enterobacter aerogenes* and *Clostridium buytricum* instead of these expensive chemical reducing agents to make process inexpensive for effective H₂ gas production by dark fermentation^{109,131}.

Various substrates have been used for dark hydrogen fermentation. Bioconversion of 1 mol of glucose yields 4 mol of H₂ gas in dark fermentation. Highest H₂ yield obtained from glucose is around 2.0-2.4 mol/mol^{132,133} mainly due to the utilization of glucose as an energy and carbon source for bacterial growth. Moreover, in the presence of other type of sugar (sucrose), a yield of 4.52 mol H₂/mol sucrose was obtained at 8 h HRT using continuously stirred tank reactor (CSTR) process¹³⁴. Optimization of C/N ratio at 47 provided efficient conversion of sucrose to H₂ gas with a yield of 4.8 mol H₂/mol sucrose¹³⁰. However, highest yield (6 mol H₂/mol sucrose) was produced by *Enterobacter cloacae* ITT-BY 08, which is highest yield among other tested carbon sources¹²⁰. Collet¹¹⁰ reported maximum H₂ yield of 3 mol H₂/mol lactose although theoretical yield is 8

mol H₂/mol lactose. The results presumably indicate that sucrose gives higher yield compare to other simple sugars, however yield per mole of hexose remains almost the same. According to the reaction stoichiometry, 1 g of starch yields 553 ml H₂ gas with acetate as a by-product¹³⁵. However, practically the yield is lower than theoretical value because of utilization of starch for cell synthesis. Maximum specific H₂ production rate of 237 ml H₂/g VSS/d was observed by *C. pasteurianum* using 24 g/l of edible corn starch¹¹¹ and 365 ml H₂/g VSS/d by *Thermoanaerobacterium* at 55°C¹³⁵. A mixed culture of *C. butyricum* and *E. aerogenes* gives better H₂ yield (2.4 mol H₂/mol glucose) obtained in long term repeated batch operations when starch residue (2.0%) containing wastewater was used¹⁰⁹.

Biohydrogen Production from Cellulosic Materials using Dark Fermentation

Biologically derived organic materials and residues currently constitute a large source of biomass¹³⁶, which includes agricultural crops and their waste byproducts, wood and wood waste, food processing waste, aquatic plants, algae, and effluents produced in the human habitat. Use of these biomass-rich resources for bioenergy and related bioproducts could contribute to displacement of fossil fuels as primary energy source and could reduce GHG emissions. Bioenergy derived from water containing biomass (sewage sludge, agricultural and livestock effluents as well as animal excreta) was mainly produced by microbial fermentation. Production of biohydrogen from renewable resources (lignocellulosic wastes) would become major and attractive future source of energy¹⁷. Bio-conversion of biomass to produce H₂ has been demonstrated utilizing anaerobic fermentation of some well-defined compounds in water^{107,137-139}. However, only limited data on H₂ yield is reported^{113,140} from wastewater sludge. In addition, many processes that produce H₂ from biomass are complementary to those producing biomaterials. Therefore, countries with large agricultural economies have potential for significant economic development through incorporation of bioenergy into bioindustry.

Main source of H₂ production during a biological fermentative process is carbohydrate, either as oligosaccharide or as its polymeric form (cellulose, hemicellulose and starch). Cellulose is predominant constituent of plant biomass and highly available in agricultural wastes and industrial effluents (pulp/paper and food industry⁸), which could be considered as a very

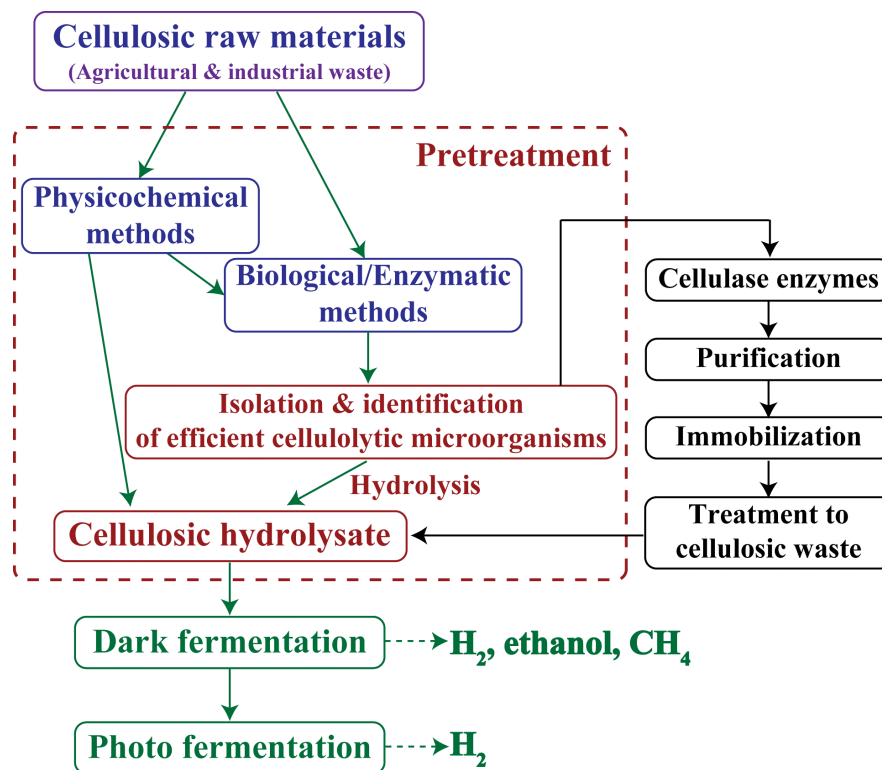


Fig. 5—A possible process configuration for conversion of lignocellulosic feedstock to bioenergy via two-stage hydrolysis and biohydrogen production processes

promising feedstock for biohydrogen production. Significant amounts of H_2 may be produced from cellulosic feedstocks (straw, woodchips, grass residue, paper waste, saw dust, etc.) using conventional anaerobic dark fermentation technology and natural mixed microflora under conditions that favor for H_2 producing acetogenic bacteria (AB) and inhibit methane-producing bacteria (MB)¹⁴¹⁻¹⁴². However, depending on metabolic shift used by organisms within consortium, H_2 yields may be variable^{88,96}. For effective H_2 yield directly from cellulose materials using dark fermentation requires pretreatment processes (delignification and hydrolysis) to dissolve organic matter from a lignocellulose complex and makes process expensive¹⁴³⁻¹⁴⁵. Moreover, microbial (efficient cellulolytic microorganisms) and enzymatic (cellulase complex) pretreatment have potential to convert cellulosic biomass into fermentable sugars and to make process cost effective. Biohydrogen production from cellulosic feedstock under dark anaerobic fermentation could be achieved by either a direct process in which cellulose is simultaneously hydrolyzed and converted into H_2 gas in a single stage or by a two-stage process where cellulose hydrolysis and biohydrogen production are carried out separately (Fig. 5).

Cellulosic Biohydrogen Production Using Direct Process

Due to tightly packed, highly crystalline and water insoluble cellulose becomes recalcitrant to hydrolysis into its individual glucose subunit. In nature, some microorganisms degrade cellulose effectively by using their cellulase enzymes and resulting hydrolyzed products (saccharides) can be converted into H_2 under dark fermentation with coexisting pure or mixed bacterial populations (Table 3). Cellulose can be degraded using cellulolytic and non cellulolytic microorganisms, thus mixed microbial consortia presenting in anaerobic digester sludge, sludge compost, soil, animal feces, etc, may be useful for direct utilization of cellulose for H_2 production¹⁴⁸. In general, anaerobic activated sludge is used for H_2 production from cellulose and biomass. By using mixed culture at thermophilic condition, Liu¹⁴⁷ reported maximum H_2 yield (102 ml H_2 /g cellulose) which is only 18% of theoretical yield due to partial hydrolysis of cellulose. Highest H_2 yield (2.8 mmol H_2 /g cellulose) was observed by using cow dung microflora in presence of cellulose as a substrate under anaerobic dark fermentation¹⁵⁰. Some mixed cultures are also useful to treat raw biomass with better H_2 yield^{65,148}; however,

Table 3—Comparison of biohydrogen production performance using cellulose or hydrolyzed cellulose as substrate under batch culture

H ₂ producer	Cellulosic substrate	Temp., °C	Initial pH	H ₂ yield
Mixed culture Anaerobic digested sludge ¹²⁶	Microcrystalline cellulose, 12.5 g/l	37	7.0	2.18 mmol H ₂ /g cellulose
Heat-shocked mixed cultures ¹⁴⁶	Microcrystalline cellulose, 25 g/l	26	6.0	1.60 mmol H ₂ /g cellulose
Sludge compost ¹⁰⁵	Cellulose, 4.7 g/l	60	NA ^b	0.02 mmol H ₂ /g cellulose
Mixed microflora ^{100*}	Cellulose, 10 g/l	60	5.5 (controlled)	0.90 mol H ₂ /mol hexose
Mixed culture ¹⁴⁷	Palm oil mill effluent	60	5.5	4708 ml H ₂ /(l POME)
Dried mixed sludge ¹⁴⁸	Cellulose	55	6.5	102 ml H ₂ /g cellulose
Heat-treated anaerobically digested sludge ¹⁴⁹	Corn stover biomass	35	5.5	2.84 and 3.0 at neutral and acidic pretreatment
Heat-treated anaerobically digested sludge ¹⁴⁹	Fodder maize	35	5.2-5.3	62.4 ml/g dry matter of fodder maize,
Heat-treated anaerobically digested sludge ¹⁴⁹	Chicory fructo oligosaccharides	35	5.2-5.3	218 ml/g chicory fructooligosaccharides
Heat-treated anaerobically digested sludge ¹⁴⁹	Perennial ryegrass (<i>Lolium perenne</i>)	35	5.2-5.3	75.6 ml H ₂ /g dry matter of wilted perennial ryegrass
Anaerobic cow dung microflora ¹⁵⁰	Cellulose, 3 g/l	55	7.0	21.8 ml H ₂ /g dry matter of fresh perennial ryegrass.
Coculture study <i>Clostridium</i> <i>acetobutylicum</i> X ₉ and <i>Ethanoigenens</i> <i>harbinense</i> B49 ¹⁵¹	Cellulose, 3 g/l	55	7.0	2.8 mmol H ₂ /g cellulose
<i>Clostridium</i> <i>thermocellum</i> JN4 and <i>Thermoanaerobac</i> <i>terium</i> <i>thermosaccharolyt</i> <i>icum</i> GD17 ¹⁰³	Microcrystalline cellulose, 10 g/l	37	5.0	3.66 mmol H ₂ /g cellulose
Individual strains <i>Clostridium</i> <i>pasteurianum</i> ¹⁵²	Microcrystalline cellulose, 10 g/l	60	7.0	10 mmol H ₂ /g glucose
<i>Ruminococcus albus</i> ¹⁵³	Corn stalk powder, 0.5%	60	7.0	16.1 mmol l ⁻¹ Corn stalk powder
<i>Thermotoga maritima</i> (DSM 3109) ¹⁵⁴	Corn cob powder, 0.5%	60	7.0	20.4 mmol l ⁻¹ Corn cob powder
<i>Thermotoga maritima</i> (DSM 3109) ¹⁵⁴	Hydrolyzed carboxymethyl cellulose, 10 g/l	35	7.0	1.09 mmol H ₂ /g glucose
<i>Ruminococcus albus</i> ¹⁵³	Sorghum extract, 3g/l	37	6.4-6.5	14.5 mmol H ₂ /g glucose
<i>Thermotoga maritima</i> (DSM 3109) ¹⁵⁴	Sorghum stalks, 3 g/l	37	6.4-6.5	17.5 mmol H ₂ /g glucose
<i>Thermotoga maritima</i> (DSM 3109) ¹⁵⁴	Sorghum residues, 3 g/l	37	6.4-6.5	14.4 mmol H ₂ /g glucose
<i>Thermotoga maritima</i> (DSM 3109) ¹⁵⁴	Cellulose, 5 g/l	80	6.5	0.96 mmol H ₂ /g cellulose ^c

Contd..

H ₂ producer	Cellulosic substrate	Temp., °C	Initial pH	H ₂ yield
	CMC, 5 g/l			3.29 mmol H ₂ /g cellulose
<i>Thermotoga neapolitana</i> (DSM 4359) ¹⁵⁴	Cellulose, 5 g/l	75	7.0	1.07 mmol H ₂ /g cellulose ^c
	CMC, 5 g/l			3.37 mmol H ₂ /g cellulose
	Glucose, 3 g/l	36	6.8	14.6 mmol H ₂ /g substrate
	Xylose, 3 g/l			16.1 mmol H ₂ /g substrate
<i>Clostridium</i> sp. strain No. 2 ¹⁴⁴	Avicel hydrolysate ^a , 3 g/l			19.6 mmol H ₂ /g substrate
	Xylan hydrolysate, 3 g/l			18.6 mmol H ₂ /g substrate
<i>Clostridium acetobutylicum</i> X ₉	microcrystalline cellulose, 10g/l	37	7.0	0.17 mmol H ₂ /g cellulose
<i>Clostridium thermocellum</i> 27405 ¹⁰⁴	Delignified wood fibers	60	7.0	1.6 mol H ₂ /mol glucose

^aObtained from enzymatic hydrolysis; ^bNot available; ^cConverted from original data; ^{*}Fed batch

compared to pure-culture systems, production yield may be lower due to interference of some MB.

Isolating strains that can effectively utilize cellulose materials to produce H₂ is of great practical interest. For example, *Clostridium thermocellum* is a gram-positive, acetogenic, thermophilic, anaerobic bacterium that degrades cellulose by using cellulosome and carries out mixed-product fermentation, generating gaseous H₂ and CO₂ products, as well as acetate, lactate and ethanol as soluble metabolites under different growth conditions¹⁵⁵⁻¹⁵⁹. Cellulosome is a complex structure located on the surface of cell containing various cellulolytic enzymes^{40,44}. During hydrolysis, bacteria attach to cellulose particles via cellulosome, and enzymes within cellulosome efficiently degrade cellulose to glucose and cellulodextrins, which are transported into cells for metabolism^{40,44}. In biological treatment, to process natural cellulosic materials without pre-treatment and/or sterilization is difficult. However, high optimal growth temperature (60°C) of *C. thermocellum* could prevent contamination of many mesophilic bacteria and there is no need for sterilization of incoming biomass, which is generally required for pure-culture fermentation process. Thermophilic operation also decreases solubility of gases, leading to more efficient removal of product gases (H₂ and CO₂⁴⁰), thereby avoiding product inhibition. *C. thermocellum* shows higher cellulose degradation rate relative to other cellulose degrading *Clostridial* species and has ability to generate H₂, CO₂ and acetate, offering the potential for H₂ production directly from cellulosic waste biomass^{158,159}. *C. thermocellum* 27405 can utilize

cellulose, shredded filter paper, and delignified wood fibers (DLWs) in batch culture under anaerobic dark fermentation¹⁰⁴. A high H₂ yield (1.6 mol H₂/mol glucose) was observed in presence of DLWs with acetate, ethanol, lactate, and formate as fermentation end products¹⁰⁴.

In addition to *C. thermocellum*, some other anaerobic thermophilic microorganisms belonging to genus *Thermoanaerobacterium* (*T. thermosaccharolyticum* and *Desulfotomaculum geothermicum*) are robust with stable cellulolytic enzymes and able to produce H₂ gas in thermophilic acidogenic culture¹¹⁵. For instance, *T. thermosaccharolyticum* gives nearly equivalent H₂ yield compared to *C. butyricum*¹³². *Thermococcus kodakaraensis* KOD1 and *C. thermolacticum* strains can produce H₂ at 85°C and 58 C¹²¹, respectively. Liu¹⁰³ reported that isolated *C. thermocellum* JN4 can degrade microcrystalline cellulose and produce H₂ (0.8 mol H₂/mol glucose) with ethanol, acetic acid and lactic acid as end products. Strain also has ability to degrade natural plant raw materials [corn stalk powder (0.5%; H₂ yield, 9.1 mmol/l) and corn cob powder (0.5%; H₂ yield, 9.4 mmol/l)]. *Ruminococcus albus*, a non spore-forming, obligatory anaerobic, coccoid, ruminant bacterium, produces extracellular hydrolytic enzymes (exoglucanases and endoglucanases), which break down cellulose and hemicellulose^{160,161} and further metabolized saccharides to give mixed fermentation products such as acetate, ethanol, formate, H₂ and CO₂ in different stoichiometric ratios depending on environmental and operating conditions¹⁶². Lay¹²⁶ observed that increasing microcrystalline cellulose concentration under mesophilic

conditions with heat-digested sludge resulted in lower H_2 yields (2.18 mol H_2 /mol cellulose) under a cellulose concentration of 12.5 g/l; yield decreased to 1.60 mmol H_2 /g cellulose when cellulose concentration was doubled at 25 g/l. In a co-culture study of *C. thermocellum* JN4 and *T. thermosaccharolyticum* GD17, in presence of microcrystalline cellulose, H_2 production yield increased about 2-fold to 1.8 mol H_2 /mol glucose in contrast to using single culture¹⁰³. This co-culture system could also effectively utilize several kinds of natural substrates [corn stalk powder (0.5%; H_2 yield, 16.1 mmol/l) and corn cob powder (0.5%; H_2 yield, 20.4 mmol/l)] as carbon sources for producing H_2 , which are more efficient when compared to individual *C. thermocellum* JN4 yield¹⁰³.

Biohydrogen Production from Cellulosic Feedstock Using Two Stage Processes

In direct cellulosic biohydrogen production process, cellulose hydrolysis and sequential H_2 yield, production is carried out by same or co-existing microorganisms. As a result, reducing sugars produced from hydrolysis of cellulose could be consumed by both H_2 -producing and non- H_2 producing microorganisms present in the culture for their growth, thereby markedly reducing H_2 yield. Pure as well as co culture study gives efficient cellulose hydrolysis but pure culture (*C. thermocellum*) usually requires thermophilic condition resulting in an increase in operation cost. For co-culture system, major problems are difficulty of achieving mutual optimal conditions for co-existing cultures as well as consumption of reducing sugar by non- H_2 producing bacteria.

On the other hand, two stage process (hydrolysis-biohydrogen process) where cellulose hydrolysis can be done by using mixed or pure microbial culture and hydrolysate (more reducing sugars) are removed after certain period (or continuously) for sequential H_2 production by using efficient H_2 producers to increase H_2 yield. Taguchi *et al*¹⁴⁵ hydrolyzed cellulose and used hydrolysate for fermentation by a *Clostridium* sp and during 81 h period of stationary culture, organisms consumed 0.92 mmol glucose/h and produced 4.10 mmol H_2 /h. Same culture was also used for H_2 production from pure xylose or glucose and enzymatic hydrolysate of Avicel cellulose or xylan. H_2 yield from hydrolysate was higher than that of carbohydrates, reaching a yield of 19.6 and 18.6 mmol H_2 per g of substrate consumed, respectively¹⁴⁵. Lo *et al*¹⁵² reported isolated microbial consortium (NS) could effectively hydrolyze pure carboxy

methyl cellulose (CMC), and raw cellulosic materials (bagasse and rice husk) under mild conditions. In contrast to thermophilic conditions often required by most chemical and enzymatic hydrolysis²⁴, their system seems to be advantageous in practical applications due to being less energy intensive. In their study, hydrolyzed CMC (10 g/l) gave better H_2 yield (1.09 cellulose/g glucose) during batch study by using *C. pasteurianum* for dark fermentation²⁴. Although two-stage process might achieve better H_2 yield due to the feasibility of optimizing hydrolysis and biohydrogen production stages individually, cost of two-stage process is often be higher than single-stage approaches.

Future Biohydrogen Production Scenario

Fermentative H_2 production from cellulosic feedstock or from lignocellulosic wastes could be competitive with fossil fuel-derived H_2 , providing a plausible approach to practical biohydrogen production. While renewable H_2 technologies that use low value waste biomass as feedstock has great potential to become cost competitive, it is currently more expensive to produce H_2 from biomass than it is to derive H_2 from natural gas. Infrastructure of H_2 storage, transportation and utilization also needs to be established. One way to achieve low-cost biohydrogen is to develop more effective and economically feasible bioprocess for H_2 production from cellulosic feedstock. Process optimization using either one-stage or two-stage conversion of cellulose to biohydrogen needs to be developed. During dark hydrogen fermentation, anaerobic bacteria could produce H_2 while converting organic substrates into volatile fatty acids and alcohols. To achieve better energy yield and lower chemical oxygen demand (COD) level in the effluent, these soluble metabolites (organic acids and alcohols) can be further utilized via photo fermentation using photosynthetic bacteria, such as purple nonsulfur bacteria, resulting in more H_2 production as well as higher COD removal. Thus, an integration process combining dark- and photo- H_2 fermentation could be effective and efficient energy process to increase H_2 production capacity and enhancing energy recovery from cellulosic feedstock in a future prospective. Of course, it still requires tremendous research work to upgrade and improve existing fermentative H_2 production processes in terms of enhancing H_2 yield and rate, along with enhancement on utilization efficiency of either raw cellulosic materials or cellulose hydrolysate.

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