Bio-Hydrogen: Technology Developments in Microbial Fuel Cells and Their Future Prospects

3

Pamidimarri D. V. N. Sudheer, Sushma Chauhan, and Balasubramanian Velramar

Abstract

The energy is the part of the human evolution; the innovation in the transportation and industrial evolution happened in this century made mankind to depend on fossil fuels invariably. The depletion of fossil fuel resources and global carbon footprint accumulation are worrying the global countries for the future environmental safety. The clear policies were amended to come out of releasing the global carbon footprint by many countries; even developing countries are making it compulsory for controlling or reducing greenhouse gases releasing in to environment. In this context hydrogen fuel is getting promising significance since it has high energy content per unit mass, and up on combustion it will not release any carbon footprint and considered to be complete green energy. Though there are many chemical and physicochemical methods available for the production of H_2, biological H_2 production will be superior since this method do not use harsh chemical process and do not need extreme conditions for the production. Hence, many research studies are put forward for the production of biological hydrogen production. In this book chapter we will have comprehensive discussion on these technologies developed for the hydrogen production till date. This chapter also included the next generation technologies which are in acceleration in engineering the strains for the enhancing the productivity and various other parameters like utilization of waste biomass and waste industrial affluent etc. This chapter also included with the list of aspects to be looked for the future development of H_2 as the next generation fuel energy.

P. D. V. N. Sudheer (✉) · S. Chauhan · B. Velramar
Institute of Biotechnology, Amity University Chhattisgarh, Raipur, Chhattisgarh, India
e-mail: spamidimarri@rpr.amity.edu

N. Kumar (ed.), *Biotechnology for Biofuels: A Sustainable Green Energy Solution*,
https://doi.org/10.1007/978-981-15-3761-5_3

Keywords

Biohydrogen · Microbial fuel cell · *E. coli* · Waste biomass and metabolic engineering

3.1 Introduction

Global demand for energy sources, depletion of the fossil fuel resources and critical worry on the greenhouse gas release pusing scientific community looking for the alternative sources of green energy which can check the environmental issues (Sudheer et al. 2010; Sudheer Pamidimarri and Reddy 2014). The global decay of the earth's environmental health and direct accumulation of the carbon footprints relesed by usage of fossil fuels; non-carbon energy source is said to be the way-out for the global crises of energy and to avoid the production of greenhouse gases (Hansel and Lindblad 1998). The non-carbon green fuels available in the present technology are hydraulic, wind, solar energies, and hydrogen fuel. Among these hydrogen fuel can readily answer the global environmental issues and have possibility to compensate the global energy demands (Dunn 2002).

Hydrogen (H_2) produced from biological sources is considered as the cleanest energy. Biological hydrogen is generated from the biological source by the process where green energy is generated by environmentally friendly way and was credited with zero emissions of pollutants. Globally at present H_2 is the most promising source in the succession of fuel evolution. Hydrogen fuel is encouraged throughout the globe because of several technical, socio-economic, and environmental benefits (Das and Veziro□lu 2001). H_2 gas is considered to be safer compared to the natural gas and better than domestic natural gas and is now universally accepted as environmentally safe. Moreover, hydrogen fuel could be generated from renewable source which can defy the greenhouse effect (Kumar and Kumar 2017). Presently, H_2 is produced from various sources like natural gas, heavy oils, naptha, coal, and electrolysis which in turn contribute to greenhouse emissions. Microbial cell factories, unlike the chemical or electrochemical counterparts, generate no effluents and are environmentally safe. Biological production of hydrogen catalyzed by microorganisms in an aqueous environment at the ambient temperature and atmospheric pressure is a complete green process (Lynd et al. 2009; Chaubey et al. 2013).

Globally, the major share of energy utilization is for the transportation and it occupies the share of 65%. Petroleum based fuels are the sole source of transportation fuel presently used, which is causing the local and comprehensive climate change and air congestion in the urbanized areas (Kumar and Kumar 2017). This is causing the alarming disturbance in the air quality and making the metro cities unsuitable for the living. If the same continue further, the future position of the urban areas in prospective of living standards will be deteriorated and countries need to spend the major section of economy for the health care. Hence, replacing the traditional transportation fuel (petroleum and coal based) with hydrogen fueled transportation system will improve the situation and can make the metro and urban

cities more human friendly (Das and Veziro?lu 2001; Maeda et al. 2012; Kumar and Kumar 2017).

In the present era of biotechnology, the concept of microbial fuel cell is rising since the biomass requirement of the microbial cells is more flexible and the productivity is reached near to the theoretical values. This whole cell based catalysis for the production of fuel energy is supposed to be the most efficient system which can answer the present energy crisis. Hydrogen production from the microbial fuel cell is said to be a good concept of green fuel since the hydrogen fuel combustion results in no greenhouse gases. Moreover, the energy content per mass of the hydrogen energy is 142 MJ kg^{-1} which is better than biofuels like bio-ethanol and biodiesel (Maeda et al. 2012). This book chapter presents the microbial hydrogen fuel cells, their significance and production mechanism, will discuss further about the different microbial sources of hydrogen production, the biomass requirement, and prospective utilization of lignocellulosic biomass or other waste biomass. A separate section is dedicated for the biotechnological approaches for the improvement of hydrogen production in *E. coli*. The concluding part will include the future prospective of the microbial fuel cells and possible strategies for enhancing the hydrogen production and aspects of hydrogen economy for the implementation.

3.2 Hydrogen Production Sources

Currently, hydrogen production is by three major processes; these include electrochemical, thermochemical, and biological process. Superiority of these methods is always under debate since each method is having its own credits and demerits (Stojić et al. 2003; Turner 2004). Biological or microbial based hydrogen fuel production is encouraged globally for their independence of non-renewable substrates. In this section brief account of each method and their merits and demerits will be discussed and detailed discussion is made on microbial based hydrogen fuel production.

3.2.1 Electrochemical Process

Electrochemical process is the first process to be designed for the production of hydrogen from the source of water via electrolysis. It is the simple splitting of the water in to corresponding components by using the electrical energy (Stojić et al. 2003). There are majorly two types of the process involved in the electrolysis; these are by alkaline electrolyzer and the polymer electrolyte membrane (PEM) electrolyzer (Marcelo and Dell'Era 2008). The efficiencies of these processes are about 56–73%. Though, the H_2 considered to be green energy source, however, the greenness of the process is mainly depending on the source of electricity utilized in the process. Hence the debate of the greenness of the process is still continuing. Utilizing solar energy for conducting the electrolysis is considered to be the best way for making whole process environmentally green. Considering the renewable source of electricity (via solar or wind power) the process can be most permissive in the

view of carbon footprint. However, the investment is needed for shifting towards hydrogen renewable energies. In economic stand point for the production, cost per unit is very high and is not a method of choice for the commercial production. Moreover, the investment needed for this is very high and this will be added to the production cost.

3.2.2 Thermochemical Process

Unlike the electrochemical process thermochemical process is more suitable for the bulk production and will have possibility of scale-up to the commercial level due to its higher productivity and efficiency (Ohta 1979; Freni et al. 2000; Funk 2001). There are various thermochemical methodologies used to produce H_2. These include thermal dissociation, thermal pretreatment (pyrolysis and gasification), and reforming. Among these three processes, only the thermal dissociation method uses direct splitting of water into corresponding elements and produces H_2 as same as in case of electrochemical process (Utgikar and Thiesen 2006). Later two methods use either hydrocarbons or organic biomass as starting material for the production of H_2 (Haryanto et al. 2005; Navarro et al. 2007). Thermal pretreatment method uses carbonaceous matter, and is first converted to smaller constituents which can be used for the production of H_2 in the second phase. Pyrolysis is the popular method for converting the rice husk or similar biomass into hydrogen. Gasification is similar to reforming, where it uses steam or oxygen for the conversion of carbonaceous material or biomass into gaseous product (Vasudeva et al. 1996; Marquevich et al. 2000; Demirbas 2004; Czernik et al. 2007). However, these methods are under debate since all these discussed methods rely on energy input which may not be from the source of green process. Hence, there are many efforts were made to integrate renewable energy like solar energy for the production of heat energy which can be used in the process (Fujishima et al. 2000). Moreover, the process reforming and pyrolysis process use the hydrocarbons as raw material whose sources are non-renewable; hence, long-term production technologies using renewable biomass must be developed for the sustainable production of H_2.

3.2.3 Biological Process

Biological production of H_2 is said to be the most prominent process since the technology involves complete green production and moreover the flexibility of starting material could be diverse based on the microbial source utilized for fermentation. The hydrogen producing microbes can be divided into two groups: photosynthetic and non-photosynthetic or fermentative hydrogen producers (Das and Veziro□lu 2001). Both processes use renewable raw material for the biomass generation and hydrogen production. Superiority of any method is not relevant since both photosynthetic and fermentative process have own advantages and

demerits. Hence the following section describes in details regarding biological H_2 production.

3.3 Microbial Hydrogen Fuel Cells

In contrast with electrochemical or thermochemical hydrogen production microbial fuel cells for the H_2 production is always given superiority because they are based on completely green process. Moreover, the process could be conducted in ambient condition without use of extreme temperatures and pressures. As mentioned earlier, among the photosynthetic and fermentative methods, much of the research is focused on the fermentative method because of the advantages like (1) this method does not depend on the presence of light for the H_2 production, (2) its higher production rates, and (3) a variety of carbon energy sources like organic matter, low-cost carbohydrates, cellulosic, lignocellulosic, cellobiose, and other waste biomass could be used as carbon source to grow the microbial cell mass for the production of H_2. In this section we will discuss both photosynthetic and fermentative methods of H_2 production.

3.3.1 Photosynthetic H_2 Production

Photosynthetic H_2 production is carried out by various bacterial, algal, and cyanobacterial species. These microbes use diverse pathways and various machinery for the generation of cellular energy and H_2 production, respectively. These photosynthetic H_2 producing bacteria can be grouped majorly into two groups based on oxygen generation. Majority of algal and cyanobacterial species use photosystems for harvesting the energy, and electrons are donated by photolysis of water, importantly the O_2 accept the electrons finally and these are called oxygenic photosynthetic H_2 producers (Barbosa et al. 2001; Kovács et al. 2006). The other group depends on various organic acids for the electron donors and use nitrogenases for the production of H_2 as a by-product during nitrogen fixation. In this section the mechanisms, advantages, technical limitations, and future prospective will be discussed in detail.

3.3.1.1 Oxygenic Photosynthetic H_2 Production

Photosynthesis is the basic functional aspect of plants, algae, and cyanobacteria. In the process of oxygenic photosynthesis H_2O is oxidized, generate O_2 and the electrons will be used by photosystems for the reduction of NADP. The protons released during photolysis combined with the electrons passed to membrane, upon electron transport by reducing NADPH or ferredoxin will be used for the production of H_2 by hydrogenases in many cyanobacteria and algae (Miyake et al. 1999) [Fig. 3.1]. In general, the photosynthetic system needs four electrons for a pair of electrons sequester from H_2O and reduce NADP or to generate couple of H_2 molecules. The major advantage of this process is, it utilizes the light energy for

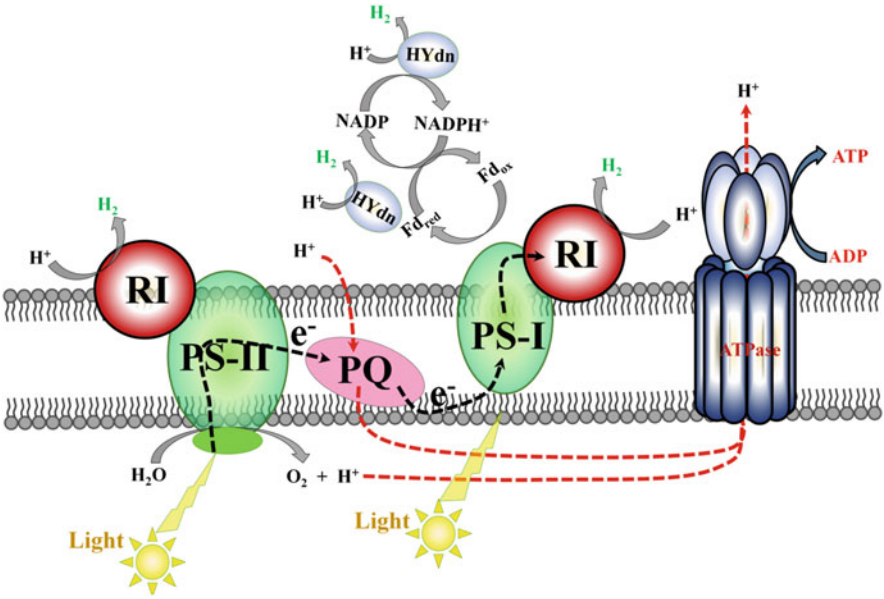

Fig. 3.1 Photosynthetic oxygenic H_2 production by microalgae and cyanobacteria. *RI* reactive intermediate, *PS-I* Photosystem 1, *PSII* Photosystem II, *PQ* Plastoquinone, *Hydn* Hydrogenase

the splitting of H_2O to O_2 and H_2 (Dutta et al. 2005; Lee et al. 2010a). This oxygen generating H_2 production system is the only green energy produced from the renewable light energy without emission of CO_2 and also it has a great importance of fixing the CO_2 and also generates the algal biomass which could be used for many biotechnological and fermentative applications (Miyake et al. 1999; Dutta et al. 2005). Although oxygenic photosynthetic H_2 production looks very promising, the major challenge in commercial implementation is especially in the context of engineering limitations for designing a suitable bioreactor for scale up to the level of industrial production. Since, the system needs the illumination of light, engineering a closed system with translucent glass reactor for the bulk production is necessary. Hence, there should be an innovative reactor model need to be designed for the bulk production and scale-up.

3.3.1.2 Non-oxygenic Photosynthetic H_2 Production

Though, the oxygenic photosynthetic hydrogen production system is under major discussion; a separate group of bacterial species called non-oxygenic photosynthetic H_2 producers comes under the group photosynthetic purple non-sulfur bacteria are also important group worth discussing in this section. The genera *Rhodobacter*, *Rhodopseudomonas*, and *Rhodospirillum* are the major representatives of photosynthetic purple bacteria that generate H_2 without generating O_2 (Lee et al. 2010a). These are the alternative photosynthetic H_2 producers in place of oxygenic photosynthetic H_2 producers. These utilize light as the energy source and organic acids

most commonly carboxylic acids as electron donors. Since H_2O does not act as the electron donor, hence no oxygen is released. The major benefit of this system is, in case of oxygenic photosynthetic H_2 production, the sensitivity of hydrogenases towards the presence of O_2 in high concentration will inhibit or lower in several folds the production efficiency. These non-oxygenic H_2 producers do not generate O_2 since this system uses nitrogenase in place of hydrogenases to generate H_2 (Masepohl et al. 2002). This system can effectively bypass the issue of hydrogenase sensitivity to the O_2 and can integrate with dark fermentation using organic acid containing effluents. This integrated system will be very valuable in harvesting energy from light; in addition it will help in effluent treatment and producing valuable green energy. The stoichiometry of moles of H_2 released during the fixation of mole of N_2 differs vastly. It ranges from 1 mol of H_2 produced while fixing 1 mol of N_2 by common Mo-containing nitrogenase to 9 mol of H_2will be produced while fixing a mole of N_2 by highly oxygen sensitive Fe-containing nitrogenase. Despite the unfavorable hydrogen production by nitrogen fixation, which may not be economically valuable; however, acceptable amount of H_2 production is possible if an efficient reactor system is developed based on the utilization of waste organic effluent. This could harvest natural light can bring an economically feasible system for H_2 production while treating effluent (Harwood 2008).

3.3.2 Hydrogen Producing Machinery (Hydrogenases/ Nitrogenases) in Photosynthetic hydrogen Production

The most common hydrogenases are Fe-Fe hydrogenases prominently present in most of the bacteria and eukaryote and followed by Ni-Fe hydrogenases present generally in *Achaea* and some species of bacteria. Among these Fe-Fe found to be more sensitive to oxygen compared to Ni-Fe hydrogenases. Fe-Fe hydrogenases are highly sensitive to oxygen and undergo denaturation even under trace concentrations of O_2 in the cell. Ni-Fe hydrogenases found to be more stable in the presence of O_2; in few cases up to minutes of exposure these remain stable and active (Stripp et al. 2009). Hence, Ni-Fe hydrogenase containing microbial source, in this case H_2 production in micro-oxygenic conditions is more preferable than Fe-Fe hydrogenases. Moreover, unlike Fe-Fe hydrogenases, Ni-Fe hydrogenases upon long time exposure to O_2 will get inactivate reversible rather than irreversible manner, hence, H_2 production can be revived by removal of O_2.However, the Fe-Fe hydrogenases have advantage of high rate of H_2 production compared to the Ni-Fe hydrogenases (Ghirardi et al. 2007). In case of scale-up production in industrial scale, the hydrogenases with O_2 stability will have better advantage, Ni-Fe hydrogenases are more preferred. These hydrogenases are taken as subject of studies in the aspects of molecular improvement and could be selected for the future protein engineering studies. The most promising virtue of enhancing the productivity is heterologous expression of more oxygen tolerant hydrogenases in efficient microbial system for the H_2 production. Introducing gene cluster of tolerant hydrogenase gene cluster into target organism can be beneficial system for enhancing H_2 production.

However, expression of active hydrogenases is very difficult since the maturation of the hydrogenase apparatus to involve multiple steps to produce active protein. Hence along with hydrogenase gene cluster, the maturation proteins also need to express in the heterologous system. Few studies reported in this regard (Maeda et al. 2008; Vardar-Schara et al. 2008); however, the successful bench scale studies need to be scale up to the industrial level for the real economic success. The reactor engineering is the major part of research to be concentrated for making these lab scale studies to get commercial success.

In evolution, purple bacteria generally produce H_2*via* nitrogen fixation; hence, the hydrogenases are replaced with nitrogenases and H_2 produced as by-product during nitrogen fixation. Nitrogenases catalyze high energy implicated, electron intensive N_2–fixation and there is no oxygen involvement in this process. Like in case of hydrogenases, nitrogenases are also oxygen sensitive and need to be protected from oxygen for their normal functions. Majorly two types of nitrogenases understood and they are Mo-containing nitrogenases and Fe-containing nitrogenases. In virtue of productivity Fe-containing nitrogenases produce high stoichiometric (9 mol) H_2production of per 1 mol of N_2 fixation. In this regard, Mo-containing nitrogen fixation found to be more energy intensive (use 16ATP) for the production of 1 mol of H_2 (Harwood 2008). Unlike in case of photo-chemical H_2 production, where the electron donor is by photolysis of water; purple bacteria needs organic acid for the electron to be provided to the microorganisms. Hence, the economic feasibility is under debate unless the carbon source is derived from the waste biomass or from organic effluent. So, key challenge here is to integrate the waste biomass and/or effluent carbon source with light harvesting bioreactor for efficient and economically viable hydrogen production by purple bacteria.

3.4 Fermentative Hydrogen Production

H_2 production via fermentation which does not need any light energy, more specifically it is also called as dark fermentation. The hydrogen is produced in the dark fermentation by taking H_2 as electron sink and is possible via anaerobic fermentation. These microbes are divided into two major groups; (1) Obligate anaerobe H_2 producers and (2) facultative anaerobe H_2 producers. The obligate anaerobes are strict anaerobes that will harvest the electron from pyruvate oxidation, then use these electrons for the oxidation of ferredoxin (Fd), further these electrons travel to the hydrogenases where H_2 will be produced. The best examples of this category are *Clostridium, Ethanoligenens*, *and Desulfovibrio*. The second group is facultative anaerobes which produce H_2 *via* formate oxidation. In this process formate is electron donor and produces hydrogen through formate hydrogen lyase. The major group of microbes fall under this system are *Enterobacter, Citrobacter, Klebsiella, Escherichia coli*, and *Bacillus* species (Brosseau and Zajic 1982; Kapdan and Kargi 2006). The dark fermentation takes up a pair of electrons and the ultimate sink of the electron is not always H_2. Only a part of electrons will be parted to produce H_2. In many cases only 17% of electrons are ended up in producing H_2 and other will be

accepted by other organic side products. The best example is, up on glucose fermentation by *E. coli* only the theoretical yields of H_2 are 2 mol per 1 mol of glucose and many other organic products act as electron sinks and will be accumulated in the culture medium. Ethanol and lactic acid are popular among those. To push maximum metabolic flux towards the H_2 production, many researchers made efforts in metabolic engineering and successfully made recombinant *E. coli* strain to make the H_2 production near to theoretical yields. Moreover, many organisms have hydrogenases which also conduct reversible reaction which utilize H_2 for the electron generation and utilize the protons for the reduction of co-factors (Hallenbeck 2012). Hence, the gene product needs to be removed in the cell via gene knockout for stabilizing the produced H_2. There are prominent studies conducted in this aspect and will be discussed in the preceding section in detail.

3.4.1 H_2 Production by Microbes and Productivity

Hydrogen energy by dark fermentation was studied from past couple of decades. However, the research was more confined to the laboratory. There are very limited studies promoted up to pilot scale level. Though the technologies demonstrated in the laboratory, the major success in scale-up will depend on the efficient bioreactor engineering. Many times though successful hydrogen is generated through the fermentation, instability to maintain the produced hydrogen is also a major issue since the microbial hydrogenases are equipped with reversible reaction to take up the H_2 back and release protons for reducing the co-factor. In nature dark fermentation occurs in a larger quantity utilizing the organic matter releasing H_2 in the environment by various processes. This process is called anaerobic digestion (Antonopoulou et al. 2008; Ren et al. 2011). During this process hydrogen is produced as a by-product; however, the produced product will be immediately utilized by other microbes producing methane and CO_2 as an end product. In this process many microbial communities are involved, namely hydrolyzers, acetogens, facultative anaerobic H_2 producers combined with methanogens and Archaea bacterial communities (Tapia-Venegas et al. 2015). Though the synthetic anaerobic digestion systems are reported for H_2 production by many researchers, these processes will be discussed in the later part of this section.

Pure cultures are always advantageous for study and implication in any microbial based fermentation system because of their consistent results, and easy for the storage and reproduction of the process. Pure cultures are significant in the aspect of metabolic control, easy for the establishing optimized conditions, also suitable for the molecular manipulations for enhancing the H_2 production by diversion of metabolic flux towards H_2 production either by addition of heterologous genes or knockout of the unwanted genes in the genome. In a dark fermentation process by a pure culture, the possible complete oxidation of glucose can result up to 12 molecules of hydrogen. However, this is true when only complete energy is released as H_2 gas. In dark fermentation the H_2 production in any microorganism is only a by-product during production of fermentation products like ethanol, acetate, formate, or butanol,

etc. In this dark fermentation the maximum yields of H_2 production can reach to 4 mole of H_2 from any hexose sugar. Moreover, sugar as a carbon source will be utilized for the biomass generation. Hence, even if theoretical stoichiometry is followed, still the H_2 productivity using glucose will not be economically feasible compared to other commercial system through which H_2 is generated presently. There should be a cheap and/or waste biomass should be implied to make the technology economic then it can compete with present technologies (Kim et al. 2006a; Ghimire et al. 2015) (Table 3.1).

3.4.2 Metabolic Pathway of H_2 Production in Microbial Cell

The simple hexose sugar glucose is a basic sugar used as carbon source by microbes. The microbes follow majorly two routes for the production of H_2. As mentioned earlier, H_2 is the by-product of dark fermentation and the final fermentation product is organic acids like acetic acid, butyric acid, lactic acid or alcohol like ethanol or butanol [Fig. 3.2]. In majority of microbes the glucose degradation leads to the pyruvate production via basic pathway of glycolysis. It results in the production of cellular energy, i.e., ATP and reduction of NAD to form NADH. This pyruvate now either converted in to acetyl-CoA and CO_2 or acetyl-CoA and formate. In the first case, the reduce ferredoxin molecule will be oxidized to produce H_2 by pyruvate ferredoxin oxidoreductase (PFOR). In the later situation, the formate was converted to H_2 and CO_2 by formate hydrogen lyase (FHL) system and whole pathway is called pyruvate formate lyase (PFL) pathway (Cai et al. 2011; Hallenbeck et al. 2012). The most popular organisms follow these pathways are *Clostridium* sp., being an obligate anaerobe follow the former one and *E. coli* as a facultative anaerobe will follow the later pathway produce H_2 from formate using FHL system. The productivity of these two pathways differs significantly. The production of H_2 by facultative anaerobes using FHL system depends on formate dependent [Fe-Fe] hydrogenases in most cases will not use NADH produced during glycolysis; hence, various products (ethanol or lactate) will be formed upon oxidizing the NAD. Hence, the final product of this pathway is only 2 moles of H_2 for 1 mole of glucose utilized. Unlike FHL system which follows PFL pathway, in case of PFOR pathway hydrogen production results by oxidation of reduced ferredoxin (Fd_{red}) with the help of ferredoxin dependent [FeFe] hydrogenase. Moreover, two more H_2 can also be generated by oxidation of NAPH with the help of NADH dependent [Fe-Fe] hydrogenase or NADH-Fd_{red} dependent [Fe-Fe] hydrogenase. Hence, here the productivity can be 2–4 mole of hydrogen from 1 mol of glucose. This shows the potentiality of the POFL pathway in efficient production of hydrogen (Tapia-Venegas et al. 2015).

Table 3.1 Yields of H_2 production using dark fermentation process with different carbon sources and different type of cultures (adopted from Łukajtis et al. 2018)

Substrate	Microorganism	Temp (°C), pH, hr. (h)	Hydrogen productivity	Hydrogen yields	Reference
Glucose (1%)	*E. cloacae*	36 °C, 6.0, 3.3 h	447 cm^3 $H_2/(dm^3.h)$	2.2 mole H_2/mole glucose	Kumar and Das (2000)
Glucose 7 g/dm^3	Mixed culture	36 °C, 5.5, 6 h	–	2.1 mole H_2/mole glucose	Kotsopoulos et al. (2006)
Glucose 4.85 g COD/dm^3	Mixed culture	70 °C, 7.2, 26.7 h	11.15 mM H_2/d	2.46 mole H_2/mole glucose	Kotsopoulos et al. (2006)
Glucose 10 g/dm^3	*Clostridiaceae* and *flexibacteraceae*	35 °C, 5.5, 3.3 h	640 cm^3 $H_2/dm^3.h$	4 mole H_2/mole glucose	Oh et al. (2004)
Glucose 10 g/dm^3	Mixed culture from compost	60 °C, 5.5	147 cm^3 $H_2/(dm^3.h)$	2.1 mole H_2/mole glucose	Morimoto et al. (2004)
Glucose 20 g/COD/ dm^3	*Clostridia sp*	32 °C, 6,6 h	7.42 mM H_2/ (gVSSh)	1.42 mole H_2/mole glucose	Lin and Chang (2004)
Lactose 29 $mmol/dm^3$	*C. termolacticum*	58 °C, 7, 35.7 h	2.58 mM H_2/ $(dm^3.h)$	1.5 mole H_2/mole hexose	Collet et al. (2004)
D-xylose 10 g/dm^3	*E. cloacae IIT-BT 08*	58 °C,7, 35.7 h	348 cm^3 $H_2/(dm^3.h)$	0.95 mole H_2/mole xylose	Kumar and Das (2000)
L-Arabinose 10 g/dm^3	*E. cloacae IIT-BT 08*	36 °C, 6, 37 h	360 $cm^3H_2(dm^3.h)$	1.5 mole H_2/mole arabinose	Kumar and Das (2000)
Sucrose 1 $g/COD/dm^3$	Mixed culture	26 °C, 6, 1 h	–	1.8 mole H_2/mole sucrose	Logan et al. (2002)
Sucrose 10 g/dm^3	*E. cloacae IIT-BT 08*	36 °C, 6	660 cm^3 $H_2/(dm^2.h)$	6 mole H_2/mole sucrose	Kumar and Das (2000))
Sucrose 20 g/dm^3	Mixed culture	35 °C, 6.7, 1 h	1.32 dm^3 $H_2/(dm^3.$ h)	–	Chang et al. (2002)
Sucrose 25 g/dm^3	Mixed culture	35 °C,5.5	1504 cm^3 H_2/h	2 mole H_2/mole sucrose	Mu et al. (2007)

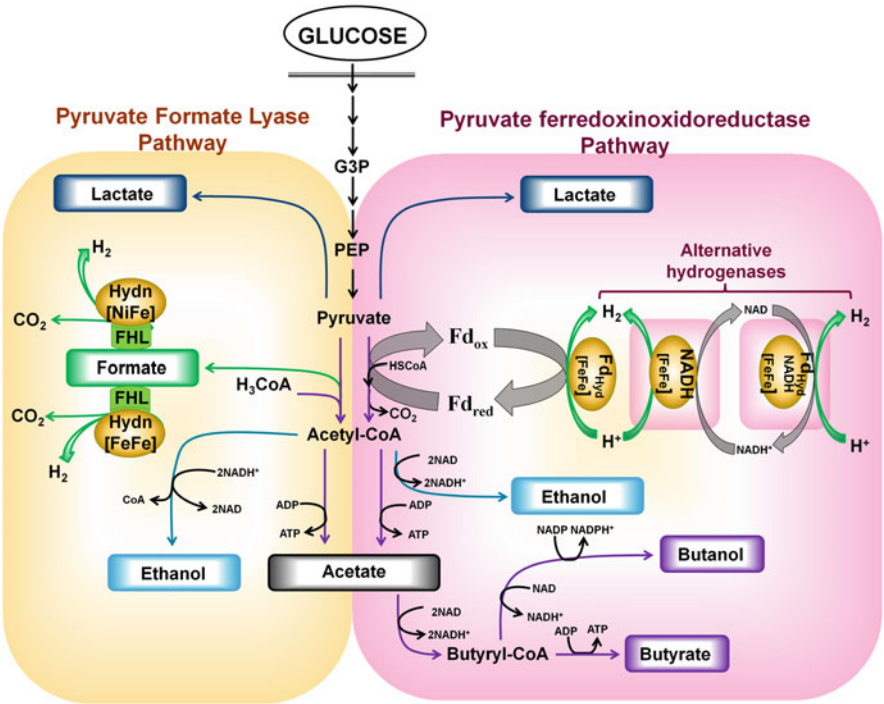

Fig. 3.2 Various pathways used by different microbial species for the production of hydrogen from basic hexose sugar (Glucose)

3.4.3 Dark Fermentation: An Economic Prospective of H_2 Production

Using simple sugars like glucose, sucrose, and lactose are generally studied in the lab scale for understanding the efficiency and stoichiometry of H_2 production. The majority reports demonstrated in the lab scale utilized these model sugars. These sugars are readily acceptable for many microbes and their utilization in metabolic pathway is well known, and manipulating the condition for the better productivity is convenient. However, in economic point of view, the production cost of H_2 using these sugars as carbon source cannot compete the present commercial H_2 production cost. Hence, the technology needs to be developed to replace these costly model sugars with low-cost renewable carbon sources. Utilization of lignocellulosic biomass, crude glycerol generated during biodiesel production, industrial waste water containing different organic acids which can directly enter into the metabolic pathway for the production of H_2, waste biomass having high content of biodegradable sugars looks very promising and many researchers conducted valuable studies utilizing these low cost or waste carbon source for the production of H_2. Dark fermentation found to be very promising concept of H_2 production by utilizing the

waste biomass for the H_2 production from industrial waste biomass [Table 3.2] (Łukajtis et al. 2018; Toledo-Alarcón et al. 2018).

Theoretically, dark fermentation is capable of producing the biological hydrogen from any waste biomass. In the literature, the reports are seen where the hydrogen was derived via biological means by dark fermentation utilizing renewable waste carbon source derived from agriculture, food industry, dairy whey, distillery industry, breviary, pulp and paper industry. Those waste biomasses are rich in starch, cellulose, and lignocelluloses which can be utilized as carbon source in anaerobic fermentation or by dark fermentation via mixed culture anaerobic digestion. The theoretical yield of the H_2 production in dark fermentation depends on the ultimate electron acceptor during the anaerobic fermentation. As shown in the Eqs. (3.1) and (3.2) theoretical yields of the H_2 production depends on the type of fermentation carried by the microorganism producing H_2.

$$C_6H_{12}O_6 + 6H_2O \rightarrow 2CO_2 + 2CH_3COOH + 4H_2 \tag{3.1}$$

$$C_6H_{12}O_6 + 6H_2O \rightarrow 2CO_2 + CH_3CH_2CH_2COOH + 2H_2 \tag{3.2}$$

Though the theoretical yields are either 4 or 2 moles of H_2 from the 1 mol of glucose, the final fermentation yields is always lower than the theoretical yields since the accumulation of different organic acids accumulated as electron acceptors. Moreover, the carbon sources are also used to build up the microbial cell biomass generation. With respective to the results obtained during the fermentation the experimental yields of the H_2 in anaerobic fermentation vary from 1 to 1.5 mole. In economic prospective the conversion of 60 to 80% biomass energy to H_2 said to be a cost-effective process. Possible use of organic acids accumulated during the fermentation for other process could decrease the cost of the production. A number of factors influence in the yields of the H_2 production and in this section few of the important factors were discussed (Levin et al. 2006; Hawkes et al. 2007).

3.4.3.1 Substrates for the Dark Fermentation

The carbohydrates are the major source for the microbes to use for their metabolism and produce H_2 in dark fermentation. Simple monosaccharides such as glucose, xylose, ribose, and disaccharides such as sucrose and lactose are the sugars readily utilized by most of the microbes and produce H_2. In the reports shows that the highest yield of 6 mole H_2 was obtained by utilizing mole of sucrose [83], in case of lactose up to 3 moles/mole of lactose. However, simple carbohydrates are not suitable carbon source in economic point of view because of their cost. Hence, use of these simple sugars makes unprofitable in industrial scale. Continuous and profitable production of the H_2 needs the use of renewable and non-edible sugars. Lignocelluloses or starch polymer derived from the various agriculture and food waste are the good source of alternative to simple sugars and also they act as renewable carbon source for the industry [Table 3.3] (Logan et al. 2002; Hawkes et al. 2007). The major hindrance in utilizing lignocelluloses is, in many instances these carbon sources are not suitable to use directly for dark fermentation due to their

Table 3.2 Hydrogen production achieved from wastewater by different industries (Adopted from Tapia-Venegas et al. 2015)

Substrate ($g_{COD}\ L^{-1}$)	Microbial species	Fermentation Temp (°C), pH, hrs condition	H_2 Production	Reference
Glycerol crude (1)	Activated sludge	40 °C; 6.5	4.90[a]	Mangayil et al. (2012)
Glycerol crude (5)	*Thermotoga neapolitana*	75 °C; 6.8	12.20[a]	Ngo et al. (2011)
Vinasse (0.25)	Hydrogen producers from a packed-bed reactor	25 °C; 5.5	24.97	Fernandes et al. (2010)
Domestic sewage	Hydrogen producers from a packed-bed reactor	25 °C; 5.5	6.01	Fernandes et al. (2010)
Glycerol crude (0.25)	Hydrogen producers from a packed-bed reactor	25 °C; 5.5	6.03	Fernandes et al. (2010)
Brewery (6.05)	Anaerobic sludge	35.9 °C; 5.95	6.12[b]	Shi et al. (2010)
Coffee drink (20)	Anaerobic digest sludge	35 °C; 5.5; 6 h	6.72[c]	Jung et al. (2010)
Cheese whey (40)	Anaerobic digest sludge	55 °C; 5.5; 3.5 h	22.00	Azbar et al. (2009)
Probiotic (9.48)	Mixed anaerobic consortia	37 °C; 5.5; 2 h	9.37[c]	Sivaramakrishna et al. (2009)
Condensed molasses (50)	Co-culture: C *sporosphaeroides*-C *pasteurianum*	35 °C; 7 2 h	9.27	Hsiao et al. (2009)
POME (100)	*C. bytyricum*	37 °C; 5.5 8 h	1.31[b]	Chong et al. (2009)
Distillery effluent (100)	Co-culture: C. *freundii*-E *aerogenes*-R. *palustris*	28–44 °C; 5–7 2 h	14.37[c]	Vatsala et al. (2008)
Cattle (2.4)	Sewage sludge	45 °C; 5.5; 2 h	13.05[b]	Tang et al. (2008)
POME (70–90)	Thermophilic microflora	60 °C; 5.5; 4d	11.66[c]	O-Thong et al. (2007)
Chemical and domestic sewage (2.75)	Anaerobic mixed microflora	29 °C; 6 h	1.25	Venkata Mohan et al. (2007b)
Dairy waste (3.5 g_{COD} $L^{-1}\ h^{-1}$)	Anaerobic mixed microflora	28 °C; 6; 24 h	0.46	Venkata Mohan et al. (2007a)
Citric acid (19.2)	Facultative anaerobic enrichment cultures	35–38 °C; 7; 12 h	4.37[c]	Yang et al. (2006)

Cheese whey (46.5)	*C. saccharoperbutylacetonicum*	30 °C; 6 h	7.03[d]	Ferchichi et al. (2005)
Confectionery (0.6) processing	Soil	23 °C; 6.1; 2 h	6.96[b]	Van Ginkel et al. (2005)
Apple processing (9)	Soil	23 °C; 6.1;	4.09[b]	Van Ginkel et al. (2005)
Potato processing (21)	Soil	23 °C; 6.1;	5.73[b]	Van Ginkel et al. (2005)
Rice whey (34)	Mixed bacteria flora	55 °C; 5.5; 2 h	11.14[c]	Yu et al. (2002)

[a]224 $g_{COD}\ mol^{-1}{}_{glycerol}$
[b]Considering a relation: V/mol = 24.44 L mol^{-1}, 25 °C and 1 atm
[c]192.06 $g_{COD}\ mol^{-1}{}_{hexose}$
[d]Considering a relation: 1.122 $g_{COD}\ g^{-1}{}_{Lactose}$

Table 3.3 Hydrogen production by dark fermentation with different renewable waste carbon resources (Adopted from Łukajtis et al. 2018)

Substrate	Organism	Liquid organic product	Temp (°C), pH condition	Hydrogen productivity	Hydrogen yield	Reference
Kitchen waste: 66% food waste, 27% vegetable waste, 0.96% tea waste, 1.09% egg shells, 1.36% packing materials, 3.61% ash	Mixed cultures	Butyric acid, acetic acid, propionic acid	pH = 5.5	N.D	72 cm^3 H_2/g VS	Jayalakshmi et al. (2009)
Organic municipal solid waste 110 g TVS/dm^3	Mixed culture	Butyric acid	50 °C, 5.5	5.7 dm^3 H_2/dm^3/d	N.D	Zahedi et al. (2013)
Organic municipal waste mixed with poultry slaughterhouse waste 70.86 g/dm^3	Mesophilic anaerobic sludge	Acetic acid	34 °C, 6.0	N.D	71.3 cm^3 H_2/g VS	Gómez et al. (2006)
Kitchen garbage	Anaerobic digester sludge	Butyric acid, acetic acid, ethanol	55 °C, 5.0	1.7 dm^3/H_2/dm^3/d	66 cm^3 H_2/g VS	Chu et al. (2012)
Synthetic food waste (Rice, vegetable, meat 30 g COD/dm^3	Anaerobic sludge from UASB treating cassava wastewater	Butyric acid, acetic acid, ethanol	37 °C, 6.0	0.9 dm^3 H_2/dm^3/d	55 cm^3 H_2/GVS	Nathao et al. (2013)
Potato steam peel 10 g glucose/dm^3	Mixed culture	Acetic acid, lactic acid	75 °C, 6.9	12.5 mM H_2/dm^3h	3.8 mole H_2/mole	Mars et al. (2010)
Kitchen waste from several cafeterias 50 g COD/dm^3	Anaerobic sludge from treatment plant	Butyric acid, lactic acid, acetic acid	55 °C, 5.5	79 mM H_2/l medium/d	N.D	Mohd Yasin et al. (2011)
Food waste: Pasta, bread, fruit, vegetable, fish, and meat	Mixed culture from aerobic sludge	Acetic acid, butyric acid	36 °C	N.D	70.34 cm^3/gVS	Alibardi and Cossu (2015)
Stimulated food waste fish 5% meat 10%; bread 10%; onion 5%; carrot 5%; cabbage 10%; potato 15%	Mixed culture from digested sludge	Acetic acid, butyric acid	34 °C, 5.5	0.23 N dm^3 H_2/kgVS	20.5 dm^3 H_2/kg VS	Redondas et al. (2012)

Mixed food waste from residential home	Anaerobic sludge from treatment plant	Butyric acid, lactic acid, acetic acid	50 °C, 7.5	54.2 cm^3 H_2/h	57 cm^3 H_2/gVS	Pan et al. (2008)
Raw cassava starch	Facultative anaerobic bacteria	Butyric acid, acetic acid, ethanol	35 °C	N.D	1.44 moles H_2/mole glucose	Wang et al. (2017)

polymer nature and slow microbial degradation process. The yields are also very slow which adds the operational cost for the production of H_2 (Hallenbeck et al. 2012). The researchers come with the idea of pretreatment using chemically or biological means. In case of biological pretreatment, the biomass is subjected to pretreatment with many fungal species that will release the simple sugars like xylose and ribose which could be easily integrated in to metabolism by microbial species for the H_2 production. The use of other waste carbon sources like organic waste derivatives, cheese whey, milk waste, crude glycerol obtained as by-product during biodiesel production could be best alternatives for the direct use in dark fermentation for the H_2 production. The glycerol after a simple purification by neutralizing with the mild acid followed by heating and filtration will result in the purified form which is devoid of contaminants derived during biodiesel preparation (Sudheer et al. 2018). In this study authors successfully demonstrated the utilization of crude glycerol generated during synthesis of biodiesel from *Jatropha* seed oil. This work proving the potential of utilization of waste crude glycerol as biomass for the many fermentation process and could be also implemented for the dark fermentation to generate H_2 gas (Hawkes et al. 2007; Ren et al. 2011).

Organic waste generated from domestic kitchen, food industry, bravery industry, and restaurants is also rich in carbon source in the form of simple sugars, cellulose, hemicelluloses, proteins, and lipid (Jayalakshmi et al. 2009). This waste biomass not only fulfills the carbon source but also some part as nitrogen supplement. These organic wastes are also very much suitable for the microbial fermentation. Moreover, the dark fermentation utilization of mixed culture fermentation results in the green manure rich in the form of simple nutrients. By using these waste biomasses to produce the H_2 gas will have two-way advantages. One is, released to environment these will be taken up by methanogens and result in release of methane which in turn increase the carbon footprint. Utilizing it for the H_2 production will result in green fuel (H_2) with nil carbon footprint upon combustion (Guo et al. 2008). Many researchers also consider the municipal waste also organic waste, since it is rich in carbohydrates, disaccharides, proteins, and peptides. In addition, sewage sludge is of rich in the microbial community and no need to add externally any microbial inoculum. However, the sludge should be pretreated to remove the hydrogen utilizers like methano-bacteria. Various methods are suggested to remove these methano-bacteria. The simple methods are treating the sludge by microwave or ultrasound, acid or alkaline treatment. Guo et al. have studied in details and found that sludge treated by microwave and ultrasound treatment provided highest yields of H_2 production (15 cm^3 H_2/g COD) (Valdez-Vazquez et al. 2005; Karlsson et al. 2008).

3.4.3.2 Microbial Type and Source

As introduced about the microbial types for the H_2 production in the earlier section, in this section the details of the microbial system for H_2 synthesis will be discussed in detail. The hydrogen gas production is purely of anaerobic fermentation and the cultures to be used should perform the anaerobic fermentation. This can be done by both obligate (strictly sensitive to oxygen) and facultative (grow in both in presence

and absence of oxygen) anaerobic bacteria. Dark fermentation can be carried in either pure cultures or mixed cultures. Both systems have their superiorities and disadvantages [Table 3.1]. The pure cultures fermentation is a single bacterial strain that will involve in the fermentation utilizing a metabolizable carbon sugars. The best example of bacterial genus is *Clostridium* sp. *Clostridium* sp. is an obligatory anaerobic bacterium that utilizes many simple sugars and produces H_2 via dark fermentation. The major characteristic feature of this species is, it performs the fermentation in variable carbon sources and also it has the ability to survive in difficult conditions such as high temperatures, pH, and presence of toxic substances. The major disadvantage with this species is; it produce the H_2 during the log phase and once reach to stationary phase the metabolic flux will be shifted towards accumulation of organic compounds. Depending on the substrate used for the fermentation, *Clostridium* produces H_2 along with accumulation of organic acids like acetic acid and butyric acid. Though wide variety of species like Methylotrophs, enteric bacteria like *E. coli*, *Enterobacter*, *Citrobacter*, *Alcaligenes*, *Bacillus* are capable of performing the dark fermentation as a pure culture; mixed culture fermentation has its superiority in H_2 production from a complex organic or carbon source derived from waste biomass (Kapdan and Kargi 2006; Hallenbeck et al. 2012; Łukajtis et al. 2018).

The mixed consortia under a strict controlled condition can perform dark fermentation on complex organic carbon source and produce H_2. These enriched consortia perform the dark fermentation utilizing broad spectrum of carbon source like industrial waste, animal waste manure, agricultural waste, sewage sludge, compost, and domestic kitchen waste. Upon the dark fermentation via mixed consortia will generate acetic acid, formic acid, butyric acid, and CO_2 along with H_2. The mixed culture fermentation has the advantage of utilizing the waste biomass like cellulose and lignocellulosic biomass directly without the pretreatment since metabolic cooperation one species with other will help in utilization of complex carbon sources. Hence, the mixed consortia based dark fermentation is the best way of utilization of waste biomass for the production of biohydrogen (Miyake et al. 1999; Logan et al. 2002; Ren et al. 2011; Łukajtis et al. 2018).

The other group of bacteria, i.e., the facultative anaerobes utilize oxygen for the generation of ATP and switch to anaerobic conditions in the absence of oxygen. The best example of hydrogen producing facultative anaerobes is Enterobacteriaceae group. The major system of hydrogen production in this group is via formate hydrogen lyase (FHL) system; where the hydrogen and CO_2 are released by utilizing formic acid as the substrate. The base pathway of formate generation studied via glucose metabolism; where maximum theoretical hydrogen yields are 2 moles of H_2 per mole of glucose. The final electron acceptor in the metabolism is most of the times organic acids or ethanol. Hence, at the end of the fermentation these organic acids are generated as end products along with hydrogen. To enhance the productivity and diverting the metabolic flux towards useful organic acids many researchers utilized molecular approaches, and details of this genetically modified strains for enhancing the hydrogen are described in the coming section (section details).

3.4.3.3 Fermentation Conditions Which Influence H_2 Production

Dark fermentation utilizing the mixed culture or pure cultures, the comprehensive reactions flow involved in the microbes for the production of hydrogen are thermodynamically favorable; however, they are controlled via biological regulators by various mechanisms in microbial cells and need to have favorable conditions to attain maximum productivity. The optimal growth and production conditions should be maintained to get maximum productivity during fermentation process. Three major factors which influence the fermentation conditions are (a) temperature, (b) pH, and (c) gas partial pressures. In this section we will give details of these conditions and how they influence the end productivity of H_2 in the fermentation.

Temperature

The crucial factor in any fermentation system is the temperature in which the fermentation system is operating. The productivity affected to the level of 100% or up to nil if favorable temperatures are not provided. There are no generalized temperatures defined for the H_2 production. It ranges from ambient (20 °C) to as high as 80 °C. The optimum temperatures depend on the type of organism and/or crucial bacterial species whose hydrogenase system responsible for the H_2production in context of mixed fermentation. Basically, bacterial species fall under three temperature groups and reports show that in each group of bacteria, ability of H_2 production is reported. The suitable growth conditions like low temperature (5–20 °C) in case of psychrophiles, ambient temperatures to moderately high temperature (25–45 °C) for mesophiles, and high temperatures (65–80 °C) for thermophiles (Levin et al. 2004).

Selection of optimum temperatures for biohydrogen production depends on species in the culture or mixed culture used for the fermentation. And also, the production of H_2 varies with the substrate used as carbon source. In many cases the cell growth and H_2 production temperatures differ since the optimum growth of the cell need not be the favorable temperature for the hydrogenase enzyme which produces H_2. Hence, crucial optimizations are very much necessary for the cell mass generation and H_2 production. Pakarinen et al. (Levin et al. 2004) found that 70 °C is the optimum temperatures for the maximum productivity of H_2 production; however, the cell mass generation is at the highest temperature of 50 °C. The multiple studies confirm that, thermophilic conditions are favorable for the substrates need to undergo hydrolysis during fermentation, and ambient conditions are sufficient for the simple sugars. This is because the high temperatures favor the hydrolyzing enzymes responsible for hydrolysis of complex substrates. One more reason for the enhanced productivity of H_2 in high temperatures is because of low solubility of gases at low temperatures; hence the growth inhibition of microbes will be minimum in low dissolved aqueous medium (Wong et al. 2014). Though in context of H_2 productivity, the high temperatures are favorable; however, in context of energy investment the profitability of process will be low (Azbar et al. 2009).

pH

In any fermentation system pH plays a significant role in cell growth and productivity, since all the metabolic processes are based on the enzyme activity of particular reaction at specific pH. The majority of the enzymes have a specific pH range; when the productivity of a target depends on multiple metabolic reactions, an optimum temperature needs to be studied to get a maximum productivity. The same concept is applicable to produce H_2. Moreover, pH affects the growth of microbes whether it is pure culture or mixed cultures. In mixed culture fermentation, lower pH value favors for the production of H_2 and limits the methanogens to utilize the produced H_2. However, maintaining at specific pH during fermentation is very important. The production of hydrogen is accompanied by the accumulation of organic acids (acetic, lactic, butyric, and propionic) which will lower the pH of the medium makes it unfavorable for hydrogenase complex to produce H_2 gas. Hence, the pH lower than 5 is not advised for the H_2 production (Bowles and Ellefson 1985). It is also noted that both initial pH and the operational pH are important; in case of batch fermentation, initial pH at neutral is favorable. In case of continuous mode, maintaining the nutral pH will favor the maximum productivity (Wang and Wan 2009; Jung et al. 2011). The initial and optimal operational pH to be maintained vary with the kind of microbial strains selected for the fermentation or source of microbial consortia (in case of mixed culture), kind of substrate selected, mode of fermentation (batch/continues) system will determine the pH to be applied for the best productivity.

In general the pH range for the H_2 production is reported to be in the range between 5.0 and 7.0 corresponding to the growth of the bacterial growth (Li and Fang 2007). The optimum pH differs with the substrate used for the fermentation; the neutral pH is suitable for the livestock waste, pH 6.5–7.0 is favorable for the crop/agriculture waste, pH 5–6 is good for the food waste (Liu and Shen 2004; Li and Fang 2007: Guo et al. 2010). However, some studies reported that 7–8 pH conditions also favorable for some mixed bacterial cultures, e.g. the studies of Liu and Shen explained that, the mixed culture fermentation of corn starch substrate gave best hydrogen production at pH 7 and 8 and the production was 103 and 120 mL H_2/g substrate, respectively.

Partial Pressure of H_2

The partial pressure of hydrogen (PPH) in the reactor is very crucial factor that affect the productivity. The hydrogen produced in the microbes is the result of the ferredoxin reduction up on oxidation enzyme hydrogenase. The hydrogenase also participates in reversible reaction up on higher availability of hydrogen gas, hence at high partial pressure of H_2 in the reactor the production rate will reduce and metabolic flux will move towards other products such as organic acids, ethanol, and butanol (Abo-Hashesh and Hallenbeck 2012; Hallenbeck 2012; Ghimire et al. 2015). There are two ways to deal with high PPH in the reactor and make system continue with high productivity. One is reducing the partial pressures of hydrogen produced in the reactor by sparging with inert gas most frequently nitrogen or removing of gas released in the system by application of vacuum. The earlier method

where reducing the PPH by sparging is effective, the results vary with the type of gas applied for sparging. Kim et al. (2006b) applied CO_2 as sparging gas and observed a better productivity compared with the nitrogen sparging. The yields were up to1.68 moles H_2/mole of $hexose_{consumed}$ compared to nitrogen sparging which yielded 0.95 moles H_2/mole of $hexose_{consumed}$. However, the major disadvantage of sparging system is the product will be diluted with the sparging gas and hydrogen separation will become tedious, time consuming, and require cost input. This all make the sparging system non-economic system which make final cost not competitive in commercial prospective. The alternative method as discussed is the removal of the generated gas in the reactor by applying vacuum. Theoretically this looks more beneficial than sparging; however, very limited studies were made in this aspect (Lee et al. 2012).

An alternative to above two methods is proposed by Teplyakov et al. (2002) and Nielsen et al. (2001) using activated selective membrane to hydrogen. The reactor equipped with the membrane system will remove the hydrogen which in turn will reduce the PPH. However, the membranes are effected with biofilms formed by microbes will have to be replaced often. Though many techniques are evolved to reduce the PPH, still much of the research is needed for handling high PPH in the reactor for the better productivity with inexpensive method which is economically competitive.

3.5 Engineered Bacterial System for Improving Hydrogen Productivity

In advance in the molecular biology, availability of genome sequencing system and evolution of various techniques for genome facilitated various researchers to engineer the available microbial sources rather than isolate new microbes with better productivity. The first choice of any researcher for microbial engineering is *E. coli* since much of the molecular information is explored and many tools were developed for the genome manipulation. Moreover, metabolic pathways were well characterized and information is available for easy manipulation for metabolic engineering. The majority of the work in strain engineering for understanding the microbial hydrogen production and/or improving the hydrogen productivity is made in *E. coli*. In this section much of the discussion will be made with the view of *E. coli*.

E. coli is a facultative anaerobe belongs to Enterobacteriaceae family have the intrinsic ability to produce hydrogen. The hydrogen producing apparatus of *E. coli* includes FHL (Formate Hydrogen Lyase) system. FHL system consists of hydrogenase 3 (*hyc*ABCDEFGHI) (Bagramyan and Trchounian 2003) and formate dehydrogenase-H (*fdh*F) (Axley et al. 1990). HycA protein acts as repressor of the FHL system. The FhlA will up regulate the FHL system and in turn will help in accumulation of H_2. However, *E. coli* consume hydrogen produced by the FHL system by hydrogenase 1 (*hya*ABCDEF) and 2 (*hyb*OABCDEFG). The efficient production of hydrogen by *E. coli* is controlled by the availability of formate to FHL

system. There are two formate dehydrogenases, such as, formate dehydrogenase-N and formate dehydrogenase-O and formate transporter (*Foc*A and *Foc*B) (Rossmann et al. 1991; Suppmann and Sawers 1994; Andrews et al. 1997). Moreover, the cells having sufficient amount of formate can divert metabolic flux to produce and enhance the H_2 productivity.The majority of the strain engineering aspects were designed based on the deletion of hydrogen utilizing genes, over expression of FhlA to upregulate the FHL system in turn to enhance the hydrogen production, and making formate available to the FHL system for increasing the productivity. In addition, the hydrogenases which utilize the produced hydrogen via the FHL system need to delete to avoid the reutilization of produced H_2.

3.5.1 Metabolic Engineering of *E. coli* for Better Productivity

Theoretically, the productivity of hydrogen is formed from basic energy molecules such as 2 mole of glucose and 1 mole of formate. Reaching to the theoretical yields in the system is practically not possible, since the microbial cell utilizes much of the carbon source for the growth and cell biomass generation. Hence, many studies are made in the view of hydrogen production always towards getting near to theoretical yields. Maeda et al. (2007a, b, 2008, 2012, 2018) contributed major input on the metabolic engineering of *E. coli* for the hydrogen production. Their studies first time reported to reach the theoretical values when formate was used as the substrate for the hydrogen production. In this study, Maeda et al. (2008, 2018) explained to the theoretical values (Maeda et al. 2012) of over-expressed *fhlA* and deleted the HycA repressor for enriching the FHL complex cell. The hydrogen uptake activity was eliminated by gene deletion of larger subunits (*hyaB* and *hybC*) of hydrogenase 1and 2, respectively. In addition the metabolic flux from formate to H_2 production was enhanced by deleting *fdoG* gene; this will inactivate the FDH which is responsible to convert formate into CO_2 without H_2 production (Maeda et al. 2008; Maeda et al. 2018).

Glucose is the being the starting carbon moiety and less expensive than formate, many researchers taken interest on metabolic engineering of *E. coli* utilizing glucose as the substrate to produce H_2. The basic principle most of the strategies were designed to increase the metabolic flux towards enhancing the formate availability to FHL system for the hydrogen production. As mentioned earlier, the base strain selected always with inactive hydrogenase 1 and 2 to eliminate reutilization of H_2 produced by FHL and FHL repressor (*hycA*) (*E. coli - hyaB*$^{-}$*, hybC*$^{-}$ *and hycA*$^{-}$). These mutations also showed that there is enhancement in H_2 production using glucose as the substrate (Penfold et al. 2003; Yoshida et al. 2006; Maeda et al. 2007; Turcot et al. 2008; Fan et al. 2009; Kim et al. 2009; Mathews et al. 2010). In addition, the H_2 production was further improved by over expressing FhlA with N-terminal truncation (Self et al. 2001; Turcot et al. 2008).

In *E. coli,* the glucose metabolism leads the formation of the phosphoenolpyruvate and pyruvate. The pyruvate is converted in to formate; subsequently, formate is transformed in to succinate and lactate as by-products. Hence, it is necessary to

divert the metabolic flux towards formate and eliminate the succinate and lactate accumulation during fermentation for enhancing H_2 production. The studies based on these were made by various researchers and enabled the recombinant *E. coli* to produce H_2 from glucose (Yoshida et al. 2006; Maeda et al. 2007; Manish et al. 2007; Fan et al. 2009; Kim et al. 2009). These studies targeted genes deletion of *ppc* encodes phosphoenolpyruvate, frd ABCD fumarate reductase, idhA lactate dehydrogenase (Maeda et al. 2007). Table 3.4 comprehended the information of various studied done in *E. coli* and productivity achieved by various engineered *E. coli* strains. In addition to these, expressing Fnr a global DNA-binding transcriptional global regulator also found to enhance the H_2 productivity (Fan et al. 2009). Comprehending all, the best H_2 productivity was obtained with the *E. coli* holding knockout of seven genes (hyaB, hybC, hycA, fdoG, ldhA, frdC, and aceE) five gene inactivation by (hyaAB, hybABC, hycA, ldhA, and frdBC/hycA, hya, hyb, ldhA, and frdAB) (Kim et al. 2009; Mathews et al. 2010) and three gene inactivation (hya, hyb, and ldhA) (Turcot et al. 2008).

As discussed in earlier section about the application of various carbon substrates like crude glycerol or lignocellulosic biomass, application of these components as carbon source will be economically beneficial. In this regard, glycerol fermentation was initially ruled out since the glycerol fermentation do not favor H_2 production. However, these studies made by Dharmadi et al. (2006) and Gonzalez et al. (2008) showed that at alkaline pH was favored the hydrogen production in the presence of potassium and phosphate. Despite the theoretical understanding of anaerobic fermentation by utilizing glycerol have the benefit of extra $NADPH^+$ generation; however, there are many genes whose expression will be shutdown which are based on glucose metabolism. Despite of handful studies on glycerol fermentation by *E. coli* are available; the information existing for hydrogen production is far from the understanding when compared to glucose and other monosaccharaides. This is because of contradictory studies by various researchers and also experimental yields are considerably limited.

Metabolic engineering is a good way to make *E. coli* to produce good amount of H_2 from glycerol. A powerful approach was made by Tran et al. (2014, 2015). In this study the knockout mutant of *E. coli* with seven genes which are mostly participating in enhancing the formate accumulation and blocking the metabolic flux in synthesis of by-products like methylglyoxal. The selected genes deleted are fumarate reductase (encoded by *frdC*), lactate dehydrogenase (*ldhA*), formate dehydrogenase (*fdnG*), phosphoenolpyruvate (*ppc*), nitrate reductase (*narG*), methylglyoxal synthase (*mgsA*), and the regulator of the transcriptional regulator FhlA (*hycA*). The resulted strain is able to produce the hydrogen near to the theoretical value (1 mole of H_2 for 1 mole of glycerol). Instead of targeted gene deletions, Tran et al. applied random mutagenesis for looking genes responsible for hydrogen production in glycerol fermentation (Tran et al. 2014, 2015). In this study four genes were identified which involved in hydrogen production. The individual mutant of the following four genes, namely *aroM, gatZ, ycgR,* and *yfgI* enhanced the hydrogen production up to 1.6fold. Moreover, the mutants not only enhanced the hydrogen production but also increased the growth rate of the mutant strains compared to wild type under glycerol fermentation in anaerobic conditions. In addition to adoptive

Table 3.4 Comparison of In-vivo Hydrogen production by engineered *E. coli* (Reproduced from Maeda et al. 2012)

Substrate	System	H_2 production rate (reported units)	H_2 production rate (converted units)
Protein engineering			
Formate	Protein engineering of HycE (truncation) of *E. coli*	9 μM H_2 (mg protein)$^{-1}$ h^{-1}	9 moles H_2 (mg protein)$^{-1}$ h^{-1}
Formate	Protein engineering of FhlA of E. coli	7 μM H_2 (mg protein)$^{-1}$ h^{-1}	7 moles H_2 (mg protein)$^{-1}$ h^{-1}
Metabolic engineering through modifying multiple native genes in E. coli			
Formate	Inactivation of HycA and overexpression of FhlA	23.6 g H_2 l^{-1} h^{-1}	254 μM H_2 (mg protein)$^{-1}$ h^{-1}
Formate	Inactivation of HyaB, HybC, HycA, FdoG and overexpression of FhlA	113 μM H_2 (mg protein)$^{-1}$ h^{-1}	113 μmol H_2 (mg protein)$^{-1}$ h^{-1}
Cheese whey	Inactivation of HycA and LacI	5.88 ml H_2 OD)$^{-1}$ h^{-1}	11 μM H_2 (mg protein)$^{-1}$ h^{-1}
Glucose	Inactivation of HycA, LdhA, FrdBC and overexpression of FhlA	13 mM (g DCW)$^{-1}$ l^{-1} h^{-1}	26 μM H_2 (mg protein)$^{-1}$ h^{-1}
Glucose	Inactivation of HyaB, HybC, HycA, FdoG, FrdC, LdhA, and AcoE	32 μM H_2 (mg protein)$^{-1}$ h^{-1}	32 μM H_2 (mg protein)$^{-1}$ h^{-1}
Glucose	Inactivation of Hyd1, hyd2, ldhA and overexpression of truncated FhlA	5.3 mM H_2 i^{-1} h^{-1}	24 μM H_2 (mg protein)$^{-1}$ h^{-1}
Glucose	Inactivation of HycA, HyaAB, HybBC, LdhA, and FrdAB	31.3 mM H_2 (gDCW)$^{-1}$ h^{-1}	63 μMl H_2 (mg protein)$^{-1}$ h^{-1}
Glucose + formate	Production of Hyd 1	3 ml H_2 100 ml^{-1}	0.8 μM H_2 (mg protein)$^{-1}$ h^{-1}
Glucose	Inactivation of HyaAB, HybABC, HycA, LdhA, and FrdBC	1.0 mM H_2(g DCW)$^{-1}$ h^{-1}	1.5 μM H_2 (mg protein)$^{-1}$ h^{-1}
Adaptive evaluation			
Glycerol	Chemical mutagenesis and adaptive evaluation	22 μM H_2 (mg protein)$^{-1}$	4 μM H_2 (mg protein)$^{-1}$ h^{-1}
Heterologous gene expression			
Glucose	Production of (Fe) hydrogenase from *E. cloacae*	0.96 mM h^{-1}	14.5 μM H_2 (mg protein)$^{-1}$ h^{-1}
Glucose	Production of HoxEFUYH hydrogenase from *Synechocystis sp.* PCC 6803	22 ± 3 μM H_2 (mg protein)$^{-1}$	4 μM H_2 (mg protein)$^{-1}$ h^{-1}
Glucose	Production of HoxEFUYH hydrogenase and the maturation proteins HypABCDEF and Hox W from Synechocystis sp. PCC 6803	8.4 μM H_2l^{-1}	0.004 μM H_2 (mg protein)$^{-1}$ h^{-1}
Glucose	Production of HydFEGA	420.3 μM H_2 min^{-1} l^{-1}	0.12 μM H_2 (mg protein)$^{-1}$ h^{-1}
Glucose	Production of HydFEGA and inactivation of lacR	1257.5 nM H_2 min^{-1} l^{-1}	0.34 μM H_2 (mg protein)$^{-1}$ h^{-1}
Glucose	Inactivation of lacR, production of hydFEGA hydrogenase from	9.6 mM H_2 (gDCW)$^{-1}$ h^{-1}	10 μM H_2 (mg protein)$^{-1}$ h^{-1}

(continued)

Table 3.4 (continued)

Substrate	System	H_2 production rate (reported units)	H_2 production rate (converted units)
	C. acetobutylicum, CpFdx ferredoxin form *C. pasteurianum* and YdbK		
Glucose	Production of HupSL hydrogenase from *Rhodobacter sphaeroides*	19.68 μl H_2 (ml culture)$^{-1}$ h^{-1}	1.1 μM H_2 (mg protein)$^{-1}$ h^{-1}
Starch	Inactivation of lacR, production of HydFEGA hydrogenase from *C. acetobutylicum*, CpFdx ferredoxin from C. Pasteurianum and YdbK pyruvate-flavodoxin oxidoreductase from *E. coli* and amyE from *B. subtilis*	30 μM H_2 culture^{-1}	0.65 μM H_2 (mg protein)$^{-1}$ h^{-1}
Sucrose	Inactivation of HycA and TatC and expression of the genes encoding ScrKYABR invertase from Bacillus subtilisG	1.38 ml H_2 (mg DCW)$^{-1}$ h^{-1}	3.9 μM H_2 (mg protein)$^{-1}$ h^{-1}
Single gene knockout or expression			
Formate	Inactivation of HycA	NA	100 μM H_2 (mg protein)$^{-1}$ h^{-1}
Formate	Production of FhlA	7 μM H_2 (mg protein)$^{-1}$ h^{-1}	7 μM H_2 (mg protein)$^{-1}$ h^{-1}
	Inactivation of HycA		
Glucose	Inactivation of FocA	14.9 μM H_2 (mg protein)$^{-1}$ h^{-1}	1.8 μM H_2 (mg protein)$^{-1}$ h^{-1}
Glucose	Inactivation of HybC	12.1 μM H_2 (mg protein)$^{-1}$ h^{-1}	1.4 μM H_2 (mg protein)$^{-1}$ h^{-1}

mutagenesis, Hu and Wood isolated a mutant strain named as HW2 which is holding the ability to produce 20 times more productivity and fivefold higher cell growth than original strain BW25113 Δ*frdC* (Hu and Wood 2010). Further transcriptome analysis of this strain showed that the isolated mutant defective in fructose-1,6-bisphosphatase (encoded by *fbp*), formate transportation (*focA*), and tagatose-1,6-bisphosphate aldolase (*gatYZ*). These studies gave a better picture on glycerol metabolism in hydrogen production; however, more comprehensive data is needed to link all these studies for elaborated understanding glycerol metabolism and hydrogen production for better productivity with less energy investment which can lead to a technology which can be as competitive as commercial production presently followed (Akhtar and Jones 2008a).

In addition, with the strategies based on the deletion of targeted genes, adoptive mutagenesis and random mutagenesis; few studies are also made for enhancing the hydrogen production by heterologous expression of various clusters of genes. Among these studies, expression of hydrogenases genes isolated from various strains in *E. coli* is important and results in enhanced H_2 production. In this regard the expression of hydrogenases derived from the microbial species like *Enterobacter cloacae* (Mishra et al. 2004; Chittibabu et al. 2006), *Ethanoligenens harbinense*

(Zhao et al. 2010), *Rhodobacter sphaeroides* (Lee et al. 2010b), *Clostridium acetobutylicum*, and *C. pasteurianum* (Akhtar and Jones 2008b, 2009) were heterologous expressed in *E. coli* BL21 with no ability to produce hydrogen. The heterologous expression of hydrogenases resulted in H_2 production by BL21 strain. Along with hydrogenases heterologous expression, few researchers also tried co-expression of other genes involved in the transportation of substrates and substrate utilizing enzymes which will divert in the core cellular metabolism tried for enhancing the hydrogen production. Few among these a significant study is expression of *scrB* (encode β-D-fructofuranosidefructohydrolase catalyzes the hydrolysis of sucrose 6-phosphate to β-D-fructose and α-D-glucose 6-phosphate) and *scrR* (encodes the negative repressor of the *scr* regulon) which enhanced the hydrogen productivity up to twofold from sucrose (Penfold et al. 2003).

3.6 Future Prospects

Hydrogen being the only green fuel which does not release any carbon footprint up on combustion is the next generation fuel for the future environmental outlook. To make this fuel as alternative fuel for the transportation and other industrial applications, the production cost must come down as competitive as commercial available hydrocarbon based fuels. The key points to be looked in the aspects of biohydrogen production is (1) innovative methodologies to be developed to utilize waste biomass and industrial waste water effluents, (2) isolating and developing efficient strains which could be used for hydrogen production utilizing more diverse carbon substrates, (3) engineering the microbial system for enhancing the productivity, resistance to growth retarding fermentative by-products, increasing growth rate, imparting ability to utilizing complex substrates, and accumulating useful by-products, and (4) innovative reactor designs. Looking at these aspects future research goals need be put forward to generate a sustainable biological hydrogen producing system prospective to forecast energy needs and for environmental safety.

Acknowledgements The authors would like to thank DBT (Department of Biotechnology), India for the funding under Ramalingaswami re-entry fellowship (Project # AUR002). The authors also thank Prof. Rajendra Kumar Pandey (Vice Chancellor), Dr. Ravi Kanth Singh (Director, AIB) for their kind support and encouragement.

References

Abo-Hashesh M, Hallenbeck PC (2012) Fermentative hydrogen production. In: Hallenbeck PC (ed) Microbial technologies in advanced biofuels production. Springer, Boston, pp 77–92

Akhtar MK, Jones PR (2008a) Deletion of iscR stimulates recombinant clostridial Fe-Fe hydrogenase activity and H2-accumulation in Escherichia coli BL21(DE3). Appl Microbiol Biotechnol 78:853–862

Akhtar MK, Jones PR (2008b) Engineering of a synthetic hydF-hydE-hydG-hydA operon for biohydrogen production. Anal Biochem 373:170–172

Akhtar MK, Jones PR (2009) Construction of a synthetic YdbK-dependent pyruvate: H2 pathway in Escherichia coli BL21(DE3). Metab Eng 11:139–147
Alibardi L, Cossu R (2015) Composition variability of the organic fraction of municipal solid waste and effects on hydrogen and methane production potentials. Waste Manag 36:147–155
Andrews SC, Berks BC, McClay J, Ambler A, Quail MA, Golby P, Guest JR (1997) A 12-cistron Escherichia coli operon (hyf) encoding a putative proton-translocating formate hydrogenlyase system. Microbiology 143:3633–3647
Antonopoulou G, Gavala HN, Skiadas IV, Angelopoulos K, Lyberatos G (2008) Biofuels generation from sweet sorghum: fermentative hydrogen production and anaerobic digestion of the remaining biomass. Bioresour Technol 99:110–119
Axley MJ, Grahame DA, Stadtman TC (1990) Escherichia coli formate-hydrogen lyase. Purification and properties of the selenium-dependent formate dehydrogenase component. J Biol Chem 265:18213–18218
Azbar N, Dokgöz FT, Keskin T, Eltem R, Korkmaz KS, Gezgin Y, Akbal Z, Öncel S, Dalay MC, Gönen Ç, Tutuk F (2009) Comparative evaluation of bio-hydrogen production from cheese whey wastewater under thermophilic and mesophilic anaerobic conditions. Int J Green Energy 6:192–200
Bagramyan K, Trchounian A (2003) Structural and functional features of Formate hydrogen Lyase, an enzyme of mixed-acid fermentation from Escherichia coli. Biochem Mosc 68:1159–1170
Barbosa MJ, Rocha JMS, Tramper J, Wijffels RH (2001) Acetate as a carbon source for hydrogen production by photosynthetic bacteria. J Biotechnol 85:25–33
Bowles LK, Ellefson WL (1985) Effects of butanol on Clostridium acetobutylicum. Appl Environ Microbiol 50:1165–1170
Brosseau JD, Zajic JE (1982) Hydrogen-gas production with Citrobacter intermedium and Clostridium pasteurianum. J Chem Technol Biotechnol 32:496–502
Cai G, Jin B, Monis P, Saint C (2011) Metabolic flux network and analysis of fermentative hydrogen production. Biotechnol Adv 29:375–387
Chang J-S, Lee K-S, Lin P-J (2002) Biohydrogen production with fixed-bed bioreactors. Int J Hydrog Energy 27:1167–1174
Chaubey R, Sahu S, James OO, Maity S (2013) A review on development of industrial processes and emerging techniques for production of hydrogen from renewable and sustainable sources. Renew Sust Energ Rev 23:443–462
Chittibabu G, Nath K, Das D (2006) Feasibility studies on the fermentative hydrogen production by recombinant Escherichia coli BL-21. Process Biochem 41:682–688
Chong M-L, Abdul Rahman NA, Rahim RA, Aziz SA, Shirai Y, Hassan MA (2009) Optimization of biohydrogen production by Clostridium butyricum EB6 from palm oil mill effluent using response surface methodology. Int J Hydrog Energy 34:7475–7482
Chu C-F, Xu K-Q, Li Y-Y, Inamori Y (2012) Hydrogen and methane potential based on the nature of food waste materials in a two-stage thermophilic fermentation process. Int J Hydrog Energy 37:10611–10618
Collet C, Adler N, Schwitzguébel J-P, Péringer P (2004) Hydrogen production by Clostridium thermolacticum during continuous fermentation of lactose. Int J Hydrog Energy 29:1479–1485
Czernik S, Evans R, French R (2007) Hydrogen from biomass-production by steam reforming of biomass pyrolysis oil. Catal Today 129:265–268
Das D, Veziro□lu TN (2001) Hydrogen production by biological processes: a survey of literature. Int J Hydrog Energy 26:13–28
Demirbas A (2004) Hydrogen-rich gas from fruit shells via supercritical water extraction. Int J Hydrog Energy 29:1237–1243
Dharmadi Y, Murarka A, Gonzalez R (2006) Anaerobic fermentation of glycerol by Escherichia coli: a new platform for metabolic engineering. Biotechnol Bioeng 94:821–829
Dunn S (2002) Hydrogen futures: toward a sustainable energy system. Int J Hydrog Energy 27:235–264

Dutta D, De D, Chaudhuri S, Bhattacharya SK (2005) Hydrogen production by cyanobacteria. Microb Cell Factories 4:36–36

Fan Z, Yuan L, Chatterjee R (2009) Increased hydrogen production by genetic engineering of Escherichia coli. PLoS One 4:e4432

Ferchichi M, Crabbe E, Gil G-H, Hintz W, Almadidy A (2005) Influence of initial pH on hydrogen production from cheese whey. J Biotechnol 120:402–409

Fernandes BS, Peixoto G, Albrecht FR, Saavedra del Aguila NK, Zaiat M (2010) Potential to produce biohydrogen from various wastewaters. Energy Sustain Dev 14:143–148

Freni S, Calogero G, Cavallaro S (2000) Hydrogen production from methane through catalytic partial oxidation reactions. J Power Sources 87:28–38

Fujishima A, Rao TN, Tryk DA (2000) Titanium dioxide photocatalysis. J Photochem Photobiol C: Photochem Rev 1:1–21

Funk JE (2001) Thermochemical hydrogen production: past and present. Int J Hydrog Energy 26:185–190

Ghimire A, Frunzo L, Pontoni L, d'Antonio G, Lens PNL, Esposito G, Pirozzi F (2015) Dark fermentation of complex waste biomass for biohydrogen production by pretreated thermophilic anaerobic digestate. J Environ Manag 152:43–48

Ghirardi ML, Posewitz MC, Maness PC, Dubini A, Yu J, Seibert M (2007) Hydrogenases and hydrogen photoproduction in oxygenic photosynthetic organisms. Annu Rev Plant Biol 58:71–91

Gómez X, Morán A, Cuetos MJ, Sánchez ME (2006) The production of hydrogen by dark fermentation of municipal solid wastes and slaughterhouse waste: a two-phase process. J Power Sources 157:727–732

Gonzalez R, Murarka A, Dharmadi Y, Yazdani SS (2008) A new model for the anaerobic fermentation of glycerol in enteric bacteria: trunk and auxiliary pathways in Escherichia coli. Metab Eng 10:234–245

Guo L, Li X-M, Bo X, Yang Q, Zeng G-M, Liao D-x, Liu J-J (2008) Impacts of sterilization, microwave and ultrasonication pretreatment on hydrogen producing using waste sludge. Bioresour Technol 99:3651–3658

Guo XM, Trably E, Latrille E, Carrère H, Steyer J-P (2010) Hydrogen production from agricultural waste by dark fermentation: a review. Int J Hydrog Energy 35:10660–10673

Hallenbeck PC (2012) Microbial technologies in advanced biofuels production. Springer Science, Business Media, LLC, New York

Hallenbeck PC, Abo-Hashesh M, Ghosh D (2012) Strategies for improving biological hydrogen production. Bioresour Technol 110:1–9

Hansel A, Lindblad P (1998) Towards optimization of cyanobacteria as biotechnologically relevant producers of molecular hydrogen, a clean and renewable energy source. Appl Microbiol Biotechnol 50:153–160

Harwood CS (2008) Nitrogenase-catalyzed hydrogen production by purple nonsulfur photosynthetic bacteria. In: Bioenergy. American Society of Microbiology Press

Haryanto A, Fernando S, Murali N, Adhikari S (2005) Current status of hydrogen production techniques by steam reforming of ethanol: a review. Energy Fuel 19:2098–2106

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

Hsiao C-L, Chang J-J, Wu J-H, Chin W-C, Wen F-S, Huang C-C, Chen C-C, Lin C-Y (2009) Clostridium strain co-cultures for biohydrogen production enhancement from condensed molasses fermentation solubles. Int J Hydrog Energy 34:7173–7181

Hu H, Wood TK (2010) An evolved Escherichia coli strain for producing hydrogen and ethanol from glycerol. Biochem Biophys Res Commun 391:1033–1038

Jayalakshmi S, Joseph K, Sukumaran V (2009) Bio hydrogen generation from kitchen waste in an inclined plug flow reactor. Int J Hydrog Energy 34:8854–8858

Jung K-W, Kim D-H, Shin H-S (2010) Continuous fermentative hydrogen production from coffee drink manufacturing wastewater by applying UASB reactor. Int J Hydrog Energy 35:13370–13378

Jung K-W, Kim D-H, Kim S-H, Shin H-S (2011) Bioreactor design for continuous dark fermentative hydrogen production. Bioresour Technol 102:8612–8620

Kapdan IK, Kargi F (2006) Bio-hydrogen production from waste materials. Enzym Microb Technol 38:569–582

Karlsson A, Vallin L, Ejlertsson J (2008) Effects of temperature, hydraulic retention time and hydrogen extraction rate on hydrogen production from the fermentation of food industry residues and manure. Int J Hydrog Energy 33:953–962

Kim D-H, Han S-K, Kim S-H, Shin H-S (2006a) Effect of gas sparging on continuous fermentative hydrogen production. Int J Hydrog Energy 31:2158–2169

Kim S, Seol E, Oh Y-K, Wang GY, Park S (2009) Hydrogen production and metabolic flux analysis of metabolically engineered Escherichia coli strains. Int J Hydrog Energy 34:7417–7427

Kim S-H, Han S-K, Shin H-S (2006b) Effect of substrate concentration on hydrogen production and 16S rDNA-based analysis of the microbial community in a continuous fermenter. Process Biochem 41:199–207

Kotsopoulos TA, Zeng RJ, Angelidaki I (2006) Biohydrogen production in granular up-flow anaerobic sludge blanket (UASB) reactors with mixed cultures under hyper-thermophilic temperature (70°C). Biotechnol Bioeng 94:296–302

Kovács KL, Maróti G, Rákhely G (2006) A novel approach for biohydrogen production. Int J Hydrog Energy 31:1460–1468

Kumar N, Das D (2000) Enhancement of hydrogen production by Enterobacter cloacae IIT-BT 08. Process Biochem 35:589–593

Kumar R, Kumar P (2017) Future microbial applications for bioenergy production: a perspective. Front Microbiol 8:450–450

Lee H-S, Vermaas WFJ, Rittmann BE (2010a) Biological hydrogen production: prospects and challenges. Trends Biotechnol 28:262–271

Lee K-S, Tseng T-S, Liu Y-W, Hsiao Y-D (2012) Enhancing the performance of dark fermentative hydrogen production using a reduced pressure fermentation strategy. Int J Hydrog Energy 37:15556–15562

Lee SY, Lee HJ, Park J-M, Lee JH, Park J-S, Shin HS, Kim Y-H, Min J (2010b) Bacterial hydrogen production in recombinant Escherichia coli harboring a HupSL hydrogenase isolated from Rhodobacter sphaeroides under anaerobic dark culture. Int J Hydrog Energy 35:1112–1116

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

Levin DB, Islam R, Cicek N, Sparling R (2006) Hydrogen production by Clostridium thermocellum 27405 from cellulosic biomass substrates. Int J Hydrog Energy 31:1496–1503

Li C, Fang HHP (2007) Fermentative hydrogen production from wastewater and solid wastes by mixed cultures. Crit Rev Environ Sci Technol 37:1–39

Lin C-Y, Chang R-C (2004) Fermentative hydrogen production at ambient temperature. Int J Hydrog Energy 29:715–720

Liu G, Shen J (2004) Effects of culture and medium conditions on hydrogen production from starch using anaerobic bacteria. J Biosci Bioeng 98:251–256

Logan BE, Oh S-E, Kim IS, Van Ginkel S (2002) Biological hydrogen production measured in batch anaerobic respirometers. Environ Sci Technol 36:2530–2535

Łukajtis R, Hołowacz I, Kucharska K, Glinka M, Rybarczyk P, Przyjazny A, Kamiński M (2018) Hydrogen production from biomass using dark fermentation. Renew Sust Energ Rev 91:665–694

Lynd LR, Larson E, Greene N, Laser M, Sheehan J, Dale BE, McLaughlin S, Wang M (2009) The role of biomass in America's energy future: framing the analysis. Biofuels Bioprod Biorefin 3:113–123

Maeda T, Sanchez-Torres V, Wood TK (2007a) Enhanced hydrogen production from glucose by metabolically engineered Escherichia coli. Appl Microbiol Biotechnol 77:879–890
Maeda T, Sanchez-Torres V, Wood TK (2007b) Escherichia coli hydrogenase 3 is a reversible enzyme possessing hydrogen uptake and synthesis activities. Appl Microbiol Biotechnol 76:1035–1042
Maeda T, Sanchez-Torres V, Wood TK (2008) Metabolic engineering to enhance bacterial hydrogen production. Microb Biotechnol 1:30–39
Maeda T, Sanchez-Torres V, Wood TK (2012) Hydrogen production by recombinant Escherichia coli strains. Microb Biotechnol 5:214–225
Maeda T, Tran KT, Yamasaki R, Wood TK (2018) Current state and perspectives in hydrogen production by Escherichia coli: roles of hydrogenases in glucose or glycerol metabolism. Appl Microbiol Biotechnol 102:2041–2050
Mangayil R, Karp M, Santala V (2012) Bioconversion of crude glycerol from biodiesel production to hydrogen. Int J Hydrog Energy 37:12198–12204
Manish S, Venkatesh KV, Banerjee R (2007) Metabolic flux analysis of biological hydrogen production by Escherichia coli. Int J Hydrog Energy 32:3820–3830
Marcelo D, Dell'Era A (2008) Economical electrolyser solution. Int J Hydrog Energy 33:3041–3044
Marquevich M, Coll R, Montané D (2000) Steam reforming of sunflower oil for hydrogen production. Ind Eng Chem Res 39:2140–2147
Mars AE, Veuskens T, Budde MAW, van Doeveren PFNM, Lips SJ, Bakker RR, de Vrije T, Claassen PAM (2010) Biohydrogen production from untreated and hydrolyzed potato steam peels by the extreme thermophiles Caldicellulosiruptor saccharolyticus and Thermotoga neapolitana. Int J Hydrog Energy 35:7730–7737
Masepohl B, Schneider K, Drepper T, Müller A, Klipp W (2002) Chapter 8 - alternative nitrogenases. In: Leigh GJ (ed) Nitrogen fixation at the millennium. Elsevier Science, Amsterdam, pp 191–222
Mathews J, Li Q, Wang G (2010) Characterization of hydrogen production by engineered Escherichia coli strains using rich defined media. Biotechnol Bioprocess Eng 15:686–695
Mishra J, Khurana S, Kumar N, Ghosh AK, Das D (2004) Molecular cloning, characterization, and overexpression of a novel [Fe]-hydrogenase isolated from a high rate of hydrogen producing Enterobacter cloacae IIT-BT 08. Biochem Biophys Res Commun 324:679–685
Miyake J, Miyake M, Asada Y (1999) Biotechnological hydrogen production: research for efficient light energy conversion. J Biotechnol 70:89–101
Mohd Yasin NH, Rahman NAA, Man HC, Mohd Yusoff MZ, Hassan MA (2011) Microbial characterization of hydrogen-producing bacteria in fermented food waste at different pH values. Int J Hydrog Energy 36:9571–9580
Morimoto M, Atsuko M, Atif AAY, Ngan MA, Fakhru'l-Razi A, Iyuke SE, Bakir AM (2004) Biological production of hydrogen from glucose by natural anaerobic microflora. Int J Hydrog Energy 29:709–713
Mu Y, Yu H-Q, Wang G (2007) Evaluation of three methods for enriching H2-producing cultures from anaerobic sludge. Enzym Microb Technol 40:947–953
Nathao C, Sirisukpoka U, Pisutpaisal N (2013) Production of hydrogen and methane by one and two stage fermentation of food waste. Int J Hydrog Energy 38:15764–15769
Navarro RM, Peña MA, Fierro JLG (2007) Hydrogen production reactions from carbon feedstocks: fossil fuels and biomass. Chem Rev 107:3952–3991
Ngo TA, Kim M-S, Sim SJ (2011) High-yield biohydrogen production from biodiesel manufacturing waste by Thermotoga neapolitana. Int J Hydrog Energy 36:5836–5842
Nielsen AT, Amandusson H, Bjorklund R, Dannetun H, Ejlertsson J, Ekedahl L-G, Lundström I, Svensson BH (2001) Hydrogen production from organic waste. Int J Hydrog Energy 26:547–550
Oh S-E, Iyer P, Bruns MA, Logan BE (2004) Biological hydrogen production using a membrane bioreactor. Biotechnol Bioeng 87:119–127

Ohta T (1979) Chapter 5 - Thermochemical hydrogen production. In: Ohta T (ed) Solar-hydrogen energy systems. Pergamon, pp 81–114

O-Thong S, Prasertsan P, Intrasungkha N, Dhamwichukorn S, Birkeland N-K (2007) Improvement of biohydrogen production and treatment efficiency on palm oil mill effluent with nutrient supplementation at thermophilic condition using an anaerobic sequencing batch reactor. Enzym Microb Technol 41:583–590

Pan J, Zhang R, El-Mashad HM, Sun H, Ying Y (2008) Effect of food to microorganism ratio on biohydrogen production from food waste via anaerobic fermentation. Int J Hydrog Energy 33:6968–6975

Penfold DW, Forster CF, Macaskie LE (2003) Increased hydrogen production by Escherichia coli strain HD701 in comparison with the wild-type parent strain MC4100. Enzym Microb Technol 33:185–189

Redondas V, Gómez X, García S, Pevida C, Rubiera F, Morán A, Pis JJ (2012) Hydrogen production from food wastes and gas post-treatment by CO2 adsorption. Waste Manag 32:60–66

Ren N, Wanqian G, Bingfeng L, Guangli C, Jie D (2011) Biological hydrogen production by dark fermentation: challenges and prospects towards scaled-up production. Curr Opin Biotechnol 22:365–370

Rossmann R, Sawers G, Böck A (1991) Mechanism of regulation of the formate-hydrogenlyase pathway by oxygen, nitrate, and pH: definition of the formate regulon. Mol Microbiol 5:2807–2814

Self WT, Hasona A, Shanmugam KT (2001) N-terminal truncations in the FhlA protein result in formate- and MoeA-independent expression of the hyc (formate hydrogenlyase) operon of Escherichia coli. Microbiology 147:3093–3104

Shi X-Y, Jin D-W, Sun Q-Y, Li W-W (2010) Optimization of conditions for hydrogen production from brewery wastewater by anaerobic sludge using desirability function approach. Renew Energy 35:1493–1498

Sivaramakrishna D, Sreekanth D, Himabindu V, Anjaneyulu Y (2009) Biological hydrogen production from probiotic wastewater as substrate by selectively enriched anaerobic mixed microflora. Renew Energy 34:937–940

Stojić DL, Marčeta MP, Sovilj SP, Miljanić ŠS (2003) Hydrogen generation from water electrolysis—possibilities of energy saving. J Power Sources 118:315–319

Stripp ST, Goldet G, Brandmayr C, Sanganas O, Vincent KA, Haumann M, Armstrong FA, Happe T (2009) How oxygen attacks [FeFe] hydrogenases from photosynthetic organisms. Proc Natl Acad Sci 106:17331–17336

Sudheer PDVN, Rahman H, Mastan SG, Reddy MP (2010) Isolation of novel microsatellites using FIASCO by dual probe enrichment from Jatropha curcas L. and study on genetic equilibrium and diversity of Indian population revealed by isolated microsatellites. Mol Biol Rep 37:3785–3793

Sudheer PDVN, Seo D, Kim E-J, Chauhan S, Chunawala JR, Choi K-Y (2018) Production of (Z)-11-(heptanoyloxy)undec-9-enoic acid from ricinoleic acid by utilizing crude glycerol as sole carbon source in engineered Escherichia coli expressing BVMO-ADH-FadL. Enzym Microb Technol 119:45–51

Sudheer Pamidimarri DV, Reddy MP (2014) Phylogeography and molecular diversity analysis of Jatropha curcas L. and the dispersal route revealed by RAPD, AFLP and nrDNA-ITS analysis. Mol Biol Rep 41:3225–3234

Suppmann B, Sawers G (1994) Isolation and characterization of hypophosphite-resistant mutants of Escherichia coli: identification of the FocA protein, encoded by the pfl operon, as a putative formate transporter. Mol Microbiol 11:965–982

Tang G-L, Huang J, Sun Z-J, Tang Q-Q, Yan C-H, Liu G-Q (2008) Biohydrogen production from cattle wastewater by enriched anaerobic mixed consortia: influence of fermentation temperature and pH. J Biosci Bioeng 106:80–87

Tapia-Venegas E, Ramirez-Morales JE, Silva-Illanes F, Toledo-Alarcón J, Paillet F, Escudie R, Lay C-H, Chu C-Y, Leu H-J, Marone A, Lin C-Y, Kim D-H, Trably E, Ruiz-Filippi G (2015) Biohydrogen production by dark fermentation: scaling-up and technologies integration for a sustainable system. Rev Environ Sci Biotechnol 14:761–785

Teplyakov VV, Gassanova LG, Sostina EG, Slepova EV, Modigell M, Netrusov AI (2002) Lab-scale bioreactor integrated with active membrane system for hydrogen production: experience and prospects. Int J Hydrog Energy 27:1149–1155

Toledo-Alarcón J, Capson-Tojo G, Marone A, Paillet F, Júnior ADNF, Chatellard L, Bernet N, Trably E (2018) Basics of bio-hydrogen production by dark fermentation. In: Liao Q, Chang J-S, Herrmann C, Xia A (eds) Bioreactors for microbial biomass and energy conversion. Springer, Singapore, pp 199–220

Tran KT, Maeda T, Wood TK (2014) Metabolic engineering of Escherichia coli to enhance hydrogen production from glycerol. Appl Microbiol Biotechnol 98:4757–4770

Tran KT, Maeda T, Sanchez-Torres V, Wood TK (2015) Beneficial knockouts in Escherichia coli for producing hydrogen from glycerol. Appl Microbiol Biotechnol 99:2573–2581

Turcot J, Bisaillon A, Hallenbeck P (2008) Hydrogen production by continuous cultures of Escherchia coli under different nutrient regimes. Int J Hydrog Energy 33:1465–1470

Turner JA (2004) Sustainable hydrogen production. Science 305:972–974

Utgikar V, Thiesen T (2006) Life cycle assessment of high temperature electrolysis for hydrogen production via nuclear energy. Int J Hydrog Energy 31:939–944

Valdez-Vazquez I, Ríos-Leal E, Esparza-García F, Cecchi F, Poggi-Varaldo HM (2005) Semi-continuous solid substrate anaerobic reactors for H2 production from organic waste: Mesophilic versus thermophilic regime. Int J Hydrog Energy 30:1383–1391

Van Ginkel SW, Oh S-E, Logan BE (2005) Biohydrogen gas production from food processing and domestic wastewaters. Int J Hydrog Energy 30:1535–1542

Vardar-Schara G, Maeda T, Wood TK (2008) Metabolically engineered bacteria for producing hydrogen via fermentation. Microb Biotechnol 1:107–125

Vasudeva K, Mitra N, Umasankar P, Dhingra SC (1996) Steam reforming of ethanol for hydrogen production: thermodynamic analysis. Int J Hydrog Energy 21:13–18

Vatsala TM, Raj SM, Manimaran A (2008) A pilot-scale study of biohydrogen production from distillery effluent using defined bacterial co-culture. Int J Hydrog Energy 33:5404–5415

Venkata Mohan S, Lalit Babu V, Sarma PN (2007a) Anaerobic biohydrogen production from dairy wastewater treatment in sequencing batch reactor (AnSBR): effect of organic loading rate. Enzym Microb Technol 41:506–515

Venkata Mohan S, Vijaya Bhaskar Y, Murali Krishna P, Chandrasekhara Rao N, Lalit Babu V, Sarma PN (2007b) Biohydrogen production from chemical wastewater as substrate by selectively enriched anaerobic mixed consortia: influence of fermentation pH and substrate composition. Int J Hydrog Energy 32:2286–2295

Wang J, Wan W (2009) Factors influencing fermentative hydrogen production: a review. Int J Hydrog Energy 34:799–811

Wang S, Ma Z, Zhang T, Bao M, Su H (2017) Optimization and modeling of biohydrogen production by mixed bacterial cultures from raw cassava starch. Front Chem Sci Eng 11:100–106

Wong YM, Wu TY, Juan JC (2014) A review of sustainable hydrogen production using seed sludge via dark fermentation. Renew Sust Energ Rev 34:471–482

Yang H, Shao P, Lu T, Shen J, Wang D, Xu Z, Yuan X (2006) Continuous bio-hydrogen production from citric acid wastewater via facultative anaerobic bacteria. Int J Hydrog Energy 31:1306–1313

Yoshida A, Nishimura T, Kawaguchi H, Inui M, Yukawa H (2006) Enhanced hydrogen production from glucose using ldh- and frd-inactivated Escherichia coli strains. Appl Microbiol Biotechnol 73:67–72

Yu H, Zhu Z, Hu W, Zhang H (2002) Hydrogen production from rice winery wastewater in an upflow anaerobic reactor by using mixed anaerobic cultures. Int J Hydrog Energy 27:1359–1365

Zahedi S, Sales D, Romero LI, Solera R (2013) Hydrogen production from the organic fraction of municipal solid waste in anaerobic thermophilic acidogenesis: influence of organic loading rate and microbial content of the solid waste. Bioresour Technol 129:85–91

Zhao X, Xing D, Zhang L, Ren N (2010) Characterization and overexpression of a [FeFe]-hydrogenase gene of a novel hydrogen-producing bacterium Ethanoligenens harbinense. Int J Hydrog Energy 35:9598–9602