

Chapter 2

Microbiology and Enzymology

2.1 Microorganisms in Hydrogen-Producing System

2.1.1 Overview

As shown in Fig. 2.1, microorganisms present in biological hydrogen production system can be categorized into hydrogen producers and non-hydrogen producers.

Taking hydrogen as target product, lots of studies have been focused on isolating and exploring the characteristics of hydrogen producers. Based on the different metabolisms in producing hydrogen, hydrogen producers include photosynthetic microorganisms, photo-fermentative microorganisms, and dark fermentative microorganisms. Photosynthetic microorganisms include cyanobacteria and green algae. They can use light as an energy source, splitting water into hydrogen and oxygen. Photo-fermentative microorganisms include purple sulfur bacteria (e.g., *Chromatium*), purple nonsulfur bacteria (*Rhodobacter*), green sulfur bacteria (e.g., *Chlorobium*), and gliding bacteria (e.g., *Chloroflexus*). These photo-fermentative microorganisms convert organic matters to hydrogen in the presence of light, and substrate in small molecules like short-chain volatile fatty acids can be used in photo fermentation system. Dark fermentative microorganisms are rich in species and widely distributed; they not only include the common strains like *Clostridium* sp. and *Enterobacter* sp., but cover the strains live in harsh conditions like the thermophiles habitat in hot spring (*Thermoanaerobacterium* sp.) and the psychrophiles live polar areas (*Polaromonas* sp.). These strains can convert organic substrate into hydrogen a series of biochemical reactions. Unlike photo fermentation, dark fermentation can be conducted in the absence of light.

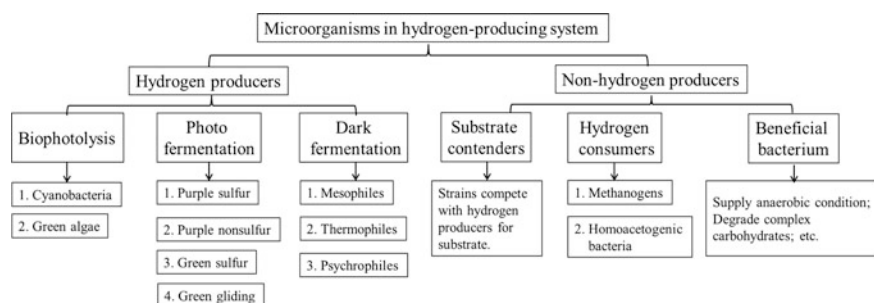


Fig. 2.1 Schematic diagram represents the diversity of microorganisms present in hydrogen-producing systems

2.1.2 Microbial Diversity in Hydrogen-Producing System

Besides the hydrogen producers, there are usually some other microorganisms present in the system, especially the mixed cultures are used as inoculum. Some of them are in demand while others are undesirable. The undesired non-hydrogen producers include the hydrogen consumers (methanogen and homoacetogenic bacteria) and the strains compete with hydrogen producers for substrate. The presence of the undesired non-hydrogen producers can lead to the low hydrogen production and hydrogen yield. Studies usually try to eliminate the undesired non-hydrogen producers through the pretreatment of inocula and operational control. Besides the undesired non-hydrogen producers, the presence of some non-hydrogen producers might provide useful combinations of metabolic pathways for the processing of complex waste material ingredients, thereby supporting the more efficient decomposition and hydrogenation of biomass. For example, some strains can improve the hydrogen production by the granular formation/retention of biomass, like *Streptococcus* sp. (Hung et al. 2011a, b); some aerobes or facultative anaerobes can help to maintain an anaerobic environment in the system (Hung et al. 2011a, b); and some cultures have the potential to increase the hydrogen production through the breakdown of macromolecular organic compounds, which is pretty helpful when complex organic wastes are used as substrate.

2.2 Inocula for Dark Fermentation

Inoculum for dark fermentative hydrogen production system can be mixed cultures, like anaerobic sludge, compost, soil, leachate, etc., or pure cultures, like *Clostridium* sp., *Enterobacter* sp., etc. In the practical application, mixed cultures are more widely used because of the broader choice of feedstock, cheaper operation, and easier control (Das 2009). It was also proved that the co-cultures of different bacteria can be more effective in hydrogen production especially when complicated

substrates are used (Hung et al. 2011a, b). On the other side, systems with pure cultures may cost more in system operation and maintenance. However, operations applying pure cultures can provide a better understanding of metabolic pathways happening during the hydrogen production process, thus revealing precious information about the ways of promoting hydrogen production rate and hydrogen yield of the system. Furthermore, the isolation and identification of effective hydrogen producers can provide valuable microbial species resources for the research on gene modification. Some studies have proved that hydrogen production can be significantly enhanced through the addition of high-efficient pure cultures to mixed-culture systems (Kotay and Das 2009).

2.2.1 Mixed Culture

Microorganisms capable of producing hydrogen are widely present in natural habitat, such as sludge, compost, soil, sediments, leachate and organic wastes, and so on. These materials can be potential sources for enriching hydrogen producers. Anaerobic sludge is the most commonly used source for hydrogen producers (Wang and Wan 2008; Abdallah et al. 2016; Yin and Wang 2016), followed by animal compost (Xing et al. 2011; Chu et al. 2012; Li et al. 2016), soil underground (García et al. 2012), seacoast sludge (Lin et al. 2013; Lee et al. 2012), and leachate (Watanabe and Yoshino 2010; Wong et al. 2014). When organic wastes were applied for hydrogen production, like waste-activated sludge, food waste, cereal, etc., the indigenous microorganisms can be used as hydrogen producers and no additional inoculum was required (Bru et al. 2012; Cui and Shen 2012; Li et al. 2012a, b; Argun and Dao 2016).

A different microbial diversity was observed from different inoculum sources. System inoculated anaerobic sludge usually dominated by *Clostridium* spp., among which *Clostridium butyricum*, *Clostridium pasteurianum*, and *Clostridium beijerinckii* were most common strains (Ren et al. 2008a, b; Chu et al. 2011a, b; Chen et al. 2012; Li et al. 2012a, b; Jeong et al. 2013). As to the system applied compost as inoculum, *Enterobacter* spp., *Bacillus* spp., and *Enterococcus* spp. were usually coexist with *Clostridium* spp. (Song et al. 2012a, b; Li et al. 2016).

When same pretreatment method is used, inoculum from different sources also showed different activities on fermentative hydrogen production. Chen et al. compared hydrogen production by heat-treated different inocula, and sludge from municipal wastewater treatment plant showed 2.2 times higher in hydrogen yield over cow dung compost at same reaction conditions (Chen et al. 2012). Indicating that waste-activated sludge had better hydrogen production ability over compost, similar conclusion was also obtained by Ghimire et al. who found that H_2 yield was doubled when heat-treated waste-activated sludge was used in comparison to buffalo manure fed digested sludge (Ghimire et al. 2016). Besides, García et al. conducted hydrogen production with heat-treated soil beneath the surface ground, but the result was not satisfied comparing with parallel tests that adopted anaerobic

sludge or compost (García et al. 2012). The indigenous bacteria in organic substrates were also studied; however, results obtained by Lay et al. showed that the hydrogen production from sweet potato with indigenous microorganisms was far behind the parallel groups with extra inocula (waste-activated sludge or cow dung compost) (Lay et al. 2012).

Therefore, the inoculum has a significant influence on hydrogen production. According to the present studies, highest hydrogen yield was usually obtained by waste-activated sludge, followed by animal compost, soil underground, and fermentation with indigenous bacteria came last.

2.2.2 Pure Culture

At present, a lot of strains have been reported to be capable of producing hydrogen. Commonly studied strains include *Clostridium* sp., like *Clostridium butyricum*, *Clostridium beijerinckii*, *Clostridium pasteurianum*, *Clostridium tyrobutyricum*, etc.; *Enterobacter* sp., like *Enterobacter aerogenes*, *Enterobacter asburiae*, *Enterobacter cloacae*, etc., and some other strains like *Ethanoligenens*, *Bacillus*, *Klebsiella*, *Thermoanaerobium*, *Rahnella*, etc., all showed capacity in hydrogen production.

According to the cultivation temperature, hydrogen producers can be categorized into mesophiles (*Clostridium* sp., *Enterobacter* sp., etc.), thermophiles (*Klebsiella* sp., *Thermoanaerobium* sp., etc.), and psychrophiles (*Rahnella* sp., *Polaromonas* sp., etc.). Mesophilic cultures are more widely used for the process employing mesophiles is more economical. Basing on the tolerance to oxygen, they can be categorized into anaerobes (*Clostridium* sp.), facultative anaerobes (*Enterobacter* sp.), and aerobes (*Bacillus licheniformis*). Anaerobes usually have higher hydrogen yield, while facultative anaerobes can help to supply anaerobic environment for anaerobes in the fermentation system. Table 2.1 shows some typical hydrogen producers and their hydrogen yields obtained in studies.

Besides the known strains, new strains of hydrogen producers are still being found. Species *Enterococcus faecium* has been detected in many hydrogen production systems in which mixed cultures were used (Liu et al. 2009; Song et al. 2012a, b; Cisneros-Pérez et al. 2015). Studies showed that species *Enterococcus faecium* is usually found in systems applying heat-treated sludge as inoculum.

Although many strains that are able to produce hydrogen have been obtained, studies searching for high-efficient hydrogen producers have never stopped. Besides trying to isolate more efficient strains, many attempts have been made focus on enhancing the hydrogen production through the engineering of strains. This includes overexpression of hydrogen-producing genes (native and heterologous), knockout of competitive pathways, creation of a new productive pathway, and creation of dual systems.

Table 2.1 Some typical hydrogen producers used in dark fermentation systems

Hydrogen producers	Characteristics	Typical species	Hydrogen yield (mol H ₂ /mol hexose)	References
<i>Mesophiles</i>				
<i>Clostridium</i> sp.	Obligate anaerobes. Spore-forming bacteria. High hydrogen yield, able to degrade a wide range of carbohydrates, fermentation condition ranges 30–43 °C, pH 5.0–8.5	<i>Clostridium butyricum</i>	0.23–3.47	(Beckers et al. 2015; Calusinska et al. 2015; Ortigueira et al. 2015; Rafeenia and Chaganti 2015)
		<i>Clostridium tyrobutyricum</i>	2	(Jo et al. 2008)
		<i>Clostridium beijerinckii</i>	0.6–2.52	(Pan et al. 2008; Zhao et al. 2011; An et al. 2014)
		<i>Clostridium pasteurianum</i>	0.96–3.0	(Cheng and Chang 2011; Hu et al. 2013)
<i>Enterobacter</i> sp.	Facultative anaerobes Supply anaerobic environment, some are aciduric, fermentation condition ranges 30–40 °C, pH 4.0–7.5	<i>Enterobacter aerogenes</i>	0.1–0.3	(Hu et al. 2013; Batista et al. 2014)
		<i>Enterobacter asburiae</i>	0.54	(Shin et al. 2007)
		<i>Enterobacter cloacae</i>	1.3–1.8	(Harun et al. 2012; Mishra and Das 2014)
<i>Bacillus</i> sp.	Facultative anaerobes. Hydrolyze substrate to simple sugars, Supply anaerobic environment,	<i>Bacillus firmus</i>	1.1–1.3	(Sinha and Pandey 2014)
		<i>Bacillus amyloliquefaciens</i>	2.26	(Song et al. 2013)

(continued)

Table 2.1 (continued)

Hydrogen producers	Characteristics	Typical species	Hydrogen yield (mol H ₂ /mol hexose)	References
<i>Mesophiles</i>				
Others	produce H ₂ , fermentation condition ranges 35–40 °C, pH 5.3–7.4			
	Obligate or facultative anaerobes. Produce H ₂ from different kinds of carbohydrates. Not widely studied	<i>Ethanoligenens harbinensis</i>	2.2–3.1	(Xie et al. 2010; Zhang et al. 2015a, b)
		<i>Citrobacter freundii</i>	0.83–2.49	(Oh et al. 2003; Hamilton et al. 2010)
		<i>Rhodopseudomonas Palustris</i>	1–2.76	(Oh et al. 2002)
<i>Thermophiles</i>				
<i>Thermoanaerobacterium</i> sp.	Obligate or facultative anaerobes. High hydrogen production rate and yield, fermentation condition ranges 40–60 °C, pH 6.2–8.0	<i>Thermoanaerobacterium thermosaccharolyticum</i>	2.42–2.53	(O-Thong et al. 2008; Ren et al. 2008a, b; Singh et al. 2014)
<i>Klebsiella</i> sp.		<i>Klebsiella pneumoniae</i>	0.43	(Chookaew et al. 2012)
<i>Psychrophiles</i>				
<i>Polaromonas</i> sp.	Obligate or facultative anaerobes. Low cost in operation without temperature control, fermentation condition ranges 20–25 °C, pH 6.5	<i>Polaromonas rhizosphaerae</i>	1.3–1.7	(Alvarez-Guzmán et al. 2016)
<i>Rahnella</i> sp.		<i>Rahnella aquatilis</i>	1.8–3.4	(Debowski et al. 2014)

Our studies have tried to obtain the high-efficient mutant of hydrogen producers through the gamma irradiation, and strains *Clostridium butyricum* INET1 and *Enterococcus faecium* INET2 were isolated from 5 kGy gamma-irradiated sludge.

2.3 Pure Culture for Hydrogen Production

2.3.1 *Clostridium butyricum* INET1

2.3.1.1 Isolation and Identification of Strain

The strain *Clostridium butyricum* INET1 (NCBI GenBank accession number: KX148520) was isolated from the 5 kGy gamma irradiation pretreated digested sludge (Yin and Wang 2016). Medium used for isolation contains 10 g/L glucose and 5 mL/100 mL nutrient solution, and the composition of nutrient solution is given in our previous study (Yin et al. 2014a, b); the initial pH of the medium was adjusted to pH 7.0. During the isolation process, 5 mL gamma irradiation pretreated sludge was transferred into 200 mL medium and cultured under anaerobic condition at 36 °C for 24 h. Then, incubated culture was diluted serially (10^{-1} , 10^{-2} , 10^{-3}) with normal saline and further processed for isolation using the roll-tube method on solid medium (1.5% agar w/v). The process was repeated until single colony was obtained. Then, the obtained strains were transferred into the fresh medium and cultured at 36 °C for 48 h. The purity of the isolates was checked through microscopic observation. Hydrogen production from glucose was investigated and strain INET1 showed the best hydrogen production.

The 16S rRNA gene of the isolated strain was amplified by PCR according to the standard method, PCR was performed in a DNA thermal cycler, and the process condition is as follows: denaturation at 96 °C for 2 min, 94 °C for 40 s (32 cycles), 54 °C for 40 s, 72 °C for 60 s, and final extension at 72 °C for 5 min. The 16S rRNA gene sequence (1344 bp) was characterized by universal primers 27F and 1492R. The PCR products were purified using DNA Fragment Purification Kit (Takara, Dalian, China). The strain was identified by China General Microbiological Culture Collection Center (CGMCC) and deposited in CGMCC numbered as CGMCC 1.5199. A phylogenetic tree was established with MEGA 6.06 using the neighbor-joining method. Credibility of the obtained phylogenetic tree was evaluated by re-sampling 1000 bootstrap trees.

In our previous study, gamma irradiation pretreated digested sludge was proved to be a good source of hydrogen producers (Yin et al. 2014a, b). Hydrogen yield of 2.15 mol H₂/mol glucose was achieved by the mixed culture, and various carbon sources were able to be used for hydrogen production. Microbial analysis demonstrated that the mixed culture was dominated by genus *Clostridium* (Yin and Wang 2016). Considering the mutation effect of gamma irradiation, we expected to obtain high-efficiency hydrogen-producing isolates from gamma-irradiated sludge.

Table 2.2 Standard biochemical analyses of strain *Clostridium butyricum* INET1

Characteristics	Results	Characteristics	Results	Characteristics	Results
Methyl red test	+	Catalase	–	Oxidase	–
<i>Utilization of</i>					
Glycerol	+	Mannitol	–	Melezitose	–
Erythritol	–	Sorbitol	–	Raffinose	+
D-Arabinose	–	α -Methyl-D-Mannitol glycosides	–	Starch	+
L-Arabinose	+	α -Methyl-D-Glucoside	+	Glycogen	+
D-Ribose	+	N-Acetyl-Glucosamine	+	Xylitol	–
D-Xylose	+	Amygdalin	+	Gentiobiose	+
L-Xylose	–	Arbutin	+	D-Turanose	+
Adon alcohol	–	Esculin	+	D-Lyxose	–
β -Methyl-D-Xyloside	–	Salicin	+	D-Tagatose	–
D-Galactose	+	Cellobiose	+	D-Fucose	–
D-Glucose	+	Maltose	+	L-Fucose	–
D-Sucrose	+	Lactose	+	D-Arabinitol	–
D-Mannose	+	Melibiose	–	L-Arabinitol	–
L-Sorbitol	–	Sucrose	+	Gluconate	–
L-Rhamnose	–	Trehalose	+	2-Keto-Gluconate	–
Dulcitol	–	Inulin	+	Inositol	–

For the strain isolation, over 10 strains were isolated from digested sludge pretreated by 5 kGy gamma irradiation. Among the isolated strains, strain INET1 showed the best hydrogen production ability both in cumulative hydrogen production (218 mL/100 mL) and hydrogen yield (2.07 mol H₂/mol glucose). Strain INET1 was identified as *Clostridium butyricum* by CGMCC according to 16S rRNA gene (1344 bp) and standard biochemical analyses using standard method (Table 2.2).

It can be seen from Table 2.2 that various carbon sources can be used by *Clostridium butyricum* INET1, including monosaccharides (like glucose, galactose, mannose), disaccharides (like sucrose, lactose, maltose, trehalose), and polysaccharides (like starch, inulin, glycogen). Sugars like inulin, arabinose, and xylose are widely present in plants, indicating that this strain can use the hydrolysate of agricultural wastes as substrate. However, for the sugar, alcohols and acids like arabinitol, inositol, and gluconate cannot be used as carbon source by strain INET1.

The 16S rRNA gene sequence (1389 bp) analysis against public gene bank (<http://www.ezbiocloud.net/eztaxon>) showed strain *Clostridium butyricum* INET1 had the highest similarity of 99.79% to strain *Clostridium butyricum* DSM 10702 T (accession No. AQQF01000149). A detailed phylogenetic tree is shown in Fig. 2.2 to describe the relationship between strain *Clostridium butyricum* INET1 and the most closely taxonomic species.

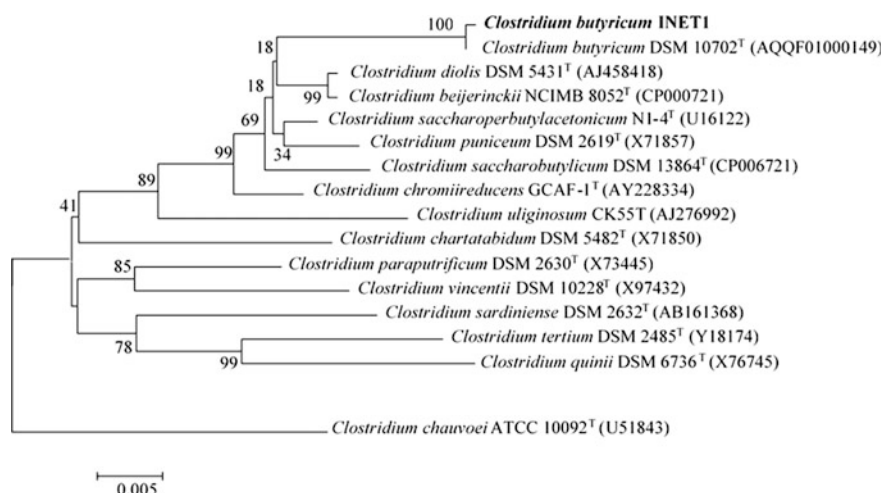


Fig. 2.2 Phylogenetic tree showing the relationships between strain *Clostridium butyricum* INET1 and related species based on 16S rRNA gene

2.3.1.2 Characteristics of Hydrogen Production

As shown in Fig. 2.3a, both maximum cumulative hydrogen production and highest hydrogen yield were obtained at 35 °C. Figure 2.3b shows that the hydrogen yield flocculated between 1.75 and 2.07 mol H₂/mol hexose when initial pH ranges from 5.0 to 7.0, then decreased with the increase of initial pH. Maximum cumulative hydrogen production of 218 mL/100 mL was obtained at initial pH 7.0. Figure 2.3c shows that the hydrogen yield decreased from 2.24 to 1.49 mol H₂/mol hexose with the increase of substrate concentration from 5 to 20 g/L glucose. The highest cumulative hydrogen production was achieved at 10 g/L glucose. Figure 2.3d shows that hydrogen yield fluctuated between 1.76 and 2.07 mol H₂/mol hexose when inoculation proportion was between 10 and 20%. The optimum inoculation proportion for cumulative hydrogen production was 10%.

In general, the maximum cumulative hydrogen production of 218 mL/100 mL was obtained at 35 °C, initial pH 7.0, substrate concentration 10 g/L glucose, and inoculation proportion 10%, at this condition hydrogen yield of 2.07 mol H₂/mol hexose was achieved. Otherwise, the highest hydrogen yield of 2.24 mol H₂/mol hexose was attained at same condition as maximum cumulative hydrogen production except initial pH 5.0. However, cumulative hydrogen production was only 138 mL/100 mL.

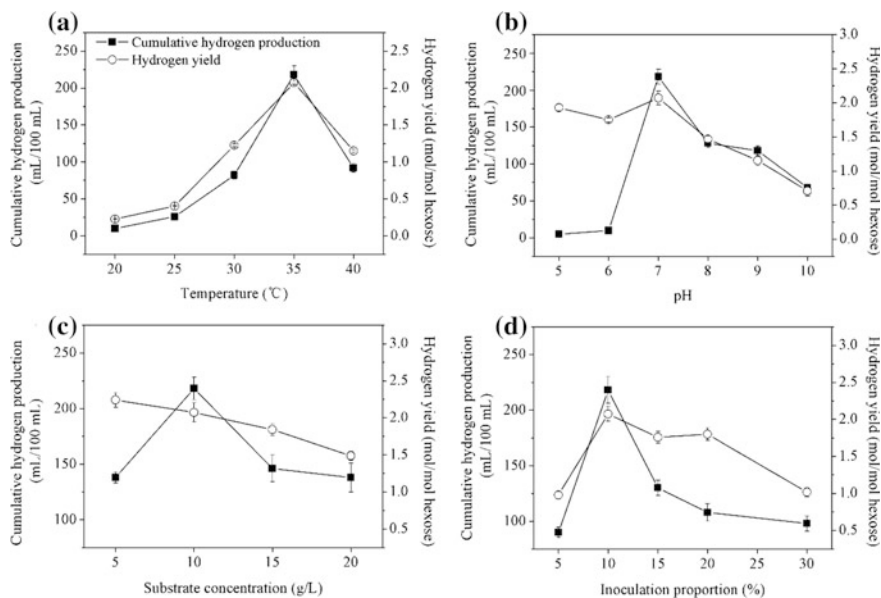


Fig. 2.3 Characteristics of hydrogen production by *Clostridium butyricum* INET1. **a**, **b**, **c**, **d** represents the effect of temperature, initial pH, substrate concentration and inoculation proportion on hydrogen production, respectively. (—■— Cumulative hydrogen production, —○— Hydrogen yield)

2.3.1.3 Optimization of Fermentative Conditions

The operational conditions, including temperature, initial pH, substrate concentration, and inoculation proportion, were optimized, and the optimal condition for hydrogen production by *Clostridium butyricum* INET1 was determined to be 35 °C, initial pH 7.0, substrate concentration of 10 g/L glucose, and inoculation proportion of 10%.

Temperature applied in fermentative hydrogen production by different *Clostridium butyricum* isolates lies in 30–40 °C (Beckers et al. 2010; Junghare et al. 2012; Pachapur et al. 2016). Junghare et al. explored the effect of temperature on hydrogen production by *Clostridium butyricum* EB6 in a range of 25–55 °C, and the maximum hydrogen production was obtained at 37 °C (Junghare et al. 2012). Chong et al. optimized hydrogen production through response surface methodology, and the optimal temperature was determined to be 36 °C (Chong et al. 2009a, b). In this study, hydrogen production by *Clostridium butyricum* INET1 showed sensitive reaction to temperature change, which may due to the inactivation and denaturation of the key enzymes at inappropriate temperature condition (Cai et al. 2013a, b).

Optimal pH for hydrogen production by *Clostridium butyricum* strains varies a lot, ranging from pH 5.2 to pH 9.0 (Abdul et al. 2013; Hiligsmann et al. 2014). In

this study, maximum hydrogen production was obtained at initial pH 7.0. Little hydrogen was produced at initial pH 5.0–6.0, which was because of the formation of VFA further decreased pH of liquid phase, leading to the inhibition of hydrogen production process. Many studies have showed that *Clostridium* species can hardly grow below a pH range pH 4.0–5.0 (Cai et al. 2013a, b). However, high hydrogen yield was obtained at initial pH range from pH 5.0 to pH 7.0. Basing on this phenomenon, many studies have tried to enhance the hydrogen yield through fixing pH of a reactor at around pH 5.5 (Calusinska et al. 2015). Both low hydrogen production and hydrogen yield decreased with the increase of initial pH, possibly because the metabolic pathways changed from hydrogen production to volatile fatty acids production at higher pH conditions.

Organic loading is a vital factor for fermentative hydrogen production process. In this study, maximum hydrogen production was obtained at 10 g/L glucose, lower or higher substrate concentration all caused a significant decrease in cumulative hydrogen production. Low substrate concentration may constrain the microbial growth, while high substrate concentration may cause a quick decrease in pH and end-product inhibition (An et al. 2014), both will result in low hydrogen production. Hydrogen yield decreased with the increase of substrate concentration, possible reason is that more energy was used for microbial growth rather than hydrogen production when substrate was abundant. Many studies also observed the decrease of hydrogen yield along with the increasing substrate concentration. Hydrogen production by *Clostridium butyricum* CGS5 from microalgal biomass showed an increase in cumulative hydrogen production at 3–9 g/L sugar concentrations and declined over 9 g/L, but hydrogen yield decreased along with the increase of sugar concentration from 3 g/L to 9 g/L. It was reported a decline in hydrogen yield when sugar concentration was over 20–25 g COD/L with *Clostridium butyricum* TISTR 1032 (Plangklang et al. 2012). *Clostridium butyricum* EB6 was reported to achieve the highest hydrogen yield at 15.7 g/L glucose concentration and higher substrate concentration resulted in significant decrease in hydrogen yield (Chong et al. 2009a, b).

Inoculation proportion also plays a crucial role in the successful operation of fermentative hydrogen production process. Proper inoculation proportion can help to achieve the quick start and high hydrogen production rate of a fermentative hydrogen production system. In this study, both highest cumulative hydrogen production and hydrogen yield were obtained at inoculation proportion of 10%. Lower or higher inoculation proportion all resulted in a decrease of both cumulative hydrogen production and hydrogen yield. Possible reason is that more energy is required for microbial reproduction when inoculation proportion is low. Since studies have found that most of hydrogen production happened at the logarithmic growth phase for *Clostridium* spp. (Patel et al. 2015); thus, much high inoculation proportion can make the bacteria in the system grow quickly into stable and decline phase, leading to a change of metabolic pathway from hydrogen production to the formation of other soluble metabolites.

2.3.1.4 Hydrogen Production from Different Substrates

Strain *Clostridium butyricum* INET1 showed the ability of producing hydrogen from different carbon sources, including monosaccharide (glucose and xylose), disaccharide (sucrose and lactose), polysaccharide (starch), and alcohol (glycerol).

It can be observed from Fig. 2.4a that highest cumulative hydrogen generation was obtained with glucose as substrate (218 mL/100 mL), followed by lactose (178 mL/100 mL), sucrose (140 mL/100 mL), starch (114 mL/100 mL), xylose (102 mL/100 mL), and glycerol (68 mL/100 mL). Kinetics of hydrogen production process was simulated by the Modified Gompertz equation (Table 2.3), hydrogen

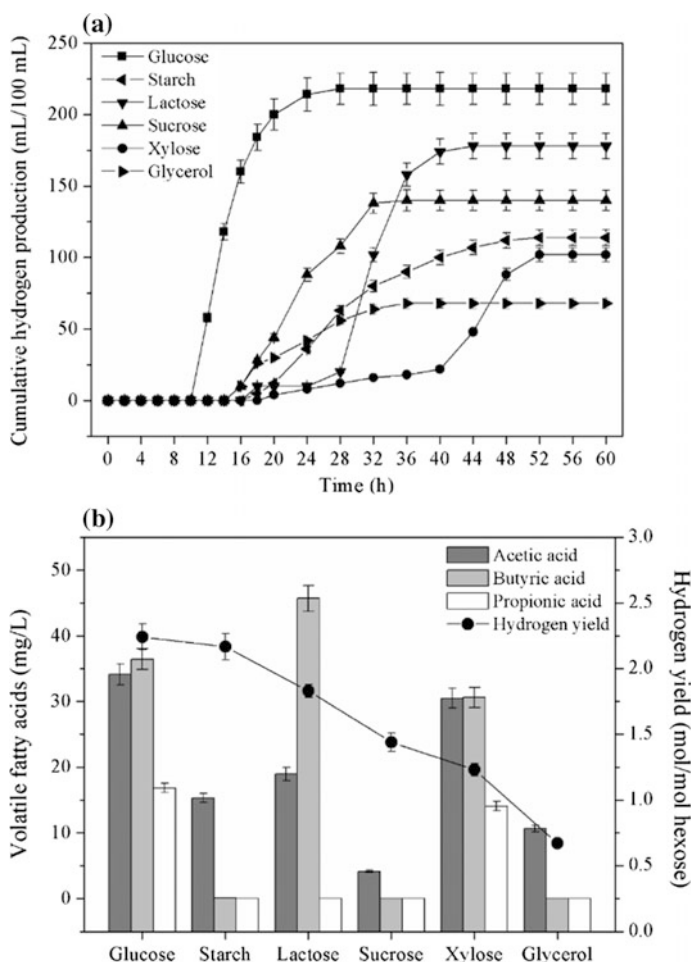


Fig. 2.4 Hydrogen production from different carbon sources by *Clostridium butyricum* INET1. **a.** Hydrogen generation during the fermentation process. **b.** Volatile fatty acids formation and hydrogen yield obtained from different substrates

Table 2.3 Parameters estimated by the modified Gompertz model

Substrates	P (mL)	R_m (mL/h)	λ (h)	R^2
Glucose	215.9	30.2	10.4	0.9955
Xylose	103.3	7.5	36.6	0.9872
Sucrose	141.2	10.6	16.4	0.9954
Lactose	177.7	22.1	27.5	0.9983
Starch	112.6	6.1	16.8	0.9946
Glycerol	67.8	4.7	14.4	0.9907

production from glucose showed both highest maximum hydrogen production rate and lowest lag time, test with xylose demonstrated the longest lag time, while test with glycerol showed the smallest maximum hydrogen production rate.

As shown in Fig. 2.4b, more VFA was formed with glucose, xylose, and lactose as substrate, followed by starch, glycerol, and sucrose. VFA was dominated by butyric acid in test with lactose as substrate, for tests with sucrose, starch, and glycerol as substrate, more acetate acid was formed, while for tests with glucose and xylose as substrate, both acetate acid and butyric acid are the main metabolic products.

Highest hydrogen yield of 2.24 mol H_2 /mol hexose was achieved with glucose as substrate, followed by 2.17 mol H_2 /mol hexose with starch. 1.23–1.83 mol H_2 /mol hexose was attained with xylose, sucrose, and lactose, while glycerol showed the lowest hydrogen yield of 0.67 mol H_2 /mol hexose.

Analysis of VFA formation shows that hydrogen production from different substrates followed different fermentation types. Hydrogen production from glucose and xylose was dominated by mixed acid type fermentation, fermentation with sucrose, starch, and glycerol as substrate followed by acetate-type fermentation, hydrogen production from lactose went through butyrate-type fermentation. Similar phenomenon was observed in hydrogen production by *Clostridium butyricum* TM-9A from different carbon sources (Junghare et al. 2012). However, Patel et al. examined hydrogen production from various carbon sources by *Clostridium* sp. IODB-O3, and all the tests were dominated by butyrate-type fermentation (Patel et al. 2015).

Table 2.4 shows the comparison of hydrogen production from various carbon sources by *Clostridium butyricum* INET1 and other *Clostridium butyricum* isolates. It can be seen from Table 2.3 that operational condition used in different studies with *Clostridium butyricum* isolates also various, the temperature ranged from 30 to 37 °C and the initial pH ranged from pH 5.5 to pH 9.0. Most commonly used condition was 37 °C and pH 7.0. Variation in operational conditions indicates that although belonging to specie *Clostridium butyricum*, characteristics of different isolates also varies in a certain extent.

For the studies used glucose, xylose, sucrose, lactose, starch, and glycerol as substrate, hydrogen yield of 0.23–3.47 mol H_2 /mol hexose, 0.59–3.12 mol H_2 /mol hexose, 0.44–1.63 mol H_2 /mol hexose, 0.69–1.83 mol H_2 /mol hexose, 0.73–3.2 mol H_2 /mol hexose, and 0.67–3.6 mol H_2 /mol hexose were obtained by different *Clostridium butyricum* isolates. Different strains showed advantage in

Table 2.4 Comparison of hydrogen production by *Clostridium butyricum* INET1 and other *Clostridium butyricum* isolates

Microorganism	Substrates	Temperature	pH	Hydrogen yield (mol H ₂ /mol hexose)	References
<i>Clostridium butyricum</i> DSM 10702	Glucose	37	7.0	3.47	Ortigueira et al. (2015)
<i>Clostridium butyricum</i> CWBI1009	Glucose (1–10 g/L)	30–37	5.2–8.0	0.23–2.4	Beckers et al. (2010; Beckers et al. 2015)
<i>Clostridium butyricum</i> W5	Glucose (5–10 g/L)	39	6.5	0.82–1.4	Wang et al. (2009)
<i>Clostridium butyricum</i>	Glucose (3 g/L)	37	6.5	2.09	Seppälä et al. (2011)
<i>Clostridium butyricum</i> IFO 3847	Glucose (9 g/L)	37	7.0	1.26	Karube et al. (1976)
<i>Clostridium butyricum</i> IAM 19002	Glucose (9 g/L)	37	7.0	1.04	Karube et al. (1976)
<i>Clostridium butyricum</i> IMA 19003	Glucose (9 g/L)	37	7.0	1.2	Karube et al. (1976)
<i>Clostridium butyricum</i> TM-9A	Glucose (10 g/L)	37	8.0	2.67–3.1	Junghare et al. (2012)
<i>Clostridium butyricum</i> A1	Glucose (10 g/L)	37	6.5	1.9	Jenol et al. (2014)
<i>Clostridium butyricum</i> DSM 10702	Glucose (10 g/L)	37	6.8	2.4–3.1	Hu et al. (2013)
<i>Clostridium butyricum</i> EB6	Glucose (15.7 g/L)	37	5.6	2.2	Chong et al. (2009a, b)
<i>Clostridium butyricum</i> INET1	Glucose (COD = 10 g/L)	35	7.0	2.24	This study
<i>Clostridium butyricum</i> DSM 10702	Xylose	37	7.0	3.12	Ortigueira et al. (2015)
<i>Clostridium butyricum</i> LMG 1213t1	Xylose (10 g/L)	–	5.5–7.0	1.94–2.48	Heyndrickx et al. (1991)
<i>Clostridium Butyricum</i> TM-9A	Xylose (10 g/L)	37	8.0	0.59	Junghare et al. (2012)
<i>Clostridium butyricum</i> INET1	Xylose (COD = 10 g/L)	35	7.0	1.23	This study

(continued)

Table 2.4 (continued)

Microorganism	Substrates	Temperature	pH	Hydrogen yield (mol H ₂ /mol hexose)	References
<i>Clostridium butyricum</i> CWBI 1009	Sucrose (4.3 g COD/L)	30	7.3	0.44	Beckers et al. (2010)
<i>Clostridium butyricum</i> TM-9A	Sucrose (10 g/L)	37	8.0	1.49	Rafeenia and Chaganti (2015)
<i>Clostridium butyricum</i> CGS2	Sucrose (COD = 20 g/L)	37	7.1	0.95	Fritsch et al. (2008)
<i>Clostridium butyricum</i> CGS5	Sucrose (COD = 20 g/L)	37	5.5	1.39	Chen et al. (2005)
<i>Clostridium butyricum</i> TISTR 1032	Sucrose (COD = 25 g/L)	37	6.5	1.52	Plangklang et al. (2012)
<i>Clostridium butyricum</i> W5	Molasses (100 g/L)	35	7.0	1.63	Wang et al. (2009)
<i>Clostridium butyricum</i> KBH1	Molasses (5.9 g/L)	37	9.0	1.49	Abdul et al. (2013)
<i>Clostridium butyricum</i> INET1	Sucrose (COD = 10 g/L)	35	7.0	1.44	This study
<i>Clostridium butyricum</i> CWBI 1009	Lactose (COD = 4.3 g/L)	30	7.3	0.69	Beckers et al. (2010)
<i>Clostridium butyricum</i> INET1	Lactose (COD = 10 g/L)	35	7.0	1.83	This study
<i>Clostridium butyricum</i> DSM 10702	Starch	37	–	3.2	Ortigueira et al. (2015)
<i>Clostridium butyricum</i> CWBI 1009	Starch (COD = 4.3 g/L)	30	7.3	0.73	Beckers et al. (2010)
<i>Clostridium butyricum</i> NCIB 9576	Starch (10 g/L)	37	–	2.58	KIM et al. (2006)
<i>Clostridium butyricum</i> CGS2	Starch hydrolysate	37	7.5	1.23–2.03	Pattra et al. (2008)
<i>Clostridium butyricum</i> INET1	Starch (COD = 10 g/L)	35	7.0	2.17	This study

(continued)

Table 2.4 (continued)

Microorganism	Substrates	Temperature	pH	Hydrogen yield (mol H ₂ /mol hexose)	References
<i>Clostridium butyricum</i>	Glycerol (5 g/L)	37	7.4	3.6	Kivisto et al. (2013)
<i>Clostridium butyricum</i>	Glycerol (20 g/L)	37	6.5	0.67	Pachapur et al. (2016)
<i>Clostridium butyricum</i> INET1	Glycerol (COD = 10 g/L)	35	7.0	0.67	This study

degrading different substrates. Highest hydrogen yields from glucose, xylose, and starch were all obtained by *Clostridium butyricum* DSM 10702 at 37 °C and initial pH 7.0 (Ortigueira et al. 2015). *Clostridium butyricum* W5 showed high efficiency in using molasses wastewater (Wang et al. 2009). Strain INET1 isolated in this study showed relatively high hydrogen yield with all the mentioned carbon sources, especially for lactose, and highest hydrogen yield among published reports was obtained. Thus, hydrogen production from dairy wastewater by this strain can be further explored in future studies.

In general, *Clostridium butyricum* INET1 showed a relative high hydrogen yield with glucose, sucrose, lactose, starch, and glycerol as substrate compared with the other *Clostridium butyricum* isolates. Especially for the lactose, few studies have reported hydrogen production from lactose based substrate by species *Clostridium butyricum*. Therefore, *Clostridium butyricum* INET1 is a potential strain for efficient hydrogen production from complex organic waste.

2.3.2 *Enterococcus faecium* INET2

2.3.2.1 Isolation of Strain

The bacterium used in this study, *Enterococcus faecium* INET2, was isolated from the gamma irradiation pretreated digested sludge (Yin et al. 2014a, b). The digested sludge used in this study was obtained from the primary anaerobic digester of a municipal wastewater treatment plant located in Beijing, China. The anaerobic digested sludge was pretreated with 5 kGy gamma irradiation to enrich hydrogen producers (Yin and Wang 2016). After the irradiation process, treated sludge was pre-cultured to enrich the hydrogen producers. Medium used for pre-culture was as follows: 50 g glucose, 10 g peptone, 0.5 g yeast extract, and 10 ml/100 mL of nutrient solution (each liter of nutrient solution contains 40 g NaHCO₃, 5 g NH₄Cl, 5 g NaH₂PO₄ · 2H₂O, 5 g K₂HPO₄ · 3H₂O, 0.25 g FeSO₄ · 7H₂O, 0.085 g MgCl₂ · 6H₂O, 0.004 g NiCl₂ · 6H₂O). Treated sludge was pre-cultured in flask

reactors, and the pre-culture process was conducted in triplicate. 10 mL of treated sludge was added in each 100 mL medium, and the initial pH of the mixture was adjusted to 7.0. The medium was flushed with pure N₂ for 3 min to create the anaerobic environment. Then, flask reactors were incubated in reciprocal shaker (100 rpm) at constant temperature of 36 °C for 36 h.

After the enrichment step, the bacterial strain was isolated according to the method described elsewhere (Archana et al. 2004; Cai et al. 2013a, b).

2.3.2.2 Identification of Strain and Phylogenetic Analysis

The chromosomal DNA was extracted from cell pellets and the 16S rRNA gene of isolated strain was amplified by PCR according to the standard method (Green and Sambrook 2012). A pair of universal primers of 27F (50-AGA GTT TGA TCC TGG CTC AG-30) and 1492R (50-TAC GGT TAC CTT GTT ACG ACT T-30) were used to obtain the 16S rRNA gene sequence (1389 bp) of strain INET2. The PCR products were purified using DNA Fragment Purification Kit (Takara, Dalian, China). The strain was identified and deposited in China General Microbiological Culture Collection Center (CGMCC1.15321). The 16S rRNA gene sequence was aligned in GenBank using BLAST program (Altschul et al. 1990). A phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei 1987), and neighbor-joining analysis was conducted with MEGA 6.06 (Tamura et al. 2013). Credibility of the obtained tree was evaluated by re-sampling 1000 bootstrap trees (Felsenstein 1985).

The pyrosequencing data of strain INET2 has been deposited in the NCBI GenBank with accession number of KU647682.

The isolated strain was identified by CGMCC, and the results indicated it belongs to genus *Enterococcus* and species *faecium*. This strain was stored in CGMCC (CGMCC 1.15321), and named as *Enterococcus faecium* INET2. *Enterococcus faecium* INET2 is a facultative anaerobic bacterium, Gram-positive, and sphere shape. The results of the standard biochemical analyses are shown in Table 2.5. It can be seen that the strain INET2 was not spore-forming bacteria. The strain was positive for the utilization (sole carbon source) of D-Glucose, D-Fructose, D-Mannose, D-Ribose, D-Galactose, L-Arabinose, lactose, sucrose, maltose, trehalose, melibiose, cellobiose, raffinose, mannitol, esculin, salicin, and amygdalin.

The 16S rRNA gene sequence (1389 bp) was deposited in Genbank under accession number KU647682, and it was aligned with public gene bank at website <http://www.ezbiocloud.net/eztaxon>. Results showed that the 16S rRNA gene sequence from strain *Enterococcus faecium* INET2 exhibited over 99% sequence identity with strain *Cedecea davisae* DSM 4568^T (ATDT01000040), *Enterobacter cancerogenus* LMG 2693^T (Z96078), *Leclercia adecarboxylata* GTC 1267^T (AB273740), and *Kluyvera cryocrescens* ATCC 33435^T (AF310218). The strain *Enterococcus faecium* INET2 had the highest similarity of 99.79% to *Enterobacter*

Table 2.5 The characteristics of the strain *Enterococcus faecium* INET2

Characteristics	Results	Characteristics	Results
Methyl red test	+	Catalase	–
Ability of forming spore	–	Oxidase	–
<i>Ability to grow</i>			
50 °C	–	45 °C	+
15 °C	+	6.5% NaCl	+
Air	+		
<i>Utilization of</i>			
D-Glucose	+	Trehalose	+
D-Fructose	+	Melibiose	+
D-Mannose	+	Cellobiose	+
D-Ribose	+	Melezitose	–
D-Xylose	–	Raffinose	+
D-Galactose	+	Sorbitol	–
D-Arabinose	–	Mannitol	+
L-Arabinose	+	Sodium gluconate	–
L-Sorbose	–	Esculin	+
L-Rhamnose	–	Salicin	+
Lactose	+	Amygdalin	+
Sucrose	+	Starch	–
Maltose	+		

asburiae JCM 6051^T (AB004744). As shown in Fig. 2.5, a phylogenetic tree was constructed to describe the relationship between strain *Enterococcus faecium* INET2 and the most closely taxonomic species based on 16S rRNA sequences. It

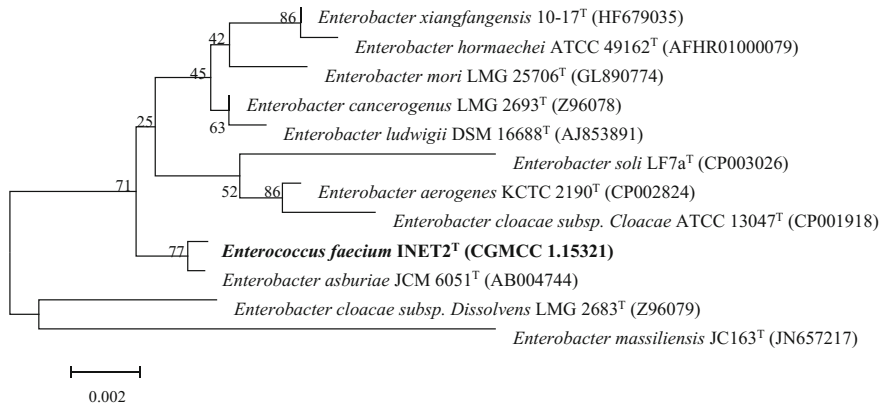


Fig. 2.5 Phylogenetic tree showing the relationships between strain *Enterococcus faecium* INET2 and related species based on 16S rDNA gene

can be seen that strain *Enterococcus faecium* INET2 was grouped together with the reference strain *Enterobacter asburiae* JCM 6051^T (AB004744). Species *Enterobacter asburiae* has also been reported to be effective in fermentative hydrogen production (Shin et al. 2007; Lee et al. 2014).

2.3.2.3 Batch Fermentation for Hydrogen Production

All batch tests were performed in 150-mL Erlenmeyer flasks with working volume of 100 mL. Neoprene rubber stoppers were used to avoid gas leakage from the flasks. Glucose was used as the sole carbon source and 10 mL of nutrient solution (as mentioned above) was added in each flask. 5 mol/L HCl and 5 mol/L NaOH were used to adjust the pH of the medium. Nitrogen gas was passed through to drive away the oxygen in the medium. Before the inoculation, mediums were sterilized at 115 °C for 30 min. Strain *Enterococcus faecium* INET2 was inoculated at its logarithmic phase.

Effect of culture temperature, initial pH, substrate concentration and inoculation proportion on hydrogen production by strain *Enterococcus faecium* INET2 was explored. Experiments were conducted at varying incubation temperature (20–40 °C), initial pH (5.0–10.0), glucose concentration (5–20 g/L), and inoculation proportion (5–30%). Flasks were cultured in constant temperature reciprocal shaker at 100 rpm until the reaction terminated. Hydrogen production by suspended and immobilized *Enterococcus faecium* INET2 under the optimized conditions (temperature 35 °C, pH 7.0, glucose concentration 15 g/L, and inoculation proportion 10%) were then studied. Modified Gompertz equation was used to describe the kinetics of hydrogen production process. All the batch tests were performed in duplicate.

The cumulative hydrogen production (mL) was calculated from the total biogas produced and the concentration of H₂ in the headspace. The hydrogen yield (mol H₂/mol glucose) was calculated using Eq. (1). The substrate degradation rate (%) was calculated by dividing the amount of glucose consumed after hydrogen production process by the amount of initial glucose added in the system:

$$\text{Hydrogen yield} = \frac{\text{Cumulative hydrogen production (mol)}}{\text{Amount of glucose consumed (mol)}}. \quad (1)$$

2.3.2.4 Effect of Fermentative Parameters on Hydrogen Production

Since fermentative hydrogen production is a complex microbial metabolic process, it can be affected by many parameters. In this study, the effects of operational conditions like temperature, initial pH, substrate concentration, and inoculation proportion were explored to obtain the optimal hydrogen production conditions.

(1) Effects of temperature

Temperature is one of the most important parameters affecting the activity of hydrogen-producing microorganism, and high temperature may damage the enzymes while low temperature may cause the low activity of microorganisms (Wang and Wan 2009). Incubation temperature used in studies produces hydrogen by species *Enterococcus faecium* ranged from 30 to 37 °C (Liu et al. 2009; Song et al. 2012a, b; Cisneros-Pérez et al. 2015). However, *Enterococcus faecium* in those studies were all present in mixed hydrogen-producing cultures, and no study has examined the effects of cultivation temperature on hydrogen production by pure stain of *Enterococcus faecium*. Thus the effects of cultivation temperature in the range of 25 to 40 °C were studied in the medium with 10 g/L glucose as sole carbon source and initial pH of the medium was adjusted to 7.0, and inoculation proportion adopted was 10%.

Figure 2.6 shows the effects of temperature on cumulative hydrogen production (mL H₂/100 mL), hydrogen yield (mol H₂/mol glucose), and substrate degradation rate (%). As shown in Fig. 2.6a, cumulative hydrogen production increased with the rise of temperature in the range of 25–35 °C, and achieved the highest point of 102 mL H₂/100 mL at 35 °C. When the temperature further increased to 40 °C, cumulative hydrogen production showed a little decrease to 85 mL H₂/100 mL, and similar trend was observed in Fig. 2.6c, which showed relation between the substrate degradation rate and fermentation temperature, highest substrate degradation rate of 89.9% was obtained at 35 °C. Similar with *Enterococcus faecium* INET1, cumulative hydrogen production as well as substrate degradation by different hydrogen-producing strains also showed sensitive reaction to temperature: An et al. and Zhang et al. examined hydrogen production by *Clostridium* strains, and the deviation of temperature from the suitable one all caused significant decrease in cumulative hydrogen, hydrogen production rate, and xylose degradation rate, which may because of the inactivation and denaturation of the key enzymes at inappropriate temperature conditions.

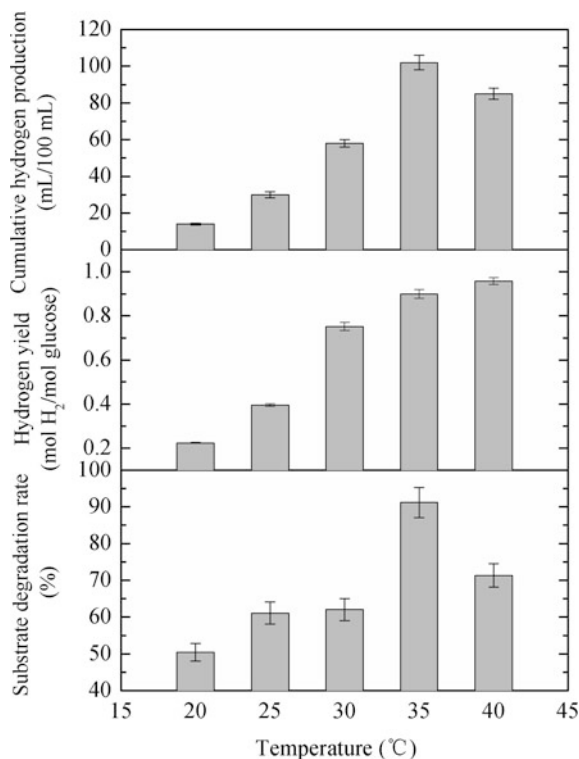
As shown in Fig. 2.6b, hydrogen yield increased gradually with the rise of temperature, and highest hydrogen yield of 0.96 mol H₂/mol glucose was attained at 40 °C, which was slightly higher than 0.90 mol H₂/mol glucose obtained at 35 °C. Possible reason was that 35 °C was more suitable for the growth of strain *Enterococcus faecium* INET2, leading to more energy consumption for microbial growth and reproduction.

It can be seen that different suitable temperatures were obtained for cumulative hydrogen production and hydrogen yield. Chookaew et al. also observed similar phenomenon that suitable temperature for hydrogen yield was higher than that for cumulative hydrogen production (Chookaew 2012).

(2) Effects of initial pH

The value of pH is another important factor that influences the fermentative hydrogen production process, as the pH changes the electric charge on the cell membrane, and then affects enzyme activity as well as the metabolism pathway. To

Fig. 2.6 Effect of temperature on hydrogen production

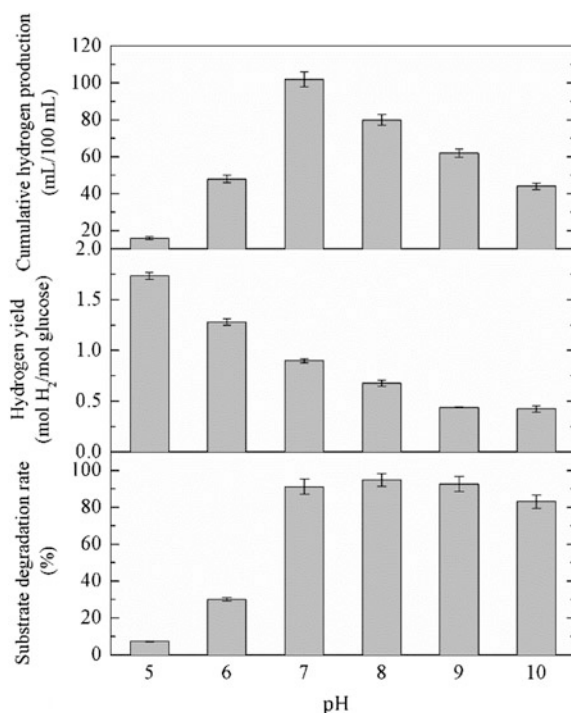


determine the optimal initial pH for hydrogen production by *Enterococcus faecium* INET2, initial pH ranged from 5 to 10 was studied. For the tests with different initial pHs, substrate concentration of 10 g/L glucose and inoculation proportion of 10% were used and batches were incubated at 35 °C.

Figure 2.7 shows the effects of different initial pHs on hydrogen production by strain *Enterococcus faecium* INET2. It can be seen that highest cumulative hydrogen production, hydrogen yield, and substrate degradation rate were obtained at initial pH 7, pH 5, and pH 8, respectively. Figure 2.7a shows that the cumulative hydrogen presented a summit at initial pH 7, lower or higher initial pH all led to the decrease of cumulative hydrogen production. As to hydrogen yield, it decreased gradually with the increase of initial pH as shown in Fig. 2.7b, and highest hydrogen yield of 1.74 mol H₂/mol glucose was obtained at initial pH 5. When it comes to Fig. 2.7c, substrate degradation rate raised when initial pH increased from 5 to 7, and then stayed stable at around 92% at initial pH range of 7 to 9, and then decreased slightly to 83% at pH 10.

For the test with initial pH 5, little hydrogen was produced because during the fermentation process, pH of the medium dropped quickly to 3.86, which constrained the further utilization of substrate and hydrogen production. Many studies have found that fermentative hydrogen production process terminated when pH of

Fig. 2.7 Effect of initial pH on hydrogen production

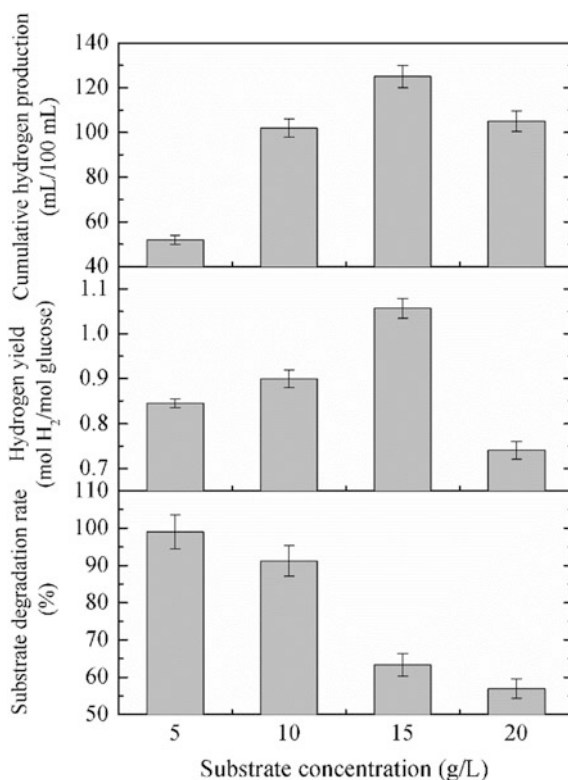


medium was decreased to a certain degree (Yin et al. 2014a, b). Considering this phenomenon, to achieve high hydrogen yield and hydrogen production, measurements can be taken to maintain the pH of medium at 5 in the future study. Cisneros-Pérez et al. applied EGSB in continuous fermentative hydrogen production, and the pH was kept at 5.5 to achieve a high hydrogen production yield and hydrogen production rate (Cisneros-Pérez et al. 2015). For the batches with initial pH 7–10, over 80% of glucose was used and pH of the medium was all ended at around 5. Thus, the glucose consumed may be transformed into volatile fatty acids, indicating that higher pH can lead to the metabolic pathways change from hydrogen production to volatile fatty acids production (Jung et al. 2015). Thus, the optimum initial pH for hydrogen production by strain *Enterococcus faecium* INET2 was 7.

(3) Effects of substrate concentration

Organic loading is a crucial factor for fermentative hydrogen production process. Studies have found that in an appropriate range, increasing substrate concentration could lead to an increase in microbial hydrogen production ability. However, substrate concentration at much higher level may constrain the hydrogen production process and even harm the microbial activity. In this study, substrate concentration in a range of 5 to 20 g/L glucose was investigated at initial pH 7, incubation temperature 35 °C, and inoculation proportion of 10%.

Fig. 2.8 Effect of substrate concentration on hydrogen production



As shown in Fig. 2.8a, b, the optimal substrate concentration for both cumulative hydrogen production and hydrogen yield was 15 g/L, and maximum cumulative hydrogen production of 125 mL H₂/100 mL and hydrogen yield of 1.06 mol H₂/mol glucose were obtained. Figure 2.8c demonstrates that the increase of substrate concentration results in the decrease of substrate degradation rate. Over 95% of glucose was degraded in batch test with 5 g/L glucose as substrate, while only 55% of the substrate was used for the test of 20 g/L glucose.

Many other studies also observed the inhibitory effect of high substrate concentration on both microbial growth and hydrogen production (Chookaew 2012; Cai et al. 2013a, b; An et al. 2014). Some studies applied load shock in selectively inhibiting microorganisms (Kannaiah Goud and Venkata Mohan 2012). On the other side, Shin et al. found that substrate degradation rate remained at a high level of over 99% when substrate concentration ranged from 2 to 50 g/L, possible reason was the addition of peptone and yeast extract in the medium, which promoted the glucose utilization and microbial growth (Shin et al. 2007).

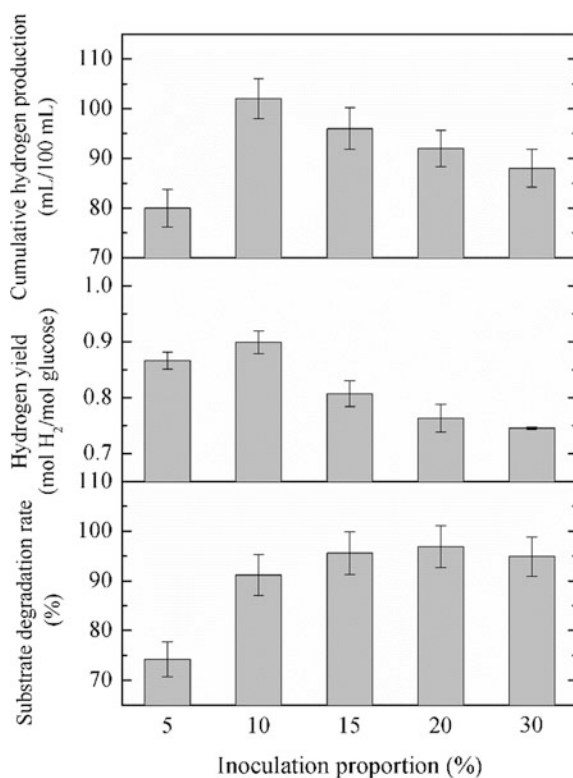
(4) Effects of inoculation size

Inoculation proportion is also a vital factor for the successful operation of fermentative hydrogen production process. Proper inoculation proportion can help the fermentative hydrogen production system start quickly and keep a high hydrogen production rate. Different inoculation proportions (5–30%) were investigated at 35 °C, initial pH 7 and substrate concentration of 10 g/L to explore the optimum inoculation proportion of strain *Enterococcus faecium* INET2.

Figure 2.9 shows the effect of inoculation proportion on hydrogen production by *Enterococcus faecium* INET2. It can be seen that both highest cumulative hydrogen production (102 mL H₂/100 mL) and hydrogen yield (0.90 mol H₂/mol glucose) were obtained at inoculation proportion of 10%, while highest substrate degradation rate (96.9%) was achieved at 20% inoculation proportion. As shown in Fig. 2.9a, b, lower inoculation proportion resulted in lower cumulative hydrogen production and hydrogen yield, which may because of more energy was used for cell growth. Furthermore, little bacteria present in a system can lead to a much longer lag time of hydrogen production process.

Many studies have attempted to shorten the lag time of a reactor through enriching hydrogen producers exist in the system (Zhu and Béland 2006; O-Thong

Fig. 2.9 Effect of inoculation ratio on hydrogen production



et al. 2009; Yin et al. 2014a, b; Yin and Wang 2016). However, for the inoculation proportion higher than 10%, both cumulative hydrogen production and hydrogen yield showed a downtrend with the increase of inoculation proportion, since studies have found that the maximum hydrogen production rate happened at the logistic phase of microbial growth (Abdeshahian et al. 2014; Singh et al. 2014). However, too much microorganism present in a system can make the bacteria grow quickly into stable and decline phase, causing less hydrogen production. Figure 2.9 shows that substrate degradation rate raised from 74.2 to 96.7% with the increase of inoculation proportion from 5 to 20%, and then declined slightly to 94.9% at inoculation amount of 30%.

2.3.2.5 Hydrogen Production at Optimized Condition

Optimized condition for fermentative hydrogen production by *Enterococcus faecium* INET2 was determined to be incubation temperature of 35 °C, initial pH of 7, substrate concentration of 15 g/L glucose, and 10% inoculation proportion. Furthermore, hydrogen production by *Enterococcus faecium* INET2 under the optimized condition was conducted, and hydrogen generation, substrate degradation, and microbial growth were examined during the fermentation process.

As shown in Fig. 2.6a, hydrogen began to evolve after 16 h incubation and the hydrogen generation process terminated at 44 h. Cumulative hydrogen production of 130 mL H₂/100 mL was obtained. Hydrogen production process could be simulated by the modified Gompertz model, and the determination of coefficient (R^2) of the regression was over 0.99. Hydrogen production potential, maximum hydrogen production rate, and the lag time obtained by the modified Gompertz model were 132.20 mL, 8.28 mL/h, and 21.86 h, respectively. It can also be seen from Fig. 2.10a that substrate was utilized since the beginning of the fermentation, and the substrate degradation rate increased gradually with the increase of fermentation time, when cumulative hydrogen production reached the maximum value at 44 h, substrate degradation rate came to 93.3%, and remained constant.

Figure 2.10b shows the microorganism growth during the fermentation process. It can be seen that after 16 h adaptation, microorganisms entered the exponential growth phase and lasted for 20 h, and then followed by stationary phase and decline phase. During the stationary phase from 36 to 44 h, little hydrogen was produced while substrate concentration decreased continuously. When the bacteria came to decline phase, both hydrogen production and substrate utilization terminated. What worth mention was that hydrogen production was mainly happened throughout the exponential phase. Same phenomenon has also been observed by many other studies (WANG et al. 2007; Abdeshahian et al. 2014; Singh et al. 2014). However, Harun et al. got highest hydrogen production rate both at exponential and stationary phase (Harun et al. 2012).

Figure 2.10c depicts the hydrogen production rate at different fermentation time intervals. The hydrogen production rate increased gradually from 16 h and achieved the highest point at 36 h. Then it decreased continuously until the termination of

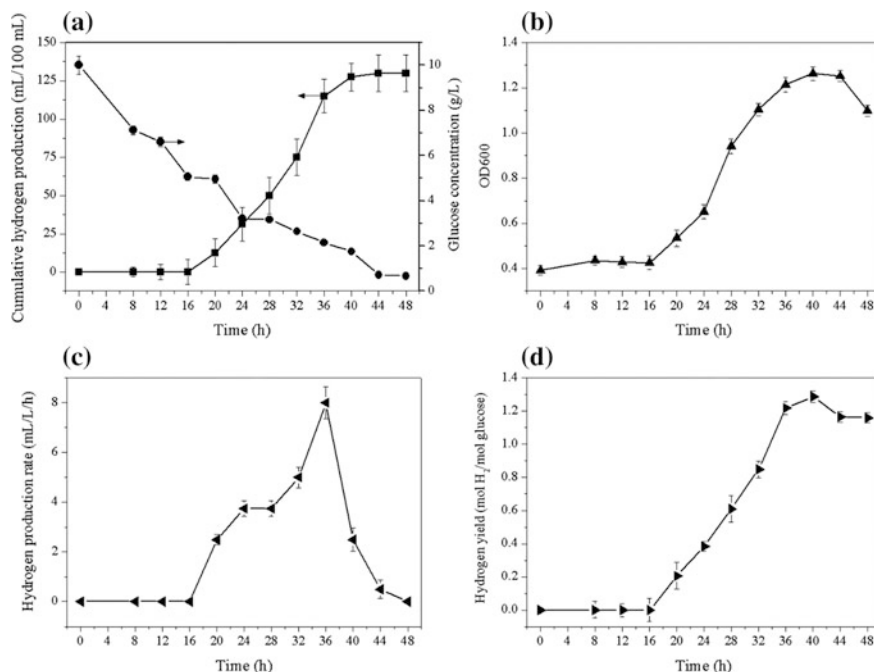


Fig. 2.10 Hydrogen production performances at the optimized condition

hydrogen production. As to the hydrogen yield (Fig. 2.10d), same trend as hydrogen production was observed in the first 40 h. However, with the further degradation of substrate and little hydrogen generation during microbial stationary phase, hydrogen yield dropped from 1.166 to 1.160 mol H₂/mol glucose from 44 h to 48 h.

Composition of volatile fatty acids formed during the fermentation process can be a good indicator of the microbial metabolic pathway. Thus, the formation of VFA as well as pH change during the hydrogen production process was examined in this study. As shown in Fig. 2.11, formic acid, acetic acid, and butyric acid were the main VFA detected during the fermentation process. In the first 12 h, little VFA was formed, consistent with little hydrogen production. Then, both of acetic acid and butyric acid showed significant increase from the 20th h, until the end of fermentation, concentration of formic acid, acetic acid, and butyric acid reached 0.44 g/L, 2.94 mg/L and 1.78 g/L, respectively. Acetic acid was the dominant VFA during the process, indicating that the hydrogen production process followed acetate-type fermentation (Yin and Wang 2016). With the accumulation of VFA, pH decreased from 7.0 to 4.42.

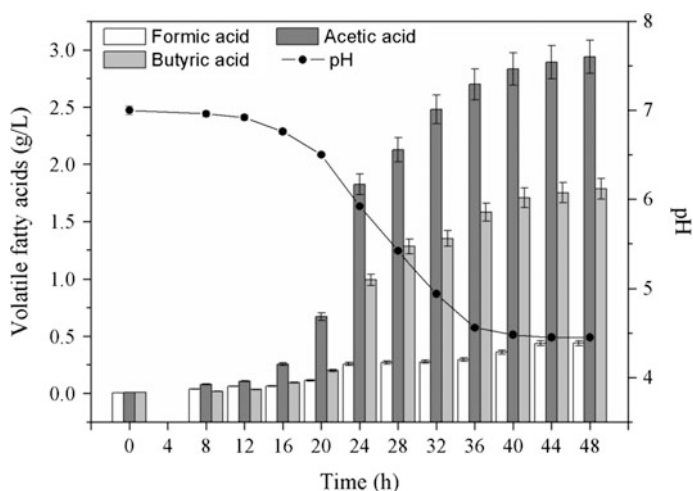


Fig. 2.11 Variation of volatile fatty acids (VFA) with time

2.3.2.6 Immobilization of *Enterococcus Faecium* INET2

Isolated *Enterococcus faecium* INET2 was enriched and centrifuged at 5000 r/min for 10 min, and then washed by 0.9% NaCl solution for 3 times before immobilization.

Polyvinyl alcohol (PVA, nominal degree of polymerization = 1750, approx. molecular weight 75,000–80,000) was dissolved in distilled water at 80 °C (10% w/v), and then sodium alginate was added and stirred until the mixture became homogenous (1% w/v). 15 mL of formed mixture was sterilized at 115 °C for 30 min, and then cooled to room temperature before being mixed thoroughly with 5 mL of microorganisms prepared previously. Then, the mixture was filled into a syringe, and dropped through a needle into saturate boric acid solution containing 2% w/v CaCl_2 to form spherical beads (about 3 mm in diameter). The formed beads were kept in the solution for 4 h to complete gelation process inside beads, and then the beads were washed by 0.9% NaCl solution for 3 times and kept at 4 °C until being used (Long et al. 2004).

Studies have found that immobilization of bacterial cells can help to relieve the end-product inhibition to biomass activity (Hawkes et al. 2002, 2005), protect microorganisms from the adverse impacts of hazardous materials existing in the substrate (Guo et al. 2008), and furthermore prevent the biomass washout from the system. Studies have figured out that PVA-sodium alginate beads possess both high activity and good mechanical properties, which is necessary for a long-term stable operation (Long et al. 2004; Zhang et al. 2007). Thus, PVA-sodium alginate was employed in this study to entrap anaerobic digested sludge for dark fermentative hydrogen production.

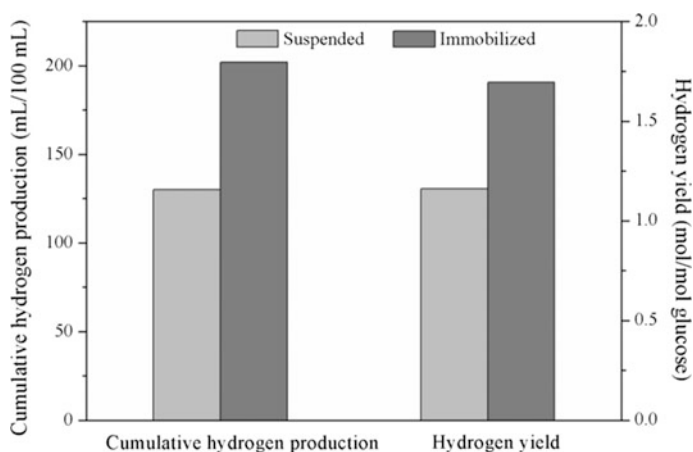


Fig. 2.12 Comparison of hydrogen production by immobilized and free cells

Hydrogen production by suspended and immobilized *Enterococcus faecium* INET2 were compared at optimized condition of 35 °C, initial pH of 7, substrate concentration of 15 g/L glucose, and 10% inoculation proportion. As shown in Fig. 2.12, the immobilized microorganisms established a better performance both in cumulative hydrogen production of 202 mL/100 mL and hydrogen yield of 1.69 mol H₂/mol glucose than suspended bacteria with 130 mL/100 mL and 1.16 mol H₂/mol glucose, respectively. Possible reason was that during the fermentation process, volatile fatty acids were formed which cause feedback inhibition to the microbes. However, immobilization of cells can reduce negative effects of metabolites and toxic substances in the liquid phase, thus enhancing the hydrogen production of system (Chu et al. 2011a, b).

2.4 Biochemistry of Hydrogen Production

2.4.1 Metabolic Pathways

Fermentative bacteria such as *Enterobacter* sp., *Bacillus* sp., and *Clostridium* sp. are capable of producing H₂ from carbohydrate-rich substrates in a dark environment. Among them, *Clostridium* sp have several advantages, for example, they have the highest H₂ yield (1.61–2.36 mol H₂/mol glucose); they are abundant in natural environments.

As shown in Fig. 2.13, *Clostridium* sp. have diverse liquid metabolites; some metabolites (acetate and butyrate) are related to H₂ production, and others are not.

Through the metabolism of bacteria present in the system, complex polymers are hydrolyzed to glucose. Subsequently, pyruvate is produced via the glycolytic

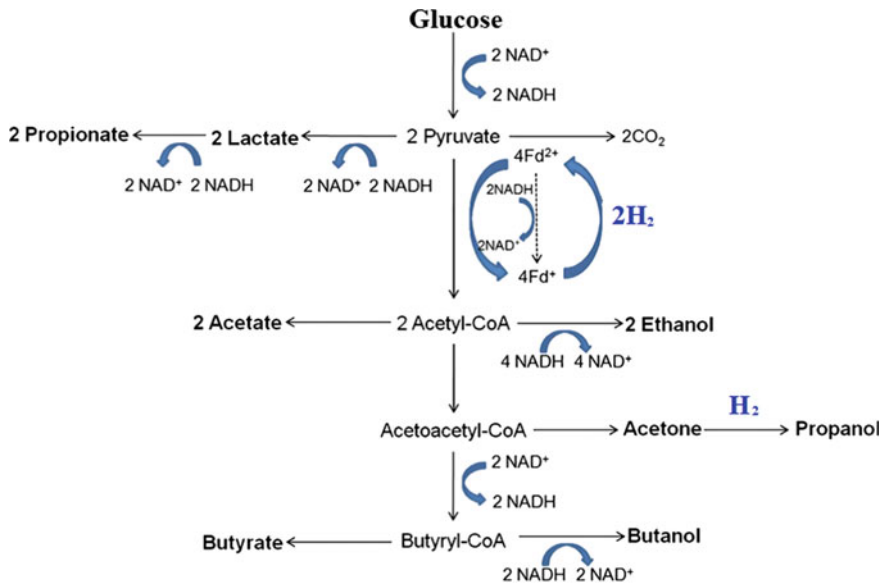
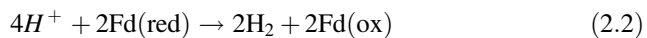


Fig. 2.13 Biological H₂ production mechanism in dark fermentation

pathway to generate adenosine triphosphate (ATP). And then, according to hydrogen-producing strains present in the system (obligate anaerobes like *Clostridia* or facultative anaerobic enteric bacteria like *E. coli.*), pyruvate is involved in two different biochemical reactions leading to the formation of hydrogen (Eqs. 2.1–2.4) (Bundhoo and Mohee 2016).



It is obvious that higher hydrogen yield can be attained through Eq. 1.1; different microbial distributions can lead to diverse hydrogen production efficiency. Studies have shown that over 2.6 mol H₂/mol hexose was obtained by genus *Clostridium* while no more than 2.0 mol H₂/mol hexose was achieved by genus *Enterobacter* and *Bacillus* (Harun et al. 2012; Junghare et al. 2012; Beckers et al. 2013; Sinha and Pandey 2014; Ortigueira et al. 2015).

Equation 1.1 mainly happens in hydrogen production by *Clostridium* sp. During this process, pyruvate is catalyzed by pyruvate dehydrogenase (PDH), and releases electrons and forms AcetylCoA. Then, with the function of Ferredoxin (FeFd), the released electrons are catalyzed by hydrogenase and united with H⁺, forms H₂.

AcetylCoA is further disintegrated into acetate and ethanol with the function of alcohol dehydrogenase (ADH) and acetate kinase (ACK).

Equation 1.2 mainly happens in hydrogen production by *Enterobacter* sp. During this process, pyruvate is catalyzed by pyruvate formate-lyase (PFL), and forms formate and AcetylCoA. Then, with the function of formate hydrogen lyase (FHL) and hydrogenase, formate is decomposed into H_2 and CO_2 .

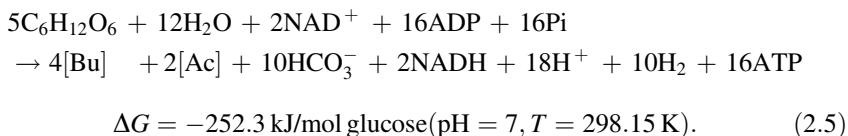
Besides, studies have found that some syntrophic acetogenic bacteria species are able to disintegrate the liquid metabolites like butyrate, propionate, and ethanol into hydrogen and acetate. However, syntrophic acetogenic bacteria species grow very slow, and the long growth cycle makes it hard for syntrophic acetogenic bacteria become dominant, especially in the systems with short hydraulic retention time.

2.4.2 Fermentation Types

Theoretically, 1 mol glucose can be converted into 12 mol H_2 . However, during the fermentation process, hydrogen production is accompanied with microbial growth and volatile fatty acid formation, leading to the maximal hydrogen yield with no more than 4 mol H_2 . Volatile fatty acids as important by-products in dark fermentation process, microbial metabolism pathways can be speculated from the composition of volatile fatty acids. According to the main volatile fatty acids, widely accepted fermentation types include butyrate-type fermentation, propionate-type fermentation, ethanol-type fermentation, and mixed-type fermentation.

2.4.2.1 Butyrate-Type Fermentation

Main volatile fatty acids for butyrate-type fermentation are butyrate acid and acetate acid. Take glucose as example, during the fermentation, glucose is degraded to pyruvate through the glycolytic pathway, and then, pyruvate is changed to AcetylCoA, H_2 , and CO_2 by the function of pyruvate dehydrogenase (PDH). Theoretically, ratio of formed acetate acid and butyrate acid is 2 (Eq. 2.5). Studies have found that butyrate-type fermentation usually happens in *Clostridium* sp.



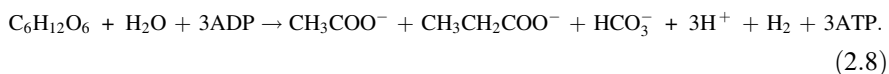
As shown in Eqs. 2.6 and 2.7, during the butyrate-type fermentation, the more acetate acid is formed, and higher hydrogen yield can be achieved. However, the accumulation of $NADH + H^+$ is accompanied with the formation of acetate acid,

leading to the significant decrease of pH. Thus, butyrate acid is usually formed in microbes to relieve the accumulation of $\text{NADH} + \text{H}^+$.



2.4.2.2 Propionate-Type Fermentation

Main volatile fatty acids for propionate-type fermentation are propionate acid and acetate acid. As shown in Eq. 2.8, glucose is degraded into acetate acid and propionate acid in the ratio of 1. It can be seen that only 1 mol H_2 is produced from 1 mol glucose in propionate-type fermentation. Thus, studies usually try to avoid the propionate-type fermentation through controlling the operational conditions:



2.4.2.3 Ethanol-Type Fermentation

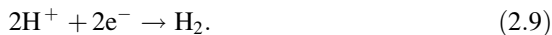
Main volatile fatty acids for ethanol-type fermentation are ethanol and acetate acid. Similar with butyrate-type fermentation, the formation of ethanol is also a way to balance the amount of $\text{NADH} + \text{H}^+$ formed in cells

2.4.2.4 Mixed-Type Fermentation

There are no significant characteristics of volatile fatty acids in mixed-type fermentation; it represents a state of the coexistence of various fermentation types. Mixed-type fermentation mainly happens at the start-up of fermentation process, since no significant dominant bacterial community is formed at the beginning. There is no theory of microbial metabolism for mixed-type fermentation; it is a representative of the uncertainty of fermentation process.

2.5 Enzymology of Hydrogen Production

The enzymes can greatly accelerate the rates of biochemical reactions. The key enzyme involved in catalyzing H_2 formation from protons or oxidation to protons is hydrogenase, which can catalyze the following reaction:



The above reaction is reversible, and its direction is dependent upon the redox potential of the components that are able to interact with hydrogenase.

In addition, nitrogenase, an enzyme that normally catalyzes the reduction of N_2 to ammonia, is able to reduce protons to H_2 as a by-product under photo-heterotrophic conditions. The knowledge of hydrogenase is essential for understanding the H_2 production mechanism, for controlling the metabolism of hydrogen-producing microorganisms, and for improving H_2 production (Kim and Kim 2011).

2.5.1 Classification of Hydrogenase

Nature has evolved plenty of hydrogenases, as shown in Fig. 2.14. Some of these hydrogenases are oxygen-sensitive, which can be irreversibly inactivated when exposed to oxygen; some of them are oxygen-resistant, they can be suppressed by oxygen but can be recovered in anaerobic condition; others are oxygen-tolerant, they are aerobically active and catalyze hydrogen oxidation. Some hydrogenases catalyze the reversible hydrogen oxidation and hydrogen formation, while others are only active in either hydrogen formation or hydrogen consumption. Some microorganisms own more than one hydrogenase, and each of them functions in different ways.

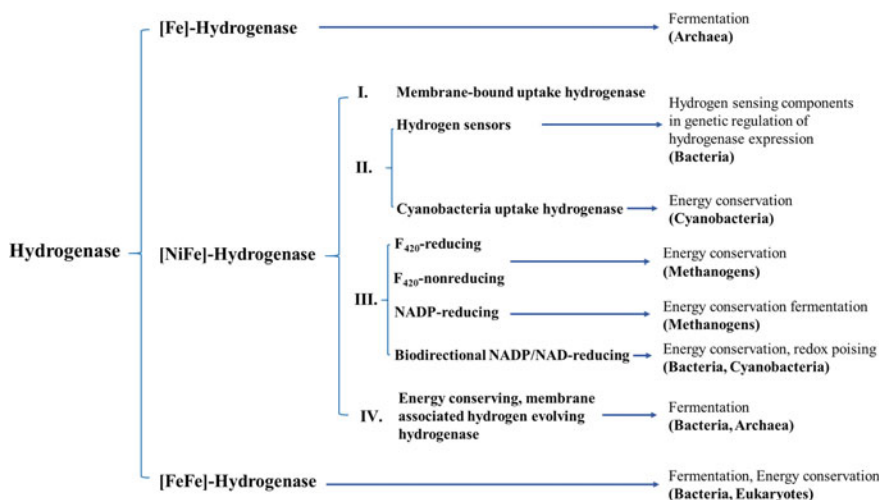


Fig. 2.14 Classification of hydrogenases

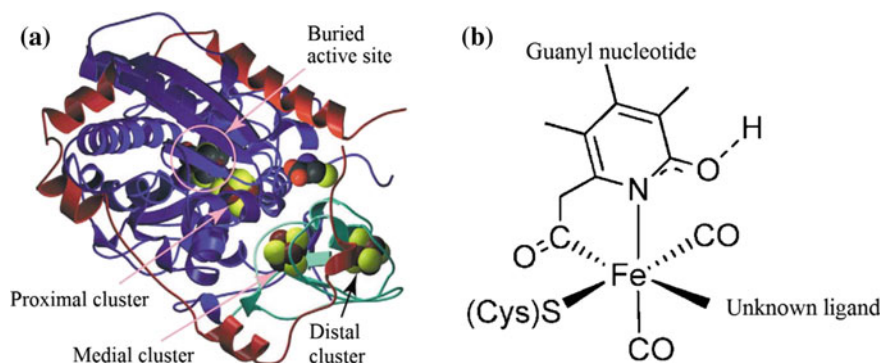


Fig. 2.15 **a** Schematic representation of the crystal structures [Fe]-hydrogenase from *Desulfovibrio desulfuricans* ATCC 7757 (Nicolet et al. 2002). **b** Structure of the active site of [Fe]-hydrogenase. (Chen et al. 2010)

According to the metal content of the active site, the hydrogenases can be categorized into three classes, [Fe]-, [FeFe]-, and [NiFe]-hydrogenases.

2.5.1.1 [Fe]-Hydrogenases

[Fe]-hydrogenase or H_2 -forming methylene-tetrahydromethanopterin dehydrogenase (Hmd) is also referred to as iron-sulfur-cluster-free hydrogenase for it is devoid of iron-sulfur clusters.

Figure 2.15a shows the structure of [Fe]-hydrogenase according to the current model; it can be seen that it contains three clusters and the active site is buried. Figure 2.15b shows the structure of the active site, in which the iron center is coordinated to a cysteine sulfur atom, two cis-CO ligands, a bidentate pyridone molecule through its nitrogen and acyl carbon atoms, and a yet unidentified ligand (Chen et al. 2010).

[Fe]-hydrogenase catalyzes the reversible reduction of methenyltetrahydromethanopterin (methenyl- H_4MPT^+) with H_2 to methylene- H_4MPT , which is an intermediate step in the reduction of CO_2 to methane by some methanogens. In the reaction, a hydride from H_2 is transferred into the pro-R position of the C (14a) methylene group of the reaction product (Schleucher et al. 1999). Figure 2.16 shows the reduction reaction of methenyl- H_4MPT^+ to methylene- H_4MPT .

2.5.1.2 [NiFe]-Hydrogenases

[NiFe]-hydrogenases catalyze the heterolytic cleavage of molecular hydrogen into two protons and two electrons. Besides, under sufficiently reducing conditions, they

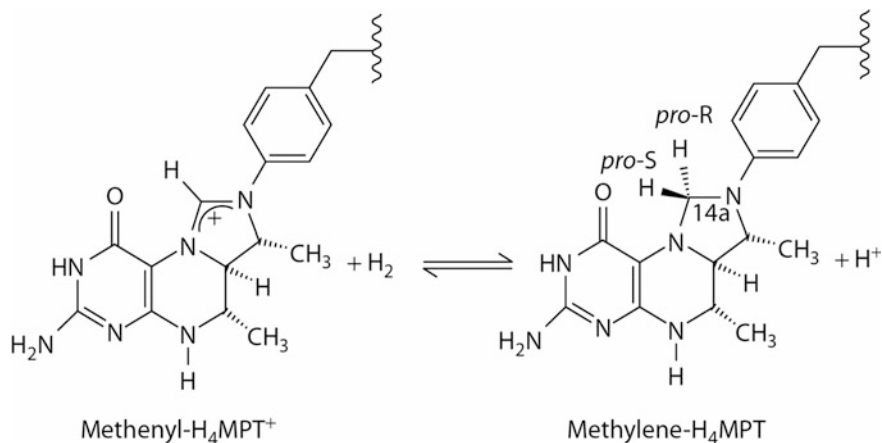


Fig. 2.16 The reversible reduction of methenyltetrahydromethanopterin (methenyl-H₄MPT⁺) with H₂ to methylene-H₄MPT catalyzed by [Fe] hydrogenase. (Vogt et al. 2008)

are also able to catalyze the production of hydrogen from two protons and two electrons. [NiFe]-hydrogenases are the most-studied classes of hydrogenases.

All [NiFe]-hydrogenases have a common heterodimeric core that resembles the first structure of the enzyme from *Desulfivibrio gigas* published by (Volbeda et al. 1995), as shown in Fig. 2.17a.

The active site of [NiFe]-hydrogenases is located in the hydrogenase large (L) subunit, which shows two strong peaks of Ni and Fe in the initial 2.85 Å resolution electron density map. Figure 2.17b shows the nickel-iron active site of *D.*

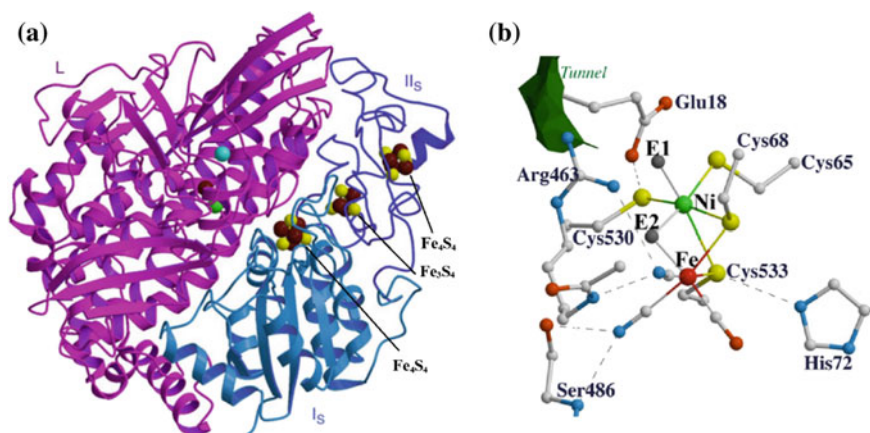


Fig. 2.17 A structure of *D. gigas* [NiFe]-hydrogenase. Arrows b-strands; Ribbons a-helices; spheres metal sites with color codes: Ni green, Fe red-brown, Mg cyan, S yellow. B-The nickel-iron active site (Fontecilla-Camps and Volbeda 2013)

gigas [NiFe]-hydrogenase. The active site contains two *cis* sites available for substrate binding: a bridging site between Fe and Ni, called E2, and a Ni-terminal one called E1. The small subunit is composed of two structural domains called I_S and II_S (Fig. 2.17a). Three FeS clusters are responsible for the transformation of electrons to and from the active site. I_S has a flavodoxin-like topology, and it binds [Fe₄S₄]; II_S lacks extensive secondary structure, and it binds the rest two FeS clusters: mesial [Fe₃S₄] and distal [Fe₄S₄]. All the remaining protein ligands to the FeS clusters are cysteine thiolates.

The active sites of [NiFe]-hydrogenases are buried in the protein. Consequently, electron and proton need to transfer between the catalytic center and the molecular surface. Thus, the consumption and generation of hydrogen also requires the molecular hydrogen access the active site or escape from it.

The oxygen tolerance of microorganisms determines their survival in aerobic environment, while the oxygen tolerance of hydrogenase determines the oxygen tolerance of microbial hydrogen production. Thus, lots of efforts have been made to understand the oxygen-tolerant hydrogenase, giving directions on molecular modification of hydrogen producers. Three typical structures have been identified responsible for the oxygen resistance of [NiFe]-hydrogenase:

(1) [NiFeSe]-hydrogenases

In some [NiFe]-hydrogenases, the mesial [Fe₃S₄] cluster is substituted by [Fe₄S₄] cluster, and one of the cysteine ligands of the Ni is replaced by a seleno-cysteine (SeCys). Then, it is named as [NiFeSe]-hydrogenases. The [NiFeSe]-hydrogenases attract attention not only for its higher catalytic activity than the [NiFe] enzymes but for its high oxygen tolerance (Baltazar et al. 2015). A possible reason for the less oxygen-sensitive is the presence of Se in the active site, which allows the transformation of hydrogen while obstructs oxygen.

(2) Hydrogen sensors related to [NiFe]-hydrogenases

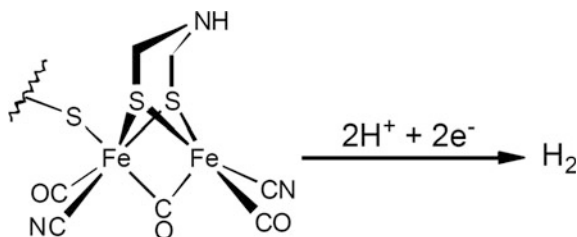
In bacteria like *Ralstonia eutropha* and *Rhodobacter capsulatus*, presence of hydrogen sensors limits the access of oxygen to active Ni–Fe site, leading to the oxygen resistance of [NiFe]-hydrogenases.

In those species, H₂-dependent transcription is directed by a signal transduction apparatus. The sensors related to [NiFe]-hydrogenases are responsible for the catalysis of hydrogen consumption, generation, and H–D exchange. Only the hydrogenases in reduced states are accessible to the sensors; thus, the oxidized hydrogenases are avoided from the sensors. Consequently, the sensors are insensitive to both oxygen and carbon monoxide.

(3) Oxygen-insensitive [NiFe]-hydrogenases

[NiFe]-hydrogenases connected to the respiratory chain in Knallgas bacterium *Ralstonia eutropha* shows high resistance to both oxygen and carbon monoxide. The enzymes connected [NiFe]-hydrogenases and respiratory chain include a b-type

Fig. 2.18 Active site biochemistry of [FeFe]-hydrogenase enzyme (Justice 2008)



cytochrome (MBH) and a cytoplasmic soluble one (SH), which are both oxygen-insensitive, and results in the oxygen resistance of [NiFe]-hydrogenases.

2.5.1.3 [FeFe]-Hydrogenases

[FeFe]-hydrogenases catalyze the interconversion of hydrogen with protons and electrons. The active site (H-cluster) is composed of a diiron core in a face-shared bioctahedral structure. H-cluster is linked to the FeS cluster, which is located at the N terminal of H-cluster and responsible for the electrons transformation to and from the active site (Fig. 2.18).

[FeFe]-hydrogenases can be categorized into two families:

- (1) Cytoplasmic, soluble, monomeric [FeFe]-hydrogenases. They are found in *Clostridium pasteurianum* (CpI hydrogenase) and *Megasphaera elsdenii* and they catalyze both hydrogen evolution and consumption. Take *Clostridium pasteurianum* as an example; during the anaerobic fermentation of organic matters, low-potential electrons are produced. Then, with the function of ferredoxin, the excess electrons are transferred to CpI hydrogenase, using protons as electron acceptors to generate hydrogen. These [FeFe]-hydrogenases are oxygen-sensitive, and can only be found in strict anaerobes.
- (2) Periplasmic, heterodimeric [FeFe]-hydrogenases. They are found in *Desulfovibrio* spp. and they mainly catalyze hydrogen oxidation. Periplasmic hydrogenases create electrons through the oxidation of hydrogen, and the electrons are transferred to the cytoplasm to reduce sulfate to sulfide or to generate reducing power for the cell.

2.5.2 Genetic Modification of Hydrogenase

To genetically and metabolically modify the hydrogenase is a very promising strategy to improve the biological hydrogen production from water or organic substances through optimizing the flow of reducing equivalents to it by redirecting the electron paths.

The deletion of the gene for H₂-uptake hydrogenase, the insertion of a gene for enzyme expression such as an overexpression or an increase of the efficiency of H₂-producing enzymes in microbial cells, and increase of the O₂ tolerance of hydrogenase will enhance the biohydrogen production.

2.5.2.1 Deletion of Hydrogen-Uptake Hydrogenase

The elimination of uptake hydrogenase, which re-oxidizes the produced hydrogen, is the main concern to achieve a satisfactory amount of hydrogen. In many studies, mutants deficient in genes for uptake hydrogenases showed an increased production of H₂ and H₂ production rate.

So far, significant research has been performed to inhibit the uptake hydrogenase activity using different approaches.

2.5.2.2 Genetic Insertion of an Enzyme to Facilitate Hydrogenase

The functional [FeFe]-hydrogenase from the strict anaerobe bacterium *Clostridium pasteurianum* was expressed in the cyanobacterium *Synechococcus* sp. to investigate the possibility for improving the hydrogen production capacity. The *Synechococcus* mutant demonstrated the possibility of introducing a foreign hydrogenase into the other species of microorganisms, resulting in a significant increase in hydrogen production capacity.

2.5.2.3 Oxygen Tolerance of Hydrogenase

Significant research has been conducted in an effort to increase the oxygen tolerance of H₂-producing enzymes, especially hydrogenases in a cyanobacterial system, by transferring the gene for O₂-tolerant NiFe-hydrogenase from *Thiocapsa roseopersicina* into cyanobacteria.

To improve the H₂ production from organic wastes using microorganisms, the contribution of the genetic engineering of enzymes responsible for H₂ production may be required to increase the efficiency of H₂ evolution. Further increases are expected by maximizing the H₂ production rate, in technical aspects, from the optimization of the biotechnology of the process.

2.5.3 Environmental Applications of Hydrogenase

Although the research on the application of hydrogenases has mostly focused on the biological hydrogen production, they have other environmental applications, such as for the bioremediation of contaminated environments. For example, hydrogenase

from *Thiocapsa roseopersicina*, due to its high hydrogen-producing activity and stability, can be used as an electrode together with electron donors and carriers in vitro systems, and in two-compartment fuel cells, to produce hydrogen, the results indicated that O_2 -tolerant hydrogenases have high potential for environmental application.

Hydrogenases have the potential to reduce halogenated pollutants such as tetrachloroethene to less chlorinated ethanes; NADPH is the cofactor that is required for the production of various types of oxidoreductase, and its formation can be catalyzed by hydrogenases. Tetrachloroethene is one of the most common contaminants in groundwater due to the presence of chlorinated compounds such as pesticides, solvents, and cooling agents. Several anaerobic bacteria can use this pollutant as terminal electron acceptor in a novel kind of respiration known as dehalorespiration. It is widely known that most of the tetrachloroethene-dehalorespiring organisms use hydrogen as electron donor. These organisms have a high affinity for hydrogen and can outcompete methanogens and homoacetogens for this substrate. For example, *Dehalobacter restrictus* and *Dehalococcoides ethenogenes* are capable of dechlorination using hydrogen as electron donor.

Besides dechlorination, hydrogenases are also known to be involved in reduction of toxic heavy metals from solution by efficient reduction to less soluble metal species. For example, the [Fe]-hydrogenase from the *Desulfovibrio vulgaris* strain has high

2.6 Microbial Modification

2.6.1 Co-cultivation

It has been confirmed that higher hydrogen yield can be obtained by pure cultures. However, with the application of complex organic wastes as substrate, mixed cultures show both higher hydrogen production and substrate degradation rate. This may be due to the biological interactions presents in mixed cultures. Since the biological community structure may be very complicate, and highly dependent on the consortium sources. Thus, the biological interactions are unclear, which may lead to poor hydrogen production effect.

To ensure a sustainable hydrogen production efficiency, synthetic microbial consortia is used. In this process, two or more known microbial populations with complementary metabolic activities are integrated. Studies have shown that the well-designed consortia will almost certainly outperform traditional monocultures (Bernstein and Carlson 2012). The discipline of synthetic microbial consortia has been widely used in medicine, food, and biofuel field. In the field of dark fermentative hydrogen production, synthetic microbial consortia can be categorized into three groups according to the functions.

2.6.1.1 Maintaining an Anaerobic Environment by Depleting Oxygen

As it is known that most hydrogenases are pretty sensitive to oxygen, the most widely used hydrogen producer *Clostridium* sp. are strict anaerobic, even the facultative anaerobes like *Enterobacter* sp. can only produce hydrogen at anaerobic environment. Thus, to maintain a sustainable operation of a hydrogen production system, it is necessary to ensure a strict anaerobic condition during the fermentation. However, with the addition of carbon sources and other nutrients, especially for the continuous mode operation, oxygen usually enters the system along with the feedstock, leading to the inhibition to hydrogen producers.

Thus, to avoid the oxygen shock, dissolved oxygen present in system need to be removed as soon as possible. Considering the industrially feasible operation, the presence of facultative anaerobes can be useful to maintain the anaerobic environment in the system. In this case, both hydrogen-producing facultative anaerobes and non-hydrogen-producing ones can be used.

For the hydrogen producers, *Enterobacter* sp., *Bacillus* sp., and *Klebsiella* sp. are co-cultured with *Clostridium* sp. to achieve a sustainable hydrogen production (Yokoi et al. 2002; Lu et al. 2007; Hung et al. 2011a, b). Besides the co-culture of pure strains, differently treated mixed cultures are also used. For example, Zhu and B  land (2006) found that heat-shock-treated sludge has little capacity to consume oxygen for only spore-forming bacteria survived. On the other side, aeration-treated mixed culture may lack high-efficient *Clostridium* sp. but rich in facultative anaerobic microbes. Thus, co-culture of heat and aeration-treated consortium can be a good choice.

2.6.1.2 Breakdown of Complex Organic Substrates

To achieve the dual benefits of energy generation and wastes management, real organic wastes are used as substrate. Thus, efforts on enhancing hydrogen production efficiency from complex organic matters are needed.

The most common and cheap organic wastes include agricultural wastes, municipal wastes, and various waste water. Macromolecules like cellulose, starch, and protein form the main components of these organic wastes. Then, the hydrolysis of these complex organic matters becomes the rate-limiting step in fermentative hydrogen production. Besides the commonly used pretreatment of wastes, strains that are efficient in hydrolyzing these macromolecules can be helpful in enhancing the hydrogen production process.

For example, when lignocellulosic wastes are used as substrate, carbon source mainly includes cellulose, cellobiose, and lignin. Zeidan and Van Niel (2009) examined the improvement of hydrogen production rate with the co-culture of *Caldicellulosiruptor saccharolyticus* and *Caldicellulosiruptor kristjanssonii*, for glucose and xylose can be simultaneously degraded. Adav et al. (2009) achieved 2.19 mol H₂/mol hexose from cellobiose with the co-culture of a cellobiose degrader *Enterococcus saccharolyticus* and hydrogen producer *C. butyricum*. Li

and Liu (2012) enhanced hydrogen yield by 94.1% from corn stalk through the co-culture of *Clostridium thermocellum* and *Clostridium thermosaccharolyticum*. Lu et al. (2009) identified diverse bacterial communities in hydrogen production from cornstalks, among which *Cytophagales* str., *Acetivibrio cellulolyticus* may be useful in degrading cellulose, while *Clostridium* sp. may be beneficial to hydrogen production. Nissilä et al. (2011) explored thermophilic hydrogen production from cellulose, and concluded that bacteria closely related to *Clostridium cellulosi* and *Clostridium stercoarium* were responsible for cellulose degradation, while bacterium closely related to *Thermoanaerobium thermosaccharolyticum* was the responsible for hydrogen production.

Besides lignocellulosic material, Yokoi et al. (2002) reported higher hydrogen production from starch by the co-culture of *C. butyricum* and *Enterobacter aerogenes*; Cheng et al. (2008) found that *Bifidobacterium* sp. broke down starch into small molecules, supplied simple sugars for *Clostridium* sp. for hydrogen production. Lay et al. (2010) found that the co-culture of *Clostridium* sp. and *Acidaminococcus* sp. can simultaneously consume the carbohydrates and monosodium glutamate present in condensed molasses, thereby enhancing hydrogen productivity.

2.6.2 Microbial Immobilization

Microbial immobilization is defined as a technique used for the physical or chemical fixation of microbial cells, organelles, enzymes, or other proteins (e.g., monoclonal antibodies) onto a solid support, into a solid matrix, or retained by a membrane, in order to increase their stability and facilitate their repeated or continued use.

Microbial immobilization can improve microbial cells or enzyme applications. This method, based on the fixation of the biocatalyst into or onto various materials, may increase robustness of the biocatalyst, allows its reuse, or improves the product yield. In recent decades, a number of immobilization techniques have been developed. They can be classified according to the used natural or synthetic material and principle of biocatalyst fixation in the particle.

The advantages of immobilization include easy separation of the biocatalyst, which allows particles reuse in repeated and continuous processes, protection of the attached biocatalyst against environmental effects, higher yields and productivity due to an increased concentration of the biocatalyst, as well as better process and storage stability. Moreover, immobilized biocatalysts have lower sensitivity to contamination, allowing in some cases non-sterile conditions.

There are four methods for microbial cell immobilization, i.e., entrapment, adsorption, aggregation, and confinement.

In entrapment method, microbial cells can be immobilized in three-dimensional matrices such as an electro-polymerized film and network. This immobilization is easy to perform. Immobilized cells based on physical entrapment are often

characterized by increased operational and storage stability. However, limitations such as the possible diffusion barriers can restrict the performances of the systems.

Microbial adsorption onto solid supports represents the easiest method of physical immobilization. The adsorption mechanisms are based on weak bonds such as Van der Waal's forces and electrostatic and/or hydrophobic interactions. This technique does not involve any functionalization of the support and is generally non-destructive for microbial activity. Although this immobilization method causes little or no microbial inactivation, this technique presents drawbacks: microbial cells are loosely bound to the support and desorption of the cells appears to be the main problem.

Wu et al. (2013) investigated the effect of different aspect ratios, height (H) to diameter (D) of 1:1, 3:1 and 5:1, of a CSTR with immobilized anaerobic sludge on hydrogen (H_2) production in order to overcome bacterial washout frequently occurs in the traditional continuous stirred tank reactor (CSTR) systems at low hydraulic retention time (HRT). They immobilized thermally treated sludge by silicone gel entrapment approach. The entrapped-sludge system operated stably at a low HRT without suffering from cell washout. Hence, the hydrogen production rate (HPR) was enhanced by increasing organic loading rates.

Han et al. (2015) developed a continuous mixed immobilized sludge reactor (CMISR) using activated carbon as support carrier for dark fermentative hydrogen production from enzymatic hydrolyzed food waste. They examined the effect of immobilized sludge packing ratio (10–20%, v/v) and substrate loading rate (OLR) (8–40 kg/m³/d) on biohydrogen production. They found that the hydrogen production rates (HPRs) with packing ratio of 15% were significantly higher than the results obtained from packing ratio of 10 and 20%.

Sun et al. (2016) developed an up-flow anaerobic sludge bed (UASB) system with sludge immobilized on granular activated carbon for continuous fermentative hydrogen production from herbal medicine wastewater at various organic loading rates.

2.6.3 Metabolic Engineering

Metabolic engineering uses systematic and quantitative analysis of pathways, and molecular biology and genomic approaches, to modify metabolic pathways to increase the biological hydrogen production. Metabolic engineering could be used to overcome limiting factors for biohydrogen production in various systems by increasing the flow of electrons to hydrogen-producing pathways, increasing substrate utilization, and engineering more efficient and/or oxygen-resistant hydrogen-evolving enzymes (Abo-Hashesh et al. 2011). In terms of dark fermentation, metabolic engineering could be used at several different levels for process improvement (Fig. 2.19).

The biofuels production scheme that relies on fixed carbon substrates can be divided into two levels: acquisition and conversion of complex substrates to key

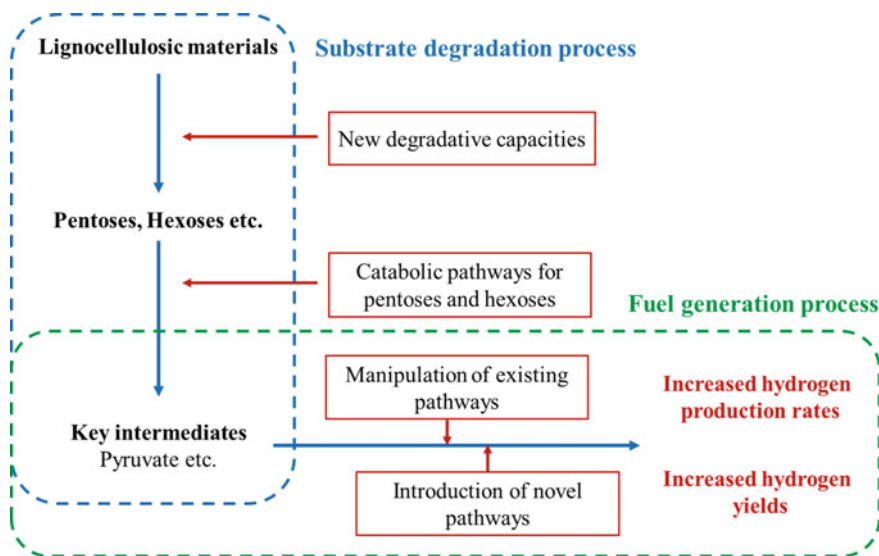


Fig. 2.19 Roles for metabolic engineering in dark fermentative hydrogen production

metabolic intermediates, and conversion of key metabolic intermediates to the desired biofuel. Metabolic engineering can play a role in several different ways:

- (1) to add pathways to an organism, enabling it to directly use a wider range of complex substrates;
- (2) to add pathways permitting the conversion of a wider range of monomers to key metabolic intermediates;
- (3) to boost production of a biofuel that is naturally produced by the organism; and
- (4) to add pathways leading to the production of a novel biofuel.

For the biological hydrogen production, metabolic engineering can be used to extend the range of substrates used by a given hydrogen-producing microorganism, necessary in many cases, if abundant lignocellulosic substrates are to be used as feedstock. Thus, microorganisms could be given the capacity to directly degrade lignocellulosic substrates, or to use the mixture of pentoses and hexoses available after enzymatic conversion of this feedstock. Finally, metabolic engineering can be used to increase the rates and/or yields of hydrogen production once the soluble sugars are converted to pyruvate, the key intermediate.

Two approaches could be taken, modification of the existing pathways, or introduction of novel hydrogen-producing pathways. A variety of tools for achieving these types of modifications are now available.

Das et al. (2001) studied the redirection of biochemical pathways for the enhancement of H_2 production by *Enterobacter cloacae*. *E. cloacae* IIT-BT 08 produces H_2 at a higher rate and yield using different carbon sources as substrate, but it was still low for commercial application. They attempted to redirect the

biochemical pathways for further improvement of the process by blocking alcohol and some of the organic acids formation in *E. cloacae* IIT-BT 08 during their metabolism because NADH is usually generated by catabolism of glucose to pyruvate through glycolysis. The conversion of pyruvate to ethanol, butanediol, lactic acid, and butyric acid involves oxidation of NADH. The concentration of NADH would be increased if the formation of these metabolites could be blocked, thus enhancing H₂ production through the oxidation of NADH.

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