Green Energy and Technology

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Biohydrogen Production from Organic Wastes

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Preface

The environment pollution and energy crisis are two vital issues for sustainable development worldwide. The use of fossil fuels (petroleum, natural gas, and coal) is considered as the cause of serious environmental problems.

With the increasing demand of energy and depleting reserves of conventional fossil fuels, there has been growing global interest in developing alternative sources of energy. Although hydrogen (H_2) is not a primary energy source, it has been considered a promising candidate as a substitute for fossil fuels because it has the potential to eliminate most of the problems caused by the fossil fuels.

From the perspective of energy source, hydrogen can be produced from organic wastes (agricultural wastes, municipal wastes, algal biomass, etc.), renewable energy (solar, wind, etc.), fossil fuel (coal, oil, natural gas, etc.), and nuclear energy. Different technologies have been used for hydrogen production, such as direct thermal, thermochemical (such as hydrocarbon reforming and coal gasification), electrochemical, and biological. Among the hydrogen production methods, hydrogen production using biological processes is new, innovative, and potentially more efficient, which has been broadly studied for its mild reaction condition and high potential environmental benefits.

Biohydrogen production is performed by hydrogen-producing microorganisms at ambient temperature and pressure. Microorganisms can recover and concentrate the energy from aqueous organic wastes, such as industrial wastewater and sludge. Biohydrogen can be produced from the biophotolysis of water using algae and cyanobacteria, the photo-decomposition of organic compounds by photosynthetic bacteria, and the dark fermentation from organic compounds with anaerobic bacteria. Among these biological processes, dark fermentation is more favorable than photo-dependent hydrogen production for its independency of light, generally high rate of hydrogen generation, simple reactor as well as easy control. In particular considering the wide range of substrates, dual benefits of clean energy generation and organic wastes management can be achieved, since hydrogen is produced from various organic wastes and wastewater enriched with carbohydrates as the substrate, decreasing the cost for hydrogen production. Thus, fermentative hydrogen

production is widely accepted as a more feasible biohydrogen production way, and gained widespread interest and attention.

Biological hydrogen production processes offer a technique through which renewable energy sources like biomass can be utilized for the generation of the cleanest energy carrier. Hydrogen-intensive research work has already been carried out on the advancement of these processes, such as the development of genetically modified microorganism, metabolic engineering, improvement of the reactor designs, use of different solid matrices for the immobilization of whole cells, biochemical-assisted bioreactor, and development of two-stage processes for higher hydrogen production rate.

The present book provides the state-of-the-art information on the status of the biohydrogen production from various organic wastes. This book has eight chapters, including the microbiology, biochemistry and enzymology of biohydrogen production, the enrichment of hydrogen-producing microorganisms, the pretreatment of various organic wastes for hydrogen production, the influence of different physicochemical factors on hydrogen production, the kinetic models and simulation of biological process of fermentative hydrogen production, the optimization of biological hydrogen production process, and the fermentative hydrogen production from sewage sludge.

The text in all the chapters is supported by numerous clear, informative figures and tables. To our knowledge, this book is a first attempt to describe the biological hydrogen production from various organic wastes, which is aimed at a wide range of readers, mainly including undergraduates, postgraduates, energy researchers engineers, and others who are interested in hydrogen production in general and biological hydrogen production in particular, as well as to industrial concerns that are looking for inexpensive hydrogen production technologies.

We warmly thank Dr. Mengchu Huang and Dr. Sivajothi Ganesarathinam, and the team of Springer Nature for their cooperation and efforts in producing this book.

We hope readers will find this book interesting and informative for their research pursuits.

Beijing, China

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Dr. Yanan Yin is currently postdoctorate researcher at Institute of Nuclear and New Energy Technology, Tsinghua University, Beijing. She received her Ph.D. degree in Environmental Engineering from Tsinghua University in 2016. Her research is focused on the environmental protection and new energy technology, such as biohydrogen production from different kinds of organic wastes. She has published more than 20 peer-reviewed journal papers.

Chapter 1
Introduction

1.1 Global Energy Development

World economic growth and rapid urbanization require more energy to fuel the increased level of activity. It has been predicted that energy consumption will increase by 35% from 2014 to 2035, and fossil fuels account for around 80% of total energy supply (BP 2016a). As shown in Fig. 1.1a, world primary energy consumption increased from around 9400 million tons oil in 2000 to over 13000 million tons oil in 2015. By 2035, fossil fuels remain the dominant source of energy powering the global economy, providing around 60% of the growth in energy and accounting for almost 80% of total energy supply. Presently, the utilization of fossil fuels are causing global climate change mainly due to the emission of pollutants like CO_x; NO_x; SO_x; C_xH_x, soot, ash, droplets of tars, and other organic compounds. A series of environmental problems have been caused by the continuous heavy dependence on fossil fuels, like global warming, air pollution and acid rain, and so on. The increase in Greenhouse Gas (GHG) emissions was estimated to have increased by 80% from 1970 to 2004 (Li and Lin 2015) and a further increase of 52% between 2005 and 2050 was predicted (De Boeck et al. 2015). Considering the far-reaching effect on world climate change, environmental problems are seriously affecting the sustainable development of human beings. Measurements must be taken to deal with the problems at the fundamental level.

On the other side, the reserves of primary energy can hardly meet the quick increasing demand. By the end of 2015, the increase of explored fossil fuel reserves was much lower than the increase of production (BP 2016b). Primary energy crisis forces people to search for renewable energy candidates.

Thus, to solve the environmental degradation and energy crisis, exploration of clean and renewable energy alternatives is extremely urgent. Quite a few renewable energy sources have been explored, like solar energy, wind energy, hydropower, and biomass. Solar energy is one of the most abundant energy resources on the surface of the earth. However, solar energy has a tiny contribution in the world's total primary

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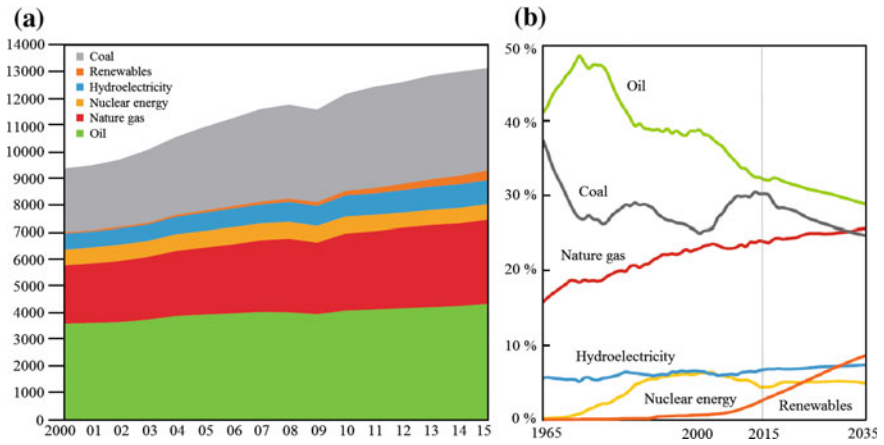

Fig. 1.1 Situation of world energy consumption. **a** World energy consumption equivalent with million tons oil (Adapted from (BP 2016b)); **b** Shares of various energy sources (Adapted from (BP 2016a))

energy supply of less than 1% (Demirbas et al. 2017). Wind energy has been used for centuries to power windmills to mill wheat or pump water. The wind energy sector is one of the fastest growing energy sectors in the world: wind power generation capacity has increased from about 24.3–337 GW in the last 50–60 years (World Wind Energy Agency 2014). The water in rivers and streams can be captured and turned into hydropower. Large-scale hydropower provides about one-quarter of the world's total electricity supply. The technically usable world's potential of large-scale hydropower is estimated to be over 2,200 GW, of which only about 25% is currently exploited (Demirbas et al. 2017). Biomass resources include various natural and derived materials. Biomass can be turned to energy through three ways: it can be burned to produce heat and electricity; changed to gas-like fuels (methane, hydrogen, carbon monoxide, etc.); and converted to a liquid fuel (alcohols, volatile fatty acids, etc.). Besides the above-mentioned renewable energy, other energy sources like tide energy, geothermal energy, etc., are also explored. There still remains great potential in the further development of renewable energy. It has been predicted that the share of primary energy will decrease gradually in the future and renewable energy will rise from around 3% today to 9% in next 20 years (Fig. 1.1b), supplying one-third of the growth in power generation (BP 2016a). According to International Renewable Energy Agency (IRENA) with policies in place and under consideration today, the global penetration of modern renewable energy will reach 14% of total final energy consumption (TFEC) by 2030 (International Renewable Energy Agency (IRENA) 2014).

However, energy sources like solar, wind and ocean projects have limited life-times and if applied globally might consume a remarkable share of construction materials (Budzianowski and Postawa 2017). The facilities for energy collection are usually in remote regions, and energy supply is heavily dependent on climate,

leading to the high cost of energy storage and transportation. Furthermore, some negative effects on environment are also exist in developing solar, wind energy and hydropower, mainly include the water and air pollution caused in equipment manufacture, ecological environmental impact caused in energy generation process (Demirbas et al. 2017).

By contrast, biogas production from biomass owns several advantages in further application: (1) dual benefits of energy generation and organic wastes management; (2) easy storage and transportation; (3) Infrastructures for natural gas are available; (4) little negative impacts on environment throughout the production and utilization process. And among methane, hydrogen and carbon monoxide, hydrogen is considered as the most potential candidate.

1.2 Hydrogen Energy

Hydrogen is the most plentiful element in the universe, making up about three-quarters of all the matters (Das and Veziroğlu 2001). The atmosphere contains about 0.07% hydrogen. At standard temperature and pressure, hydrogen is a colorless, odorless, tasteless, nontoxic, diatomic gas. While on the earth's surface, most of the hydrogen exists in molecular forms such as water or organic compounds, occupies around 0.14% of all the compounds.

Hydrogen has extensive applications, such as:

(1) Hydrogen is an important raw material in chemical industries.
Over 60% of hydrogen produced all over the world is used in ammonia production. Hydrogen is also used as a hydrogenating agent in increasing the level of saturation of unsaturated fats, oils, and alcohol production.
(2) Hydrogen is an important raw material in petroleum and coal industry.
Hydrogen is used in upgrading the fossil fuels. For example, the hydrodealkylation, hydrodesulfurization, and hydrocracking process in the petrochemical plant; coal gasification; desulfurization and denitrification of fossil fuels, etc.
(3) Hydrogen has wide applications in engineering.
Hydrogen is used as a shielding gas in welding process, as the rotor coolant in electrical generators and so on.
(4) Hydrogen is a potential energy carrier.
With a pretty high energy density of 142 kJ/g, hydrogen has been used to store energy from wind, solar, nuclear, and so on.
(5) Hydrogen is an ideal fuel.
Hydrogen is highly flammable and can burn in air at a very wide range of concentrations between 4 and 75% by volume. The enthalpy of combustion is −286 kJ/mol, and the combustion forms only water.

$$H_2 + \frac{1}{2}O_2 \rightarrow H_2O \quad \Delta G = -286\,\mathrm{kJ/mol} \tag{1.1}$$

From the evolution of fuel, mankind's fuels have continually evolved as better, more efficient, safer, and cleaner fuels. From wood, to animal fat, to coal, to petroleum, to natural gas, shows a clear trend to lighter and cleaner fuels, and low-carbon energy becomes more and more preferable (Das 2009). Hydrogen, as the only carbon-free energy, is the direction of energy evolution.

Hydrogen owns several benefits as a promising fuel candidate, for example:

(1) it has the highest energy yield of 142.35 kJ/g;
(2) it is totally clean with water as sole product;
(3) it is easy for the storage and transportation;
(4) the rapid development of fuel cell technology further facilitates the application of hydrogen in engines and electric power system.

Hydrogen is universally accepted as an environmentally safe, renewable energy resource, and an ideal alternative to fossil fuels.

1.3 Hydrogen Production

Hydrogen can be produced from many ways. From the perspective of energy source, hydrogen can be produced from biomass (agricultural wastes, municipal wastes, algae, etc.), renewable energy (solar, wind, etc.), fossil fuel (coal, oil, natural gas, etc.) and nuclear energy (Fig. 1.2).

Hydrogen production methods mainly include four categories:

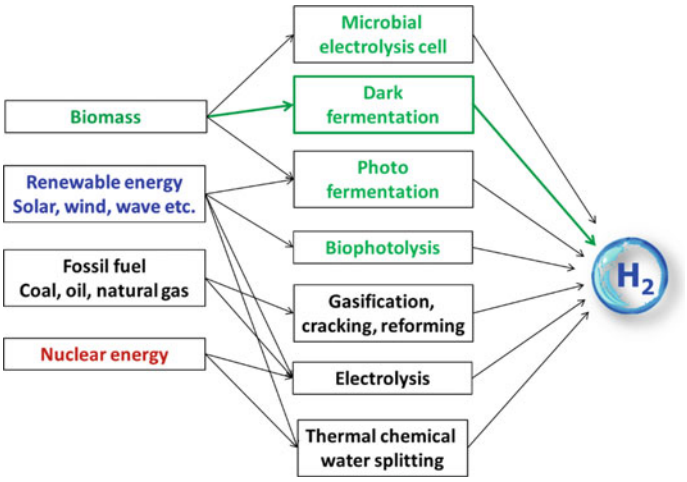

Fig. 1.2 Sources and methods of hydrogen production

1.3.1 Steam Reforming

At present, steam reforming is the most widely and commercially used method in hydrogen production. In the steam reforming of natural gas, steam (water vapor) reacts with methane at high temperatures (700–1100 °C), following reactions happen (Oxtoby et al. 2015):

$$CH_4 + H_2O \rightarrow CO + 3H_2 \tag{1.2}$$

$$CH_4 \rightarrow C + 2H_2 \tag{1.3}$$

$$CO + H_2O \rightarrow CO_2 + H_2. \tag{1.4}$$

Besides steam reforming of natural gas, hydrogen is also produced through the partial oxidation of hydrocarbons (Williams 1968) and coal reaction (Oxtoby et al. 2015):

$$2CH_4 + O_2 \rightarrow 2CO + 4H_2 \tag{1.5}$$

$$C + H_2O \rightarrow CO + H_2 \tag{1.6}$$

1.3.2 Electrolysis

Hydrogen can be produced from the electrolysis of water:

$$2H_2O(l) \rightarrow 2H_2(g) + O_2(g) \quad E_0 = +1.229\,\mathrm{V} \tag{1.7}$$

A DC electrical power source is connected to two electrodes placed in the water. Hydrogen will appear at the cathode, and oxygen will appear at the anode. Assuming an ideal state, the amount of hydrogen generated is twice the amount of oxygen, and proportional to the total electrical charge conducted by the solution. Considering the electrical conductivity, electrolysis of pure water requires excess energy in the form of over potential to overcome the activation barriers. Thus, studies usually increase the efficiency of electrolysis through the addition of an electrolyte (such as a salt, acid, base, etc.) and the use of electrocatalysts.

Currently, about 4% of hydrogen gas produced worldwide is generated by electrolysis, and normally used onsite. Electricity used may come from fossil energy, nuclear energy, and renewable. Especially for wind and solar, hydrogen is used as energy storage media to match the variation of production and demand.

1.3.3 Thermal Chemical

Thermal chemical hydrogen production mainly refers to the thermal decomposition of water.

At elevated temperatures, water molecules can split into hydrogen and oxygen. Thermal water splitting has been investigated for hydrogen production since the 1960s (Funk 2001). As the temperatures needed to obtain substantial amounts of hydrogen is pretty high (over 3000 °C), requirements on the materials used are very severe, which inhibited the direct application of thermal chemical methods in hydrogen production. However, the development of catalysts effectually reduces the temperature required and makes thermal chemical hydrogen production focus in the area of the catalysis and thermochemical cycles. Among the available thermochemical cycles, iodine–sulfur cycle, copper–chlorine cycle, iron oxide cycle, cerium (IV) oxide–cerium (III) oxide cycle, zinc zinc-oxide cycle, and hybrid sulfur cycle are commonly studied. Both solar energy and nuclear energy are used as heat source for thermal chemical hydrogen production.

For the hydrogen production from nuclear plant, an important advantage of hydrogen production from nuclear energy is that a nuclear reactor can shift between producing electricity and hydrogen, which can both match the electrical demand variation and achieve excess energy storage. Besides nuclear energy, the high temperatures needed to split water can be achieved through the use of concentrating solar power. Material constraints due to the required high temperatures are reduced by the design of a membrane reactor with simultaneous extraction of hydrogen and oxygen. Thermal chemical hydrogen production from solar energy is completely clean. With concentrated sunlight as heat source and only water in the reaction chamber, the produced gases are very clean with the only possible contaminant being water.

1.3.4 Biological

Biological hydrogen production is defined as hydrogen produced through the metabolism of microorganisms, mainly including algae, bacteria, and archaea.

Based on the different energy sources, biological hydrogen production can be categorized as photo-dependent (biophotolysis and photo fermentation) and photo-independent (dark fermentation). Figure 1.3 shows the overview of biological hydrogen production processes.

1.3.4.1 Biophotolysis

Producing hydrogen through biophotolysis of water catches researchers' high attention for it shows the potential to generate a clean fuel from water and light,

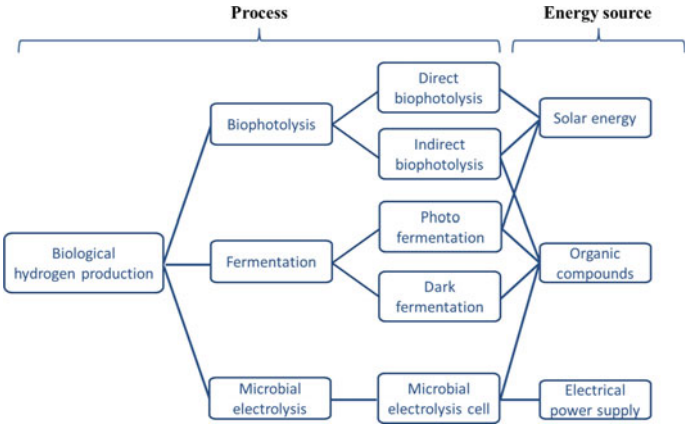

Fig. 1.3 Overview of biological hydrogen production processes

which is plentiful in the nature. It can be achieved by either isolated cellular components or algae cultures, and studies of microorganisms that produce hydrogen through biophotolysis of water have been focused on green algae and cyanobacteria.

In the green algae, energy of sunlight in photosynthesis is used to extract electrons from water, generating O_2 on the oxidizing side of photosystem II(PSII), and producing H_2 on the reducing side of photosystem I(PSI) (Fig. 1.4). However, since the reversible hydrogenase is sensitive to O_2 and the production of O_2 seriously inhibits H_2 production, it extremely restricted the application of H_2 production by biophotolysis process (Gaffron 1942). Until in 1998, Wykoff et al. (1998) found that sulfur starvation can dramatically limit O_2 generation in PSII. This breakthrough discovery leads people to turn to the indirect biophotolysis, in which H_2O oxidation and hydrogen generation is spatially or temporally separated.

As to the cyanobacteria, there are three pathways of producing hydrogen. The first pathway is catalyzed by [MoFe]-nitrogenase and can only be found in nitrogen-fixing cyanobacteria, this way is energetically inefficient for it requires two ATP molecules for per electron to be transferred. Furthermore, uptake [NiFe]-hydrogenases also take part in this process to consume some hydrogen generated. In the second pathway, water acts as electron donor like in algae, but as cyanobacteria do not depend on Fd- as the sole electron donor, the efficiency of hydrogen production in this way is higher than in algae. In the third way, external carbon is required to metabolize in the presence of light. Hydrogen is produced from exogenous carbohydrates, and the reaction is catalyzed by nitrogenase or hydrogenase.

In theory, biophotolysis would only be limited by the inherent maximal efficiency of photosynthesis which can convert up to 33% of absorbed light into chemical energy. Since somewhat less than half the sunlight energy is in the visible,

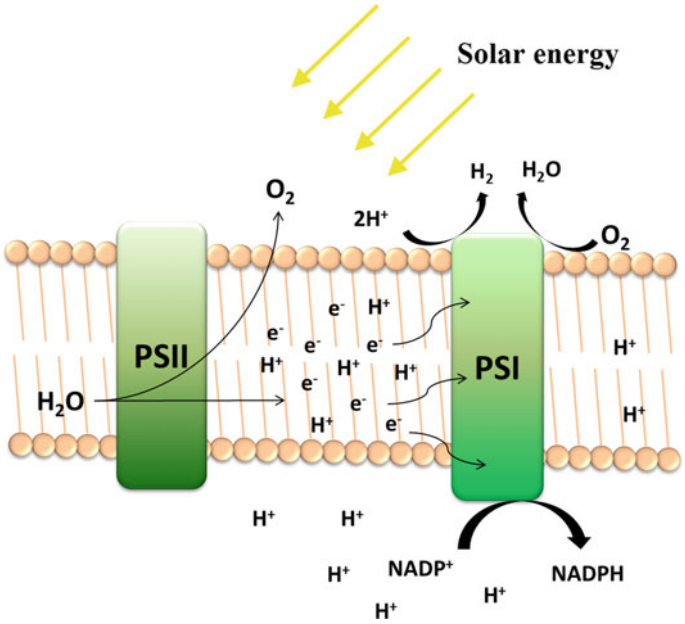

Fig. 1.4 Water is photosynthetically oxidized on PSII, producing O_2, H^+ and electrons, then the electrons are transferred to PSI, and finally go into three ways: the formation of H_2, the reproduction of H_2O with O_2 and the synthesis of NADPH

photosynthetically active region and since there are unavoidable losses, the theoretical maximal efficiency for photosynthesis is generally given as about 6%.

At present, development of practical biophotolysis systems is limited by the low efficiency of photosynthesis, lack of scientific knowledge and economic constraints.

1.3.4.2 Photofermentation

The concept of photofermentation was initially proposed by Benemann et al. (Benemann et al. 1973). Photofermentative bacteria essentially belong to two groups: Purple and Green. The purple bacteria can be further subdivided into purple sulfur (e.g., *Chromatium*) and purple nonsulfur bacteria (*Rhodobacter*), while the green bacteria are further subdivided into green sulfur (e.g., *Chlorobium*) and gliding bacteria (e.g., *Chloroflexus*). These photofermentative bacteria have evolved light-harvesting complexes akin to photosynthetic organism. Light energy is converted to chemical energy via photophosphorylation.

As shown in Fig. 1.5, take purple sulfur bacteria as example. Sulfide or organic substrate is used as electron donors, and hydrogen production is accompanied with

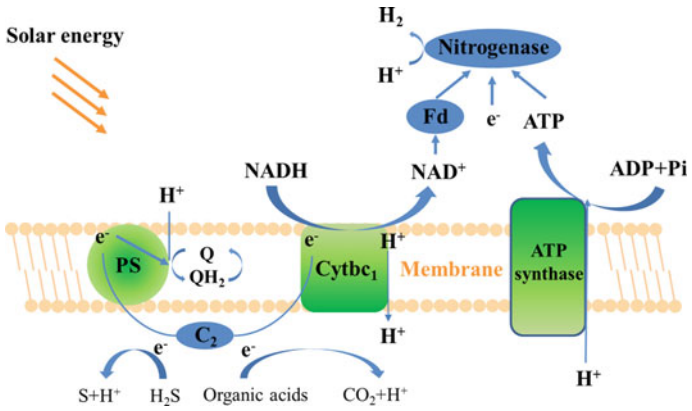

Fig. 1.5 Hydrogen production in purple sulfur bacteria (Modified from Das et al. (2014))

inorganic (sulfur-containing compounds) or organic substrate-driven reserve electron flow. NAD^+ is reduced to NADH through a reversed electron flow. Then, electrons are transferred toward nitrogenase via ferredoxin, ATP is consumed and hydrogen is produced. 1 mol hydrogen production through nitrogenase requires 4 ATP consumption, which is pretty expensive for microbes.

As to the purple nonsulfur bacteria, hydrogen is produced to supply electron for photosynthesis, in which process CO_2 is fixed via Calvin cycle and oxygen is formed as terminal electron acceptor. However, the presence of oxygen is toxic to enzyme nitrogenase, and hydrogen formation is suppressed consequently. Thus, to produce hydrogen using purple nonsulfur bacteria, absence of oxygen is necessary. In this case, light energy is only used for ATP generation, and electrons are obtained from the oxidation of organic substrate. Then, under nitrogen starvation, nitrogenase catalyzes the formation of molecular hydrogen from protons instead of NH_3. The overall reaction is stated as follows:

$$CH_3COOH + 2H_2O \rightarrow 4H_2 + 2CO_2 \tag{1.8}$$

Similar to purple nonsulfur bacteria, green sulfur bacteria can fix nitrogen by the enzyme nitrogenase, and hydrogen can be production at limited N_2 conditions. For the green gliding bacteria, the metabolic pathways are not completely known yet, and very few studies have been conducted for the hydrogen production from gliding bacteria.

Photo fermentative hydrogen production appears promising because of the possibility of achieve hydrogen production from free solar light and organic wastes. However, its application is still far from being practical, low light conversion efficiencies, low hydrogen production rate, and the high-cost photo bioreactors, etc., a lot of work is needed in enhancing hydrogen production rate and light absorption efficiency.

1.3.4.3 Dark Fermentation

Dark fermentation is more favorable than photo-dependent hydrogen production for its independency of light, generally high rate of hydrogen generation, simple reactor as well as easy control. Especially considering the wide range of substrate, dual benefits of clean energy generation and organic wastes management can be achieved. Thus, fermentative hydrogen production is widely accepted as a more feasible biohydrogen production way, and gained widespread interest and attention in recent years.

Metabolic pathways leading to hydrogen production from organic wastes are shown in Fig. 1.6. Taking the catabolism of carbohydrate as example, pyruvate is formed from the glycolytic pathway. Then, pyruvate can then be further converted to acetyl coenzyme A (acetyl-CoA), carbon dioxide, and hydrogen. Acetyl-CoA is a central intermediate, and it can be dissimilated into a variety of soluble metabolites, like acetate, butyrate, and ethanol and so on.

Although dark fermentation is more feasible than biophotolysis and photo fermentation, the low hydrogen yield and substrate degradation rate still barriers its application. Thus, breakthroughs are needed in process optimization, expansion and so on.

1.3.4.4 Microbial Electrolysis

Bioelectrochemical systems involve the use of exoelectrogenic microbes to produce current in conjunction with the oxidation of reduced compounds. This current can be used directly for power in a microbial fuel cell (MFC). Then, by exploiting the low redox potential produced by exoelectrogens at the anode, cathodic proton reduction can be accomplished with a little extra power supply (PS). This system is

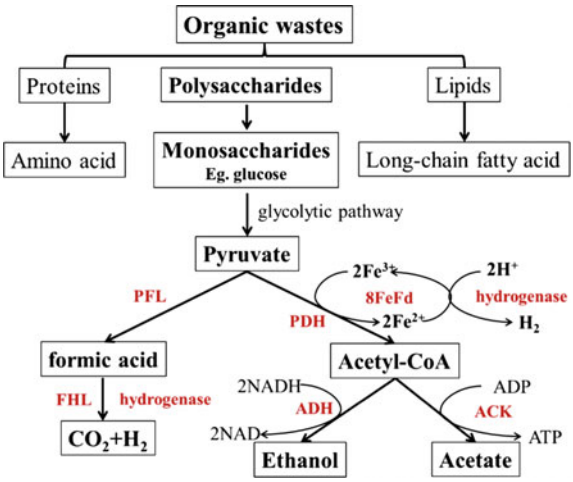

Fig. 1.6 Metabolic pathways in dark fermentative hydrogen production process (*PFL* Pyruvate formate-lyase; *FHL* Formate hydrogen lyase; *PDH* Pyruvate dehydrogenase; *ADH* Alcohol dehydrogenase; *ACK* Acetate Kinase)

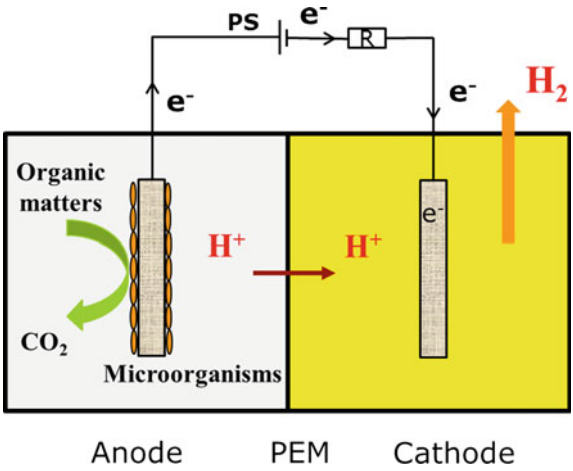

Fig. 1.7 Schematic of microbial electrolytic system. Anode and cathode are separated by a proton exchange membrane (PEM) with a power supply as the driving force for electrons flow from anode to cathode

called microbial electrolysis cell (MEC), which is capable of producing hydrogen from organic matters. The MEC efficiency relative to the electrical input has reached over 400% (Call and Logan 2008), proving that the electrical energy needed (typically > 0.2 V applied) is much less than that used for water electrolysis (>1.6–1.8 V applied) (Lalaurette et al. 2009). Unlike dark fermentation, MEC systems are not subject to the hydrogen yield constraints of 4 mol H2/mol hexose, while they owns the benefits of organic wastes degradation and high hydrogen production rate.

Figure 1.7 shows the schematic of MEC systems. The essential physical components include an anode, a cathode, a membrane, electrochemically active microrganisms, and a PS. During the hydrogen production process, organic matters are degraded in the anode chamber by the exoelectrogenic microbes, releasing the electrons and protons. Then, the electrons travel through the wire driven by the PS, and combine with the protons at the cathode chamber to form hydrogen. The protons formed in anode chamber with travel through the proton exchange membrane (PEM) to the cathode chamber.

Taking acetate as example, the electrode reactions can be written as follows:

$$\text{Anode}: CH_3COO^- + 4H_2O \rightarrow 2HCO_3^- + 9H^+ + 8e^- \tag{1.9}$$

$$\text{Cathode}: 2H^+ + 2e^- \rightarrow H_2 \tag{1.10}$$

$$\text{Overall reaction}: CH_3COO^- + 4H_2O \rightarrow 2HCO_3^- + H^+ + 4H_2 \tag{1.11}$$

It requires the electrochemically active microbes to achieve the transformation of electrons from organic matters to anode, hydrogen production efficiency in MEC is closely related with the ability of the exoelectrogenic microbes. Microorganism with electron transfer activity to anodes include b–*Proteobacteria* sp. (*Rhodoferax*), g-*Proteobacteria* sp. (*Shewanella* and *Pseudomonas*), d-*Proteobacteria* sp. (*Aeromonas*,

Geobacter, *Geopsychrobacter*, *Desulfuromonas*, *Desulfobulbus*), *Firmicutes* sp. (*Clostridium*), and *Acidobacteria* sp. (*Geothrix*), etc. (Hallenbeck 2010).

Besides the microorganisms, hydrogen production efficiency by MEC is also affected by the exchange efficiency of PEM, electrical conductivity and chemical stability of anode and cathode. For the application of MECs in biohydrogen production, continuous development in reactor design focus on reducing system internal resistance, decreasing the material costs and increasing the biomass concentration in anode chambers are needed.

1.3.4.5 Combined Systems for Biohyrogen Production

Hydrogen production in single biological ways usually have some limitations. For the photo-dependent hydrogen production, the low production rate and light conversion efficiency hinders its application. Dark fermentation can hardly achieve hydrogen yield over 4 mol H_2/mol hexose, which is only 33% of the 12 mol of hydrogen possible based on stoichiometric conversion of glucose to hydrogen. The residual organic matters present in liquid phase (include acetic and butyric acids, and ethanol, etc.) can hardly be further converted, leading to the energy dissipation. MECs are believed to be able to produce hydrogen from any biodegradable organic matters, but studies have found that the conversation rates from complex organic wastes (like cellulose) is pretty low compared to small molecules (like VFAs) (Cheng and Logan 2007).

To make biological hydrogen production processes more practical, various two-stage systems have been proposed. The combined systems usually take an optimized dark fermentation as the first stage, and the second stages include a methane digestion of the endproduct of dark fermentation, photo fermentation and MECs to further recover the energy remained in liquid metabolites.

(1) Combined dark fermentation and methane production
In this approach, dark fermentative hydrogen production and methane production are conducted in separated reactors, effluent from hydrogen fermentation system is used as substrate for methane production. Both reactors are operated under their optimized conditions, respectively. Studies have found that both energy extraction and wastes degradation rate are enhanced in two-stage system. Hydrogen production system facilitated the hydrolysis of complex substrates, leading to higher methane yield and more complete substrate degradation. Thus, it is more important to optimize hydrogen productivity than yields in hydrogen production system.
Thus, although not generating a pure hydrogen stream, a two-stage approach, with an acidogenic hydrogen fermentation in the first stage and a second stage with a methane generating anaerobic digestion of the effluent from the first stage can achieve both enhanced waste treatment and energy recovery.

(2) Combined dark and photo fermentative hydrogen production
Similar with combined dark fermentation and methane production, a two-stage process combining dark and photo hydrogen fermentation also take dark fermentation as the first stage and the liquid metabolites in the first stage is used as substrate for the second stage. Therefore, the combination of the dark and photo fermentation could achieve a theoretically maximum yield of 12 mol H_2/mol hexose, which is an efficient system to increase biohydrogen yield, enhance energy recovery, and benefit the subsequent process of organic metabolites (Chen et al. 2008).
However, the practical application of the combined system still has a number of problems to be overcome. Since different microorganisms are used in two stages, the microbial biomass in the effluent of first stage may need to be separated or sterilized before starting the second stage. Besides, effluent from dark fermentation may need to be pretreated like pH adjustment, unfavorable components separation (such as sulfur or nitrogen). Furthermore, when complex organic wastes are used as substrate for dark fermentation, the effluent may be dark colored and rich in suspended particles, which strongly affect the light conversation efficiency for microbes in photo fermentation system (Hallenbeck 2010).

(3) Combined dark fermentation and MECs hydrogen production
Similarly, in the combined dark fermentation and MECs system, MECs systems are used to further convert dark fermentation metabolites into hydrogen with the application of an additional voltage. Such a process can theoretically derive complete conversion of a hexose and achieve a maximum yield of 12 mol H_2/mol hexose with only a minimal (0.14 V) input of electrical energy. Studies have proved the significant enhancement of hydrogen yields to 67–90% of theoretical value (Cheng and Logan 2007).
There are several advantages of this combined system over the others. It is more practical in using a wide range of organic wastes for hydrogen generation, higher hydrogen production rate and efficiency, and relatively abundant energy input (electricity). However, there are also some obstacles. Since MECs systems are sensitive to the pH change, effluent from dark fermentation need to be buffered before the electrolysis process, which can be pretty costly. Besides, improvements on MECs systems are still required like the development of low-cost electrode materials, efficient system control, etc.

(4) Combined dark fermentation and MFCs
In microbial fuel cells (MFCs), organic matters are oxidized by the microorganisms, generating electrons and transferred from the anode (biological compartment) to a cathode through an external circuit, where electricity is produced from the electron flow. Then, the protons transferred from the anode to a cathode through PEM, combined with oxygen generating water (Fig. 1.8). Similar with MECs, small molecules are more easily used in MFCs. Through

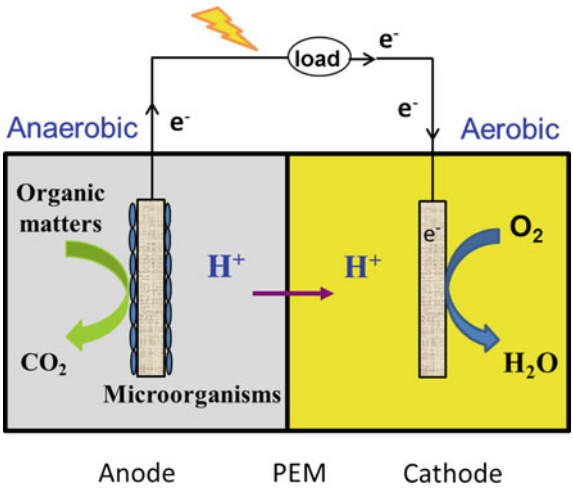

Fig. 1.8 Schematic of microbial fuel system

the combination of dark fermentation and MFCs, the metabolites can be further used and turned into electricity power. In this case, particulate organic matters can be hydrolyzed in dark fermentation system, producing hydrogen and enhancing the adequate MFCs operation (De Gioannis et al. 2013).

The application of MFCs is hindered by similar problems with MECs, including the cost of material, internal resistance, microbial density, and the pH sensitivity. Furthermore, lots of efforts are still need to achieve a constant sufficiently high catalytic density.

Hydrogen generation is attracting worldwide attention. According to survey, the global hydrogen production increased from USD 87.5 billion in 2011 to USD 118 billion by 2016, and it was estimated to reach USD 152.09 billion by 2021, at a compound annual growth rate (CAGR) of 5.2% from 2016 to 2021 (Markets and Markets 2016). To achieve a long-term decarbonisation, the share of hydrogen was predicted to reach 5–6% by 2050 (16000 PJ), and be dominated by low-carbon hydrogen production technologies (Sgobbi et al. 2016).

However, at present, over 90% of the hydrogen production still relies on the fossil energy, among which 40% is produced from natural gas, 30% from heavy oils and naphtha, 18% from coal, and 4% from electrolysis. Only about 1% is produced from biomass (Das 2009), which can hardly realize the sustainable hydrogen production (Fig. 1.9). To maintain a sustainable development of hydrogen society, hydrogen production from renewable sources attracts more attention. With mild reaction condition, high potential environmental benefits and low-cost substrate, dark fermentation supplies a promising way for hydrogen production (Urbaniec and Bakker 2015). Furthermore, hydrogen production from biomass supplies dual benefits of energy generation and wastes treatment. Thus, the biological hydrogen production makes more sense in developing hydrogen society.

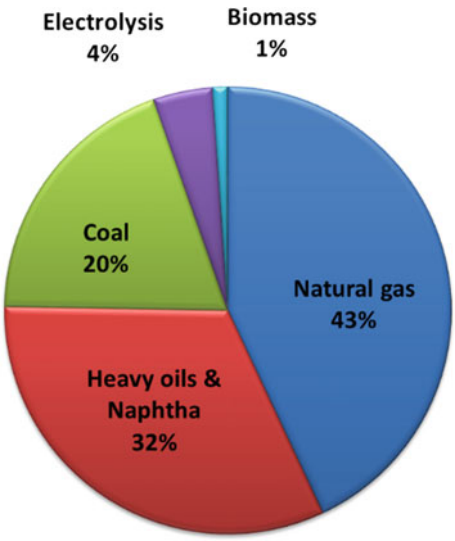

Fig. 1.9 Hydrogen production from various resources

1.4 Overview of This Book

Biological hydrogen production is considered as the most environmentally friendly alternatives for satisfying future hydrogen demands. Biohydrogen production from organic wastes obviously offers advantages for environmental protection over the existing physicochemical methods.

In this book, we summarized the principles and applications of dark fermentative hydrogen production from various kinds of organic wastes. This chapter introduces the hydrogen production from different technologies. Chapter 2 reviews the microbiology, biochemistry, and enzymology involved in the biological process of dark fermentative hydrogen production. Chapter 3 introduces the various treatment methods for the enrichment of hydrogen-producing microorganisms. Chapter 4 outlines the pretreatment technologies of organic wastes for biological hydrogen production. Chapter 5 summarizes the different factors influencing fermentative hydrogen production. Chapter 6 focuses on the kinetic models for biological process of fermentative hydrogen production. Chapter 7 deals with the optimization of biological hydrogen production process. Finally, Chap. 8 reviews the current states of fermentative hydrogen production from sewage sludge.

To our knowledge, this book is a first attempt to describe the biological hydrogen production from various organic wastes, which is aimed at a wide range of readers, mainly including undergraduates, postgraduates, energy researchers engineers, and others who are interested in hydrogen production in general and biological hydrogen production in particular, as well as to industrial concerns that are looking for inexpensive hydrogen production technologies.

We hope readers will find this book interesting and informative for their research pursuits.

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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.

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Green Energy and Technology, DOI 10.1007/978-981-10-4675-9_2

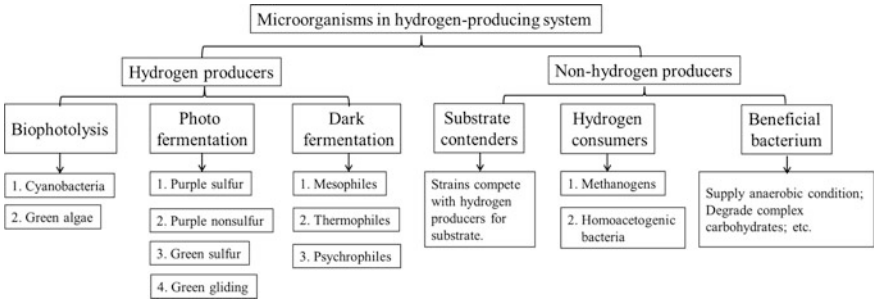

Fig. 2.1 Schematic diagram represents the diversity of microorganisms present in hydrogen-producing systems

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 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 lichenformis*). 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
Mesophiles				
Clostridium 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	*Clostridium butyricum*	0.23–3.47	(Beckers et al. 2015; Calusinska et al. 2015; Ortigueira et al. 2015; Rafieenia and Chaganti 2015)
		Clostridium tyrobutyricum	2	(Jo et al. 2008)
		Clostridium beijerinckii	0.6–2.52	(Pan et al. 2008; Zhao et al. 2011; An et al. 2014)
		Clostridium pasteurianum	0.96–3.0	(Cheng and Chang 2011; Hu et al. 2013)
Enterobacter sp.	Facultative anaerobes Supply anaerobic environment, some are aciduric, fermentation condition ranges 30–40 °C, pH 4.0–7.5	*Enterobacter aerogenes*	0.1–0.3	(Hu et al. 2013; Batista et al. 2014)
		Enterobacter asburiae	0.54	(Shin et al. 2007)
		Enterobacter cloacae	1.3–1.8	(Harun et al. 2012; Mishra and Das 2014)
Bacillus sp.	Facultative anaerobes. Hydrolyze substrate to simple sugars, Supply anaerobic environment,	*Bacillus firmus*	1.1–1.3	(Sinha and Pandey 2014)
		Bacillus amyloliquefaciens	2.26	(Song et al. 2013)

(continued)

Table 2.1 (continued)

Hydrogen producers	Characteristics	Typical species	Hydrogen yield (mol H_2/mol hexose)	References
Mesophiles				
	produce H_2, fermentation condition ranges 35–40 °C, pH 5.3–7.4			
Others	Obligate or facultative anaerobes. Produce H_2 from different kinds of carbohydrates. Not widely studied	*Ethanoligenens harbinens*	2.2–3.1	(Xie et al. 2010; Zhang et al. 2015a, b)
		Citrobacter freundii	0.83–2.49	(Oh et al. 2003; Hamilton et al. 2010)
		Rhodopseudomonas Palustris	1–2.76	(Oh et al. 2002)
Thermophiles				
Thermoanaerobacterium sp.	Obligate or facultative anaerobes. High hydrogen production rate and yield, fermentation condition ranges 40–60 °C, pH 6.2–8.0	*Thermoanaerobacterium thermosaccharolyticum*	2.42–2.53	(O-Thong et al. 2008; Ren et al. 2008a, b; Singh et al. 2014)
Klebsiella sp.		*Klebsiella pneumoniae*	0.43	(Chookaew et al. 2012)
Psychrophiles				
Polaromonas sp.	Obligate or facultative anaerobes. Low cost in operation without temperature control, fermentation condition ranges 20–25 °C, pH 6.5	*Polaromonas rhizosphaerae*	1.3–1.7	(Alvarez-Guzmán et al. 2016)
Rahnella sp.		*Rahnella aquatilis*	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 H_2/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	–
Utilization of					
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 H_2/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.

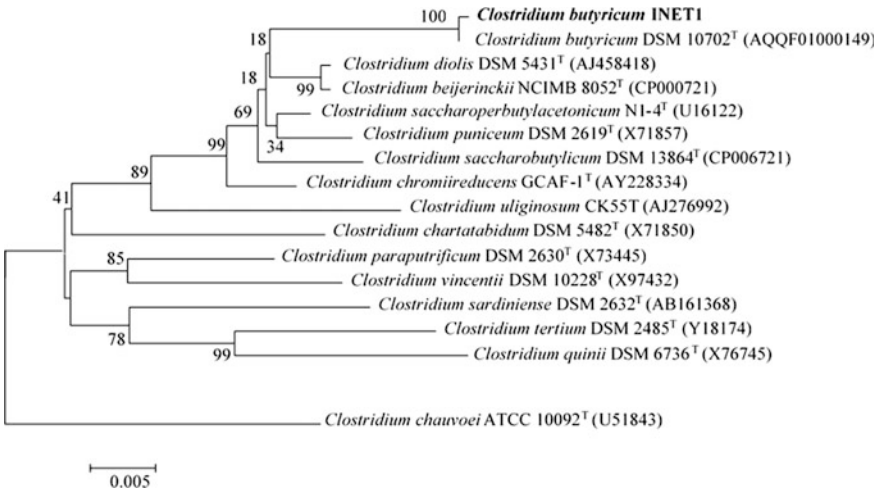

Fig. 2.2 Phylogenetic tree showing the relationships between strain *Clostridium butyricum* INET1 and related species based on 16S rRNA gene

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 H_2/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 H_2/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 H_2/mol hexose was achieved. Otherwise, the highest hydrogen yield of 2.24 mol H_2/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.

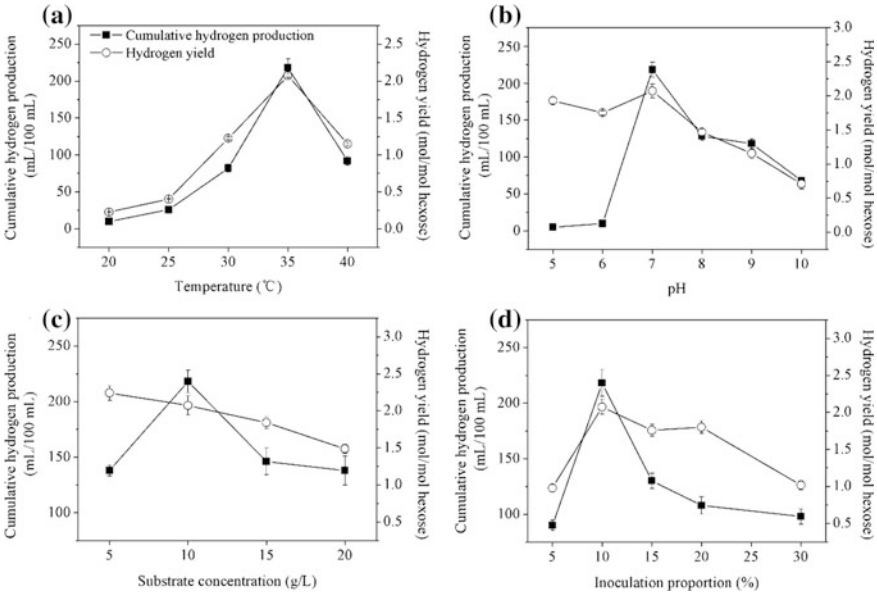

Fig. 2.3 Characteristics of hydrogen production by *Clostridium butyricum* INET1. **a**, **b**, **c**, **d** represents the effect of temperature, initial pH, substrate concentration and inoculation proportion on hydrogen production, respectively. (–■– Cumulative hydrogen production, –○– Hydrogen yield)

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

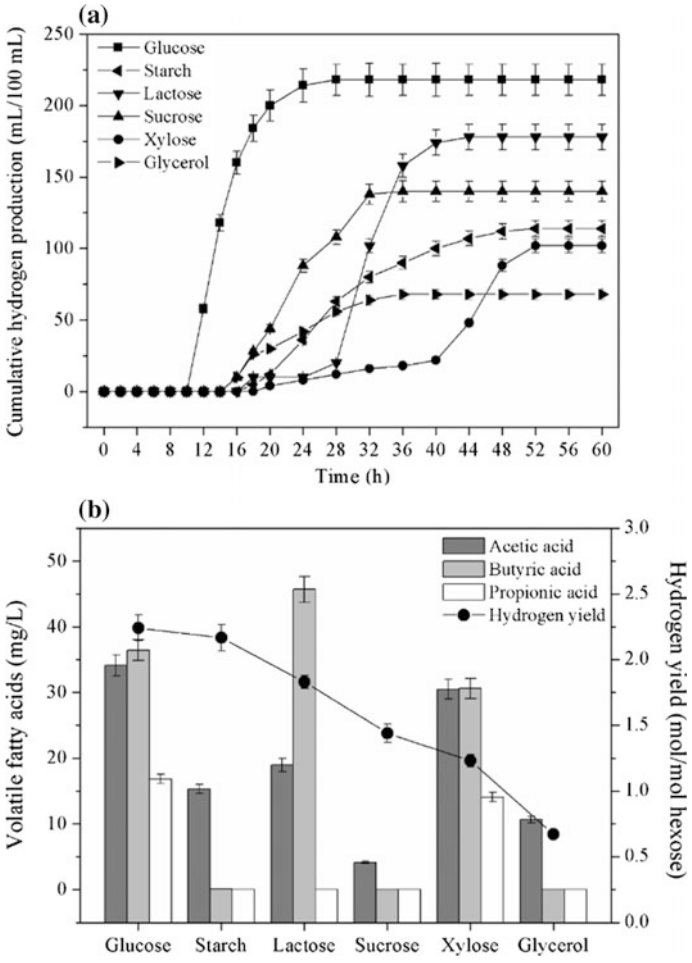

Fig. 2.4 Hydrogen production from different carbon sources by *Clostridium butyricum* INET1. **a**. Hydrogen generation during the fermentation process. **b**. Volatile fatty acids formation and hydrogen yield obtained from different substrates

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 H2/mol hexose)	References
Clostridium butyricum DSM 10702	Glucose	37	7.0	3.47	Ortigueira et al. (2015)
Clostridium butyricum CWBI1009	Glucose (1–10 g/L)	30–37	5.2–8.0	0.23–2.4	Beckers et al. (2010; Beckers et al. 2015)
Clostridium butyricum W5	Glucose (5–10 g/L)	39	6.5	0.82–1.4	Wang et al. (2009)
Clostridium butyricum	Glucose (3 g/L)	37	6.5	2.09	Seppälä et al. (2011)
Clostridium butyricum IFO 3847	Glucose (9 g/L)	37	7.0	1.26	Karube et al. (1976)
Clostridium butyricum IAM 19002	Glucose (9 g/L)	37	7.0	1.04	Karube et al. (1976)
Clostridium butyricum IMA 19003	Glucose (9 g/L)	37	7.0	1.2	Karube et al. (1976)
Clostridium butyricum TM-9A	Glucose (10 g/L)	37	8.0	2.67–3.1	Junghare et al. (2012)
Clostridium butyricum A1	Glucose (10 g/L)	37	6.5	1.9	Jenol et al. (2014)
Clostridium butyricum DSM 10702	Glucose (10 g/L)	37	6.8	2.4–3.1	Hu et al. (2013)
Clostridium butyricum EB6	Glucose (15.7 g/L)	37	5.6	2.2	Chong et al. (2009a, b)
***Clostridium butyricum* INET1**	**Glucose (COD = 10 g/L)**	**35**	**7.0**	**2.24**	**This study**
Clostridium butyricum DSM 10702	Xylose	37	7.0	3.12	Ortigueira et al. (2015)
Clostridium butyricum LMG 1213t1	Xylose (10 g/L)	–	5.5–7.0	1.94–2.48	Heyndrickx et al. (1991)
Clostridium Butyricum TM-9A	Xylose (10 g/L)	37	8.0	0.59	Junghare et al. (2012)
***Clostridium butyricum* 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 H2/mol hexose)	References
Clostridium butyricum CWBI 1009	Sucrose (4.3 g COD/L)	30	7.3	0.44	Beckers et al. (2010)
Clostridium butyricum TM-9A	Sucrose (10 g/L)	37	8.0	1.49	Rafieenia and Chaganti (2015)
Clostridium butyricum CGS2	Sucrose (COD = 20 g/L)	37	7.1	0.95	Fritsch et al. (2008)
Clostridium butyricum CGS5	Sucrose (COD = 20 g/L)	37	5.5	1.39	Chen et al. (2005)
Clostridium butyricum TISTR 1032	Sucrose (COD = 25 g/L)	37	6.5	1.52	Plangklang et al. (2012)
Clostridium butyricum W5	Molasses (100 g/L)	35	7.0	1.63	Wang et al. (2009)
Clostridium butyricum KBH1	Molasses (5.9 g/L)	37	9.0	1.49	Abdul et al. (2013)
***Clostridium butyricum* INET1**	**Sucrose (COD = 10 g/L)**	**35**	**7.0**	**1.44**	**This study**
Clostridium butyricum CWBI 1009	Lactose (COD = 4.3 g/L)	30	7.3	0.69	Beckers et al. (2010)
***Clostridium butyricum* INET1**	**Lactose (COD = 10 g/L)**	**35**	**7.0**	**1.83**	**This study**
Clostridium butyricum DSM 10702	Starch	37	–	3.2	Ortigueira et al. (2015)
Clostridium butyricum CWBI 1009	Starch (COD = 4.3 g/L)	30	7.3	0.73	Beckers et al. (2010)
Clostridium butyricum NCIB 9576	Starch (10 g/L)	37	–	2.58	KIM et al. (2006)
Clostridium butyricum CGS2	Starch hydrolysate	37	7.5	1.23–2.03	Pattra et al. (2008)
***Clostridium butyricum* 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 H2/mol hexose)	References
Clostridium butyricum	Glycerol (5 g/L)	37	7.4	3.6	Kivisto et al. (2013)
Clostridium butyricum	Glycerol (20 g/L)	37	6.5	0.67	Pachapur et al. (2016)
***Clostridium butyricum* 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 $NaHCO_3$, 5 g NH_4Cl, 5 g $NaH_2PO_4 \cdot 2H_2O$, 5 g $K_2HPO_4 \cdot 3H_2O$, 0.25 g $FeSO_4 \cdot 7H_2O$, 0.085 g $MgCl_2 \cdot 6H_2O$, 0.004 g $NiCl_2 \cdot 6H_2O$). 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 N_2 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	–
Ability to grow			
50 °C	–	45 °C	+
15 °C	+	6.5% NaCl	+
Air	+		
Utilization of			
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

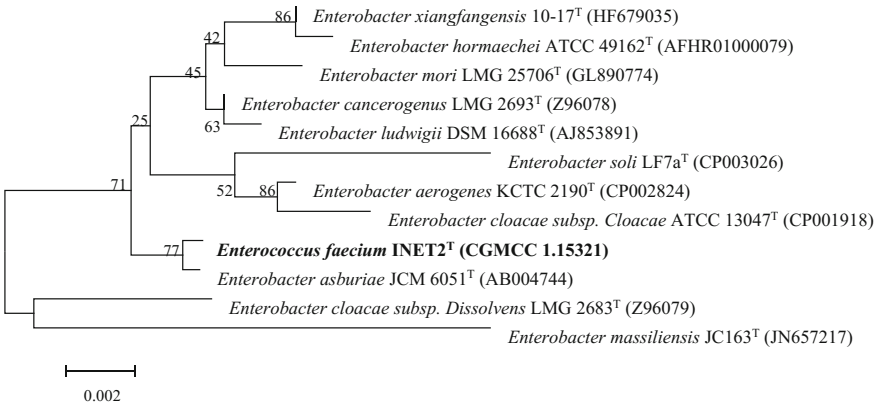

Fig. 2.5 Phylogenetic tree showing the relationships between strain *Enterococcus faecium* INET2 and related species based on 16S rDNA gene

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 H_2 in the headspace. The hydrogen yield (mol H_2/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:

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

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 H_2/100 mL), hydrogen yield (mol H_2/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 H_2/100 mL at 35 °C. When the temperature further increased to 40 °C, cumulative hydrogen production showed a little decrease to 85 mL H_2/100 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 H_2/mol glucose was attained at 40 °C, which was slightly higher than 0.90 mol H_2/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

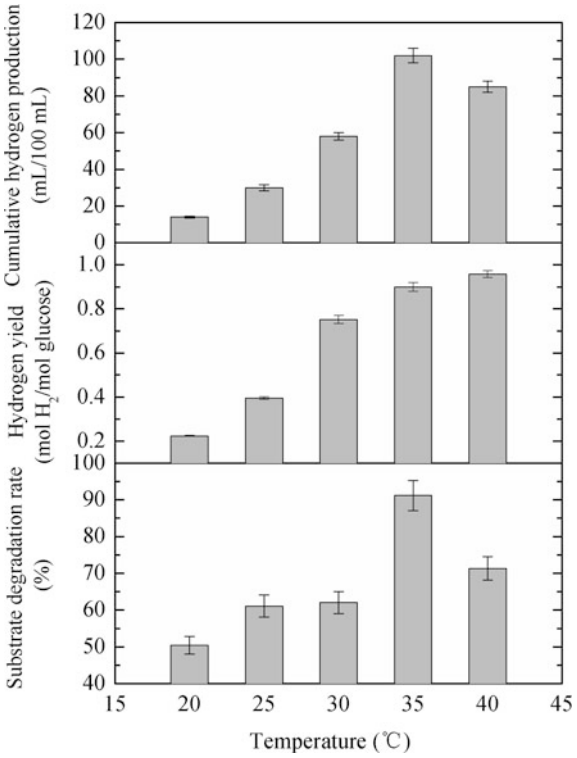

Fig. 2.6 Effect of temperature on hydrogen production

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 H_2/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

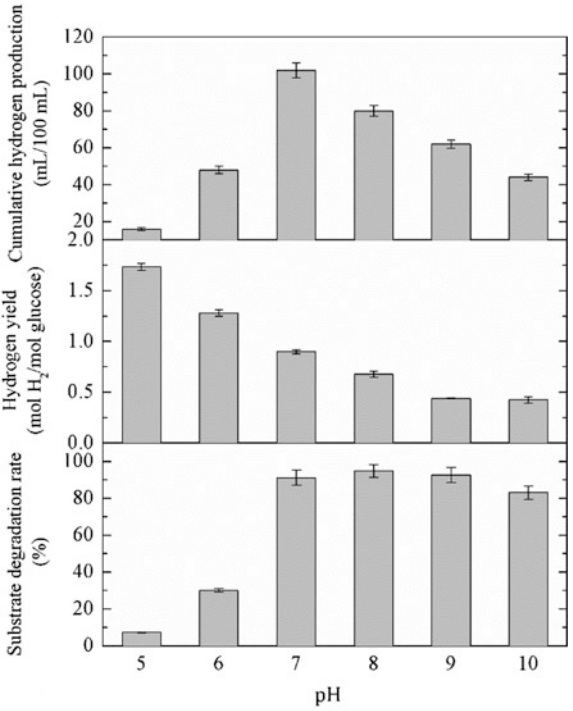

Fig. 2.7 Effect of initial pH on hydrogen production

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%.

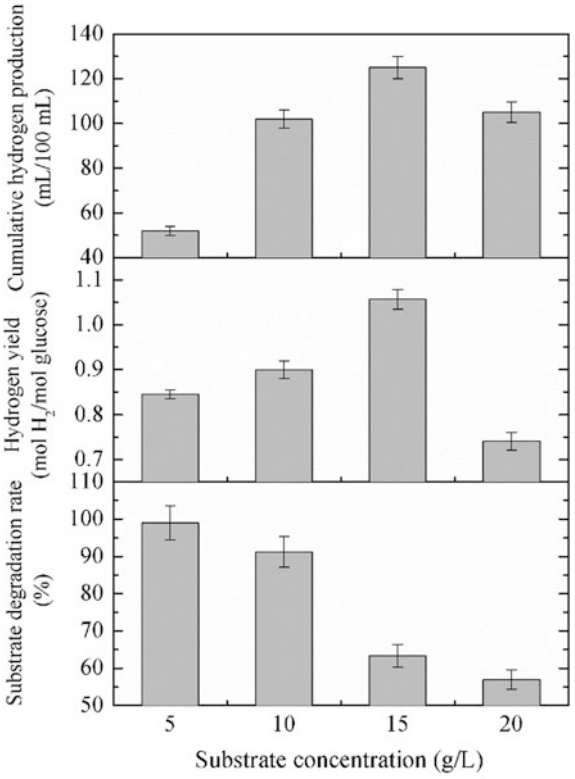

Fig. 2.8 Effect of substrate concentration on hydrogen production

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 H_2/100 mL and hydrogen yield of 1.06 mol H_2/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 H_2/100 mL) and hydrogen yield (0.90 mol H_2/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

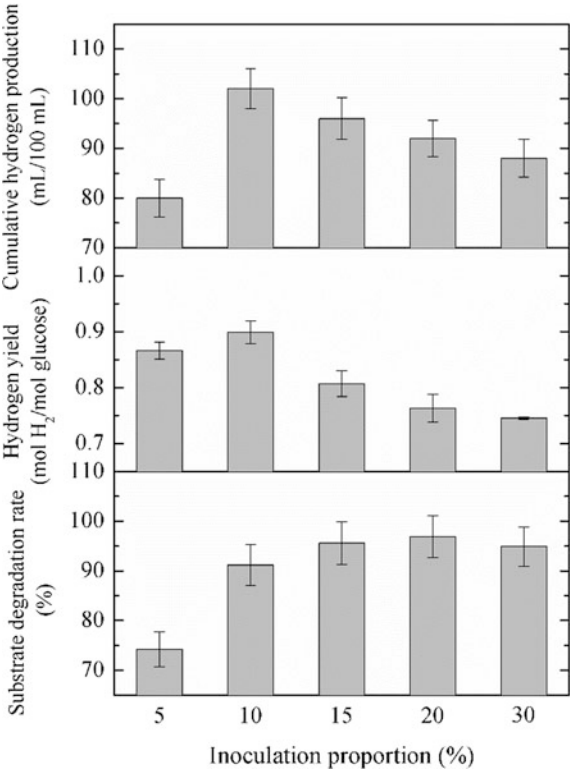

Fig. 2.9 Effect of inoculation ratio on hydrogen production

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 H_2/100 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

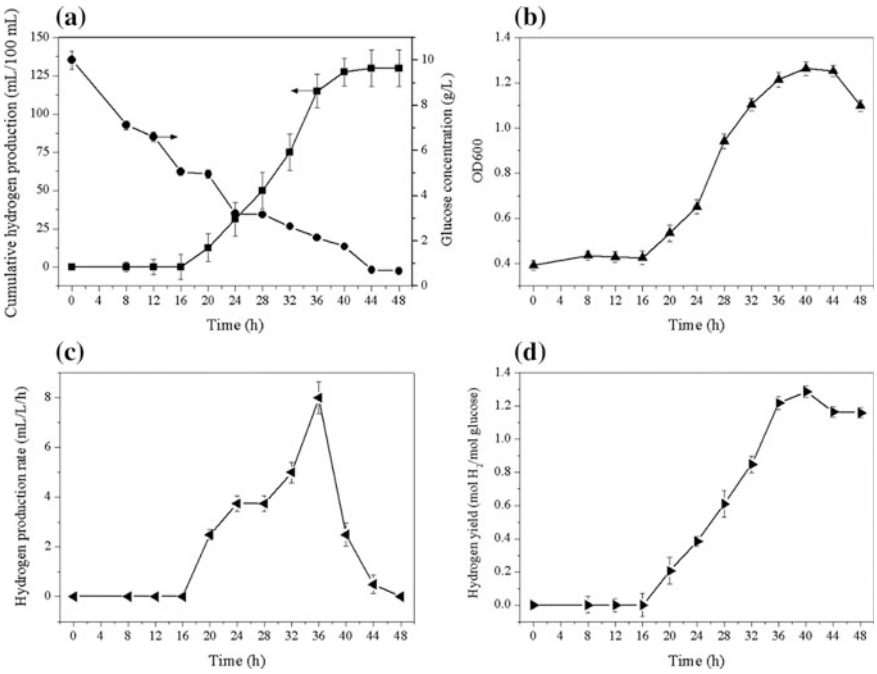

Fig. 2.10 Hydrogen production performances at the optimized condition

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 H_2/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.

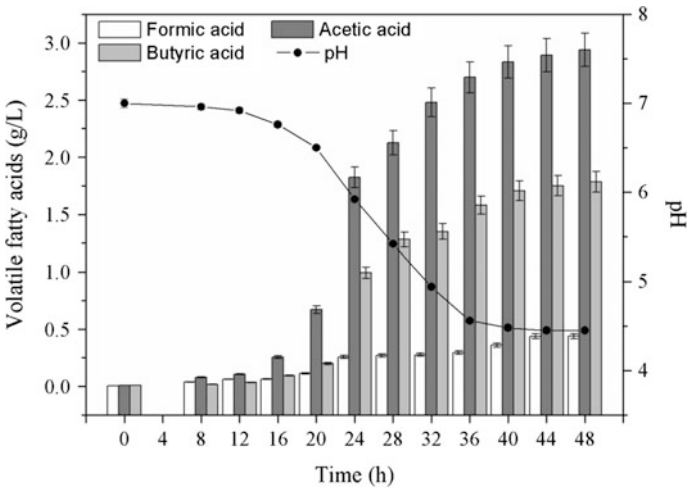

Fig. 2.11 Variation of volatile fatty acids (VFA) with time

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.

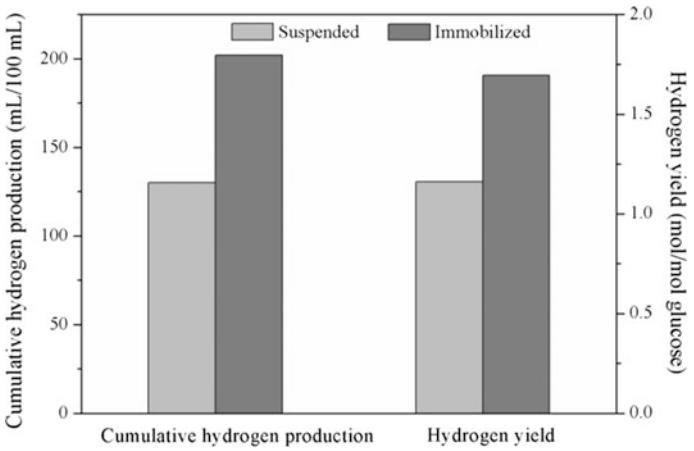

Fig. 2.12 Comparison of hydrogen production by immobilized and free cells

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 H_2/mol glucose than suspended bacteria with 130 mL/100 mL and 1.16 mol H_2/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 H_2 from carbohydrate-rich substrates in a dark environment. Among them, *Clostridium* sp have several advantages, for example, they have the highest H_2 yield (1.61–2.36 mol H_2/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 H_2 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

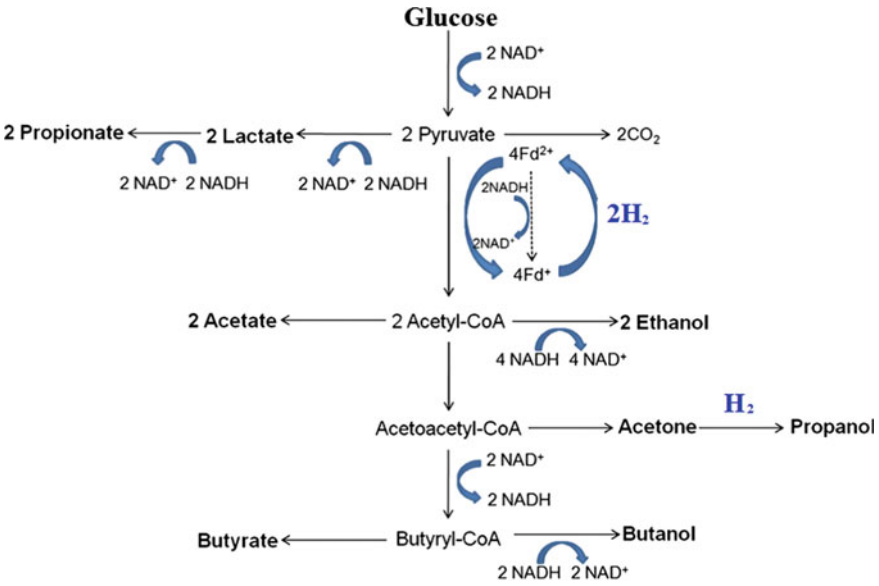

Fig. 2.13 Biological H_2 production mechanism in dark fermentation

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).

$$\text{Pyruvate} + \text{CoA} + 2\text{Fd}(ox) \rightarrow \text{AcetylCoA} + 2\text{Fd}(\text{red}) + \text{CO}_2 \quad (2.1)$$

$$4H^+ + 2\text{Fd}(\text{red}) \rightarrow 2\text{H}_2 + 2\text{Fd}(\text{ox}) \quad (2.2)$$

$$\text{Pyruvate} + \text{CoA} \rightarrow \text{AcetylCoA} + \text{HCOOH} \quad (2.3)$$

$$\text{HCOOH} \rightarrow \text{CO}_2 + \text{H}_2. \quad (2.4)$$

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 H_2/mol hexose was obtained by genus *Clostridium* while no more than 2.0 mol H_2/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 H_2.

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.

$$\begin{aligned} &5C_6H_{12}O_6 + 12H_2O + 2NAD^+ + 16ADP + 16Pi \\ &\rightarrow 4[Bu] \quad + 2[Ac] + 10HCO_3^- + 2NADH + 18H^+ + 10H_2 + 16ATP \end{aligned}$$

$$\Delta G = -252.3\,\text{kJ/mol glucose}(\text{pH} = 7, T = 298.15\,\text{K}). \tag{2.5}$$

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 NADH + H^+.

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 4H_2 + 2CO_2 \quad (2.6)$$

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2CH_2COOH + 2H_2 + 2CO_2. \quad (2.7)$$

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:

$$C_6H_{12}O_6 + H_2O + 3ADP \rightarrow CH_3COO^- + CH_3CH_2COO^- + HCO_3^- + 3H^+ + H_2 + 3ATP. \quad (2.8)$$

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 NADH + 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:

$$2H^+ + 2e^- \rightarrow H_2. \tag{2.9}$$

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.

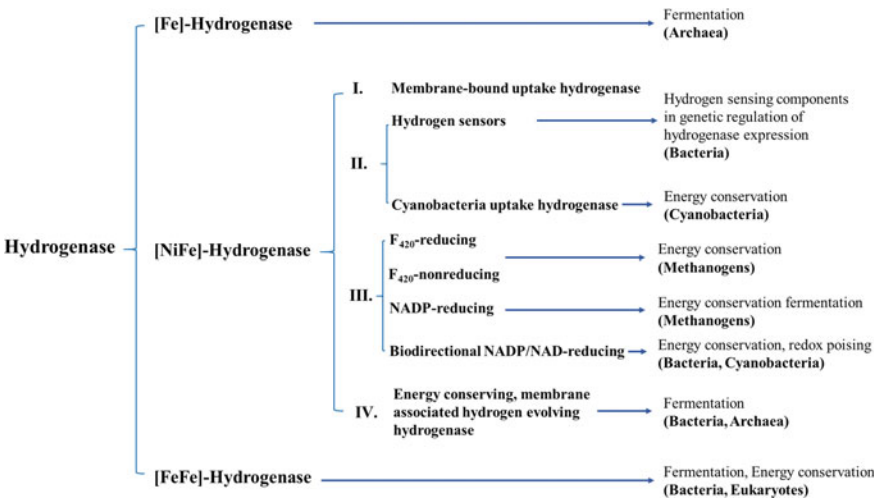

Fig. 2.14 Classification of hydrogenases

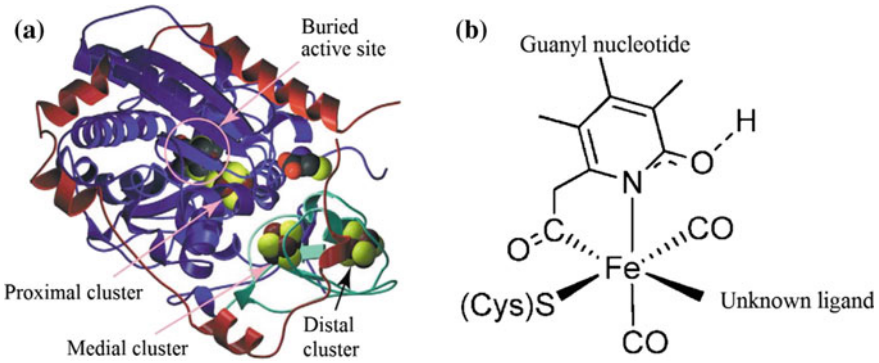

Fig. 2.15 **a** Schematic representation of the crystal structures [Fe]-hydrogenase from *Desulfovibrio desulfuricans* ATCC 7757 (Nicolet et al. 2002). **b** Structure of the active site of [Fe]-hydrogenase. (Chen et al. 2010)

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

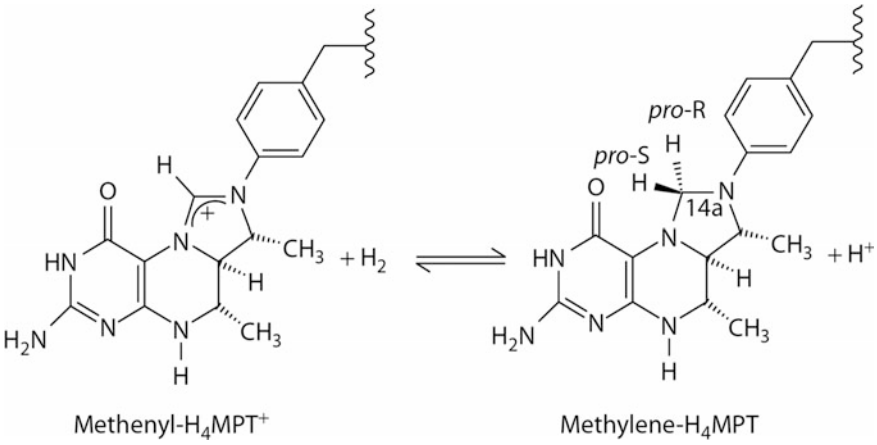

Fig. 2.16 The reversible reduction of methenyltetrahydromethanopterin (methenyl-H_4MPT^+) with H_2 to methylene-H_4MPT catalyzed by [Fe] hydrogenase. (Vogt et al. 2008)

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.*

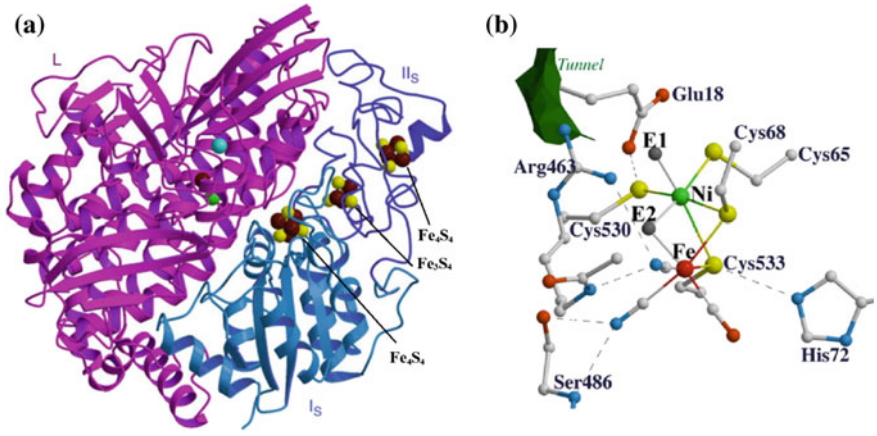

Fig. 2.17 A structure of *D. gigas* [NiFe]-hydrogenase. *Arrows* b-strands; *Ribbons* a-helices; spheres metal sites with color codes: Ni *green*, Fe *red-brown*, Mg *cyan*, S *yellow*. B-The nickel–iron active site (Fontecilla-Camps and Volbeda 2013)

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 $[Fe_4S_4]$; II_S lacks extensive secondary structure, and it binds the rest two FeS clusters: mesial $[Fe_3S_4]$ and distal $[Fe_4S_4]$. 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 $[Fe_3S_4]$ cluster is substituted by $[Fe_4S_4]$ 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, H_2-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

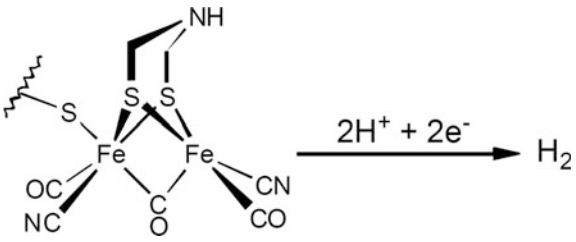

Fig. 2.18 Active site biochemistry of [FeFe]-hydrogenase enzyme (Justice 2008)

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 H_2-uptake hydrogenase, the insertion of a gene for enzyme expression such as an overexpression or an increase of the efficiency of H_2-producing enzymes in microbial cells, and increase of the O_2 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 H_2 and H_2 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 H_2-producing enzymes, especially hydrogenases in a cyanobacterial system, by transferring the gene for O_2-tolerant NiFe-hydrogenase from *Thiocapsa roseopersicina* into cyanobacteria.

To improve the H_2 production from organic wastes using microorganisms, the contribution of the genetic engineering of enzymes responsible for H_2 production may be required to increase the efficiency of H_2 evolution. Further increases are expected by maximizing the H_2 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 Béland (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 H_2/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 stercorarium 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/m(3)/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

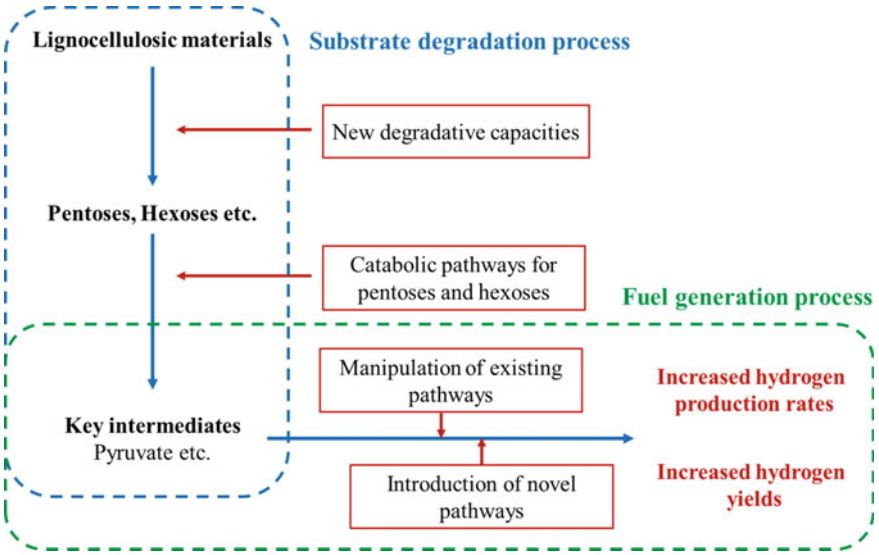

Fig. 2.19 Roles for metabolic engineering in dark fermentative hydrogen production

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 H_2 production through the oxidation of NADH.

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Chapter 3
Enrichment of Hydrogen-Producing Microorganisms

3.1 Overview

It is known that various kinds of microorganisms, including hydrogen producers and nonhydrogen producers, even hydrogen consumer, coexist in the mixed cultures. The hydrogen producers can produce hydrogen from various carbohydrates. However, during the fermentation process, nonhydrogen producers may compete with hydrogen producers for substrates, leading to low hydrogen conversion efficiency. Furthermore, some hydrogen consumers can also convert the hydrogen produced to other products, like methane, acetate acid, and ethanol.

With the application of complex organic wastes as substrate in dark fermentation, studies have found that some nonhydrogen producers can degrade the complex organic matters (cellulose, starch, etc.) into small molecules like glucose, xylose, etc. Table 3.1 shows some typical nonhydrogen producers present in dark fermentation systems.

To eliminate the undesired nonhydrogen producers, especially hydrogen consumers, mixed cultures are usually pretreated before the inoculation. The application of pretreatment methods aims at eliminating the hydrogen consumers while preserving the hydrogen producers. To achieve this target, characteristics of microorganisms present in mixed cultures can be considered. For example, some hydrogen-producing species like *Clostridium* spp. are able to form spores as a reaction to adverse environmental conditions, pretreatments can be conducted basing on their larger chance to survive in harsh conditions. Some hydrogen producers like *Enterobacter* spp. and *Bacillus* spp. are facultative anaerobic bacteria, better oxygen tolerance can help them to be preserved in treatment process. Methanogens are obligate anaerobes and were proved to be active in a relatively narrow pH range (6.8–7.2), indicating that they can be inhibited by either aeration or pH adjustment.

Figure 3.1 summarizes the different pretreatment methods used for enriching hydrogen producers for fermentative hydrogen production. As shown in Fig. 3.1a,

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Table 3.1 Typical nonhydrogen producers present in dark fermentation systems

Nonhydrogen producers	Characteristics	Typical species	References
Methanogens	Obligate anaerobes; Mesophilic bacteria; Active in a narrow pH range. Consume H_2 for methane production.	Methanobacterium sp. Methanococcus sp., etc.	(1993; Whitman et al. 2006; Ray et al. 2009)
Homoacetogenic bacteria	Obligate anaerobes; Spore-forming bacteria. Utilize H_2 as electron donors for volatile fatty acids generation, reduction of carboxylic acids into their corresponding alcohols	*Clostridium carboxidivorans Clostridium ragsdalei Clostridium ljungdahlii,* etc.	Kundiyana et al. (2011), Perez et al. (2013), Ramachandriya et al. (2013), Phillips et al. (2015)
Substrate contenders	Obligate or facultative anaerobes. Compete for substrate with hydrogen producers	*Enterococcus* sp. *Thermoanaerobacterium* sp., etc.	Lu et al. (2007), Adav et al. (2009), Li and Liu (2012), Valdez-Vazquez et al. (2015)
Beneficial bacterium	Obligate or facultative anaerobes. Supply anaerobic environment in the fermentation system; Degrade complex carbohydrates to simple organic compounds	*Bifidobacterium* sp. *Enterococcus* sp. *Bifidobacterium* sp., etc.	Hung et al. (2011)

heat treatment has been the most widely used method, followed by acid and base treatment, chemical inhibitors and aeration. Combination of different treatment methods also attracted quite a few interests. Figure 3.1b shows some methods that were not commonly used or newly developed, which make up "others" present in Fig. 3.1a. Detail description of different pretreatment methods is as follows.

3.2 Heat Treatment

Temperature is a vital factor affecting the microbial survival. According to the different optimum temperature conditions, microbes can be categorized into psychrophiles (0–20 °C), mesophiles (20–45 °C), and thermophiles (45–122 °C). High temperature usually disrupts the chemical bonds of the cell wall and membrane,

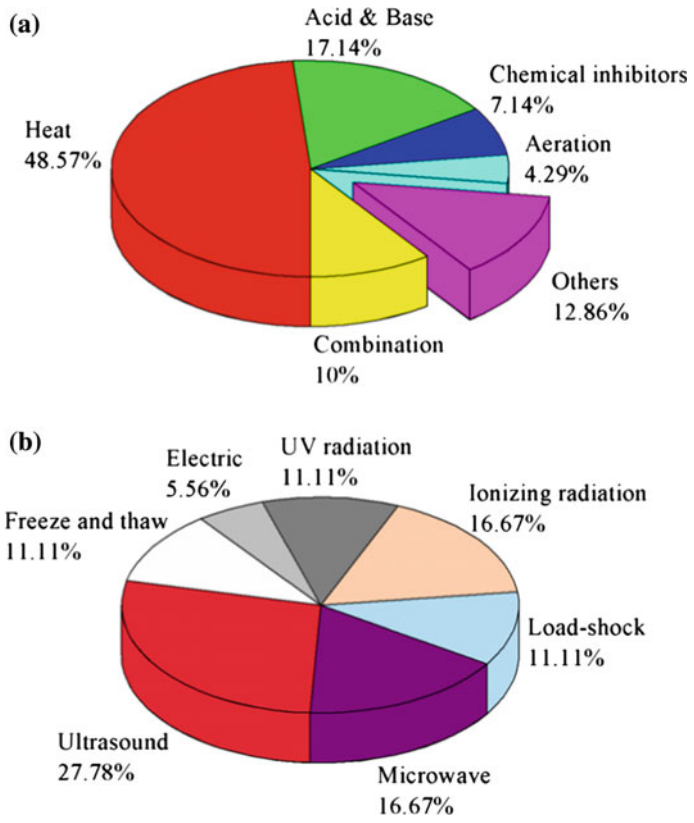

Fig. 3.1 Pretreatment methods used for enriching hydrogen producers

solubilizes the cell components and causes deterioration of microbial protein (Appels et al. 2008). Considering the higher heat resistance of spore-forming hydrogen producers, heat treatments have been used for killing non spore-forming methanogens from the mixed cultures.

As shown in Table 3.2, heat treatment has been applied on different inoculum sources, include various kinds of sludge, compost, and organic wastes, etc., the treating temperature ranged from 65 to 121 °C, and duration time from 10 min to 10 h. Among the reviewed studies, highest hydrogen yield of 2.49 mol/mol hexose was obtained by anaerobic sludge treated at 100 °C for 60 min.

Figure 3.2 shows the frequency of heating temperature and duration used in reviewed studies. It can be seen that the most commonly used condition was 100 °C and 60 min. Treating duration was also affected by inoculum source. For the anaerobic sludge, no more than 1 h was more frequently used while for the compost, 2 h was the most commonly used duration. Possible reason for the difference may be the different cell density and microbial diversity present in different sources.

Table 3.2 Heat treatments for enriching hydrogen-producing bacteria

Inoculum source	Temperature	Treating time	Substrate	Operational conditions	Hydrogen yield[a]	References
Anaerobic sludge	65–121 °C	10–90 min	Glucose (2-25 g/L)	30–40°C, pH 5.5–7.5	0.9–2.41	Davila-Vazquez et al. (2008), Wang and Wan (2008), Wu et al. (2008), Cavalcante De Amorim et al. (2009), Elbeshbishy et al. (2010), Baghchehsaraee et al. (2011), de Sá et al. (2013), Kan (2013), Yin et al. (2014), Abdallah et al. (2016)
Compost	105 °C	2 h	Glucose (COD = 14 g/L)	33.5 °C, pH 5.0	2.15	Wu et al. (2010)
Anaerobic sludge	100 °C	60 min	Fructose (10 g/L)	35 °C, pH 5.5	2.09	de Sá et al. (2013)
Anaerobic sludge	100 °C	60 min	Sucrose (10–20 g/L)	35–60 °C, pH 5.5	1.16–2.49	Lin et al. (2006a, b), O-Thong et al. (2009), de Sá et al. (2013)
Compost	100–104 °C	2 h	Sucrose (8.9–10 g/L)	22–36 °C, pH 7-8.5	2.15–2.22	Gadhamshetty et al. (2009), Song et al. (2012a, b)
Seacoast sludge	95–100 °C	30–60 min	Sucrose (17.8–39.3 g/L)	35–37 °C, pH 5.5–7.6	0.52–2.25	Zhang et al. (2005), Davila-Vazquez et al. (2008), Lin et al. (2010), Lin et al. (2006a, b), Lee et al. (2012a, b)
Anaerobic sludge	100 °C	40 min	Lactose (5 g/L)	36 °C, pH 7.5	1.8	Davila-Vazquez et al. (2008)
Anaerobic sludge	100 °C	60 min	Xylose (10 g/L)	35 °C, pH 5.5	2.26	de Sá et al. (2013)
Anaerobic sludge	121 °C	30 min	Arabinose (10 g/L)	37 °C, pH 6.5	2.38	Danko et al. (2008)
Anaerobic sludge	90–100 °C	15–20 min	Distillery wastewater (COD = 40–120 g/L)	37 °C, pH 5.5	1.3–2.09	Searmsirimongkol et al. (2011), Gadhe et al. (2014)
Anaerobic sludge	95–100 °C	60 min	Textile wastewater (Total sugar = 20 g/L)	37 °C, pH 7.0	1.37	Li et al. (2012)

(continued)

Table 3.2 (continued)

Inoculum source	Temperature	Treating time	Substrate	Operational conditions	Hydrogen yield[a]	References
Anaerobic sludge	95–100 °C	60 min	Cassava starch (COD = 32)	37 °C, pH 6.0	1.81	Lee et al. (2008)
Anaerobic sludge	100 °C	45 min	Molasses (COD = 40)	35 °C, pH 5.5	2.1	Lay et al. (2010)
Anaerobic sludge	100°C	5 h	Wheat powder solution (COD = 10–20 g/L)	37 °C, pH 7.0	0.69–2.96	Argun et al. 2008a, b, Oztekin et al. (2008), Argun et al. (2009)
Anaerobic sludge	105 °C	90 min	Palm oil mill effluent (COD = 38.4)	44 °C, pH 7.0	0.13	Leaño et al. (2012)
Anaerobic sludge	105°C	2 h	Corn stover	35°C, pH 5.5	3	Datar et al. (2007)
Anaerobic sludge	105–121 °C	30 min–3 h	Activated sludge	30–35 °C, pH 5.5–6.9	0.95–1.43	Wang et al. (2003), Assawamongkholsiri et al. (2013)
Anaerobic sludge	105 °C	90 min	Food waste	55 °C, pH = 4.5	60.6 mL/g VS	Ghimire et al. (2016)
Anaerobic sludge	90 °C	10 min	Whear straw	37 °C, pH 5.5	19.63 mL/g VS	Quéméneur et al. (2012)
Anaerobic sludge	90 °C	20 min	Microalgal biomass (Dry cell weight = 76)	35 °C, pH 7.4	31.2 mL/g Dry cell weight	Yun et al. (2012)
Anaerobic sludge	95 °C	40 min	Rice straw (TS = 90 g/L))	55 °C, pH = 6.5	24.8 mL/g TS	Chen et al. (2012)
Cracked cereal	100 °C	30 min	Grass	35 °C, pH 7.0	72.2 mL/g dry grass	Cui and Shen (2012)
Compost	100 °C	45 min	Sweet potato solution (COD = 150 g/L)	35 °C, pH 6.5	1.31 mmol/g potato	Chu et al. (2012)
Compost	100 °C	2 h	Wheat straw	36 °C, pH 7.0	68.1 mL/g TVS	Fan et al. (2006)

[a]mol H_2/mol hexose except for mentioned

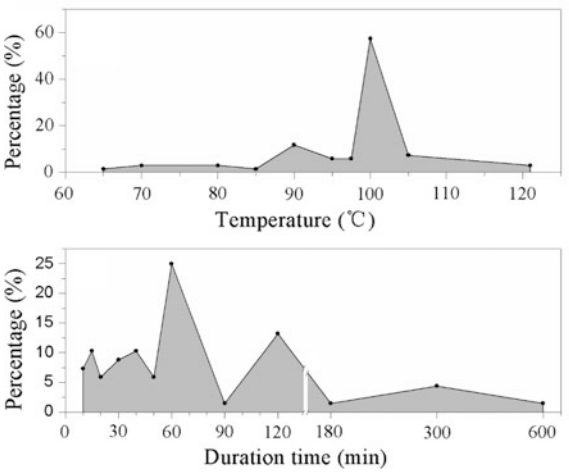

Fig. 3.2 Conditions used in heat treatment method

The reason for the wide application of heat treatment may be its simple operation and easy control. The equipment investment can be very low, a heater, or an oven is enough. Their effect on enhancing hydrogen production ability of inoculum is also significant. Hydrogen consumers like methanogens are inhibited effectively, hydrogen producers *Clostridium* spp. become dominant, resulting in high hydrogen yield (Baghchehsaraee et al. 2011; Cheng et al. 2011; Lay et al. 2012). However, spore-forming homoacetogenic bacteria, which are also a main kind of hydrogen consumer, can hardly be inhibited by heat treatment (Oh et al. 2003). Furthermore, other microorganisms that are helpful to hydrogen production are also inhibited, like hydrogen producers *Bacillus* spp. and *Enterobacter* spp., cellulose degrading microbes, and so on.

3.3 Acid/Alkaline Treatment

Different pH changes the electric charge on the cell membrane, which affects enzyme activity and nutrient absorption of microbes. Studies have found that methanogens were active in a narrow pH range. Both of acid and base treatments have been used to curtail methanogenic activity of the seed sludge. As shown in Table 3.3, for acid treatment, pH ranged from 2 to 4, and duration time ranged from 30 min to 24 h, the most commonly used condition was pH = 3 for 24 h. For base treatment, pH applied fall between 10 to 12, and duration from 30 min to 24 h, the most frequently used condition was pH = 10 for 24 h. Highest hydrogen yield of 3.06 mol H_2/mol hexose was obtained with base pretreated inoculum (pH = 10, 30 min) (Zhu and Béland 2006). However, there is no consistent conclusion on whether acid or base is more efficient in enriching hydrogen producers. Chang et al. (2011) observed better hydrogen production with acid treated inoculum while

Table 3.3 pH treatments for enriching hydrogen-producing bacteria

Inoculum sources	pH	Treating time	Substrate	Operational conditions	Hydrogen yield[a]	References
Anaerobic sludge	2	24 h	Glucose (30 g/L)	35 °C	–	Lee et al. (2012a, b)
Anaerobic sludge	3	24 h	Glucose (8–20 g/L)	35–37 °C pH 6.5-7.0	0.27–1.51	Ren et al. (2008), Wang and Wan (2008), Elbeshbishy et al. (2010), Chang et al. (2011), Kan (2013), Yin et al. (2014a, b)
Anaerobic sludge	3	30 min–24 h	Sucrose (10–22 g/L)	35 °C pH 4.9–7.0	1.26–2.25	Zhu and Béland (2006), Wu and Chang (2007), Chen et al. (2009), de Sá et al. (2013)
Rumen fluid	3	24 h	Cellulose (2 g/L)	37 °C pH 7.0	18.5 mmol/g cellulose	Ratti et al. (2014)
Fresh cattle dung	4	24 h	Starch	30 °C pH 7.0	255 mL/g starch	Sen and Suttar (2012)
Anaerobic sludge	10	30 min	Sucrose (10 g/L)	35 °C pH 7.0	3.06	Zhu and Béland (2006)
Anaerobic sludge	10	24 h	Glucose (8-20 g/L)	35–37 °C pH 6.5-7.0	0.25–1.72	Wang and Wan (2008), Elbeshbishy et al. (2010), Chang et al. (2011), Kan (2013), Yin et al. (2014a, b)
Anaerobic sludge	11	24 h	Glucose (10 g/L)	35 °C pH 6.8	1.08	Ren et al. (2008)
Anaerobic sludge	12	60 min–24 h	Sucrose (10–20 g/L)	35–60 °C pH 5.5	0.51–1.96	O-Thong et al. (2009), de Sá et al. (2013)

[a]mol H_2/mol hexose except for mentioned

(Wang and Wan 2008; Yin et al. 2014a, b) and (Zhu and Béland 2006) showed both higher cumulative hydrogen production and hydrogen yield with base treated inoculum. Possible reason was the different microbial distributions present in the inoculum. Besides a few studies achieved higher hydrogen yield with pH treatment over heat treatment (Zhu and Béland 2006; Yin et al. 2014a, b), it is widely accepted that heat treatment is more effective in enhancing hydrogen production ability of inoculum.

3.4 Chemical Inhibitors

Some chemical inhibitors can selectively inhibit methanogenic activity without impacting the hydrogen production. Commonly used chemical inhibitors include bromoethanesulphonic acid (BESA), chloroform, iodopropane, and fatty acids. It has been verified that Methyl–coenzyme M reductase (MCR), the key enzyme responsible for the microbial formation of methane (Ermler et al. 1997), can be inhibited by certain amount of BESA, acetoclastic methane production is totally inhibited by 1 mmol BESA and blockage of H_2 reduction can be achieved by 50 mmol BESA (Zhu and Béland 2006). Chloroform has been reported to have a strong inhibition to methanogens, because chloroform can block the function of corrinoid enzymes and inhibit MCR (Hu and Chen 2007). High hydrogen production could be reached with iodopropane pretreated seed sludge, but the good operation did not last long (Zhu and Béland 2006). In addition, some environmental-friendly oleo chemicals were also reported to inhibit methanogens. Through disrupting cellular functions like membrane transport and metabolic enzymes, long chain fatty acids were able to control the growth of hydrogen-consuming microorganisms (Ray et al. 2009). The suppression of different fatty acids on methanogenesis was studied (Dohme et al. 2001; Shanmugam et al. 2014).

Table 3.4 summarizes the chemical inhibitors used in inhibiting hydrogen-consuming microorganisms. Chemicals studied include chloroform, 2-bromoethanesulfonic acid (BESA), iodopropane, and unsaturated fatty acids. Chloroform was the most widely studied inhibitor. Highest hydrogen yield of 2.82 mol H_2/mol hexose was achieved by with iodopropane as inhibitor. However, most of the inhibitors are not only inhibitive to hydrogen consumers but toxic to hydrogen producers and the environment, which restricts their wide application. Thus, the development of environmental-friendly deserves more attention.

3.5 Aeration

Aeration is used basing on microbial different tolerance to oxygen. As methanogens are strictly anaerobic bacteria while hydrogen producers like *Enterobacter* spp. are facultative anaerobic bacteria, controlling proper dissolved oxygen (DO) through aeration can help to inhibit methanogens and preserve hydrogen producers.

As shown in Table 3.5, the aeration conditions used include continuous aeration and repeated aeration, and aeration time ranges from 30 min to 4 d. Since the supplied oxidative stress by aeration can also inhibit strict anaerobic hydrogen producers like *Clostridium* spp., and it has also been reported that continuous aeration can hardly repress methanogenic activity completely (Zhu and Béland 2006). Lower hydrogen production was usually achieved comparing with heat-shock treated inoculum (Wang and Wan 2008; Song et al. 2012a, b). However,

Table 3.4 Chemical inhibitors for enriching hydrogen-producing bacteria

Inoculum sources	Chemical inhibitors	Treating time	Substrate	Operational conditions	Hydrogen yield[a]	References
Anaerobic sludge	Chloroform (0.05%)	17 h	Wheat (COD = 20 g/L)	37 °C, pH 7.0	0.19	Argun and Kargi (2009)
Anaerobic sludge	Chloroform (0.1%)	24 h	palm oil mill effluent (COD = 49 g/L)	35 °C, pH 5.5	0.04	Mohammadi et al. (2011)
Anaerobic sludge	Chloroform (1%)	24 h	Glucose (10 g/L)	35 °C, pH 7.0	0.61	Chang et al. (2011)
Anaerobic sludge	Chloroform (2%)	24 h	Glucose (10 g/L)	37 °C, pH 7.0	0.66	Wang and Wan (2008)
Fresh cattle dung	BESA (0.001 mol/L)	24 h	Sago starch	30 °C, pH 7.0	1.5	Sen and Suttar (2012)
Anaerobic sludge	BESA (0.01 mol/L)	30 min	Sucrose (10 g/L)	35 °C, pH 7.0	2.64	Zhu and Béland (2006)
Anaerobic sludge	BESA (0.2 mol/L)	30 min	Sucrose (20 g/L)	60°C, pH 7.0	1.01	O-Thong et al. (2009)
Anaerobic sludge	Iodopropane (0.01 mol/L)	30 min	Sucrose (10 g/L)	35 °C, pH 7.0	2.82	Zhu and Béland, (2006)
Anaerobic sludge	Unsaturated long fatty acids (C_{18}, 2 g/L)	During fermentation	Glucose (5 g/L)	21 °C, pH 7.6–8.0	1.1–1.3	Ray et al. (2009)

[a]mol H_2/mol hexose except for mentioned

Table 3.5 Aeration as treatment method for enriching hydrogen-producing bacteria

Inoculum sources	Treating conditions	Substrate	Hydrogen yield[a]	References
Anaerobic sludge	Control DO < 0.5 mg/L 12 h	Glucose (10 g/L)	1.96	Ren et al. (2008)
Anaerobic sludge	Continuous aeration 24 h	Glucose (10 g/L)	0.86	Wang and Wan (2008)
Anaerobic sludge	Continuous aeration 30 min	Sucrose (10 g/L)	1.86	Zhu and Béland (2006)
Compost	Continuous aeration 72 h	Sucrose (10 g/L)	1.95	Song et al. (2012a, b)
Compost	Continuous aeration 24 h	Corn stalk	205 mL/g TVS	Xing et al. (2011)
Compost	Continuous aeration 4 d	Stale corn	250 mL/g substrate	Wang et al. (2012)

[a]mol H_2/mol hexose except for mentioned

Ren et al. explored the effect of controlling dissolved oxygen (DO) through repeated aeration on hydrogen production, achieved hydrogen yield of 1.96 mol H_2/mol glucose and examined novel genus *Ethanoligenens*. Indicating some specific hydrogen-producing genus can be enriched through aeration treatment.

Bellucci et al. (2016) applied different treatments to suppress the hydrogen consumers in inoculum and examined the inhibition effect of 5-hydroxymethylfurfural (HMF) on hydrogen production. As shown in Fig. 3.3, hydrogen production in aeration treated system was the most efficient, and the HMF had little effects in acid treated system. In this case, aeration showed better effect in enhancing the hydrogen production by mixed culture than acid and heat treatment.

3.6 Other Treatments

Some other pretreatment methods were also studied to enhance hydrogen production ability of inoculum. These methods are not as widely used as the methods described above, but these explorations can also give us inspirations in choosing pretreatment methods.

3.6.1 Ultrasonication

Ultrasonication is the act of applying sound energy to agitate particles in a sample. Figure 3.4a shows the details of typical 20 kHz piezoelectric ultrasonic system, and it works when it is immerged into the samples.

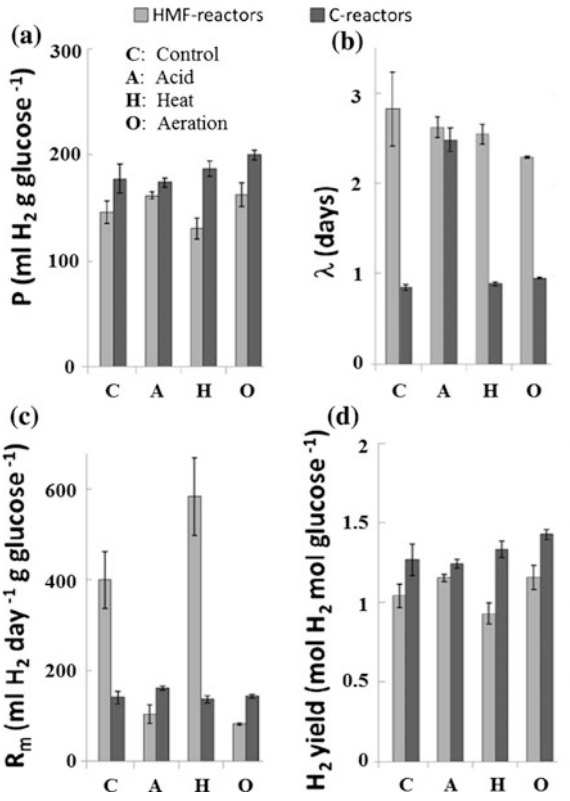

Fig. 3.3 Maximum cumulative H_2 production (P) (**a**), lag phase (λ) (**b**), maximum H_2 production rate (Rm) (**c**), and H_2 yield (**d**). C reactors: Control; HMF reactors: HMF inhibited. (Bellucci et al. 2016)

Ultrasonication is a mechanical pretreatment method, but it owns both physical and chemical effects. When ultrasound wave propagates in a medium, acoustic streaming forms, leading to formation and collapse of micro bubbles, generating shear forces, high localized temperature (5000 K) and pressure (180 MPa), and highly active radicals. As shown in Fig. 3.4b, acoustic streaming forms in three regions. Region I is the largest region, and it is the furthest from the work face of ultrasonic system. Circulating currents formed in region I are defined by the shape of the container and the size of the wavelength of the acoustic wave in the liquid. Region II is near the work face, and the size and shape of circulating currents defined by the acoustic tooling. Region III is adjacent to the fluid acoustic boundary layer. In this region, the tangential fluid velocity is near the velocity of the work face of ultrasonic system. All three regions play a critical role in mixing of the fluid.

Ultrasonication has been used for solubilizing solid wastes for further degradation (Tiehm et al. 1997; Kim et al. 2003; Guo et al. 2008; Kotay and Das 2009; Wang et al. 2010). Thus, by controlling the energy input to the inoculum, ultrasonication was used in suppressing methanogenic bacteria and preserving spore-forming hydrogen producers (Elbeshbishy et al. 2010; Dhar et al. 2012).

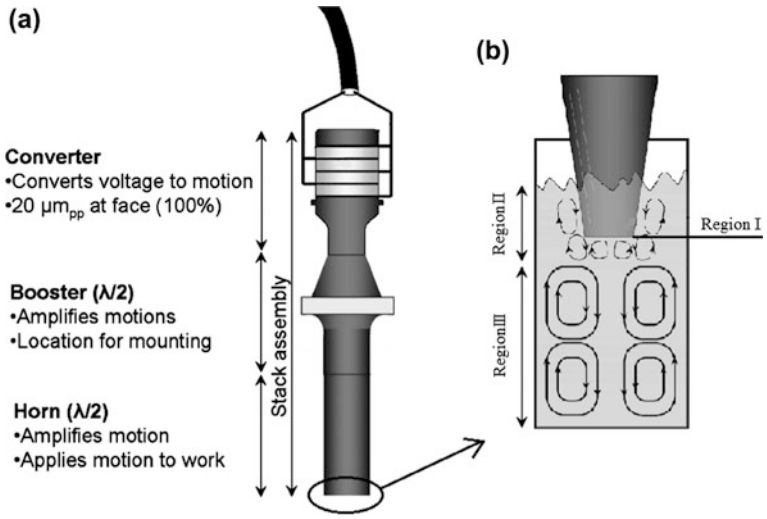

Fig. 3.4 Details of typical 20 kHz piezoelectric ultrasonic system

Boboescu et al. (2014) enriched anaerobic mixed consortia sampled from various environments and used as inocula for hydrogen production. The hydrogen generation capability showed dependence on both consortia source and pretreatment methods. Sludge from wastewater treatment plant and ultrasonication treatment was proved to be the best inoculum source and pretreatment method for enriching the hydrogen producers.

Elbeshbishy et al. (2010) examined the effect of treating time and temperature on enrichment efficiency of ultrasonication. As shown in Fig. 3.5a, cumulative hydrogen production increased with the increase of sonication time from 0 to 40 min, but when sonication time over 20 min, enhancement became less. Figure 3.5b shows that temperature control during the sonication can significantly enhance the ultrasonication efficiency in treating mixed culture, and it is prior than commonly used heat, acid, and base treatment.

3.6.2 Freezing and Thawing

Freezing and thawing involves freezing sludge at extreme temperature and thawing at room temperature for several cycles. Since strong fluctuations in temperature can lead to the intracellular formation of ice crystals and cell swelling, freezing, and thawing can damage microbial cells and disrupt cell aggregates (Sawicka et al. 2010). However, low hydrogen yield of 0.04–0.17 mol H_2/mol hexose was obtained by freezing and thawing treated mixed culture (Table 3.6). Mohammadi et al. and Liu et al. compared a serious pretreatment methods include freezing and

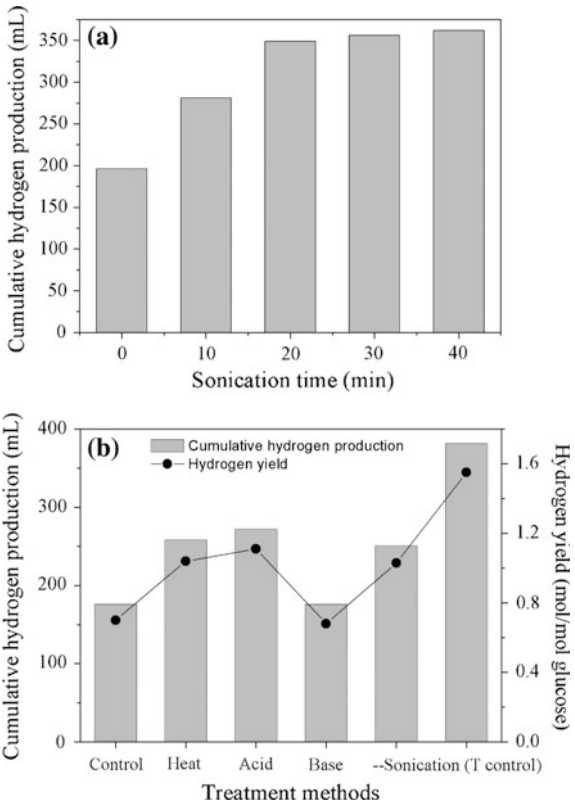

Fig. 3.5 Cumulative hydrogen production for optimizing the sonication time (**a**); Hydrogen production for different treatment methods (**b**). (Elbeshbishy et al. 2010)

thawing, chemical, acid, base and heat-shock, freezing, and thawing treated sludge showed no advantage in hydrogen production (Liu et al. 2009a, b; Mohammadi et al. 2011).

3.6.3 Electric Treatment

Electric current shocking was found to have the capacity of destructing cell membranes (Zimmermann et al. 1974). Although little is known about what exactly happened inside cells, a widely accepted hypothesis is that when critical electrical field strength is exceeded, pores form in cell membranes, leading to the microbial permeabilization (Lojewska et al. 1989). It is generally accepted that the critical electrical field strength causing microbial inactivation is about 1 V (Rowan et al. 2000). Park et al. examined the lethal effects of low-amperage electric treatment (0.5 A, 12 V) on microorganisms (Park et al. 2003). Roychowdhury observed the inactivation of methane production when low-voltage (3.0–4.5 V) electric current was applied to the anaerobically decomposed organic materials (Roychowdhury

Table 3.6 Other treatment methods for enriching hydrogen-producing bacteria

Inoculum sources	Treating methods	Treating conditions	Substrate	Operational conditions	Hydrogen yield[a]	References
Anaerobic sludge	Ultrasonication	20–79 kJ/g TS	Glucose (8–8.5 g/L)	37 °C, pH = 6.5–6.8	1.03–1.7	Elbeshbishy et al. (2010), Dhar et al. (2012)
Anaerobic sludge	Freeze and thaw	–25 °C (24 h); Room temperature (5 h)	Glucose (20 g/L)	37 °C, pH = 7.2	0.17	Liu et al. (2009a, b)
Anaerobic sludge	Freeze and thaw	–10 °C (24 h); 30 °C to Room temperature	Palm oil mill effluent (COD = 49 g/L)	35 °C, pH = 5.5	0.04	Mohammadi et al. (2011)
Anaerobic sludge	Electric current	10 V, 10 min	Glucose (20 g/L)	37 °C, pH = 7.0	1.43	Jeong et al. (2013)
Anaerobic sludge	Load-shock	COD = 83 g/L, 2d	Sucrose (20 g/L)	60 °C, pH = 5.5	1.57	O-Thong et al. (2009)
Anaerobic sludge	Load-shock	COD = 50 g/L, 3 d	Waste water (COD = 3 g/L)	28 °C, pH = 6.0	3.17	Kannaiah Goud and Venkata Mohan (2012)
Anaerobic sludge	Microwave	900 W, 2 min	Waste sludge (COD = 19.5 g/L)	35 °C, pH = 7.9	14.2 mL/g VSS	Guo et al. (2015)
Compost	Microwave	2450 W, 1.5 min	Maize straws (20 g/L)	36 °C, pH = 7.0	144.3 mL/g substrate	Song et al. (2012a, b)
Compost	Microwave	325 W, 5 min	Petha waste (Sugar = 14 mg/L)	Room temperature, pH = 7.0	14 mmol/mol sugar	Singhal and Singh (2014)
Anaerobic sludge	UV radiation	15 min	Apple pomace (15 g/L)	36 °C, pH = 5.0	107 mL/g TS	Wang et al. (2010)
Compost	UV radiation	3 h	Stale corn (10 g/L)	36 °C, pH = 7.0	257 mL/g substrate	Wang et al. (2012)

[a]mol H_2/mol hexose except for mentioned

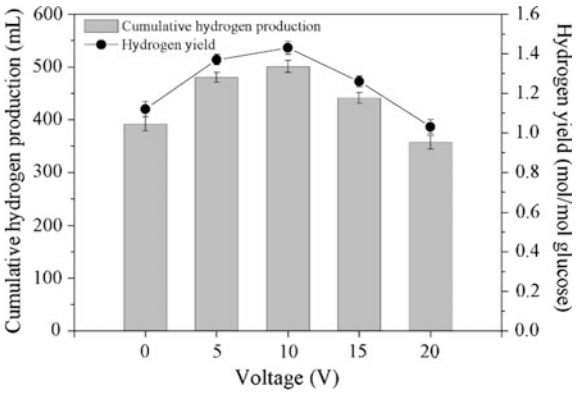

Fig. 3.6 Cumulative H_2 production from batch fermenter treated by different pretreatments (Jeong et al. 2013)

2006). Then, Jeong verified the feasibility of electric current as a pretreatment method for inoculum preparation in dark fermentative hydrogen production, and highest hydrogen yield of 1.43 mol H_2/mol hexose was obtained at 10 V (Jeong et al. 2013).

Jeong et al. (2013) examined the effect of various voltages (5–20 V) on electric treatment in enriching hydrogen producers and compared it with heat treatment. The results showed that both the highest H_2 yield of 1.43 mol H_2/mol hexose and highest production rate 101.4 mL H_2/L/h were obtained at 10 V. Microbial analysis confirmed that only hydrogen-producing bacteria was detected, indicating the electric current can suppress nonhydrogen producers efficiently (Fig. 3.6).

3.6.4 Microwave Treatment

Microwaves are a kind of electromagnetic radiation with frequencies range from 0.3 GHz to 300 GHz and wave lengths in air from 1 to 0.0001 m. When microwaves pass through a product, a "dielectric" is formed inside the product, polar molecules inside the product (like water) try to align themselves with the polarity of the electric field. A chaotic movement of molecules happens when the polarity keeps changing. Then, the kinetic energy and friction caused by collisions between adjacent molecules generates heat within the product (Piyasena et al. 2003; Hong et al. 2006; Dańczuk and Łomotowski 2010). Microwaves have been proved to be effective in inactivating microorganisms, and its influence on microorganisms comprises thermal and nonthermal effect (Vela and Wu 1979; Jeng et al. 1987).

For the thermal effect, as shown in Fig. 3.7, conventional heating works by creating high temperature, but uneven heating usually happens, either insufficient heat inside sample or overheating on the surface happens. Otherwise, for the microwave irradiation, heat is generated throughout the sample, which has higher efficiency in achieving the required heat. Furthermore, the nonthermal effect also benefits in suppressing microbes. Kuglarz et al. compared microwave and thermal

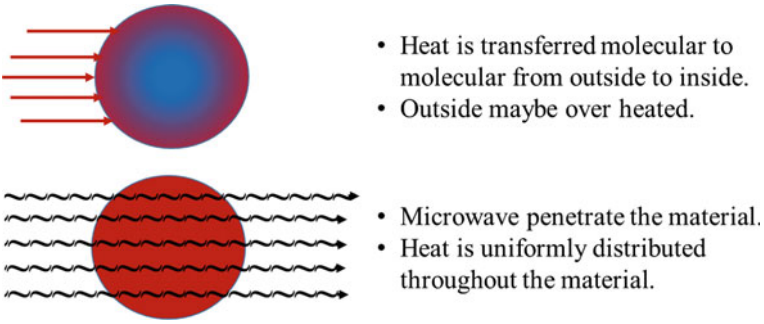

Fig. 3.7 Difference between conventional heating and microwave heating

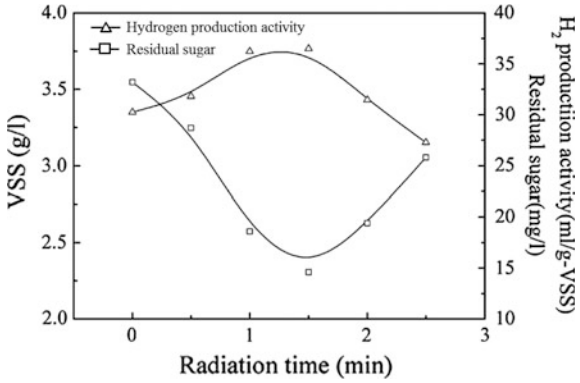

Fig. 3.8 Effect of microwave radiation time on various fermentation parameters

treatment at same temperature, and microwave treatment showed superior effect over thermal treatment with respect to cell solubilization (Kuglarz et al. 2013).

The effectiveness of microwaves in destructing microorganisms depends on the time of exposure and the power of the electromagnetic field used. Frequency of microwave used in enriching hydrogen producers is 2450 MHz, treating power and time varies according to different characteristics of inoculum sources, and *Clostridium* species were found to be dominant in microwave-treated mixed culture (Song et al. 2012a, b; Singhal and Singh 2014; Guo et al. 2015). Song et al. (2012a, b) examined the effect of reaction time on treatment efficiency of microwave, the results showed that VSS was significant increased with the increase of treating time, while best hydrogen production was obtained at 1.5 min (Fig. 3.8). Guo et al. compared effect of different treatment methods include microwave, thermal and multi-enzyme on hydrogen production from waste sludge, highest hydrogen yield of 14.2 mL H_2/g VSS was obtained from microwave treated sludge (Guo et al. 2015). However, microwave is not widely used in inactivating nonhydrogen

producers, possible reason is that microwave can cause the extraction of toxic contaminants exist in the inocula, which is deleterious to hydrogen-producing bacteria (Guo et al. 2008).

3.6.5 *Ionizing Radiation Treatment*

Ionizing radiation comprises gamma rays, X-rays, and the higher ultraviolet part of the electromagnetic spectrum. Ionizing radiation has both direct and indirect effect on microorganism. As shown in Fig. 3.9, when microbes are irradiated by ionizing radiation, high-energy rays may directly break microbial genes and enzymes, leading to the death, or mutation. Besides the direct action, when high-energy rays go through water or aqueous solutions, high reactive chemical species are formed (Eq. 3.1), these highly reactive products can react with other substances exist in the solutions, including the cellular material present in liquid phase.

$$H_2O \underset{100eV}{\rightarrow} [2.7] \bullet OH + [2.6]H_3O^+ + [0.7]H_2O_2 + [0.45]H_2 \tag{3.1}$$

Due to the low core water content of the spores, less hydroxyl radicals are generated inside microbes during ionizing irradiation process, thus, spore-form bacteria are expected to be more likely to survive under ionizing irradiation (Gould 1983; Setlow 2006; Skowron et al. 2014). Yin et al. verified the feasibility of gamma irradiation as a pretreatment method in enriching hydrogen producers, and 5 kGy gamma irradiation dose treated sludge showed superiority over widely used

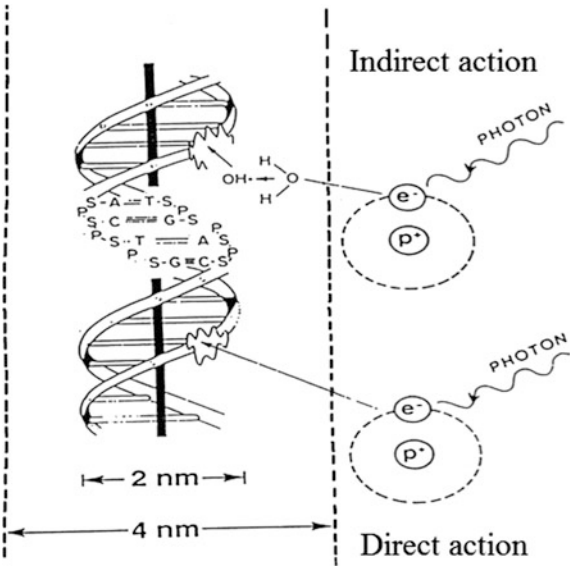

Fig. 3.9 Effect of ionizing radiation on microorganisms

heat-shock, acid and base treatment in both hydrogen yield and cumulative hydrogen production (Yin et al. 2014a, b; Yin and Wang 2016a, b). Microbial analysis demonstrated the treated sludge was dominated by genus *Clostridium* (Yin and Wang 2016a, b).

3.6.6 Ultraviolet (UV) Radiation

Ultraviolet (UV) radiation can denature the DNA of microorganisms, leading to the death or inactivation of cells (Sharrer et al. 2005). Inactivation of microorganisms by UV radiation follows approximately first-order kinetics with respect to UV intensity (White 2010). Studies found that proper dose of UV irradiation could kill the methanogens and other hydrogen-consuming bacteria while preserving the spore-forming hydrogen producers, and UV radiation showed superiority over ultrasonication and aeration (Wang et al. 2010; Wang et al. 2012).

3.6.7 Load-Shock Treatment

Load-shock treatment works by exposing inoculum to a high organic loading for a couple of days, leading to a rapid generation, and accumulation of organic acids and pH decrease. During this process, methanogens are inhibited from two aspects: the feed-back inhibition of methanogenesis caused by the accumulation of methanogenic substrates (various VFA, CO_2, and H_2); low pH inhibition. Comparing with the widely used treatment methods like heat-shock, pH adjustment, etc., methanogens are indirectly inhibited in load-shock process, higher quantity of microorganisms was observed (Kannaiah Goud and Venkata Mohan 2012). Basing on this phenomenon, highest hydrogen production rate was observed by load-shock treated inoculum (O-Thong et al. 2009). However, Goud and Mohan found that load-shock cannot eliminate methanogens completely, methanogenic activity of system regained after 160 d operation (Kannaiah Goud and Venkata Mohan 2012).

3.6.8 Operational Condition Control

Besides treating the inoculum previously, inactivation of nonhydrogen producers can also be achieved during the fermentation process by controlling operational conditions. On the basis of methanogenic biomass has longer generation time comparing with hydrogen producers, low hydraulic retention time (HRT) was adopted in continuous operation to wash out the methanogens. Tapia et al. applied HRT from 14 to 6 h, and highest hydrogen yield was 2.7 mol H_2/mol glucose at an HRT = 12 h (Tapia-Venegas et al. 2013). Since methanogens could be suppressed

by product inhibition during the fermentation: Bastidas et al. operated the reactor without pH control for a week until the methane production stopped, a hydrogen yield of 3.25 mol H_2/mol glucose was obtained in the subsequent operation, which was close to the theoretical value of 4 mol H_2/mol glucose (Bastidas-Oyanedel et al. 2010; Bastidas-Oyanedel et al. 2012). Hydrogen production operated in thermophilic (55 °C) and hyperthermophilic (70 °C) conditions usually omit the inoculum treatment process for the reason that methanogens can be suppressed in these harsh operational conditions (Calli et al. 2008; Liu et al. 2008; Zhao et al. 2009; Kargi et al. 2012a, b).

3.7 Combined Treatments

As it is difficult to eliminate hydrogen consumers with a single pretreatment method in some cases, combination of different methods are required. As shown in Table 3.7, heat-shock is the most popularly used in combined treatments. Mohan et al. found that heat-shock combined with acid treatment achieved higher hydrogen yield over heat-shock and acid alone as treatment method (Mohan et al. 2008). However, quite a few studies observed negative effect of other treatment on heat-shock treated inoculum. Argun and Kargi compared heat-shock, chloroform, and combination of heat-shock and chloroform, combined treatment showed no advantage over heat-shock treatment (Argun and Kargi 2009). Elbeshbishy et al. combined heat-shock and ultrasonication as treatment method, but hydrogen yield was significantly decreased comparing with tests used heat-shock and ultrasonication alone (Elbeshbishy et al. 2010). Studies by Mohan et al. also observed inhibition of hydrogen production when heat-shock was combined with chemical treatment (Mohan et al. 2008). Boboescu et al. found that the hydrogen production ability of the group treated by combined methods did not recover even after the enrichment process (Boboescu et al. 2014), indicating that the combined strengths of different treatments can cause irreversible deterioration to microorganisms as well as hydrogen producers, affecting the hydrogen production ability of treated inocula.

Mohan et al. (2008) evaluated the influence of different combined treatment methods on anaerobic mixed inoculum for selectively enriching the hydrogen producers. The results showed that the efficiency of hydrogen evolution and substrate removal efficiency were dependent on the treatment methods. Among the studied pretreatment methods including pH, heat, chemical, combined pH-heat, chemical-heat, pH-chemical and pH-heat-chemical, chemical pretreatment (2-bromoethane sulphonic acid sodium salt) enabled the highest hydrogen yield and substrate removal efficiency. In the case of combined treatment, integration of pH (pH 3) and chemical pretreatment evidenced higher hydrogen production.

Table 3.7 Combined treatment methods for enriching hydrogen-producing bacteria

Inoculum source	Combined treatment	Substrate	Hydrogen yield[a]	References
Anaerobic sludge	Heat-shock: 100 °C, 15 min Aeration: 4 min	Glucose (7.5 g/L)	1.83	LI et al. (2008)
Anaerobic sludge	Heat-shock: 77 °C, 20 min Ultrasounication: 79 kJ/g TS	Glucose (10 g/L)	1.03	Elbeshbishy et al. (2010)
Anaerobic sludge	Heat-shock: 121 °C, 15 min Base: pH = 8.93, 24 h	Glucose (10 g/L)	1.35	Faloye et al. (2013)
Anaerobic sludge	Heat-shock: 89 °C, 63 min Base: pH = 8.36, 24 h	Glucose (10 g/L)	0.78	Faloye et al. (2013)
Anaerobic sludge	Heat-shock: 100 °C, 30 min Base: pH = 10, 24 h	Glucose (10 g/L)	2.45	Liu et al. (2012)
Anaerobic sludge	Heat-shock: 100 °C, 15 min Base: pH = 10, 24 h	Glucose (20 g/L)	0.6	Kan (2013)
Anaerobic sludge	Heat-shock: 100 °C, 15 min Acid: pH = 3, 24 h	Glucose (20 g/L)	0.85	Kan (2013)
Anaerobic sludge	Heat-shock: 100 °C, 1 h Acid: pH = 3, 24 h	Dairy wastewater (COD = 10.4 g/L)	0.004	Mohan et al. (2008)
Anaerobic sludge	Heat-shock: 100 °C, 1 h Acid: pH = 5.9, 1 h	Hydrolyzed wheat starch (20 g/L)	2.4	Cakır et al. (2010)
Anaerobic sludge	Heat-shock: 100 °C, 1 h Chemical: BESA, 0.2 g/L, 24 h	Dairy wastewater (COD = 10.4 g/L)	0.002	Mohan et al. (2008)
Anaerobic sludge	Heat-shock: 100 °C, 5 h, repeated 2 times Chemical: chloroform, 0.05%, 17 h	Waste wheat (20 g/L)	0.44-0.51	Argun and Kargi (2009)
Anaerobic sludge	Heat-shock 100 °C, 15 min Freeze and thaw: –20 °C to frozen; defrost and keep at 4 °C for 2 h	Glycerol (10 g/L)	0.70	Seifert et al. (2009)
Anaerobic sludge	Base: pH = 11, 24 h Microwave: 860 W, 2 min	Glucose (10 g/L)	1.78	Faloye et al. (2014)
Anaerobic sludge	Acid: pH = 3, 24 h Chemical: BESA, 0.2 g/L, 24 h	Dairy wastewater (COD = 10.4 g/L)	0.006	Mohan et al. (2008)
Anaerobic sludge	Heat-shock: 70 °C, 1 h Acid: pH = 3, 24 h Ultrasounication: 24 kHz, 30 min	Synthetic wastewater (Glucose = 3.7 g/L)	–	Boboescu et al. (2014)

(continued)

Table 3.7 (continued)

Inoculum source	Combined treatment	Substrate	Hydrogen yield[a]	References
Anaerobic sludge	Heat-shock: 100 °C, 2 h Acid: pH = 3, 24 h Chemical: BESA, 0.2 g/L, 24 h	Citrus limetta peelings (COD = 52–60 g/L)	0.20–0.68	Venkata Mohan et al. (2009)
Anaerobic sludge	Heat-shock: 100 °C, 1 h Acid: pH = 3, 24 h Chemical: BESA, 0.2 g/L, 24 h	Dairy wastewater (COD = 10.4 g/L)	0.002	Mohan et al. (2008)
Cow manure	Heat-shock: 100 °C, 30 min Acid: 0.2% HCl	Cow manure (TS = 100 g/L)	18 mL/g TVS	Xing et al. (2010)
Cow manure	Heat-shock: 100 °C, 30 min Base: 0.2% NaOH	Cow manure (TS = 100 g/L)	14 mL/g TVS	Xing et al. (2010)
Swine manure	Microwave: 140 °C, 15 min Acid: 1% H_2SO_4	Swine manure (TS = 50 g/L)	71.8 mL/g TVS	Cheng et al. (2014)

[a]mol H_2/mol hexose except for mentioned

3.8 Effect of Pretreatment Methods on Microbial Community

Since hydrogen production efficiency depends on the metabolic pathway happened in microbes, which is closely related to the microbial distribution present in the system. Many studies have examined the relationship between structure of microbial communities and the performance of hydrogen production process (de Sa et al. 2013; Dhar et al. 2012; Dohme et al. 2001; Elbeshbishy et al. 2010; Ermler et al. 1997; Faloye et al. 2013, 2014). Through exploring the effects of pretreatment methods on dominant microbial community, more knowledge can be obtained to help us choose an appropriate treatment method.

Table 3.8 summarizes different treatment methods and the corresponding dominant microbial communities present in hydrogen production systems. It can be seen that *Clostridium* species can survive most treatment methods like heat-shock, pH adjustment, aeration, gamma radiation, and so on. *Enterobacter* species mainly present in heat-shock and aeration treated inoculum. *Bacillus* species were detected in heat-shock, acid, chemical, and microwave treated system. It worth mentioning that besides the treatment methods, inoculum source also has a great effect on microbial distribution. For example, *Enterobacter* species are discovered in all treated samples using compost as inoculum.

Table 3.8 Dominant microbial community after different pretreatment methods

Sludge sources	Treatment method	Dominant microbial community	References
Anaerobic sludge	Heat-shock: 65–100 °C, 20–30 min	*Clostridium* sp.	Gould 1983a, b, 175]
Anaerobic sludge	Heat-shock: 95–100 °C, 1 h	*Clostridium butyricum*; *Klebsiella oxytoca*	Dhar et al. (2012)
Cow dung compost	Heat-shock: 100 °C, 30 min	*Clostridium* sp.; *Enterobacter* sp.	Faloye et al. (2014)
Cow dung compost	Heat-shock: baked in an infrared oven for 2 h	*Clostridium* sp.; *Enterobacter* sp.	Faloye et al. (2014)
Anaerobic sludge	Heat-shock: 121, 20 min	*Bacillus coagulans*; *Thermoanerobacter* sp.; *Alicyclobacillus acidocalvarius*	Kapdan and Kargi (2006)
Anaerobic sludge	Acid: pH 2–4, 24 h	*Clostridium* sp.	[88, 176]
Anaerobic sludge	Acid: pH 3–4, 24 h	*Bacillus coagulans*; *Acetivibrio cellulolyticus*; *Acetivibrio. acidocalvarius*	Kapdan and Kargi (2006)
Anaerobic sludge	Acid: pH 3, 24 h	*Clostridium tyrobutyricum*; *Clostridium longisporum*	Ermler et al. (1997)
Anaerobic sludge	Base: pH 12, 24 h	*Clostridium* sp.	Liu et al. (2009a)
Anaerobic sludge	Base: pH 10, 24 h	*Ruminococcus gnavus*; *Clostridium pasteurinum*; *Clostridium ramosum*	[177]
Anaerobic sludge	Base: pH 11, 24 h	*Clostridium tyrobutyricum*; Clostridium *vincentii*; *Bacteroides vulgatus*	Ermler et al. (1997)
Anaerobic sludge	Chemical: BESA, 10 mmol/L, 30 min	*Bacillus* sp	Liu et al. (2009a)
Cow dung compost	Aeration: Continuous, 72 h	*Clostridium* sp.; *Enterobacter* sp.	Faloye et al. (2014)
Anaerobic sludge	Aeration: Continuous, 7 d	*Clostridium* sp.; *Bacteriodes* sp.; *Propionibacterium* sp.; *Fusobacterium* sp.	[178]
Anaerobic sludge	Aeration: Repeated DO < 0.5 mg/L, 12 h	*Ethanoligenens harbinens*; *Enterobacter aerogenes*; *Ethanoligenens harbinens*; *Bacteroides vulgatus*	Ermler et al. (1997)
Cow dung compost	Microwave: 2450 W, 1.5 min	*Bacillus amyloliquefaciens*; *Bacillus licheniformis*; *Bacillus subtilis*; *Enterococcus faecium*	Faloye et al. (2013)
Anaerobic sludge	Electric current: 10 V, 10 min	*Clostridium sardiniense*; *Clostridium saccharobutylicum*; *Clostridium butyricum*; *Clostridium beijerinckii*; *Clostridium saccharobutylicum*; *Clostridium* sp.	de Sa et al. (2013)
Anaerobic sludge	Load-shock: 50 g COD/L, 3 d	*Firmicutes*	Liu et al. (2009a)
Anaerobic sludge	Gamma radiation: 5 kGy	*Clostridium* sp.	Chen et al. (2012)

(continued)

Table 3.8 (continued)

Sludge sources	Treatment method	Dominant microbial community	References
Anaerobic sludge	Microwave: 860 W, 2 min Base: pH = 11, 24 h	*Clostridium* sp.	[172]
Anaerobic sludge	Heat-shock: 121, 20 min Chemical: BESA, 10 mmol/L, 24 h	*Bacillus coagulans*	Kapdan and Kargi (2006)
Anaerobic sludge	Heat-shock Acid	*Clostridium pasteurianum*; *Streptococcus* sp.; *Propionibacterium* sp.	Elbeshbishy et al. (2010)

3.9 Comparison of Different Pretreatment Methods

It can be concluded that heat, acid and base treatments were the most commonly used methods, followed by aeration and methanogens inhibitors. Newly developed methods and reaction conditions control were not widely used, but have potential for further development. Since no agreement is reached on the best treatment method. Many studies have tried to compare different treatment methods. Table 3.9 summarizes some comparisons of different treatment methods.

Table 3.9 Comparison of different pretreatment methods

Hydrogen yield[a]					References
Heat	Acid	Base	Chemical inhibitors	Others	
1.16	0.65	0.51	1.01 (BESA)	1.96 (Load shock)	O-Thong et al. (2009)
1.52	0.42	1.08	1.80 (Aeration)	–	Ren et al. (2008)
1.78	0.80	1.10	0.66(chloroform)	0.86 (Aeration)	Wang and Wan (2008)
1.59	–	3.06	1.82 (BESA) 1.20 (Iodopropane)	–	Zhu and Béland (2006)
1.04	1.11	0.68	–	1.55 (Ultrasounication)	Elbeshbishy et al. (2010)
1.39	1.19	1.72		2.15 (Ionizing radiation)	Yin et al. (2014a, b)
0.9	0.27	0.25	–	–	Kan (2013)
2.34	1.84	–	1.61 (BESA)	–	Sen and Suttar (2012)
1.00	1.00	–	–	–	Penteado et al. (2013)
0.52	–	–	0.19 (Chloroform)	–	Argun and Kargi (2009)
2.22	–	–	–	1.96 (Aeration)	Song et al. (2012a, b)
–	–	–	–	257 mL/g stale corn (UV radiation) 250 mL/g stale corn (Aeration)	Wang et al. (2012)

[a]mol H_2/mol hexose except for mentioned

Wang et al. compared the effect of heat treatment, acid, base, aeration, and chloroform treatment on hydrogen production, better performance was obtained from heat treated group (Wang and Wan 2008). Similar conclusions were obtained by different researchers (Argun and Kargi 2009; Sen and Suttar 2012; Song et al. 2012a, b; Kan 2013). However, although heat treatment can lead to the repression of methanogenic activity, it can also partially repress hydrogen producers. Furthermore, spore-forming homoacetogenic bacteria may still remain active in heat treated cultures (Zhu and Béland 2006; Argun and Kargi 2009). Zhu et al. found that when the treated inocula were used in second batch, performance of all inocula dropped except heat and base treated ones. Hydrogen yield of base treated group increased dramatically from 1.44 to 6.12 mol/mol sucrose, indicating that base treated inocula were more preferable in long time repeated use (Zhu and Béland 2006; Argun and Kargi 2009). Besides heat-shock and base, different pretreatment methods were also regarded as the best one in different studies, such as acid (Ratti et al. 2014), chloroform (Hu and Chen 2007), aeration (Ren et al. 2008), UV radiation (Wang et al. 2012), load-shock (O-Thong et al. 2009) and ionizing radiation (Yin et al. 2014a, b), which may due to the different inoculum sources (Boboescu et al. 2014). Therefore, to evaluate the performance of pretreatment methods, quite a few factors need to be considered, like inoculum sources, substrate types, and operational conditions.

Besides the hydrogen production performance, operability is very important considering their industrial applications. For industrial scale application, the economics of heat treatment still need to be evaluated. The acid and base treated inocula need to be neutralized before the utilization. Some inhibitors like chloroform, BESA are not practical for their contamination of receiving water bodies, environmental-friendly fatty acids that are capable of inhibiting methanogens seems to have more development potential. Aeration is a good choice for their economics and easy operation. The application of ionizing radiation is restricted by limitation of the radiation source. Newly developed methods like ultrasonication, microwave, UV radiation and load-shock owns quite a few advantages in practical application, and further studies are advised.

Although many pretreatment methods have been extensively studied, there is no agreement on which method is universally appropriate, lots of research works are still needed.

Heat treatment is the most widely studied one, but the energy consumption and low efficiency in eliminating homoacetogens are the “limiting factors” in its application. The combination of different treatments can remedy the limitation of sole treatment. Like combination of heat-shock and acid/base treatment, heat-shock and methanogen inhibitors, microwave, and base treatment, and so on. Combinations of heat-shock and chemical methods showed more potential, which can not only reduce the temperature or duration needed for heat-shock, reducing the energy consumption, but can eliminate hydrogen consumers more efficiently. However, conditions of combined treatment suitable for different inoculum sources are still variable. To give references in determining treatment conditions, more efforts on mechanisms of different treatment methods are recommended, especially

the effects of pretreatment on the variation of microbial communities. Changes in microbial distribution and metabolic pathways brought by pretreatments can be further studied using modern molecular biological approaches.

3.10 Gamma Irradiation for Enriching Hydrogen-Producer

3.10.1 Overview

Pretreatment methods have been reported for enriching hydrogen-producing bacteria from seed sludge mainly include heart-shock (Davila-Vazquez et al. 2008; Wang and Wan 2008; Chu et al. 2012; de Sá et al. 2013; Kan 2013); addition of chemical inhibitors such as acid (Chen et al. 2009; La Licata et al. 2011; Lee et al. 2012a, b), alkali (Wang and Wan 2008; O-Thong et al. 2009; de Sá et al. 2013), chloroform (Wang and Wan 2008; Argun and Kargi 2009), 2-bromoethanesulfonic acid (BESA) (O-Thong et al. 2009), etc.; ultrasonication (Elbeshbishy et al. 2010; Dhar et al. 2012) and aeration (Wang and Wan 2008; Xing et al. 2011; Song et al. 2012a, b).

Nowadays, gamma irradiation is more and more widely used in the field of environmental governance (Luchini et al. 1999; Solpan and Guven 2002; Liu et al. 2011; Zheng et al. 2011).

When pure water or aqueous solutions are irradiated by gamma irradiation, reaction (3-1) happens (Spinks and Woods 1990). High reactivity is characteristic of these radiolysis products, these products' reactions with other substances typically require less than 1 us.

Researches have shown that gamma irradiation is an effective method for the removal of some species (Wang and Wang 2007; Wang and Xu 2012) and our research group has studied applying gamma irradiation to the degradation of chlorophenols (CPs) (Hu et al. 2006; Xue and Wang 2008; Peng et al. 2012)and sulfamethazine(Liu and Wang 2013). However, an extensive literature search indicating that there is little research about adopting ionizing radiation as pretreatment method for enriching hydrogen-producing bacteria from seed sludge.

Similar to their resistance to harsh conditions, spore-form bacteria are expected to be more likely to survive under gamma irradiation. Although the reasons for spores' resistance to ionizing radiation is not all clear, it may be spores' low core water content reduces the ability of gamma irradiation to generate damaging hydroxyl radicals(Setlow 2006), researches have shown that spores are more resistant to ionizing (γ) radiation than growing cells (Roberts and Hitchins 1969; Gould 1983; Setlow 2006).

3.10.2 *Effect of Dose on Hydrogen Production*

Figure 3.10 the effect of different irradiation doses pretreated seed sludge on the cumulative hydrogen production from glucose at substrate concentration of 10 g/L and initial pH 7.0. The result demonstrates that after 48 h reaction, cumulative hydrogen production of all tests reached the maximum. Basing on the data showed in Fig. 3.10, the kinetic of hydrogen production can be fitted with the modified Gompertz model, and the main results are listed in Table 3.10.

The gaseous products of tests pretreated with different irradiation doses were examined with Gas Chromatogram and it showed that the process of anaerobic

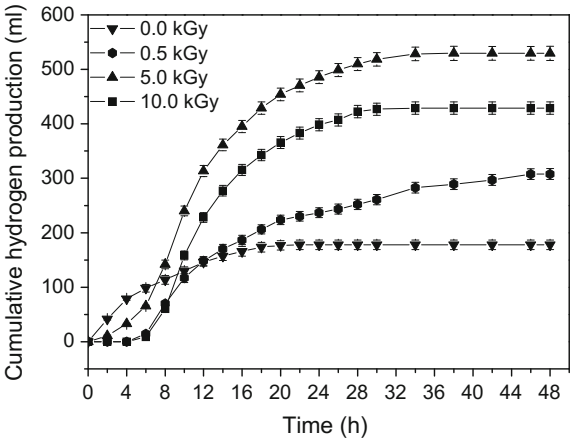

Fig. 3.10 Cumulative hydrogen production for different irradiation doses pretreatment Of all the tests, irradiation pretreatment with 5.0 kGy dose achieved maximum cumulative hydrogen production 529.4 ml and highest maximum hydrogen production rate of 37.25 ml/h, followed by 10.0 kGy (429.0 ml, 35.15 ml/h) and 0.5.0 kGy (307.8 ml, 15.40 ml/h), the control test with 0.0 kGy dose has got the least cumulative hydrogen production and lowest production rate with 177.9 ml and 14.33 ml/h. The effects of different irradiation doses of seed sludge on hydrogen production ability ranked as: 5.0 kGy > 10.0 kGy > 0.5 kGy > 0.0 kGy. That is to say irradiation with 5.0 kGy dose is more effective on enriching hydrogen-producing bacteria. The dose of 10 kGy was too high to be used in seed sludge pretreatment, it probably constrained hydrogen-producing bacteria along with inactivating hydrogen-consuming bacteria. Otherwise, the dose of 0.5 kGy apparently was too low to achieve the goal of inhibiting hydrogen-consuming organisms ultimately

Table 3.10 Parameters of the modified Gompertz model for hydrogen production

Pretreatment method (kGy)	P(ml)	R_m(ml/h)	λ(h)	R^2
0.0	178.64	14.33	0	0.988
0.5	291.94	15.40	3.66	0.981
5.0	524.97	37.25	3.98	0.997
10.0	425.34	35.15	5.97	0.996

fermentation only produced hydrogen and carbon dioxide, no methane was observed. One possible reason is that seed sludge used in this experiment came from a primary anaerobic digester in which the dominant organisms were hydrolytic bacteria and fermentative bacteria, methanogens had lower activity. Furthermore, after 48 h preculture, system had adapted to the hydrogen production process, hydrogen-producing bacteria became dominant. That is why no detectable methane was produced even in 0.0 kGy irradiation test (control). Similar phenomenon was found in O-Thong and Prasertsan's work in which two consecutive batch experiments with differently pretreated seed sludge were conducted, and no detectable methane contained in some sets of their experiment(O-Thong et al. 2009), in research of Zhu and Béland, no methane was observed in all second batch tests(Zhu and Béland 2006). However, Argun, H. and F. Kargi's work on biohydrogen production by dark fermentation with precultured granular anaerobic sludge also examined certain amount of methane (Argun and Kargi 2009).

The data obtained illustrates that dark fermentation with seed sludge pretreated by different irradiation doses all show better hydrogen production ability than control test both in hydrogen production potential and maximum hydrogen production rates. This indicates that hydrogen-producing organisms dominated by spore-forming bacteria, chiefly *Clostridium* and *Enterobacter* do have stronger survivability under gamma irradiation than hydrogen-consuming bacteria, mainly methanogens and homoacetogens. Spore-forming hydrogen-producing bacteria can be protected from the damage of gamma irradiation and radiolysis products while hydrogen-consuming bacteria without such capability can be seriously damaged or destroyed. Thus, gamma irradiation can be a good choice for seed sludge pretreatment to inactivate hydrogen-consuming bacteria without adversely impact spore-forming hydrogen-producing bacteria, reducing the hydrogen consumption during hydrogen-producing process and enhance the hydrogen-producing rate and potential further. Whereas, what is need to be noted is the selection of irradiation doses, inappropriate doses can hardly achieve the desired effect: too low may not able to suppress hydrogen-consuming bacteria effectively while doses too high can also inhibit hydrogen-producing bacteria.

As to the lag time (λ), from Table 3.10 we can see that it is also affected by different doses pretreatment. 10.0 kGy dose test came to the longest lag time with 5.97 h, 5.0 kGy with 3.98 h and 0.5 kGy with 3.66 h follows while there was no lag time for 0.0 kGy test. It is reasonable, for irradiation will definitely inhibit microbial activity more or less. More doses, more serious inhibition and more time for restoration. It is worth noting that the lag time in this study is much shorter than other researches (usually more than 10 h) (Datar et al. 2007; Lo et al. 2008; Wang and Wan 2008). That is because of the pretreated seed sludge was precultured for 48 h before hydrogen-producing process to enrich the microbial biomass, certain time of incubation before the experiment can largely shorten the lag time of hydrogen production process. Similar phenomenon was also observed in Argun, H. and F. Kargi's research (Argun and Kargi 2009). Besides, Chen and Chen et al.'s

research also shows that the fermentation of complex substrates (food waste or nonfat dry milk) can achieve shorter lag time comparing with pure substrate (sucrose) (Chen et al. 2006).

3.10.3 *Effect of Dose on Substrate Degradation and Hydrogen Yield*

The substrate degradation efficiency was estimated by dividing the amount of glucose consumed after hydrogen production process by the amount of initial glucose added in the system. Figure 3.11 demonstrates difference of the glucose degradation rate of the seed sludge pretreated by different doses after 48 h reaction.

The result showed that the substrate degradation efficiency of 5.0 kGy pretreated seed sludge and 10.0 kGy were equivalent, 98.9% and 99.0%, respectively, which was higher than the control test when 10 g/L glucose was used as substrate, while the degradation rate of seed sludge pretreated with 0.5 kGy dose was lower than that of control test. The high substrate degradation efficiency obtained by 5.0 kGy and 10.0 kGy irradiation pretreatment was higher than most of the research done by others (Lo et al. 2008; Wang and Wan 2008; de Sá et al. 2013).

The hydrogen yield was calculated by dividing the hydrogen production potential by the glucose consumed in each batch test. Figure 3.12 shows the difference of hydrogen yield with seed sludge pretreated by different irradiation doses after 48 h hydrogen production process. It can be seen that after pretreated with different irradiation doses, seed sludge exerts better hydrogen yield than that without irradiation pretreatment when 10 g/L glucose was used as substrate. Among all these tests, seed sludge irradiated with 5.0 kGy dose occupied the highest hydrogen yield with 267 ml/g glucose (2.14 mol/mol glucose), which was higher than our previous work done with heat treated seed sludge that achieved

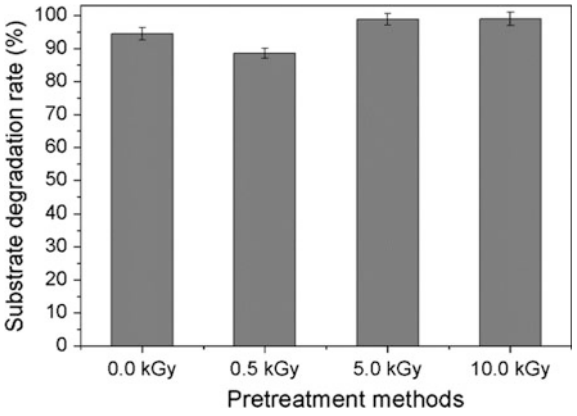

Fig. 3.11 Substrate degradation efficiency for different irradiation doses pretreatment

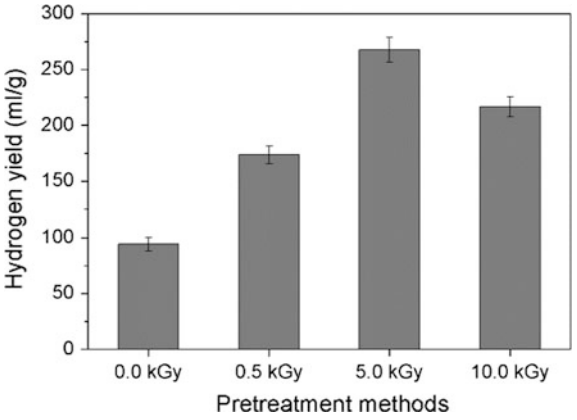

Fig. 3.12 Hydrogen yield for different irradiation doses pretreatment

221.5 ml/g glucose (Wang and Wan 2008). Comparing with other pretreatment methods: Kargi and Argun got maximum hydrogen yield of 1.00 mol/mol glucose with heat pretreated seed sludge (Argun and Kargi 2009), Zaiat and Penteado et al. accomplished hydrogen yield of 2 mol/mol sucrose with acid treated sludge (Penteado et al. 2013), Ren and Guo et al. achieved highest hydrogen production yield of 1.96 mol/mol glucose with repeated aeration pretreatment (Ren et al. 2008), Elbeshbishy and Hafez et al. adopted sonication with temperature control as pretreatment method and achieved maximum hydrogen yield of 1.55 mol/mol glucose(Elbeshbishy et al. 2010), Prasertsan and O-Thong attained highest hydrogen yield of 1.96 mol/mol hexose with load-shock treated sludge(O-Thong et al. 2009), the hydrogen yield of the seed sludge pretreated by 5.0 kGy irradiation in this study was much higher than other studies that using other pretreatment methods.

As we all know, in strict anaerobic environment, a theoretical maximum of 4 mol of hydrogen can be obtained per mole of glucose in dark fermentation. According to this, the conversion rate of glucose in our study is 53.79%, 43.52%, 35.12%, and 18.91%, respectively for 5.0 kGy, 10.0 kGy, 0.5 kGy, and 0.0 kGy as pretreatment dose. Glucose conversion rate of seed sludge pretreated by 5.0 kGy irradiation was pretty high.

3.10.4 Effect of Dose OnVolatile Fatty Acids

In anaerobic dark fermentation, the production of hydrogen accompanied with the generation of soluble metabolites, which mainly consist of volatile fatty acids (VFA) and some solvent products (Levin et al. 2004). The amount of hydrogen yielded by glucose depends on its fermentation type and end-products, thus, the identification of VFA formed in hydrogen-producing process can supply useful information for identifying the microbial metabolism ways. The major VFA

Table 3.11 soluble metabolites for different pretreatment method

Treatment method	Degree of acidification				
	Acetic acid	Butyric acid	Propionic acid	Hydrogen	HAc/HBu
0.5 kGy	0.25	0.39	0.21	0.12	0.64
5 kGy	0.04	–	–	0.20	–
10 kGy	0.14	0.16	0.05	0.16	0.83
Control	0.12	0.28	0.08	0.07	0.45

detected in this study include acetate (HAc), butyrate acid (HBu), and propionic acid (HPr). The degree of acidification is defined as the ratio of the COD equivalent of the acidogenic products (including organic acids and hydrogen) to the initial SCOD. As depicted in Table 3.11, seed sludge pretreated with 0.5 kGy dose irradiation along with 10.0 kGy and no irradiation showed their dominant soluble metabolites were acetate, butyrate acid and propionic acid, and the difference of their content was not significant, which indicated that their metabolic pathway belong to the mixed-type fermentation. For 5.0 kGy irradiation pretreatment, no detectable butyrate and propionic acid was produced, just a small amount of acid generated, and considering its excellent hydrogen production and yield, it may be dominated by acetate-type and ethanol-type fermentation (Ren et al. 2006; Ren et al. 2007).

A theoretical hydrogen yield of 4 mol/mol glucose can be obtained when acetate acid is the end product while 2 mol/mol glucose for butyrate acid as end product, therefore, the ratio of acetate to butyrate generated in the anaerobic digestion can reflect the hydrogen yield to a certain extent. Accordingly,we calculated HAc/HBu ratio of different pretreatment methods in this study. Obviously, 5.0 kGy has the highest acetate to butyrate since no butyric acid was detected in this set. Then, the acetate to butyrate of 0.83 was achieved in 10.0 kGy, and followed by 0.64 in 0.5 kGy and 0.45 in control test. It just matches the order of hydrogen yield: the highest HAc/HBu ratio was associated with the highest hydrogen yield and cumulative hydrogen production. Other researchers have also come to similar conclusions: study done by de Sá et al. got 4.62 mol H_2/mol sucrose under HAc/HBu ratio of 1.14 with heat pretreated inoculum (de Sá et al. 2013), O-Thong and Prasertsan achieved hydrogen yield of 1.57 mol H_2/mol hexose while the HBu/HAc ratio was quite low (0.9–1.3) when seed inocula was pretreated with load-shock (O-Thong et al. 2009), Elbeshbishy and Hafez found that there is a linear growth relationship between the hydrogen yield and HAc/HBu ratio (Elbeshbishy et al. 2010).

The effects of different doses of irradiation pretreatment on pH value are established in Fig. 3.13. It shows that after 48 h anaerobic digestion, the final pH of all tests dropped below the initial pH 7.0, ranging from 4.5 to 5.0. Among them, 5.0 kGy pretreated test shows the highest pH with 5.0, then pH 4.9, pH 4.6 and pH 4.5 for 10.0 kGy, 0.5 kGy and control test, respectively. Similar results were got by other researchers, Nakhla and Elbeshbishy got final pH range from 4.4 to 5.5, Sen

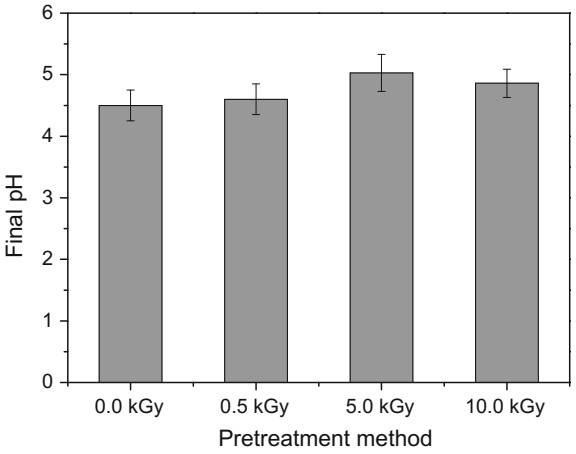

Fig. 3.13 Final pH for different irradiation doses pretreatment

and Suttar achieved highest hydrogen yield with heat pretreatment ended with pH 5.24 (Sen and Suttar 2012), Prasertsan and O-Thong reported final pH of 4.8 with load-shock as pretreatment method (O-Thong et al. 2009). It has been considered that the acidic pH (5.0–5.5) is ideal for hydrogen production with dark fermentation, for it can repress methanogens and benefit the development of hydrogen-producing bacteria (Zhu and Béland 2006). Yet, highly acidic pH (4.5–5.5) is treated to be injurious to hydrogen generation for its inhibition effect on hydrogen producers (Dabrock et al. 1992).

3.10.5 Conclusions

Four gamma irradiation doses 0.0 kGy (control test), 0.5 kGy, 5.0 kGy, and 10.0 kGy were used to estimate the suitability of ionizing irradiation in enhancing hydrogen-producing ability of primary anaerobic digested sludge in batch tests, the experimental results revealed that gamma irradiation with 5.0 kGy dose did a great job in enriching hydrogen-producing microorganisms. Basing on outcomes of this study, following conclusions can be drawn:

Comparing with control test, seed sludge irradiated with different doses all show better capacity in hydrogen generation at 36 °C, with initial pH 7.0 and 10 g/L glucose as substrate. Irradiation at 5.0 kGy demonstrates superiority over the other two doses used in this study with the maximum cumulative hydrogen production, supreme hydrogen yield, highest hydrogen production rate and great substrate degradation efficiency, which were 529.4 ml, 267.7 ml/g glucose, 37.25 ml/h, and 98.9%, respectively. It shows superiority over the conventional pretreatment methods including heat-shock, acid, base, aeration and chloroform studied in our

previous work (Wang and Wan 2008). Irradiation with proper dose has the potential to be as an optimal pretreatment method for enriching hydrogen-producing bacteria from digested sludge.

3.11 Hydrogen Production Performance by Different Pretreated Sludge

3.11.1 Effect on Hydrogen Production

Biogas produced in each batch was examined with Gas Chromatogram and the results showed that only carbon dioxide and hydrogen was observed. Possible reason of no detectable methane is that seed sludge of different pretreatment including control test was obtained from a primary anaerobic digester, which was dominated by the hydrolytic bacteria and fermentative bacteria, methanogens were not active in that environment. In addition, digested sludge were precultured for 48 h before the hydrogen-producing process, cultures for inoculum were adapted to the anaerobic dark fermentation. Similar phenomenon was found in researches done by Wang and Wan, in which no methane was detected in all sets (Wang and Wan 2008). Researches done with two consecutive batch experiments can also come to such phenomenon (Zhu and Béland 2006; O-Thong et al. 2009), but certain amount of methane can also be detected in some work with precultured seed sludge (Argun and Kargi 2009).

Effects of different pretreatment methods on cumulative hydrogen production during the dark fermentation process are presented in Fig. 3.14. The result shows that hydrogen-producing process in all batches came to an end in 48 h, tests with seed sludge pretreated by different methods all have higher cumulative hydrogen production than the control test, which verifies the studied pretreatment methods' enhancement on the hydrogen production capacity of seed sludge.

Basing on the data shown in Fig. 3.14, we used a modified Gomperz model to analyze the kinetics of hydrogen production during the dark fermentation, and the key results are listed in Table 3.12.

In all tests, ionizing irradiation with 5 kGy pretreatment method obtained both the maximum hydrogen production potential 525.6 ml and the highest hydrogen-producing rate 37.2 ml/h, which indicate that spores of spore-forming hydrogen-producing organisms, mainly *Clostridium* and *Enterobacter,* provided valid protection against ionizing irradiation. Meanwhile, hydrogen-consuming bacteria like methanogens and homoacetogens were suppressed effectively. Other researches have also shown that spores have stronger resistance to ionizing irradiation than growing cells (Roberts and Hitchins 1969; Gould 1983; Nicholson et al. 2000; Setlow 2006), however, the exact cause of spores' resistance to ionizing irradiation is not all clear, but one factor that plays an important role may be the low core water content, which presumably reduces the chance of damage caused by

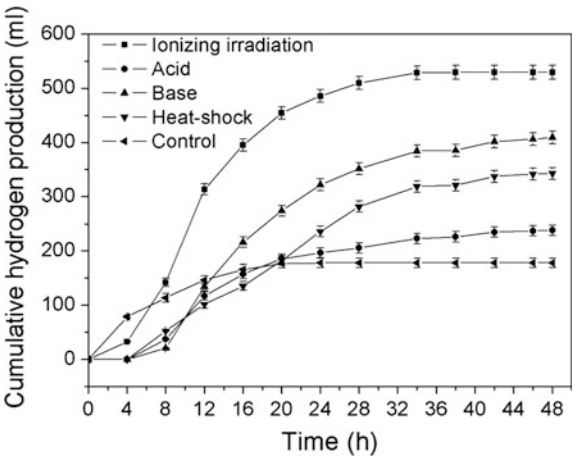

Fig. 3.14 Cumulative hydrogen production for different pretreatment methods

Table 3.12 Parameters of modified Gompertz model for different pretreatment method

Pretreatment method	P(ml)	R_m(ml/h)	λ(h)	R^2
Ionizing irradiation	525.6	37.2	4.0	0.998
Acid	227.2	15.0	5.1	0.988
Base	402.6	22.2	6.7	0.996
Heat-shock	356.9	13.7	5.6	0.994
Control	178.6	14.3	0	0.988

reactive radicals generated during irradiation process (Setlow 2006). Thus, we can see that ionizing irradiation can be a promising pretreatment method for the enrichment of hydrogen-producing bacteria in digested sludge.

Besides ionizing irradiation pretreatment method, seed sludge pretreated by base holds the highest hydrogen production potential and maximum hydrogen-producing rate, which were 402.6 ml and 22.2 ml/h. Heat-shock pretreated seed sludge has the lowest maximum hydrogen-producing rate (13.7 ml/h) even when control test was factored in (14.3 ml/h). Comparing with acid, base pretreatment and control test, quite a few researches have got the highest hydrogen production potential as well as maximum hydrogen-producing rate with heat-shock pretreatment method (Wang and Wan 2008; O-Thong et al. 2009; de Sá et al. 2013). It may because of the preculture process before dark fermentation, the fermentation products generated during the preculture process caused feed-back inhibition to hydrogen producers in heat-shock pretreated seed sludge. Similar phenomenon can be found in research done by Zhu and Béland, in their secondary cultivation, base pretreated seed sludge showed significant increase in hydrogen-producing ability while seed sludge pretreated by heat-shock displayed low activity in hydrogen production (Zhu and Béland 2006). However, study done by O-Thong, Prasertsan et al. demonstrated that the capacity of hydrogen production with different pretreatment methods were

all improved to some degree in the second batch fermentation, even heat-shock pretreated test shows good ability in hydrogen producing(O-Thong et al. 2009). One possible reason is that seed sludge in their study was boiled at 100 °C for 1 h for heat-shock pretreatment, which is much longer than Zhu and Béland's 20 and 15 min in our study. Thus, longer time of heat-shock can suppress more unwanted organisms for hydrogen production, avoid unnecessary metabolites and alleviate feed-back inhibition effect.

Effects of different pretreatment methods on the lag time show that base>heat-shock>acid>ionizing irradiation>control. Seed sludge without pretreatment does not show any lag time during the hydrogen-producing process, for it is clear of the hurt from pretreatment methods, and precultured to adapt to the dark fermentation, so it started working as soon as the process started. It is worth noting that the lag time of all tests was less than 10 h, which is the average value of lag time in other studies (Datar et al. 2007; Wang and Wan 2008; La Licata et al. 2011). Similar phenomenon can be found in batch studies with preculture process or the batches after the first of more than one batch tests (Zhu and Béland 2006; Argun and Kargi 2009; O-Thong et al. 2009). Thus, we can come to a conclusion that proper preculture before dark fermentation can not only improve the density of available microorganisms but also help hydrogen-producing bacteria recover the activity more quickly, leading to the enhancement of the whole hydrogen-producing process.

3.11.2 Effect on Substrate Degradation and Hydrogen Yield

As glucose was used as the sole carbon source for hydrogen production in our study, substrate degradation efficiency was calculated by dividing the amount of glucose consumed after dark fermentation process by the amount of initial glucose added in the system. The effects of different pretreatment methods on hydrogen production with 10 g/L glucose as substrate are shown in Fig. 3.15.

For the effects on substrate degradation efficiencies by different pretreatment methods, both ionizing irradiation and heat-shock pretreatment methods achieved the highest substrate degradation rate with 98.9%, followed by base pretreatment with 95.6% and control test with 94.5%, acid pretreated seed sludge had the lowest substrate degradation with 80.2%. The degradation rates of ionizing irradiation, base and heat-shock pretreated seed sludge achieved in this study were pretty higher than studies done with similar pretreatment methods (Wang and Wan 2008; O-Thong et al. 2009).

Hydrogen yield was estimated by dividing the hydrogen production potential by the glucose consumed after 48 h dark fermentation. Figure 3.16 demonstrates the effects of different pretreatment methods on the hydrogen yield of each batch test. Data in Fig. 3.16 shows that seed sludge pretreated by different pretreatment methods all accomplished higher hydrogen yield comparing with control test. Of all the tests, seed sludge pretreated by 5 kGy ionizing irradiation employed the highest

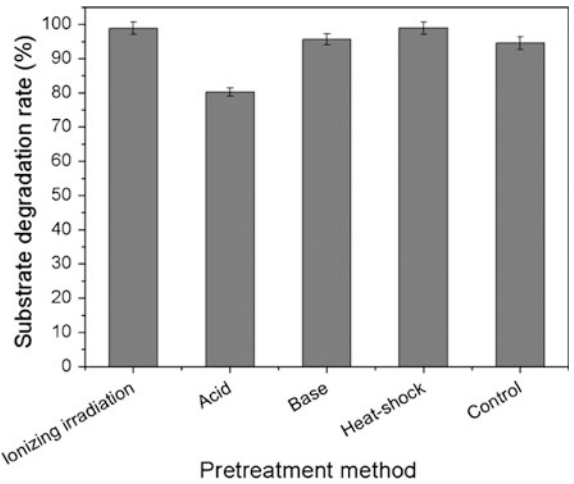

Fig. 3.15 Substrate degradation efficiencies for different pretreatment methods

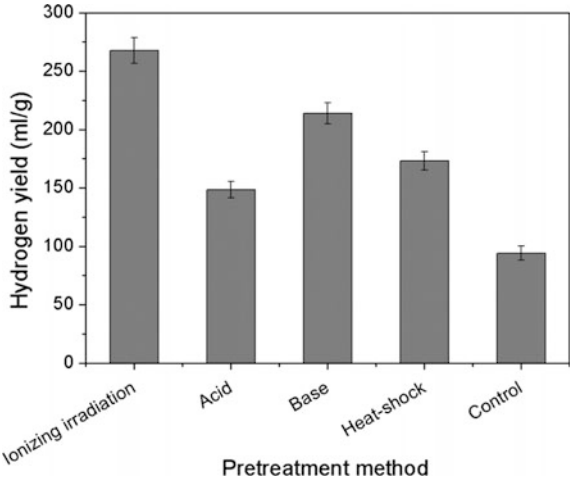

Fig. 3.16 Hydrogen yield for different pretreatment methods

hydrogen yield with 267.7 ml/g glucose (2.15 mol/mol glucose), followed by base pretreatment method with 214.0 ml/g glucose (1.72 mol/mol glucose), heat-shock with 173.2 ml/g glucose (1.39 mol/mol glucose), acid with 148.4 ml/g glucose (1.19 mol/mol glucose) and finally control test with 94.1 ml/g glucose (0.76 mol/mol glucose). Hydrogen yield obtained by base and acid pretreatment method in this study are all higher than our previous work (Wang and Wan 2008), and studies by other researchers (Elbeshbishy et al. 2010; Kan 2013). Hydrogen yield of heat-shock pretreated seed sludge showed lower hydrogen-producing ability than our previous study, which was 221.5 ml/g glucose (Wang and Wan

2008), but it is also higher than quite a few studies (Elbeshbishy et al. 2010; Baghchehsaraee et al. 2011; Kan 2013).

Besides hydrogen production potential and maximum hydrogen-producing rate, ionizing irradiation pretreated seed sludge also showed the highest substrate degradation rate and hydrogen yield per gram of glucose. Comparing with other studies with various pretreatment methods including heat-shock, acid (Penteado et al. 2013), base, aeration (Ren et al. 2008), ultrasonication (Elbeshbishy et al. 2010), load-shock (O-Thong et al. 2009) and so on, pretreatment method of ionizing irradiation with 5 kGy also shows great advantage in improving hydrogen-producing ability of digested sludge. All these indicates that for the pretreatment of seed sludge aiming at hydrogen production, ionizing irradiation pretreatment method deserves further study.

3.11.3 *Effect on Volatile Fatty Acids and Final pH*

Hydrogen-producing process with dark fermentation is accompanied with the generation of soluble metabolites, of which the main compositions are volatile fatty acids (VFA) and some other solvent products (Levin et al. 2004). The analysis of soluble metabolites can help us identify the fermentation type in the reactor. The VFA detected in the culture after fermentation is shown in Table 3.13.

The results showed that VFA generated in dark fermentation mainly comprised acetic acid, butyric acid and propionic acid, among which the acetic acid and butyric acid accounted for a higher proportion while propionic acid was found to be a lesser extent. For seed slugged pretreated by ionizing irradiation, no detectable VFA was examined and maximum hydrogen production was achieved in this group, thus it can be classified into ethanol-type fermentation (Ren et al. 2006; Ren et al. 2007; Ren et al. 2008; Liu et al. 2009a, b). As to acid and heat-shock pretreated seed sludge, it showed that their dominant VFA was acetic acid, with no other VFA was detected. Considering the small amount of acetic acid examined and relatively high hydrogen production expressed in these two groups, they may be dominated by acetate-type and ethanol-type fermentation. For base pretreated group and control test, all three VFA was found and there was no significant difference in their content, indicating the fermentation process belongs to the mixed-type fermentation. Furthermore, for the

Table 3.13 Soluble metabolites for different pretreatment method

Preatment methods	Volatile fatty acids (mmol/L)			
	Acetic acid	Butyric acid	Propionic acid	HAc/HBu
Ionizing irradiation	–	–	–	–
Acid	0.2	–	–	–
Base	2.3	1.2	2.1	1.92
Heat-shock	1.5	–	–	–
Control	19.8	13.6	11.0	1.46

acetate-type fermentation, a theoretical hydrogen yield of 4 mol/mol glucose can be achieved when the end product is acetate acid, while for the butyric acid-type fermentation, in which butyrate acid is the end product, a theoretical hydrogen yield of 2 mol/mol glucose can be obtained. Accordingly, the ratio of acetate to butyrate generated in mixed-type fermentation can reflect the hydrogen yield to some extent. From Table 3.13, we can see that seed sludge pretreated by base occupied higher HAc/HBu ratio of 1.92 comparing with control test with 1.46, which matches their hydrogen-producing ability: 402.6 ml of cumulative hydrogen production and hydrogen yield with 214.0 ml/g glucose for base pretreated set while 178.6 ml, 94.1 ml/g glucose for control test. Similar connections have also been observed in other researches (Elbeshbishy et al. 2010).

As a result of the generation of VFA, final pH of the medium all dropped below the initial value of pH 7. Figure 3.17 shows the effects of different pretreatment methods on the pH value, ranging from 4.5 to 5.0. Ionizing pretreated set showed the highest final pH with 5.0, followed by base and heat-shock pretreat sets with pH 4.8, acid pretreated set with pH 4.7, and control test with pH 4.5. It's known that acidic pH range from 5.0 to 5.5 is ideal for hydrogen generation for its repression of methanogens while preserving the activity of hydrogen producers, however, highly acidic pH range from 4.0 to 4.5 is considered to be detrimental to hydrogen-producing process (O-Thong et al. 2009). That is one reason that ionizing pretreated seed sludge owned the highest cumulative hydrogen production and hydrogen yield while the other four sets had relatively low hydrogen yield.

According to the study above, ionizing irradiation pretreatment method achieved the best hydrogen production, and we also compared hydrogen production with different pretreatment methods in this study and other studies. According to the theoretical maximum hydrogen production with 1 mol glucose and sucrose are 4

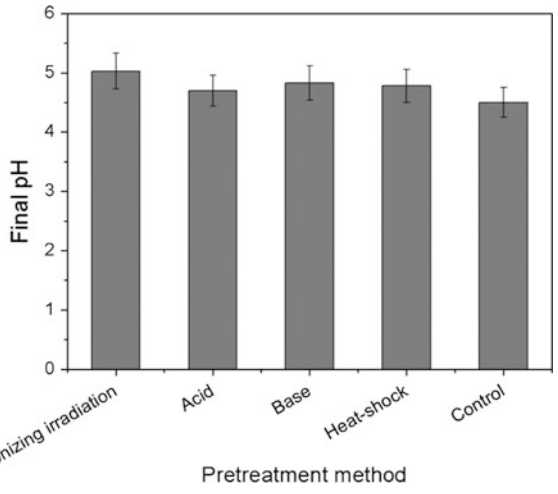

Fig. 3.17 Final pH for different pretreatment methods

Table 3.14 Comparison of the pretreatment methods

Seed	Pretreatment methods	Substrate	Hydrogen yield (mol H_2/mol substrate)	Hydrogen conversion rate (%)	References
Digested sludge	100 °C for 15 min	Glucose (10 g/L)	1.39	34.8	Yin et al. (2014a)
Digested sludge	100 °C for 15 min	Glucose (10 g/L)	1.78	44.5	Wang and Wan (2008)
Anaerobic sludge	100 °C for 15 min	Glucose (20 g/L)	0.9	22.5	Kan (2013)
Anaerobic sludge	65 °C for 30 min	Glucose (20 g/L)	1.36	34.0	Baghchehsaraee et al. (2011)
Anaerobic sludge	100 °C for 40 min	Glucose (5 g/L)	1.46	36.5	Davila-Vazquez et al. (2008)
Anaerobic sludge	100 °C for 60 min	Glucose (10 g/L)	2.19	54.8	de Sá et al. (2013)
Anaerobic sludge	100 °C for 60 min	Sucrose (10 g/L)	4.62	57.8	de Sá et al. (2013)
Digested sludge	pH 3 for 24 h	Glucose (10 g/L)	1.19	29.8	Yin et al. (2014a)
Digested sludge	pH 3 for 24 h	Glucose (10 g/L)	0.8	20.0	Wang and Wan (2008)
Anaerobic sludge	pH 3 for 24 h	Glucose (8 g/L)	1.11	27.8	Elbeshbishy et al. (2010)
Anaerobic sludge	pH 2 for 1 h	Sucrose (10 g/L)	3.85	48.1	de Sá et al. (2013)
Anaerobic sludge	pH 3 for 24 h	sucrose (25 g COD/L)	2.53	31.6	Chen et al. (2009)
Digested sludge	pH 10 for 24 h	Glucose (10 g/L)	1.72	43.0	Yin et al. (2014a)
Digested sludge	pH 10 for 24 h	Glucose (10 g/L)	1.09	27.2	Wang and Wan (2008)
Anaerobic sludge	pH 10 for 24 h	Glucose (20 g/L)	0.25	6.2	Kan, (2013)
Anaerobic sludge	pH 12 for 1 h	Sucrose (10 g/L)	3.93	49.1	de Sá et al. (2013)
Digested sludge	5 kGy dose gamma irradiation	Glucose (10 g/L)	2.15	53.5	Yin et al. (2014a)

and 8 mol hydrogen in dark fermentation, respectively, the conversion rate of substrate can be calculated. The hydrogen yield and hydrogen conversion rate with different pretreatment methods in different studies are listed in Table 3.14.

3.12 Changes in Microbial Community During Biohydrogen Production

3.12.1 Seed Sludge and Fermentation Conditions

Seed sludge used was the anaerobic digested sludge collected from a municipal sewage treatment plant located in Beijing (China). Raw sludge was pretreated by 5 kGy gamma irradiation as described in the previous study (Yin et al. 2014a) and then stored at 4 °C until being used.

Before being used as inoculum, gamma irradiated seed sludge was precultured. After the cultivation, the mixtures were centrifuged at 4000 rpm for 5 min to get the microbial biomass, then washed by 0.9% NaCl solution for 3 times before being used as inoculum. Batch fermentation was conducted for hydrogen production.

3.12.2 DNA Extraction and PCR Amplification

To analyze the structure of bacterial communities of sludge at different stages, raw sludge, gamma irradiated sludge, and sludge after batch tests were collected and kept at –80 °C until DNA extraction. DNA samples were extracted using the E.Z.N. A. soil DNA extraction kit (Omega Bio-tek, Norcross, GA, U.S.) according to manufacturer's protocols and confirmed using 1% agarose gel electrophoresis. PCR amplification of partial 16S rRNA gene (V4-V5 region) was performed from the obtained DNA, two primers were used: 515F (5'-barcode- GTGCCAGC MGCCGCGG-3') and 907R (5'-CCGTCAATTCMTTTRAGTTT-3'), and for each sample, barcode is a unique eight-base sequence. PCR reactions were carried out in a total volume of 20 uL mixture containing 10 ng of template DNA, 4 μL of 5 × FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM) and 0.4 μL of FastPfu Polymerase. PCR conditions were 95 °C for 2 min, 95 °C for 30 s repeated 25 cycles, 55 °C for 30 s, 72 °C for 30 s and a final extension at 72 °C for 5 min. All the reactions were in triplicate. PCR products were detected by electrophoresis in a 2% agarose gel and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, U.S.) according to the manufacturer's instructions. According to the results obtained from electrophoresis, PCR products were further quantified using QuantiFluor™ -ST (Promega, U.S.).

3.12.3 MiSeq Sequencing and Data Analysis

The Illumina MiSeq PE250 was applied to perform amplicon sequencing by Shanghai Majorbio Bio-pharm Biotechnology (Shanghai, China). Raw fastq files were demultiplexed and quality-filtered using QIIME (version 1.17). Optional Units

(OTUs) were clustered at a 97% similarity level using UPARSE(version 7.1)and chimeric sequences were identified and removed using UCHIME. As to the phylogenetic affiliation, 16S rRNA gene sequences were analyzed by RDP Classifier against the silva (SSU115)16S rRNA database at a 70% confidence threshold (Amato et al. 2013). Phylogenetic tree was constructed by MEGA 6 (Tamura et al. 2013). Venn diagram and heat map were drawn using the R package (http://www.R-project.org/).

3.12.4 Hydrogen Production Progress

As shown in Fig. 3.18, fermentative hydrogen production process terminated in 24 h, and the maximum cumulative hydrogen production reached 300 mL per 100 ml of mixture. Hydrogen production potential, maximum hydrogen production rate, and the lag time obtained were 300.90 mL, 19.87 mL/h, and 0 h, respectively. The degradation of substrate was accompanied with the production of hydrogen. In the end of the fermentation, substrate degradation rate and hydrogen yield reached 78.1% and 1.81 mol H_2/mol glucose, respectively.

Accumulation of hydrogen was accompanied by the formation of acidic metabolites. Figure 3.19 depicts the change of pH value and volatile fatty acids concentration during the fermentation process. It can be seen that the pH dropped significantly from 7.92 to 4.77 in the first 6 h, then it showed a gradual decline in the following 20 h and stayed consistent at around 4.5. Similar phenomenon has been reported in literature (Harun et al. 2012; Singh et al. 2014).

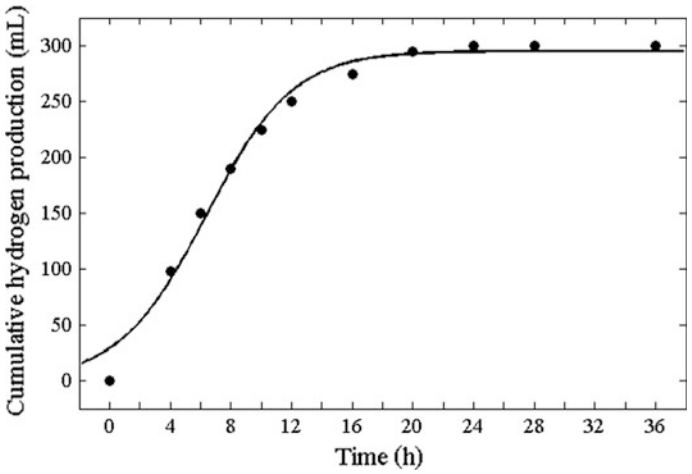

Fig. 3.18 Cumulative hydrogen production over time

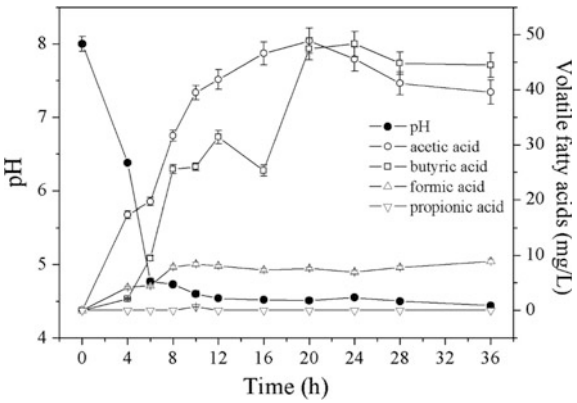

Fig. 3.19 The change of pH and volatile fatty acids (VFA) over time

The generation of volatile fatty acids showed that in the first 10 h, formic acid, acetic acid, propionic acid, and butyric acid were all produced and accumulated, which indicates that the culture followed mixed-acid pathway during this period. Then, in the following 14 h, it transformed to acetate butyrate pathway, during which acetic acid and butyrate acid were accumulated as the main soluble metabolites. The change may because of the accumulation of volatile fatty acids as well as the drop of pH value inhibited certain enzyme or microbial activity. Studies have reported that different dominant microbial species can lead to different fermentation type (Lo et al. 2008; Harun et al. 2012).

3.12.5 Microbial Diversity Characteristics

To reveal the bacterial structure of seed sludge at different stages, samples from raw sludge (raw), 5 kGy gamma irradiation pretreated sludge (irradiated) and sludge after fermentation (fermented) were used to pyrosequence the former region of 16S rDNA gene using the 454 GS-FLX sequencer.

As shown in Table 3.15, Ace and Chao1 are richness estimators, which are commonly used in ecology to estimate the total number of species. Greater value of Ace and Chao1 indicates larger variety of species exist in a sample. Shannon and Simpson are diversity indexes for an OTU definition, larger value of Shannon and

Table 3.15 Characteristics of microbial phylotype diversity of 16S rRNA gene libraries

Sludge samples	Reads	OTU[a]	Ace	Chao1	Shannon	Simpson
Raw sludge	11772	215	223	229	3.94	0.0423
Irradiated sludge	16737	207	220	225	3.41	0.0643
Fermented sludge	11722	29	75	68	1.11	0.4081

[a]The operational taxonomic units (OTU) were defined with 3% dissimilarity

smaller value of Simpson means higher community diversity of a sample (Schloss et al. 2011). It can be seen from Table 3.15 that raw sludge owns both largest number of microbial species and highest community diversity while sludge after fermentation has the lowest richness and biodiversity. It means that gamma irradiation inhibited some bacteria, caused microbial diversity and richness decrease. Furthermore, fermentation conditions supplied in this experiment further selected microorganisms suitable for the environment. It can be indicated from the four estimators in Table 3.15 that reaction environment can cause greater influence on microbial community than pretreatment. Similar phenomenon was also observed by Im et al. (2012), who adopted sewage sludge and food waste for biohydrogen production.

As shown in Table 3.15, 215, 207, and 29 species-level OTUs were found in raw sludge, gamma irradiation pretreated sludge and sludge after fermentation, respectively.

Figure 3.20 shows the comparison of microbial sequences of sludge at three different stages, and the total number of bacterial species-level OTUs was 232. Gamma irradiated sludge shared 191 OTUs with raw sludge and the rest 16 were novel ones which may be mutations of strains in raw sludge. Only 29 OTUs were left after fermentation process. On one side, the fermentation process supplied a certain environment that only suitable to some species; on another side, during the gamma irradiation pretreatment, not only a group of species were killed directly, many remaining species were injured to some extent, even after the pre-culture process, some of them can hardly revive. Sludge after fermentation and raw sludge shared 27 species, which occupied 93.10% of species exist in fermented sludge. This phenomenon implies that there is a large chance that most of the hydrogen producers were from the raw sludge but not the mutations, which means the mechanism of gamma irradiation as a good pretreatment method is mainly because it can inhibit hydrogen consumers efficiently while preserve hydrogen producers coexist in the mixed culture.

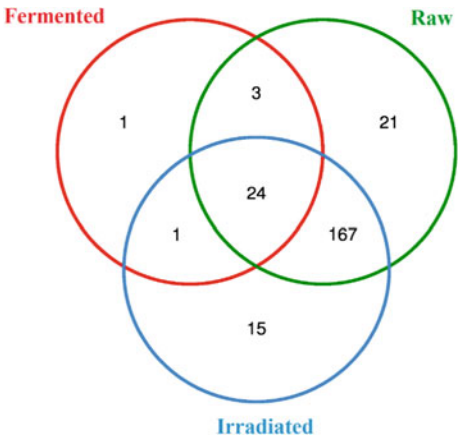

Fig. 3.20 Comparison of microbial sequences of sludge at three different stages

3.12.6 Microbial Diversity at Different Stages

In order to observe the microbial diversity in detail, bacterial community and relative abundances by phylum and genus were shown in Fig. 3.21. It can be seen that the microbial community was very diverse in raw sludge, sequences belong to more than 20 phylum and 100 genus were detected, among which genus *Candidate division OD1* was the most abundant (1830 sequences, 15.55% of total bacteria), followed by *Pseudomonas* (983, 8.35%), *W5* (821, 6.97%), *Lactococcus* (550, 4.67%), and *Longilinea* (513, 4.36%). The other divisions were all present in minor components in no more than 2%. After gamma irradiation pretreatment, microbial

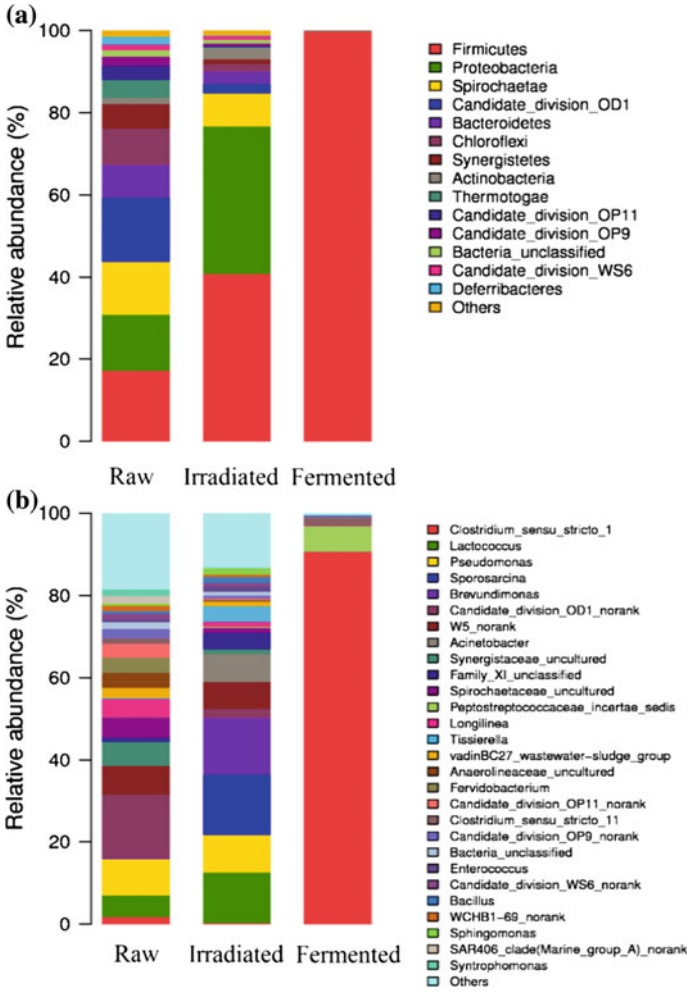

Fig. 3.21 Bacterial community and relative abundance by phylum (**a**) and genus (**b**)

community was dominated by phylum *Firmicutes* (40.74%) and *Proteobacteria* (35.92%), among which genus Lactococcus (1835, 10.96%), *Pseudomonas* (1386, 8.28%), and *W5* (1095, 6.54%) still remained plenty while the others dominant in raw sludge were inhibited significantly. Besides, some other genus become prevailing from trace, like *Brevundimonas* (2310, 13.80%), *Sporosarcina* (1704, 10.18%), and *Acinetobacter* (1137, 6.79%), which were all reported to have high survival rates under radiation conditions. As to the sludge after fermentation, 99.66% of microbes were occupied by phylum *Firmicutes*, among which genus *Clostridium* sensu stricto *1* took up 90.57% (10617 sequences) and become the predominant genus, followed by *Peptostreptococcaceae* (768, 6.55%) and *Clostridium* sensu stricto *11* (242, 2.06%). The rest ones were all present in less than 0.5%. Obviously, *Clostridium* sensu stricto *1* genus has made main contributions to good hydrogen production in this system.

Clostridium genus has been widely studied in fermentative hydrogen production. For the studies adopted mixed cultures as inoculum, researchers have found a relationship between high hydrogen production and the dominant presence of *Clostridium* genus (Lin et al. 2006a, b; Jeong et al. 2013). *Clostridium* spp. have been widely accepted as potential microorganisms for satisfactory hydrogen producers from various organic materials (Lee et al. 2011). In this study, genus *Clostridium* sensu stricto *1* comprised 2 species. One was identified as *Clostridium butyrium*, which was one of the most common hydrogen producers identified in fermentative hydrogen production process, it occupied 39.00% of total bacteria. The other one was identified as a new species (*Clostridium unclassified*), occupied 59.17% of all bacteria in fermented sludge.

To further understand the characteristics of strain *Clostridium unclassified*, the 16S rDNA gene sequence (424 bp) was aligned with public gene bank at website of Ezbiocloud (http://www.ezbiocloud.net/eztaxon), The 16S rDNA gene sequence

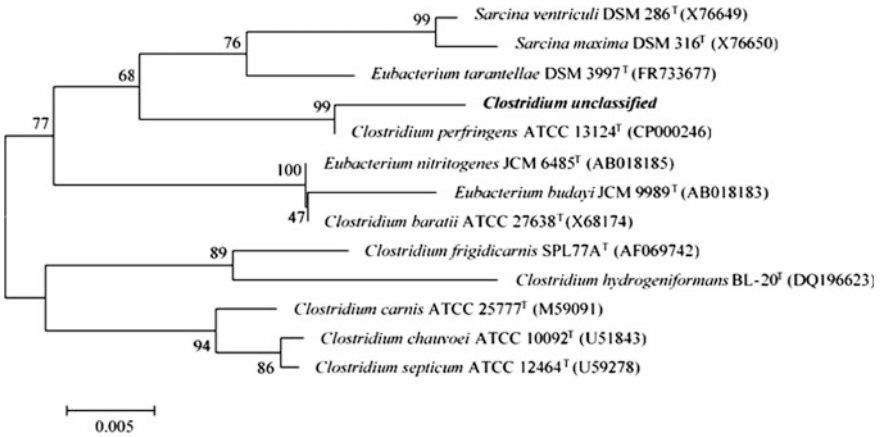

Fig. 3.22 Phylogenetic tree showing the relationships between strain *Clostridium* unclassified and related species based on 16S rRNA gene

showed 99% sequence identity with *Clostridium perfringens* ATCC 13124^T (CP000246). A phylogenetic tree was constructed to describe the relationship between *Clostridium unclassified* and the most closely taxonomic species based on 16S rDNA sequences (Fig. 3.22).

It worth mentioning that the two species dominant in hydrogen production system were all negligible but present in both raw and gamma irradiated sludge samples. It means that the mechanism of gamma irradiation in enriching hydrogen producers from mixed cultures was its selective inhibition to hydrogen consumers but not mutation effect.

As the upmost microbe present in the dark fermentation system was not identical to taxonomically validated strains, further work relating to its isolation and identification is deserved.

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Chapter 4
Pretreatment of Organic Wastes for Hydrogen Production

4.1 Overview

Organic wastes refer to the wastes rich in organic matters, which can be broken down into carbon dioxide, water, methane, or simple organic molecules by micro-organisms. Increasing living standard brings the increase of wastes generation. Take solid wastes as example, in US alone, municipal wastes generation increased from 88.1 million tons in 1965 to 258.5 million tons by 2014; in China, waste activated sludge production has increased from 3.25 to 6.25 million tons dry solids in 6 years by 2013 (Yang et al. 2015). Direct disposal of organic wastes may pollute water, air, and impair the living quality of human beings. Increasing amount of organic wastes is becoming a serious problem. The European Council Directive on the Landfill of Wastes 1999/31/EC provided that within 2016, landfilled biowaste production should be reduced to 35% of the amount produced in 1995 (Cesaro and Belgiorno 2014). Considering the energy contained in organic matters, energy recovery from organic wastes is attracting people's attention.

Quite a few studies have explored the feasibility of using various organic wastes as substrate for hydrogen production. The organic wastes used in dark fermentative hydrogen production mainly comprise waste activated sludge, algae biomass, cellulose-base biomass, food waste and organic wastewater. During the dark fermentation process, complex organic matters are firstly disintegrated into soluble matters, which mainly include proteins, carbohydrates and lipids. Then, these molecules are further hydrolyzed to smaller molecules like amino acids, monosaccharaides and long chain fatty acids. Then, it comes to the acidogenesis and acetogenesis process, besides the target product H_2, a wide range of intermediates and byproducts include soluble metabolites and CO_2 are formed. The terminal liquid products are mainly composed of acetate acid, butyrate acid, ethanol and propionic acid. The composition depends on microbial species, fermentation conditions as well as substrate sources and pretreatment process (Fig. 4.1).

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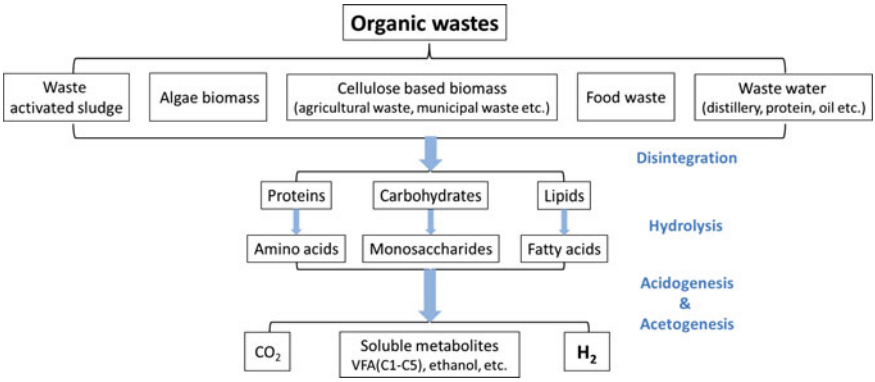


Fig. 4.1 Biodegradation steps and biological processes involved in fermentative hydrogen production from organic wastes

4.2 Main Structural Components of Organic Wastes

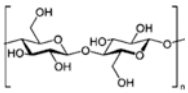
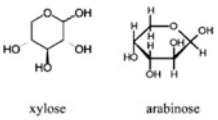
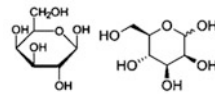
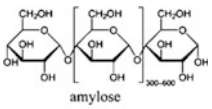
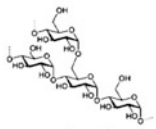
Composition of organic wastes is complex (Table 4.1), while anaerobic cultures are usually inefficient in hydrolyzing macromolecules. Thus, pretreatment is usually necessary to promote the disintegration and hydrolysis of biomass. During the pretreatment process, complex structures of biomass are collapsed, leaving free cells. Then, cell walls and membranes are solubilized, releasing the trapped components. Subsequently, crystal and polymeric structures of macromolecules are destroyed, generating molecules readily available for hydrogen producers. Extensive studies have verified the enhancing effect of pretreatment on hydrogen production from biomass (Taherzadeh and Karimi 2008; Haghighi Mood et al. 2013; Monlau et al. 2013; Bundhoo et al. 2015).

Application of organic wastes in biohydrogen production is a promising way for sustainable energy generation. Thus, this chapter will provide an insight on recent developments and present status of fermentative hydrogen production from organic wastes, hence facilitate the future studies.

4.3 Types and Compositions of Organic Wastes

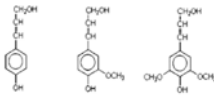
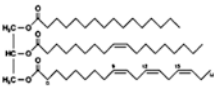
Organic wastes have been considered as an important energy source for it can be efficiently converted to energy through a series of physical or chemical methods. Among all the conversion processes, biological hydrogen production process is preferred for its mild reaction condition, economic feasibility, and environmental benefits.

Table 4.1 Main structural components of organic wastes and their properties (Pavlovič et al. 2013)

Component	Chemical structure and monomers/oligomers formula	Main properties	The main sources in agro- and food industry waste
Cellulose	 cellobiose $[C_6H_{10}O_5]_n$ n~100-10 000	Homopolymer of glucose units joined by β-1-4-glycoside bonds with crystalline ribbon-like structure	Cereals straw, wine shoots, sunflower stalks, sugarcane bagasse, cotton stalks
		Cellulose is not soluble in water at standard conditions, but starts dissolving at 180 °C and completely dissolves at around 330 °C.	
Hemicellulose	$[C_5H_{10}O_5]$  xylose arabinose $[C_6H_{12}O_6]$  galactose mannose	Heteropolymer of pentoses (xylose and arabinose) and hexoses (glucose, galactose, mannose), highly substituted with sugar acids (acetic acid)	Sugarcane bagasse, corn cobs, sunflower seed hulls and stalks
		Due to amorphous structure it is easily hydrolysed in waster at temperatures above 160 °C to monomers, which could be, at acid water conditions, further transformed to chemicals	
Starch	 amylose  amylopectin	Polymer consisted from 10–35% of linear chain α-amylopectin (branched chain of basic repeating units of 1,4 linked glucose with branches of 1,6 linked glucose)	Potato, cereal grains
		Hydrolyses very easily in hot water to glucose and further to chemicals (5-HMF, fufural, etc.)	

(continued)

Table 4.1 (continued)

Component	Chemical structure and monomers/oligomers formula	Main properties	The main sources in agro- and food industry waste
Lignin	 p-coumaryl coniferyl alcohols sinapyl	Heteropolymer consisting of three hydroxycinnamyl alcohol monomers (C9) differing in their degree of methoxylation: p-coumaryl, coniferyl and sinapyl alcohols.	Drup endocarp (coconut shell, walnut shell, olive shell, etc).
		Lignin is chemically the most resistant component of lignocellulose. Dissolution and hydrolysis to monomers starts in near- and supercritical water	
Lipids/fats	 Triglycerides	Nonpolar aliphatic compounds composed of triglycerides (TGAs)-esters of fatty acids and glycerol	Oilseed cakes, slaughterhouse waste, algae, meat food waste, etc.
		They are insoluble in water at normal temperatures, but at HT conditions hydrolyse to fatty acids and glycerol and further to hydrocarbon like substances (higher alkanes, acrolein, etc.)	
Proteins	 Peptide bonds [NHCH(R)C(O)]n n=50-2000, R-various side groups of amino acids	Built from amino acids linked together by peptide bonds	Meat waste (blood, fats), fish waste oil seeds, etc.
		Not easily hydrolyzed by HT reactions, but degrade slowly to amino acids, which further rapidly degrade to hydrocarbons, amines, ammonia, aldehydes and acids	

Various organic wastes, including waste activated sludge, algal biomass, cellulose-based biomass, starch-based biomass, food waste and wastewater, have been studied as feedstock for fermentative hydrogen production.

4.3.1 Waste Activated Sludge

Biological wastewater treatment process is the most widely used wastewater treatment process. During this process, organic matters present in wastewater are turned into CO_2 and microbial biomass. With the increasing amount of wastewater produced, large amount of waste activated sludge is formed. Waste activated sludge is a kind of solid–liquid mixture with water content of 95–99.5%.

The composition of waste activated sludge can be categorized into four groups:

(1) nontoxic carbon sources, mainly composed of microbial cells and extracellular polymeric substances. It accounts for around 60–70% of total dry weight;
(2) inorganic nutrients include nitrogen- and phosphorous-containing compounds;
(3) toxic inorganic and organic pollutants, like heavy metals, pesticides, estrogens, and pathogenic microorganisms, etc.;
(4) inorganic compounds like silicates, calcium- and magnesium-containing components (Rulkens 2008).

Large amount, high water content, and the presence of poisonous substances make the treatment and dumping of waste activated sludge difficult and costly. The treatment of waste activated sludge usually composes over 50% of operating cost in wastewater treatment plant (Cai et al. 2004). Treatment and disposal of waste activated sludge have become an urgent environmental problem (Yang et al. 2015). Existing sludge disposal includes sanitary landfill, incineration, agricultural use like sludge compost (Listed 1990), construction use like building materials (Okuno et al. 2004), and energy recovery (Tyagi and Lo 2013). Considering the rich content of organic substances, waste activated sludge is receiving attention for its potential application for renewable fuel production (Wang et al. 2003; De Gioannis et al. 2013; Guo et al. 2013). Productions of biofuel include: methane, hydrogen, syngas (H_2+CO) (Lv et al. 2007), bio-oil, and biodiesel (Dufreche et al. 2007) from waste activated sludge have been explored. Among them, fermentative hydrogen production owns more environmental benefits and has a potential for sustainable development.

However, up to now, the low yield of hydrogen production during the fermentation process hinders its further application. To enhance the energy recovery efficiency, following studies have been conducted:

(1) Pretreatment of waste activated sludge.
Since most of organic matters are encapsulated in microbial cells, pretreatment is necessary to disrupt microbial membranes and release the organic substances. Thus, both energy recovery rate and sludge degradation rate can be significantly enhanced. Lin et al. (2013a, b) achieved 50.21% enhancement in hydrogen production through chemical treatment of waste activated sludge. Kuglarz et al. (2013) reported 35% more biogas production with microwave-treated sludge.

(2) Supplementary carbon source.
Proper C/N ratio can significantly enhance hydrogen production efficiency. Thus, adjusting C/N ratio of waste activated sludge through adding carbon sources like sugars and food wastes were explored. Both Kim et al. (2013) and Liu et al. (2013) increased hydrogen production through co-digestion of sludge with rice straw and food wastes, respectively. Yin and Wang (2015, 2016), found that though the addition of glucose to fermentation system, reaction period, maximum hydrogen production rate, and hydrogen yield were significantly enhanced.
(3) Two/Three-stage fermentation.
Dark fermentative hydrogen production was followed by photofermentation and methane production, both hydrogen yield and energy recovery was improved (Mishra et al. 2016). Besides the above-mentioned measurements, inoculation of robust hydrogen producers, optimization of both treatment conditions and operational conditions can also enhance hydrogen production process. Thus, with extensive explorations, waste activated sludge is a potential feedstock for hydrogen production.

4.3.2 Algal Biomass

Algal biomass are simple chlorophyll containing organisms, they have high photosynthetic efficiencies in converting atmosphere CO_2 into a wide range of organic substances like proteins, polysaccharides, and lipids (Luque 2010). They vary greatly in size—unicellular of 3–10 μm to giant kelps up to 70 m long and growing at up to 50 cm per day. Algae can be found everywhere where there is a light to carry out photosynthesis: in the sea, rivers and lakes, on soil and walls, in even in animal and plants (as symbionts-partners). Algae can be categorized into macroalgae and microalgae. General composition of macroalgae and microalgae used for fermentative hydrogen production is shown in Table 4.2.

Macroalgae is also known as seaweeds, prevalently present in marine ecosystem, and mainly grow on rocky substrates. They are excellent in producing and storing carbon sources because of their high growth rate and big size of up to 60 m in length (Sambusiti et al. 2015). Chemical compositions of macroalgae are affected by their culturing environment like light, temperature, salinity, pollution, and motion of tides. Macroalgae have a high content of water, minerals, and carbohydrates. They have been used as food, crude drugs (like iodine deficiency), a source of additional vitamins, hypocholesterolemic and hypoglycemic agents and as vermifuges. For example, the red algae *Kappaphycus* and *Betaphycus* are the most important sources of carrageenan, a commonly used ingredient in food industry (yogurt, chocolate milk, puddings, etc.); *Gracilaria*, *Gelidium,* and *Pterocladia* are used in manufacturing agar, which is widely used as a growth medium for

Table 4.2 General composition of different algae cells (% of dry matter)

	Protein	Polysaccharides	Lipid
Microalgae			
Chlorella sp.	51–58	12–17	2–63
Scenedesmus sp.	8–56	10–50	11–55
Nannochloropsis sp.	50–67	10–20	5–56
Arthrospira sp.	33–70	10–23	6–13
Dunaliella sp.	50–60	12–40	6–67
Chlamydomonas sp.	48–60	13–20	21–70
Spirulina sp.	43–71	13–16	2–17
Anabaena cylindrica	43–56	25–30	4–7
Macroalgae			
Laminaria japonica	12–55	10–61	0.6–4
Gelidium amansii	19–42	30–60	0.8–2

microorganisms; *Laminaria japonica*, *longleaf luckyweed* flower and many other marine algae are made into popular dishes.

Macroalgae can be divided into green algae (Fig. 4.2a), red alga (Fig. 4.2b), and brown algae (Fig. 4.2c) according to their different colors. Carbohydrates content of green algae ranges between 25 and 60%, which is mainly composed of mannan, ulvan, starch, and cellulose. For the red algae, carbohydrates content is around 30–60%, which is dominated by carrageenan (up to 75%) and agar (up to 52%). As to brown algae, carbohydrates content varies between 30 and 50% and the main components include alginate, laminarin, and cellulose (Becker 1994; Jung et al. 2013; Sambusiti et al. 2015). Brown algae like *Laminaria japonica* has been widely studied as feedstock for anaerobic fermentation due to its high carbohydrates content and low requirement for pretreatment (Park et al. 2009).

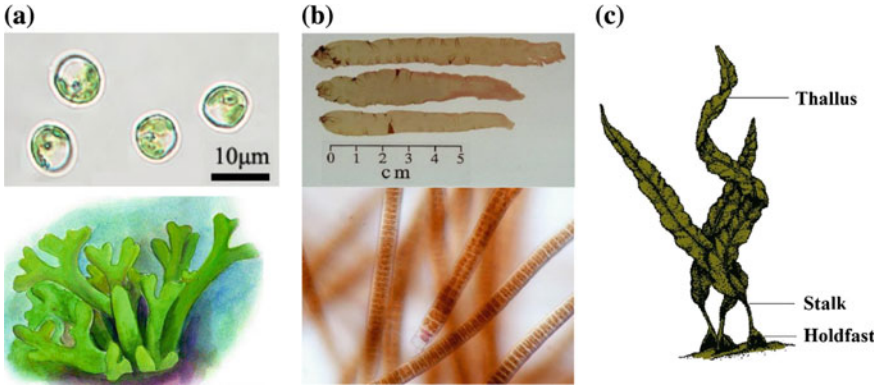


Fig. 4.2 The diversity of macroalgae (**a**: Green algae e; **b**: Red alga; **c**: Brown algae)

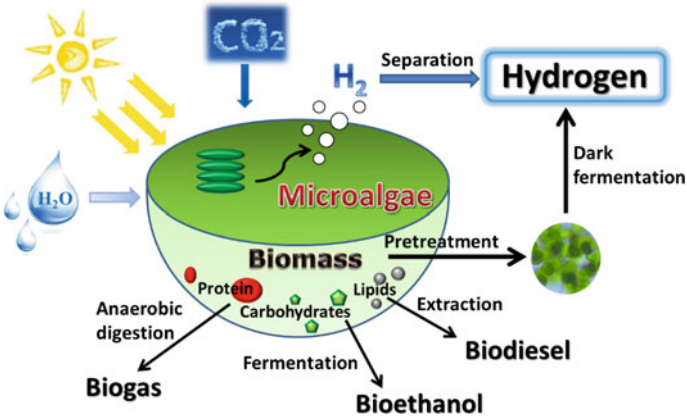


Fig. 4.3 The composition and applications of microalgae

Microalgae are unicellular or simple multicellular microorganisms. Microalgae are adaptive to various environment conditions, and can be cultivated in freshwater, seawater, and wastewater. Components of microalgae vary according to their species and cultivation environment, distribution of biochemical fractions of a microalgae cell is as follows: proteins 40–60%, carbohydrates 5–60%, lipids 8–30%, and nucleic acids 5–10% (Becker 1994; Uggetti et al. 2014).

Microalgae have been widely used in biofuel production (Fig. 4.3). Anaerobic fermentation is widely applied to treat organic wastes as well as produce biogas. In recent years, interest has grown in anaerobic digestion of microalgae biomass. Due to the specific cell wall properties and components variety, anaerobic digestion efficiency is heavily dependent on microalgae strain species. A significant variability of the methane yield from 140 to 400 mL CH_4/g VS has been observed in the literature (Uggetti et al. 2014). Besides the generation of biogas, anaerobic digestion of microalgae can achieve the mineralization of microalgae, releasing the organic nitrogen and phosphorus present in microalgae cells to the liquid phase, which can be further processed to produce fertilizers. Taking advantage of high carbohydrates content, low percentage of lignin and hemicellulose, microalgae show significant potential in the application in bioethanol production. The generation methods are not only restricted to fermentation, but include the gasification and thermochemical processes. *Chlorococcum* sp. and *Chlorella* sp. are particularly suitable for bioethanol production (Harun et al. 2010; Uggetti et al. 2014). Microalgae is also an ideal feedstock for biodiesel production for the high oil content, which may exceed 80% while agricultural oil crops can hardly achieve 5% (Amaro et al. 2011). Microalgae oil production is significantly more efficient than conventional oil crops (oil palm, jatropha, soybean, etc.), which provides higher oil yields and lower land area utilization (Luque 2010).

Besides biofuel, microalgae is also capable of producing a wide range of high-value products, like healthy food, aquaculture and animal feed,

pharmaceutical, medical products, pigments, and so on. Furthermore, it is also used in environmental processes like wastewater treatment and mitigation of CO_2 emissions (Misra et al. 2016).

As a kind of newly explored energy crop, algae biomass has many advantages: 1. Rapid growth rate. They can proliferate rapidly and be obtained in large amount easily. 2. Benefit to the environment. They are quite efficient in utilizing inorganic carbon sources to synthesize cell biomass, fixing CO_2. Furthermore, they can be cultured in wastewater, which can be combined with wastewater treatment. 3. Strong adaptation to various environments without competing with fertile soils for agriculture. 4. High carbohydrate content which is helpful in enhancing the hydrogen production efficiency. 5. Lack of hemicellulose and lignin, thus, the required pretreatments can be milder.

Considering the advantages mentioned above, feasibility of hydrogen production from algae biomass has been explored. *Chlorella* sp., *Scenedesmus* sp., and *Saccharina* sp. were the widely studied strains in fermentative hydrogen production, and hydrogen yield of 0.4–760 mL H_2/g volatile solids (VS) (Sun et al. 2011; Roy et al. 2014; Wieczorek et al. 2014), 0.42–113.1 mL H_2/g VS (Yang et al. 2011; Batista et al. 2014; Ortigueira et al. 2015) and 23.4–159.6 mL H_2/g total solids (TS) (Jung et al. 2011a, b; Shi et al. 2013; Jung et al. 2015) were obtained, respectively. However, there still remain some obstacles hindering the wide application of algae as substrate for hydrogen production. For example, the problems associated with biomass culturing and harvesting. The efficient mass production of algae still needs to be developed; the cost of collection and concentration of algae biomass needs to be reduced (Uggetti et al. 2014). Furthermore, since the components of algae are highly affected by cultivation environment, various compositions of differently cultured algae cause difficulties in choosing efficient treatment method, and also affect stable operation of hydrogen production system.

4.3.3 Cellulose-Based Biomass

Cellulose-based biomass mainly comprises agricultural and municipal wastes, like straws, stalks, forest residues, yard clippings, grass, wood chips, and so on. Cellulose-based biomass is composed of three main biopolymers: cellulose (30–70%), hemicelluloses (15–30%) and lignin (10–25%), the specific composition varies along with different plant species (Table 4.3). Figure 4.4 shows the cellulose–hemicellulose–lignin network in plant. It can be seen that cellulose and lignin fibers are connected by hemicellulose, and hemicellulose is covalently bonded with lignin through lignin–carbohydrate complex (Björkman 1957). As the major component of cellulose-based biomass, cellulose is insoluble in water and most organic solvents, and has been verified to be biodegradable. It can be broken down chemically into its glucose units by treating it with concentrated acids at high temperature. Many properties of cellulose depend on its chain length, crystallinity, or degree of polymerization. Different from the crystalline and strong resistance to

Table 4.3 Compositions of cellulose-based biomass

Cellulose-based biomass	Cellulose	Hemicellulose	Lignin	References
Wheat straw	30–40	20–50	10–21	Thomsen et al. (2006), Taherzadeh and Karimi (2008), Kaparaju et al. (2009), Drapcho et al. (2015)
Rice straw	32–47	18–27	5–24	(Persson et al. 2009; Binod et al. 2010; Harun and Geok 2016)
Barley straw	37–38	25–35	16	Akpinar et al. (2009), Jin et al. (2009)
Corn stalk	38	28	15	Jin et al. (2009)
Corn stover	37	31	13–26	Pordesimo et al. (2005), Sun et al. (2005), Templeton et al. (2009)
Bagasse	45–55	20–25	18–24	Adamovska et al. (2016)
Poplar	44.5	22.5	19.5	Guerra et al. (2006)

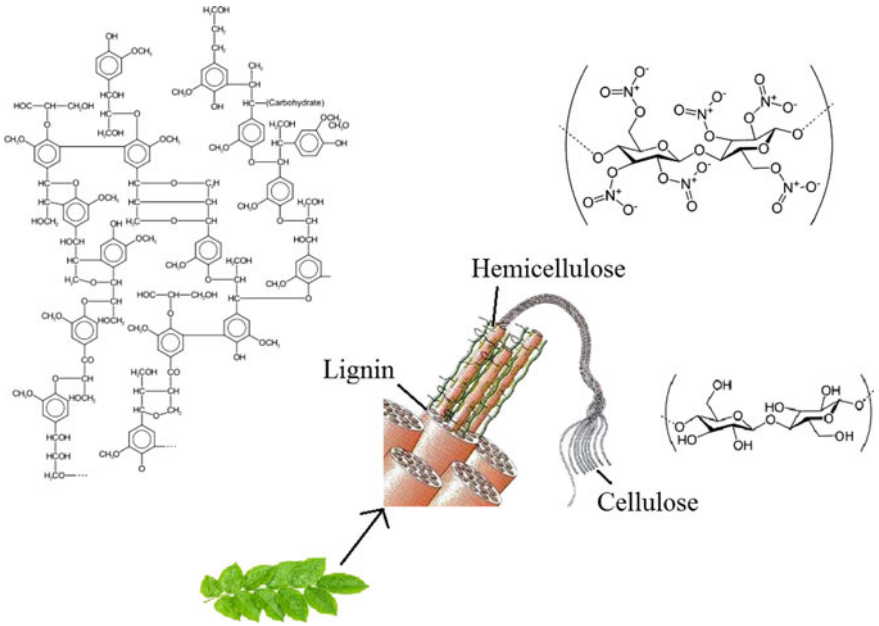


Fig. 4.4 Simplified scheme of the lignification, supramolecular organization, and composition of plant cell walls in a lignocellulosic matrix

hydrolysis of cellulose, hemicelluloses have a random, amorphous structure with little strength, and have a lower molecular weight than cellulose. The dominant component of hemicelluloses can be xylan or glucomannan. Hemicelluloses are the most thermal chemically sensitive part of cellulose-based biomass. Lignin is a cross-linked network hydrophobic polymer, it is not only insoluble but resistant to

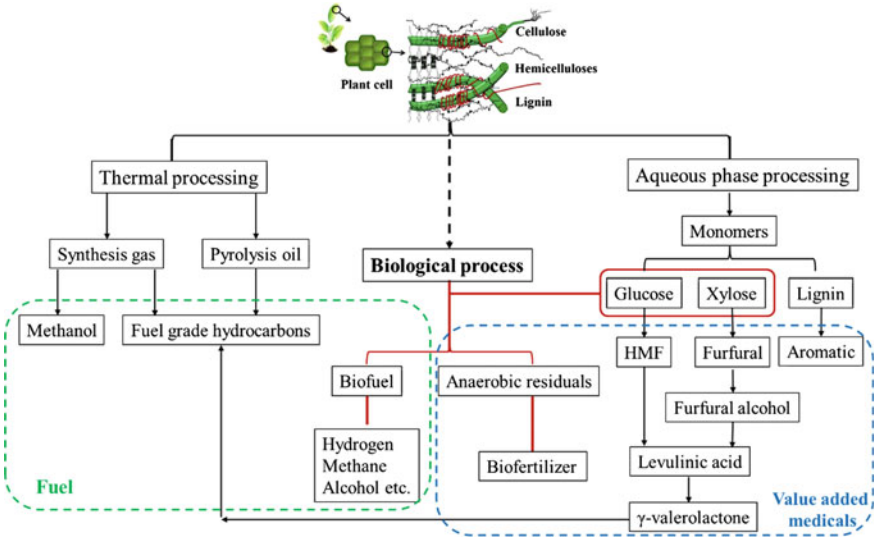


Fig. 4.5 Diagram of the conversion of cellulose-based biomass to fuels and value-added chemicals. (*HMF* 5-hydroxymethylfurfural) (Modified from Yan et al. (2015))

biodegradation. Its main purpose is to give the plant structural rigidity, impermeability, and resistance against microbial attack and oxidative stress. Thus, lignin is the main hindering factor for fermentative hydrogen production from cellulose-based biomass (Monlau et al. 2013a, b).

Cellulose-based biomass is by far the most abundant raw material and wide applications have been explored. Figure 4.5 shows the conversion of cellulose-based biomass to value-added chemicals and fuel. Cellulose-based biomass is considered as a potential fuel source. the annual yields from all over the world are estimated to exceed 220 billion tons (Kumar et al. 2015), equivalent to 60–80 billion tons of crude oil (Ren et al. 2009). Till now, it accounts for 14% of the world's energy supply, serving as the fourth largest source of energy after coal, petroleum, and natural gas (Saxena et al. 2009). Cellulose-based biomass has been used as source for fuel generation through various ways like: biomass gasification (Alauddin et al. 2010), catalytic production of diesel fuels and value-added chemicals (Yan et al. 2015), fermentative production of ethanol (Srivastava et al. 2015), methane (Chandra et al. 2012) and hydrogen (Kumar et al. 2015), and so on. Biological conversion processes are preferred for their environmental benefits.

To enhance the digestibility of cellulose-based biomass, pretreatment methods are required to remove or solubilize the lignin present in cellulose-based biomass. Besides, pretreatment is also beneficial in reducing the crystallinity of cellulose and enlarging the available surface area, further enhance the biodegradability of cellulose-based biomass. Motte et al. (2014) found that hydrogen yield from wheat straw can be significantly increased from 3.4 to 15.3 mL/g TS to 20–35 mL/g TS

by grinding process. Ozkan et al. (2011) also enhanced hydrogen production from sugar beet pulp through various treatment methods. However, Kumar et al. (2015) reviewed 22 studies about the effect of treatment on hydrogen production from lignocellulosic biomass, and concluded that the average hydrogen yields for treated and untreated biomass were quite similar.

4.3.4 Starch-Based Biomass

Starch-based biomass refers to the biomass rich in starch, such as wheat, corn, rice, cassava and potatoes. Starch is a kind of polysaccharides produced by plants to store energy. Similar with cellulose-based biomass, starch-based biomass is pretty plentiful, and accounts for the second most abundant organic compound on earth. However, different with the hydrolyze-resistant cellulose, starch is polymerized with a large number of glucose by alpha bonds, which can be much easily hydrolyzed comparing with the resistant beta bonds present in cellulose-based biomass. Thus, starch-based biomass is more biodegradable than cellulose-based biomass, it can be easily hydrolyzed into simple sugars (mainly include glucose and maltose) by enzymatic or acid saccharification. For the application in anaerobic fermentation, the pretreatment process is not necessary and can be much milder. In this case, starch-based biomass is more preferred in biological applications and shows great potential for economical biofuel production.

Besides the well-developed methane and ethanol fermentation, starch-based biomass has been proved to be a potential substrate for hydrogen production. Wheat starch, rice starch, potato starch, cassava starch, cornstarch as well as some other residues rich in starch (bread, sago, and brewery residues) are all explored as substrate for hydrogen production.

4.3.5 Food Waste

Food industry is the most important industry closely related to our everyday life. With the development of society, a wide range of food products are developed. However, numerous causes like the perishability of food products, waste behavior by consumers and inefficiencies in food transportation all result in the discard of food, generating food waste (Zhang et al. 2016). It has been estimated that the food waste produced each year can account for 1/3–1/2 of all food produced (2016).

Composition of food waste is pretty complex, depending on different food industry processes. It may contain grain flours, rice, vegetables, meats, fruits, and so on. Basing on the constituent, it can be categorized mainly into four groups: carbohydrates (sugars, starch, cellulose, and hemicelluloses), proteins, lipids, and organic acids (Table 4.4).

Table 4.4 Major food products and processing wastes/by-products (Zhang et al. 2016)

Major food products		Processing wastes/by-products	
Type	Annual yield, million tons (year)	Type (major composition)	Annual yield, million tons (% of total product yield)[c]
Oil (palm)	54.4 (2013)[a]	Palm empty fruit bunch (lignocellulose)	163.2 (~300%)
Oil (soybean)	42.6 (2013)[a]	Soybean meal (protein, carbohydrate)	170.4 (~400%)
Oil (rapeseed)	24.7 (2013)[b]	Rapeseed meal (protein, carbohydrate)	32.1 (~130%)
Rice	740.9 (2013)[a]	Rice hull (lignocellulose, ash)	148.2 (~20%)
Wheat	715.9 (2013)[a]	Bran (arabinoxylan, cellulose, protein)	143.2 (~20%)
Potatoes	376.4 (2013)[a]	Potato peel and other processing waste (lignocellulose, starch)	75.3 (~20%)
Banana	106.7 (2013)[a]	Rejected banana (lignocellulose, pectin, starch)	32.0 (~30%)
Apple	81.0 (2013)[a]	Rejected apples (fucogalactoxyloglucan, lignocellulose, glucose, fructose)	24.3 (~30%)
Raw sugar	178.9 (2013)[a]	Molasses (sucrose, glucose and fructose)	35.8 (~20%)
Beer (barley)	189.1 (2013)[a]	Brewery waste (carbohydrate, protein, organic acids, high COD)	851.0 (~450%, wastewater)
Wine	27.4 (2013)[a]	Brewery waste (carbohydrate, organic acids, high COD)	164.4 (~600%, wastewater)
Cheese	21.3 (2013)[a]	Whey (lactose and protein)	191.7 (~900%)
Beef	59.7 (2014)[b]	Slaughter waste (animal fat, protein)	29.8 (~50%)
Pork (2014)	110.5 (2014)[b]	Animal fats, protein	29.8 (~20%)

[a]Data from Food and Agricultural Organization, www.fao.org
[b]Data from the US Department of Agriculture, www.usda.gov
[c]Estimated data based on the approximate waste (by-production)/product ratio in literature

To manage the increasing amount of food waste, landfilling is a common practice for its disposal. However, as food waste is pretty rich in organic matters and nutrients, direct dumping or landfilling may cause various environmental problems like greenhouse gases emission, groundwater contamination and odor problems. Thus, applications other than disposal of food waste are developed. One good way is the animal feed, include feeding swine, poultry, and farming maggot to be fed to other animals. Besides, food waste can also be used as compost directly or through vermicomposting (Singh et al. 2013). With the development of anaerobic digestion process, conversion of food waste into biofuel as well as other

value-added products attracts wide attention and is considered as the most appropriate method for food waste management (Sen et al. 2016). During the anaerobic fermentation process, bioenergy (methane, hydrogen, ethanol, etc.), liquid valued medicals (acetate, butyrate, valerate, etc.) can be obtained. Furthermore, the solid residue after fermentation can be further used as fertilizer. Obviously, more values can be recovered from food waste through fermentation process.

Quite a few studies have conducted hydrogen production from food waste through dark fermentation. Food wastes used in hydrogen production are mainly obtained from dining hall or restaurant waste, which are cooked and the pretreatment process can be omitted (Yasin et al. 2013a, b). Considering the complex composition of food waste, explorations about the effects of lipid, protein, and carbohydrates on hydrogen production are conducted, and it is believed that carbohydrate fraction in food waste plays an important role in hydrogen production.

4.3.6 Wastewater

Agriculture, industry, and domestic practices all around the world are producing increasing amount of wastewater, which contains multiple compounds and becomes an increasing environmental concern (Grandclément et al. 2017). It has been estimated that the wastewater comprises around 50–100% of lost waste resources, which can be a great pollution source or a valuable resource carrier (Puyol et al. 2017). With the increasing requirement of sustainable society in both economy and environment, the focus on wastewater treatment has shifted from pollution control to resource recovery (Angenent et al. 2004).

While lots of new technologies are being developed for the resource recovery from wastewater, biological methods attract more interests for the sustainable and efficient recovery. Microorganisms have been applied in organics recovery, metal recovery, valuable chemicals recovery as well as energy recovery.

The organic-rich wastewater is more commonly used for the bioenergy recovery. Biofuel generation from organic wastewater has been widely explored, including the anaerobic digestion process, microbial fuel cell, and algae cultivation for biodiesel generation. Biofuels include methane, hydrogen, alcohols, and biodiesel are obtained.

For the dark fermentative hydrogen production, organic-rich wastewater like distillery wastewater, beverage wastewater, palm oil mill effluent, and dairy wastewater, etc., have been used as substrate. Hydrogen production process is significantly affected by the compositions of organic wastewater. Table 4.5 shows the characteristics of various wastewaters (Mohammadi et al. 2012; Karadag et al. 2014; Sivagurunathan et al. 2014; Tikariha and Sahu 2014; Monti et al. 2015; Kyzas et al. 2016; Lofrano and Meric 2016).

Table 4.5 Chemical characteristics of various wastewaters

	Type of wastewater					
Parameter	Distillery	Beverage	Palm oil mill	Olive mill	Molasses	Dairy
pH	3.0–4.1	2.6–3.5	4.2–6.4	4.5	5.2	6.1–7.7
Alkalinity (meq/L)	–	–	–		6,000	198.45–376.80
EC (S/cm)	346	–	–		–	–
Phenol (mg/L)	103–735	–	–	740–1120	450	–
VFAs (g/L)	1.6	–	–	3.4–6.0	8.5	–
COD_T (g/L)	100–120	760–900	49.8–51.5	40–60	80–350	–
COD_S (g/L)	–	–	21.95	–	–	1–10
BOD_5 (g/L)	30	–	22.5–49.5	–	–	0.3–9.0
TOC (mg/L)	2674	–	–	–	–	–
Protein (mg/L)	–	–	–	0.3–0.4	165550	13.78–72.12
Carbohydrates (mg/L)	–	660–750	–	6.5–7.7	29400	0.101–0.296
Lipids (%)	–	–	2.1–4.3	4.8–6.0	0.95	0.01–0.06
VS (g/L)	50	–		–	79	–
VSS (g/L)	2.8	–	13.3	–	2.5	–
TS (g/L)	51.5–100	–	36.7	–	109	3.3–57.0
TSS (g/L)	0.48–1.26	–	18.8	–	–	–
MS (g/L)	–	–	–	–	30	–
MSS (mg/L)	–	–	–	–	1,100	–
TN (g/L)	1.35	–	0.43	–	1.8	–
NH_4^+ (mg/L)	0–45	–	–	–	–	2.1–6.5
NO_3–(mg/L)	4,900	–	–	–	–	10.24–15.52
TP (g/L)	–	–	0.084	–	–	18.0–26.4

To enhance the hydrogen production from wastewater, different types of wastewater require specific treatment. For example, distillery and beverage wastewater are rich in carbohydrates while have little proteins, while dairy wastewater and cheese whey effluent are rich in proteins. Thus, to achieve a proper C/N ratio, extra nitrogen source or carbohydrates source are needed. The main carbon source of biodiesel effluent is glycerol, which occupies over 40% of biodiesel wastewater. Thus, microbes capable of degrading glycerol are preferred in this system.

4.4 Pretreatment of Organic Wastes

Since low hydrolytic enzymatic activity is observed with the anaerobic cultures, a pretreatment step is often required for the hydrolysis of biomass, consequently enhance the hydrogen production efficiency. During the pretreatment process, trapped components were released through cell wall lysis and delignification of lignocellulosic biomass. Subsequently, pretreatment process can destroy the crystal structure of macromolecular substances and release their polymerization degree. Consequently, a higher proportion of readily fermentable substances are made accessible for microorganisms.

Pretreatment methods can be divided into four categories: physical treatment, chemical treatment, biological treatment, and a combination of different treatments. Table 4.6 shows the commonly used pretreatment methods and their applications in treating biomass.

4.4.1 Physical Treatment

Physical treatment methods have been studied for biomass pretreatment include mill, grind, ultrasonication, heat, freeze and thaw, microwave and ionizing radiation as illustrated in Table 4.6.

4.4.1.1 Mechanical Treatment

Mechanical treatment methods like milling, grinding, and chipping are usually used as the first step to reduce the particle size, create higher accessible surface area and decrease crystallinity (Taherzadeh and Karimi 2008; Haghighi Mood et al. 2013). Mechanical treatment is usually combined with other physical treatment methods and chemical, biological treatment methods. Studies have figured out that mechanical treatment can efficiently enhance the treatment efficiency of the followed treatment methods (Ntaikou et al. 2010). Yeh et al. (2010) observed that the enzymatic hydrolysis rate of cellulose was increased by more than fivefolds after a milling treatment. Mais et al. (2002) achieved 100% hydrolysis of lignocellulosic substrate with a minimum enzyme loading by using simultaneous ball milling.

4.4.1.2 Heat Treatment

Heat treatment is the most widely used method in solubilizing biomass for fermentative hydrogen production. High temperature can disrupt the chemical bonds of the cell wall and membrane, leading to the solubilization of cell components and deterioration of microbial protein (Appels et al. 2008). Besides its high efficiency in

Table 4.6 Commonly used methods for biomass pretreatment

Category			Applications
Physical treatment	Mechanical treatment	Milling; grinding; chipping	First step of treating Cellulose-based biomass and algae
		Ultrasonication	More used in treating waste activated sludge
	Temperature control	Heat treatment	Most commonly used treatment method in treating various organic waste
		Freeze and thaw	Waste activated sludge, but not commonly used
	Irradiation	Microwave	More used in treating waste activated sludge
		Ionizing radiation: gamma ray; electron beam	Waste activated sludge
	Electric current		Algae
Chemical treatment	pH control	Acid: HCl; H_2SO_4; H_3PO_4	Commonly used in treating various organic waste
		Base: NaOH; NH_4OH; $Ca(OH)_2$	Commonly used in treating various organic waste, especially effective in solubilizing waste activated sludge
	Oxidizing agent	Ozone; H_2O_2	Algae and waste activated sludge
	Methanogenic inhibitors	$CHCl_3$; BESA	Waste activated sludge
Biological treatment	Enzyme	Cellulase;Viscozyme L; Endoxylanase; Glucosidase; Bromelain; Lysozyme; Amylase; Composite enzyme, etc.	Most commonly used in Cellulose-based biomass
	Bacterial hydrolysis	Fungi (White-rot, brown-rot, soft-rot)	Cellulose-based biomass
		Methane fermentation	Cellulose-based biomass
		Aerobic thermophilic digestion	Waste activated sludge

biomass disintegration, the wide application of heat treatment also owes to its simple operation and easy control. It is essential to choose an appropriate temperature in heat treatment. Low temperatures may lead to insufficient disintegration, while too high temperatures can result in the excessive degradation of organic matters in biomass, reduce the value of biomass as an organic source for fermentative hydrogen production (Bertanza et al. 2015). Furthermore, too high treatment temperatures may also cause the formation of refractory compounds, which are

inhibitory to fermentative hydrogen production (Bougrier et al. 2007). Shanableh (2000) examined the effect of temperature on hydrothermal treatment of waste activated sludge, and the results showed the residual sludge COD decreased from approximately 9000–10,000 mg/L to 1000–3000 mg/L as the reaction temperature increased from 280 to 460 °C and the most of the residual COD was in the soluble form. Hydrothermal treatment at 300–350 °C was more effective in destroying sludge solids than in achieving COD removal.

Temperature adopted in disintegrating biomass varies a lot according to different substrates. For the waste activated sludge treated for biohydrogen production, treating temperature ranges from 100 to 175 °C and duration time from 15 min to 60 min. The most widely used condition is 121 °C for 20–30 min. For the algae biomass, treating temperature ranges from 65 to 180 °C and duration time from 15 min to 60 min. Similar with waste activated sludge, the most commonly used condition is 121 °C for 15–20 min. As to the cellulose-based biomass, treating temperature ranges from 50 to 220 °C and duration time from 90 s to 90 min. Since heat treatment is usually combined with other treatment methods in treating cellulose-based biomass, the temperature adopted varies a lot according to the combined treatment methods.

4.4.1.3 Freeze and Thaw

Freeze and thaw is mainly applied in treating waste activated sludge. It involves freezing the sludge at extreme temperature and thawing at room temperature for several cycles. During these strong fluctuations in temperature, cell swelling is caused by intracellular ice crystals formation. Consequently, cell aggregates are damaged and disrupted (Sawicka et al. 2010). Kotay and Das (2009) observed 40% enhancement in hydrogen yield from freeze and thaw treated waste activated sludge. Wang et al. (2003) achieved 1.5–2.5 times increase of hydrogen yield with freeze and thaw treatment. However, both Kotay and Wang et al. reported no enhancement in sludge solubilization after freeze and thaw treatment. Besides waste activated sludge, Chang et al. (2011) first explored the effects of freeze and thaw treatment on rice straw, and a significant increase of enzyme digestibility from 48% to 84% was obtained. Indicating that freeze and thaw can be a good candidate when combined with enzyme treatment.

4.4.1.4 Electric Current

Electric current was found to be able to destruct cell membranes and lead to microbial permeabilization (Zimmermann et al. 1974; Lojewska et al. 1989). It has been used in inactivating microbes but very few studies have applied it in treating feedstock. Jeong et al. (2015) examined the feasibility of electric current (20–100 V) as a pretreatment method to enhance hydrogen production from *Laminaria japonica*. As shown in Fig. 4.6, with the increase of voltage from 0 V to 60 V,

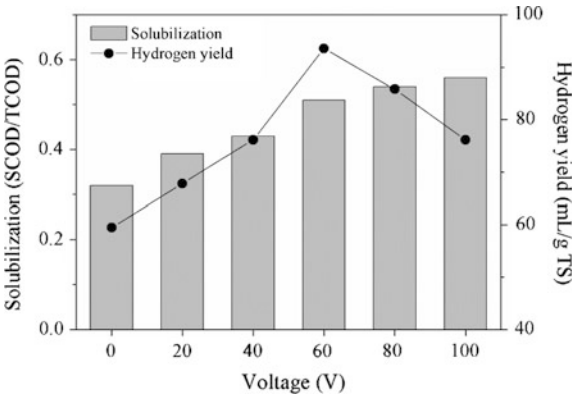


Fig. 4.6 *Laminaria japonica* solubilization and hydrogen production with voltage treatment

algae solubilization increased from 0.32 to 0.56, highest hydrogen yield of 93.6 mL/g TS was obtained at 60 V. With the further optimization, hydrogen yield was increased by 72.6% at 58.5 V for 30 min, revealing that the electric current has a potential as an alternative method for feedstock preparation.

4.4.1.5 Radiation

Radiation can be divided into ionizing radiation and nonionizing radiation (Fig. 4.7) (Rendic and Peter Guengerich 2012).

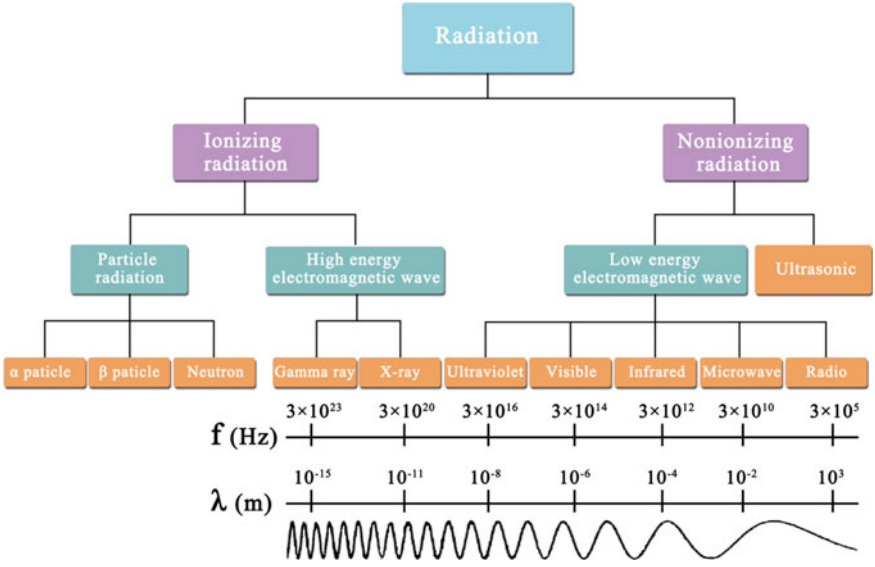


Fig. 4.7 Various types of ionizing radiation and nonionizing radiation

For the treatment of biomass for hydrogen production, microwave ultrasonication and ionizing radiation have been used.

(1) Microwave
Microwave irradiation is a kind of electromagnetic radiation with frequencies range from 0.3 GHz to 300 GHz. Effects of microwave irradiation on microorganisms comprises thermal and nonthermal effect (Vela and Wu 1979; Jeng et al. 1987). When microwave pass through a product, a chaotic movement of molecules happens. With the continuous rotating electric field, alignment and realignment of polar molecules (like water) occurs, leading to the heat generation, rupture of hydrogen bonds and modification of hydration zone (Piyasena et al. 2003; Hong et al. 2006; Dańczuk and Łomotowski 2010; Bundhoo et al. 2015).

The effectiveness of microwave irradiation in destructing cells is affected by the treating temperature and the electromagnetic field power. As shown in Fig. 4.8, Xia et al. (2013) treated algae biomass by microwave, the results showed that the reducing sugar yield was increased with the treating temperature from 80 to 140 °C, and further increase of temperature caused the decrease of sugar yield. When the treating temperature was fixed, microwave time had little effect on sugar release. Kuglarz et al. (2013) compared sludge treated by microwave of 700 W and 900 W at same temperature, better performance was obtained by 900 W. To figure out the nonthermal effect of microwave, Guo et al. (2015) compared the effect of microwave and heat treatment on sludge disintegration at same temperature, solubilization of microwave treated sludge was more than three times over heat treated sludge. Thus, microwave is a quite potential treatment method in disintegrating biomass for fermentative hydrogen production.

(2) Ultrasonication
Ultrasonication is also considered as a mechanical pretreatment method, but it owns both physical and chemical effects. During the ultrasonication process,

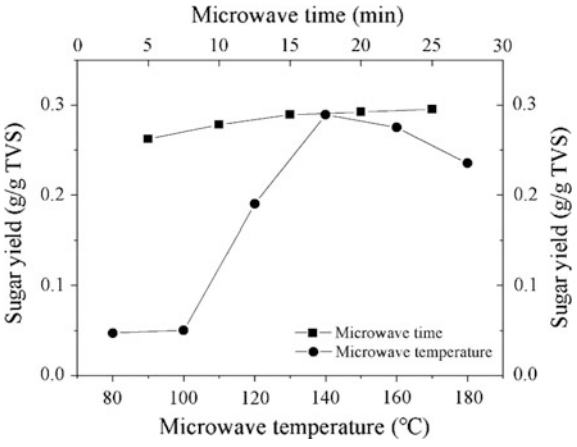


Fig. 4.8 Effects of microwave heating on the reducing sugar yield from *Nannochloropsis oceanica* biomass. Effect of microwave temperature (microwave time: 15 min); Effect of microwave time (microwave temperature: 140 °C)

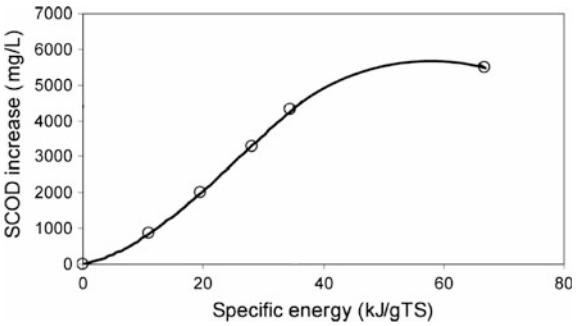


Fig. 4.9 Effect of specific energy input on SCOD increase of waste activated sludge (frequency: 20 kHz; maximum power: 1.5 kW; TS content: 3%; and ultrasound density: 1.07 W/ml)

shear forces, high localized temperature (5000 K) and pressure (180 MPa), and highly active radicals are generated in the medium, resulting in cell disruption and particle solubilization. Ultrasonication is more commonly used in solubilizing waste activated sludge. Studies have found that the sludge disintegration degree increased with the energy density and treating time of ultrasonication (Yang et al. 2012). As shown in Fig. 4.9, with the energy input from 0 to 60 kJ/g TS, SCOD of waste activated sludge was significantly increased (Khanal et al. 2007). Kotay and Das (2009) treated waste activated sludge by ultrasonication and enhanced COD solubilization by 5% and hydrogen yield by 60%. Guo et al. (2008) improved COD solubilization of waste activated sludge by 87% using ultrasonication treatment. Nguyen et al. (2010) achieved the enhanced hydrogen production by over 25% from sonicated algae biomass.

(3) Ionizing radiation

Ionizing radiation is several kinds of high-energy particle flows (e.g. α particles, β particles) and electromagnetic radiation (e.g. X ray, gamma ray) given off by nuclear reactions, radiation producing machines and radioactive material, which could cause radiation effects of radiated substances through energy transformation (Azzam et al. 2012). In the electromagnetic spectrum, X ray radiation occurs in wavelengths of 10^{-10}–10^{-8} m. Gamma ray occurs in wavelengths of 10^{-10}–10^{-12} m. Since X ray, α particle and β particle flows have limited penetration capability, gamma ray and accelerated electrons are more commonly used.

^{60}Co and ^{137}Cs are usually applied as two gamma radiation sources for sludge handling. ^{60}Co emits gamma rays with two radiation energies of 1.33 and 1.17 meV, respectively (Meeroff 2001). The radiation energy of ^{137}Cs is 0.66 meV. The half-value thickness of ^{60}Co gamma rays is about 28 cm in water, and no less than 25 cm in sludge (Wang and Wang 2007). The half-value thickness of ^{137}Cs is 24 cm in water (Wang and Wang 2007). Clearly, ^{60}Co gives higher energy and penetrating capacity than ^{137}Cs do, ensuring the radiation impact on sludge layer. ^{60}Co and ^{137}Cs have half-lives of 5.26 and 30 years, respectively. ^{137}Cs has a longer half-life than ^{60}Co. However, caesium chloride is soluble in water, which

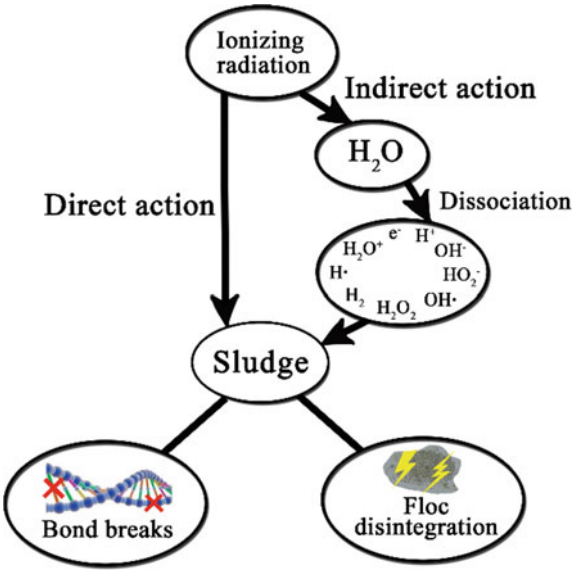


Fig. 4.10 Mechanisms of ionizing radiation treating sludge

restrains its wide application since it may cause the leak problem or other accident. Thus, ^{60}Co is the most commonly applied as the gamma radiation source for sludge handling.

Accelerated electrons are emitted by various kinds of electron beam accelerators. These accelerators accelerate charged particles by electric and magnetic field in a single direction. The energy of accelerated electrons can achieve 3 MeV with conventional machines (Wang and Wang 2007). High-energy electrons are usually produced by more complicated and expensive linear accelerators. The penetrating capacity of electron beam is about 3 mm/MeV in water (Borrely et al. 1998).

Regarding above two radiation systems, the main merit of electron beam over gamma radiation is its absence of radioactive source, which significantly avoid some security issues. Another merit of electron beam radiation over gamma radiation is its facilitating control method, because it can be turned on and off instantaneously. However, electron beam radiation is limited by its rapid energy loss with the penetration depth in aqueous media. So homogenous condition and the distribution of absorbed dose is vital for applying electron beam to organic wastes treatment.

When organic wastes are irradiated by ionizing radiation, high-energy irradiation can change physical or chemical properties of target substance through both direct action and indirect action (Fig. 4.10). Direct action refers to the atoms in molecules absorb the energy, which is sufficient to remove the electrons from the atoms and result in bond breaks. Indirect action is mediated by radiolysis products of H_2O. The comprehensive reaction of H_2O radiolysis can be described as

$$H_2O \rightarrow 2.7\ OH + 2.6\ e_{aq}^{-} + 0.6\ H + 2.6\ H_3O^{+} + 0.7\ H_2O_2 + 0.45\ H_2. \quad (4.1)$$

These highly active radicals are usually generated when water is irradiated by ionizing radiation, and they can react with substances present in the liquid phase in less than 1 us. Both direct and indirect effects can result in cell rupture and biomass solubilization.

Ionizing radiation has been widely used in treating waste activated sludge for various purposes include sludge disinfection, hazardous materials elimination, sludge dewatering, etc. (Borrely et al. 1998; Zheng et al. 2001; Al-Bachir et al. 2003; Wang and Wang 2007). Both Kim et al. (2009) and Chu et al. (2011) found that sludge solubilization increased with ionizing radiation dose lower than 50 kGy. Bak et al. (2009) examined the effect of electron beam radiation on enzyme digestibility of rice straw, the results showed that glucose yield increased with the increase of electron beam from 0 kGy to 80 kGy but declined when the treating dose increased to 90 kGy. Yin and Wang (2015, 2016, b) explored hydrogen production from gamma radiation treated waste activated sludge, and the highest hydrogen yield of 10.5 mL/g SCOD was achieved from sludge treated by alkali-irradiation at pH = 12 and 20 kGy.

4.4.2 Chemical Treatment

Chemical treatment methods have been studied for biomass treatment comprises acid and alkaline treatment, oxidizing agent addition and methanogenic inhibitors addition as illustrated in Table 4.3.

4.4.2.1 Acid and Base Treatment

Acid treatment is also a commonly used treatment method for feedstock preparation. It disrupts cells through changing the electric charge on the cell membrane. HCl, H_2SO_4 as well as H_3PO_4 are applied. For waste activated sludge treatment, HCl is used for the treatment. The treating pH ranges from 3 to 4, and treating time ranges from 0 to 24 h. Assawamongkholsiri et al. (2013) explored waste activated sludge treated by 0.5% (w/v) HCl for 0–24 h, and the maximum hydrogen production was obtained from the sludge treated for 6 h. As to algae biomass and cellulose-based biomass, acid treatment is usually combined with heat treatment. H_2SO_4 is more widely used and the concentrations range between 0.5% and 6% according to the followed heating temperature. Although it is believed that concentrated acid accompanied with lower temperature is more economic (Haghighi Mood et al. 2013), diluted acid with higher temperature is more widely used for its better performance. Park et al. (2013) studied hydrogen production from red algal biomass treated by combined acid and heat treatment, both H_2SO_4 concentration

(0.5–1.5%) and heat temperature (120–180 °C) were examined. The results revealed that the hydrolysis temperature was the most significant factor, and the highest hydrogen production was obtained at 0.5% H_2SO_4 and 161–164 °C hydrolysis. Besides, 0.5% H_2SO_4 and 121 °C hydrolysis is the most commonly used its easier operation and relatively high efficiency (Pattra et al. 2008; Song et al. 2012; Brynjarsdottir et al. 2013; Sinha and Pandey 2014).

Base treatment works through the dissolution and saponification of ester bonds, leading to cell membrane dissolution, polymerisation decrease and cellulose crystallinity destruction. NaOH, NH_4OH, and $Ca(OH)_2$ are used in base treatment. Waste activated sludge can be effectively solubilized at pH 10–12 for 24 h. Cai et al. (2004) achieved 82.4% enhancement of hydrogen production from waste activated sludge through base treatment at pH 12 for 24 h. Both Carver et al. (2011) and Lakaniemi et al. (2011) enhanced hydrogen production from *Chlorella vulgaris* biomass through base treatment at pH 9.5. As to the cellulose-based feedstock, Ozkan et al. (2011) compared base treatment, combined base-heat treatment and combined base-microwave treatment, best hydrogen production was obtained from the base treated beet-pulp. However, in most studies, base treatment is used in combination with heat or biological treatment (de Vrije et al. 2009; Cheng et al. 2011; El-Bery et al. 2013; Monlau et al. 2013a, b; Phummala et al. 2014).

Acid and base treatments are usually conducted at room temperature. Besides heat treatment, they are the most commonly used treatment methods for the convenient operation and high efficiency. Cheap and efficient acid or alkali can easily promote their application. However, both acid and base treatment requires long resident time when they are used alone. Furthermore, inhibitory compounds were also detected after the treatment process (Jonsson et al. 2013). Another limiting factor is the pH adjustment required after the treatment process, which is neither economic nor environmentally friendly. The addition of chemical reagents creates more work for the subsequent processing.

4.4.2.2 Oxidizing Agent

For the ozonolysis process, ozone gas is used to solubilize biomass for its strong oxidizing property and high solubility. Unlike acid or base treatment, no inhibitory compounds are formed during the ozonolysis process and no residual chemical reagents are left after the ozonolysis process (Taherzadeh and Karimi 2008). Treatment efficiency of ozonolysis process is affected by aeration rate of ozone, processing time, and water content of feedstock. Yang et al. (2012) examined sludge disintegration by ozone dose range 0.05–0.2 g O_3/g dry solid and time duration 0–60 min, the highest sludge disintegration degree was obtained at 0.15 g O_3/g dry solid for 60 min. Wu et al. (2013a, b) revealed that with the increasing ozone dose from 0 to 6.5 mg O_3/g straw, and ozonation time from 0 to 150 min, lignin contents of the wheat straw and barley straw decreased gradually. However, inhibitory effect on hydrogen yield was observed when ozonolysis process was over 90 min. Ozonolysis has showed its potential application in treating biomass for its

high efficiency. But considering the large amount of ozone required in large scale application, economical efficiency of this technology still need to be further discussed.

H_2O_2 can generate nascent oxygen that breaks the glycosidic bonds, disintegrates complex carbohydrates to simple sugars. H_2O_2 treatment is not widely used in feedstock treatment. Roy et al. (2014) explored hydrogen production from algae biomass treated by H_2O_2, and compared it with different treatment methods include autoclave, sonication, and acid treatment. A better performance in hydrogen production was observed in H_2O_2 treatment test than autoclave and sonication tests.

4.4.2.3 Methanogenic Inhibitors

For the feedstock treatment, BESA and $CHCl_3$ have been applied in suppressing the methanogenic bacteria while solubilizing organic matters (Wang et al. 2003; Kotay and Das 2009). Wang et al. (2003) obtained treated waste activated sludge through adding 1 mol/L BESA, and obtained significant SCOD/TCOD increase and methane yield inhibition. However, hydrogen yield was also suppressed compared with raw sludge. Kotay and Das (2009) revealed that both BESA and $CHCl_3$ had no effect on sludge solubilization, hydrogen yield was slightly stimulated by BESA while suppressed by $CHCl_3$. Wongthanate et al. (2014) also showed an inhibitory effect of BESA on hydrogen production from food waste and starch waste. Basing on the studies mentioned above, methanogenic inhibitors can hardly be considered as a good treatment method for feedstocks.

4.4.3 Biological Treatment

Enzymatic treatment is commonly used in treating cellulose-based biomass and algae biomass, and it is usually conducted after physical or chemical treatment. Various enzymes have been used in enzymatic treatment (Table 4.6), cellulase is the most frequently used enzyme. The disintegration effect of enzymatic treatment is affected by enzyme species, substrate characteristics, enzyme dose, and hydrolyzing duration. Guo et al. (2015) hydrolyzed waste activated sludge by adding multienzyme in the ratio of 1:50 (v:v), dissolved organic matters were significantly increased and achieved a better performance than microwave and heating. However, hydrogen yield from sludge hydrolyzed by enzyme showed no advantage than sludge hydrolyzed by physical treatments. Cheng et al. (2014) explored the effect of cellulase and glucoamylase on sugar yields of algae biomass. The application of both enzymes showed significant advantage in sugar release than cellulose alone. Quéméneur et al. (2012) compared the effect of different CEP enzyme concentrations on hydrogen production from wheat straw, and revealed a positive correlation between hydrogen yield and enzyme dose.

Besides enzymatic treatment, bacterial hydrolysis is also applied in decomposing feedstock. The commonly known bacterial hydrolysis is the disruption of lignocellulosic biomass by fungal treatment. Among the fungi species include white-rot, brown-rot and soft-rot fungi, white-rot fungi *Phanerochaete chrysosporium* is the most widely used. Zhao et al. (2012), (2013) enhanced enzymatic saccharification of corn stalk and hydrogen production. Besides the fungal degradation of biomass, other microbes were also used. Guo et al. (2015) and Sittijunda et al. (2010) explored sludge disintegration by aerobic thermophilic digestion, through controlling a proper digestion condition and duration, both studies achieved considerate enhancement in hydrogen production. Carver et al. (2011) successfully increased hydrogen production from algae biomass by inoculating cellulose degrading strain TC60 to the system.

Biological treatment is usually conducted in mild conditions, low energy consuming and requires no subsequent processing. However, because of the low hydrolysis rate, most biological treatment methods are time-consuming and hard to be commercialized.

4.4.4 Combined Treatment

To disintegrate biomass more efficiently and take advantage of various treatment methods, the combination of different methods is studied. Most combined treatment methods comprise a physical treatment method and a chemical treatment method.

Combined heat and acid treatment is the most commonly used method. Assawamongkholsiri et al. (2013) compared the activated sludge treated by acid, heat and combined acid and heat, the highest SCOD, protein and carbohydrate concentrations were all obtained from combined acid and heat treated sludge. Roy et al. (2014) examined effect of different treatment methods on algae biomass disintegration and hydrogen production, best performance was observed with combined acid and heat treated microalgae. Combined acid and heat treatment is also frequently applied in disintegrating cellulose-based biomass include straw (Nasirian et al. 2011; Pawar et al. 2013; De Sá et al. 2015), stalk (Song et al. 2012), bagasse (Pattra et al. 2008; Fangkum and Reungsang 2011; Saripan and Reungsang 2014; Sinha and Pandey 2014) and grass (Brynjarsdottir et al. 2013; Veeravalli et al. 2014), etc.

Besides acid treatment, heat treatment has also been combined other methods such as base treatment, enzyme treatment, and oxidizing agent addition. Kang et al. (2012) applied combined base and heat treatment to sewage sludge, solubilization ratio of 85% was achieved, which was much higher than using heat or base treatment alone. Ozkan et al. (2011) obtained significant solubilization of beet pulp with combined base and heat treatment. Enzyme hydrolysis after heat treatment has been proved to be able to significantly enhance the hydrogen yield. Massanet-Nicolau et al. (2008), Massanet-Nicolau et al. (2010) and Yasin et al. (2013a, b) added cellulase hydrolysis after heat treatment to enhance the sludge

solubilization. Li and Chen (2007) and Prasad et al. (Kaparaju et al. 2009) also achieved an increase in hydrogen production from straw by cellulase addition.

Other combination of treatment methods are also Studied. Yang et al. (2012) and Wu et al. (2013) combined ozone with ultrasonication and enzyme hydrolysis, respectively. Cheng et al. (2011) and Xia et al. (2013) combined microwave with base and acid treatment, respectively. Yin and Wang (2015, 2016, b) and Kim et al. (2009) examined the combination of ionizing radiation and base treatment. All of them achieved enhanced hydrogen production from treated biomass wastes. In some cases, combinations of three or more treatment methods were used. Like acid-heat-enzyme treatment (Wang et al. 2010; Kumar et al. 2016), acid-microwave-enzyme treatment (Cheng et al. 2014), base-heat-enzyme treatment (Phummala et al. 2014), and so on.

4.4.5 *Comparison of Different Treatment Methods*

Different treatment methods have different effects on biomass disintegration.

Figure 4.11 shows the scanning electron microscope (SEM) images of waste activated sludge treated by different physical treatment methods. Figure 4.11a–f show the raw sludge and sludge treated by ultrasonication, microwave and ionizing radiation, respectively. It can be seen that all the treatment methods have an apparent effects on microbial cell disruption and sludge solubilization.

Yasin et al. (2013a, b) examined the effect of enzyme treatment on sludge before and after fermentation process. SEM micrograph showed that after the enzyme hydrolysis, a hole was observed in sludge structure. Sludge with enzyme treatment was degraded to smaller structures after fermentation while sludge without enzyme treatment still showed complex structure after fermentation.

Liu et al. (2008) explored waste activated sludge treated by different combined treatments. The SEM images showed that ultrasonicalkaline treated sludge had the smallest particle size, followed by thermoalkaline. Neither thermoacid nor thermoalkaline was efficient in sludge disintegration, indicating that base and ultrasonication treatments are more efficient in destroying cell walls than acid and thermal treatments.

Roy et al. (2014) examined the algae biomass treated by different treatment methods including 50 dm^3/m^3 HCl-heat treatment, 200 dm^3/m^3 HCl-heat treatment, H_2O_2 treatment, autoclave, and ultrasonication. The results showed that before the treatment process, intact algae cells were clearly displayed. Higher acid concentration brought out more cell solubilization. For the H_2O_2 treated algae, quite a few intact cells were still present after treatment, and physical treatment like autoclave and ultrasonication led to the less cell debris. This result indicated that combined treatment had the strongest disintegration effect on cells, followed by chemical treatment. Physical treatment showed to be the least effective.

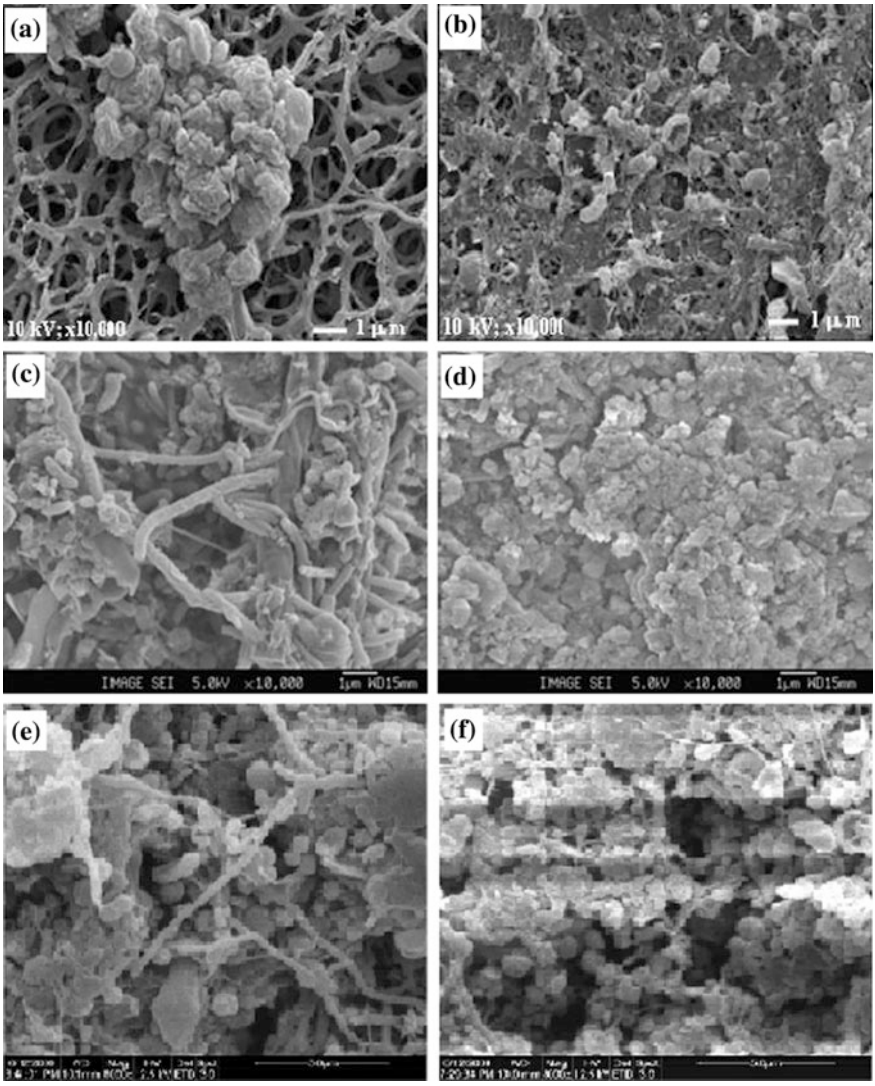


Fig. 4.11 Waste activated sludge treated by different physical treatment methods. **a** Raw sludge, **b** Ultrasonication treated sludge, from Ref. (Khanal et al. 2007); **c** Raw sludge, **d** Microwave treated sludge, from Ref. (Zhou et al. 2010); **e** Raw sludge, **f** Ionizing radiation treated sludge, from Ref. (Chu et al. 2011)

Basing on the different effects of treatment methods, their application also differs according to the feedstock species. Articles focused on dark fermentative hydrogen production from biomass in recent 10 years are reviewed, and accordingly, the distributions of different treatment methods on biomass are summarized in Fig. 4.12. For the treatment applied in all kinds of biomass, combined methods

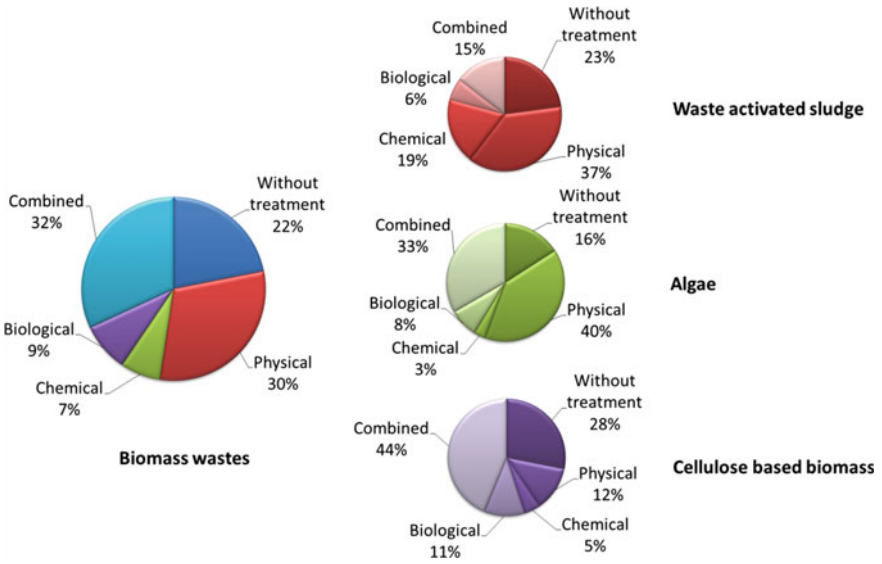


Fig. 4.12 Number of article published on the subject of applying various treatment methods on treating different kinds of biomass

were mostly used, accounting for about 32% of all reports, followed by physical treatment with 30%. Chemical treatment was the least used method. However, when it comes to different kinds of biomass, treatment methods present different distributions. For the waste activated sludge and algae biomass, physical treatment was the most commonly used, accounts for 37% and 40%, respectively. The reason is that both waste activated sludge and algae biomass have high water content, physical treatment like heat, microwave, and ultrasonication can be easily and efficiently conducted. Comparing with waste activated sludge, algae biomass and cellulose-based biomass are more refractory. Thus, the combined methods are more widely used with 33% and 44%, respectively. It can be seen that cellulose-based material without treatment also occupied a big part with 28%, which was mainly contributed by studies used chemicals like carboxymethyl cellulose to represent the natural cellulose material.

It is hard to conclude which treatment method is the best fit. In some cases, the best disintegration does not mean the best hydrogen production. Severe treatment conditions bring out good dissolution performance as well as the loss of organic matters and the formation of refractory substances (Yin and Wang 2016, b). Thus, the review of pretreatment supplies reference for selecting treatment method, specific treatment conditions still need to be explored according to the specific circumstances.

4.5 Hydrogen Production from Organic Wastes

Fermentative hydrogen production is affected by many factors like substrate type and concentration, pretreatment methods, inoculum source, fermentation temperature, initial pH, and so on. For hydrogen production from biomass wastes, variation of substrate composition requires different operational and bioprocessing conditions for efficient hydrogen production.

4.5.1 Hydrogen Production from Waste Activated Sludge

Hydrogen production through dark fermentation from waste activated sludge is summarized in Table 4.7. Treatment process is a critical step before hydrogen production from waste activated sludge, not only disintegrating sludge, but inhibiting non-hydrogen producers present in waste activated sludge. Thus, studies are classified and summarized according to the treatment methods applied.

Total solid concentration of sludge varies significantly according to the different sources and retention time. Inoculum is not essential when waste activated sludge is used as substrate since it is pretty rich in microorganisms. However, in some studies, hydrogen producers like *Clostridium* sp. and *Enterobacter* sp. were inoculated into the fermentation system to enhance the hydrogen production efficiency (MIAH et al. 2004). Initial pH 5.0–11.0 was used, which is pretty diverse. It owed to the different pretreatment methods used. For the treatment with the addition of acid or alkali, acidic, or alkaline conditions were adopted to save the cost for pH adjustment (Cai et al. 2004; Assawamongkholsiri et al. 2013). Both mesophilic (30–37 °C) and thermal (55–78 °C) conditions were studied, and mesophilic fermentation was more commonly used for the lower operational cost.

4.5.2 Hydrogen Production from Algal Biomass

Table 4.8 shows the fermentative hydrogen production from various algae biomass. Since the composition of algae biomass varies significantly along with its species, studies are summarized and listed according to the algae species. It can be seen that microalgae is more used as substrate than macroalgae. This may because the microalgae biomass has simpler structure than macroalgae, thus simpler pretreatments are required. Besides, microalgae can be cultivated in various conditions and more easily obtained. Furthermore, high salinity of macroalgae cultivated in marine ecosystem inhibited its application in fermentative hydrogen production.

Among microalgae strains, *Chlorella* sp. was the most widely studied. Highest hydrogen yield of 958 mL H_2/g VS was obtained from acid-heat treated *Chlorella sorokiniana* biomass (Roy et al. 2014). *Scenedesmus* sp. was also extensively

Table 4.7 Bio-hydrogen production from waste activated sludge

Treatment method	Concentration of Waste activated sludge (g/L TS)	Inoculum	Operational conditions	Hydrogen yield (mL/g TS)	References
Without treatment					
–	3.0	–	pH 7, 55 °C; Batch	9.7[c]	Lin et al. (2012)
–	10.36	–	pH 11, 36 °C; Batch	8.1	Cai et al. (2004)
–	10.6	–	pH 10, 37 °C; Batch	19.2[b]	Wang et al. (2015a, b)
–	10.6	–	pH 10, 55 °C; Batch	80.1[b]	Wang et al. (2015a, b)
–	14.3	–	pH 6.8, 37 °C; Batch	0.35[b]	Xiao and Liu (2009)
–	14.3[a]	–	pH 10, 37 °C; Batch	58.7[b]	Wang et al. (2015a, b)
–	50	–	pH 5.5, 37 °C; Batch	8.83	Sittijunda et al. (2010)
–	16.5	*Clostridium bifermentans*	pH 6, 35 °C; Batch	20.2	Wang et al. (2003)
–	22.8	*Caldicellulosiruptor bescii*	pH 6.3, 78 °C; Batch	86	Yilmazel et al. (2015)
–	100	Anaerobic sludge	pH 5.5, 30 °C; Batch	9[b]	Assawamongkholsiri et al. (2013)
–	28.7 (addition of Gelatin solid waste, paperboard mill sludge)	Anaerobic sludge	pH 6, 55 °C; Batch	144.9[b]	Elsamadony and Tawfik (2015)

(continued)

Table 4.7 (continued)

Treatment method	Concentration of Waste activated sludge (g/L TS)	Inoculum	Operational conditions	Hydrogen yield (mL/g TS)	References
Physical treatment					
Heat: 100 °C, 30 min	13.4	–	35 °C; Batch	15.53[b]	Guo et al. (2015)
Heat: 100 °C, 60 min	5.2 (Addition of rice straw)	–	pH 5, 35 °C; Batch	14.7[b]	Alemahdi et al. (2015)
Heat: 102 °C, 30 min	17.95[a]	–	pH 6–10, 37 °C; Batch	7.4–100.6[c]	Chen et al. (2012)
Heat: 110 °C, 15–60 min	100	Anaerobic sludge	pH 5.5, 30 °C; Batch	2–11[b]	Assawamongkholsiri et al. (2013)
Heat: 121 °C, 20 min	15.42	*Pseudomonas* sp. GZ1	35 °C; Batch	30.38	Guo et al. (2008)
Heat: 121 °C, 20 min	120.5	*Enterobacter cloacae* IIT-BT 08; *Citrobacter freundii* IITBT L139; *Bacillus coagulans* IIT-BT S1	–	14[c]	Kotay and Das (2009)
Heat: 121 °C, 30 min	14.3	–	pH 6.8, 37 °C; Batch	16.26[b]	Xiao and Liu (2009)
Heat: 121 °C, 30 min	16.5	*Clostridium bifermentans*	pH 6, 35 °C; Batch	70.6	Wang et al. (2003)
Heat: 175 °C, 30 min	16.4	Anaerobic sludge	pH 7, 36 °C; Batch	55.4[c]	Yin and Wang (2016, b)
Microwave: 560 W, 2 min	15.42	*Pseudomonas* sp. GZ1	35 °C; Batch	18.28	Guo et al. (2008)
Microwave: 600 W, 2 min	120.5	*Enterobacter cloacae* IIT-BT 08; *Citrobacter freundii* IITBT L139; *Bacillus coagulans* IIT-BT S1	–	8.5[c]	Kotay and Das (2009)
Microwave: 900 W, 2 min	13.4	–	35 °C; Batch	14.2[b]	Guo et al. (2015)

(continued)

Table 4.7 (continued)

Treatment method	Concentration of Waste activated sludge (g/L TS)	Inoculum	Operational conditions	Hydrogen yield (mL/g TS)	References
Ultrasonication: 1.4 W/mL	120.5	*Enterobacter cloacae* IIT-BT 08; *Citrobacter freundii* IITBT L139; *Bacillus coagulans* IIT-BT S1	–	7.8[c]	Kotay and Das (2009)
Ultrasonication: 1.5 W/mL	17.4	–	pH 7.5, 35 °C; Batch	7.24	Yang et al. (2012)
Ultrasonication: 2 W/mL	15.42	*Pseudomonas* sp. GZ1	35 °C; Batch	5.4	(Guo et al. 2008)
Ultrasonication: 20 kHz, 20 min	16.5	*Clostridium bifermentans*	pH 6, 35 °C; Batch	<20.2	(Wang et al. 2003)
Freeze-thaw: –20 °C/25 °C, 6 h, two cycles	120.5	*Enterobacter cloacae* IIT-BT 08; *Citrobacter freundii* IITBT L139; *Bacillus coagulans* IIT-BT S1	–	7.6[c]	Kotay and Das (2009)
Freeze-thaw: –17 °C/25 °C, 24 h/12 h	16.5	*Clostridium bifermentans*	pH 6, 35 °C; Batch	50.4	Wang et al. (2003)
Chemical treatment					
Acid: pH 3, 6 h	16.5	*Clostridium bifermentans*	pH 6, 35 °C; Batch	20.2	Wang et al. (2003)
Acid: pH 3–4, 24 h	120.5	*Enterobacter cloacae* IIT-BT 08; *Citrobacter freundii* IITBT L139; *Bacillus coagulans* IIT-BT S1	–	7.5[c]	(Kotay and Das 2009)
Acid: HCl 0.5%, 0–24 h	100	Anaerobic sludge	pH 5.5, 30 °C; Batch	12–41[b]	Assawamongkholsiri et al. (2013)
Base: pH 10–11, 24 h	120.5	*Enterobacter cloacae* IIT-BT 08; *Citrobacter freundii* IITBT L139; *Bacillus coagulans* IIT-BT S1	–	8[c]	Kotay and Das (2009)

(continued)

Table 4.7 (continued)

Treatment method	Concentration of Waste activated sludge (g/L TS)	Inoculum	Operational conditions	Hydrogen yield (mL/g TS)	References
Base: pH 12, 24 h	10.97	–	pH 11, 36 °C; Batch	16.9	(Cai et al. 2004)
Chemical: $CHCl_3$ 1%, 24 h	120.5	*Enterobacter cloacae* IIT-BT 08; *Citrobacter freundii* IITBT L139; *Bacillus coagulans* IIT-BT S1	–	4[c]	Kotay and Das (2009)
Chemical: BESA 10 mmol/L, 24 h	120.5	*Enterobacter cloacae* IIT-BT 08; *Citrobacter freundii* IITBT L139; *Bacillus coagulans* IIT-BT S1	–	6[c]	Kotay and Das (2009)
Chemical: BESA 1 mmol/L	16.5	*Clostridium bifermentans*	pH 6, 35 °C; Batch	<20.2	Wang et al. (2003)
Chemical: Ozone 0.15 g/g DS	17.4	–	pH 7.5, 35 °C; Batch	3.59	Yang et al. (2012)
Biological treatment					
Composite enzyme: bromelain, lysozyme = 1:50, 30 °C, 5 h	13.4	–	35 °C; Batch	12[b]	Guo et al. (2015)
Aerobic thermophilic digestion: 65 °C, 12 h	13.4	–	35 °C; Batch	14[b]	Guo et al. (2015)
Aerobic thermophilic digestion: 55 °C, 48 h	50	–	pH 5.5, 37 °C; Batch	136.9	Sittijunda et al. (2010)
Combined treatment					
Acid-Heat: HCl 0.5%, 110 °C, 15–60 min	100	Anaerobic sludge	pH 5.5, 30 °C; Batch	12–23[b]	Assawamongkholsiri et al. (2013)

(continued)

Table 4.7 (continued)

Treatment method	Concentration of Waste activated sludge (g/L TS)	Inoculum	Operational conditions	Hydrogen yield (mL/g TS)	References
Base: pH 13, 2 h; Heat: 150 °C, 30 min	27.88[a]	Anaerobic sludge	pH 5.5, 30 °C; Batch	7.9[c]	Kang et al. (2012)
Heat: 70 °C, 1 h; Biological: cellulase 13 L, 5%	36.7	Anaerobic sludge	pH 5.5, 55 °C; Batch	18.14	(Massanet-Nicolau et al. 2008)
Heat: 121 °C, 40 min; Biological: amylase, cellulase 10 U/mL, 2 h	80 (Addition of oil palm frond juice)	*Escherichia coli*	pH 6, 37 °C; Batch	1.5[d]	(Yasin et al. 2013)
Heat: 70 °C, 1 h; Biological: cellulase 13 L, 5%	36.7	Anaerobic sludge	pH 5.5, 55 °C; CSTR, HRT = 24 h	21.9[b]	Massanet-Nicolau et al. (2010)
Chemical: Ozone 0.15 g/g DS; Ultrasonication: 1.5 W/mL	17.4	–	pH 7.5, 35 °C; Batch	9.28	Yang et al. (2012)
Gamma irradiation: 20 kGy; Base: pH 12, 24 h	12.4	Anaerobic sludge	pH 7, 36 °C; Batch	10.5[c]	Yin and Wang (2016, b)

Table 4.8 Bio-hydrogen production from algae biomass

Substrate	Substrate concentration (g TS/L)	Treatment method	Inoculum	Operational condition	Hydrogen yield (mL H_2/g VS)	References
Microalgae						
Chlorella sorokiniana	14	Heat:121 °C, 20 min	Anaerobic sludge	pH = 6.5, 60 °C; Batch	338	Roy et al. (2014)
	14	Acid-Heat: HCl 5%, 121 °C, 20 min	Anaerobic sludge	pH = 6.5, 60 °C; Batch	760	Roy et al. (2014)
	14	Chemical: H_2O_2 2%, 20 min	Anaerobic sludge	pH = 6.5, 60 °C; Batch	63	Roy et al. (2014
	14	Acid-Heat: HCl 25%, 121 °C, 20 min	Anaerobic sludge	pH = 6.5, 60 °C; Batch	958	Roy et al. (2014)
	10	Heat: 121 °C, 20 min; Acid: HCl 2.0%, 12 h	*Enterobacter cloacae* IIT-BT 08	pH = 7.0, 37 °C; Batch	201.6[d]	Kumar et al. (2013)
Chlorella vulgaris	5–30	–	Anaerobic sludge	pH = 7.5, 60 °C; Batch	1.75–19	Wieczorek et al. (2014)
	20–117	–	Anaerobic sludge	pH = 4.2–9.8, 35 °C; Batch	14.6–31.2[e]	(Yun et al. 2012)
	0.14[a]	–	Compost	37 °C; Batch	11	Carver et al. (2011)

(continued)

Table 4.8 (continued)

Substrate	Substrate concentration (g TS/L)	Treatment method	Inoculum	Operational condition	Hydrogen yield (mL H_2/g VS)	References
	5	–	Anaerobic sludge	pH = 7.0, 37 °C; Batch	10.8	Lakaniemi et al. (2011)
	10	Biological: Onozuka R-10	Anaerobic sludge	pH = 7.5, 60 °C; Batch	39	Wieczorek et al. (2014)
	10	Biological: Macerozyme R-10	Anaerobic sludge	pH = 7.5, 60 °C; Batch	62	Wieczorek et al. (2014)
	10	Biological: Onozuka R-10 + Macerozyme R-10	Anaerobic sludge	pH = 7.5, 60 °C; Batch	135	Wieczorek et al. (2014)
	20	Acid-Heat: H_2SO_4 0.1 mmol/L, 108 °C, 30 min	*Clostridium acetobutylicum* B-1787	pH = 6.8, 37 °C; Batch	6[e]	Efremenko et al. (2012)
Chlorella pyrenoidosa	20	Acid-Heat: H_2SO_4 1%, 135 °C, 15 min	*Clostridium butyricum*	pH = 6.0, 35 °C; Batch	81.2	Xia et al. (2014)
	10 (additional cassava starch 10 g/L)	Acid-Heat: H_2SO_4 1%, 135 °C, 15 min	*Clostridium butyricum*	pH = 6.0, 35 °C; Batch	276.2	Xia et al. (2014)
Chlorella sp.	4–40	–	Anaerobic sludge	pH = 6.5, 35 °C; Batch	0.37–7.13	Sun et al. (2011)

(continued)

Table 4.8 (continued)

Substrate	Substrate concentration (g TS/L)	Treatment method	Inoculum	Operational condition	Hydrogen yield (mL H_2/g VS)	References
Scenedesmus obliquus	10–50	Milling	*Clostridium butyricum* DSM 10702	pH = 7.0, 37 °C; Batch	28.1–35.0	Ortigueira et al. (2015)
	10–50	Milling	Anaerobic sludge	pH = 7.0, 37 °C; Batch	5.4–34.8	Ortigueira et al. (2015)
	10–50	Milling	Anaerobic sludge	pH = 7.0, 58 °C; Batch	0.7–15.3	Ortigueira et al. (2015)
	10–50	Milling	Anaerobic sludge + *Clostridium butyricum* DSM 10702	pH = 7.0, 58 °C; Batch	32.7–48.9	Ortigueira et al. (2015)
	2.5–50	Heat: 121 °C, 15 min	*Enterobacter aerogenes* ATCC 13048	pH = 6.8, 30 °C; Batch	10.8–56.5	Batista et al. (2014)
	2.5–50	Heat: 121 °C, 15 min	*Clostridium butyricum* DSM 10702	pH = 6.8, 37 °C; Batch	94.3–113.1	Batista et al. (2014)
	–	Acid-Heat: H_2SO_4 0.5 mol/L, 100 °C, 30 min;	*Clostridium butyricum*	pH = 7.0, 37 °C; Batch	2.9[f]	Ferreira et al. (2013)
Scenedesmus sp.	4.5-45	Lipid-extracted	Anaerobic sludge	pH = 5.0–7.0, 37 °C; Batch	0.42–40.27	Yang et al. (2011)

(continued)

Table 4.8 (continued)

Substrate	Substrate concentration (g TS/L)	Treatment method	Inoculum	Operational condition	Hydrogen yield (mL H_2/g VS)	References
Nannochloropsis oceanica	50	Acid: H_2SO_4 0–2.0%; Microwave: 80–180 °C, 5–25 min	Anaerobic sludge	pH = 6.0, 35 °C; Batch	2–39	Xia et al. (2013)
Nannochloropsis sp.	20	Acid-Heat: H_2SO_4 0.1 mmol/L, 108 °C, 30 min	*Clostridium acetobutylicum* strain B-1787	pH = 6.8, 37 °C; Batch	0–16.25[e]	Efremenko et al. (2012)
	2.5–10	Lipid-extracted	*Enterobacter aerogenes* ATCC 13048	30 °C; Batch	26.4–60.6[e]	Nobre et al. (2013)
Arthrospira platensis	20	Acid-Heat: H_2SO_4 0.1 mmol/L, 108 °C, 30 min	*Clostridium acetobutylicum* strain B-1787	pH = 6.8, 37 °C; Batch	3.75–13.75[e]	Efremenko et al. (2012)
	10–40	Acid-Microwave: H_2SO_4 0.2 mL, 140 °C, 15 min; Biological: Glucoamylase 0.2%	Anaerobic sludge	pH = 6.5, 35 °C; Batch	86.5–96.6[e]	Cheng et al. (2012)
Dunaliella tertiolecta	0.094[a]	Base: NaOH pH = 9.5	Compost	37 °C; Batch	13	Carver et al. (2011)
	5	Base: NaOH pH = 9.5	Anaerobic sludge	pH = 7.0, 37 °C; Batch	12.6	Lakaniemi et al. (2011)
Chlamydomonas reinhardtii	50	–	*Clostridium butyricum* NCBI 9576	pH = 6.0, 37 °C; Batch	16.6[e]	KIM et al. (2006)

(continued)

Table 4.8 (continued)

Substrate	Substrate concentration (g TS/L)	Treatment method	Inoculum	Operational condition	Hydrogen yield (mL H_2/g VS)	References
	5	Sonication; 130 W, 10 min; Chemical: methanol 30%, 60 min	*Thermotoga neapolitana* DSM 4359	–	1.8–2.2[f]	Nguyen et al. (2010)
Spirulina platensis	10	Acid-Heat: H_2SO_4 0.5%, 121 °C, 60 min	*Bacillus firmus* NMBL-03	pH = 6.5, 38 °C; Batch	0.38[f]	Sinha and Pandey (2014)
Mixed algae	25	Acid-Microwave: pH 1.4, 140 °C, 15 min; Biological: Cellulase 0.05 g/g TVS, 48 h; Glucoamylase 0.05 g/g TVS, 24 h	Anaerobic sludge	pH = 6.0, 35 °C; Batch	42.4–47.07	Cheng et al. (2014)
Mixed algae	25	Acid-Heat: pH 1.4, 140 °C, 15 min; Biological: Cellulase 0.05 g/g TVS, 48 h; Glucoamylase 0.05 g/g TVS, 24 h	Anaerobic sludge	pH = 6.0, 35 °C; Batch	33.56–43.84	Cheng et al. (2014)
Mixed algae	5[b]	Acid-Heat: H_2SO_4 3%, 121 °C, 60 min	Anaerobic sludge	pH = 6.0, 29 °C; Batch	122[e]	Subhash and Mohan (2014)
Macroalgae						
Laminaria japonica	50	Heat: 65 °C, 20 min	Anaerobic sludge	pH = 7.5, 35 °C; Batch	28[e]	Park et al. (2009)
	20[b]	Heat: 170 °C, 20 min;	Anaerobic sludge	pH = 8.0, 35 °C; Batch	109.6[d]	Jung et al. (2011a, b)
	20[b]	Milling	Anaerobic sludge	pH = 7.5, 35 °C; Batch	71.4[e]	Liu and Wang (2014)

(continued)

Table 4.8 (continued)

Substrate	Substrate concentration (g TS/L)	Treatment method	Inoculum	Operational condition	Hydrogen yield (mL H_2/g VS)	References
	35	Milling	Anaerobic sludge	pH = 8.0, 35 °C; Batch	23.38–45.75	(Jung et al. 2015)
	30	Electric: 20–30 V, 30 min	Anaerobic sludge	pH = 5.5, 37 °C; Batch	59.5–102.7[e]	Jeong et al. (2015)
	20[b]	Acid-Heat: HCl 4.8%, 93 °C, 23 min	Anaerobic sludge	pH = 8.0, 35 °C; Batch	159.6[e]	Jung et al. (2011a, b)
	20[b]	–	Anaerobic sludge	pH = 8.0, 35 °C; Batch	69.1[d]	Jung et al. (2011a, b)
	5–40	–	Anaerobic sludge	pH = 7.5, 35 °C; Batch	71.4[e]	Shi et al. (2011)
	OLR = 3.4 g COD/L/d	Milling	Anaerobic sludge	pH = 5.5, 35 °C; ASBR	61.3[e]	Shi et al. (2013)
Gelidium amansii	15[c]	Acid-Heat: H_2SO_4 0.5–1.5%, 150–180 °C, 15 min	Anaerobic sludge	pH = 5.3, 35 °C; Batch	16.7–33.5[e]	Park et al. (2009)
Codium fragile	20[b]	–	Anaerobic sludge	pH = 8.0, 35 °C; Batch	3.7[d]	(Jung et al. 2011a, b)

(continued)

Table 4.8 (continued)

Substrate	Substrate concentration (g TS/L)	Treatment method	Inoculum	Operational condition	Hydrogen yield (mL H_2/g VS)	References
Gelidium amansii	20[b]	–	Anaerobic sludge	pH = 8.0, 35 °C; Batch	2.54[d]	Jung et al. (2011a, b)
Porphyra tenera	20[b]	–	Anaerobic sludge	pH = 8.0, 35 °C; Batch	1.06[d]	Jung et al. (2011a, b)
Gracilaria verrucosa	20[b]	–	Anaerobic sludge	pH = 8.0, 35 °C; Batch	3.56[d]	Jung et al. (2011a, b)
Hizikia fusiforme	20[b]	–	Anaerobic sludge	pH = 8.0, 35 °C; Batch	1.19[d]	Jung et al. (2011a, b)
Ecklonia stolonifera	20[b]	–	Anaerobic sludge	pH = 8.0, 35 °C; Batch	3.29[d]	Jung et al. (2011a, b)
Undaria pinnatifida	20[b]	–	Anaerobic sludge	pH = 8.0, 35 °C; Batch	1.77[d]	Jung et al. (2011a, b)

studied for its high carbohydrates content (Becker 1994), highest hydrogen yield of 113.1 mL H_2/g VS was achieved from heat treated *Scenedesmus* sp. (Batista et al. 2014). As to the macroalgae, *Laminaria japonica* was the most commonly studied for its high carbohydrates content and low requirement of treatment (Park et al. 2009). Besides *Laminaria japonica,* other macroalgae species were only explored in a few studies, which was far from the application.

As to the operational conditions, substrate concentration varied from 5 g TS/L to 117 g TS/L, commonly used inoculum was anaerobic sludge and *Clostridium butyricum* strains. Initial pH range between 6.0 and 8.0 Except for hydrogen production from *Chlorella* sp. explored thermal conditions (58–60 °C), most studies were conducted at mesophilic conditions (29–38 °C).

4.5.3 Hydrogen Production from Cellulose-Based Biomass

Cellulose-based biomass has been widely used as feedstock for fermentative hydrogen production. To explore hydrogen production process, some studies used cellulose to simulate natural cellulose materials. Besides, cellulose-based biomass has also been extensively studied, including straws (rice straw, wheat straw, corn straw, sugarcane straw, etc.), stalks (corn stalk, sunflower stalk, etc.), corn stover and cob, bagasse, grass, leaves, and so on.

Table 4.9 demonstrates the hydrogen production from simulated cellulose materials. Cellulose concentration from 0.2 g/L to 10 g/L was studied. Since the bought cellulose is usually present in powder, besides the studies without treatment, commonly used treatment include enzyme treatment, bacterial hydrolysis and combined acid-heat treatment. Pure strains were more widely used as inoculum than mixed cultures like anaerobic sludge and compost. Thermophilic bacteria species were especially preferred among the pure strains. Accordingly, operational temperature range from 55 to 70 °C was used. For the studies conducted at mesophilic conditions, 37 °C was used. Besides the temperature, pH 5.5-8.0 was adopted. Hydrogen yield of 0.6-19 mmol H_2/g cellulose was achieved, and higher yield was obtained from thermophilic fermentation systems.

As to the natural cellulose-based biomass, various straws were most widely studied for hydrogen production. As shown in Table 4.10, 10–340 g TS/L straw was used as substrate after pretreatment. Mixed cultures like anaerobic and manure were preferred, since it is hard to maintain the system uninfected when real wastes are used as substrate. pH ranged from 4.1 to 8.0 and pH 6–7 was the most widely used. Temperature between 35–40 and 55–80 °C were studied, and 35 °C was the most commonly used. Hydrogen yield of 3.49–93.4 mL H_2/g TS was achieved, and hydrogen yield obtained from treated straw was significantly higher than untreated tests.

Besides straws, many other cellulose-based biomass was also studied. As shown in Table 4.11, most of the biomass was from agricultural wastes, like corn wastes (cornstalk, corn stover, corn cob, and corn silage), tree wastes (poplar leaves and

Table 4.9 Bio-hydrogen production from cellulose and cellulosic hydrolysate

Substrate	Concentration of substrate (g/L)	Treatment method	Inoculum	Operational condition	Hydrogen yield (mmol H_2/g cellulose)	References
Cellobiose	0.1–4.5	–	*Clostridium thermocellum* 27405	pH = 7; Batch	0.99–2.32[a]	Levin et al. (2006)
Cellobiose	2.5	–	*Clostridium thermocellum* 27405	pH = 6.8, 55 °C; Batch	1.64[a]	Lalaurette et al. (2009)
Cellulose	0.25	–	Elephant dung	pH = 7, 55 °C; Batch	7.22	Saripan and Reungsang (2014)
Cellulose	5	–	Anaerobic sludge	pH = 8, 37 °C; CSTR, HRT = 10 d	0.6	Gadow et al. (2012)
Cellulose	5	–	Anaerobic sludge	pH = 8, 55 °C; CSTR, HRT = 10 d	15.2	Gadow et al. (2012)
Cellulose	5	–	Anaerobic sludge	pH = 8, 80 °C; CSTR, HRT = 10 d	19	Gadow et al. (2012)
Cellulose	9	–	*Clostridium thermocellum* DSM 1237, *Clostridium thermopalmarium* DSM 5974	pH = 7, 55 °C; Batch	1.36[a]	Geng et al. (2010)
Cellulose	10	–	*Clostridium butyricum* CGS5	pH = 7.5, 37 °C; Batch	4.79	Lo et al. (2009)
Cellulose	10	–	*Clostridium acetobutylicum* X9, *Ethanoigenens harbinense* B49	pH = 5, 37 °C; Batch	3.37–8.08	Wang et al. (2008)
Cellulose	10	–	Anaerobic sludge	pH = 5.5–6, 70 °C; CSTR, HRT = 10 d	7.1	Gadow et al. (2013)

(continued)

Table 4.9 (continued)

Substrate	Concentration of substrate (g/L)	Treatment method	Inoculum	Operational condition	Hydrogen yield (mmol H_2/g cellulose)	References
Cellulose	10	–	Anaerobic sludge	pH = 5.5–6, 55 °C; CSTR, HRT = 10 d	15.4	Jiang et al. (2015)
Cellulose	10	–	Anaerobic sludge	pH = 5.5, 55 °C; Batch	4.94	Jiang et al. (2015)
Cellulose	10	–	Cow dung	pH = 7.6–8.0, 55 °C; Batch	2.8	Lin and Hung (2008)
Cellulose	10	–	*Caldicellulosiruptor Saccharolyticus* DSM 8903	pH = 7.2, 65 °C; Batch	11.63	Talluri et al. (2013)
Cellulose	10	Biological: bacterial hydrolysis with NS sludge, 35 °C, pH = 7	*Clostridium pasteurianum* CH7	pH = 7.5, 37 °C; Batch	1.21[a]	Lo et al. (2008)
Cellulose	4.5	Acid-Heat: H_2SO_4 0.5%, 121 °C, 1 h; Biological: Celluclase, 45 °C, 68 h	*Thermoanaerobacter* GHL15	pH = 6–7, 65–70 °C; Batch	2.3[a]	Brynjarsdottir et al. (2013)
Cellulose	4.5	Acid-Heat: H_2SO_4 0.5%, 121 °C, 1 h; Biological: Celluclase, 45 °C, 68 h	*Caldicellulosiruptor saccharolyticus*	pH = 6–7, 65–70 °C; Batch	2[a]	Brynjarsdottir et al. (2013)
Cellulose	2–4	Biological: cellulase 7000 units/L, during fermentation process	Rumen fluid	pH = 7, 37 °C; Batch	9.6–18.5	Ratti et al. (2014)
Cellulose	2.5–10	Biological: cellulase 2100 units/g cellulose, during fermentation process	Leachate	pH = 7, 37 °C; Batch	0.6–2.3[a]	Ratti et al. (2013)

Table 4.10 Bio-hydrogen production from straws

Substrate	Concentration of substrate (g/L TS)	Treatment method	Inoculum	Operational condition	Hydrogen yield (mL H_2/g TS)	References
Rice Straw	90	–	Anaerobic sludge	pH = 6.5, 55 °C; Batch	24.8	Chen et al. (2012a, b)
Rice Straw	200	Heat: 210 °C	Anaerobic sludge	pH = 7, 35 °C; Batch	28[b]	He et al. (2014)
Rice Straw	1.1[a]	Base-Heat: NH_4OH 0.3%, 100 °C, 60 min	Anaerobic sludge	pH = 6.8, 35 °C; ABR, HRT = 20 h	0.97–1.19[c]	El-Bery et al. (2013)
Rice Straw	10	Base-Microwave: NaOH 0.5%, 140 °C, 15 min; Biological: cellulase 40 °C, 96 h	Anaerobic sludge	pH = 6.5, 35 °C; Batch	155[b]	Cheng et al. (2011)
Wheat Straw	5	–	*Thermoanaerobacterium thermosaccharolyticum* M18	pH = 7, 60 °C; Batch	3.49–3.53	Cao et al. (2014)
Wheat Straw	100–340	–	Anaerobic sludge	pH = 5.5, 55 °C; Batch	3.4–15.3	Motte et al. (2014)
Wheat Straw	10	–	Anaerobic sludge	pH = 5.5, 37 °C; Batch	5.2–10.5[b]	Quéméneur et al. (2012)
Wheat Straw	100–330	Mechanical: Grind to particles 0.4–1 mm	Anaerobic sludge	pH = 4.1–5.7., 35 °C; Batch	20–35	Motte et al. (2013)
Wheat Straw	22	Heat: 80 °C, 20 min; 180 °C, 15 min; 195 °C, 3 min	Anaerobic sludge	pH = 4.9, 70 °C; Batch	318.4[d]	Kongjan et al. (2010)
Wheat Straw	88	Heat: 80 °C, 20 min; 180 °C, 15 min; 195 °C, 3 min	Anaerobic sludge	pH = 8, 80 °C; CSTR, HRT = 3 d	178[d]	Kongjan et al. (2010)
Wheat Straw	80	Base: $Ca(OH)_2$ 7.4%, 16 d	Anaerobic sludge	pH = 6.25, 35 °C; Batch	58.8[b]	Reilly et al. (2014)

(continued)

Table 4.10 (continued)

Substrate	Concentration of substrate (g/L TS)	Treatment method	Inoculum	Operational condition	Hydrogen yield (mL H_2/g TS)	References
Wheat Straw	10	Biological: CEP enzyme, 37/50 °C, 24–50 h	Anaerobic sludge	pH = 5.5, 37 °C; Batch	11.1–19.6[b]	Quéméneur et al. (2012)
Wheat Straw	5	Biological: *Phanerochaete chrysosporium*, 30 °C, 21 d	*Clostridium perfringens* ATCC 13124	pH = 6.5, 40 °C; Batch	78.5	Zhi and Wang (2014)
Wheat Straw	196[a]	Acid-Heat: H_3PO_4 0.5%, 50 °C, 72 min;	*Caldicellulosiruptor saccharolyticus*	pH = 7, 70 °C; Batch	2.08–3.43[c]	Pawar et al. (2013)
Wheat Straw	3.3	Acid-Heat: H_2SO_4 2%, 120 °C, 90 min;	Anaerobic sludge	35 °C; Batch	6.4–168.4[b]	Nasirian et al. (2011)
Wheat Straw	176	Heat: 80 °C, 6 min; 180 °C, 15 min; 195 °C, 3 min; Biological: cellulase, 50 °C, 24 h	Anaerobic sludge	pH = 8, 80 °C; CSTR, HRT = 3 d	178[d]	Kaparaju et al. 92009)
Wheat Straw	50	Chemical: Ozone, 15–90 min; Biological: cellulase 5%, endoxylanase 0.2%, β-glucosidase 0.6%	Cow manure	pH = 6, 35 °C; Batch	30–90	Wu et al. (2013)
Sugarcane Straw	250	Acid-Heat: H_2SO_4 2.9%, 130 °C, 20 min	Anaerobic sludge	pH = 5.5, 35 °C; Batch	1.91[c]	De Sá et al. (2015)
Oat Straw	10	Heat: 1.5 MPa, 10 min; Biological: cellulase, 50 °C, 48 h	*Clostridium butyricum* AS1.209	pH = 7, 35 °C; Batch	68	Li and Chen (2007)
Corn Straw	40	Mechanical: Grind to particles<1 mm	Anaerobic sludge	pH = 7, 35 °C; Batch	41.6[b]	Li et al. (2016)
Corn Straw	50	Chemical: Ozone, 15–90 min; Biological: cellulase 5%, endoxylanase 0.2%, β-glucosidase 0.6%	Cow manure	pH = 6, 35 °C; Batch	69.6–93.4	Wu et al. (2013)
Barley Straw	5 COD	Acid-Heat: HCl 2%, 90 °C, 2 h; Base: NaOH 1%, 24 h; Celluclase, 45 °C, 10 h	Anaerobic sludge	pH = 5.5, 35 °C; ASBR	2[c]	Arreola-Vargas et al. (2013)

Table 4.11 Bio-hydrogen production from various cellulose based biomass

Substrate	Concentration of substrate (g/L TS)	Treatment method	Inoculum	Operational condition	Hydrogen yield (mL H_2/g TS)	References
Corn stalk	5	–	*Thermoanaerobacterium thermosaccharolyticum* M18	pH = 7, 60 °C; Batch	3.28–3.47	Cao et al. (2014)
Corn stalk	20	Mechanical: Grind to particles through 40 mesh	*Clostridium butyricum* FS3	pH = 5.6, 36 °C; Batch	92.9	Song et al. (2014)
Corn stalk	60	Mechanical: Grind to particles through 40 mesh	*Bacillus* sp. FS2011	pH = 7.5, 36 °C; Batch	79.8	Guo et al. (2014)
Corn stalk	20	Acid-Heat: H_2SO_4 0.5%, 121 °C, 60 min	Anaerobic sludge	pH = 7, 36 °C; Batch	144.3	Song et al. (2012)
Corn stalk	350	Biological: Phanerochaete chrysosporium 29 °C, 3 d; Cellulase, 50 °C, 4 d	*Thermoanaerobacterium thermosaccharolyticum* W16	pH = 7, 60 °C; CSTR, HRT = 20 h	1.9[d]	Zhao et al. (2013)
Corn stalk	35	Biological: Phanerochaete chrysosporium, 29 °C, 15 d	*Thermoanaerobacterium thermosaccharolyticum* W16	pH = 7, 60 °C; Batch	80.3	Zhao et al. (2012)
Corn stalk	20	Acid-Heat: HCl 0.6%, 90 °C, 2 h; Biological: cellulase, 50 °C, 72 h	Anaerobic sludge	pH = 7, 36 °C; Batch	146.9	Wang et al. (2010)
Corn stover	26	Heat: 220 °C, 3 min	Anaerobic sludge	pH = 5.5, 35 °C; Batch	2.84[d]	Datar et al. (2007)
Corn stover	2.5	Acid-Heat: H_2SO_4 1.08%, 190 °C, 90 s	*Clostridium thermocellum* 27405	pH = 6.8, 55 °C; Batch	1.67[d]	Lalaurette et al. (2009)
Corn stover	26	Acid: H_2SO_4 1.2%, 2 h; Heat: 190 °C, 2 min	Anaerobic sludge	pH = 5.5, 35 °C; Batch	3[d]	Datar et al. (2007)

(continued)

Table 4.11 (continued)

Substrate	Concentration of substrate (g/L TS)	Treatment method	Inoculum	Operational condition	Hydrogen yield (mL H_2/g TS)	References
Corn stover	5[b]	Acid-Heat: H_2SO_4 1.7%, 121 °C, 20 min	Anaerobic sludge	pH = 7, 37 °C; Batch	64.3–112.7[e]	Zhang et al. (2011)
Corn cob	5	–	*Thermoanaerobacterium thermosaccharolyticum* M18	pH = 7, 60 °C; Batch	3.23–3.27	Cao et al. (2014)
Corn cob	250	Acid-Heat: H_2SO_4 2.9%, 130 °C, 20 min	Anaerobic sludge	pH = 5.5, 35 °C; Batch	1.75[d]	De Sá et al. (2015)
Corn silage	800	Biological: Methane fermentation	Anaerobic sludge	pH = 4, 37 °C; Batch	55.6–59.4[c]	Nkemka et al. (2015)
Bagasse	10	Acid-Heat: H_2SO_4 0.5%, 121 °C, 60 min	*Bacillus firmus* NMBL-03	pH = 6.5, 38 °C; Batch	1.29[d]	Sinha and Pandey (2014)
Bagasse	20[a]	Acid-Heat: H_2SO_4 0.5%, 121 °C, 60 min	*Clostridium butyricum* TISTR 1032	pH = 5.5, 37 °C; Batch	1.73[d]	Pattra et al. (2008)
Bagasse	66.7	Acid-Heat: H_2SO_4 1%, 121 °C, 60 min	Elephant dung	pH = 7, 37 °C; Batch	0.84[d]	Fangkum and Reungsang (2011)
Bagasse	0.4	Acid-Heat: H_2SO_4 1%, 121 °C, 60 min	Elephant dung	pH = 7, 55 °C; Batch	7.1[d]	Saripan and Reungsang (2014)
Bagasse	66.7	Acid-Heat: H_2SO_4 2.3%, 115 °C, 114.2 min	*Thermoanaerobacterium aotearoense* SCUT27/Δldh.	pH = 6.8, 55 °C; Batch	1.86[d]	Lai et al. (2014)
Bagasse	250	Acid-Heat: H_2SO_4 2.9%, 130 °C, 20 min	Anaerobic sludge	pH = 5.5, 35 °C; Batch	2.99[d]	De Sá et al. (2015)
Sugar beet-pulp	20[a]	–	Anaerobic sludge	pH = 6, 35 °C; Batch	90.1[f]	Ozkan et al. (2011)

(continued)

Table 4.11 (continued)

Substrate	Concentration of substrate (g/L TS)	Treatment method	Inoculum	Operational condition	Hydrogen yield (mL H_2/g TS)	References
Sugar beet-pulp	20[a]	Base: pH = 12, 30 min	Anaerobic sludge	pH = 6, 35 °C; Batch	115.6[f]	Ozkan et al. (2011)
Sugar beet-pulp	20[a]	Base: pH = 12, 30 min; Heat: 121 °C, 30 min	Anaerobic sludge	pH = 6, 35 °C; Batch	108.2[f]	Ozkan et al. (2011)
Sugar beet-pulp	20[a]	Base: pH = 12, 30 min; Microwave: 170 °C, 30 min;	Anaerobic sludge	pH = 6, 35 °C; Batch	66.7[f]	Ozkan et al. (2011)
Grass silage	25	–	–	pH = 7, 37 °C; Batch	37.8	Li et al. (2012)
Grass	15	Acid-Heat: HCl 4%, 100 °C, 30 min	Cracked cereal	pH = 7, 35 °C; Batch	72.2	Cui and Shen (2012)
Grass	4.5	Acid-Heat: H2SO4 0.5%, 121 °C, 1 h; Biological: Celluclase, 45 °C, 68 h	*Thermoanaerobacter* GHL15	pH = 6–7, 65–70 °C; Batch	1.2[d]	Brynjarsdottir et al. (2013)
Grass	4.5	Acid-Heat: H_2SO_4 0.5%, 121 °C, 1 h; Biological: Celluclase, 45 °C, 68 h	*Caldicellulosiruptor saccharolyticus*	pH = 6–7, 65–70 °C; Batch	1.5[d]	Brynjarsdottir et al. (2013)
Switchgrass	100	Acid: H_2SO_4 1%, 12 h; Heat: 190 °C, 10 min	Anaerobic sludge	pH = 5, 36 °C; UASBR, HRT = 10 h	99.86[c]	Veeravalli et al. (2014)
Switchgrass	30	–	*Caldicellulosiruptor Saccharolyticus* DSM 8903	pH = 7.2, 65 °C; Batch	203.4–310.3	Talluri et al. (2013)
leaf-shaped vegetable refuses + potato peels	117	–	–	pH = 5, 28–37 °C; Batch	3.3–19[c]	Marone et al. (2014)

(continued)

Table 4.11 (continued)

Substrate	Concentration of substrate (g/L TS)	Treatment method	Inoculum	Operational condition	Hydrogen yield (mL H_2/g TS)	References
Leaf-shaped vegetable refuses	53	–	–	pH = 5, 28–37 °C; Batch	8.8–24[c]	Marone et al. (2014)
Birch pulp	1.1	–	Compost	pH = 6, 37 °C; Batch	560	Nissilä et al. (2012)
Cattail	290	Biological: Methane fermentation	Anaerobic sludge	pH = 5.8, 37 °C; Batch	1–104[c]	Nkemka et al. (2015)
Delignified wood	0.1–4.5	Mechanical: Grind to powders	*Clostridium thermocellum* 27405	pH = 7; Batch	2.5–7.8	Levin et al. (2006)
Citrus limetta peelings	1.17–4.69[a]	Heat: 121 °C, 20–40 min	Anaerobic sludge	pH = 6, 32 °C; Batch	23.5–80.4[f]	Mohan et al. (2009)
Conifer pulp	5	Acid: H_2SO_4 55%, 180 min	Compost	pH = 5–9, 37 °C; Batch	0.09–0.77[d]	Nissilä et al. (2012)
Poplar leaves	12.5	Acid: HCl 4%,	Cracked cereal	pH = 7, 35 °C; Batch	33.45	Cui et al. (2010)
Poplar leaves	12.5	Biological: Viscozyme L 2%,	Cracked cereal	pH = 7, 35 °C; Batch	44.92	Cui et al. (2010)
Cocoa husk	250	Acid-Heat: H_2SO_4 2.9%, 130 °C, 20 min	Anaerobic sludge	pH = 5.5, 35 °C; Batch	1.65[d]	De Sá et al. (2015)
Empty palm fruit bunch	10	Acid-Heat: H_2SO_4 5%, 121 °C, 60 min	Anaerobic sludge	pH = 7.0–7.5, 35 °C; Batch	0.96[d]	Gonzales et al. (2016)
Oil palm empty fruit bunch	5[b]	Acid-Heat: H_2SO_4 6%, 120 °C, 15 min	Anaerobic sludge	pH = 5.5, 35 °C; Batch	1.98[d]	Chong et al. (2013)
pine tree wood	10	Acid-Heat: H_2SO_4 5%, 121 °C, 60 min	Anaerobic sludge	pH = 7.0–7.5, 35 °C; Batch	0.99[d]	Gonzales et al. (2016)

(continued)

Table 4.11 (continued)

Substrate	Concentration of substrate (g/L TS)	Treatment method	Inoculum	Operational condition	Hydrogen yield (mL H_2/g TS)	References
rice husk	10	Acid-Heat: H_2SO_4 5%, 121 °C, 60 min	Anaerobic sludge	pH = 7.0–7.5, 35 °C; Batch	1.25[d]	Gonzales et al. (2016)
Rice bran	10	H_2SO_4 1%; 121 °C, 1 h	*Clostridium acetobutylicum* YM1	pH = 5.5, 35 °C	0.37–0.94	Azman et al. (2016)
De-oiled jatropha waste	24.6[b]	Acid-Heat: HCl 2%, 115 °C, 114.2 min; Biological: Viscozyme L, 5%, 50 °C, 3 h	Anaerobic sludge	pH = 5.5–6.0, 37 °C; CSTR, HRT = 12 h	150[e]	Kumar et al. (2016)
Sunflower Stalks	35	Base: NaOH 4%, 24 h; Heat: 55/170 °C, 1 h	Anaerobic sludge	pH = 5.5, 35 °C; Batch	2.3–59.5[c]	Monlau et al. (2013)
Miscanthus	17	Base: Ca(OH)2 9–12%, 85 °C, 16 h; Biological: Cellulase, 50 °C, 24 h	*Caldicellulosiruptor saccharolyticus; Thermotoga neapolitana*	pH = 6.8, 80 °C; Batch	2.9–3.4[d]	de Vrije et al. (2009)
Wooden chopsticks	70	Base: NaOH 2%; Heat: 100 °C, 60 min; Biological: Cellulase, 20 U/g, 50 °C, 12 h	Hot spring sediment	pH = 7.5, 50 °C; Batch	195[e]	Phummala et al. (2014)

pine tree wood), grass, bagasse, and so on. Substrate concentration of 10–340 g TS/L was used. Similar with hydrogen production from straws, anaerobic sludge was the most commonly used inoculum. For the studies conducted at thermal conditions, thermophilic bacteria strains other than mixed bacteria were more used as inoculum. This was because the risk of infection was reduced significantly at thermal conditions, thus, pure strains were preferred to achieve a higher hydrogen yield (de Vrije et al. 2009; Zhao et al. 2013; Cao et al. 2014; Lai et al. 2014). pH ranged from 4.0 to 7.5 while pH 6–7 was the most commonly used. Mesophilic reactions were conducted at 28–38 °C, and 35 °C was the most widely used. Thermal fermentations were performed at 50–80, and 60 °C was the most widely used.

4.5.4 Hydrogen Production from Starch-Based Biomass

Similar with cellulose-based biomass, starch-based biomass is readily available and inexpensive. Differently, Starch-based biomass can be more easily hydrolyzed into simple sugars than biomass mentioned above. Thus, it offers special advantages for hydrogen production. Table 4.12 summarizes hydrogen production from starch-based biomass. It can be seen that pretreatment is omitted in most studies, and the conditions applied for treatment are much milder than cellulose-based biomass. Both mixed culture and pure culture are used as inoculum. Mesophilic conditions were commonly used, however, studies have also shown that higher hydrogen yield can be achieved from thermophilic fermentation (Cakır et al. 2010). Operational pH ranged from 5.5 to 7.2, and acidic environment (pH 5.2–6.5) is more commonly used. Hydrogen yield varied from 0.22 to 3.4 mol H_2/mol hexose, which is affected by starch source, inoculum, and operational conditions.

4.5.5 Hydrogen Production from Food Wastes

Hydrogen production from food wastes is summarized in Table 4.13. Since food wastes are cooked, few studies applied pretreatment before the fermentation process. Anaerobic is preferred than pure cultures, which may because of the complex compositions of food wastes. Mesophilic conditions were adopted and the temperature ranges from 34 to 39 °C. Operational pH various from 4.0 to 7.2 and acidic environment is more widely used. Hydrogen yield is affected by both food wastes concentration and operational conditions.

Table 4.12 Hydrogen production from Starch-based biomass

Substrate	Treatment method	Inoculum	Operational condition	Hydrogen yield (mol/mol hexose)	References
Corn starch (Sugar 10 g/L)	–	*Bacillus* sp. A1, *Brevumdimonas* sp. B1.	pH = 6.5, 35 °C	1.88	Wang et al. (2016)
Cassava starch COD 4 g/L)	–	Anaerobic sludge	pH = 5.5, 28 °C	0.13–1.91	Amorim et al. (2014)
Cassava starch (COD 20 g/L)	–	Anaerobic sludge	pH = 5.5, 37 °C	1.41–3.32	Sreethawong et al. (2010)
Cassava starch (TS 10 g/L)	Hydrolyzed with a-amylase and glucoamylase	Anaerobic sludge	pH = 7.0, 35 °C	1.74–2.00	Su et al. (2009)
Cassava starch (10 g/L) +Chlorella pyrenoidosa Ts 10 g/L	Acid-Heat: H_2SO_4 1%, 135 °C, 15 min	*Clostridium butyricum*	pH = 6.0, 35 °C	276.2 mL/g VS	Xia et al. (2014)
Potato waste (TS 10 g/L)	homogenized, 70 °C, a few minutes	Anaerobic sludge	pH = 5.5, 35 °C	68 mL/g TS	Zhu et al. (2008)
Sweet potato (TS 150 g/L)	–	Cow dung	pH = 6.5, 35 °C	0.22	Chu et al. (2012)
Sweet potato (TS 150 g/L)	–	Indigenous microbes	pH = 6.7, 37 °C	1.24	Lay et al. (2012)
Sweet Potato Starch Residue	–	*Clostridium butyricum*, *Enterobacter aerogenes*	pH = 5.2, 37 °C	2.4	Yokoi et al. (2001)
Durum wheat	–	Anaerobic sludge	pH = 7.2, 35 °C	0.35	Giordano et al. (2014)
Common wheat	–	Anaerobic sludge	pH = 7.2, 35 °C	0.22	Giordano et al. (2014)
Waste wheat (sugar 5 g/L)	pH 3, 121 °C, 30 min	Anaerobic sludge	pH = 7.0, 30 °C	3.4	Sagnak and Kargi (2011)
Wheat starch (TS 10 g/L)	100 °C, 1.5 h	*Clostridium butyricum*-NRRL 1024, *Clostridium pasteurianum*-NRRL B-598	pH = 5.5, 30 °C	0.79	Ozmihci and Kargi (2011)
Wheat starch (TS 20 g/L)	pH 2.5, 121 °C, 30 min	Anaerobic sludge	pH = 7.0, 55 °C	2.4	Cakır et al. (2010)

Table 4.13 Hydrogen production from food wastes

Substrate	Treatment method	Inoculum	Operational condition	Hydrogen yield (mol/mol hexose)	References
Food waste (COD 8.8 g/L)	–	Anaerobic sludge	pH = 5.5, 34 °C	20.6 mL/g VS	Redondas et al. (2012)
Food waste (COD 30 g/L)	pH 11, 6 h	Indigenous microbes	pH = 6.0, 37 °C	1.57	Jang et al. (2015)
Food waste (COD 62.5 g/L)	–	Anaerobic sludge	pH = 6.7, 35 °C	1.24	Tawfik et al. (2015)
Food waste (VS 2.54 g/L)	–	Anaerobic sludge	pH = 7.2, 30 °C	104.58 mL/g VS	Sreela-or et al. (2011)
Food waste (VS 40–70.2 g/L)	–	Anaerobic sludge	pH = 6.5, 39 °C	56.7–117.6 mL/g VS	Cappai et al. (2014)
Food waste (TS 50 g/L)	Solid-state fermentation using *Aspergillus awamori* and *Aspergillus oryzae*	*Biohydrogenbacterium* R3.	pH = 4.0–4.6, 37 °C	52.4 mL/g TS	Han et al. (2015)
Food waste	–	*Enterobacter aerogenes*	pH = 5–6, 37 °C	155.2 mL/g VS	Xiao et al. (2013)
Food waste + pulp and paper sludge (TS 28 g/L)	–	Anaerobic sludge	pH = 5.5, 37 °C	64.48 mL/g VS	Lin et al. (2013)

4.5.6 Hydrogen Production from Wastewater

Several kinds of wastewater contain high levels of organic matters that usually require special treatment. With the increase of people's environmental concept, the focus on wastewater treatment is shifting from pollution control to resource recovery. Thus, various organic wastewaters are used as substrate for hydrogen production.

Table 4.14 Hydrogen production from distillery wastewater

Distillery wastewater (COD g/L)	Inoculum	Operational condition	Hydrogen yield (mol/mol hexose)	References
3	Anaerobic sludge	pH 6.0, 28 °C	1.18	Mohan et al. (2008)
3.9	Anaerobic sludge	pH 7.0, 28 °C	0.07	Venkata Mohan et al. (2011)
15	Anaerobic sludge	pH 5.0, 55 °C	0.38	Santos et al. (2014)
40	Anaerobic sludge	pH 5.5, 37 °C	1.3	Searmsirimongkol et al. (2011)
60	Anaerobic sludge	pH 5.5, 55 °C	0.24–0.98	Intanoo et al. (2012)
90–96	Anaerobic sludge	pH 5.8	0.23–0.30	Akil and Jayanthi (2014)
10 (+sucrose 5 g COD/L)	Anaerobic sludge	30 °C	0.06	Lazaro et al. (2015)
22.9 (+sucrose 5.6 g/L)	Anaerobic sludge	pH 5.1–5.2, 55 °C	1.6–2.8	Fuess et al. (2016)
32.7 (VS = 28.9 g/L)	Anaerobic sludge	pH 7.0, 70 °C	172–196 mL/g VS	Qiu et al. (2011)

Table 4.14 demonstrates the hydrogen production from distillery wastewater. Chemical oxygen demand (COD) concentration ranges from 3 g/L to 96 g/L, and in some cases, sucrose was added to enhance the carbon source concentration. Since distillery wastewater is usually rich in microorganisms, few studies have inoculated pure cultures for hydrogen production. Anaerobic sludge is more widely used as inoculum. Both mesophilic and thermophilic conditions were used, and operational temperature ranged from 28 to 70, 28 °C and 55 °C are the most widely used temperature for mesophilic and thermophilic operation, respectively. Besides the temperature, pH 5.0–7.0 was adopted. Hydrogen yield of 0.07–2.8 mol H_2/mol hexose was achieved.

Wastewater rich in protein like cheese whey wastewater, dairy wastewater, and textile wastewater is also used as substrate for hydrogen production. As shown in Table 4.15, the COD concentration studied varies from 4.52 g/L to 88 g/L, anaerobic sludge was preferred as inoculum while some pure cultures like gene modified *Escherichia coli ΔhycA ΔlacI* and strain *Thermotoga neapolitana* are used. The operational temperature between 29 °C and 77 °C are used 35–37 °C was the most commonly used. pH ranged from 5.5 to 8.5 and pH 6–7 was the most widely adopted. Hydrogen yield of 0.0003–2.3 mol H_2/mol hexose was achieved, and the hydrogen yield was significantly affected by the characteristics of wastewater.

Besides protein wastewater, sugar-based waster is also a good substrate for hydrogen production. Beverage wastewater is rich in mixed micromolecule sugars

Table 4.15 Hydrogen production from protein-based wastewater

Substrate	Inoculum	Operational condition	Hydrogen yield (mol/mol hexose)	References
Cheese whey				
Cheese whey (12.5 g/L)	*Thermotoga neapolitana*	pH 8.5, 77 °C	0.99	Frascari et al. (2013)
Cheese whey (20.0 g/L)	*Escherichia coli ΔhycA ΔlacI*	pH 7.5, 37 °C	1.37	Alvarado-Cuevas et al. (2013)
Cheese whey (15 g/L)	Anaerobic sludge	pH 6.0, 37 °C	1.55	Castello et al. (2009)
Cheese whey (lactose 10 g/L)	*Enterobacter aerogenes* MTCC 2822	pH 6.8, 30 °C	1.02–1.75	Rai et al. (2012)
Cheese whey (lactose 20 g/L)	Anaerobic sludge	pH 7.0, 37 °C	2.06	Romao et al. (2014)
Cheese whey (COD 88 g/L)	Anaerobic sludge	pH 8.0, 36 °C	0.94–1.65	Seo et al. (2015)
Dairy wastewater				
Dairy wastewater (COD 15.3 g/L)	Anaerobic sludge	pH 5.5, 37 °C	2.3	Karadag et al. (2014)
Dairy wastewater (COD 10.4 g/L)	Anaerobic sludge	pH 6.3, 29 °C	0.0003–0.005	Venkata Mohan et al. (2008)
Textile wastewater				
Textile wastewater (hexose 33.1 g/L)	Anaerobic sludge	pH 5.5, 35 °C	0.27–0.97	Lay et al. (2014)
Textile wastewater (sugar 20 g/L)	Anaerobic sludge	pH 7.0, 37 °C	1.37	Li et al. (2012)
Silk wastewater (COD 4.52 g/L)	Anaerobic sludge	pH 7.0, 36 °C	111.3 mL/g protein	Xiao et al. (2013)
Others				
Tofu-processing wastewater (COD 20 g/L)	Anaerobic sludge	pH 5.5–6.0, 35 °C	0.81	Lay et al. (2013)

like glucose, sucrose, etc.; molasses wastewater is mainly composed of sucrose; leachate is composed of complex organic molecules and various nutrients, but before being used as substrate, simple sugars are usually added to enhance the carbohydrates concentration. As shown in Table 4.16, carbohydrates concentration varies from 5 g/L to 40 g/L, similarly anaerobic sludge was the most commonly used inoculum. Most studies were conducted at mesophilic conditions and 35–37 °C was the most widely used. pH ranged from 5.0 to 9.0 and pH 5.0 was the most commonly used. Hydrogen yield obtained ranged from 0.3 to 3.57 mol H_2/mol hexose, both the lowest and highest hydrogen yield were obtained from the

Table 4.16 Hydrogen production from sugar-based wastewater

Substrate	Inoculum	Operational condition	Hydrogen yield (mol/mol hexose)	References
Beverage wastewater				
Sugarcane juice (Carbohydrates 13.42 g/L)	Faeces	pH 7.0, 37 °C	1.83–3.57	Sydney et al. (2014)
Beverage wastewater (Sugar 5 g/L)	Anaerobic sludge	pH 5.5, 37 °C	0.3	Liu et al. (2016)
Beverage wastewater (Sugar 20 g/L)	Compost	pH 5.5, 45 °C	1.68	Sivagurunathan et al. (2014)
Molasses wastewater				
Molasses (5.9 g/L)	*Clostridium butyricum* KBH1	pH 9.0, 37 °C	1.49	Abdul et al. (2013)
Molasses (20 g/L)	*Thermotoga neapolitana*	pH 8.5, 77 °C	1.69	Frascari et al. (2013)
Molasses (40 g/L)	Anaerobic sludge	pH 5.5, 35 °C	0.8-2.1	Lay et al. (2010)
Sugar refinery wastewater (COD 15 g/L)	Anaerobic sludge	pH 5.5, 31 °C	0.48	Won et al. (2013)
Leachate				
Leachate (COD 7.5 g/L)	Anaerobic sludge	pH 7.0, 37 °C	2.8	Hafez et al. (2010)
Leachate (COD 3.38 g/L + glucose 6.2 g/L)	Anaerobic sludge	pH 5.0, 35 °C	1.95	Liu et al. (2015)
Leachate (COD 3.38 g/L + glucose 6.2 g/L)	Anaerobic sludge	pH 9.0, 35 °C	1.4	Liu et al. (2012)

beverage wastewater, indicating that for the sugar-based substrate, operational conditions and inoculum may have great effect on hydrogen production.

Furthermore, some industrial wastewater like oil mill wastewater and biodiesel wastewater are also used as substrate for hydrogen production. Oil mill effluent contains a high level of organic acids, carbohydrate, lipids, minerals, and proteins that can serve as growth nutrients for the microorganisms (Mishra et al. 2016); biodiesel wastewater is pretty rich in glycerol, which is also a kind of biodegradable carbohydrate. Thus, both oil mill wastewater and biodiesel wastewater are suitable substrates for the hydrogen production

As shown in Table 4.17, COD concentration of oil mill effluent ranges from 3 g/L to 80 g/L, anaerobic sludge is more commonly used as inoculum. All the studies were conducted at mesophilic conditions with temperature range from 35 to

Table 4.17 Hydrogen production from industrial wastewater

Substrate	Inoculum	Operational condition	Hydrogen yield (mol/mol hexose)	References
Oil effluent				
Palm oil mill effluent (COD 3 g/L)	Anaerobic sludge	pH 5.5, 38 °C	2.11	Mohammadi et al. (2012)
Palm oil mill effluent (COD 30 g/L)	*Clostridium butyricum* LS2	pH 5.5, 37 °C	2.88	Singh et al. (2013)
Palm oil mill effluent (COD 49.5 g/L)	*Clostridium butyricum* LS2	pH 5.5, 37 °C	0.28	Mishra et al. (2016)
Palm oil mill effluent (COD 80 g/L)	Anaerobic sludge	pH 5.8, 36 °C	0.12	Rasdi et al. (2012)
Olive mill wastewater (COD 52 g/L)	Anaerobic sludge	pH 7.0, 35 °C	0.36	Monti et al. (2015)
Biodiesel wastewater				
Biodiesel wastes (glycerol 1 g/L)	Anaerobic sludge	pH 6.5, 40 °C	1.32	Mangayil et al. (2012)
Biodiesel wastes (glycerol 2.5 g/L)	*Thermotoga maritima*	pH 7–7.5, 40 °C	2.89–3.41	Maru et al. (2013)
Biodiesel wastes (glycerol 2.5 g/L)	*Thermotoga maritima*	pH 7.0, 80 °C	3.3	Maru et al. (2012)
Biodiesel wastes (glycerol 2.5 g/L)	*Thermotoga neapolitana*	pH 7.0, 80 °C	3.18	Maru et al. (2012)
Biodiesel wastes (glycerol 10 g/L)	*Clostridium pasteurianum*	pH 7.0, 35 °C	0.06–0.92	Lo et al. (2013)
Biodiesel wastes glycerol (1.7–25 g/L)	*Enterobacter aerogenes* HU-101	pH 6.8, 37 °C	0.85–1.34	Ito et al. (2005)

38 °C and pH 5.5–5.8 is widely adopted. Hydrogen yield varies a lot from 0.12 to 2.88 mol H_2/mol hexose, and the lower hydrogen yield is accompanied with high substrate concentration. As to the biodiesel wastewater, glycerol concentration ranged from 1 g/L to 25 g/L. Most studies were conducted with pure cultures, and temperature ranged from 35 to 80 °C. Neutral condition (pH 6.5–7.5) was preferred. Hydrogen yield ranged from 0.06 to 3.41 mol H_2/mol hexose, and higher hydrogen yield was obtained at thermophilic conditions.

4.6 Concluding Remarks and Perspectives

Hydrogen production from fossil fuel is presently dominant for its applicable technologies and high efficiency. However, the pollutant formation and energy consumption dramatically decreased the environmental benefits of hydrogen produced. Thus, development of sustainable hydrogen production processes is

necessary. Dark fermentative hydrogen production from organic wastes achieves dual benefits of hydrogen generation and wastes treatment, maps a promising future for hydrogen society. Five categories of organic wastes were considered in this chapter, including waste activated sludge, algae biomass, cellulose-based biomass, starch-based biomass, food waste, and wastewater. Waste activated sludge is becoming a serious environmental problem. Algae biomass attracts attention recently for its CO_2 fixation and easy cultivation. Cellulose-based biomass is the most widely studied for its abundant sources. Starch-based biomass along with food waste has high carbohydrates content and rich nutrients, which are considered as optimal substrate for hydrogen production. Wastewater usually requires no pretreatment and can be easily applied in fermentation process. A large amount of studies have indicated the feasibility of dark fermentative hydrogen production from organic wastes. However, it appears that the technology is still in its infancy, the low hydrogen yield and substrate degradation rate hindered the extensive application. Thus, further studies in quite a few areas are needed to assess the implementation potential of fermentative hydrogen production process.

(1) For the hydrogen production from actual organic wastes, mixed cultures have been more commonly used as inoculum. Various sources of mixed cultures may lead to the variation of hydrogen production performance, especially for the systems using complex substrate. A few studies have tried to enhance hydrogen yield through adding functional microbes into the system, like robust hydrogen producers, cellulose-decomposing microorganisms, lignocellulolytic microorganisms, and so on. Nevertheless, maintaining the desired microbial cultures through controlling fermentation conditions deserve further investigations.
(2) Pretreatment is a critical process for fermentative hydrogen production from complex organic wastes. It can not only affect the biodegradability, but the composition of organic matters in feedstock. Studies have shown that heat treatment was the most widely used, and the combinations of different treatment methods were increasingly explored. Different treatment methods were preferred by different kinds of wastes, and cellulose-based biomass usually requires more severe treatment. Since the treatment process determines the cost of the whole hydrogen production process, more explorations are required to make the process more efficient and economical, and offer the guide for choosing a proper treatment according to biomass characteristics and compositions.
(3) Besides the microbes and substrate, the biological processes are highly dependent on the fermentation conditions, like temperature, pH, composition of inorganic matters, partial pressure of hydrogen, etc. These operational parameters affect not only hydrogen yield, but also the by-product formation.
(4) During the fermentation process, a wide range of intermediates and by-products are formed besides the target product H_2. Energy conversion ratio to hydrogen is usually lower than 40%, the other 60% is mainly turned into volatile fatty acids formation and microbial growth. To recover the energy remained in liquid

phase and minimize the wastes, downstream processes are recommended. A few studies have been conducted to convert the residual volatile fatty acids and alcohols to bioenergy, like photo fermentative hydrogen production, methanogenic fermentation, electricity generation through microbial fuel cells and value-added chemicals production through chain elongation. However, all above processes are still in their infancy stage and need to be further explored.

(5) Till now, hydrogen production in bath mode has been extensively studied, while the studies on fermentation in continuous mode are far from enough. Both the design and operation of high rate reactors deserve further studies.

For such reasons, to achieve a full-scale application of fermentative hydrogen production from organic wastes, considerable efforts are still needed from both technical and managing aspects.

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Chapter 5
Influencing Factors for Biohydrogen Production

5.1 Introduction

Among various hydrogen production processes, biological method is known to be less energy intensive, for it can be carried out at ambient temperature and pressure (Nishio and Nakashimada 2004; Kraemer and Bagley 2007). Biological method mainly includes photosynthetic hydrogen production and fermentative hydrogen production. Compared with the photosynthetic hydrogen production, fermentative hydrogen production is more feasible and thus widely used because fermentative hydrogen production has the advantages of rapid hydrogen production rate and simple operation. Moreover, it can use various organic wastes as substrate for fermentative hydrogen production. It is of great significance to produce hydrogen from organic wastes by fermentative hydrogen production, because it can not only treat organic wastes, but also produce very clean energy. Therefore fermentative hydrogen production has been received increasing attention in recent years (Li and Fang 2007a, b).

Fermentative hydrogen production is very common under anoxic conditions. When bacteria degrade organic substrates, electrons which need to be disposed of to maintain electrical neutrality, are produced. In anoxic environments, protons can act as electron acceptor to produce molecular hydrogen (Das and Veziroglu 2008). Hydrogen can be produced from various substrates by hydrogen-producing bacteria. When glucose is used as the model substrate for fermentative hydrogen production, it is first converted by hydrogen-producing bacteria to pyruvate, producing the reduced form of nicotinamide adenine dinucleotide (NADH) via the glycolytic pathway. Pyruvate can then be further converted to acetylcoenzyme A (acetyl-CoA), carbon dioxide, and hydrogen by pyruvate-ferredoxin oxidoreductase and hydrogenase. Pyruvate may also be further converted to acetyl-CoA and formate, which may be readily converted to hydrogen and carbon dioxide. Acetyl-CoA is finally converted into some soluble metabolites such as acetate, butyrate, ethanol, and so on (Hawkes et al. 2007; Li and Fang 2007a, b).

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Moreover, fermentative hydrogen production is a very complex process and influenced by many factors such as inoculum, substrate, reactor type, nitrogen, phosphate, metal ion, temperature, and pH. And the effects of these factors on fermentative hydrogen production have been reported by a great number of studies throughout the world in the last few years (Nishio and Nakashimada 2004; Hawkes et al. 2007; Li and Fang 2007a, b). This chapter attempts to summarize the above factors influencing fermentative hydrogen production. In this review, the effect of each factor on fermentative hydrogen production and the advance in the research of the effect were briefly introduced and discussed, followed by some suggestions for the future work of fermentative hydrogen production.

5.2 Effect of Inoculum

5.2.1 Pure Cultures

A lot of pure cultures of bacteria have been used to produce hydrogen from various substrates. Table 5.1 summarizes a lot of studies using pure cultures for fermentative hydrogen production. As is shown in Table 5.1, *Clostridium* and *Enterobacter* were most widely used as inoculum for fermentative hydrogen production. Species of genus *Clostridium* are gram-positive, rod-shaped, strict anaerobes, and endospore formers, whereas *Enterobacter* are gram-negative, rod-shaped, and facultative anaerobes (Li and Fang 2007a, b). Most of the studies using pure cultures of bacteria for fermentative hydrogen production were conducted in batch mode and used glucose as substrate; however, it is more desirable to produce hydrogen from organic wastes using pure cultures in continuous mode, because continuous fermentative hydrogen production from organic wastes is more feasible for industrialization to realize the goal of waste reduction and energy production. Thus more researches using pure cultures for continuous fermentative hydrogen production from organic wastes are recommended (Li and Fang 2007a, b).

5.2.2 Mixed Cultures

The bacteria capable of producing hydrogen widely exist in natural environments such as soil, wastewater sludge, compost, and so on (Wang and Wan 2008a, b, c, d). Thus these materials can be used as inoculum for fermentative hydrogen production. At present, the mixed cultures of bacteria from anaerobic sludge, municipal sewage sludge, compost, and soil have been widely used as inoculum for fermentative hydrogen production (Li and Fang 2007a, b). Fermentative hydrogen

Table 5.1 The pure bacterial cultures for fermentative hydrogen production

Inoculum	Substrate	Reactor type	Maximum hydrogen yield	References
Clostridium acetobutylicum	Glucose	Batch	2.0 mol/mol glucose	Chin et al. (2003)
Clostridium acetobutylicum ATCC 824	Glucose	Continuous	1.08 mol/mol glucose	Zhang et al. (2006)
Clostridium butyricum CWBI 1009	Glucose	Continuous	0.23–1.95 mol/mol glucose	Calusinska et al. (2015)
Clostridium butyricum CGS5	Xylose	Batch	0.73 mol/mol xylose	Lo et al. (2008)
Clostridium butyricum CGS2	Starch	Batch	9.95 mmol/g COD	Chen et al. (2007)
Clostridium butyricum DSM 10702	Starch	Batch	3.2 mol/mol hexose	Ortigueira et al. (2015)
Clostridium beijerinckii DSM 791	Glucose	Batch	0.6–1.6 mol/mol glucose	Hu et al. (2013)
Clostridium beijerinckii YA001	Xylose	Batch	2.31 mol/mol xylose	An et al. (2014)
Clostridium pasteurianum DSM 525	Glucose	Batch	1.8–3.0 mol/mol glucose	Hu et al. (2013)
Clostridium pasteurianum CH4	Sucrose	Batch	2.07 mol/mol hexose	Lo et al. (2008)
Clostridium paraputrificum M-21	Chitinous wastes	Batch	2.2 mol/mol substrate	Evvyernie et al. (2001)
Clostridium thermocellum 27405	Cellulosic biomass	Batch	2.3 mol/mol glucose	Levin et al. (2006)
Clostridium thermolacticum	Lactose	Continuous	3.0 mol/mol lactose	Collet et al. (2004)
Clostridium sp. strain no. 2	Cellulose	Continuous	0.3 mol/mol glucose	Taguchi et al. (1996)
Clostridium sp. Fanp2	Glucose	Batch	0.2 mol/L medium	Pan et al. (2008)
Clostridium sp. IODB-O3	Wheat straw	Batch	2.54–2.61 mol/mol hexose	(Patel et al. 2015)
Enterobucter aerogencs HO-39	Glucose	Batch	1.0 mol/mol glucose	Yokoi et al. (1995)
Enterobacter aerogenes NBRC 13534	Glucose	Batch	0.05 mol/L medium	Ogino et al. (2005)
Enterobacter aerogenes	Glucose	Batch	–	Jo et al. (2008)
Enterobacter aerogenes HU-101	Glycerol	Batch	0.6 mol/mol glycerol	Nakashimada et al. (2002)
Enterobacter aerogenes	Starch	Batch	1.09 mol/mol starch	Fabiano and Perego (2002)
Enterohacter aerogenes E 82005	Molasses	Continuous	3.5 mol/mol sugar	Tanisho and Ishiwata (1995)

(continued)

Table 5.1 (continued)

Inoculum	Substrate	Reactor type	Maximum hydrogen yield	References
Enterobacter aerogenes ATCC 13048	Scenedesmus obliquus	Batch	57.6 mL/g VS	Batista et al. (2014)
Enterobacter cloacae IIT-BT 08	Glucose	Continuous	–	Kumar and Das (2001)
Enterobacter cloacae IIT-BT 08	Sucrose	Batch	6 mol/mol sucrose	Kumar and Das (2000)
Enterobacter cloacae IIT-BT 08	Cellobiose	Batch	5.4 mol/mol cellobiose	Kumar and Das (2000)
Escherichia coli MC13-4	Glucose	Batch	1.2 mol/mol glucose	Ishikawa et al. (2006)
Escherichia coli	Glucose	Batch	2.0 mol/mol glucose	Bisaillon et al. (2006)
Escherchia coli	Glucose	Continuous	2.0 mol/mol glucose	Turcot et al. (2008)
Bacillus firmus NMBL-03	bagasse hydrolysate	Continuous	1.29 mol/mol sugar	Sinha and Pandey (2014)
Pseudomonas sp. GZ1	Waste sludge	Batch	0.007 mol/g TCOD	Guo et al. (2008)
Thermoanaerobacterium thermosaccharolyticum KU001	Glucose	Batch	2.4 mol/mol glucose	Ueno et al. (2001)
Thermoanaerobacterium thermosaccharolyticum TERI S7	Xylose	Batch	2.2 mol/mol xylose	Singh et al. (2014)
Thermococcus kodakaraensis KOD1	Starch	Continuous	–	Kanai et al. (2005)
Thermotoga elfii	Glucose	Batch	84.9 mmol/L medium	Van Niel et al. (2002)
Hydrogen-producing bacterial B49	Glucose	Batch	0.1 ml/L culture	Wang et al. (2007)
Ruminococcus albus	Glucose	Batch	2.52 mol/mol glucose	Ntaikou et al. (2008)
Hafnia alvei	Glucose	Batch	–	Podestá et al. (1997)
Citrobacter amalonaticus Y19	Glucose	Batch	8.7 mol/mol glucose	Oh et al. (2008)
Ethanoligenens harbinense YUAN-3	Glucose	Continuous	1.93 mol/mol glucose	Xing et al. (2008a, b)
Klebsiella pneumoniae TR17	Crude glycerol	Batch	0.25 mol/mol glycerol	Chookaew (2012)

production processes using mixed cultures are more practical than those using pure cultures, because the former are simpler to operate and easier to control, and may have a broader source of feedstock (Li and Fang 2007a, b). However, in a fermentative hydrogen production process using mixed cultures, the hydrogen

produced by hydrogen-producing bacteria may be consumed by hydrogen-consuming bacteria. In addition, when mixed cultures are treated under harsh conditions, hydrogen-producing bacteria would have a better chance than some hydrogen-consuming bacteria to survive. Thus, in order to harness hydrogen from a fermentative hydrogen production process, the mixed cultures can be pretreated by certain methods to suppress as much hydrogen-consuming bacterial activity as possible while still preserving the activity of the hydrogen-producing bacteria (Wang and Wan 2008a, b, c, d). The optimal index is highest hydrogen yield.

The pretreatment methods reported for enriching hydrogen-producing bacteria from mixed cultures mainly include heat-shock, acid, base, aeration, freezing and thawing, chloroform, sodium 2-bromoethanesulfonate or 2-bromoethanesulfonic acid and iodopropane (Wang and Wan 2008a, b, c, d). Different pretreatment methods have different property and comparison of different pretreatment methods to obtain a better pretreatment method for a given fermentative hydrogen production process was conducted by many studies (Wang and Wan 2008a, b, c, d). Table 5.2 summarizes several studies comparing various pretreatment methods for enriching hydrogen-producing bacteria from mixed cultures.

As shown in Table 5.2, there exists certain disagreement on the optimal pretreatment method for enriching hydrogen-producing bacteria from mixed cultures (Cheong and Hansen 2006; Hu and Chen 2007; Wang and Wan 2008a, b, c, d). The possible reason for this disagreement was the difference among these studies in the terms of inoculum, pretreatment method studied, specific condition of each pretreatment method and the kind of substrates.

Even though heat-shock was the most widely used pretreatment method for enriching hydrogen-producing bacteria from inoculum (Li and Fang 2007a, b), it is not always effective for enriching hydrogen-producing bacteria from mixed culture inoculum compared with other pretreatment methods, for it may inhibit the activity of some hydrogen-producing bacteria (Wang and Wan 2008a, b, c, d).

In addition, in the reviewed studies, the comparisons of various pretreatment methods for enriching hydrogen-producing bacteria from mixed culture inoculum were all conducted in batch mode, and conducting these comparisons in continuous mode is recommended. Furthermore, most of the comparisons were conducted using glucose as substrate, and more comparisons conducted using organic wastes as substrate are recommended.

Moreover, some microbial analysis methods such as PCR-DGGE have been used to determine the community structure of mixed cultures during fermentative hydrogen production (Shin et al. 2004; Kim et al. 2006; Kim and Shin 2008). And they can also be used to detect the changes in the community structure of mixed cultures after certain pretreatment. For example, using PCR-DGGE technique, Kim and Shin reported that base pretreatment of mixed cultures would prevent the microbial population shift to non-H_2-producing acidogens, thus was beneficial for fermentative hydrogen production (Kim and Shin 2008).

Table 5.2 Comparison of various pretreatment for enriching hydrogen-producing bacteria

Inoculum	Inoculum pretreatment method studied	Substrates	Reactor type	Maximum hydrogen yield	Optimal pretreatment method	References
Digested sludge	Acid, base, heat-shock, aeration and chloroform	Glucose	Batch	1.8 mol/mol glucose	Heat-shock	Wang and Wan (2008a, b, c, d)
Cattle manure sludge	Freezing and thawing, acid, heat-shock, and sodium 2-bromoethanesulfonate	Glucose	Batch	1.0 mol/mol glucose	Acid	Cheong and Hansen (2006)
Methanogenic granules	Acid, heat-shock and chloroform	Glucose	Batch	1.2 mol/mol glucose	Chloroform	Hu and Chen (2007)
Digested wastewater sludge	Heat-shock, aeration, acid, base, 2-bromoethanesulfonic acid and iodopropane	Sucrose	Batch	6.12 mol/mol sucrose	Base	Zhu and Béland (2006)
Anaerobic sludge	Sodium 2-bromoethanesulfonate, acid, heat-shock and their four combinations	Dairy wastewater	Batch	0.0317 mmol/g COD	Sodium 2-bromoethanesulfonate	Mohan et al. (2008)
Cow dung compost	Heat-shock, infrared drying aeration	Sucrose	Batch	1.96 molg/mol hexose	Aeration	Song et al. (2012)
Digested sludge	Heat-shock, acid, base, ultrasonication	Glucose	Batch	1.55 mol/mol glucose	Ultrasonication	Elbeshbishy et al. (2010)
Digested sludge	Heat-shock, acid, base, ionizing radiation	Glucose	Batch	2.15 mol/mol glucose	Ionizing radiation	Yin et al. (2014)

5.3 Effect of Substrate

5.3.1 Overview

A lot of substrates have been used for fermentative hydrogen production. Table 5.3 summarizes a lot of studies using various substrates for fermentative hydrogen production. As is shown in Table 5.3, glucose, sucrose, and starch were most widely used substrate for fermentative hydrogen production. However, in recent years, a few studies have begun to use organic wastes as substrate for hydrogen production (Kapdan and Kargi 2006). In addition, most of the studies on fermentative hydrogen production were conducted in batch mode, and more studies conducted in continuous mode are recommended.

It has been demonstrated that in an appropriate range, increasing substrate concentration could increase the ability of hydrogen-producing bacteria to produce hydrogen during fermentative hydrogen production, but substrate concentrations at much higher levels could decrease it with increasing levels (Zhu and Béland 2006; Lo et al. 2008). Furthermore, there exists certain disagreement on the optimal concentration of a given substrate for fermentative hydrogen production. For example, the optimal sucrose concentration for fermentative hydrogen production reported by van Ginkel et al. was 7.5 g COD/L (Ginkel et al. 2001), while that reported by Lo et al. was 40 g COD/L (Lo et al. 2008). The possible reason for this disagreement was the difference among these studies in the terms of inoculum and substrate concentration range studied.

Some complex substrates are not ideal for fermentative hydrogen production due to their complex structures; however, after being pretreated by some methods, they can be easily used by hydrogen-producing bacteria. For example, Zhang et al. (2007) reported that the hydrogen yield from cornstalk wastes after acidification pretreatment was much larger than that from cornstalk wastes without any pretreatment.

Waste activated sludge from wastewater treatment plants contains high levels of organic matter and thus is a potential substrate for hydrogen production. After appropriate pretreatments such as ultrasonication, acidification, freezing and thawing, sterilization, methanogenic inhibitor, and microwave, the ability of hydrogen-producing bacteria to produce hydrogen from it can be improved (Wang et al. 2003; Ting and Lee 2007). Different substrate pretreatment methods have different property and comparison of various substrate pretreatment methods was conducted by several studies. Table 5.4 summarizes several studies comparing various substrate pretreatment methods for fermentative hydrogen production from wastewater sludge.

As shown in Table 5.4, among the substrate pretreatment methods studied, freezing and thawing and sterilization are superior pretreatment methods of

Table 5.3 Comparison of various substrates used for fermentative hydrogen production

Inoculum	Substrates	Reactor type	Substrate concentration (g COD/L)		Optimal index (value)	References
			Range studied	Optimal		
Clostridium butyricum CGS5	Xylose	Batch	5–40	20	Maximum hydrogen production potential (172.9 mL)	Lo et al. (2008)
Municipal sewage sludge	Xylose	Continuous	10–100	20	Maximum hydrogen yield (2.25 mol/mol xylose)	Lin and Cheng (2006)
Anaerobic sludge	Glucose	Batch	0.27–4.3	1.1	Maximum hydrogen production rate (0.13 mL/h)	Hang et al. (2008)
Digested sludge	Glucose	Batch	1.1–320	2.1	Maximum hydrogen yield (3.1 mol/mol glucose)	Wang and Wei (2008a, b, c, d)
Anaerobic digester sludge	Glucose	Continuous	6.5–51.4 g COD/L/d	25.7 g COD/L/d	Maximum hydrogen yield (2 mol/mol hexose)	Nunes Ferraz Junior et al. (2014)
Clostridium acetobutylicum ATCC 824	Glucose	Continuous	1.1–11.2	11.2	Maximum specific hydrogen production rate (1270 mL/g glucose-L reactor)	Zhang et al. (2006)
Ethanoligenens harbinense YUAN-3	Glucose	Batch	5.3–21.3	10.7	Maximum hydrogen yield (1.93 mol/mol glucose)	Xing et al. (2008)
Thermoanaerobacterium thermosaccharolyticum PSU-2	Sucrose	Batch	5.6–56	5.6	Maximum hydrogen yield (6 mol/mol sucrose)	O-Thong et al. (2008)
Mixed cultures	Sucrose	Batch	1.5–44.8	7.5 g	Maximum hydrogen yield (38.9 mL/(g COD-L culture)	Ginkel et al. (2001)
Municipal sewage sludge	Sucrose	Batch	10–30	10	Maximum hydrogen yield (2.46 mol/mol sucrose)	Wang et al. (2006)
Clostridium butyricum CGS5	Sucrose	Batch	5–30	20	Maximum hydrogen yield (2.78 mol/mol sucrose)	Chen et al. (2005)

(continued)

Table 5.3 (continued)

Inoculum	Substrates	Reactor type	Substrate concentration (g COD/L)		Optimal index (value)	References
			Range studied	Optimal		
Anaerobic digester sludge	Sucrose	Continuous	10–60	30	Maximum hydrogen yield (1.22 mol/mol hexose)	Kim et al. (2006)
Clostridium pasteurianum CH4	Sucrose	Batch	5–40	40	Maximum hydrogen yield (2.07 mol/mol hexose)	Lo et al. (2008)
Cracked cereals	Starch	Batch	2.1–34.1	2.1	Maximum hydrogen yield (194 mL/g starch)	Liu and Shen (2004)
Anaerobic sludge	Starch	Batch	9.8–39.0	9.8	Maximum hydrogen yield (67 mL/g starch)	Zhang et al. (2003)
Anaerobic sludge	Starch	Batch	5–60	20	Maximum hydrogen yield (2.2 mol/mol hexose)	Lin et al. (2008a, b)
Municipal sewage sludge	Starch	Batch	8–32	32	Maximum hydrogen yield (11.25 mmol/g starch)	Lee et al. (2008)
Cow dung compost	Cornstalk wastes	Batch	5.3–42.7	16	Maximum hydrogen yield (149.69 mL/TVS)	Zhang et al. (2007a, b)
Anaerobic digester sludge	Rice slurry	Batch	2.9–23.6	5.9	Maximum hydrogen yield (346 mL/g carbohydrate)	Fang et al. (2005)
Cow dung compost	Beer lees	Batch	5.3–53.3	21.3	Maximum hydrogen yield (68.6 mL/TVS)	Fan et al. (2006)
Fermented soybean-meal	Bean curd manufacturing waste	Batch	1.1–6.9	4.0	Maximum hydrogen production rate (130 mL/h-L culture)	Mizuno (2000)
Anaerobic digester sludge	Food waste	Batch	0–32.3	4.6	Maximum hydrogen yield (101 mL/g COD)	Chen et al. (2006)

(continued)

Table 5.3 (continued)

Inoculum	Substrates	Reactor type	Substrate concentration (g COD/L)		Optimal index (value)	References
			Range studied	Optimal		
Anaerobic sludge	Food waste	Batch	3.2–10.7	6.4	Maximum hydrogen yield (1.8 mol/mol hexose)	Shin et al. (2004)
Anaerobic digester sludge	Non-fat dry milk	Batch	0–96	4	Maximum hydrogen yield (119 mL/g COD)	Chen et al. (2006)
Waste activated sludge	Food wastewater	Batch	10–160	40	Maximum hydrogen yield (47.1 mmol/g COD)	Wu et al. (2004)
Municipal sewage sludge	Rice winery wastewater	Continuous	14–36	14	Maximum hydrogen yield (1.9 mol/mol hexose)	Yu et al. (2002)
Anaerobic digester sludge	Gelatinaceous wastewater	Batch	0.25–7 g COD/g VSS	1 g COD/g VSS	Maximum hydrogen yield (79.2 mL/g COD)	Mostafa et al. (2016)

Table 5.4 Various pretreatment methods for waste activated sludge

Inoculum	Reactor type	Substrate pretreatment method	Optimal pretreatment method	Optimal index (value)	References
Clostridium bifermentans	Batch	Freezing and thawing, ultrasonication, acidification, sterilization and methanogenic inhibitor	Freezing and thawing	Maximum hydrogen yield (2.1 mmol/g COD)	Wang et al. (2003)
Clostridium bifermentans	Batch	Freezing and thawing, sonication, acidification and sterilization	Freezing and thawing	Maximum hydrogen yield (4.1 g/Kg DS)	Ting and Lee (2007)
Pseudomonas sp. GZ1	Batch	Sterilization, microwave and ultrasonication	Sterilization	Maximum hydrogen yield (15.02 ml/g TCOD)	Guo et al. (2008)
Digested sludge	Batch	Without treatment, heat, acid, combined heat and acid	Acid	Maximum hydrogen yield (41 ml/g VS)	Assawamongkholsiri et al. (2013)
Indigenous microbes	Batch	Heat, microwave, enzyme, aerobic digestion	Heat	Maximum hydrogen yield (15.53 ml/g VS)	Guo et al. (2015)

wastewater sludge for fermentative hydrogen production. It is worth noting that when using *Clostridium bifermentans* as inoculum, freezing and thawing was the optimal pretreatment methods for waste activated sludge (Wang et al. 2003; Ting and Lee 2007), while when *Pseudomonas* sp. GZ1 as inoculum, sterilization was the optimal pretreatment methods for waste activated sludge (Guo et al. 2008). This demonstrates that the optimal pretreatment methods for waste activated sludge may be dependent on the inoculum used for fermentative hydrogen production.

In addition, various substrate pretreatment methods for waste activated sludge were compared in batch mode, and conducting these comparisons in continuous mode is recommended. Furthermore, they were compared using pure cultures as inoculum, and using mixed cultures as inoculum is recommended. Moreover, comparison of various substrate pretreatment methods for other complex organic wastes besides waste activated sludge is recommended.

5.3.2 *Effect on Substrate Degradation Efficiency*

Figure 5.1 shows the effect of substrate concentration on substrate degradation efficiency.

The results showed that the substrate degradation efficiency changed a little with increasing substrate concentration from 1 to 25 g/L, and a similar trend was also reported by Yu and Mu (2006). However, the substrate degradation efficiency decreased with increasing substrate concentration from 25 to 300 g/L. This demonstrated that in a lower range, increasing substrate concentration had little impact on the ability of mixed cultures to degrade substrate during the fermentative hydrogen production, but substrate at much higher concentration could decrease it.

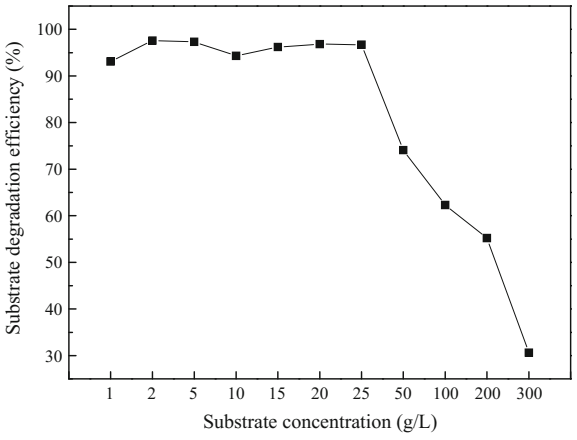

Fig. 5.1 Effect of substrate concentration on substrate degradation efficiency

5.3.3 *Effect on Hydrogen Production*

Figure 5.2 shows the effect of substrate concentration on hydrogen production potential.

The results showed that the hydrogen production potential increased with increasing substrate concentration from 0 to 25 g/L, however, it decreased when substrate concentration increased from 25 to 300 g/L. The maximum hydrogen production potential was 426.8 mL at substrate concentration of 25 g/L.

Figure 5.3 shows the effect of substrate concentration on hydrogen yield.

The results showed that the hydrogen yield increased with increasing substrate concentration from 1 to 2 g/L, however, it decreased with further increasing substrate concentration from 2 to 300 g/L. Yu et al. (2002) found that the hydrogen yield decreased with increasing substrate concentration, and the possible reason was

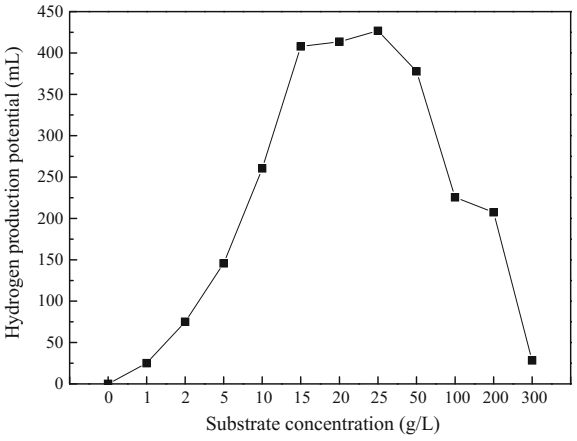

Fig. 5.2 Effect of substrate concentration on hydrogen production potential

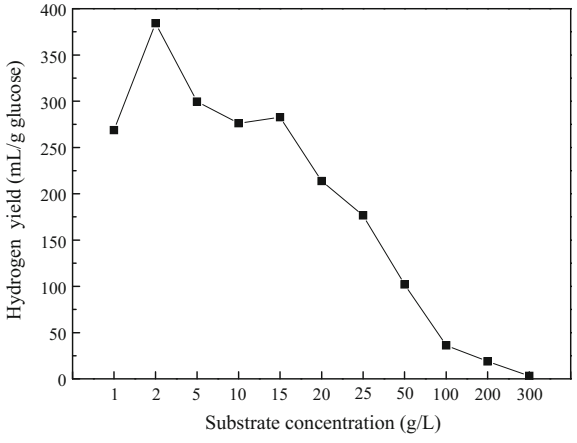

Fig. 5.3 Effect of substrate concentration on hydrogen yield

Table 5.5 Comparison of the maximum hydrogen yield from glucose by mixed cultures

Substrate concentration (g/L)	Maximum hydrogen yield (mol/mol glucose)	References
2	3.09	Wan and Wang (2008a)
10	2.5	Wan and Wang (2008b)
10	2.19	Yin et al. (2014)
10	2.15	de Sá et al. (2013)
10	1.67	Mu et al. (2006)
2.8	0.97	Oh et al. (2003)
9.5	1.8	Iyer et al. (2004)
18.75	1.17	Hu and Chen (2007)
10	2.1	Morimoto et al. (2004)
7	2.1	Fang and Liu (2002)

that the substrate concentration range they studied was over the optimal substrate concentration. The maximum hydrogen yield was 384.3 mL/g glucose (3.09 mol/mol glucose) at the substrate concentration of 2 g/L.

Table 5.5 summarizes the maximum hydrogen yield, indicating that the maximum hydrogen yield here is a little higher than those of other studies.

5.3.4 *Effect on Hydrogen Production Rate*

Figure 5.4 shows the effect of substrate concentration on hydrogen production rate.

The results showed that the hydrogen production rate increased with increasing substrate concentration from 0 to 25 g/L, however, it decreased with further increasing substrate concentration from 25 to 300 g/L.

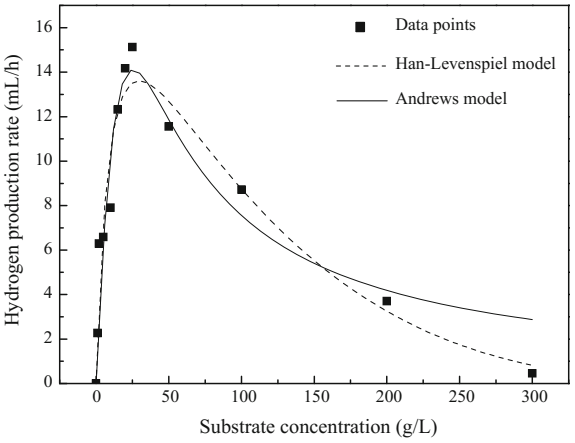

Fig. 5.4 Effect of substrate concentration on hydrogen production rate

The Han-Levenspiel model and Andrews model were used to fit the hydrogen production rate data using software Origin 7.5. The fitting results are as follows:

Han-Levenspiel model: $r = 19.2 \cdot \left(1 - \frac{S}{520.0}\right)^{3.7} \cdot \frac{S}{S + 9.0 \cdot (1 - S/520.0)^{13.2}}$ $R^2 = 0.932$

Andrews model: $r = \frac{67.1 \cdot S}{47.7 + S + S^2/13.5}$ $R^2 = 0.902$

The inhibition of the fermentative hydrogen production by substrate at higher concentration can be classified as noncompetitive inhibition, competitive inhibition, uncompetitive inhibition, and mixed inhibition, according to the specific values of n and m obtained from the Han-Levenspiel model. The values of n and m obtained from the Han-Levenspiel model were all positive, suggesting that the inhibition of the fermentative hydrogen production by substrate at higher concentration was uncompetitive inhibition. However the inhibition of the fermentative hydrogen production by substrate at higher c, van Niel et al. (2003) reported that concentration was noncompetitive inhibition. The different cultivation conditions could lead to different inhibition of the fermentative hydrogen production by substrate at higher concentration.

5.3.5 *Effect on Soluble Metabolites Distribution*

Table 5.6 summarizes the effect of substrate concentration on distributions of the soluble metabolites.

The total concentration of the soluble metabolites and the total concentration of the volatile fatty acids increased with increasing substrate concentration from 1 to 300 g/L. With increasing substrate concentration from 1 to 10 g/L, the fractions of the soluble metabolites had the following order: acetic acid > butyric acid > propionic acid or ethanol; with increasing substrate concentration from 15 to 20 g/L,

Table 5.6 Effect of substrate concentration on distribution of the soluble metabolites

Substrate concentration (g/L)	Ethanol + VFAs (mmol/L)	VFAs (mmol/L)	Fractions of the soluble metabolites (%)			
			Ethanol	HAc	HPr	HBu
1.0	8.1	7.8	3.8	79.4	4.6	12.2
2.0	10.1	10.1	0.6	72.4	7.3	19.7
5.0	16.0	15.2	5.2	52.3	2.4	40.1
10.0	59.1	56.9	3.7	47.2	8.0	41.1
15.0	45.9	44.9	2.2	34.6	0.3	62.9
20.0	57.4	56.4	1.8	27.2	4.3	66.7
25.0	92.5	90.6	2.0	65.0	0.2	32.8
50.0	129.0	122.8	4.8	56.8	0.9	37.5
100.0	110.8	102.1	7.9	69.0	20.2	2.9
200.0	139.0	129.1	7.1	57.9	32.7	2.3
300.0	119.2	116.9	1.9	81.3	14.9	1.9

the fractions of the soluble metabolites had the following order: butyric acid > acetic acid > propionic acid or ethanol; with increasing substrate concentration from 25 to 50 g/L, the fractions of the soluble metabolites had the following order: acetic acid > butyric acid > ethanol > propionic acid; with increasing substrate concentration from 100 to 300 g/L, the fractions of the soluble metabolites had the following order: acetic acid > propionic acid > ethanol > butyric acid. The changes in the fractions of the soluble metabolite with increasing substrate concentration resulted from the metabolic pathway shift induced by the different bacteria dominant at different substrate concentration.

With increasing substrate concentration from 100 to 300 g/L, the fraction of propionic acid in the soluble metabolites was relatively high, but the hydrogen yield was very low. One possible reason was that, propionic acid was produced from glucose without hydrogen production, and sometimes the production of propionic acid was hydrogen-consuming process (Noike et al. 2005; Li and Fang 2007a, b).

5.3.6 *Effect on Final pH*

Figure 5.5 shows the effect of substrate concentration on final pH.

The results showed that due to fermentation, the final pH in all the tests were lower than the initial pH 7.0. The final pH in batch tests trended to decrease with increasing substrate concentration from 0 to 300 g/L. However, Wu et al. (2004) found that although total concentration of volatile fatty acids increased with increasing substrate concentration, the final pH did not change very much, compared with initial pH.

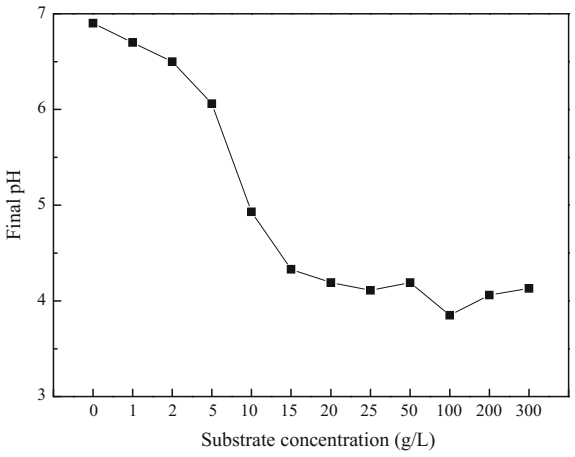

Fig. 5.5 Effect of substrate concentration on final pH

5.4 Effect of Reactor Type

As shown in 5.1–5.3, most of the studies on fermentative hydrogen production were conducted in batch mode due to its simple operation and control. However, large-scale operations would require continuous production processes for practical engineering reasons. Table 5.7 summarizes a lot of studies using continuous reactors for fermentative hydrogen production. As shown in Table 5.7, the continuous stirred tank reactor (CSTR) was widely used for continuous fermentative hydrogen production.

In a conventional CSTR, biomass is well suspended in the mixed liquor, which has the same composition as the effluent. Since biomass has the same retention time as the HRT, washout of biomass may occur at shorter HRT. In addition, biomass concentration in the mixed liquor and the hydrogen production is limited. Immobilized-cell reactors provide an alternative to a conventional CSTR, because they are capable of maintaining higher biomass concentrations and could operate at shorter HRT without biomass washout (Li and Fang 2007a, b). Biomass immobilization can be achieved through forming granules, biofilm, or gel-entrapped bioparticles (Li and Fang 2007a, b). For example, Zhang et al. found that the formation of granular sludge facilitated biomass concentration up to 32.2 g VSS/L and enhanced hydrogen production (Zhang et al. 2007).

It has been demonstrated that in an appropriate range, increasing HRT could increase the ability of hydrogen-producing bacteria to produce hydrogen during fermentative hydrogen production, but HRT at much higher levels could decrease it with increasing levels [69]. Furthermore, there exists certain disagreement on the optimal HRT for continuous fermentative hydrogen production reactors, even for the same type reactor. For example, the optimal HRT for a CSTR reported by Zhang et al. was 0.5 h (Zhang et al. 2007), while the optimal HRT for a CSTR using reported by Arooj et al. was 12 h (Arooj et al. 2008). The possible reason for this disagreement was the difference among these studies in the terms of inoculum, substrate and HRT range studied.

As shown in Table 5.5, glucose and sucrose were most widely used substrate for continuous fermentative hydrogen production. Thus, more studies on continuous fermentative hydrogen production using organic wastes as substrate are recommended.

Moreover, different reactors have different property and comparison of various reactors was conducted by several studies. For example, Zhang et al. compared a biofilm-based reactor and a granule-based reactor and concluded that the granule-based reactor was better than the biofilm-based reactor for continuous fermentative hydrogen production, because the granule-based reactor has a better ability of biomass retention (Zhang et al. 2008).

Table 5.7 The continuous reactors used for fermentative hydrogen production

Inoculum	Substrates	Reactor type	Hydraulic retention time (h)		Optimal index (value)	References
			Range studied	Optimal		
Municipal sewage sludge	Glucose	CSTR	0.5–2	0.5	Maximum hydrogen yield (1.81 mol/mol glucose)	Zhang et al. (2007a, b)
Anaerobic sludge	Glucose	CSTR	2–12	4	Maximum hydrogen production rate (115.68 mmol/d)	Gavala et al. (2006)
Municipal sewage sludge	Sucrose	CSTR	2–12	4	Maximum hydrogen yield (4.70 mol/mol sucrose)	Chen et al. (2008)
Municipal sewage sludge	Sucrose	CSTR	2–13.3	8	Maximum hydrogen yield (4.52 mol/mol sucrose)	Chen and Lin (2003)
Municipal sewage sludge	Fructose	CSTR	2–8	8	Maximum hydrogen yield (1.68 mol/mol hexose)	Lee et al. (2007)
Anaerobic sludge	Starch	CSTR	2–12	12	Maximum hydrogen yield (1.5 mol/mol hexose)	Lin et al. (2008a, b)
Anaerobically digested sludge	Glucose	CSTR	6–12	10	Maximum hydrogen yield (1.95 mol/mol glucose)	Zhang et al. (2006)
Anaerobic sludge	Glucose	CSTR	4–12	10	Maximum hydrogen yield (1.63 mol/mol glucose)	Wu et al. (2008a, b)
Municipal sewage sludge	Xylose	CSTR	4–12	12	Maximum hydrogen yield (1.63 mol/mol xylose)	Wu et al. (2008a, b)
Municipal sewage sludge	Glucose	CSTR	4–12	12	Maximum hydrogen yield (1.36 mol/mol hexose)	Lee et al. (2007)
Municipal sewage sludge	Sucrose	CSTR	2–12	12	Maximum hydrogen yield (1.60 mol/mol hexose)	Lee et al. (2007)
Anaerobic digester sludge	Starch	CSTR	4–18	12	Maximum hydrogen yield (0.92 mol/mol glucose)	Arooj et al. (2008)

(continued)

Table 5.7 (continued)

Inoculum	Substrates	Reactor type	Hydraulic retention time (h)		Optimal index (value)	References
			Range studied	Optimal		
Anaerobic sludge	Cellulose	CSTR	–	10	Maximum hydrogen yield (15.4 mol/kg cellulose)	Jiang et al. (2015)
Municipal sewage sludge	Sucrose	UASB	4–24	8	Maximum hydrogen yield (1.5 mmol/mol sucrose)	Chang and Lin (2004)
Anaerobic sludge	Glucose	UASB	2–12	12	Maximum hydrogen production rate (96.0 mmol/d)	Gavala et al. (2006)
Sewage sludge	Sucrose	UASB	6–24	8	Maximum hydrogen yield (3.6 mol/mol sucrose)	Chang and Lin (2006)
Anaerobically digested sludge	Glucose	Anaerobic biofilm fluidized bed reactors	0.125–3	0.25	Maximum hydrogen yield (1.7 mol/mol glucose)	Zhang et al. (2008)
Anaerobically digested sludge	Glucose	Anaerobic granule fluidized bed reactors	0.125–3	0.25	Maximum hydrogen yield (1.6 mol/mol glucose)	Zhang et al. (2008)
Municipal sewage sludge	Sucrose	Carrier-induced granular sludge bed bioreactor	0.25–4	0.5	Maximum hydrogen yield (3.3 mol/mol sucrose)	Lee et al. (2004)
Municipal sewage sludge	Xylose	Powder activated carbon-assisted agitated granular sludge bed reactor	2–4	4	Maximum hydrogen yield (0.7 mol/mol xylose)	Wu et al. (2008a, b)
Municipal sewage sludge	Sucrose	Packed-bed bioreactor	0.5–4	4	Maximum hydrogen yield (3.9 mol/mol sucrose)	Lee et al. (2003)
Municipal sewage sludge	Glucose	Membrane bioreactor	1–4	4	Maximum hydrogen yield (1.72 mol/mol hexose)	Lee et al. (2007)
Municipal sewage sludge	Xylose	Immobilized-cell continuously stirred anaerobic reactor	2–6	6	Maximum hydrogen yield (0.8 mol/mol xylose)	Wu et al. (2008a, b)

CSTR continuous stirred tank reactor
UASB upflow anaerobic sludge blanket reactor

5.5 Effect of Nitrogen and Phosphate

5.5.1 Overview

Since nitrogen is a very important component for proteins, nucleic acids and enzymes that are of great significance to the growth of hydrogen-producing bacteria, it is one of the most essential nutrients needed for the growth of hydrogen-producing bacteria. Thus, an appropriate level of nitrogen addition is beneficial to the growth of hydrogen-producing bacteria and to fermentative hydrogen production accordingly (Bisaillon et al. 2006). Table 5.8 summarizes several studies investigating the effect of nitrogen concentration on fermentative hydrogen production.

As shown in Table 5.8, ammonia nitrogen was the most widely investigated nitrogen source for fermentative hydrogen production. Thus, more investigations of the effect of other nitrogen source concentration besides ammonia concentration on fermentative hydrogen production are recommended.

In addition, there exists certain disagreement on the optimal ammonia nitrogen concentration for fermentative hydrogen production. For example, the optimal ammonia nitrogen concentration for fermentative hydrogen production reported by Bisaillon et al. was 0.01 g N/L (Bisaillon et al. 2006), while that reported by Salerno et al. was 7.0 g N/L (Salerno et al. 2006). The possible reason for this disagreement was the difference among these studies in the terms of inoculum and ammonia nitrogen concentration range studied.

As is shown in Table 5.8, glucose was the most widely used substrate during the investigation of the effect of nitrogen concentration on fermentative hydrogen production. Thus, more investigations of the effect of nitrogen concentration on fermentative hydrogen production using organic wastes as substrate are recommended. In addition, as is shown in Table 6, all the reviewed studies investigating the effect of nitrogen concentration on fermentative hydrogen production were conducted in batch mode, and conducting such studies in continuous mode is recommended.

Phosphate is needed for hydrogen production due to its nutritious value as well as buffering capacity. It has been demonstrated that in an appropriate range, increasing phosphate concentration could increase the ability of hydrogen-producing bacteria to produce hydrogen during fermentative hydrogen production, but phosphate concentrations at much higher levels could decrease it with increasing levels (Lay et al. 2005; Bisaillon et al. 2006).

It had been shown that an appropriate C/N and C/P is fundamental for fermentative hydrogen production. Table 5.9 summarizes several studies investigating the effect of C/N and C/P on fermentative hydrogen production.

As shown in Table 5.9, there exists certain disagreement on the optimal C/N and C/P for fermentative hydrogen production. For example, the optimal C/N and C/P for fermentative hydrogen production reported by Argun et al. (2008) were 200 and 1000, respectively, while those reported by O-Thong et al. (2008) were 74 and 559,

Table 5.8 The effect of nitrogen concentration on fermentative hydrogen production

Inoculum	Substrates	Reactor type	Nitrogen source	Nitrogen concentration		Optimal index (value)	References
				Range studied	Optimal		
Escherichia coli	Glucose	Batch	NH_4Cl	0–0.2 g N/L	0.01 g N/L	Maximum hydrogen yield (1.7 mol/mol glucose)	Bisaillon et al. (2006)
Dewatered and thickened sludge	Glucose	Batch	NH_4Cl	0.5–10 g N/L	7 g N/L	Maximum hydrogen production (150 mL)	Salerno et al. (2006)
Grass compost	Food wastes	Batch	NH_4HCO_3	0–0.6 g N/L	0.4 g N/L	Maximum hydrogen yield (77 mL/g TVS)	Lay et al. (2005)
Cracked cereals	Starch	Batch	NH_4HCO_3	0.1–2 g N/L	1 g N/L	Maximum hydrogen yield (146 mL/g starch)	Liu and Shen (2004)
Compost	Glucose	Batch	Yeast extract	2–8% yeast extract	4% yeast extract	Maximum hydrogen production (70 mmol)	(Morimoto et al. (2004)
Enterobacter aerogenes HO-39	Glucose	Batch	Polypepton	0–5% polypepton	2% polypepton	Maximum hydrogen production (58 mL)	Yokoi et al. (1995)

Table 5.9 The effect of C/N and C/P on fermentative hydrogen production

Inoculum	Substrates	Reactor type	C/N		C/P		Optimal index (value)	References
			Range studied	Optimal	Range studied	Optimal		
Wasted activated sludge	Sucrose	Batch	40–130	47	–	–	Maximum hydrogen yield (4.8 mol/mol sucrose)	Lin (2004)
Anaerobic sludge	Sucrose	Continuous	40–190	137	–	–	Maximum hydrogen yield (3.5 mol/mol sucrose)	Anzola-Rojas et al. (2015)
Anaerobic sludge	Wheat powder	Batch	20–200	200	50–1000	1000	Maximum hydrogen yield (281 mL/g starch)	Argun et al. (2008)
Anaerobic sludge	Palm oil mill effluent	Batch	45–95	74	450–650	559	Maximum hydrogen yield (6.33 L/L substrate)	O-Thong et al. (2008a, b)

respectively. The possible reason for this disagreement was the difference among these studies in the terms of substrate, C/N range and C/P range studied.

In addition, all the reviewed studies investigating the effect of C/N and C/P on fermentative hydrogen production were conducted in batch mode, and conducting such studies in continuous mode is recommended.

5.5.2 *Effect of Ammonia Concentration*

Nitrogen is beneficial to the growth of hydrogen-producing bacteria and to fermentative hydrogen production accordingly (Morimoto et al. 2004; Lin and Lay 2005; Li and Fang 2007a, b). Among various nitrogen sources, ammonia nitrogen is widely used for fermentative hydrogen production as nitrogen source.

In an appropriate concentration range, ammonia nitrogen is beneficial to fermentative hydrogen production, while at a much higher concentration, ammonia nitrogen could inhibit fermentative hydrogen production, for it may change the intracellular pH of hydrogen-producing bacteria, increase the maintenance energy requirement for hydrogen-producing bacteria or inhibit specific enzymes related to fermentative hydrogen production (Bisaillon et al. 2006; Salerno et al. 2006; Chen et al. 2008).

5.5.2.1 Kinetic Models

Zwietering et al. (1990) developed a modified Logistic model (Eq. 5.1) to describe the progress of the bacterial growth in batch tests.

$$\int_0^t \frac{dX}{dt} dt = \frac{A}{1 + \exp[4\mu_m \cdot (\lambda - t)/A + 2]}, \tag{5.1}$$

where X is the bacterial growth value at cultivation time t, A is the maximum bacterial growth value, μ_m is the maximum bacterial growth rate and λ is the lag time of the bacterial growth in batch tests.

Moreover, Lo et al. (2008) showed that under certain conditions, the amount of hydrogen produced by per unit of hydrogen-producing bacteria was a constant, which can be expressed as Eq. 5.2.

$$\frac{dH}{dX} = \alpha, \tag{5.2}$$

where α is the hydrogen yield coefficient.

The progress of cumulative hydrogen production in batch tests can be described by Eq. 5.3.

$$H = \int_0^t \frac{dH}{dt}dt = \int_0^t \frac{dH}{dX} \cdot \frac{dX}{dt}dt = \alpha \cdot \int_0^t \frac{dX}{dt}dt = \frac{\alpha \cdot A}{1 + \exp[4(\alpha \cdot \mu_m) \cdot (\lambda - t)/(\alpha \cdot A) + 2]} \tag{5.3}$$

The term $\alpha \cdot A$ can be replaced by P, which is defined as the hydrogen production potential, and the term $\alpha \cdot \mu_m$ can be replaced by R_m, which is defined as the maximum hydrogen production rate. And then Eq. 5.3 can be expressed as Eq. 5.4.

$$H = \frac{P}{1 + \exp[4R_m \cdot (\lambda - t)/P + 2]}, \tag{5.4}$$

where H (mL) is the cumulative hydrogen production at the reaction time t (h), P (mL) is the hydrogen production potential, R_m (mL/h) is the maximum hydrogen production rate, and λ (h) is the lag time.

The modified Logistic model (Eq. 5.4) was used to fit the cumulative hydrogen production data to obtain H, R_m and λ. Once the three parameters were obtained, Eq. 5.5 was used to calculate the average hydrogen production rate in each batch test.

$$R = \frac{P}{\lambda + P/R_m} \tag{5.5}$$

The Han-Levenspiel model (Eq. 5.6) was adapted to describe the effect of ammonia concentration on average hydrogen production rate (Han and Levenspiel 1988).

$$R = k \times \left(1 - \frac{C}{C_{\max}}\right)^m \times \frac{S}{S + K_s \times (1 - C/C_{\max})^n}, \tag{5.6}$$

where R (mL/h) is the average hydrogen production rate, k (mL/h) is a constant, C (g/L) is the ammonia nitrogen concentration, $C_{\max}$ (g/L) is the ammonia nitrogen concentration at which the average hydrogen production rate is zero, S (g/L) is the substrate concentration, which is 10 g/L, K_s (g/L) is the saturation constant, m and n are exponent constants.

5.5.2.2 Effect on Substrate Degradation Efficiency

Figure 5.6 shows the effect of ammonia concentration on substrate degradation efficiency.

The results showed that the substrate degradation efficiency increased with increasing ammonia concentration from 0 to 0.01 g/L, while it changed little

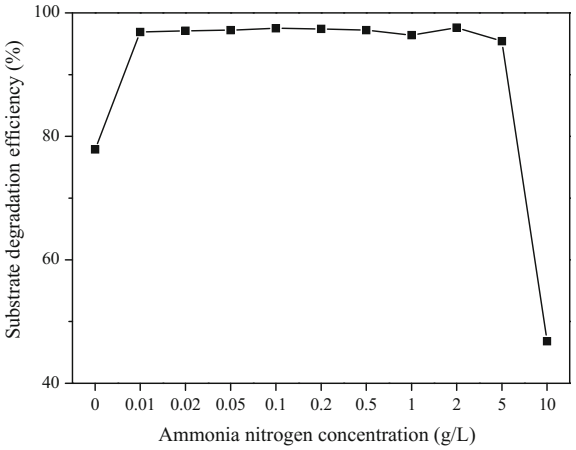

Fig. 5.6 Effect of ammonia concentration on substrate degradation efficiency

(around 96.0%) with further increasing ammonia concentration from 0.01 to 5 g/L, and it decreased sharply with further increasing ammonia concentration from 5 to 10 g/L.

5.5.2.3 Effect on Hydrogen Production

Figure 5.7 shows the effect of ammonia concentration on hydrogen production potential.

The results showed that the hydrogen production potential increased with increasing ammonia concentration from 0 to 0.1 g/L, however, it decreased with further increasing ammonia concentration from 0.1 to 10 g/L. In this study, the maximum hydrogen production potential of 291.4 mL was obtained at the ammonia

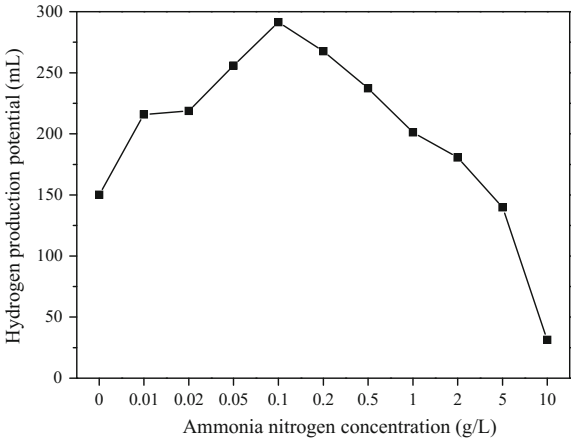

Fig. 5.7 Effect of ammonia concentration on hydrogen production potential

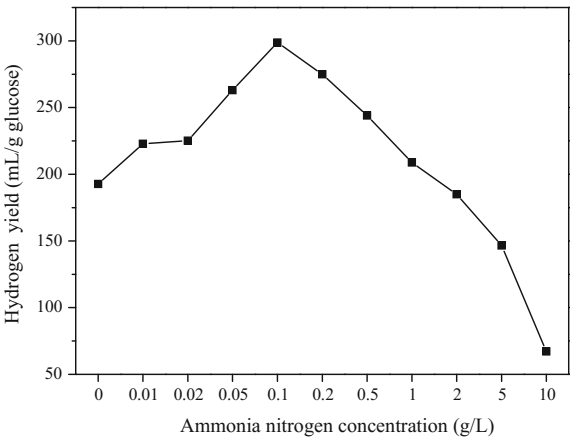

Fig. 5.8 Effect of ammonia concentration on hydrogen yield

nitrogen concentration of 0.1 g/L. This demonstrated that in an appropriate range, increasing ammonia concentration could increase the hydrogen production potential, but ammonia nitrogen at much higher concentrations could decrease it, which was also shown by Liu and Shen (2004) and Salerno et al. (2006), respectively.

Figure 5.8 shows the effect of ammonia concentration on hydrogen yield.

The results showed that the hydrogen yield increased with increasing ammonia concentration from 0 to 0.1 g/L, however, it decreased with further increasing ammonia nitrogen concentration from 0.1 to 10 g/L. The maximum hydrogen yield of 298.8 mL/g glucose was obtained at the ammonia nitrogen concentration of 0.1 g/L.

Figure 5.9 shows the effect of ammonia concentration on average hydrogen production rate.

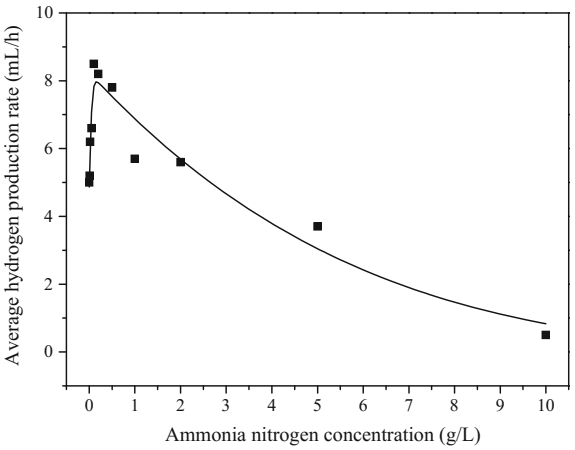

Fig. 5.9 Effect of ammonia concentration on hydrogen production rate

The results showed that the average hydrogen production rate increased with increasing ammonia concentration from 0 to 0.1 g/L, however, it decreased with further increasing ammonia concentration from 0.1 to 10 g/L. The maximum average hydrogen production rate of 8.5 mL/h was obtained at the ammonia nitrogen concentration of 0.1 g/L.

The Han-Levenspiel model was used to fit the average hydrogen production rate data. The fitting result is as follows:

$$R = 8.2 \times \left(1 - \frac{C}{26.1}\right)^{4.7} \times \frac{10}{10 + 7.0 \times (1 - C/26.1)^{775.3}} \tag{5.7}$$

The coefficient of determination (R^2) of the fitting was 0.94, with the significance level being less than 0.05, indicating that the Han-Levenspiel model could describe the effect of ammonia concentration on average hydrogen production rate.

The ammonia nitrogen concentration at which the average hydrogen production rate is zero obtained from the above fitting was 26.1 g/L, which is quite reasonable, because according to Figs. 5.7, 5.8 and 5.9, the ability of hydrogen-producing bacteria to produce hydrogen decreased rapidly with increasing ammonia nitrogen concentration from 5 to 10 g/L, thus it is reasonable to predict that the activity of hydrogen-producing bacteria will be inhibited completely when ammonia concentration further increased to 26.1 g/L, and the fermentative hydrogen production will stop and then the average hydrogen production rate will be zero accordingly.

5.5.2.4 Effect on Soluble Metabolites Distribution

Table 5.10 summarizes the effect of ammonia concentration on distributions of the soluble metabolites.

Ethanol was dominant in the soluble metabolite when ammonia concentration increased from 0.02 to 0.2 g/L and from 2 to 5 g/L, while acetic acid was dominant in the soluble metabolite when ammonia concentration was 10 g/L. In addition, propionic acid was dominant in the soluble metabolite when ammonia concentration increased from 0 to 0.01 g/L. Butyric acid was dominant when ammonia concentration was 0.5 g/L. However, Liu and Shen (2004) reported that acetic acid was dominant in the soluble metabolite with increasing ammonia concentration from 0.1 to 2 g/L, and Salerno et al. (2006) reported that butyric acid was dominant in the soluble metabolite with increasing ammonia concentration from 0.8 to 5.3 g/L.

5.5.2.5 Comparison of Optimal Ammonia Concentration

Table 5.11 summarizes the main experimental conditions and conclusions.

Table 5.10 Effect of ammonia concentration on distribution of the soluble metabolites

Ammonia concentration (g N/L)	Distribution of the soluble metabolites (%)			
	Ethanol	HAc	HPr	HBu
0	15.1	19.1	55.8	10.0
0.01	27.9	3.5	64.2	4.4
0.02	88.2	3.5	7.1	1.2
0.05	81.0	5.1	11.4	2.5
0.1	66.8	11.1	17.3	4.8
0.2	87.1	3.0	3.2	6.7
0.5	12.2	26.2	10.6	51.0
1	21.5	27.6	50.5	0.4
2	87.9	2.3	5.2	4.6
5	63.9	16.7	11.1	8.3
10	7.1	57.9	32.7	2.3

Table 5.11 Comparison of the optimal ammonia concentration

Seed sludge	Substrates	Ammonia concentration studied	Optimal ammonia concentration	References
Digested sludge	Glucose	0–10 g/L	0.1 g/L	Wang et al. (2009)
Escherichia coli	Glucose	0–0.2 g/L	0.01 g/L	Bisaillon et al. (2006)
Clostridium butyricum	Glucose	0.014–0.14 g/L	0.014 g/L	Zhu et al. (2001)
Grass compost	Food wastes	0–0.583 g/L	0.418 g/L	Lay et al. (2005)
Cracked cereals	Starch	0.1–2 g/L	1 g/L	Liu and Shen (2004)
Dewatered and thickened sludge	Glucose	0.5–10 g/L	7 g/L	Salerno et al. (2006)
Agricultural soil	Glucose	0.8–7.8 g/L	0.8 g/L	Salerno et al. (2006)
Cow dung	Cow dung	1–4 g/kg cow dung	1 g/kg cow dung	Yokoyama et al. (2007)

As shown in Table 5.11, the optimal ammonia concentration for fermentative hydrogen production differed considerably.

5.5.3 Effect of Nitrate Concentration

5.5.3.1 Effect on Substrate Degradation Efficiency

Figure 5.10 shows the effect of nitrate concentration on substrate degradation efficiency.

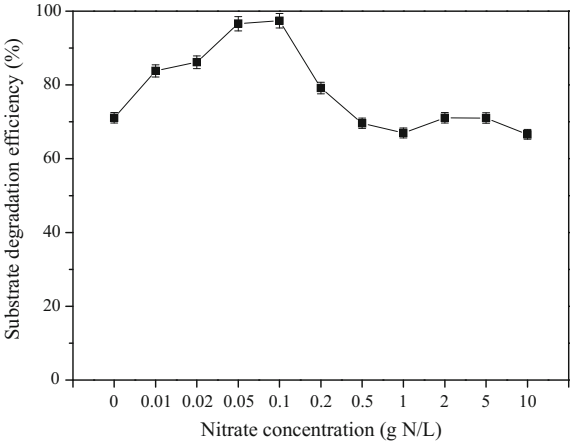

Fig. 5.10 Effect on substrate degradation efficiency

The results showed that the substrate degradation efficiency increased with increasing nitrate concentration from 0 to 0.1 g N/L, however, it decreased with further increasing nitrate concentration from 0.1 to 0.5 g N/L, and then it changed little (around 70.0%) with further increasing nitrate concentration from 0.5 to 10 g N/L. The maximum substrate degradation efficiency of 97.4% was obtained at the nitrate concentration of 0.1 g N/L, which is much higher than that reported by Mohan et al. (2008); they obtained the maximal substrate degradation rate of 87.0%.

5.5.3.2 Effect on Hydrogen Production

Figure 5.11 shows the effect of nitrate concentration on hydrogen production potential.

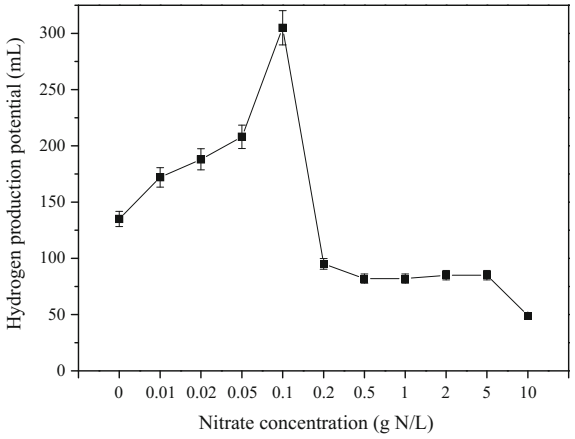

Fig. 5.11 Effect on hydrogen production potential

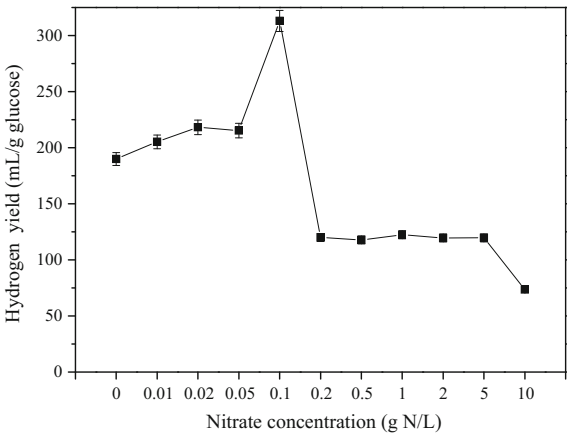

Fig. 5.12 Effect of nitrate concentration on hydrogen yield

The results showed that the hydrogen production potential in batch tests increased with increasing nitrate concentration from 0 to 0.1 g N/L, however, it dramatically decreased with further increasing nitrate concentration from 0.1 to 0.2 g N/L, while it changed little (around 85 mL) with increasing nitrate concentration from 0.2 to 5 g N/L, and then it decreased with further increasing nitrate concentration from 5 to 10 g N/L. The maximum hydrogen production potential of 305.0 mL was obtained at the nitrate concentration of 0.1 g N/L. It should be noted that superfluous nitrate nitrogen has a further inhibitive effect on the hydrogen production potential when the concentration is over 5 g N/L.

Figure 5.12 shows the effect of nitrate concentration on hydrogen yield.

The results showed that the hydrogen yield in batch tests trended to increase with increasing nitrate concentration from 0 to 0.1 g N/L, however, it dramatically decreased with further increasing nitrate concentration from 0.1 to 0.2 g N/L, while it changed little (around 120 mL) with further increasing nitrate concentration from 0.2 to 5 g N/L, and then it decreased with further increasing nitrate concentration from 5 to 10 g N/L.

Table 5.12 summarizes the maximum hydrogen yield from glucose.

Figure 5.13 shows the effect of nitrate concentration on average hydrogen production rate.

The results showed that the hydrogen production rate increased with increasing nitrate concentration from 0 to 0.1 g N/L, and it decreased with further increasing nitrate concentration from 0.1 to 0.2 g N/L, while it changed little (around 4.8 mL/h) with further increasing nitrate concentration from 0.2 to 2 g N/L, and then it decreased with further increasing nitrate concentration from 2 to 10 g N/L. I

Table 5.12 Comparison of the maximum hydrogen yield

Seed sludge	Maximum hydrogen yield (mol/mol glucose)	References
Mixed culture	2.3	Wang et al. (2009)
Mixed culture	0.7	Li et al. (2008)
Mixed culture	1.0	Oh et al. (2003)
Mixed culture	1.7	Mu et al. (2006)
Mixed culture	2.15	Yin et al. (2014)
Mixed culture	2.1	Fang and Liu (2002)
Mixed culture	3.25	Bastidas-Oyanedel et al. (2012)
Citrobacter sp.	2.5	Oh et al. (2003)
Escherichia coli	3.0	Chittibabu et al. (2006)
Enterococcus faecium	1.69	Yin and Wang (2016)

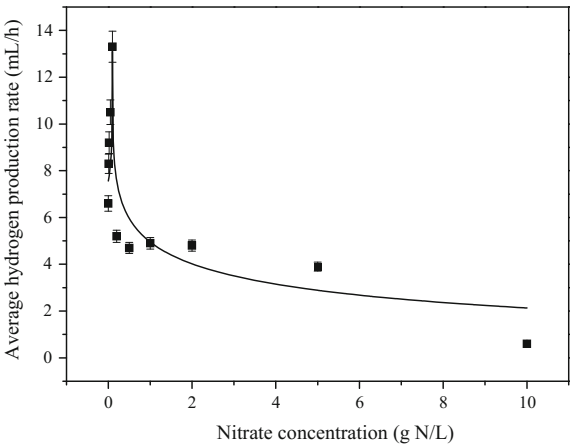

Fig. 5.13 Effect of nitrate concentration on average hydrogen production rate

5.5.3.3 Effect on Soluble Metabolites Distribution

Table 5.13 summarizes the effect of nitrate concentration on distributions of the soluble metabolites. As shown in Table 5.13, during the anaerobic fermentation process, any other solvent products like propionic acid and butyric acid were not detected. It is the biggest difference from other similar reports for the distribution of soluble metabolites (Liu and Shen 2004; Salerno et al. 2006).

5.5.3.4 Effect on Final pH and Biomass Concentration

Figure 5.14 shows the effect of nitrate concentration on the final pH. The results showed that, with the progress of gas evolution and substrate decomposition, the

Table 5.13 Effect of nitrate concentration on distribution of the soluble metabolites

Nitrate concentration (g N/L)	Distribution of the soluble metabolites (%)	
	Ethanol	HAc
0	74.5	25.5
0.01	59.5	40.5
0.02	68.2	31.8
0.05	37.0	63.0
0.1	41.4	58.6
0.2	4.6	95.4
0.5	1.2	98.8
1	0.1	99.9
2	0.5	99.5
5	0.3	99.7
10	0.0	100.0

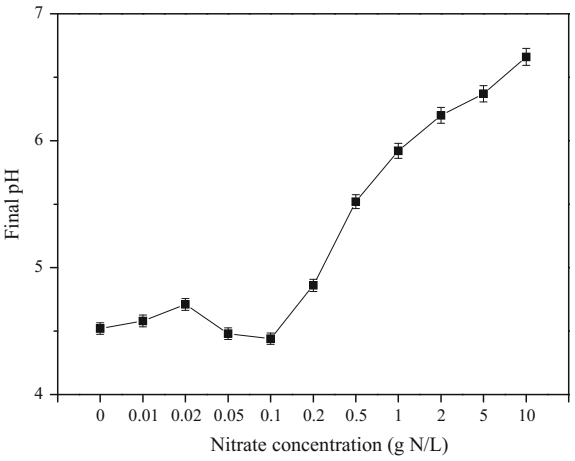

Fig. 5.14 Effect of nitrate concentration on the final pH

final pH value decreased to less than 7.0, ranging from 4.4 to 6.7. The lowest final pH of 4.4 was obtained when nitrate concentration was 0.1 g N/L, which was higher than that (about 4.0) obtained by Morimoto et al. (2004). The favorable pH for fermentative hydrogen production are between 5.2 and 7.0, while much lower or much higher pH can repress the activity of hydrogen-producing bacteria, which is harmful for fermentative hydrogen production (Li and Fang 2007a, b).

Figure 5.15 shows the effect of nitrate concentration on the final biomass concentration. The final biomass concentration decreased slowly with increasing nitrate concentration from 0 to 0.02 g N/L, while it increased with further increasing nitrate concentration from 0.02 to 0.1 g N/L, and then it decreased with further increasing nitrate concentration from 0.1 to 10 g N/L.

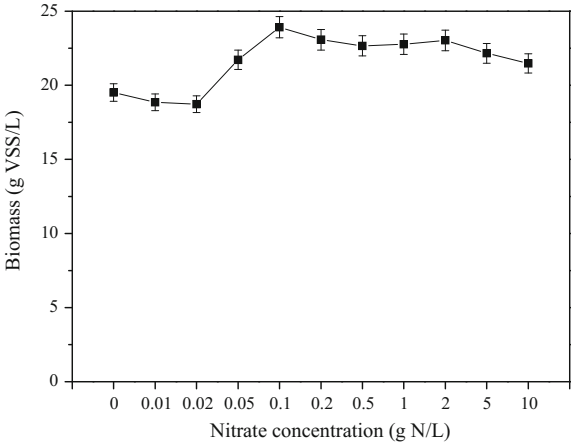

Fig. 5.15 Effect of nitrate concentration on the final biomass concentration

5.6 Effect of Trace Heavy Metal Ion

5.6.1 Importance of Heavy Metal Ions

Even though at a higher concentration, metal ion may inhibit the activity hydrogen-producing bacteria, a trace level of metal ion is required for fermentative hydrogen production (Li and Fang 2007a, b). Table 5.14 summarizes the effect of metal ion concentration on fermentative hydrogen production.

As shown in Table 5.14, Fe^{2+} was the most widely investigated metal ion for fermentative hydrogen production, probably because its presence is essential for hydrogenase (Wan and Wang 2008). Thus, more investigations of the effect of other metal ion concentration besides Fe^{2+} concentration on fermentative hydrogen production are recommended.

In addition, there exists certain disagreement on the optimal Fe^{2+} concentration for fermentative hydrogen production. For example, the optimal Fe^{2+} concentration for fermentative hydrogen production reported by Liu and Shen (2004) was 10 mg/L, while that reported by Zhang et al. (2005) was 589.5 mg/L. The possible reason for this disagreement was the difference among these studies in the terms of inoculum, substrate, and Fe^{2+} concentration range studied.

As shown in Table 5.8, glucose and sucrose were the most widely used substrate during the investigation of the effect of metal ion on fermentative hydrogen production. Thus, investigating the effect of nitrogen concentration on fermentative hydrogen production using organic wastes as substrate is recommended. In addition, as is shown in Table 5.8, most of the reviewed studies investigating the effect of metal ion concentration on fermentative hydrogen production were conducted in batch mode, and more studies conducted in continuous mode are recommended.

Several studies also investigated the toxicity of heavy metals to fermentative hydrogen production. For example, Li and Fang found that the relative toxicity of

Table 5.14 The effect of metal ion concentrations on fermentative hydrogen production

Inoculum	Substrates	Reactor type	Mental ion	Concentration (mg/L)		Optimal index (value)	References
				Range studied	Optimal		
Cracked cereals	Starch	Batch	Fe^{2+}	1.2–100	10	Maximum hydrogen yield (140 mL/g starch)	Liu and Shen (2004)
Anaerobic sludge	Starch	Batch	Fe^{2+}	0–1473.7	55.3	Maximum hydrogen yield (296.2 mL/g starch)	Yang and Shen (2006)
Grass compost	Food wastes	Batch	Fe^{2+}	0–250	132	Maximum hydrogen yield (77 mL/g TVS)	Lay et al. (2005)
Anaerobic sludge	Palm oil mill effluent	Batch	Fe^{2+}	2–400	257	Maximum hydrogen yield (6.33 L/L substrate)	O-Thong et al. (2008)
Digested sludge	Glucose	Batch	Fe^{2+}	0–1500	350	Maximum hydrogen yield (311.2 mL/g glucose)	Wan and Wang (2008)
Anaerobic sludge	Sucrose	Batch	Fe^{2+}	0–1763.8	352.8	Maximum hydrogen yield (131.9 mL/g sucrose)	Lee et al. (2001)
Cracked cereals	Sucrose	Batch	Fe^{2+}	0–1842.1	589.5	Maximum hydrogen yield (2.73 mol/mol sucrose)	Zhang et al. (2005)
Anaerobic sludge	Starch	Batch	Fe^{0}	0–50	37.5	Maximum hydrogen yield (149.8 mL/g VS)	Taherdanak et al. (2015)
Anaerobic sludge	Glucose	Batch	Cu^{2+}	0–400	400	Maximum hydrogen yield (1.74 mol/mol glucose)	Zheng and Yu (2004)
Anaerobic sludge	Glucose	Batch	Zn^{2+}	0–500	250	Maximum hydrogen yield (1.73 mol/mol glucose)	Zheng and Yu (2004)
Hydrogen-producing bacterial B49	Glucose	Batch	Mg^{2+}	1.2–23.6	23.6	Maximum hydrogen yield (2360.5 mL/L culture)	Wang et al. (2007)
Digested sludge	Glucose	Batch	Ni^{2+}	0–50	0.1		

(continued)

Table 5.14 (continued)

Inoculum	Substrates	Reactor type	Mental ion	Concentration (mg/L)		Optimal index (value)	References
				Range studied	Optimal		
						Maximum hydrogen yield (296.1 mL/g glucose)	Wang and Wan (2008a, b, c, d)
Digested sludge	Glucose	Batch	Ni^{2+}	0–50	25	Maximum hydrogen yield (383 mL/g glucose)	Taherdanak et al. (2016)
Anaerobic sludge	Starch	Batch	Ni^{0}	0–50	37.5	Maximum hydrogen yield (149.8 mL/g VS)	Taherdanak et al. (2015)
Digested sludge	Sucrose	Continuous	Ca^{2+}	0–300	150	Maximum hydrogen yield (3.6 mol/mol sucrose)	Chang and Lin (2006)
Municipal sewage sludge	Sucrose	Continuous	Ca^{2+}	0–27.2	27.2	Maximum hydrogen yield (2.19 mol/mol sucrose)	Lee et al. (2004)

six electroplating metals to fermentative hydrogen production was in the following order: Cu > Ni ~ Zn > Cr > Cd > Pb (Li and Fang 2007a, b), while Lin and Shei reported that the relative toxicity of three heavy metals to fermentative hydrogen production was in the following order: Zn > Cu > Cr (Lin and Shei 2008).

5.6.2 *Effect of Fe^{2+}*

5.6.2.1 Overview

Iron is essential to the fermentative hydrogen production by anaerobic bacteria, because hydrogenases that are able to catalyze the oxidation of hydrogen or the reduction of proton are classified into two major families: nickel-iron (Ni–Fe) hydrogenases and "iron only" (Fe–Fe) hydrogenases, according to the metal content of their respective catalytic centers (Frey 2002). Therefore, iron can influence the fermentative hydrogen production by influencing the activity of hydrogenases. Although, there have been several studies on how the external iron concentration affect the fermentative hydrogen production by mixed cultures, these research results differed considerably. For example, Lee et al. (2001) studied the effect of the Fe^{2+} concentrations ranging from 0 to 1763.8 mg/L on the fermentative hydrogen production from sucrose (10 g/L) in batch tests by mixed cultures at 37 °C and initial pH 6.0, obtaining the maximum hydrogen yield of 131.9 mL/g sucrose at the Fe^{2+} concentration of 352.8 mg/L. In addition, Yang and Shen (2006) studied the effect of the Fe^{2+} concentrations ranging from 0 to 1473.7 mg/L on the fermentative hydrogen production from starch (10 g/L) in batch tests by mixed cultures at 35 °C and initial pH 7.0, obtaining the maximum hydrogen yield of 296.2 mL/g starch at the Fe^{2+} concentration of 55.3 mg/L. Moreover, Ding et al. (2004) studied the effect of the Fe^{2+} concentrations ranging from 0 to 1473.7 mg/L on the fermentative hydrogen production from glucose (5 g/L) in batch tests by mixed cultures at 35 °C and initial pH 4.7, obtaining the maximum hydrogen yield of 143.7 mL/g glucose at the Fe^{2+} concentration of 200 mg/L. The possible reasons why these research results were different greatly are the differences of the substrates, their concentrations, initial pH value, and the seed sludge and so on in these tests.

5.6.2.2 Effect on Hydrogen Production

Figure 5.16 illustrates the effect of Fe^{2+} concentration on the cumulative hydrogen quantity. The results showed that the hydrogen fermentation ceased within 36 h. At the first 12 h of the hydrogen fermentation, the slopes of the curves increased with increasing Fe^{2+} concentrations from 0 to 250 mg/L, but when Fe^{2+} concentration was higher than 250 mg/L, the slope of the curves decreased, indicating that in certain concentration range, Fe^{2+} was able to enhance the hydrogen production rate,

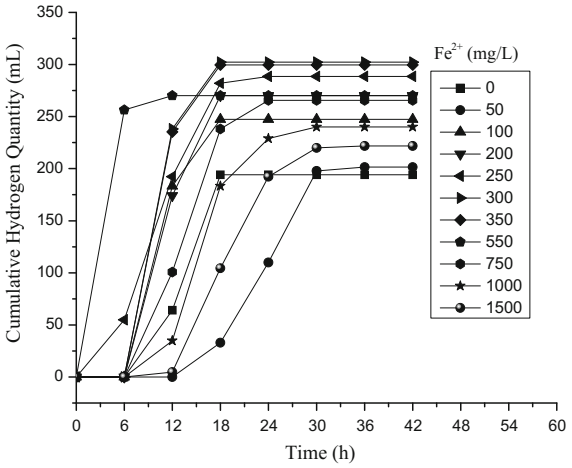

Fig. 5.16 The effect of Fe^{2+} concentration on the cumulative hydrogen quantity

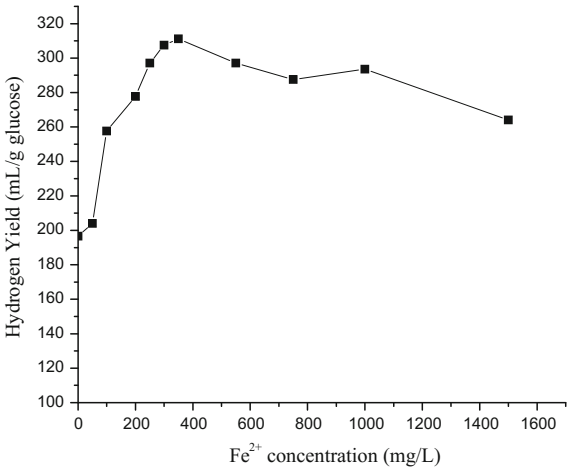

Fig. 5.17 The effect of Fe^{2+} concentration on hydrogen yield

while much lower or much higher Fe^{2+} concentration is not favorable to hydrogen production.

Figure 5.17 shows the effect of the Fe^{2+} concentration on hydrogen yield. The results showed that hydrogen yield increased with increasing Fe^{2+} concentration from 0 to 350 mg/L; however, when Fe^{2+} concentration was higher than 350 mg/L, hydrogen yield decreased. The maximum hydrogen yield was 311.2 mL/g glucose.

The electron carrier ferredoxin in hydrogenases plays an important role in the fermentative hydrogen production. Iron is a fundamental component making up the ferredoxin. Only in proper concentration range will Fe^{2+} increase the activity of hydrogenases, which can increase fermentative hydrogen production by mixed

cultures in turn, while much lower or much higher Fe^{2+} concentration will decrease the activity of hydrogenases (Ding et al. 2004; Yang and Shen 2006).

It is worth noting that the maximum hydrogen yield here is much higher than that of other related studies. For example, Yokoi et al. (1995) obtained the maximum hydrogen yield of 124.4 mL/g glucose; Ding et al. (2004) obtained the maximum hydrogen yield of 143.7 mL/g glucose; Morimoto et al. (2004) obtained the maximum hydrogen yield of 261.3 mL/g glucose.

5.6.2.3 Effect on Soluble Metabolite Yield

Table 5.15 showed the effect of Fe^{2+} concentration on soluble metabolite yield.

The results showed that ethanol yield increased slightly with increasing Fe^{2+} concentration from 0 to 50 mg/L, and continued to increase with increasing Fe^{2+} concentrations from 100 to 300 mg/L, however, when Fe^{2+} concentration was higher than 300 mg/L, the ethanol yield decreased. The maximum ethanol yield of 794.9 mmol/mol glucose was obtained at Fe^{2+} concentration of 300 mg/L. The acetic acid yield fluctuated when Fe^{2+} concentration increased from 0 to 1500 mg/L, and the maximum acetic acid yield of 314.7 mmol/mol glucose was obtained at Fe^{2+} concentration of 100 mg/L.

5.6.2.4 Effect on Substrate Conversion Rate and Biomass Yield

Figure 5.18 showed the effect of Fe^{2+} concentration on glucose degradation efficiency. The results showed that glucose degradation efficiency decreased with increasing Fe^{2+} concentration from 0 to 1500 mg/L, which was similar to the result of Ding et al. (2004).

Table 5.15 The effect of Fe^{2+} concentration on the soluble metabolite yield

Fe^{2+} concentration (mg/L)	Soluble metabolite yields (mmol/mol glucose)			
	Ethanol	Acetic acid	Propionic acid	Butyric acid
0	605.0	22.1	–	–
50	666.6	51.4	–	207.5
100	61.7	314.7	20.8	594.4
200	145.1	180.5	–	374.8
250	198.4	221.0	–	331.5
300	794.9	239.2	–	425.5
350	51.2	161.0	–	346.5
550	63.3	259.6	–	482.0
750	0.2	3.4	–	581.3
1000	2.3	79.0	–	440.5
1500	–	8.6	–	618.9

"-" stands for not detectable

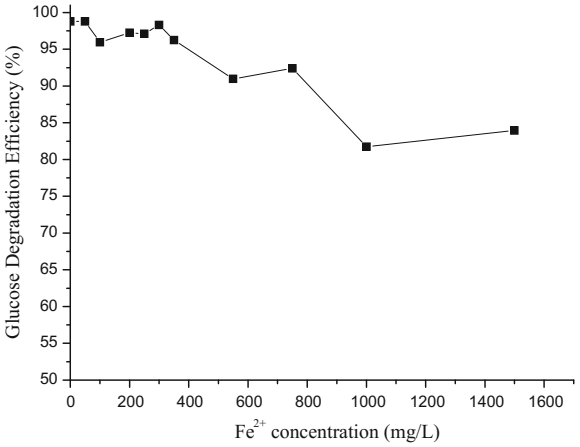

Fig. 5.18 The effect of Fe^{2+} concentration on glucose degradation efficiency

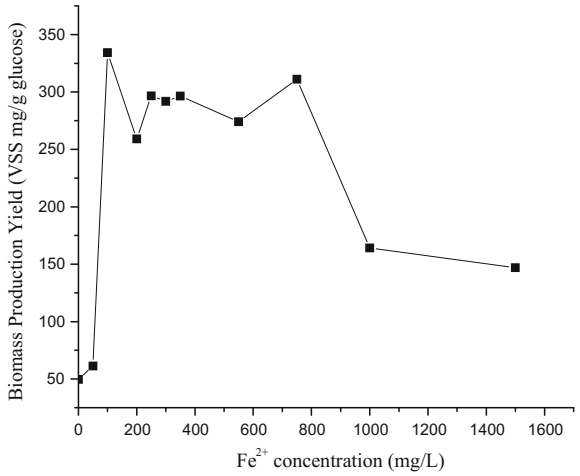

Fig. 5.19 The effect of Fe^{2+} concentration on biomass production yield

When Fe^{2+} concentration was lower than 350 mg/L, glucose degradation efficiency was between 96.25% and 98.78%. When Fe^{2+} concentration was higher than 350 mg/L, glucose degradation efficiency was relatively low (less than 92.40%).

Figure 5.19 showed the effect of Fe^{2+} concentration on biomass production yield.

The results showed that the biomass production yield increased slightly with increasing Fe^{2+} concentration from 0 to 50 mg/. When Fe^{2+} concentration was between 100 and 750 mg/L, the biomass production yield ranged from 259.2 to 334.2 mg/g glucose. When Fe^{2+} concentration was higher than 750 mg/L, the biomass production yield decreased with increasing Fe^{2+} concentration. The maximum biomass production yield of 334.2 mg/g glucose was obtained at the Fe^{2+} concentration of 100 mg/L.

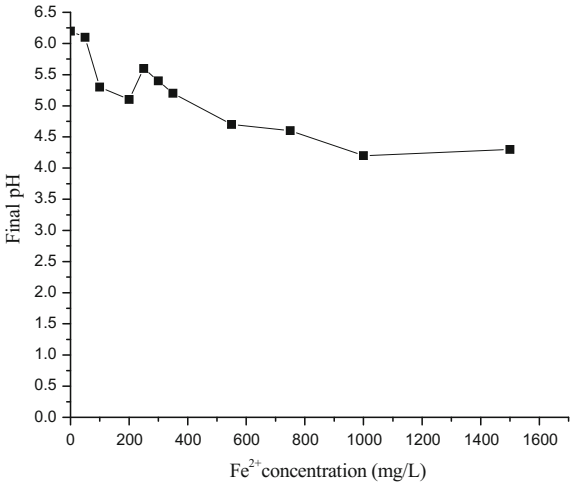

Fig. 5.20 The effect of Fe^{2+} concentration on final pH

5.6.2.5 Effect on Final pH

Figure 5.20 showed the effect of the Fe^{2+} concentration on final pH. The results showed that, due to fermentation, the final pH value decreased to less than 7.0.

5.6.3 Effect of Mg^{2+}

5.6.3.1 Overview

Magnesium is one of the necessary trace elements for various anaerobic microorganisms and it may relate to the bacterial enzyme cofactor, transport processes, and dehydrogenase. In addition, most enzymes that are essential to fermentative hydrogen production need to be activated by Mg^{2+}. Therefore, Mg^{2+} can influence the fermentative hydrogen production by regulating the activity of the enzymes. It has been shown that in appropriate concentration range, hydrogen yield increased with increasing Mg^{2+} concentration, but Mg^{2+} at much higher concentration could decrease it (Lin and Lay 2005).

5.6.3.2 Effect on Hydrogen Production

Figure 5.21 illustrates the effect of Mg^{2+} concentration on hydrogen production potential.

The results showed that the hydrogen production potential in batch tests increased with increasing Mg^{2+} concentration from 0 to 1 mg/L, and it changed little with further increasing Mg^{2+} concentration from 2 to 50 mg/L. However,

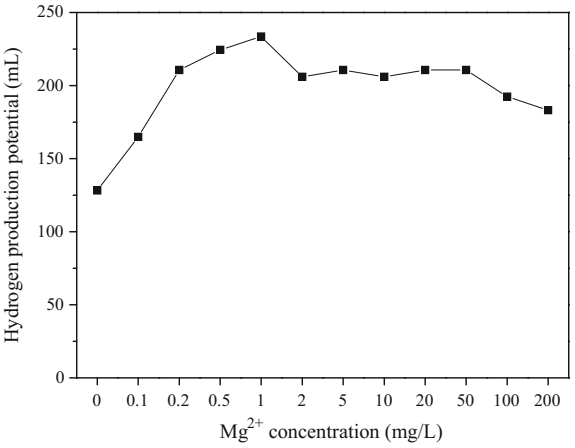

Fig. 5.21 Effect of Mg^{2+} concentration on hydrogen production potential

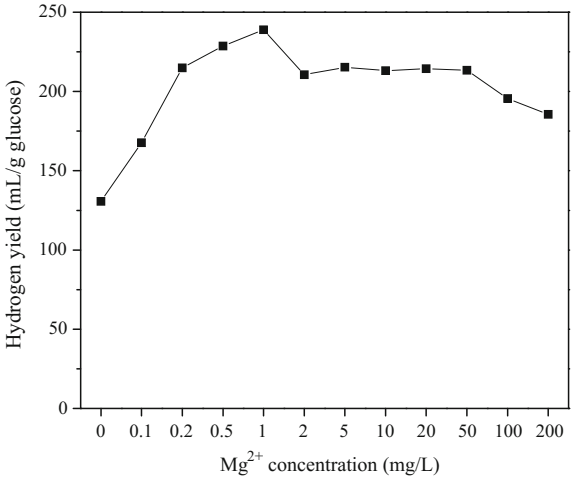

Fig. 5.22 Effect of Mg^{2+} concentration on hydrogen yield

hydrogen production potential decreased with increasing Mg^{2+} concentration from 50 to 200 mg/L. The maximum hydrogen production potential of 233.6 mL was obtained at the Mg^{2+} concentration of 1 mg/L.

Figure 5.22 shows the effect of Mg^{2+} concentration on hydrogen yield.

The results showed that the hydrogen yields increased with increasing Mg^{2+} concentration from 0 to 1 mg/L, and it changed little with further increasing Mg^{2+} concentration from 2 to 50 mg/L. However, hydrogen yield decreased with increasing Mg^{2+} concentration from 50 to 200 mg/L. The maximum hydrogen yield of 238.9 mL/g glucose (1.92 mol/mol glucose) was obtained when Mg^{2+} concentration was 1 mg/L, indicating that in an appropriate concentration range, Mg^{2+} is

Table 5.16 Comparison of maximum hydrogen yield from glucose by mixed cultures

Mg^{2+} concentration (mg/L)	Maximum hydrogen yield (mol/mol glucose)	References
14.8	1.67	Mu et al. (2006)
35.5	0.97	Oh et al. (2003)
9.8	0.92	Logan et al. (2002)
46.8	1.8	Iyer et al. (2004)
11.8	1.17	Hu and Chen (2007)
–	0.005	Liang et al. (2002)
–	2.1	Morimoto et al. (2004)
31.2	2.1	Fang and Liu (2002)

able to increase the ability of hydrogen-producing bacteria to produce hydrogen (Lin and Lay 2005).

Table 5.16 summarizes the maximum hydrogen yield from glucose by mixed cultures.

Figure 5.23 shows the effect of Mg^{2+} concentration on the average hydrogen production rate.

The results showed that the average hydrogen production rate in batch tests increased with increasing Mg^{2+} concentrations from 0 to 2 mg/L, however, it decreased with further increasing Mg^{2+} concentrations from 2 to 200 mg/L. In this study, the maximum average hydrogen production rate of 19.8 mL/h was obtained when Mg^{2+} concentration was 2 mg/L.

The Han-Levenspiel model was used to fit the average hydrogen production rate data. The fitting result is as follows:

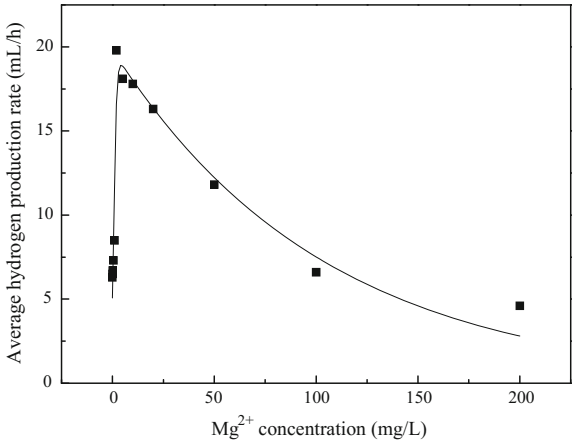

Fig. 5.23 Effect of Mg^{2+} concentration on average hydrogen production rate

$$R = 19.8 \times \left(1 - \frac{C}{6600.5}\right)^{63.6} \times \frac{10}{10 + 29.1 \times (1 - C/6600.5)^{9324.5}} \quad (5.8)$$

The coefficient of determination (R^2) of the fitting was 0.92, with the significance level being less than 0.05, indicating that the Han-Levenspiel model could describe the effect of Mg^{2+} concentration on average hydrogen production rate.

Mg^{2+} concentration at which the average hydrogen production rate is zero obtained from the above fitting was 6600.5 mg/L, which is quite reasonable, because according to Figs. 5.21, 5.22 and 5.23, the ability of hydrogen-producing bacteria to produce hydrogen decreased with increasing Mg^{2+} concentration from 100 to 200 mg/L, thus it is reasonable to predict that the activity of hydrogen-producing bacteria will be inhibited completely when Mg^{2+} concentration increased to 6600.5 mg/L, and the fermentative hydrogen production will stop, and then the average hydrogen production rate decreased to zero accordingly.

Table 5.17 Effect of Mg^{2+} concentration on the soluble metabolites distribution

Mg^{2+} concentration (mg/L)	Distribution of the soluble metabolites (%)			
	Ethanol	HAc	HPr	HBu
0	65.3	33.9	0.3	0.5
0.1	52.5	26.4	20.9	0.2
0.2	70.8	28.3	0.4	0.4
0.5	66.9	32.2	0.5	0.3
1	64.3	34.8	0.5	0.4
2	66.2	32.9	0.8	0.0
5	86.7	11.6	0.9	0.8
10	62.0	36.2	0.9	0.9
20	58.4	40.7	0.3	0.6
50	45.4	30.1	23.2	1.4
100	65.9	23.3	10.6	0.2
200	60.4	23.3	16.1	0.2

5.6.3.3 Effect on Soluble Metabolites Distribution

Table 5.17 summarizes the effect of Mg^{2+} concentration on distribution of the soluble metabolites.

Table 5.17 showed that ethanol was dominant in the soluble metabolite when Mg^{2+} concentration increased from 0 to 200 mg/L, followed by acetic acid, propionic acid and butyric acid.

Chen et al. (2002) found that the soluble metabolites contained much higher concentration of propionic acid and butyric acid. Higher concentration of butyric acid can repress the activity of hydrogen-producing bacteria and thus is not favorable for fermentative hydrogen production (Zheng and Yu 2005; Mohan et al. 2008).

5.6.3.4 Effect on Substrate Degradation Efficiency

Figure 5.24 shows the effect of Mg^{2+} concentration on substrate degradation efficiency. It can be seen that the substrate degradation efficiency was between 96.7% and 98.8% with increasing Mg^{2+} concentration from 0 to 200 mg/L, demonstrating that Mg^{2+} concentration had little effect on the substrate degradation efficiency.

5.6.3.5 Effect on Biomass Yield

Figure 5.25 shows the effect of Mg^{2+} concentration on the biomass production yield.

The results showed that the biomass production yield increased with increasing Mg^{2+} concentration from 0 to 1 mg/L, but it decreased with further increasing Mg^{2+}

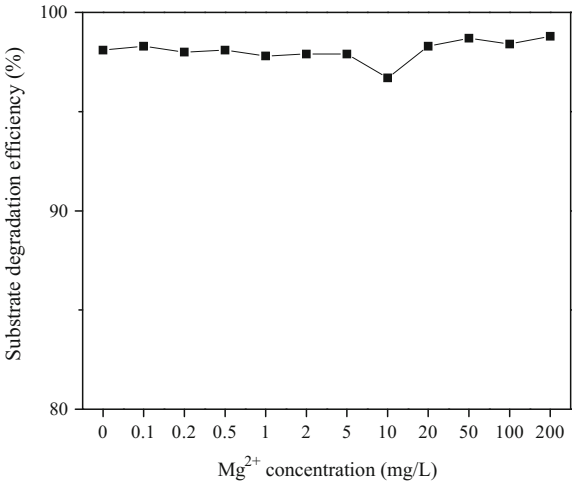

Fig. 5.24 Effect of Mg^{2+} concentration on substrate degradation efficiency

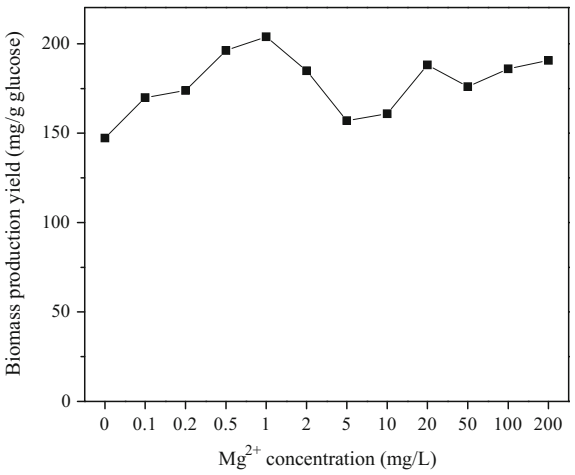

Fig. 5.25 Effect of Mg^{2+} concentration on the biomass production yield

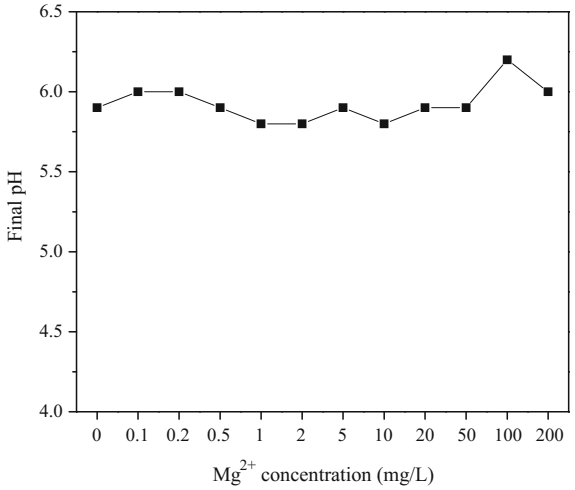

Fig. 5.26 Effect of Mg^{2+} concentration on the final pH

concentration from 1 to 10 mg/L. The maximum biomass production yield was 204.0 mg/g glucose when Mg^{2+} concentration was 1 mg/L.

5.6.3.6 Effect on Final pH

Figure 5.26 shows the effect of Mg^{2+} concentration on final pH.

The final pH changed little with increasing Mg^{2+} concentration from 0 to 200 mg/L and it was around 6.0, which was a little higher than that (around 4.6) reported by Zhang et al. (2005).

5.6.4 *Influence of Ni^{2+}*

5.6.4.1 Overview

Hydrogenases capable of catalyzing the oxidation of hydrogen or the reduction of proton are classified into two major families: the [Ni–Fe] hydrogenases and the [Fe–Fe] hydrogenases, according to the metal content at their active site (Frey 2002). Among them, the [Ni–Fe] hydrogenases are widely distributed among bacteria, whereas the [Fe–Fe] hydrogenases are restricted to a few bacteria. Moreover, the [Ni–Fe] hydrogenases have a higher substrate affinity than the [Fe–Fe] hydrogenases (Casalot and Rousset 2001). The [Ni–Fe] hydrogenases basically consist of two subunits, a large one and a small one, and contain one nickel atom and usually about 12 iron atoms per molecule, and most [Ni–Fe] hydrogenases possess two [4Fe-4S] clusters and often a [3Fe-4S] cluster (Albracht et al. 1995).

During a hydrogen production process catalyzed by the [Ni–Fe] hydrogenases, electrons are transported through an intramolecular electron transfer chain from the redox partner of the [Ni–Fe] hydrogenases (such as NADH or NADPH) to the active site, meanwhile, protons are also transferred to the active site, and then the protons are reduced by the electrons at the active site to produce hydrogen (Lacey et al. 2005; Kim et al. 2008).

Nickel is a fundamental component making up [Ni–Fe] hydrogenases; it may influence the fermentative hydrogen production by influencing the activity of [Ni–Fe] hydrogenases and thus may play an important role in fermentative hydrogen production. Even though at a higher concentration, nickel may inhibit the activity of [Ni–Fe] hydrogenases, a trace level of nickel is required for activation or function of [Ni–Fe] hydrogenases and thus is conducive to fermentative hydrogen

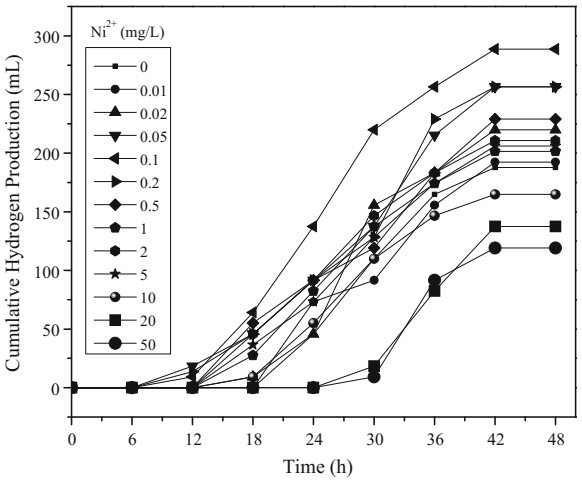

Fig. 5.27 Effect of Ni^{2+} concentration on the cumulative hydrogen production

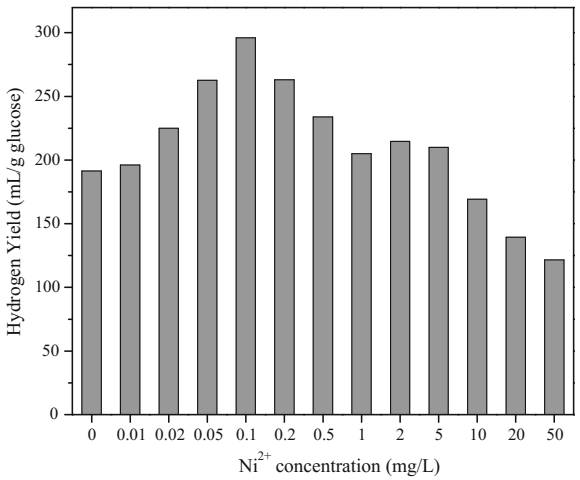

Fig. 5.28 Effect of Ni^{2+} concentration on hydrogen yield

production (Idania et al. 2005; Lin and Lay 2005; Kim et al. 2006; Levin et al. 2006; Lin et al. 2006; Li and Fang 2007; Yokoyama et al. 2007a).

5.6.4.2 Effect on Hydrogen Production

Figure 5.27 illustrates the effect of the fermentation time on the cumulative hydrogen production under different Ni^{2+} concentration. The results showed that the fermentative hydrogen production finished within 48 h. At first 18 h of fermentation, hydrogen production rate increased with increasing Ni^{2+} concentration from 0 to 0.2 mg/L, but it decreased with further increasing Ni^{2+} concentration from 0.2 to 50 mg/L.

Table 5.18 Comparison of the maximum hydrogen yield

Ni^{2+} concentration (mg/L)	Maximum hydrogen yield (mol/mol glucose)	References
0.1	2.38	Wang and Wan (2008a, b, c, d)
0.12	1.67	Mu et al. (2006)
6.09	0.97	Oh et al. (2003a, b)
–	0.92	Logan et al. (2002)
18.27	1.8	Iyer et al. (2004)
–	1.17	Hu and Chen (2007)
–	0.005	Liang et al. (2002)
–	2.1	Morimoto et al. (2004)
12.18	2.1	Fang and Liu (2002)
25	383 mL/g VS	Taherdanak et al. (2016)

Figure 5.28 shows the effect of Ni^{2+} concentration on hydrogen yield.

The results showed that hydrogen yield increased with increasing Ni^{2+} concentration from 0 to 0.1 mg/L, but it decreased with further increasing Ni^{2+} concentration from 0.1 to 1 mg/L. The hydrogen yield changed little with increasing Ni^{2+} concentration from 1 to 5 mg/L and it decreased with further increasing Ni^{2+} concentration to 50 mg/L. The maximum hydrogen yield of 296.1 mL/g glucose (2.38 mol/mol glucose) was obtained when Ni^{2+} concentration was 0.1 mg/L.

Table 5.18 summarizes the maximum hydrogen yield.

5.6.4.3 Effect on Soluble Metabolite Yield

Figure 5.29 shows the effect of Ni^{2+} concentration on the soluble metabolite yield.

The results showed that the ethanol yield decreased with increasing Ni^{2+} concentration from 0 to 0.02 mg/L and from 0.05 to 0.5 mg/L, however, it increased with further increasing Ni^{2+} concentration from 1 to 5 mg/L and from 10 to 20 mg/L. The ethanol yield decreased with increasing Ni^{2+} concentration from 20 to 50 mg/L. The maximum ethanol yield of 550.2 mmol/mol glucose was obtained. The maximum acetic acid yield of 164.4 mmol/mol glucose was obtained when Ni^{2+} concentration was 0.1 mg/L.

5.6.4.4 Effect on Substrate Degradation Efficiency

The substrate degradation efficiency was about 98.0% with increasing Ni^{2+} concentration from 0 to 50 mg/L, indicating that Ni^{2+} had little effect on the substrate

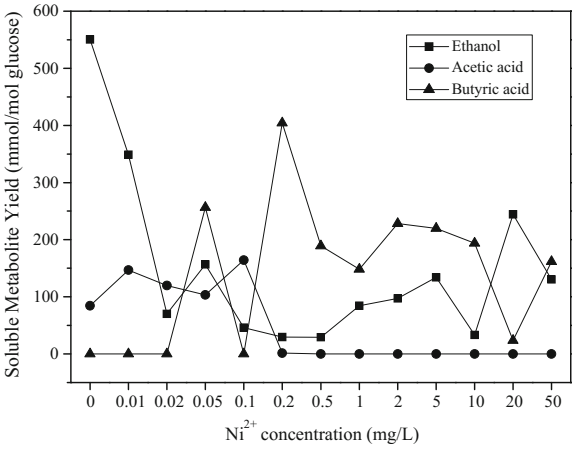

Fig. 5.29 Effect of Ni^{2+} concentration on the soluble metabolite yield

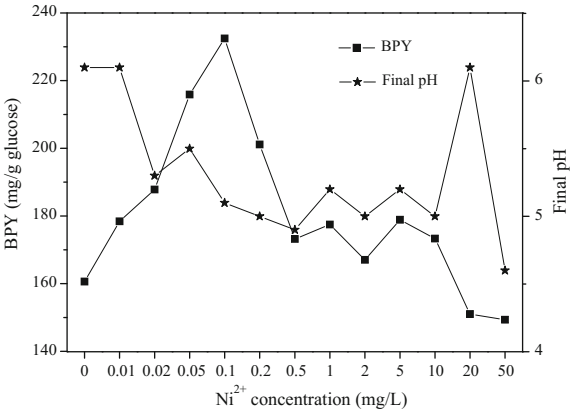

Fig. 5.30 Effect of Ni^{2+} concentration on the biomass production yield (BPY) and final pH

degradation efficiency at concentration of 0 to 50 mg/L, which is different from the result obtained by Li and Fang (2007a, b), who showed that substrate degradation efficiency decreased with increasing Ni^{2+} concentration from 0 to 50 mg/L.

5.6.4.5 Effect on Biomass Yield

Figure 5.30 shows the effect of Ni^{2+} on the biomass production yield and final pH.

The biomass production yield increased with increasing Ni^{2+} concentrations from 0 to 0.1 mg/L, but it decreased with further increasing Ni^{2+} concentration from 0.1 to 0.5 mg/L. The biomass production yield changed little with increasing Ni^{2+} concentration from 0.5 to 10 mg/L and it decreased with further increasing Ni^{2+} concentration from 10 to 50 mg/L. The maximum biomass production yield of 232.5 mg/g glucose was obtained when Ni^{2+} concentration was 0.1 mg/L.

5.6.4.6 Effect on Final pH

The final pH decreased with increasing Ni^{2+} concentration from 0 to 0.05 mg/L and it changed little with further increasing Ni^{2+} concentration from 0.1 to 10 mg/L. The final pH increased with increasing Ni^{2+} concentration from 10 to 20 mg/L and it decreased with increasing Ni^{2+} concentration from 20 to 50 mg/L. The lowest final pH of 4.6 was obtained when Ni^{2+} concentration was 50 mg/L, which was low enough to repress the activity of hydrogen-producing bacteria, so the biomass production yield decreased, which directly caused the hydrogen production potential and hydrogen yield.

5.7 Effect of Temperature

5.7.1 Overview

Temperature is one of the most important factors that influence the activities of hydrogen-producing bacteria and the fermentative hydrogen production. It has been demonstrated that in an appropriate range, increasing temperature could increase the ability of hydrogen-producing bacteria to produce hydrogen during fermentative hydrogen production, but temperature at much higher levels could decrease it with increasing levels. Table 5.19 summarizes several studies investigating the effect of temperature on fermentative hydrogen production. As shown in Table 5.19, even though the optimal temperature reported for fermentative hydrogen production was not always the same, it fell into the mesophilic range (around 37 °C) and thermophilic range (around 55 °C), respectively (Li and Fang 2007a, b).

As is shown in Table 5.19, glucose and sucrose were the most widely used substrate during the investigation of the effect of temperature on fermentative hydrogen production. Thus, investigating the effect of temperature on fermentative hydrogen production using organic wastes as substrate is recommended. In addition, most of the reviewed studies investigating the effect of temperature on fermentative hydrogen production were conducted in batch mode, and more studies conducted in continuous mode are needed.

5.7.2 Effect on Substrate Degradation Efficiency

Figure 5.31 showed the effect of temperature on substrate degradation efficiency. The results showed that the substrate degradation efficiency increased with increasing temperatures from 20 to 40 °C, however, it decreased with further increasing temperature from 40 to 55 °C. This demonstrated that in appropriate range, temperature can enhance the ability of mixed cultures to degrade substrate with increasing temperature during the fermentative hydrogen production, which was also shown by other studies (Lee et al. 2006; Mu et al. 2006a, b, c). The maximal substrate degradation efficiency was 98.1% at 40 °C.

5.7.3 Effect on Hydrogen Production

Figure 5.32 showed the progress of cumulative hydrogen production at different temperatures.

Figures 5.33, 5.34 and 5.35 showed the effect of temperature on the hydrogen production potential, the maximum hydrogen production rate and the lag time, respectively.

Table 5.19 The effect of temperature on fermentative hydrogen production

Inoculum	Substrates	Reactor type	Temperature (°C)		Optimal index (value)	References
			Range studied	Optimal		
Ethanoligenens harbinense YUAN-3	Glucose	Batch	20–44	37	Maximum hydrogen yield (1.34 mol/mol glucose)	Xing et al. (2008)
Enterococcus faecium INET2	Glucose	Batch	20–40	35	Maximum hydrogen yield (1.69 mol/mol glucose)	Yin and Wang (2016)
Anaerobic sludge	Glucose	Batch	25–55	40	Maximum hydrogen yield (275.1 mL/g glucose)	Wang and Wan (2008a, b, c, d)
Anaerobic sludge	Glucose	Batch	33–41	41	Maximum hydrogen yield (1.67 mol/mol glucose)	Mu et al. (2006)
Anaerobic sludge	Sucrose	Batch	25–45	35.1	Maximum hydrogen yield (3.7 mol/mol sucrose)	Wang et al. (2005)
Anaerobic sludge	Sucrose	Batch	25–45	35.5	Maximum hydrogen yield (252 mL/g sucrose)	Mu et al. (2006)
Anaerobic digester sludge	Rice slurry	Batch	37–55	37	Maximum hydrogen yield (346 mL/g carbohydrate)	Fang et al. (2005)
Municipal sewage sludge	Sucrose	Continuous	30–45	40	Maximum hydrogen yield (3.88 mol/mol sucrose)	Lee et al. (2006)
Thermoanaerobacterium thermosaccharolyticum PSU-2	Sucrose	Batch	40–80	60	Maximum hydrogen yield (2.53 mol/mol hexose)	O-Thong et al. (2008)
Municipal sewage sludge	Starch	Batch	37–55	55	Maximum hydrogen yield (1.44 mmol/g starch)	Lee et al. (2008)
Municipal sewage sludge	xylose	Continuous	30–55	50	Maximum hydrogen yield (1.4 mol/mol xylose)	Lin et al. (2008)
Cow dung	Cow dung	Batch	3 7–75	60	Maximum hydrogen yield (743 mL/kg cow dung)	Yokoyama et al. (2007)

(continued)

Table 5.19 (continued)

Inoculum	Substrates	Reactor type	Temperature (°C)		Optimal index (value)	References
			Range studied	Optimal		
Cow waste slurry	Cow waste slurry	Batch	37–85	60	Maximum hydrogen yield (392 mL/L slurry)	Yokoyama et al. (2007)
Anaerobic digester sludge	Organic waste	Semi-continuous	37–55	55	Maximum hydrogen yield (360 mL/g VS)	Valdez-Vazquez et al. (2005)
Anaerobic digester sludge	Glucose	Batch	40–70	58.4	Maximum hydrogen yield (2.7 mol/mol glucose)	Roy et al. (2012)
Anaerobic sludge	Corn stover	Batch	30–70	55	Maximum hydrogen yield (7.74 mmol/g sugar)	Zhang et al. (2015)
Klebsiella pneumoniae ECU-15	Glucose	Batch	30–40	37	Maximum hydrogen yield (1.2 mol/mol glucose)	Xiao et al. (2013)

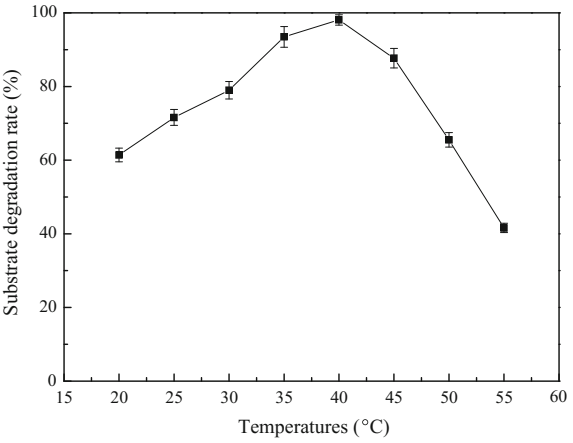

Fig. 5.31 Effect of temperature on substrate degradation efficiency

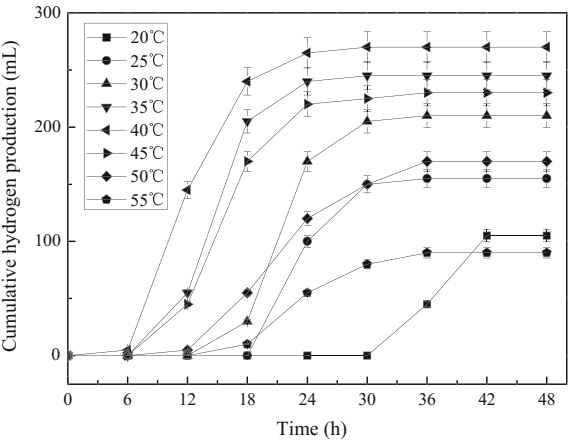

Fig. 5.32 Progress of cumulative hydrogen production at different temperatures

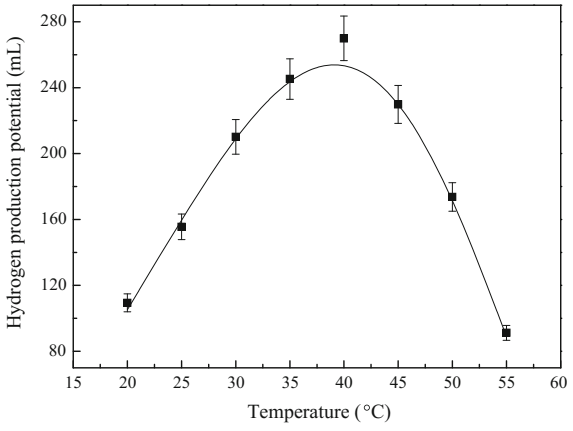

Fig. 5.33 Effect of temperature on hydrogen production potential

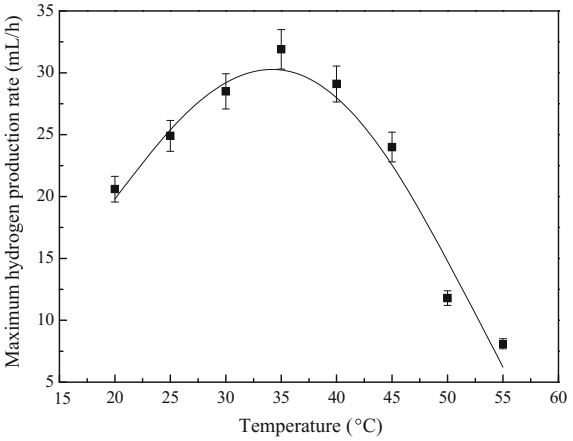

Fig. 5.34 Effect of temperature on maximum hydrogen production rate

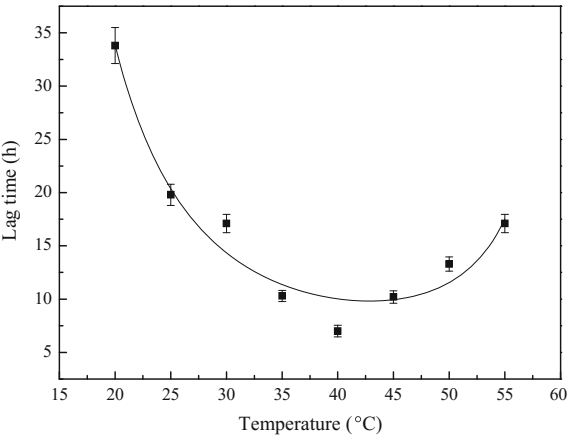

Fig. 5.35 Effect of temperature on the lag time during fermentative hydrogen production

The results showed that the hydrogen production potential increased with increasing temperature from 20 to 40 °C, however, it decreased with further increasing temperature from 40 to 55 °C. The maximal hydrogen production potential of 269.9 mL was obtained at 40 °C.

The expanded Ratkowsky models (Eqs. 5.9, 5.10) were used to describe the effect of temperature on the hydrogen production potential, maximum hydrogen production rate, while the inverted form of the expanded Ratkowsky model (Eq. 5.11) was used to describe the effect of temperature on the lag time (Ratkowsky et al. 1983; Zwietering et al. 1991).

$$P = [A_1 \cdot (T - T_{\min})]^2 \cdot \{1 - \exp[B_1 \cdot (T - T_{\max})]\}^2 \tag{5.9}$$

Table 5.20 Parameters of the expanded Ratkowsky models

Parameters	A_i	T_{min} (°C)	B_i(1/°C)	T_{max} (°C)	R^2	Optimal temperature (°C)
P	0.802 $mL^{0.5}$/°C	3.67	0.0362	62.2	0.993	39.3
R_m	0.453 $mL^{0.5}$/(°C·$h^{0.5}$)	−0.0351	0.0162	61.5	0.963	34.2
λ	0.0133 1/(°C·$h^{0.5}$)	5.26	0.0466	64.6	0.956	42.8

$$R_m = [A_2 \cdot (T - T_{\min})]^2 \cdot \{1 - \exp[B_2 \cdot (T - T_{\max})]\}^2 \quad (5.10)$$

$$\lambda = [A_3 \cdot (T - T_{\min})]^{-2} \cdot \{1 - \exp[B_3 \cdot (T - T_{\max})]\}^{-2} \quad (5.11)$$

where: P (mL) is the hydrogen production potential;

R_m (mL/h) is the maximum hydrogen production rate;

λ (h) is the lag time;

A_1 ($mL^{0.5}$/°C), A_2 ($mL^{0.5}$/(°C·$h^{0.5}$)), A_3 (1/(°C·$h^{0.5}$)), B_1(1/°C), B_2(1/°C) and B_3(1/°C) are parameters of Ratkowsky model.

T_{min} (°C) and T_{max}(°C) are minimal and maximal temperature, at which the fermentative hydrogen production by mixed cultures is observed, respectively.

The main fitting results using these models were listed in Table 5.20.

Taking into consideration the appropriate temperature for the fermentative hydrogen production by mixed cultures in terms of the hydrogen production potential, the maximum hydrogen production rate and the lag time, the range of temperature for fermentative hydrogen production by mixed cultures was from 5.3 to 61.5 °C, the optimal temperatures reported for fermentative hydrogen production by mixed cultures were also in this range (Liang et al. 2002; Yu et al. 2002; Morimoto et al. 2004; Lee et al. 2006; Mu et al. 2006; Zhang and Shen 2006; Hu and Chen 2007).

Table 5.21 Effect of temperature on the concentrations of the end metabolites

Temperature (°C)	Soluble metabolite concentration (mmol/L)			
	Ethanol	Acetic acid	Propionic acid	Butyric acid
20	0.7	3.1	0.6	–
25	1.4	4.5	0.8	0.2
30	2.9	6.4	0.8	–
35	8.1	16.3	–	–
40	5.9	15.7	–	0.9
45	3.8	8.1	1.2	–
50	1.5	5.4	0.7	–
55	0.9	6.9	0.5	–

"-" stands for not detected

5.7.4 Effect on Soluble Metabolites Concentration

Table 5.21 summarizes the effect of temperature on the concentration of the soluble metabolites.

The results showed that the concentration of ethanol and acetic acid increased with increasing temperature from 20 to 35 °C, but it decreased with further increasing temperature from 35 to 55 °C. The concentration of propionic acid and butyric acid changed a lot with increasing temperatures from 20 to 55 °C, but they were very low, even not detectable. The concentration of ethanol and acetic acid accounted for over 86% of the total concentration of the soluble metabolites, thus it belonged to ethanol-type fermentation (Ren et al. 1997; Ren et al. 1997; Ren et al. 2007; Xing et al. 2008).

5.7.5 Effect on Biomass Concentration

Figure 5.36 showed the effect of temperature on biomass concentration.

The results showed that the biomass increased with increasing temperature from 20 to 35 °C, but it decreased with further increasing temperature from 35 to 55 °C.

5.7.6 Effect on Final pH

Figure 5.37 showed the effect of temperature on the final pH. The final pH decreased to lower than 7.0, ranging from 3.34 to 5.11.

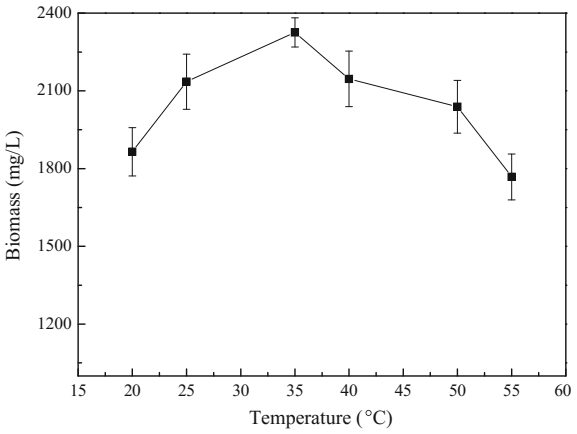

Fig. 5.36 Effect of temperature on biomass

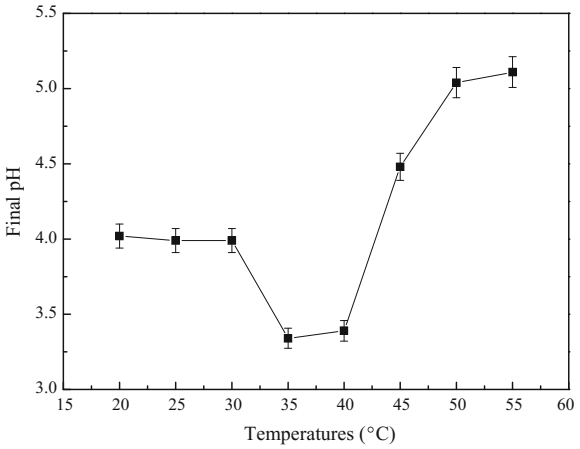

Fig. 5.37 Effect of temperature on final pH

5.8 Effect of pH

5.8.1 Overview

pH is another important factor that influences the activities of hydrogen-producing bacteria, and the fermentative hydrogen production, because it may affect the hydrogenase activity as well as the metabolism pathway. It has been demonstrated that in an appropriate range, increasing pH could increase the ability of hydrogen-producing bacteria to produce hydrogen during fermentative hydrogen production, but pH at much higher levels could decrease it with increasing levels. Since most studies were conducted in batch mode without pH control, only the effect of initial pH on fermentative hydrogen production was investigated in these studies. Table 5.22 summarizes several studies investigating the effect of initial pH on fermentative hydrogen production in batch mode.

As shown in Table 5.22, there exists certain disagreement on the optimal initial pH for fermentative hydrogen production. For example, the optimal initial pH for fermentative hydrogen production reported by Khanal et al. (2004) was 4.5, while that reported by Lee et al. (2002) was 9.0. The possible reason for this disagreement was the difference among these studies in the terms of inoculum, substrate, and initial pH range studied.

In addition, sucrose was the most widely used substrate during the investigation of the effect of initial pH on fermentative hydrogen production. Thus, investigating the effect of initial pH on fermentative hydrogen production using organic wastes as substrate is recommended.

Since some studies on fermentative hydrogen production were conducted in batch mode with pH control, while some others were conducted in continuous

Table 5.22 The effect of initial pH on fermentative hydrogen production

Inoculum	Substrates	Initial pH		Optimal index (value)	References
		Range studied	Optimal		
Compost	Sucrose	4.5–6.5	4.5	Maximum hydrogen yield (214 mL/g COD)	Khanal et al. (2004)
Anaerobic sludge	Starch	5.0–7.0	5.0	Maximum hydrogen yield (1.1 mol/mol hexose)	Lin et al. (2008)
Clostridium butyricum CGS5	Sucrose	5.0–6.5	5.5	Maximum hydrogen yield (2.78 mol/mol sucrose)	Chen et al. (2005)
Waste activated sludge	Food wastewater	4.0–8.0	6.0	Maximum hydrogen yield (47.1 mmol/g COD)	Wu et al. (2004)
Anaerobic sludge	Starch	4.0–9.0	6.0	Maximum hydrogen yield (92 mL/g starch)	Zhang et al. (2003)
Thermoanaerobacterium thermosaccharolyticum PSU-2	Sucrose	4.0–8.5	6.2	Maximum hydrogen yield (2.53 mol/mol hexose)	O-Thong et al. (2008)
Municipal sewage sludge	Xylose	5.0–9.5	6.5	Maximum hydrogen yield (2.25 mol/mol xylose)	Lin and Cheng (2006)
Municipal sewage sludge	Xylose	5.0–8.0	6.5	Maximum hydrogen yield (1.3 mol/mol xylose)	Lin et al. (2006)
Cow dung compost	Cornstalk wastes	4.0–9.0	7.0	Maximum hydrogen yield (149.69 mL/TVS)	Zhang et al. (2007)
Cow dung sludge	Cellulose	5.5–9.0	7.5	Maximum hydrogen yield (2.8 mmol/g cellulose)	Lin and Hung (2008)
Municipal sewage sludge	Sucrose	5.5–8.5	7.5	Maximum hydrogen yield (2.46 mol/mol sucrose)	Wang et al. (2006)
Anaerobic granular sludge	Glucose	3.88–8.12	7.5	Maximum hydrogen yield (1.46 mol/mol glucose)	Davila-Vazquez et al. (2008)
Cracked cereals	Starch	4.0–9.0	8.0	Maximum hydrogen yield (120 mL/g starch)	Liu and Shen (2004)
	Sucrose	3.0–12.0	9.0		Lee et al. (2002)

(continued)

Table 5.22 (continued)

Inoculum	Substrates	Initial pH		Optimal index (value)	References
		Range studied	Optimal		
Anaerobic digester sludge				Maximum hydrogen yield (126.9 mL/g sucrose)	
Enterococcus faecium INET2	Glucose	5.0–10.0	7.0	Maximum hydrogen yield (1.69 mol/mol glucose)	Yin and Wang (2016)
Anaerobic sludge	*Chlorella vulgaris*	4.2–9.8	7.4	Maximum hydrogen yield (31.2 mL/g TS)	Yun et al. (2012)
Indigenous microbes	Waste activated sludge	6.0–10.0	8.0	Maximum hydrogen yield (100.6 mL/g COD)	(Chen et al. 2012)

mode, in these cases, the effect of pH on fermentative hydrogen production was investigated. Table 5.23 summarizes several studies investigating the effect of pH on fermentative hydrogen production.

As shown in Table 5.11, there exists certain disagreement on the optimal pH for fermentative hydrogen production. For example, the optimal pH for fermentative hydrogen production reported by Mu et al. (2006) was 4.2, while that reported by Zhao and Yu (2008) was 7.0. The possible reason for this disagreement was the difference among these studies in the terms of inoculum, substrate, and pH range studied.

In addition, sucrose was the most widely used substrate during the investigation of the effect of pH on fermentative hydrogen production. Thus, investigating the effect of pH on fermentative hydrogen production using organic wastes as substrate is recommended.

5.8.2 *Effect on Substrate Degradation Efficiency*

Figure 5.38 shows the effect of initial pH on substrate degradation efficiency.

The results showed that the substrate degradation efficiency in batch tests increased with increasing initial pH from 4.0 to 7.0, but it changed little with further increasing initial pH from 7.0 to 10.0.

The maximum substrate degradation efficiency was 97.0% at initial pH = 9.0, at which Lee et al. (2002) also obtained the maximum substrate degradation efficiency, while Wang et al. (2007) obtained the maximum substrate degradation efficiency at initial pH of 8.0.

Table 5.23 The effect of pH on fermentative hydrogen production

Inoculum	Substrates	Reactor type	pH		Optimal index (value)	References
			Range studied	Optimal		
Anaerobic digester sludge	Rice slurry	Batch	4.0–7.0	4.5	Maximum hydrogen yield (346 mL/g starch)	Fang et al. (2005)
Anaerobic sludge	Sucrose	Batch	4.7–6.3	5.5	Maximum hydrogen yield (3.7 mol/mol sucrose)	Wang et al. (2005)
Anaerobic sludge	Sucrose	Batch	4.5–6.5	5.5	Maximum hydrogen yield (252 mL/g sucrose)	Mu et al. (2006)
Enterobacter cloacae IIT-BT 08	Sucrose	Batch	4.5–7.5	6.0	Maximum hydrogen production rate (29.63 mmol/g dry cell-h)	Kumar and Das (2000)
Mixed cultures	Sucrose	Continuous	3.4–6.3	4.2	Maximum hydrogen yield (1.61 mol/mol glucose)	Mu et al. (2006)
Anaerobic sludge	Glucose	Continuous	4.0–7.0	5.5	Maximum hydrogen yield (2.1 mol/mol glucose)	Fang and Liu (2002)
Mixed cultures	Sucrose	Continuous	6.1–9.5	7.0	Maximum hydrogen yield (1.61 mol/mol glucose)	Zhao and Yu (2008)

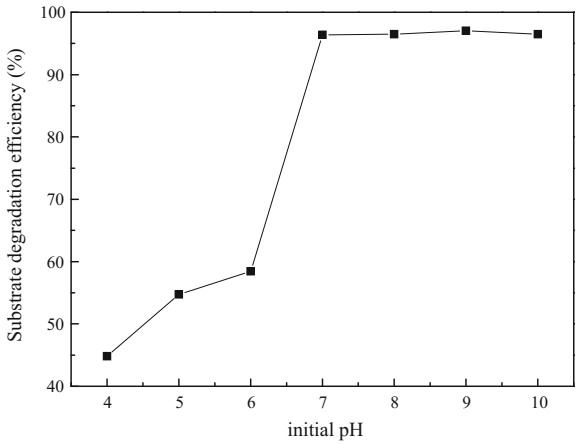

Fig. 5.38 Effect of initial pH on substrate degradation efficiency

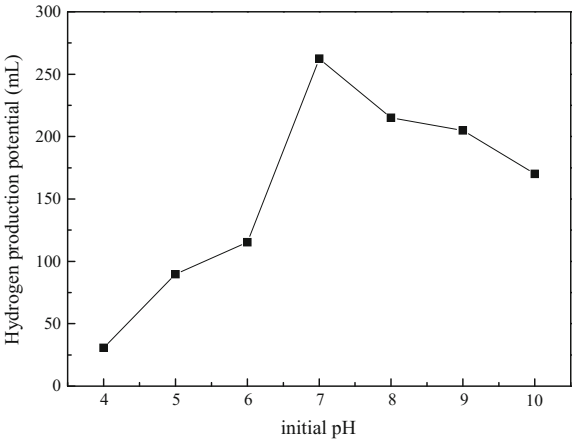

Fig. 5.39 Effect of initial pH on hydrogen production potential

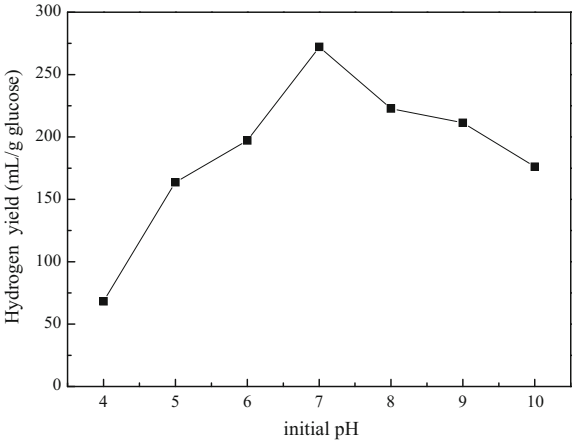

Fig. 5.40 Effect of initial pH on hydrogen yield

5.8.3 Effect on Hydrogen Production

Figure 5.39 shows the effect of initial pH on hydrogen production potential.

The results showed that the hydrogen production potential increased with increasing initial pH from 4.0 to 7.0, however, it decreased with further increasing initial pH from 7.0 to 10.0.

Figure 5.40 shows the effect of initial pH on hydrogen yield.

The results showed that the hydrogen yield increased with increasing initial pH from 4.0 to 7.0, however, it decreased with further increasing initial pH from 7.0 to 10.0. The maximum hydrogen yield of 272.2 mL/g glucose (2.19 mol/mol glucose)

Table 5.24 Comparison of the maximum hydrogen yield

pH	Maximum hydrogen yield (mol H_2/mol glucose)	References
5.5	1.67	Mu et al. (2006)
6.2(initial)	0.97	Oh et al. (2003)
5.5	1.8	Iyer et al. (2004)
–	1.17	Hu and Chen (2007)
6.0(initial)	0.66	Li et al. (2008)
–	2.1	Morimoto et al. (2004)
5.5	2.1	Fang and Liu (2002)

was obtained at initial pH of 7.0, while Khanal et al. (2004) and Lee et al. (2002) obtained the maximum hydrogen yield at initial pH of 4.5 and 9.0, respectively.

Table 5.24 summarizes the maximum hydrogen yield.

5.8.4 *Effect on Hydrogen Production Rate*

Figure 5.41 shows the effect of initial pH on hydrogen production rate.

The results showed that the hydrogen production rate in batch tests increased with increasing initial pH from 4.0 to 8.0, however, it decreased with further increasing initial pH from 8.0 to 10.0.

The modified Ratkowsky model was used to fit the hydrogen production rate data. The fitting result is as follows:

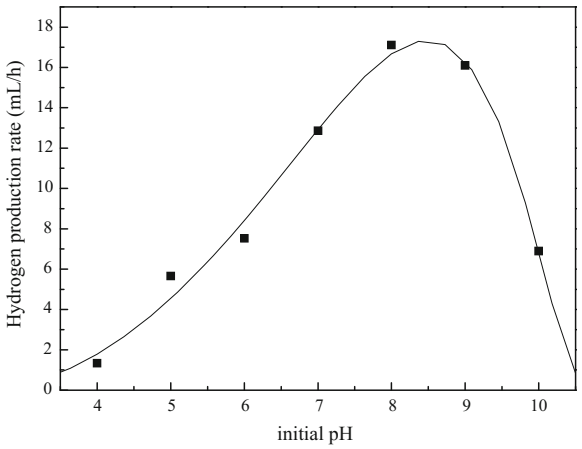

Fig. 5.41 Effect of initial pH on hydrogen production rate

$$R = [0.8 \cdot (pH - 2.3)]^2 \cdot \{1 - \exp[0.8 \cdot (pH - 10.6)]\}^2 \quad (5.12)$$

The coefficient of determination R^2 of the fitting was 0.988, which indicated that the modified Ratkowsky model could describe the effect of initial pH on the hydrogen production rate.

5.8.5 *Effect on Soluble Metabolites Distribution*

Table 5.25 summarizes the effect of initial pH on distributions of the soluble metabolites

The fraction of butyric acid in the soluble metabolites decreased with increasing initial pH from 4.0 to 10.0, while the fraction of acetic acid and propionic acid in the soluble metabolites increased with increasing initial pH from 4.0 to 10.0. This

Table 5.25 Effect of initial pH on the soluble metabolites

Initial pH	Fractions of soluble metabolites (%)	HAc	HPr	HBu
	Ethanol			
4.0	4.1	6.4	0.2	89.3
5.0	2.9	8.4	4.8	83.9
6.0	6.4	6.5	5.3	81.8
7.0	14.7	7.2	7.6	70.5
8.0	34.1	7.3	8.6	50.0
9.0	28.5	18.8	6.8	45.9
10.0	23.9	18.4	11.8	46.0

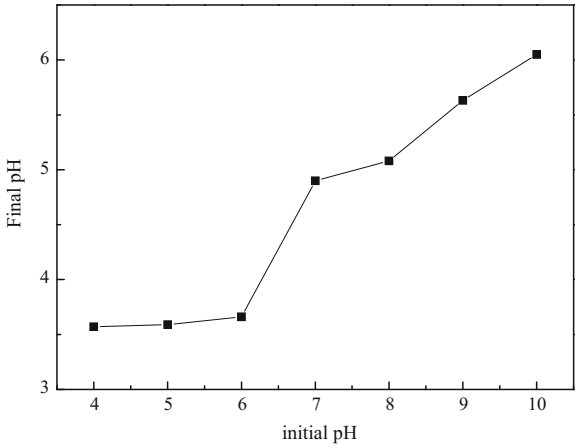

Fig. 5.42 Effect of initial pH on final pH

Table 5.26 The optimal initial pH reported for maximum hydrogen yield

Seed sludge	Substrate	Range of the initial pH studied	Optimal initial pH	Reference
Enterococcus faecium INET2	Glucose	5.0–10.0	7.0	Yin and Wang (2016)
Activated sludge	Molasses wastewater	4.0–8.0	6.0	Wu et al. (2004)
Anaerobic sludge	Starch	4.0–9.0	6.0	Zhang et al. (2003)
Compost	Sucrose	4.5–6.5	4.5	Khanal et al. (2004)
Digested sludge	Sucrose	3.0–12.0	9.0	Lee et al. (2002)
Cracked cereals	Starch	4.0–9.0	8.0	Liu and Shen (2004)
Sewage sludge	Xylose	5.0–8.0	6.5	Lin et al. (2006)
Sewage sludge	Starch	5.0–9.0	8.0	Wang et al. (2007)

demonstrated that lower pH favored butyric acid production and higher pH favored acetic acid and propionic acid production.

5.8.6 *Effect on Final pH*

Figure 5.42 shows the effect of initial pH on final pH. The results showed that due to fermentation, the final pH was in range of 3.6–6.0.

5.8.7 *Comparison of the Optimal Initial pH*

Table 5.26 summarizes the optimal initial pH reported for fermentative hydrogen production by mixed cultures.

As shown in Table 5.26, the optimal initial pH for fermentative hydrogen production by mixed cultures obtained in this study is 7.0, which is close to those (from 6.0 to 8.0) obtained in most other studies, but it is much higher than that reported by Khanal et al. (2004), and it is much lower than that reported by Lee et al. (2002). The possible reason for this difference is the differences among this study and their studies in terms of the seed sludge, substrates, or the ranges of the initial pH studied.

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Chapter 6
Kinetic Models for Hydrogen Production

List of Symbols

HPB	Hydrogen-producing bacteria
H	Cumulative value
$H_{\max}$	Maximum cumulative value
R	Rate
$R_{\max}$	Maximum rate
λ	Lag time
t	Cultivation time
X	Biomass
$X_{\max}$	Maximum biomass
X_0	Initial biomass
S	Substrate concentration
S_0	Initial substrate concentration
S_{Crit}	Critical substrate concentration
P	Product
C	Inhibitor concentration
C_{Crit}	Critical inhibitor concentration
$Y_{X/S}$	Biomass yield coefficient
$Y_{P/S}$	Product yield coefficient
$Y_{P/X}$	Growth-associated product yield coefficient
β	Nongrowth-associated product yield coefficient
k_c	Apparent specific growth rate
K_S	Half-saturation constant
K_I	Inhibition constant
K_C	Constant
k_d	Biomass decay constant
K_a	Constant
K_b	Constant
m	Constant
n	Constant
A	Constant
B	Constant

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I_{pH}	pH inhibition constant
pH_{UL}	Higher pH limit
pH_{LL}	Lower pH limit
$pH_{\min}$	Minimum pH
$pH_{\max}$	Maximum pH
T	Temperature
$T_{\min}$	Minimum temperature
T_{opt}	Optimal temperature
$T_{\max}$	Maximum temperature
E_a	Activation energy
R_g	Ideal gas constant
$[H^+]$	H^+ concentration
D	Dilution rate

6.1 Introduction

During fermentative hydrogen production, when substrate is degraded, the growth of hydrogen-producing bacteria (HPB) occurs simultaneously with the production of hydrogen, as well as some soluble metabolites. Some kinetic models such as the modified Gompertz model have been proposed to describe the progress of substrate degradation, HPB growth, hydrogen production and some soluble metabolite formation in a batch fermentative hydrogen production process. Such kinetic models can be used to predict the substrate degradation, HPB growth, hydrogen production, and some soluble metabolite formation at a given time in a batch fermentative hydrogen production process, which can help to elucidate such process (Wang et al. 2008; Wang and Wei 2008).

In addition, many factors such as substrate concentrations, inhibitors, temperatures, pH, and dilution rate can influence the fermentative hydrogen production (Wang and Wan 2009a, b; Hsia and Chou 2014). Some kinetic models have also been proposed to describe the effects of these factors on the rates of substrate degradation, HPB growth, hydrogen production, and some soluble metabolite production, as well as the concentrations of substrate, biomass, hydrogen, and some soluble metabolites. Such kinetic models could be used to explain the effects of these factors on the fermentative hydrogen production quantitatively. In addition, the kinetic constants obtained from these models can provide useful information for the analysis, design, and operation of a fermentative hydrogen production process (van Niel et al. 2003; Mu et al. 2006; Wang and Wei 2009; Boboescu et al. 2014).

Moreover, there usually exist some relationships among the substrate degradation rate, the HPB growth rate, and the product formation rate. Some kinetic models have also been proposed to describe these relationships.

This chapter attempts to summarize the kinetic models, which have been proposed to describe the progress of batch fermentative hydrogen production process, the effects of various factors on fermentative hydrogen production process, and the relationships among the substrate degradation rate, the HPB growth rate, and the product formation rate.

6.2 The Progress of Hydrogen Production Process

During fermentative hydrogen production, substrate concentrations, HPB growth, hydrogen, and some soluble metabolites change regularly. Some kinetic models have been proposed to describe such changes. Among them the modified Gompertz model (Eq. 6.1) developed by Zwietering et al. (1990) was widely used to describe the progress of substrate degradation, HPB growth, hydrogen production, and some soluble metabolite production in a batch fermentative hydrogen production process (Lay et al. 1999; Wu et al. 2004; Fang et al. 2005; Cheong and Hansen 2007; Lin et al. 2008a, b).

$$H = H_{\max} \cdot \exp\left\{-\exp\left[\frac{\mathrm{R}_{\max} \cdot e}{H_{\max}} \cdot (\lambda - t) + 1\right]\right\} \tag{6.1}$$

$$H = \frac{H_{\max}}{1 + \exp[4R_{\max} \cdot (\lambda - t)/H_{\max} + 2]} \tag{6.2}$$

When Eq. (6.1) was used to describe the progress of substrate degradation in batch tests, H and $H_{\max}$ denote the cumulative degraded substrate value and the maximum degraded substrate value, respectively. When Eq. (6.1) and (6.2) were used to describe the progress of HPB growth in batch tests, H and $H_{\max}$ denote the cumulative HPB growth value and the maximum HPB growth value, respectively.

As shown in Fig. 6.1, in a batch test, H increases very slowly with increasing cultivation time from 0 to λ, and then increases rapidly almost at the rate of $R_{\max}$ and finally reaches an asymptotic value $H_{\max}$ with further increasing the cultivation time.

Table 6.1 summarizes several studies using the modified Gompertz model to describe the progress of a batch fermentative hydrogen production process.

Recently, the modified Logistic model (Eq. 6.2), whose curve is very similar to that of the modified Gompertz model, was used by Wang and Wan to describe the progress of hydrogen production in the batch tests using glucose as substrate. In addition, it was also used by Mu et al. (2007) to describe the progress of HPB growth in batch tests.

Furthermore, Mu et al. compared the ability of the modified Gompertz model, modified Logistic model, and modified Richards to describe the progress of HPB growth in batch tests and concluded that the modified Gompertz model was the most suitable one (Mu et al. 2007).

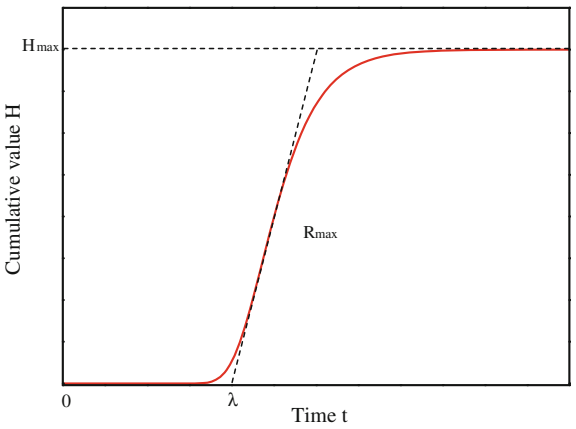

Fig. 6.1 A curve for modified Gompertz model

In addition, a Logistic model (Eq. 6.3) was also used by Mu et al. (2006) to describe the progress of HPB growth in the batch tests.

$$X = \frac{X_0 \cdot \exp(k_c \cdot t)}{1 - (X_0/X_{\max}) \cdot (1 - \exp(k_c \cdot t))} \tag{6.3}$$

Compared with the Logistic model (Eq. 6.3), the modified Logistic model (Eq. 6.2) can obtain the lag time of HPB growth directly by fitting the experimental data, thus using it to describe the progress of HPB growth in the batch tests is recommended.

$$X = X_0 + Y_{X/S} \cdot (S_0 - S) \tag{6.4}$$

$$\frac{\mathrm{d}S}{\mathrm{d}t} = \frac{-1}{Y_{X/S}} \cdot \frac{R_{\max} \cdot S}{K_S + S} \cdot X \tag{6.5}$$

$$\frac{\mathrm{d}S}{\mathrm{d}t} = \frac{-1}{Y_{X/S}} \cdot \frac{R_{\max} \cdot S}{K_S + S - S^2/K_I} \cdot X \tag{6.6}$$

$$\frac{\mathrm{d}S}{\mathrm{d}t} = \frac{-1}{Y_{X/S}} \cdot \frac{R_{\max} \cdot S}{K_S + S + S^2/K_I} \cdot X \tag{6.7}$$

where $R_{\max}$ is the specific HPB growth rate.

Kumar et al. (2000)compared the ability of two groups of models developed from a classical Monod model (Eqs. 6.4 and 6.5) and a modified Andrew model (Eqs. 6.4 and 6.6) to describe the progress of glucose degradation and *Enterobacter cloacae* IIT-BT 08 growth in batch tests and concluded that the latter was the most suitable one. In addition, Nath et al. (2008) also compared the ability of two groups of models developed from a classical Monod model (Eqs. 6.4 and 6.5) and an Andrew model (Eqs. 6.4 and 6.7) to describe the progress of glucose degradation

Table 6.1 Several studies using modified Gompertz model

Seed	Substrates	Described objectives	Correlation coefficient	References
Digester sludge	Glucose	Hydrogen	0.968	Yin and Wang (2016)
Digested sludge and soy bean-meal silo	Organic municipal solid waste	Hydrogen	Over 0.90	Lay et al. (1999)
Wasted activated sludge	Molasses	Hydrogen	0.993–1.0	Wu et al. (2004)
Municipal sewage sludge	Glucose	Hydrogen	0.977–1.0	Chen et al. (2002)
Anaerobic digester sludge	Rice slurry	Hydrogen	Over 0.98	Fang et al. (2005)
Cattle manure sludge	Glucose	Hydrogen	0.990–1.0	Cheong and Hansen (2006)
Clostridium pasteurianum CH4	Hydrolyzed starch	Hydrogen	–	Chen et al. (2007)
Clostridium butyricum CGS2	Hydrolyzed starch	Hydrogen	–	Chen et al. (2007)
Anaerobic sludge	Starch	Hydrogen	Over 0.95	Zhang et al. (2003)
Wasted activated sludge	Xylose	Hydrogen	0.987–0.999	Lin et al. (2008)
Anaerobic sludge	Sucrose	Hydrogen	0.990–1.0	Lee et al. (2001)
Wasted activated sludge	Sucrose	Hydrogen	0.996–1.0	Lin and Lay (2004)
Cracked cereals	Starch	Hydrogen	0.964–0.999	Liu and Shen (2004)
Sewage sludge	Xylose	Hydrogen	0.994–0.999	Lin et al. (2006)
Cow dung compost	Cornstalk wastes	Hydrogen	0.989	Zhang et al. (2007)
Sewage sludge	Sewage sludge	Hydrogen	0.991–1.0	Cai et al. (2004)
Cattle manure sludge	Synthetic wastewater	Hydrogen	0.995–1.0	Cheong and Hansen (2007)
Municipal sewage sludge	Starch	Hydrogen	0.976–1.0	Wang et al. (2007)
Municipal sewage sludge	Pineapple waste	Hydrogen	0.982–0.996	Wang et al. (2006)
Sewage sludge	Starch	Hydrogen	0.997–0.999	Lin et al. (2008)
Wasted activated sludge	Sucrose	Hydrogen	0.955–1.0	Lin and Shei (2008)
Clostridium saccharoper-butylacetonicum	Cheese whey	Hydrogen	0.989–0.996	Ferchichi et al. (2005)
Granular sludge	Sucrose	Hydrogen	Over 0.95	Li and Fang (2007)
Digester sludge	Microcrystalline cellulose	Hydrogen	Over 0.90	Lay (2001)
Anaerobic sludge	Sucrose	Hydrogen	0.999	Mu et al. (2006)
Anaerobic sludge	Sucrose	Hydrogen	0.999	Mu et al. (2007)
Mixed microbial consortium	Beer-brewing wastewater	Hydrogen	–	Boboescu et al. (2014)
Clostridium sp. FS3	Corn stalk	Hydrogen	–	Song et al. (2014)
Digested sludge	Waste activated sludge	Hydrogen	Over 0.937	Yin and Wang (2015)
Enterobacter aerogenes and *Clostridium butyricum*	Biodiesel waste	Hydrogen and substrate degradation	0.95	Pachapur et al. (2016)
Anaerobic sludge	Sucrose	Substrate degradation	0.994	Mu et al. (2007)
Anaerobic sludge	Glucose	HPB growth	0.937–0.994	Mu et al. (2006)

(continued)

Table 6.1 (continued)

Seed	Substrates	Described objectives	Correlation coefficient	References
Anaerobic sludge	Sucrose	HPB growth	0.998	Mu et al. (2007)
Anaerobic sludge	Sucrose	Acetate	0.999	Mu et al. (2006)
Anaerobic sludge	Sucrose	Acetate	0.992	Mu et al. (2007)
Anaerobic sludge	Sucrose	Butyrate	0.997	Mu et al. (2006)
Anaerobic sludge	Sucrose	Butyrate	0.997	Mu et al. (2007)
Digester sludge	Microcrystalline cellulose	VFA[a]	–	Lay (2001)
Digester sludge	Microcrystalline cellulose	Alcohol[b]	–	Lay (2001)

[a]VFA is the total of acetate, propionate and butyrate
[b]Alcohol is the total ethanol, propanol and butanol

and *Enterobacter cloacae* DM11 growth in batch tests and concluded that the latter was the most suitable one. The possible reason for this was that the models developed from a modified Andrew model and Andrew model took into consideration the effects of substrate inhibition, while the models developed from a classical Monod model did not take into consideration the effects of substrate inhibition.

$$I_{pH} = \exp\left[-3 \cdot \left(\frac{pH - pH_{UL}}{pH_{UL} - pH_{LL}}\right)^2\right] \tag{6.8}$$

$$\frac{dS}{dt} = \frac{-1}{Y_{X/S}} \cdot \frac{R_{\max} \cdot S}{K_S + S} \cdot X \cdot I_{pH} \tag{6.9}$$

$$\frac{dX}{dt} = \frac{R_{\max} \cdot S}{K_S + S} \cdot X \cdot I_{pH} - k_d \cdot X \tag{6.10}$$

where $R_{\max}$ is the specific HPB growth rate.

Two models developed by Ntaikou et al. from a modified Monod model incorporating low pH inhibition and the biomass decay (Eqs. 6.8, 6.9 and 6.10) were used to describe the progress of glucose degradation and *Ruminococcus albus* growth in batch tests (Ntaikou et al. 2008).

In addition, the anaerobic digestion model No. 1 (ADM1) developed by the International Water Association (IWA) task group was modified by Lin et al. (2007) to describe the progress of glucose degradation, *Clostridium* growth, and the productions of hydrogen, butyrate, acetate, and ethanol in batch tests.

In general, the modified Gompertz model can be easily used to describe the progress of substrate degradation, HPB growth, hydrogen production and some soluble metabolite production in a batch fermentative hydrogen production process, which makes it nearly an omnipotent model. Moreover, using it some constants that

have biological meanings, which may be of great importance to a better understanding of a process, can be obtained.

Even though the modified Logistic model has a similar property as the modified Gompertz model and using it can also obtain some constants that have biological meanings, it has been not used widely as the modified Gompertz model. Thus, using it to describe the progress of a batch fermentative hydrogen production process is recommended.

Even though the models developed by Kumar et al. (2000), Nath et al. (2008) and Ntaikou et al. (2008) took into consideration the effects of some inhibitions or biomass decay, they were only used to describe the progress of substrate degradation and HPB growth in batch tests, and thus using them to describe the progress of hydrogen production and some soluble metabolite production to examine their suitability for such applications is recommended.

Even though the modified ADM1 developed by Lin et al. could also be used to describe the progress of substrate degradation, HPB growth, hydrogen production, and some soluble metabolite production in a batch fermentative hydrogen production process, the development and the application of the model are very complex, which may limit its application.

In addition, the studies on the comparison of the ability of different models to describe the progress of a batch fermentative hydrogen production process are limited, thus more researches to compare them are recommended.

6.3 The Effect of Substrate Concentration on Hydrogen Production

Substrate is usually carbohydrates that can provide carbon and energy sources for HPB, thus is it of great importance to HPB growth and thus for fermentative hydrogen production. Some kinetic models have been proposed to describe the effects of substrate concentrations on the rates of substrate degradation, HPB growth and hydrogen production. Among them the classical Monod model (or Michaelis–Menten model) (Eq. 6.11) was widely used.

$$R = \frac{R_{\max} \cdot S}{K_S + S} \tag{6.11}$$

As shown in Fig. 6.2, R increases with increasing S and finally reaches an asymptotic value $R_{\max}$. It also suggests that at lower substrate concentration (relative to the half-saturation constant), R is approximately proportional to substrate concentration (first order in substrate concentration), while at higher substrate concentration, R is independent of substrate concentration (zero order in substrate concentration). Table 6.2 summarizes several studies using the Monod model (or Michaelis–Menten model) to describe the effects of substrate concentrations on the rates of substrate degradation, HPB growth, and hydrogen production.

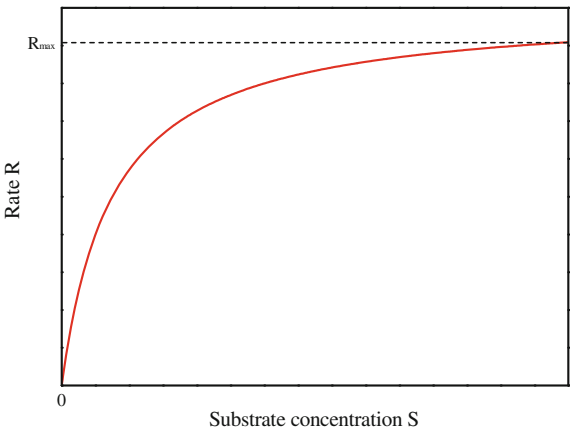

Fig. 6.2 A curve for the Monod model

When a substrate inhibits a fermentative hydrogen production process at much higher concentrations, the classical Monod model becomes unsatisfactory. In this case, modified Monod models with the item of substrate inhibition can be used to describe the effects of substrate concentrations on the hydrogen production rate and specific HPB growth rate. Among these models, the Andrew model (Eq. 6.12) was most widely used (Table 6.3).

$$R = \frac{R_{\max} \cdot S}{K_S + S + S^2/K_I} \tag{6.12}$$

$$R = \frac{R_{\max} \cdot S}{K_S + S - S^2/K_I} \tag{6.13}$$

In addition, Kumar et al. (2000) used a modified Andrew model (Eq. 6.13) to describe the effects of substrate concentrations on specific *Enterobacter cloacae* IIT-BT 08 growth rate in batch tests.

Moreover, Wang and Wan (2008) used the Han–Levenspiel model (Eq. 6.14), an extended Monod model, to describe the effects of glucose concentrations on hydrogen production rate in batch tests. In addition, Wang and Wan also compared the ability of the Andrew model and the Han–Levenspiel model to describe the effects of glucose concentrations on hydrogen production rate in batch tests and concluded that the Han–Levenspiel model was the most suitable one.

$$R = \frac{R_{\max} \cdot S \cdot \left(1 - \frac{S}{S_{Crit}}\right)^m}{S + K_S \cdot (1 - S/S_{Crit})^n} \tag{6.14}$$

$$R = \frac{R_{\max} \cdot S \cdot \left(1 - \frac{S}{S_{Crit}}\right)^m}{S + K_S} \tag{6.15}$$

Table 6.2 Several studies using the Monod model

Reactor type	Seed	Substrates	Described objectives	Correlation coefficient	References
Batch	*Enterobacter cloacae* IIT-BT 08	Glucose	Specific HPB growth rate	–	Kumar et al. (2000)
Batch	*Enterobacter cloacae* DM11	Glucose	Specific HPB growth rate	–	Nath et al. (2008)
Batch	*Clostridium butyricum* CGS5	Xylose	Specific HPB growth rate	0.881	Lo et al. (2008)
Batch	*Clostridium pasteurianum* CH4	Sucrose	Specific HPB growth rate	0.970	Lo et al. (2008)
Batch	*Enterobacter cloacae* IIT-BT 08	Glucose	Specific HPB growth rate	–	Kumar and Das (2000)
Batch	*Escherichia coli* BL-21	Glucose	Specific HPB growth rate	–	Chittibabu et al. (2006)
Batch	*Thermoanaerobacterium thermosaccharolyticum* PSU-2	Sucrose	Specific HPB growth rate	–	O-Thong et al. (2008)
Batch	Anaerobic digested sludge	Sucrose	Hydrogen production rate	0.858	Chen et al. (2006)
Batch	Anaerobic digested sludge	Nonfat dry milk	Hydrogen production rate	0.980	Chen et al. (2006)
Batch	Anaerobic digested sludge	Food waste	Hydrogen production rate	0.976	Chen et al. (2006)
Batch	*Clostridium butyricum* CGS5	Xylose	Specific hydrogen production rate	0.952	Lo et al. (2008)
Batch	*Clostridium pasteurianum* CH4	Sucrose	Specific hydrogen production rate	0.935	Lo et al. (2008)
Continuous	Municipal sewage sludge	Sucrose	Specific hydrogen production rate	0.94	Lin et al. (2006)
Batch	Municipal sewage sludge	Starch	Volumetric hydrogen production rate	0.973	Lee et al. (2008)
Continuous	Municipal sewage sludge	Sucrose	Volumetric hydrogen production rate	0.90	Lin et al. (2006)
Batch	Anaerobic sludge	Sucrose	Specific substrate degradation rate	0.963	Mu et al. (2006)
Continuous	Anaerobic sludge	Gelatin	Specific substrate degradation rate	–	Fang and Yu (2002)
Batch	Anaerobic sludge	Dairy wastewater	Specific HPB growth rate	0.997	Gadhe et al., (2014)

Table 6.3 Several studies using the Andrew model

Reactor type	Seed	Substrates	Described objectives	Correlation coefficient	References
Batch	Anaerobic digested sludge	Glucose	Hydrogen production rate	0.902	Wang and Wei (2008)
Batch	Anaerobic sludge	Glucose	Hydrogen production rate	–	Hang et al. (2008)
Batch	*Enterobacter cloacae* DM11	Glucose	Specific HPB growth rate	–	Nath et al. (2008)
Batch	Anaerobic sludge	Glucose	Specific HPB growth rate	–	Majizat et al. (1997)
Batch	Anaerobic sludge	Dairy wastewater	Specific HPB growth rate	0.980	Gadhe et al. (2014)

$$R = \frac{R_{\max} \cdot S}{K_S + S} \cdot X \cdot I_{pH} \tag{6.16}$$

In addition, van Niel et al. (2003) used a modified Han-Levenspiel model (Eq. 6.15) to describe the effects of sucrose concentrations on hydrogen production rate in batch tests.

Sometimes low pH will inhibit HPB growth and will inhibit their ability to degrade substrate accordingly, thus a modified Monod model incorporating low pH inhibition may describe the effects of substrate concentrations on the substrate degradation rate and HPB growth rate better. In addition, biomass decay may also affect the activity of HPB, and a modified Monod model incorporating biomass decay may be a better choice in such cases.

Ntaikou et al. (2008) used a modified Monod model incorporating low pH inhibition and biomass decay (Eq. 6.10) to describe the effects of glucose concentrations on the *Ruminococcus albus* growth rate. In addition, Ntaikou et al. (2008) and Lin et al. (2007) used a modified Monod model (Eq. 6.16) incorporating low pH inhibition to describe the effects of glucose concentrations on glucose degradation rate.

In general, the classical Monod model can be used easily to describe the effects of substrate concentrations on the rates of substrate degradation, HPB growth, and hydrogen production. In addition, some terms such as various inhibitions or biomass decay can be added to this model when necessary, which can make it describe the effects of substrate concentrations on the rates of substrate degradation and HPB growth better. Furthermore, different modified Monod models may have different property, thus, comparison of them to obtain the most suitable model for a given fermentative hydrogen production process is recommended.

So far, however, to the best of our knowledge, the classical Monod model and its modified forms have not been used to describe the effects of substrate concentrations on some soluble metabolite production rate during fermentative hydrogen production, thus more researches in this aspect are recommended.

6.4 The Effect of Inhibitor Concentration on Hydrogen Production

It has been demonstrated that some salts or hydrogen may change the intracellular pH of HPB, increase the maintenance energy requirement of HPB or inhibit some specific enzymes related to fermentative hydrogen production and thus they can inhibit HPB growth and then inhibit the fermentative hydrogen production.

So far, some kinetic models have been proposed to describe the inhibitory effects of some salt concentrations or hydrogen on the fermentative hydrogen production. Among them, the modified Han–Levenspiel model (Eq. 6.17) was widely used. As shown in Fig. 6.3, R value decreases from $R_{\max}$ to zero with increasing inhibitor concentrations from 0 to C_{Crit}.

$$R = R_{\max} \cdot \left(1 - \frac{C}{C_{Crit}}\right)^{m} \tag{6.17}$$

$$R = \frac{R_{\max}}{1 + (C/K_C)^{m}} \tag{6.18}$$

$$R = \frac{R_{\max} \cdot K_C}{K_C + C} \tag{6.19}$$

$$R = R_{\max} \cdot \frac{S}{K_S + S} \cdot \left(1 - \frac{S}{S_{Crit}}\right)^{m} \cdot \left(1 - \frac{C}{C_{Crit}}\right)^{n} \tag{6.20}$$

Table 6.4 summarizes several studies using the modified Han–Levenspiel model to describe the inhibitory effects of some salts or hydrogen on the hydrogen production rate and specific HPB growth rate.

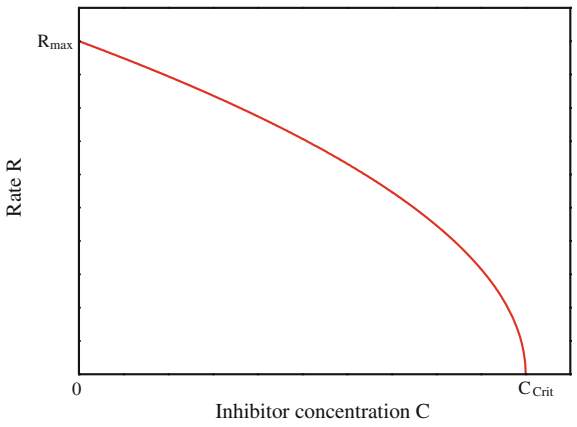

Fig. 6.3 A curve for modified Han–Levenspiel model

In addition, Wang et al. (2008) used Eq. (6.18) to describe the inhibitory effects of sodium acetate concentrations on the specific rates of sucrose degradation and hydrogen production in batch tests. Moreover, Liu et al. (2006) used Eq. (6.19) to describe the inhibitory effects of butyrate concentrations on specific growth rates of wild *Clostridium tyrobutyricum ack* and deleted mutant of *Clostridium tyrobutyricum* in fed-batch tests.

Furthermore, van Niel et al. (2003) used Eq. (6.20) to describe the combined inhibitory effects of sucrose and sodium acetate concentrations on specific growth rate of *Caldicellulosiruptor saccharolyticus* in batch tests. In addition, van Niel et al. (2003) also developed a model (not shown) incorporating cell lysis to describe the inhibitory effects of sodium acetate concentrations on specific growth rate of *Caldicellulosiruptor saccharolyticus* in batch tests.

So far, the description of the inhibitory effects of some salt concentrations or hydrogen on the rates of hydrogen production, substrate degradation, and HPB growth using these models were mostly made for batch tests; thus, the description of the inhibitory effects for continuous tests using these models is recommended.

The modified Han–Levenspiel model was only used to describe the inhibitory effects of some salt concentrations or hydrogen on hydrogen production rate. The description of the inhibitory effects of some salt concentrations or hydrogen on the rates of substrate degradation, HPB growth, and some soluble metabolite production using this model is recommended.

In addition, to the best of our knowledge, up to now, there have been no studies using models to describe the inhibitory effects of ethanol or propionate on fermentative hydrogen production. However, in some cases, ethanol can be dominant in the soluble metabolites (Wang et al. 2007), and in other cases, propionate can be dominant in the soluble metabolites (Khanal et al. 2004). At a high concentration, ethanol and propionate may also inhibit HPB growth and then inhibit the fermentative hydrogen production accordingly, thus the description of the inhibitory effects of ethanol or propionate concentrations on fermentative hydrogen production using certain models is recommended.

Moreover, the studies on the comparison of the ability of different models to describe the inhibitory effects of various inhibitors on fermentative hydrogen production are limited, thus more researches in this aspect are recommended.

6.5 The Effect of Temperature on Hydrogen Production

Temperature is one of the most important factors influencing fermentative hydrogen production, because temperature can affect the activity of HPB considerably by influencing the activity of some essential enzymes such as hydrogenases.

So far, Arrhenius model (Eq. 6.21) has been used a lot to describe the effects of temperatures on fermentative hydrogen production.

Table 6.4 Several studies using modified Han–Levenspiel model

Reactor type	Seed	Substrates	Inhibitor	Described objectives	Correlation coefficient	References
Batch	*Caldicellulosiruptor saccharolyticus*	Sucrose	Sodium acetate	Hydrogen production rate	0.99–1.0	van Niel et al. (2003)
Batch	*Caldicellulosiruptor saccharolyticus*	Sucrose	Sodium chloride	Hydrogen production rate	0.98–1.0	van Niel et al. (2003)
Batch	*Caldicellulosiruptor saccharolyticus*	Sucrose	Sodium lactate	Hydrogen production rate	0.90	van Niel et al. (2003)
Batch	*Caldicellulosiruptor saccharolyticus*	Sucrose	Potassium acetate	Hydrogen production rate	0.81	van Niel et al. (2003)
Batch	*Caldicellulosiruptor saccharolyticus*	Sucrose	Potassium chloride	Hydrogen production rate	0.98	van Niel et al. (2003)
Batch	*Caldicellulosiruptor saccharolyticus*	Sucrose	Hydrogen	Hydrogen production rate	0.79–0.98	van Niel et al. (2003)
Batch	Anaerobic sludge	Glucose	Sodium butyrate	Specific hydrogen production rate	0.989	Zheng and Yu (2005)

$$R = A \cdot \exp\left(-\frac{E_a}{R_g \cdot T}\right) \tag{6.21}$$

where T is the absolute temperature.

Table 6.5 summarizes several studies using the Arrhenius model to describe the effects of temperature on fermentative hydrogen production.

In addition, the Arrhenius model was only used to describe the effects of temperatures on hydrogen production rate and HPB growth rate, using it to describe the effects of temperatures on the substrate degradation rate and some soluble metabolite production rate is recommended.

Table 6.5 Several studies using the Arrhenius model

Reactor type	Seed	Substrates	Described objectives	Correlation coefficient	References
Batch	Anaerobic sludge	Glucose	Hydrogen production rate	0.945	Mu et al. (2006)
Batch	*Enterobacter cloacae* IIT-BT 08	Glucose	Hydrogen production rate	–	Kumar and Das (2000)
Continuous	Municipal sewage sludge	Xylose	Hydrogen production rate	0.98	Lin et al. (2008)
Batch	*Enterobacter aerogenes*	Starch hydrolysate	Maximum hydrogen production rate	0.97–0.99	Fabiano and Perego (2002)
Batch	Anaerobic sludge	Glucose	HPB growth rate	0.984	Mu et al. (2006)

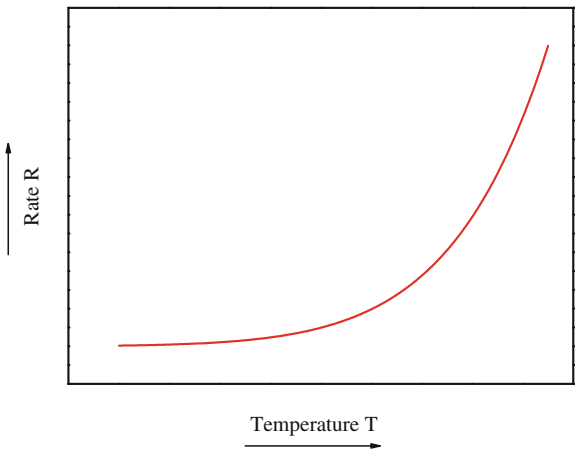

Fig. 6.4 A curve for the Arrhenius model

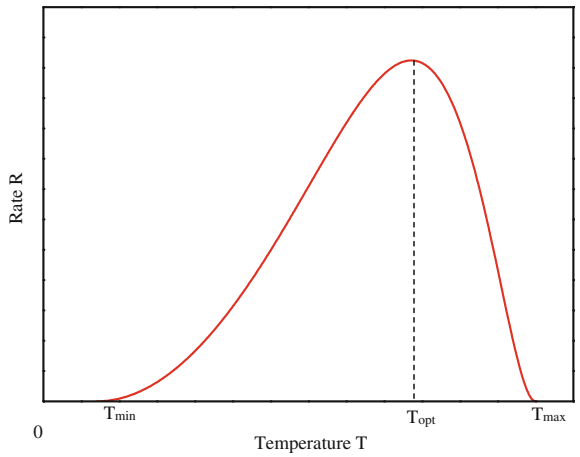

Fig. 6.5 A curve for the Ratkowsky model

One drawback of the Arrhenius model is that it cannot account for the decrease in the R with increasing temperatures above the optimal temperatures, because as shown in Fig. 6.4, R increases with increasing temperatures all the time. Thus, using models that can describe the effects of temperature on fermentative hydrogen production throughout the entire biokinetic temperature range is recommended. For such purposes, the Ratkowsky model (Eq. 6.22) may be a better choice. For R increases with increasing temperatures from $T_{\min}$ to T_{opt} and then decreases with further increasing temperatures from T_{opt} to $T_{\max}$, as shown in Fig. 6.5.

$$R = [A \cdot (T - T_{\min})]^2 \cdot \{1 - \exp[B \cdot (T - T_{\max})]\}^2 \tag{6.22}$$

6.6 The Effects of pH on Hydrogen Production

pH is another important factor influencing fermentative hydrogen production, because it can affect the activity of HPB considerably by influencing the ionization states of the active components of the cells and substrates (Mu et al. 2007).

The Andrew model (Eq. 6.23) was adopted to describe the effects of H^+ concentration on the specific hydrogen production rate (Wang and Wei 2009). In addition, using it to describe the effects of H^+ concentration on the rates of substrate degradation, HPB growth, and some soluble metabolite production is recommended.

$$R = \frac{R_{\max} \cdot [\mathrm{H}^+]}{K_a + [\mathrm{H}^+] + [\mathrm{H}^+]^2 / K_b} \tag{6.23}$$

As shown in Fig. 6.6, R value increases first and then decreases with increasing H^+ concentration.

In practice, it may be convenient to use pH rather than H^+ concentration in the model. In addition, the Ratkowsky model (Eq. 6.24) may also be a good candidate to describe the effects of pH on R.

$$R = [A \cdot (pH - pH_{\min})]^2 \cdot \{1 - \exp[B \cdot (pH - pH_{\max})]\}^2 \tag{6.24}$$

6.7 The Effect of Dilution Rate on Hydrogen Production

Dilution rate is a very important factor influencing fermentative hydrogen production in a continuous test, because it can affect the ability of HPB to degrade substrate and thus can influence the fermentative hydrogen production process.

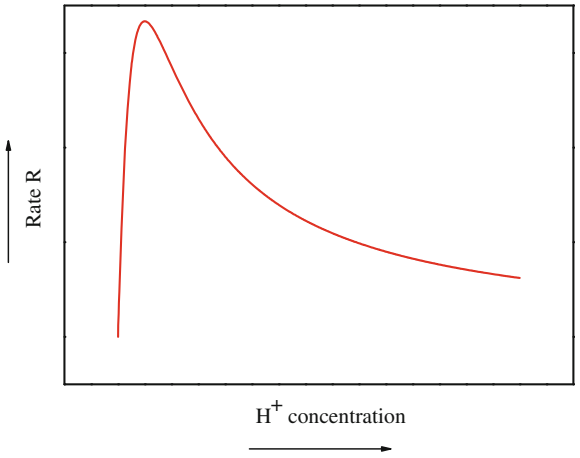

Fig. 6.6 A curve for the Andrew model

Some models have been proposed to describe the effects of dilution rates on hydrogen production rate, hydrogen production, and concentrations of substrate, biomass, and some soluble metabolites in a continuous fermentative hydrogen production process (Chen et al. 2001; Whang et al. 2006).

$$S = \frac{D \cdot K_S}{R_{\max} - D} \tag{6.25}$$

$$S = \frac{(D + k_d) \cdot K_S}{R_{\max} - D - k_d} \tag{6.26}$$

$$X = Y_{X/S} \cdot (S_0 - S) \tag{6.27}$$

$$P = Y_{P/X} \cdot X \tag{6.28}$$

where $R_{\max}$ is the specific HPB growth rate.

As shown in Fig. 6.7, S value increases with increasing dilution rate from 0 to $R_{\max}$ and is a constant with further increasing the dilution rate.

Chen et al. (2001) used the single-substrate models without biomass decay (based on Eqs. 6.25, 6.27 and 6.28) to describe the effects of dilution rates on hydrogen production and concentrations of sucrose, biomass, acetate, propionate, butyrate, and ethanol in a continuous stirred tank reactor for hydrogen production.

Moreover, Whang et al. (2006) compared the ability of three different models (based on Eqs. 6.25, 6.26, 6.27 and 6.28), namely the single-substrate model without biomass decay, the single-substrate model with biomass decay, and the dual-substrate model with biomass decay, to describe the effects of dilution rates on the hydrogen production rate and the concentrations of glucose, peptone, biomass, ammonium nitrogen, formate, acetate, and butyrate in a continuous stirred tank

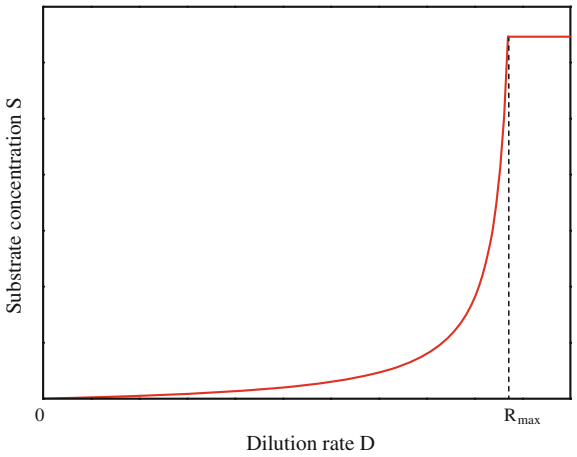

Fig. 6.7 Effect of dilution rates on substrate concentration in a continuous test

reactor for hydrogen production, and concluded that the dual-substrate model with biomass decay was the most suitable one.

In addition, other continuous hydrogen production reactors such as the packed-bed reactors, trickling biofilter, fluidized-bed reactors, and membrane bioreactors may have different property from a continuous stirred tank reactor, thus using these models to describe the effects of dilution rates on such continuous hydrogen production reactors is recommended (Wang and Wan 2009a, b).

In addition, Chang and Lin (2004) used Eq. (6.29) to describe the effects of dilution rates on the specific sucrose degradation rate in an up-flow anaerobic sludge blanket reactor for hydrogen production.

$$R = \frac{D + k_d}{Y_{X/S}} \tag{6.29}$$

6.8 The Relationship Among Substrate Degradation Rate, HPB Growth and Product Formation

The Leudeking–Piret model (Eq. 6.30) and its modified form (Eq. (6.31)) were widely used to describe the relationship between HPB growth rate and product formation rate.

$$\frac{dP}{dt} = Y_{P/X} \cdot \frac{dX}{dt} + \beta \cdot X \tag{6.30}$$

$$\frac{dP}{dt} = Y_{P/X} \cdot \frac{dX}{dt} \tag{6.31}$$

$$\frac{dP}{dt} = -Y_{P/S} \cdot \frac{dS}{dt} \tag{6.32}$$

$$\frac{dX}{dt} = -Y_{X/S} \cdot \frac{dS}{dt} \tag{6.33}$$

Table 6.6 summarizes several studies using the Leudeking–Piret model and its modified form to describe the effects of temperature on fermentative hydrogen production.

Mu et al. (2006) used Eq. (6.32) to describe the relationship between the rate of substrate degradation and the rates of hydrogen production, acetate production and butyrate production, while van Niel et al. (2002) used Eq. (6.33) to describe the relationship between substrate degradation rate and the growth rates of *Caldicellulosiruptor saccharolyticus* and *Thermotoga elfii*.

In addition, since sometimes propionate, ethanol, or formate are formed as soluble metabolites during fermentative hydrogen production, using Eq. (6.32) to

Table 6.6 Several studies using the Leudeking–Piret model and its modified form

Reactor type	Seed	Substrate	Described objective	Correlation coefficient	Reference
Batch	Anaerobic sludge	Sucrose	Hydrogen production rate	0.834	Mu et al. (2006)
Batch	*Clostridium butyricum* CGS5	Sucrose	Hydrogen production rate	Over 0.910	Lo et al. (2008)
Batch	*Clostridium pasteurianum* CH4	Xylose	Hydrogen production rate	Over 0.910	Lo et al. (2008)
Continuous	Municipal sewage sludge	Sucrose	Hydrogen production rate	0.799	Chen et al. (2001)
Batch	*Enterobacter cloacae* IIT-BT 08	Glucose	Specific hydrogen production rate	–	Kumar et al. (2000)
Continuous	Anaerobic sludge	Glucose and peptone	Formate production rate	–	Whang et al. (2006)
Continuous	Anaerobic sludge	Glucose	Formate production rate	–	Whang et al. (2006)
Batch	Anaerobic sludge	Sucrose	Acetate production rate	0.890	(Mu et al. 2006)
Continuous	Municipal sewage sludge	Sucrose	Acetate production rate	0.960	Chen et al. (2001)
Continuous	Anaerobic sludge	Glucose	Acetate production rate	–	Whang et al. (2006)
Continuous	Anaerobic sludge	Glucose and peptone	Acetate production rate	–	Whang et al. (2006)
Batch	*Thermotoga elfii*	Glucose	Acetate production rate	–	Niel et al. (2002)
Batch	*Caldicellulosiruptor saccharolyticus*	Sucrose	Acetate production rate	–	Niel et al. (2002)
Continuous	Municipal sewage sludge	Sucrose	Propionate production rate	0.824	Chen et al. (2001)
Batch	Anaerobic sludge	Sucrose	Butyrate production rate	0.964	Mu et al. (2006)
Continuous	Municipal sewage sludge	Sucrose	Butyrate production rate	0.957	Chen et al. (2001)
Continuous	Anaerobic sludge	Glucose	Butyrate production rate	–	Whang et al. (2006)
Continuous	Anaerobic sludge	Glucose and peptone	Butyrate production rate	–	Whang et al. (2006)
Continuous	Municipal sewage sludge	Sucrose	Ethanol production rate	0.941	Chen et al. (2001)
Batch	Anaerobic sludge	Dairy wastewater	Acidogenic products	0.980	Gadhe et al. (2014)

describe the relationship between the rate of substrate degradation and the production rates of propionate, ethanol or formate is recommended.

Moreover, mixed cultures may have different property from pure cultures, thus using Eq. (6.32) to describe the relationship between substrate degradation rate and some product formation rates by pure cultures and using Eq. (6.33) to describe the relationship between substrate degradation rate and the growth rate of some mixed cultures are recommended.

6.9 Conclusions

Some kinetic models, which were proposed to describe the progress of a batch fermentative hydrogen production process, the effects of substrate concentrations, inhibitor concentrations, temperatures, pH, and dilution rates on a fermentative hydrogen production process, and the relationships among the substrate degradation rate, the hydrogen-producing bacteria growth rate, and the product formation rate have been reviewed. The following conclusions can be drawn from this review.

The modified Gompertz model was widely used to describe the progress of a batch fermentative hydrogen production process, while the Monod model was widely used to describe the effects of substrate concentrations on the rates of substrate degradation, hydrogen-producing bacteria growth and hydrogen production. Arrhenius model was used a lot to describe the effects of temperatures on fermentative hydrogen production, while modified Han–Levenspiel model was used a lot to describe the effects of inhibitor concentrations on fermentative hydrogen production. The Andrew model was used a lot to describe the effects of H^+ concentration on the specific hydrogen production rate, while the Leudeking–Piret model and its modified form were widely used to describe the relationship between hydrogen-producing bacteria growth rate and product formation rate. And more researches on these kinetic models have been recommended.

In addition, a further survey of the literature showed the lack of models that incorporate important parameters affecting hydrogen production like hydrogen partial pressure and regulation mechanisms, such as $NADH/NAD^+$. Thus more researches in this respect should be carried out in the future.

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Chapter 7
Optimization of Hydrogen Production Process

7.1 Overview

Environmental pollution due to the use of fossil fuels as well as their shortfall makes it necessary to find alternative energy sources that are environmentally friendly and renewable. Hydrogen satisfies the above requirements because it produces only water, when it is combusted as a fuel or converted to electricity. Among various hydrogen production processes, biological method is known to be less energy intensive, for it can be carried out at ambient temperature and pressure. Biological method mainly includes photosynthetic hydrogen production and fermentative hydrogen production. The efficiency of photosynthetic hydrogen production is low and it cannot be operated in the absence of light, while fermentative hydrogen production can produce hydrogen continuously without light using various kinds of substrates such as organic wastes. Moreover, compared with photosynthetic hydrogen production, fermentative hydrogen production has higher hydrogen production efficiency, higher hydrogen production stability, higher feasibility for industrialization, simpler control requirement, and lower operating costs. Thus fermentative hydrogen production is more feasible and widely used. In addition, it is of great significance to produce hydrogen from organic wastes by fermentative hydrogen production, because it plays the dual role of waste reduction and energy production. Therefore, fermentative hydrogen production has been received increasing attention in recent years (Li and Fang 2007; Wang and Wan 2008; Wang and Wan 2008; Wang and Wei 2008).

A fermentative hydrogen production process can be conducted using either pure cultures or mixed cultures. However, in a fermentative hydrogen production process using mixed cultures, the hydrogen produced by hydrogen-producing bacteria can be consumed by hydrogen-consuming bacteria. Thus, in order to harness hydrogen from a fermentative hydrogen production process, the seed sludge often needs a pretreatment to suppress as much hydrogen-consuming bacterial activity as

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possible while still preserving the activity of the hydrogen-producing bacteria (Wang and Wan 2008).

Experimental design can be regarded as a process by which certain factors are selected and deliberately varied in a controlled manner to obtain their effects on a response of interest, often followed by the analysis of the experimental results. According to the number of the factors to be investigated at a time, the experimental design can be classified into two categories: one-factor-at-a-time design (single-factor design) and factorial design (multiple-factor design) (Olin 1998).

Experimental design is of great importance to a fermentative hydrogen production process, because the process is very complex and influenced by many factors such as hydrogen-producing bacteria, substrates, inorganic nutrients, operational conditions of the bioreactors, and so on; thus an appropriate experimental design can be used to study the effects of various factors on the process to make it better understood and even optimized to improve its performance.

In this chapter, we will attempt to summarize the experimental design that was used to investigate the effects of various factors on fermentative hydrogen production processes. The experimental design included one-factor-at-a-time design, full factorial design, Taguchi design, Plackett–Burman design, Central Composite Design, and Box–Behnken design. Each design was briefly introduced, followed by the introduction of its analysis and application to the study of fermentative hydrogen production.

7.2 One-Factor-at-a-Time Design

One-factor-at-a-time design is a traditional design, which investigates one factor at a time, while keeping the levels of other factors constant. The level of the factor to be investigated is then changed over a desired range to study its effects on a response. After the experimental results are obtained, certain graphs are usually constructed showing how a response is affected by the one factor studied. Since one-factor-at-a-time design is easy to operate and analyze, it has been widely used to study the effects of various factors on fermentative hydrogen production processes. Table 7.1 summarizes a number of studies using one-factor-at-a-time design to study the effects of various factors on fermentative hydrogen production processes. For example, Kim et al. investigated the effects of sucrose concentration on fermentative hydrogen production using one-factor-at-a-time design, with several graphs being plotted to show the effects of sucrose concentration on hydrogen yield, hydrogen production rate, and specific hydrogen production rate, and then concluded that the optimal sucrose concentration for fermentative hydrogen production was 30 g COD/L. Since they investigated only one factor, namely sucrose concentration, at a time in that study, while keeping the levels of other factors constant, it was easy for them to conduct the experimental design and analyze the obtained results (Kim et al. 2006).

Table 7.1 One-factor-at-a-time design for fermentative hydrogen production processes

Inoculum	Substrate	Factor studied	Reference
Digested sludge	Glucose	Fe^{2+} concentration	Wan and Wang (2008)
Digested sludge	Glucose	Inoculum pretreatment method	Wang and Wan (2008)
Digested sludge	Glucose	Ni^{2+} concentration	Wang and Wan (2008)
Digested sludge	Glucose	Substrate concentration	Wang and Wei (2008)
Pure cultures	Glucose	Inoculum type	Ito et al. (2004)
Recombinant *Escherichia coli* BL-21	Glucose	Inoculum size	Chittibabu et al. (2006)
Escherichia coli MC13-4	Glucose	Cell density	Ishikawa et al. (2008)
Anaerobic sludge	Glucose	Temperature	Mu et al. (2006)
Thermoanaerobacterium thermosaccharolyticum PSU-2	Carbohydrate	Substrate type	O-Thong et al. (2008)
Anaerobic digester sludge	Sucrose	Substrate concentration	Kim et al. (2006)
Cracked cereals	Citric acid wastewater	Organic loading rate	Yang et al. (2006)
Clostridium butyricum CGS5	Carbohydrate	Medium composition	Chen et al. (2005)
Anaerobic digester sludge	Cheese whey permeate powder	Food to microorganism ratio	Yanga et al. (2007)
Cracked cereals	Starch	Nitrogen concentration	Liu and Shen (2004)
Cracked cereals	Starch	Iron concentration	Liu and Shen (2004)
Cracked cereals	Starch	Initial pH	Liu and Shen (2004)
Cracked cereals	Starch	Substrate concentration	Liu and Shen (2004)
Sewage digester sludge	Glucose	Nitrate concentration	Kim et al. (2006)
Wasted activated sludge	Sucrose	C/N ratio	Lin (2004)
Citrobacter sp. Y19	Glucose	Phosphate concentration	Oh et al. (2003)
Digester sludge	Sucrose	Iron concentration	Lee et al. (2001)

(continued)

Table 7.1 (continued)

Inoculum	Substrate	Factor studied	Reference
Fermentative bacteria B49	Glucose	Magnesium concentration	Wang et al. (2007)
Fermentative bacteria B49	Glucose	Iron concentration	Wang et al. (2007)
Fermentative bacteria B49	Glucose	Sparging gas type	Wang et al. (2007)
Pseudomonas sp. GZ1	Wastewater sludge	Substrate pretreatment method	Guo et al. (2008)
Escherichia coli MC13-4	Glucose	Immobilized gel bead size	Ishikawa et al. (2006)
Ruminococcus albus	Glucose	Formate concentration	Ntaikou et al. (2008)
Anaerobic sludge	Glucose	Butyrate concentration	Zheng and Yu (2005)
Compost	Sucrose	Initial pH	Khanal et al. (2004)
Anaerobic sludge	Glucose	pH	Fang and Liu (2002)
Municipal sewage sludge	Xylose	Temperature	Lin et al. (2008)
Clostridium butyricum CGS2	Hydrolyzed starch	Hydraulic retention time	Chen et al. (2008)
Clostridium paraputrificum M-21	N-acetyl-D-glucosamine	Agitation speed	Evvyernie et al. (2000)
Clostridium thermolacticum	Lactose	Dilution rate	Collet et al. (2004)
Wastewater sludge	Sucrose	Liquid reflux	Lee et al. (2004)
Wastewater sludge	Sucrose	Gas reflux	Lee et al. (2004)
Enterobacter cloacae IIT-BT 08	Glucose	Recycle ratio	Kumar and Das (2001)
Enterococcus faecium INET2	Glucose	Temperature, pH, substrate concentration, inoculation proportion	Yin and Wang (2016a, b, c, d)
Citrobacter freundii CWBI952	Glucose	pH	Hamilton et al. (2010)
Clostridium sp. IODB-O3	Wheat straw	Temperature, pH	Patel et al. (2015)

However, one-factor-at-a-time design has two main drawbacks. For one thing, it does not take into consideration the interactions among different factors, which make it not guarantee the optimal conditions identified by it to be optimal, especially when the interactions among different factors are significant. For example, Kim et al. (2006) investigated the effects of only one factor, namely sucrose

concentration on fermentative hydrogen production using one-factor-at-a-time design, and ignored the interactions between sucrose concentration and other factors such as temperature. For another, it involves a relatively large number of experiments, which makes it laborious and time-consuming to carry out the experiments, especially when the number of factors is large (Kennedy and Krouse 1999). For example, Chittibabu et al. (2006) investigated respectively the effects of inoculum size, initial medium pH, initial substrate concentration, temperature, and dilution rate on hydrogen productivity using one-factor-at-a-time design, with around 30 runs of experiments being conducted.

7.3 Factorial Design

On the contrary, factorial design is able to study the effects of more than one factor at two or more levels. The experimental design generally includes various combinations of different factor levels, which enables it to depict the interactions among different factors and to be more efficient to deal with a large number of factors, compared with one-factor-at-a-time design. Factorial design can be classified into two categories: full factorial design and fractional factorial design (Kennedy and Krouse 1999).

Since coded factor levels provide a uniform framework to investigate the effects of a factor in any experimental context, while the actual factor levels depend on a particular factor to be studied, factorial design is usually given in the form of coded factor levels (Anderson and Whitcomb 2001). One can assign each actual factor level to the corresponding coded factor level of a factorial design when using it. The analysis and the model-fitting for a factorial design can be performed based on either the coded factor levels or the actual factor levels. However, in almost all situations, the coded factor level analysis is preferable, because in a coded factor level analysis, the model coefficients are dimensionless and thus directly comparable, which make it very effective to determine the relative size of factor effects (Dean and Voss 1999). In this review, the models are expressed based on coded factor levels. Such models can be expressed based on actual factor levels when necessary.

7.3.1 Full Factorial Design

In a full factorial design, every combination of each factor level is tested. For example, the number of runs for a three-factor full factorial design is $a \times b \times c$, which indicates that the first factor is tested at a levels, the second factor is tested at b levels, while the third factor is tested at c levels. The number of runs for a full factorial design of n factors, each at a levels, is a^n. The most commonly used full factorial design is two-level design, which can be denoted by 2^n when there are n factors (Kennedy and Krouse 1999). Sometimes, an appropriate polynomial

model can be used to describe the effects of the factors studied on a response and then optimize the response when necessary.

Since with a full factorial design, all possible combinations of the factor levels can be investigated, it has been used a lot to study the effects of several factors simultaneously on fermentative hydrogen production processes. Table 7.2 summarizes a lot of studies using full factorial design to study the effects of various factors on fermentative hydrogen production processes. For example, Chou et al. investigated the effects of pH (at 4 levels) and stirring speed (at 6 levels) on fermentative hydrogen production using full factorial design with 24 runs of experiment, with two second-order polynomial models being constructed to describe the effects of the two factors on hydrogen yield and specific hydrogen production rate, and then concluded that the optimal pH and stirring speed for fermentative hydrogen production were 6.0 and 120 rpm, respectively. Since they examined every combination of each pH and stirring speed level, the interactions between the two factors were depicted (Chou et al. 2008).

The number of runs for a full factorial design increases geometrically as the number of factors increases. For example, Espinoza-Escalante et al. investigated the effects of alkalinization, thermical treatment, and sonication (each at 2 levels) on fermentative hydrogen production using full factorial design. If they examined the effects of only two factors on fermentative hydrogen production using full factorial design, 2^2 runs of experiment were required, and if they examined the effects of the three factors on fermentative hydrogen production using full factorial design, 2^3 runs of experiment were required, that is when a factor with 2 levels was added to the full factorial design, the runs of experiment doubled (Espinoza-Escalante et al. 2008). In many instances, when the effects of a large number of factors are to be studied simultaneously, a great many runs of experiment are required. Generally, this will constitute a larger experiment that is not economically and practically feasible.

7.3.2 *Fractional Factorial Design*

It turns out, however, that when the number of runs for a full factorial design is relatively large, the desired information can often be obtained by performing only a fraction of the full factorial design, which is often referred to as fractional factorial design to distinguish it from the full factorial design. In other words, fractional factorial design provides an alternative when the number of runs for a full factorial design is too large to be practicable. With a fractional factorial design, the effects of certain factors on a response can be studied under an economical and practical condition (Olin 1998).

Taguchi design, Plackett–Burman design, central composite design, and Box–Behnken design are fractional factorial designs that were used a lot for fermentative hydrogen production processes. Table 7.3 summarizes some studies using fractional factorial design to study the effects of various factors on fermentative hydrogen production processes.

Table 7.2 Full factorial design for fermentative hydrogen production processes

Inoculum	Substrate	Factors studied	Reference
Municipal sewage sludge	Glucose	Cultivation pH and enrichment pH	Chen et al. (2002)
Anaerobic sludge	Sucrose	Reactor condition and pH	Wang et al. (2007)
Municipal sewage sludge	Sucrose	Temperature and initial pH	Wu and Chang (2007)
Anaerobic digester sludge	Cellulose	Cellulose concentration and sludge density	Lay (2001)
Sewage digester sludge	Sucrose	Hydraulic retention time and calcium concentration	Chang and Lin (2006)
Sludge compost	Garbage slurry	Hydraulic retention time and pH	Ueno et al. (2006)
Thermotoga elfii	Glucose	Glucose, yeast extract and tryptone concentrations	Van Niel et al. (2002)
Anaerobic sludge	Starch	Iron concentration and initial pH	Yang and Shen (2006)
Mixed cultures	Organic solid waste	Inoculum type, inoculum pretreatment method and cultivation temperature	Valdez-Vazquez et al. (2006)
Cracked cereals	Sucrose	Temperature and iron concentration	Zhang and Shen (2006)
Mixed cultures	Carbohydrate	Substrate type and inoculum type	Kalogo and Bagley (2008)
Clostridium thermocellum 27405	Cellulosic biomass	Substrate type and concentration	Levin et al. (2006)
Sewage digester sludge	Glucose	Solid retention time and pH	Lin and Chang (2015)
Dewatered and thickened sludge	Glucose	Ammonia concentration and pH	Salerno et al. (2006)
Mixed and pure cultures	Starch residue	Substrate concentration and inoculum type	Yokoi et al. (2001)
Municipal sewage sludge	Sucrose	Substrate concentration and cell immobilization method	Wu et al. (2002)
Digested sludge	Tequila's stillages	Alkalinization, thermical treatment and sonication	Espinoza-Escalante et al. (2008)
Compost	Spent grains	pH and stirring speed	Chou et al. (2008)
Mixed cultures	Glucose	Inoculum type and heat-shock time	Hu and Chen (2007)
Municipal sewage sludge	Carbohydrate	Hydraulic retention time and substrate type	Chen and Lin (2003)
Municipal sewage sludge	Carbohydrate	Upflow velocity and substrate type	Wu et al. (2007)

Table 7.3 Fractional factorial design for fermentative hydrogen production processes

Inoculum	Substrate	Design	Factors studied	Reference
Wasted activated sludge	Sucrose	Taguchi	A nutrient formulation, 3 carbonate sources, and 3 phosphate sources	Lin and Lay (2004)
Wasted activated sludge	Sucrose	Taguchi	Concentrations of 13 nutrients	Lin and Lay (2005)
Clostridium sp. Fanp2	Glucose	Plackett–Burman	Concentrations of 7 nutrients and initial pH	Pan et al. (2008)
Mixed cultures	Sucrose	Central composite	initial pH and substrate concentration	Ginkel et al. (2001)
Anaerobic sludge	Wheat powder	Central composite	C/N and C/P ratio	Argun et al. (2008)
Mixed cultures	Food residues and manure	Central composite	Hydraulic retention time, temperature, and N_2-flow rate	Karlsson et al. (2008)
Anaerobic digester sludge	Food waste with residual blood	Central composite	Solid content in the feed, proportion of residues, and hydraulic retention time	Cuetos et al. (2007)
Compost	Food wastes	Central composite	PO_4^{3-} , Fe^{2+}, and NH_4^+ concentrations	Lay et al. (2005)
Mixed cultures	Organic municipal solid waste	Central composite	Amounts of hydrogen-producing bacteria, pretreated anaerobic digestion sludge, and organic municipal solid waste	Lay et al. (1999)
Anaerobic digested sludge	Starch	Central composite	Hydraulic retention time and pH	Lay (2000)
Anaerobic sludge	Sucrose	Central composite	Substrate concentration and hydraulic retention time	Zhao et al. (2008)
Cow dung compost	Sucrose	Central composite	Substrate concentration and initial pH	Fan et al. (2004)
Anaerobic sludge	Palm oil mill effluent	Central composite	Fe^{2+} concentration, C/N ratio, and C/P ratio	O-Thong et al. (2008)
Anaerobic sludge	Sucrose	Central composite	pH, temperature, and substrate concentration	Mu et al. (2006)
Anaerobic sludge	Sucrose	Central composite	pH, temperature, and substrate concentration	Wang et al. (2005)
Clostridium sp. Fanp2	Glucose	Box–Behnken	Glucose, phosphate buffer, and vitamin concentrations	Pan et al. (2008)
Enterobacter aerogenes	Glucose	Box–Behnken	pH, temperature, and substrate concentration	Jo et al. (2008)

(continued)

Table 7.3 (continued)

Inoculum	Substrate	Design	Factors studied	Reference
Anaerobic sludge	Glucose	Box–Behnken	pH, temperature, and substrate concentration	Yin and Wang (2016a, b, c, d)
Caloranaerobacter azorensis H53214	Glucose	Plackett–Burman	pH; Temperature; substrate, NaCl, yeast, tryptone, Fe^{2+}, Mg^{2+} concentration	Jiang et al. (2014)

7.3.2.1 Taguchi Design

Taguchi design, which is a fractional factorial design using orthogonal array, allows the effects of many factors with two or more levels on a response, to be studied in a relatively small number of runs. In addition, the orthogonal array facilitates the analysis of the design. When used properly, Taguchi design may provide a powerful and efficient method to find an optimal combination of factor levels that may achieve optimum. Usually, with the aid of range analysis, analysis of variance, or analysis of signal-to-noise ratio, the key factors that have significant effects on a response can be identified and the best factor levels for a given process can be determined from the pre-determined factor levels (Antony 2006).

As shown in Table 7.3, among the reviewed studies, two studies used Taguchi design. For example, Lin and Lay (2005) studied the effects of 13 nutrient concentrations on fermentative hydrogen production using a Taguchi design. Based on the analysis of the experimental results, they determined that magnesium, sodium, zinc, and iron were important trace metals affecting hydrogen production and identified the best nutrient levels for the fermentative hydrogen production process from the pre-determined factor levels. However, the true optimal factor levels may not be guaranteed using Taguchi design, because the true optimal factor levels may be different from the corresponding pre-determined factor levels (Antony 2006).

7.3.2.2 Plackett–Burman Design

In reality, there may be a great number of factors influencing a process, but it does not mean that all the factors have significant effects on it. More often than not, the factors that influence the process greatly may be paid greater attention than those that influence it slightly, because the former are essential to the successful operation of the process. Thus, the first step to optimize a process is to identify which factors have significant effects on the process.

Plackett–Burman design, which is a two-level fractional factorial design developed by Plackett and Burman (1946), has been extensively used to screen important factors for further investigation. In addition, the number of runs for a Plackett–Burman design is equal to a multiple of 4. Plackett–Burman design can examine up to $n = N - 1$ factors in an experiments with N runs and it works for all

such N up to 100, except for 92. If the number of factors to be examined is less than $n = N - 1$, a subset of Plackett–Burman design for N runs can be used. Sometimes, some replications are performed to estimate the experimental errors.

A first-order polynomial model (Eq. (7.1)) is usually used to describe the effects of various factors on it based on the experimental results from a Plackett–Burman design:

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i, \tag{7.1}$$

where y is the response, β_0 is the constant, β_i is the linear coefficient, and x_i is the coded factor levels.

Based on the analysis of variance (ANOVA) of the estimated model, the significant factors can be identified (Plackett and Burman 1946; Weuster-Botz 2000).

As shown in Table 7.3, among the reviewed studies, only the study by Pan et al. (2008) used Plackett–Burman design to study the effects of eight factors on fermentative hydrogen production and then screened three factors (glucose, phosphate buffer, and vitamin solution) that had significant effects on the specific hydrogen production potential for further study based on analysis of the experimental results.

7.3.2.3 Method of Steepest Ascent

Frequently, the initial estimate of the optimal conditions for a bioprocess is far from the actual optimum. Thus, the second step for optimization is to locate the region of factor levels that produce optimal conditions. The method of steepest ascent is a simple and economically efficient procedure developed to move the experimental region of a response in the direction of the maximum change toward the optimum. Of course, if minimization of a response is desired, then this method is referred to as the method of steepest descent. The factors screened by the Plackett–Burman design can be further investigated using this method.

In order to obtain the path of steepest ascent for various factors, a first-order polynomial model (Eq. (7.1)) is usually used to fit the experimental data obtained from a factorial design such as a Plackett–Burman design. The path of steepest ascent is perpendicular to the contour plots of the response based on the estimated first-order polynomial model, and moves β_i units in the x_i direction for every β_j units in the x_j direction. Equivalently, the path has a movement of β_j/β_i units in x_j for every 1 unit movement in x_i. Figure 7.1 shows the contour plot of a response with varying only two factor levels, while keeping other factor levels constant, and the corresponding path of steepest ascent (Anderson and Whitcomb 2001).

The path of steepest ascent starts from the design center of the factorial design building the first-order polynomial model and ends until no further improvement can be achieved in the response, which indicates that the region of optimal response is in the neighborhood of that condition (Anderson and Whitcomb 2001).

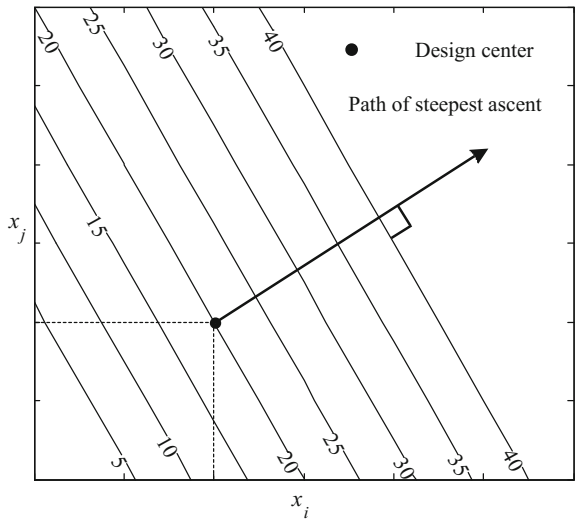

Fig. 7.1 Contour plot of a response and the path of steepest ascent

Among the reviewed studies, only the studies by Pan et al. (2008) and Lay (2000) used the method of steepest ascent to search the region of factor levels that produce optimal conditions for further optimization of fermentative hydrogen production processes. For example, Pan et al. (2008) used the method of steepest ascent to find the design centers of glucose, phosphate buffer, and vitamin solution for further optimization.

7.3.2.4 Central Composite Design and Box–Behnken Design

Once the region of optimal response is identified by the method of steepest ascent, it is often necessary to characterize the response in that region. Central composite design and Box-Behnken design are widely used experimental designs for response surface methodology to estimate a second-order polynomial approximation to a response in that region.

Central composite design is a five-level fractional factorial design developed by Box and Wilson (1951). The design usually consists of a 2^n full factorial design, $2 \times n$ axial designs and m central designs. The axial design is identical to the central design except for one factor, which will take on levels either above the high level or below the low levels of the 2^n full factorial design (Anderson and Whitcomb 2001). For example, O-Thong et al. (2008) studied the effects of Fe^{2+} concentration, C/N ratio, and C/P ratio on fermentative hydrogen production using a central composite design. They concluded that the presence of 257 mg Fe^{2+}/L, C/N ratio of 74, and C/P ratio of 559 were optimal for simultaneous hydrogen production and COD (chemical oxygen demand) removal, and Fe^{2+} concentration

and C/N ratio had the greatest interactive effect on hydrogen production, while C/N and C/P ratio gave more profound interactive effect on COD removal.

Box–Behnken design is a three-level fractional factorial design developed by Box and Wilson (1951). The design can be thought of as a combination of a two-level factorial design with an incomplete block design. In each block, a certain number of factors are put through all combinations for the factorial design, while other factors are kept at the central levels. It usually includes some central designs. For example, Pan et al. (2008) studied the effects of glucose, phosphate buffer, and vitamin solution on fermentative hydrogen production using a Box–Behnken design. They concluded that glucose and vitamin solution, and glucose and phosphate buffer had interactive effects on hydrogen production and the optimal conditions were glucose 23.75 g/L, phosphate buffer 0.159 mol/L, and vitamin solution 13.3 mL/L. Box–Behnken design provides an economical alternative to the central composite design, because it has less factor levels than the central composite design and does not contain extreme high or extreme low levels. For example, Pan et al. (2008) studied the effects of 3 factors, namely glucose, phosphate buffer, and vitamin solution (each at 3 levels), on fermentative hydrogen production using a Box–Behnken design in 15 runs of experiment, while O-Thong et al. (2008) studied the effects of three factors, namely Fe^{2+} concentration, C/N ratio, and C/P ratio (each at 5 levels), on fermentative hydrogen production using a central composite design in 20 runs of experiment.

For response surface methodology, a second-order polynomial model (Eq. (7.2)) is usually proposed to describe the effects of various factors on a response based on experimental results from a central composite design or Box–Behnken design:

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i<j} \beta_{ij} x_i x_j, \tag{7.2}$$

where y is the response, β_0 is the constant, β_i is the linear coefficient, β_{ii} is quadratic coefficient, β_{ij} is the interactive coefficient, and x_i is the coded factor level.

As shown in Fig. 7.2, the estimated second-order polynomial model can be displayed as a surface plot and a contour plot, by varying only two factor levels, while keeping other factor levels constant.

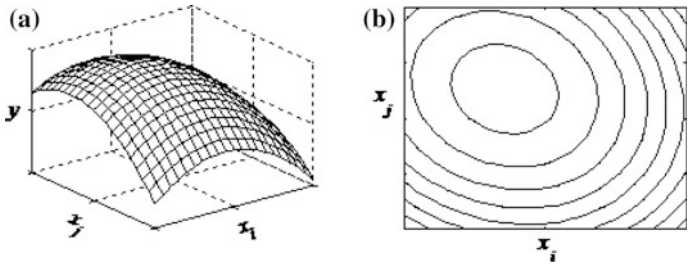

Fig. 7.2 Surface plot (**a**) and contour plot (**b**) for a response

The surface plot and contour plot will visually show the response over a region of interesting factor levels. In addition, they will indicate how sensitive the response is to the change of each factor levels and to what degree the factors interplay as they affect the response.

Based on the analysis of variance (ANOVA) of the estimated model, terms which have significant effects on the response can be determined. In addition, with the aid of the regression model, the optimal response can be estimated by calculating the derivatives of the model.

For example, Jo et al. investigated the effects of glucose concentration, temperature, and pH on the hydrogen production using a Box–Behnken design for response surface methodology. A second-order polynomial model was used to describe the effects of the three factors on the hydrogen production rate. Several surface plots and contour plots were plotted to visually show the effects of the three factors on the hydrogen production rate. Based on the analysis of variance of the estimated model, they concluded that glucose concentration, temperature, and pH all had interactive effects on the hydrogen production rate. In addition, with the aid of the regression model, the optimum conditions obtained by them were glucose concentration 118.06 mmol/L, temperature 38 °C, and pH 6.13 (Jo et al. 2008).

As shown in Table 7.3, central composite design has been used more widely for fermentative hydrogen production processes, compared with Box–Behnken design. Since Box–Behnken design provides an economical alternative to the central composite design, using it in the study of fermentative hydrogen production is recommended.

7.3.2.5 Neural Network and Genetic Algorithm

In recent years, as a mathematical representation of the neurological functioning of a brain, neural network, which is able to describe the interactive effects of various factors on a complicated process, has been applied successfully in a multivariate nonlinear process as a useful tool to construct models. It has been shown that a neural network model is more accurate than a second-order polynomial model as it represents the nonlinearities in a much better way (He et al. 2008).

A neural network model can be considered as the objective function for the purpose of optimization. However, using conventional optimization techniques such as gradient-based methods to optimize a neural network model is not a simple task because it is difficult to calculate the derivatives of the model. Genetic algorithm, which is based on the principles of evolution through natural selection, that is, the survival of the fittest strategy, has established itself as a powerful search and optimization technique to solve problems with objective functions that are not continuous or differentiable. In recent years, genetic algorithm based on a neural network model has been applied successfully to optimize complicated bioprocesses (Nagata and Chu 2003; He et al. 2008).

In addition, Nagata and Chu (2003) showed that the optimal solution identified by response surface methodology was not guaranteed to be optimal due to the poor modeling ability of the second-order polynomial model, while a neural network model had a much higher modeling ability than it, and the optimal solution identified by the genetic algorithm based on a neural network model was much better than that identified by response surface methodology.

In a word, the genetic algorithm based on a neural network model is a better optimization method than response surface methodology. To the best of our knowledge, however, the genetic algorithm based on a neural network model has not been used to optimize a fermentative hydrogen production process, thus using it for such purpose is recommended.

7.3.2.6 Multiple-Response Optimization

Moreover, many experiments involve the optimization of two or more conflicting responses, that is, the optimization of one response usually worsens the optimization of other responses. Simultaneous optimization of multiple responses involves first building an appropriate model for each response and then trying to find a set of operating conditions that in some sense optimizes all responses or at least keeps them in desired ranges.

One useful approach to multiple-response optimization is the method of desirability function (Dean and Voss 1999). The general approach is to first convert each response y_i into an individual desirability function d_i that ranges from 0 to 1. If the response y_i is at its goal or target, then $d_i = 1$, while if the response is outside an acceptable range, then $d_i = 0$. Then the design factor levels are chosen to maximize the overall desirability D (Eq. (7.3)), which is the geometric mean of all the individual desirability functions:

$$D = (d_1 \times d_2 \times \cdots \times d_m)^{1/m}. \tag{7.3}$$

In other words, the simultaneous optimization of several responses can be achieved by determining the maximum of the overall desirability. Thus, the simultaneous optimization of several responses can be reduced to maximizing a single response: the overall desirability.

Among the reviewed studies, Espinoza-Escalante et al. (2008) and Cuetos et al. (2007) used the method of desirability function to optimize several responses simultaneously for fermentative hydrogen production processes, while most other studies optimized several responses separately for fermentative hydrogen production processes. For example, Espinoza-Escalante et al. (2008) optimized several responses, namely COD increment, total sugar consumption, acetic acid increment rate, propionic acid increment rate, butyric acid increment rate, and hydrogen accumulated production simultaneously for a fermentative hydrogen production

process using the method of desirability function. Several second-order polynomial models were used to describe the effects of alkalinization, thermical treatment, and sonication on the above responses, and then each response was converted into an individual desirability function. Subsequently, the geometric mean of the individual desirability functions was built to form the overall desirability. In the end, it was observed that the higher overall desirability value was achieved when Tequila's stillages were pretreated at the alkalinization of 7, thermical treatment of 150 ° C/30 min and sonication of 47 kHz/30 min, which were the global optimal conditions for the above responses obtained by them (Espinoza-Escalante et al. 2008). Otherwise, without multiple-response optimization, they would have had to optimize the above responses separately.

Thus, when there are many responses to be optimized, using the method of desirability function to optimize several responses simultaneously for fermentative hydrogen production processes is highly recommended.

7.4 Recommended Experimental Design Strategy

From the above analysis in this review, the following experimental design strategy for optimizing a fermentative hydrogen production process is highly recommended.

First of all, Plackett–Burman design is used to screen the key factors of a fermentative hydrogen production process for further study. And then, the method of steepest ascent is used to approach the vicinity of the optimal conditions. Subsequently, central composite design or Box–Behnken design for response surface methodology can be used to estimate the relationship between a response and these key factors at the vicinity of optimum and then locate the optimal conditions based on a second-order polynomial model (Dean and Voss 1999).

Among the reviewed studies, only the study by Pan et al. (2008) first used Plackett–Burman design to study the effects of 8 factors on fermentative hydrogen production and then screened three key factors (glucose, phosphate buffer, and vitamin solution). And then they used the method of steepest ascent to find the design centers of the three factors for Box–Behnken design. Subsequently, they used Box–Behnken design for response surface methodology to study the effects of the three factors on fermentative hydrogen production and concluded that the optimal conditions for fermentative hydrogen production were glucose 23.75 g/L, phosphate buffer 0.159 mol/L, and vitamin solution 13.3 mL/L.

Moreover, the genetic algorithm based on a neural network model can be used for optimizing a fermentative hydrogen production process when necessary. In addition, if there are many responses to be optimized for the process, optimizing simultaneously these responses is highly recommended.

7.5 Software Packages for Factorial Design and Analysis

So far, several commercial software packages such as Design-Expert (Stat-Ease, Inc., USA), Minitab (Minitab, Inc., USA), and so on are able to conduct the above-mentioned factorial design such as Taguchi design, Plackett–Burman design, central composite design, and Box–Behnken design and their analysis.

Take using Minitab for example, as for the Plackett–Burman design, one can first use Minitab to generate a Plackett–Burman design with the corresponding high levels and low levels for each factor. And then one can perform the experiment and collect the response data. After that, one can fit the response data using a first-order polynomial model and then analyze the model to determine which factors have significant effects on the responses for further optimization. As for the Box–Behnken design, one can first use Minitab to generate a Box–Behnken design with the corresponding high levels and low levels for each factor. And then one can perform the experiment and collect the response data. After that, one can fit the response data using a second-order polynomial model and then analyze the model to determine which factors have significant effects on the response. If one tries to optimize one response or multiple responses at the same time, one can first set the goal (such as maximum and minimum) for each response to be optimized and then conduct the optimization.

For example, Pan et al. (2008) conducted a Plackett–Burman design and analysis, as well as Box–Behnken design and analysis using Minitab. Each software package has its unique character, thus it is up to the user to decide which one is more suitable.

The training of a neural network and the optimization of a fermentative hydrogen production process by genetic algorithm based on a neural network model can be performed by the software package of Matlab (Mathworks, Inc., USA) using its neural network toolbox and genetic algorithm toolbox, respectively.

In addition, multiple-response optimization for response surface methodology by the method of desirability function can be performed either by the software package of Design-Expert or the software package of Minitab.

Furthermore, multiple-response optimization for several responses based on neural network models can be carried out by genetic algorithm using the software package of Matlab.

7.6 Optimization of Hydrogen Production by RSM

Since fermentative hydrogen production is a complex metabolic process, which can be affected by many factors, such as temperature, pH, substrate concentration, C/N ratio, and various trace elements (Wang and Wan 2009). Appropriate temperature can promote hydrogen production rate, and suitable pH can help improve hydrogen yield through affecting microbial metabolism pathway. Substrate concentration and

the C/N ratio can affect both microbial diversity and metabolic pathway. Various trace elements are vital constitutes of essential enzymes for hydrogen production.

Lots of studies have been conducted to optimize the fermentation conditions (Wang and Wan 2008; Anzola-Rojas et al. 2015; Taherdanak et al. 2016). The optimization methods include one-factor experimental design and multifactor experimental design. Multifactor experimental designs like Orthogonal design, the Plackette–Burman design, and response surface methodology are used in hydrogen production process because it can be less laborious and time-consuming considering various influencing factors. Furthermore, to determine the interaction effects among variables and give closer confirmation of the influencing factors, response surface methodology has been extensively used in optimizing hydrogen production process. Most widely used designs of response surface methodology include central composite design (CCD) and the Box–Behnken design (BBD) (Varrone et al. 2012; Taherdanak et al. 2015; Zhang et al. 2015).

7.6.1 Three-Factor Box–Behnken Design and Response Surface Analysis

Three-factor Box–Behnken design was used to examine the interaction effect of independent variables on response. Temperature (X_1), initial pH (X_2), and substrate concentration (X_3) were taken as independent variables, while cumulative hydrogen production was chosen as the response variable. The levels of the variables and the experimental design are shown in Table 7.4.

A quadratic model (Eq. (7.4)) was used to fit the experimental data obtained from Table 7.4:

$$Y = A_0 + A_1X_1 + A_2X_2 + A_3X_3 + A_{12}X_1X_2 + A_{13}X_1X_3 + A_{23}X_2X_3 + A_{11}X_1^2 + A_{22}X_2^2 + A_{33}X_3^2, \tag{7.4}$$

where Y is the corresponding response variable, X_i (i = 1, 2, 3) are the actual values of the independent variables. A series of designed experiments were conducted to obtain an optimal response and determine the values of A_n (n = 0, 1, 2, 3, 12, 13…). The Design-Expert (Version 8.0.6, Stat-Ease Inc., Minneapolis, USA) software package was used for experimental design, regression, and response surface analysis.

7.6.2 Optimization Using Box–Behnken Design (BBD)

Three factors with three levels of Box–Behnken design (BBD) were adopted to investigate and optimize the effect of process variables on the cumulative hydrogen production. The design matrix of the variables (temperature (X_1/x_1), initial pH

Table 7.4 Experimental design for optimizing fermentative hydrogen production process and the corresponding experimental results

Run	Temperature (°C)		Initial pH		Glucose concentration (g/L)		Cumulative hydrogen production (mL/L)
	X_1	x_1	X_2	x_2	X_3	x_3	Y
1	40	1	10	0	12.5	−1	0
2	25	−1	7.5	1	20	0	1100
3	25	0	5	0	12.5	0	0
4	40	1	7.5	−1	5	0	850
5	40	−1	5	−1	12.5	0	0
6	32.5	0	7.5	1	12.5	1	2680
7	25	−1	7.5	0	5	−1	770
8	32.5	1	5	1	20	0	520
9	32.5	0	10	−1	5	−1	220
10	40	0	7.5	0	20	0	1750
11	32.5	−1	10	0	20	1	2200
12	25	0	10	−1	12.5	1	280
13	32.5	0	7.5	0	12.5	0	2700
14	32.5	1	7.5	0	12.5	1	2750
15	32.5	0	5	1	5	−1	250

(X_2/x_2), and glucose concentration (X_3/x_3)) along with the experimental values of the corresponding response variable (cumulative hydrogen production (Y)) in the uncoded and coded units are shown in Table 7.4.

The response function in terms of actual factors (Eq. (7.5)) was obtained using Eq. (7.4) to fit the experimental data of cumulative hydrogen production:

$$\begin{aligned} Y = {} & -3288.67 + 133.85X_1 + 347.28X_2 - 0.53X_3 - 0.37X_1X_2 + 0.25X_1X_3 + 2.28X_2X_3 \\ & - 2.05X_1^2 - 23.60X_2^2 - 0.76X_3^2. \end{aligned} \tag{7.5}$$

7.6.3 *Analysis of Variance (ANOVA)*

Analysis of variance (ANOVA) was used to examine the significance of the fitting model, along with the linear effect, quadratic effect, and interactive effect of the variables. Higher F-value indicates an adequate description of the variation about its mean. P-values (Prob > F) less than 0.0500 indicate model terms are significant while greater than 0.1000 indicate the model terms are insignificant.

Table 7.5 ANOVA of the fitting model for cumulative hydrogen production

Source	Sum of squares	Degree of freedom	Mean square	F-value	P-value (Prob > F)
Model	152114.00	9	16901.55	17.08	0.0030
X_1	253.12	1	253.125	0.26	0.6345
X_2	4656.12	1	4656.125	4.71	0.0822
X_3	15138.00	1	15138.00	15.30	0.0113
X_1X_2	196.00	1	196.00	0.20	0.6749
X_1X_3	812.25	1	812.25	0.82	0.4065
X_2X_3	7310.25	1	7310.25	7.39	0.0419
X_1^2	49683.69	1	49683.69	50.21	0.0009
X_2^2	80876.31	1	80876.31	81.73	0.0003
X_3^2	6906.69	1	6906.692	6.98	0.0459
Residual	4947.75	5	989.55		
Lack of fit	4921.75	3	1640.583	126.20	0.0079
Pure error	26.00	2	13.00		
Total	157061.70	14			

As shown in Table 7.5, the model F-value of 17.08 and P-value of 0.0030 imply the model was significant. There was only 0.30% chance that a "Model F-value" this large could occur due to the noise. Coefficient of determination (R^2) was 0.9685, which can explain 96.85% variability of the response variable. Thus, Eq. (7.5) could be used in this study to describe the effect of temperatures, initial pH, and substrate concentrations on cumulative hydrogen production significantly.

ANOVA of the fitting model also showed that the linear effect of substrate concentration, interactive effect between initial pH and substrate concentration, and quadratic effect of all three variables had a great impact on cumulative hydrogen ($P < 0.05$). However, linear effect of temperature and initial pH, interactive effect between temperature and initial pH, and between temperature and substrate concentration on cumulative hydrogen production were not significant ($P > 0.05$), indicating that these terms held little influence on cumulative hydrogen production.

Subsequently, the maximum cumulative hydrogen production of 2853 mL/L medium was estimated from Eq. (7.5) at the temperature of 32.9 °C, the initial pH of 7.92, and the glucose concentration of 17.0 g/L. The optimal conditions for hydrogen production were different from our previous study that used heat-shock-treated digested sludge as inoculum (Wang and Wan 2008), which may due to the difference of dominant microorganisms present in differently pretreated mixed cultures.

7.6.4 Response Surface Analysis

Response surface analysis shows the interactions between two variables by keeping the other one at its optimum level for hydrogen production. As shown in Fig. 7.3, A_1 and A_2, B_1 and B_2, and C_1 and C_2 were plotted with substrate concentration, initial pH, and temperature being kept constant at 17.0 g/L, 7.92, and 32.9 °C, respectively. A clear peak point can be found in each response surface plot, which indicates the maximum cumulative hydrogen production could be achieved inside the design boundary of all three variables.

A critical analysis of the response surface plots reveals a significant interaction between initial pH and temperature on cumulative hydrogen production (Fig. 7.3A), which means that different temperatures were favored for hydrogen

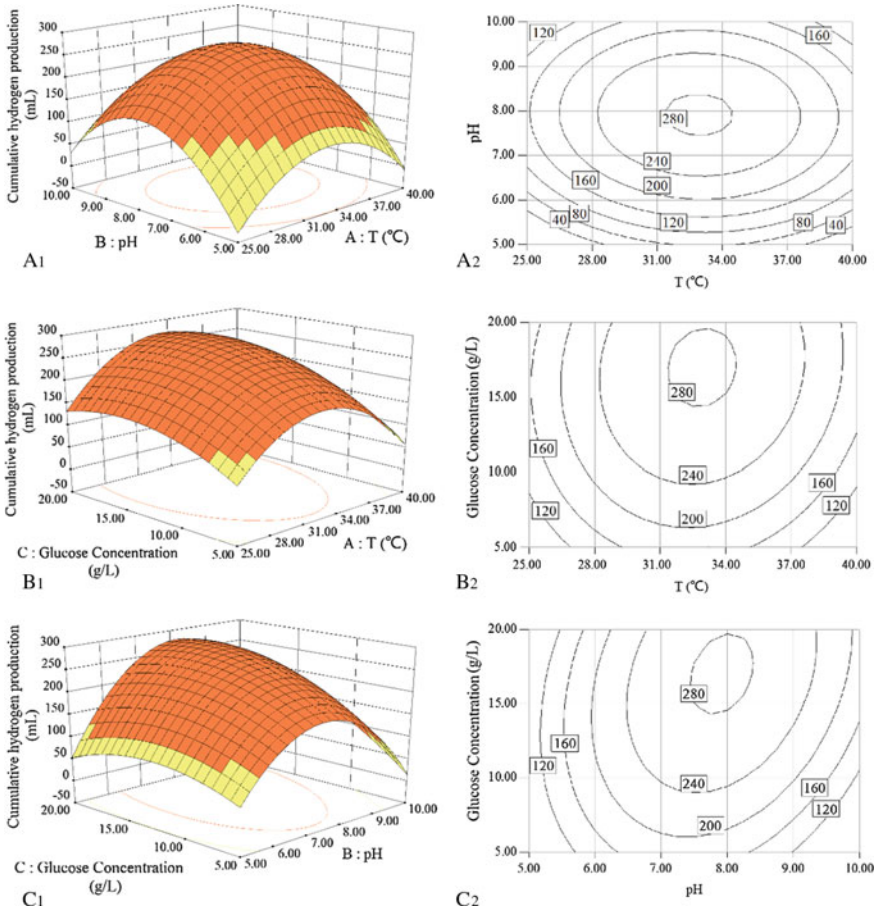

Fig. 7.3 Response surface plot and corresponding contour plot for cumulative hydrogen production

production when the mixed cultures were set into different initial pH environment. In case of the interaction between substrate concentration and temperature, cumulative hydrogen production was observed to increase with the increase of glucose concentration, and reached its maximum at glucose concentration of 17.0 g/L, as shown in Fig. 7.3B, and further increase of substrate concentration led to a little decrease in cumulative hydrogen production, which may be because of the substrate inhibition. A similar conclusion was also drawn by Wang and Wan (2008). It is worth mentioning that the lower glucose concentration preferred for hydrogen production at lower temperature, while higher glucose concentration preferred at higher temperature. One possible reason is that different temperatures are favored by different microbial species and enzymes, resulting in the change of metabolism pathway of the mixed consortia and further led to the different hydrogen production process. Similar phenomenon was observed in case of interaction between substrate concentration and initial pH. As shown in Fig. 7.3C, when the initial pH of the medium was at a low level, maximum cumulative hydrogen production was obtained at lower glucose concentration. However, highest hydrogen production was achieved at maximal glucose concentration when initial pH was 10. This phenomenon indicates that higher initial pH can help decrease the effect of substrate inhibition. Since the fermentative hydrogen production is accompanied with the accumulation of volatile fatty acids (VFA), which can lead to the pH decrease (Dahiya et al. 2015). Higher initial pH can help to dissolute the formed VFA, leading to the alleviation of product inhibition.

From Fig. 7.3 we can see that temperature, initial pH, and substrate concentration all had significant influence on hydrogen production. Temperature affects the microbial activity greatly, and low temperature may inhibit the vital enzymes for hydrogen production and lead to both low hydrogen production rate and low substrate utilization rate. Although there is no final conclusion of optimal temperature for fermentative hydrogen, best hydrogen productions were always obtained at around 37 °C for mesophilic reactions and 55 °C for thermophilic reactions (Wang and Wan 2009). The value of pH affects the electric charge on the cell membrane, which influences both microbial enzyme activity and nutrient absorption. The optimum pH for fermentative hydrogen production ranges from pH 4.5 to 9, pH lower than 4.5 can lead to the deterioration of microorganisms and further suppress the hydrogen production process (Ghimire et al. 2015). Thus, with a low initial pH, the decrease of pH can easily prevent substrate from being further used for hydrogen production. Increase of substrate concentration to a certain extent can usually lead to the increase of hydrogen production. However, too high concentration results in quick pH decrease, accumulation of VFA, and other metabolites that may inhibit hydrogen producers, leading to low hydrogen production.

7.6.5 Hydrogen Production at Optimal Conditions

Based on the experiments described above, fermentative hydrogen production by gamma irradiation pretreated digested sludge was carried out under optimized conditions (temperature 32.9 °C, pH 7.92, glucose concentration 17.0 g/L) in batch mode.

As shown in Fig. 7.4a, fermentation process finished in 24 h fermentation, maximum cumulative hydrogen production of 3000 mL/L medium, and hydrogen yield of 1.81 mol H_2/mol glucose was achieved. Substrate utilization was accompanied with hydrogen production and the degradation rate reached 78.1% at the end of fermentation. The maximum cumulative hydrogen production was higher than we have obtained (2647 mL/L) in the previous study since the operation conditions were optimized (Yin et al. 2014a, b). However, the hydrogen yield and substrate degradation rate were all lower; possible reason is high substrate concentration resulted in the incomplete degradation and conversion of glucose to hydrogen. Many studies have found that the improvement of substrate concentration can lead to the decrease of hydrogen yield and substrate degradation (Kim et al. 2006; Varrone et al. 2012; Robledo-Narváez et al. 2013).

Figure 7.4b depicts the microorganism growth during the fermentation process. The cell growth was consistent with the hydrogen generation and substrate

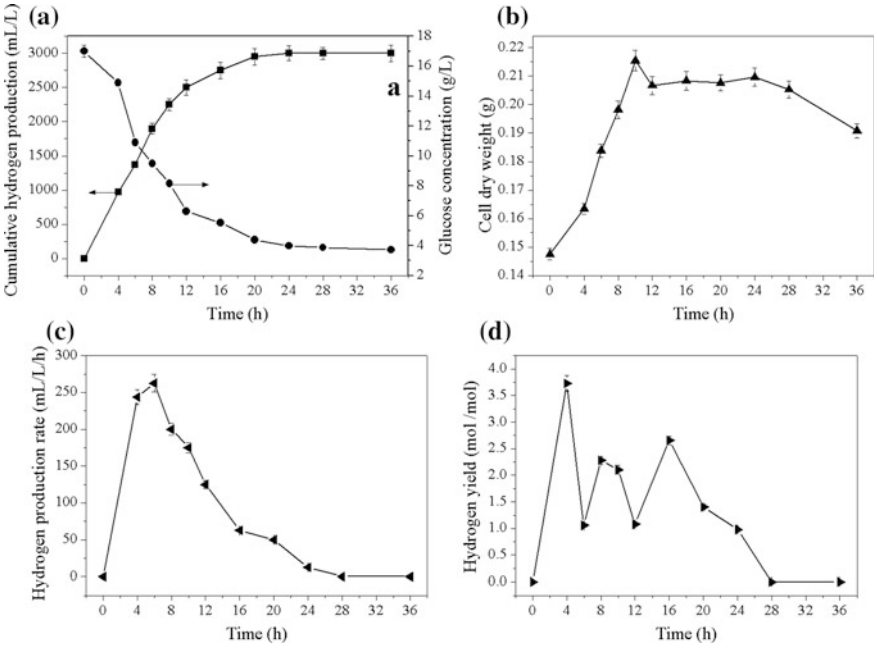

Fig. 7.4 The profile of hydrogen production at optimal conditions: **a** cumulative hydrogen production and substrate degradation; **b** cell growth; **c** hydrogen production rate; **d** hydrogen yield

consumption. The bacteria entered the exponential growth phase directly without experiencing a lag phase; it may because of the inoculum was precultured for 36 h before being inoculated for hydrogen production. Similar phenomenon was also observed by Harun et al. (2012) and Abdeshahian et al. (2014). Our previous studies also found that lag time of hydrogen production can be shortened prominently through preculturing the inoculum (Yin et al. 2014a, b). Besides, the optimized conditions and sufficient nutrients in culture medium could also attribute to early exponential phase (Harun et al. 2012; Gadhe et al. 2014).

The exponential phase continued up to 10 h and followed by stationary phase, which lasted for 14 h. The hydrogen production was consistently maintained throughout the exponential and stationary phase, and higher hydrogen production rate was obtained when the microorganisms were at their exponential phase (Fig. 7.4c). Studies done by Abdeshahian et al., Singh et al., and Wang et al. had come to the same conclusion with fermentative hydrogen production inoculated a *Clostridium* strain, *thermosaccharolyticum* strain, and a newly isolated hydrogen-producing strain, respectively (Wang et al. 2007; Abdeshahian et al. 2014; Singh et al. 2014). However, a different phenomenon was observed by Harun et al. who employed *Enterobacter cloacae* as inoculum, got highest hydrogen production rate both at exponential and stationary phase (Harun et al. 2012). After 24 h, the bacteria entered the decline phase and hydrogen production terminated accordingly.

Figure 7.4d depicts the hydrogen yield at different fermentation time intervals. The highest hydrogen yield was achieved in the first 4 h, and fluctuated in the following 20 h. The significant difference of hydrogen yield can be attributed to the change of metabolic pathways by the mixed cultures at different time intervals.

As demonstrated in Fig. 7.5a, with the accumulation of hydrogen, formation of acidic metabolites and decrease of pH happened correspondingly. The pH showed a significant decline from 7.92 to 4.77 during the first 6 h, and then dropped gradually to around 4.5 in the following 20 h and stayed consistent. Similar trend has also been observed by Harun et al. (2012) and Singh et al. (2014), which may due to the accumulation of volatile fatty acids. Over the time, drop of pH inhibited both microorganism growth and hydrogen production; no more hydrogen was produced when pH value achieved 4.5. Similar phenomenon has been reported in literature (O-Thong et al. 2008; Ren et al. 2008; Harun et al. 2012).

Figure 7.5b depicts the generation of volatile fatty acids at different time intervals during the hydrogen production process. It can be seen that in the first 10 h, formic acid, acetic acid, propionic acid, and butyric acid were all produced during this period, indicating that the culture followed mixed acid pathway. Then, in the following 14 h, acetic acid and butyrate acid were accumulated as the main soluble metabolites, indicating that the fermentation transformed to acetate butyrate pathway. Possible reason is that in the first 10 h, nutrients and pH conditions are suitable for microbial growth and metabolism; the culture was rich in biodiversity, leading to various metabolism pathway and mixed acids generation. However, after 10 h fermentation, with the accumulation of soluble metabolites, pH decreased and substrate depleted, and lots of microbes were inhibited. As demonstrated in our

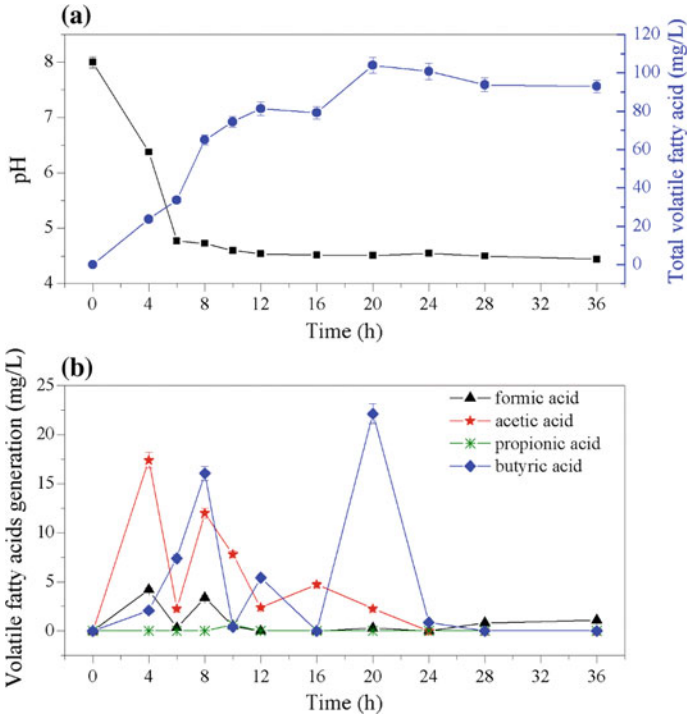

Fig. 7.5 The profile of soluble metabolites during hydrogen production: **a** total volatile fatty acid and pH changes; **b** volatile fatty acids generation at different time intervals

previous study, the mixed culture showed great microbial diversity after gamma irradiation pretreatment while after fermentation process, *Clostridium* species became dominant and occupied over 90% (Yin and Wang 2016a, b, c, d), which has been reported to undergo butyrate-type fermentation (Chen et al. 2005; Lo et al. 2008; Abdeshahian et al. 2014; Yin and Wang 2016a, b, c, d). Thus, the formation of various acids in the first 10 h shows the active effect of diverse bacteria, and acetate butyrate pathway in the late phase was due to the dominant performance of *Clostridium* species. No generation of formic acid after 8 h may be due to the low pH induced activity of formate-hydrogen lyase (Hakobyan et al. 2005).

Lots of studies have proved the relationship between metabolites generation and hydrogen production process (Barca et al. 2015; Dahiya et al. 2015). Studies done by Badiei et al. have shown a positive relationship between hydrogen and butyrate generation (Badiei et al. 2011), and in this study, highest hydrogen production rate of 262.5 mL/L/h was achieved when butyrate acid was continuously generated (Fig. 7.4c). Furthermore, hydrogen yield (Fig. 7.4d) also showed a similar trend with the generation of acetic acid. Highest hydrogen yield was corresponded with the peak generation of acetic acid during the process. As widely accepted that the

theoretical maximum hydrogen production of 4 mol can be produced from 1 mol of glucose in acetate type fermentation, thus it is reasonable for the positive correlation between hydrogen yield and acetate generation.

7.7 Genetic Algorithm for H_2 Production Optimization

Neural network (NN) is able to depict the interactive effect among the variables in complicated bioprocess, which has been applied successfully in multivariate nonlinear bioprocesses as a useful tool to construct models. Usually, neural network is a superior and more accurate modeling technique compared to the response surface methodology method as it represents the nonlinearities in a much better way (Liu et al. 1999; Nagata and Chu 2003; He et al. 2008).

A neural network model can be considered as the objective function for the purpose of optimization. However, using conventional optimization techniques such as gradient-based methods to optimize a neural network model is not a simple task because it is difficult to calculate the derivatives of the model. Genetic algorithm (GA), which is based on the principles of evolution through natural selection, that is, the survival of the fittest strategy, has established itself as a powerful search and optimization technique to solve problems with objective functions that are not continuous or differentiable. In recent years, genetic algorithm based on neural network models has been applied successfully to optimize complicated bioprocesses (Nagata and Chu 2003; He et al. 2008).

The modeling abilities of RSM models and NN models, as well as the optimizing abilities of RSM and the genetic algorithm based on neural network models, were compared during medium optimization. Nagata and Chu (2003) reported that NN models had a higher modeling ability than RSM models and the genetic algorithm based on neural network models had a higher optimizing ability that RSM. To the best of our knowledge, however, such a comparison is not available during the optimization of fermentative hydrogen production process. The effects of temperatures, initial pH, and glucose concentrations on fermentative hydrogen production by mixed cultures were investigated in batch tests and then the modeling abilities of RSM models and NN models, as well as the optimizing abilities of RSM and the genetic algorithm based on a neural network model, were compared, with the purpose of obtaining the best optimization method for fermentative hydrogen production process.

7.7.1 Experimental Design and Procedures

A three-factor central composite design (CCD) was used to design the experiment for constructing models. Hydrogen yield was chosen as the response variable, while temperatures (X_1), initial pH (X_2), and glucose concentrations (X_3) were chosen as

Table 7.6 Independent variables and experimental design levels for CCD

Independent variables	Level				
	−1.682	−1	0	1	1.682
Temperature (°C)	31.6	35	40	45	48.4
Initial pH	5.3	6.0	7.0	8.0	8.7
Glucose concentration (g/L)	16.6	20.0	25.0	30.0	33.4

three independent variables. Table 7.6 summarizes the experimental design levels for CCD. The experimental design is shown in Table 7.7.

According to the experimental design in Table 7.7, batch tests were conducted for hydrogen production. The modified Logistic model was used to fit the cumulative hydrogen production data obtained from each batch test to obtain hydrogen production potential (Wang and Wei 2008).

Table 7.7 Experimental design for constructing RSM and NN models

Run	Temperature (°C)	Initial pH	Glucose concentration (g/L)	Hydrogen yield (mL/g glucose)		
				Experimental	Predicted by RSM	Predicted by NN
1	35.0	6.0	20.0	123.1	118.3	122.6
2	35.0	6.0	30.0	71.6	88.1	71.4
3	35.0	8.0	20.0	200.9	208.9	200.4
4	35.0	8.0	30.0	169.3	180.3	169.2
5	45.0	6.0	20.0	75.1	88.8	75.0
6	45.0	6.0	30.0	37.7	54.6	37.6
7	45.0	8.0	20.0	94.8	103.0	93.5
8	45.0	8.0	30.0	40.5	70.4	40.3
9	31.6	7.0	25.0	131.9	143.6	131.5
10	48.4	7.0	25.0	30.1	26.5	29.6
11	40.0	5.3	25.0	49.0	56.0	48.8
12	40.0	8.7	25.0	145.4	146.6	144.1
13	40.0	7.0	16.6	195.0	207.4	194.4
14	40.0	7.0	33.4	158.6	154.6	158.6
15	40.0	7.0	25.0	282.3	279.8	270.3
16	40.0	7.0	25.0	254.4	279.8	270.3
17	40.0	7.0	25.0	279.5	279.8	270.3
18	40.0	7.0	25.0	268.6	279.8	270.3
19	40.0	7.0	25.0	268.6	279.8	270.3
20	40.0	7.0	25.0	270.7	279.8	270.3
RMSE					12.8	5.0
SEP					8.1%	3.2%

7.7.2 Response Surface Methodology

Equation (7.6) was used to fit the experimental data of hydrogen yield to construct the RSM model (Argun et al. 2008; Jo et al. 2008; O-Thong et al. 2008; Pan et al. 2008):

$$Y = A_0 + A_1X_1 + A_2X_2 + A_3X_3 + A_{12}X_1X_2 + A_{13}X_1X_3 + A_{23}X_2X_3 + A_{11}X_1^2 + A_{22}X_2^2 + A_{33}X_3^2, \tag{7.6}$$

where X_1, X_2, and X_3 are the actual values of the independent variables; Y is the corresponding response variable; A_0 is the constant; A_1, A_2, and A_3 are the linear coefficients; A_{12}, A_{13}, and A_{23} are the interactive coefficients; and A_{11}, A_{22}, and A_{33} are the quadratic coefficients.

Subsequently, the maximum response variable and the corresponding variables were estimated from Eq. (7.6).

7.7.3 Neural Network

A feed-forward neural network with back propagation (BP) algorithm was used (Nagata and Chu 2003). In this training process, the error between the experimental data and the corresponding predicted data is calculated and propagated backward through the network. The algorithm adjusts the weights in each successive layer to reduce the error. This procedure is repeated until the error between the experimental data and the corresponding predicted data satisfies certain error criterion.

Root mean squares error (RMSE) and standard error of prediction (SEP) were calculated to evaluate the modeling abilities of RSM model and NN model (Miller 1959). RMSE was calculated by Eq. (7.7), while SEP was calculated by Eq. (7.8):

$$\mathrm{RMSE} = \sqrt{\frac{\sum_{i=1}^{n}\left(Y_{i,e} - Y_{i,p}\right)^2}{n}} \tag{7.7}$$

$$\mathrm{SEP} = \frac{\mathrm{RMSE}}{\overline{Y}_e} \times 100\%, \tag{7.8}$$

where $Y_{i,e}$ is the experimental data, $Y_{i,p}$ is the corresponding data predicted, $\overline{Y}_e$ is the mean value of experimental data, and n is the number of the experimental data. Generally speaking, the smaller the RMSE and the SEP, the higher the modeling ability a given model has.

In addition, the data predicted by the RSM model and NN model were plotted against the corresponding experimental data to visualize the modeling abilities of the RSM model and NN model. The much closer to the line of perfect prediction

(the line on which the data predicted by a model are all equal to the corresponding experimental data) for the data points, the higher the modeling ability a given model has.

7.7.4 Genetic Algorithm

The genetic algorithm explores all regions of the solution space using a population of individuals. Each individual represents a set of independent variables. The individual chosen in this study was a set of temperature, initial pH and glucose concentration. Initially, a population of individuals is formed randomly. The fitness of each individual is evaluated using a fitness function. Upon completion of the fitness evaluation, genetic operations such as mutation and crossover are applied to individuals selected according to their fitness to produce the next generation of individuals for fitness evaluation. This process continues until an optimal solution is found.

The NN model was used as the fitness function for genetic algorithm to optimize fermentative hydrogen production process.

The modified Logistic model was used to fit the cumulative hydrogen production data, and the hydrogen yield was calculated and shown in Table 7.7.

7.7.5 Comparison of the Modeling Abilities of RSM Model and NN Model

The data of experimental hydrogen yield in Table 7.7 were used in constructing the RSM model. The RSM model (Eq. (7.9)) was obtained using Eq. (7.6) to fit the experimental data of hydrogen yield:

$$Y = -8962.22 + 241.57X_1 + 1042.08X_2 + 67.90X_3 - 3.82X_1X_2 - 0.04X_1X_3 + 0.08X_2X_3 - 2.76X_1^2 - 61.76X_2^2 - 1.40X_3^2, \tag{7.9}$$

where X_1, $X2$, and X_3 are the actual values of temperature (°C), initial pH, and glucose concentration (g/L), respectively. Y is the corresponding hydrogen yield (mL/g glucose).

Analysis of variance of the RSM model showed that the RSM model was significant ($p < 0.05$), which indicated that it could describe the effect of temperatures, initial pH, and glucose concentrations on the hydrogen yield of this study very well. And then, the data of hydrogen yield predicted by RSM model are listed in Table 7.7.

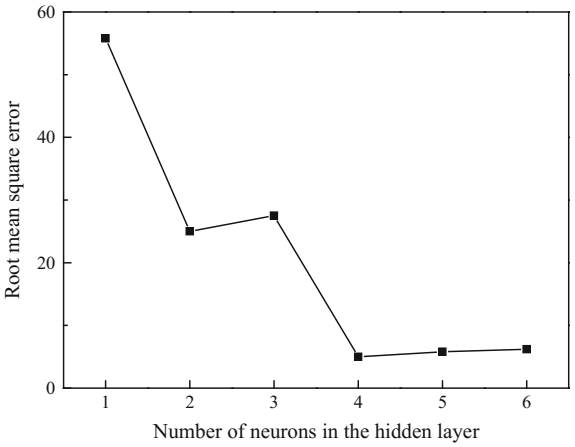

Fig. 7.6 Effect of number of neurons in the hidden layer on the root square error

The inputs chosen in this study were temperatures, initial pH, and glucose concentrations, respectively, while the output was hydrogen yield. All the inputs and output were normalized within a uniform range to ensure that they receive equal attention during the training process (Maier and Dandy 2000).

The first step in training a neural network is to design the topology of the neural network. The number of neurons in the input layer is fixed by the number of inputs, whereas the number of neurons in the output layer equals the number of outputs. The critical aspect is the choice of the number of neurons in the hidden layers (Maier and Dandy 2000). To obtain the optimal number of neurons in the hidden layer of the neural network, the number of neurons in the hidden layer was investigated.

Figure 7.6 shows the root mean square error between the experimental data and the corresponding predicted data at different numbers of neurons in the hidden layer.

The RMSE between experimental data and the corresponding predicted data decreased with increasing the number of neurons in the hidden layer from one to four, while increased slightly with further increasing of the neuron number from four to six. This indicated that when the number of neurons in the hidden layer was four, the NN model could model the data of hydrogen yield data better. Thus, four neurons were chosen in the hidden layer for the neural network. The neural network architecture in this study consists of three neurons (temperature, initial pH, and glucose concentration) in the input layer, four neurons in the hidden layer, and one neuron (hydrogen yield) in the output layer (topology 3-4-1). Training a neural network is accomplished by adjusting the weight coefficients in each successive layer to minimize the root mean square error between experiment data and the corresponding predicted data.

The data of experimental hydrogen yield in Table 7.7 used in constructing the RSM model were selected for training the NN model. And then, the data of hydrogen yield predicted by NN model were also listed in Table 7.7.

The RMSE and SEP for the RSM model and NN model were calculated by Eqs. 7.3 and 7.4, respectively, and listed in Table 7.7. As shown in Table 7.7, the RMSE (12.8) and SEP (8.1%) for the RSM model were both much larger than those (5.0 and 3.2%, respectively) for the NN model, indicating that the NN model had a much higher modeling ability than the RSM model.

The data of hydrogen yield predicted by RSM model and NN model were plotted against the corresponding experimental data of hydrogen yield, as shown in Fig. 7.7. It is obvious that the neural network predictions were much closer to the line of perfect prediction than the RSM predictions, indicating that the NN model had a much higher modeling ability than the RSM model, which was also reported by other studies (Liu et al. 1999; He et al. 2008).

Another four runs of experiment (Table 7.8) were carried out to test the RSM model and the NN model.

The RMSE and SEP for the RSM model and NN model were calculated by Eq. (7.7) and Eq. (7.8), respectively, and listed in Table 7.8. As shown in Table 7.8, the RMSE (38.4) and SEP (16.6%) for the RSM model were both much

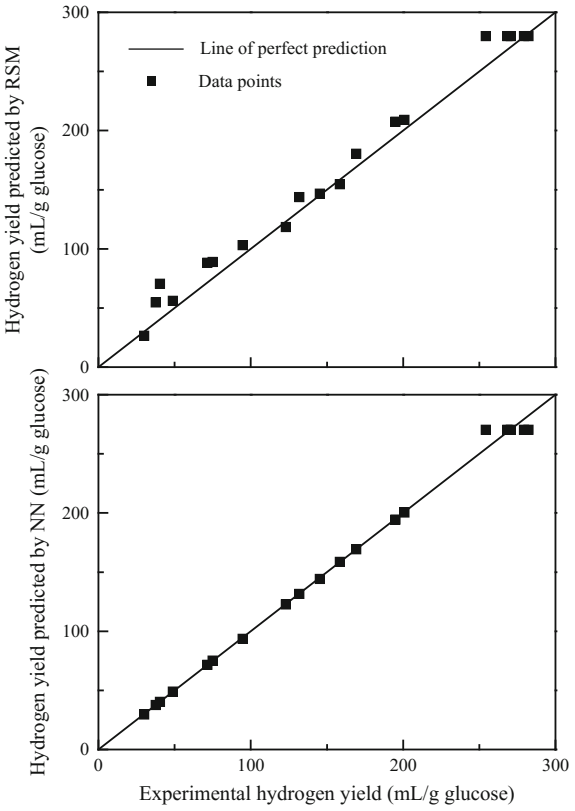

Fig. 7.7 Hydrogen yield predicted by RSM and by NN model in model constructing set

Table 7.8 Experimental design for testing the RSM and NN models

Run	Temperature (°C)	Initial pH	Glucose concentration (g/L)	Hydrogen yield (mL/g glucose)		
				Experimental	Predicted by RSM	Predicted by NN
1	37.5	6.5	22.5	220.4	245.4	214.1
2	37.5	7.5	27.5	233.6	266.4	210.6
3	42.5	6.5	27.5	175.4	204.2	153.8
4	42.5	7.5	22.5	295.6	237.5	310.7
RMSE					38.4	17.8
SEP					16.6%	7.7%

larger than those (17.8 and 7.7%, respectively) for the NN model, also indicating that the NN model had a much higher modeling ability than the RSM model.

The data of hydrogen yield predicted by RSM model and NN model were plotted against the corresponding experimental data of hydrogen yield, as shown in Fig. 7.8. It is obvious that the neural network predictions are much closer to the line of perfect prediction than those for RSM prediction, indicating that the NN model had a much higher modeling ability than the RSM model.

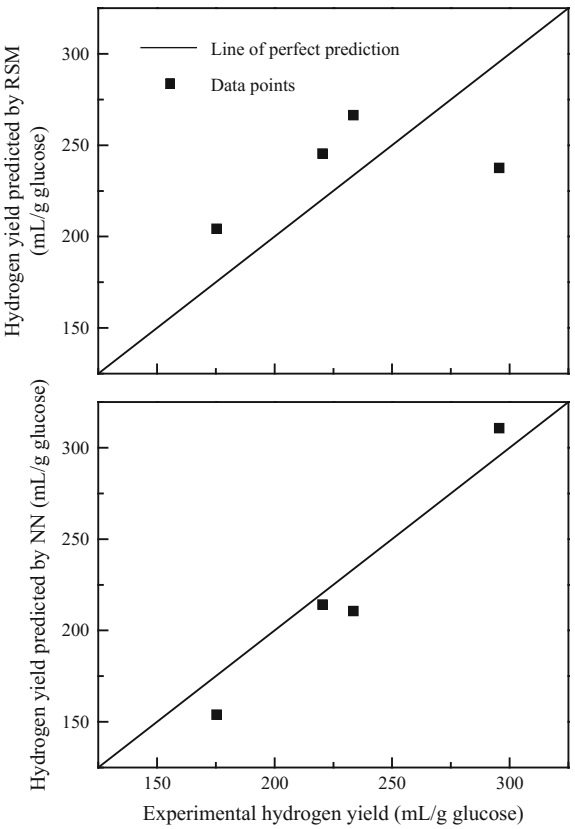

Fig. 7.8 Hydrogen yield predicted by RSM and by NN model in model testing set

7.7.6 Comparison of the Optimizing Abilities of RSM and GA Based on a NN Model

By calculating the derivatives of the RSM model, the maximum hydrogen yield of 289.8 mL/g glucose was estimated at the temperature of 38.6 °C, the initial pH of 7.2, and the glucose concentration of 23.9 g/L, which is shown in Table 7.9.

Once a satisfactory neural network model was created over the ranges of independent variables of interest, it can be used for optimization. For the fermentative hydrogen production process examined in this study, the optimal value of hydrogen yield was obtained using the genetic algorithm to optimize the input space of the neural network model developed. The results obtained are shown in Table 7.9 together with the input conditions that result in the maximum hydrogen yield.

As can be seen from Table 7.9, the maximum hydrogen yield of 289.8 mL/g glucose identified by RSM was a little lower than that of 360.5 mL/g glucose identified by the genetic algorithm based on a neural network, indicating that the genetic algorithm based on a neural network had a much higher optimizing ability than the RSM. Another 2 runs of experiment (Table 7.9) were carried out to validate the optimal conditions identified by RSM and the genetic algorithm based on a neural network. The experimental hydrogen yield under the optimal conditions identified by the genetic algorithm based on a neural network was 355.9 mL/g glucose, which was higher than the experimental hydrogen yield of 285.7 mL/g glucose under the optimal conditions identified by RSM, indicating that the genetic algorithm based on a neural network model had a much higher optimizing ability than the RSM.

These maximum hydrogen yield identified by the genetic algorithm based on a neural network was higher than that identified by RSM, indicating that the optimal solution obtained by RSM was not guaranteed to be optimal, which was also reported by other studies (Nagata and Chu 2003; He et al. 2008).

In addition, genetic algorithm was used to optimize the RSM model and the optimal solution identified was the same as that obtained by calculating the derivatives of the RSM model. This showed that it was the poor modeling ability of the RSM model, but not the method of optimization used (derivative estimation or genetic algorithm) that made the optimal solution identified by RSM be not guaranteed to be optimal.

Table 7.9 Experimental design for validating the optimal conditions identified by RSM and GA

Run	Temperature (°C)	Initial pH	Glucose concentration (g/L)	Hydrogen yield (mL/g glucose)		
				Experimental	Identified by RSM	Identified by GA
1	38.6	7.2	23.9	285.7	289.8	289.8
2	39.2	7.8	20.8	355.9	–	360.5

Although RSM have been used for fermentative hydrogen production process optimization (Argun et al. 2008; Jo et al. 2008; O-Thong et al. 2008; Pan et al. 2008), the optimal solution identified by RSM is not guaranteed to be optimal due to its poor modeling ability. However, a neural network model had a much higher modeling ability than the RSM model, and the optimal solution identified by the genetic algorithm based on a neural network model was much better than that identified by RSM.

The root mean square error and standard error of prediction for the neural network model were much smaller than those for the response surface methodology model, indicating that the neural network model had a much higher modeling ability than the response surface methodology model. The maximum hydrogen yield of 289.8 mL/g glucose identified by response surface methodology was a little lower than that of 360.5 mL/g glucose identified by the genetic algorithm based on a neural network model, indicating that the genetic algorithm based on a neural network model had a much higher optimizing ability than the response surface methodology. Thus, the genetic algorithm based on a neural network model is a better optimization method than response surface methodology and is recommended to be used during the optimization of fermentative hydrogen production process.

7.8 Optimization by Desirability Function Based on NN

Neural network can be considered as the objective function for optimization by genetic algorithm, which has established itself as a powerful search and optimization technique to solve problems with objective functions that are not continuous or differentiable. It has been reported that the genetic algorithm based on neural network had a higher optimizing ability than response surface methodology (Liu et al. 1999; Nagata and Chu 2003; He et al. 2008). Moreover, the method of desirability function can be used to optimize several responses simultaneously, when there are many responses to be optimized for a bioprocess. Otherwise, without multiple-response optimization, several responses would have to be optimized separately (Cuetos et al. 2007; Li et al. 2007; Espinoza-Escalante et al. 2008). Simultaneous optimization of multiple responses by the method of desirability function involves first building an appropriate model for each response and then trying to find a set of operating conditions that in some sense optimizes all responses or at least keeps them in desired ranges. The effects of temperature, initial pH, and glucose concentration on fermentative hydrogen production by mixed cultures were investigated in batch tests and described by neural network, and then the process was optimized by the method of desirability function based on neural network, with the purpose of obtaining the optimal conditions for the fermentative hydrogen production process.

7.8.1 Experimental Design and Procedures

A three-factor fractional factorial design was used to design the experiment for constructing neural network. Temperature, initial pH, and glucose concentration were chosen as the factors, while substrate degradation efficiency, hydrogen yield, and average hydrogen production rate were chosen as the responses. The experimental design is shown in Table 7.10.

Table 7.10 Experimental design for constructing neural network

Temperature (°C)	Initial pH	Glucose concentration (g/L)	Substrate degradation efficiency (%)	Hydrogen yield (mL/g glucose)	Average hydrogen production rate (mL/h)
30.0	6.0	10.0	70.5	236.8	8.4
30.0	7.0	10.0	80.7	262.0	9.8
30.0	8.0	10.0	75.8	224.7	9.1
30.0	9.0	10.0	52.5	136.5	6.3
31.6	7.0	25.0	79.0	131.9	12.3
35.0	6.0	10.0	82.6	278.8	9.7
35.0	6.0	20.0	74.8	123.1	12.1
35.0	6.0	30.0	83.0	71.6	12.2
35.0	8.0	10.0	83.6	264.6	10.5
35.0	8.0	20.0	75.0	200.9	15.6
35.0	8.0	30.0	83.0	169.3	18.9
35.0	9.0	10.0	64.6	160.7	7.3
40.0	5.3	25.0	79.6	49.0	8.3
40.0	6.0	10.0	80.4	277.9	9.8
40.0	7.0	16.6	70.5	195.0	9.6
40.0	7.0	25.0	95.2	282.3	23.6
40.0	7.0	33.4	84.4	158.6	16.5
40.0	8.0	10.0	87.5	263.7	10.7
40.0	8.7	25.0	80.5	145.4	9.5
40.0	9.0	10.0	60.2	160.2	7.4
45.0	6.0	10.0	71.0	228.2	8.3
45.0	6.0	20.0	75.7	75.1	5.8
45.0	6.0	30.0	81.7	37.7	4.5
45.0	7.0	10.0	80.0	252.5	9.7
45.0	8.0	10.0	72.2	216.5	9.0
45.0	8.0	20.0	73.6	94.8	5.7
45.0	8.0	30.0	78.2	40.5	5.3
45.0	9.0	10.0	52.3	131.5	6.3
48.4	7.0	25.0	79.8	30.1	3.5

According to the experimental design in Table 7.10, batch tests were conducted for hydrogen production.

The modified Logistic model (Eq. 7.1) was used to fit the cumulative hydrogen production data using nonlinear regression by software Origin 7.5.

7.8.2 Neural Network

In general, feed-forward neural network with one hidden layer containing certain hidden neurons has been shown to be capable of providing accurate approximations to many nonlinear functions (Nagata and Chu 2003). Thus, feed-forward neural network with back propagation algorithm was used here. The inputs chosen were temperature, initial pH, and glucose concentration, respectively, while the outputs were substrate degradation efficiency, hydrogen yield, and average hydrogen production rate, respectively. The transfer functions in the hidden layer and the output layer were logistic function and linear function, respectively. All the inputs and outputs were normalized within a uniform range of (0.1, 0.9) to ensure that they receive equal attention during the training process (Maier and Dandy 2000; He et al. 2008). The new scaled variables were calculated by Eq. (7.10). These values were rescaled by Eq. (7.11):

$$X^* = 0.8 \times \frac{X - X_{\min}}{X_{\max} - X_{\min}} + 0.1 \tag{7.10}$$

$$X = \frac{(X_{\max} - X_{\min})(X^* - 0.1)}{0.8} + X_{\min}, \tag{7.11}$$

where X is the variable in a group to be scaled, X_{min} is the minimum variable in a group to be scaled, X_{max} is the maximum variable in a group to be scaled, and X^* is the scaled variable.

The first step in training neural network is to design the topology of the neural network. The number of neurons in the input layer is fixed by the number of inputs, whereas the number of neurons in the output layer equals the number of outputs. The critical aspect is the choice of the number of neurons in the hidden layers (Maier and Dandy 2000). To obtain the optimal number of neurons in the hidden layer of the neural network, the number of neurons in the hidden layer was investigated. In the training process, the mean square error between the experimental data and the corresponding predicted data is calculated and propagated backward through the network. The back propagation algorithm adjusts the weights in each successive layer to reduce the error. This procedure is repeated until the error between the experimental data and the corresponding predicted data satisfies certain error criterion.

Equation (7.12) was used to calculate the coefficient of determination (R^2) of the neural network:

$$R^2 = \frac{\sum_{i=1}^{k} \left(y_i' - \overline{y}\right)^2}{\sum_{i=1}^{k} \left(y_i - \overline{y}\right)^2}, \tag{7.12}$$

where y_i is the experimental data, y_i' is the corresponding predicted data by neural network, and $\overline{y}$ is the mean of all the experimental data.

R^2 can be interpreted as the proportion of variability that can be accounted for by the neural network around the mean for the responses. R^2 equals 0 when the values of the factors do not allow any prediction of the responses, and equals 1 when the neural network can perfectly predict the responses from the factors studied. In other words, R^2 is the measure of how well the neural network describes the experimental data. The much closer of R^2 to 1, the higher the modeling ability the neural network has.

In addition, the data predicted by neural network were plotted against the corresponding experimental data to visualize the modeling ability of the neural network. The much closer to the line of perfect prediction (the line on which the data predicted by neural network are all equal to the corresponding experimental data) of the data points for the experimental and predicted data, the higher the modeling ability the neural network has.

7.8.3 Method of Desirability Function

The method of desirability function was used to obtain the maximum substrate degradation efficiency, hydrogen yield, and average hydrogen production rate simultaneously in this study. For the application of the method of desirability function, each response y_i was converted into an individual desirability function d_i that ranges from 0 to 1, according to Eq. (7.13):

$$d_\mathrm{i} = \begin{cases} 0 & \text{if} \quad y_\mathrm{i} \le L \\ \left(\frac{y_\mathrm{i}-L}{U-L}\right)^w & \text{if} \quad L < y_\mathrm{i} < U \\ 1 & \text{if} \quad y_\mathrm{i} \ge U \end{cases}, \tag{7.13}$$

where y_i is the response to be optimized, L is the minimum acceptable value of y_i, U is the maximum value beyond which improvements would serve no further benefit, and w is a weight factor.

Then the optimal conditions for several responses are obtained by maximizing the overall desirability D, which is the geometric mean of all the individual desirability functions (Eq. (7.14)):

$$D = (d_1 \times d_2 \times \cdots \times d_\mathrm{k})^{\frac{1}{k}} = \left(\prod_{i=1}^{k} d_\mathrm{i}\right)^{\frac{1}{k}}. \tag{7.14}$$

In other words, the simultaneous optimization of several responses can be achieved by determining the maximum of the overall desirability.

7.8.4 Genetic Algorithm

Genetic algorithm is based on the principles of evolution through natural selection, that is, the survival of the fittest strategy. It explores all regions of the solution space using a population of individuals. Each individual represents a set of factors. The individual chosen in this study was a set of temperature, initial pH, and glucose concentration. Initially, a population of individuals is formed randomly. The fitness of each individual is evaluated using a fitness function. Upon completion of the fitness evaluation, genetic operations such as mutation and crossover are applied to individuals selected, according to their fitness, to produce the next generation of individuals for fitness evaluation. This process continues until an optimal solution is found (Nagata and Chu 2003).

The desirability function based on neural network was used as the fitness function for genetic algorithm to optimize the fermentative hydrogen production process.

The modified Logistic model was used to fit the cumulative hydrogen production data obtained from each batch test. The coefficients of determination (R^2) of all the fittings were close to 1.000, which indicated that the modified Logistic model could describe the progress of cumulative hydrogen production in the batch tests of this study successfully. And the calculated experimental results of substrate degradation efficiency, hydrogen yield, and average hydrogen production rate are shown in Table 7.10.

7.8.5 Effects of Temperature, Initial pH, and Substrate Concentration

The experimental data of substrate degradation efficiency, hydrogen yield, and average hydrogen production rate in Table 7.10 were used to train the neural network for them. To obtain the optimal number of neurons in the hidden layer of the neural network for them, the number of neurons in the hidden layer was investigated. Figures 7.9, 7.10, and 7.11 show the mean square error between the experimental data and the corresponding predicted data at different number of neurons in the hidden layer of the neural network for substrate degradation efficiency, hydrogen yield, and average hydrogen production rate, respectively.

The mean square error between the experimental data and the corresponding predicted data for substrate degradation efficiency and hydrogen yield decreased with increasing the number of neurons in the hidden layer of the neural network for

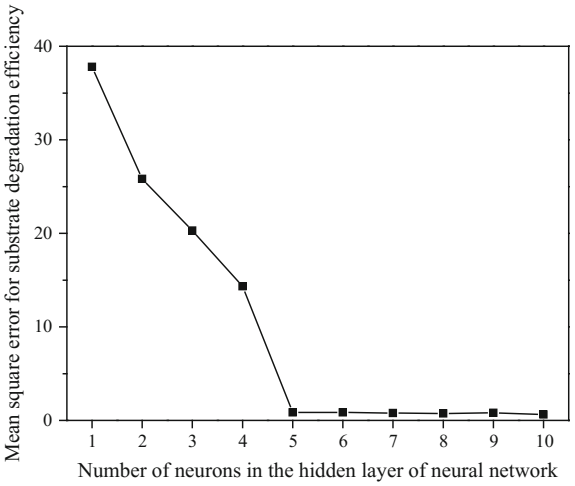

Fig. 7.9 Effect of number of neurons in hidden layer of neural network on mean square error for substrate degradation efficiency

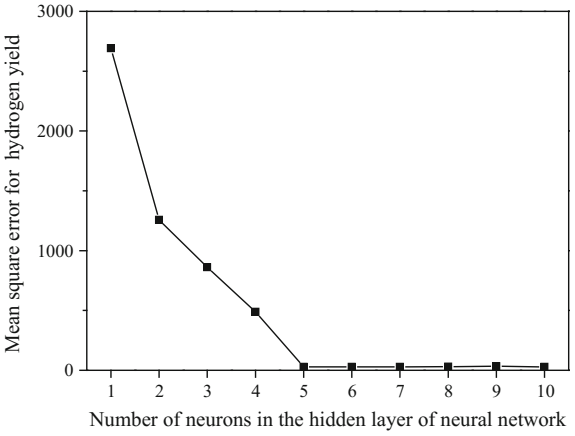

Fig. 7.10 Effect of number of neurons in hidden layer of neural network on mean square error for hydrogen yield

them from 1 to 5, while it changed little with further increasing the neuron number from 5 to 10. This indicated that when the number of neurons in the hidden layer was from 5 to 10, the neural network could model the experimental data of substrate degradation efficiency and hydrogen yield data better. In addition, when the number of the neurons in the hidden layer of the neural network is larger, the neural network is very complex and it will take a longer time to train the neural network. Thus, five neurons were chosen in the hidden layer of the neural network for substrate degradation efficiency and hydrogen yield, respectively. The neural network architecture for substrate degradation efficiency in this study consisted of three neurons (temperature, initial pH and glucose concentration) in the input layer, five neurons in the hidden layer, and one neuron (substrate degradation efficiency and hydrogen yield, respectively) in the output layer (topology 3-5-1). In a similar way,

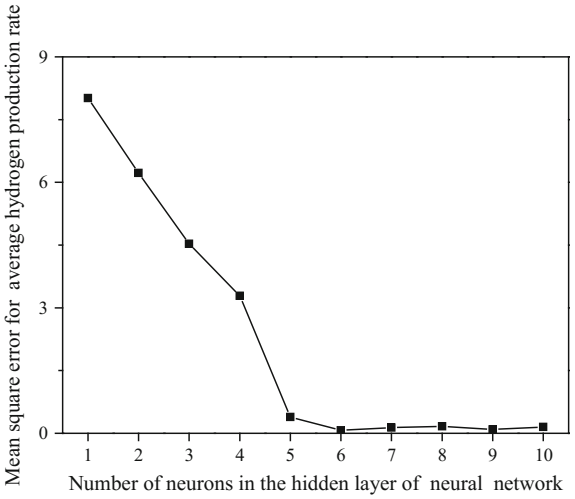

Fig. 7.11 Effect of number of neurons in hidden layer of neural network rate on mean square error for average hydrogen production

six neurons were chosen in the hidden layer of the neural network for average hydrogen production rate. Training a neural network is accomplished by adjusting the weight coefficients in each successive layer to minimize the mean square error between experiment data and the corresponding predicted data of substrate degradation efficiency, hydrogen yield, and average hydrogen production rate, respectively.

Coefficients of determination of the neural network for substrate degradation efficiency, hydrogen yield, and average hydrogen production rate were calculated as 0.984, 0.994, and 0.984, respectively. In addition, as shown in Figs. 7.12, 7.13, 7.14, the experimental and predicted data points of them were very close to the line of perfect prediction. All these indicated that the neural network for substrate degradation efficiency, hydrogen yield, and average hydrogen production rate could

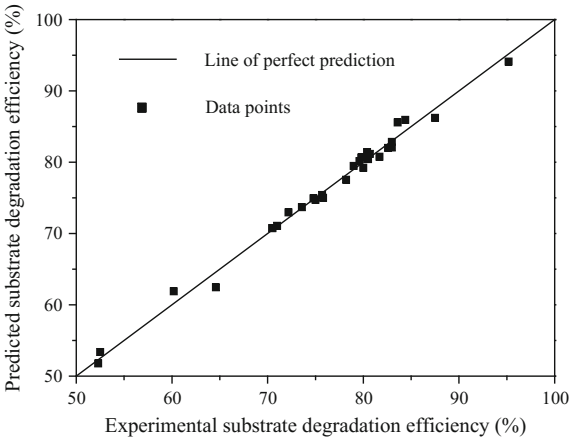

Fig. 7.12 Experimental and predicted data of substrate degradation efficiency

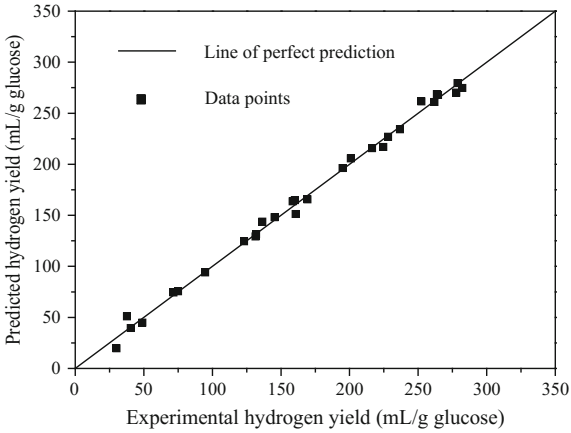

Fig. 7.13 Experimental and predicted data of hydrogen yield

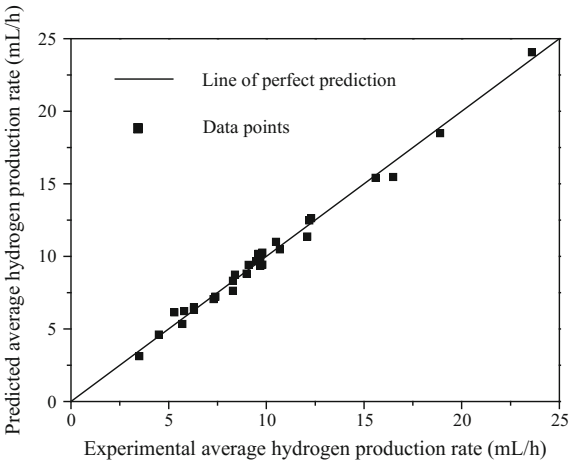

Fig. 7.14 Experimental and predicted data of average hydrogen production rate

describe the effects of temperature, initial pH, and glucose concentration on them of this study successfully.

7.8.6 Optimized Parameters by Desirability Function

The method of desirability function was used to obtain the maximum substrate degradation efficiency, hydrogen yield, and average hydrogen production rate simultaneously in this study. The parameters for optimization by method of desirability function are shown in Table 7.11.

When y_i is between L and U, the overall desirability can be expressed by Eq. (7.15):

Table 7.11 Parameters for optimization by method of desirability function

Response	y_i	L	U	w
Substrate degradation efficiency (%)	y_1	90.0	96.0	1
Hydrogen yield (mL/g glucose)	y_2	230.0	310.0	1
Average hydrogen production rate (mL/h)	y_3	20.0	25.0	1

$$D = \left(\frac{y_1 - 90}{96 - 90} \cdot \frac{y_2 - 230}{310 - 230} \cdot \frac{y_3 - 20}{25 - 20}\right)^{\frac{1}{3}}. \tag{7.15}$$

Subsequently, the overall desirability was optimized by genetic algorithm and the maximum overall desirability of 0.819 was estimated at the temperature of 39.0 °C, the initial pH of 7.0, and the glucose concentration of 24.6 g/L. Accordingly, the maximum substrate degradation efficiency of 94.5%, hydrogen yield of 307.0 mL/g glucose, and average hydrogen production rate of 23.8 mL/h were predicted at the temperature of 39.0 °C, the initial pH of 7.0, and the glucose concentration of 24.6 g/L.

The optimal temperature for fermentative hydrogen production obtained in this study was close to those (35–40 °C) reported by Wang and Wan (2008), Lin et al. (2008), and Zhang and Shen (2006). However, it was much lower than those (50–60 °C) reported by Lin et al. (2008), Yokoyama et al. (2007), and Valdez-Vazquez et al. (2005). The optimal initial pH for fermentative hydrogen production obtained in this study was close to that (6.5) reported by Lin et al. (2006), but it was much lower than that (9.0) reported by Lee et al. (2002), and it was much higher than that (4.5) reported by Khanal et al. (2004). The optimal glucose concentration for fermentative hydrogen production obtained in this study was close to that (23.8 g/L) reported by Pan et al. (2008), but it was much higher than that (1 g/L) reported by Zheng et al. (2008).

Another several experiments (Table 7.12) were carried out to validate the optimal conditions identified by the method of desirability function based on neural network.

Table 7.12 Experimental design for validating the optimized conditions identified by desirability function based on neural network

Response	Temperature (°C)	Initial pH	Glucose concentration (g/L)	Identified	Experimental
Substrate degradation efficiency (%)	39.0	7.0	24.6	94.5	95.3
Hydrogen yield (mL/g glucose)	39.0	7.0	24.6	307.0	305.3
Average hydrogen production rate (mL/h)	39.0	7.0	24.6	23.8	23.9

As shown in Table 7.12, under the optimal conditions identified, the experimental data were very close to those identified by the method of desirability function based on neural network, indicating that the method of desirability function based on neural network was a useful tool to optimize the fermentative hydrogen production process.

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Chapter 8
Sewage Sludge for Hydrogen Production

8.1 Introduction

Energy supply is one of the most important aspects for the sustainable development. At present, more than 80% of the energy is derived from the combustion of fossil fuels (Li and Fang 2007). However, the fossil fuels are finite and will become depleted in the near future (Demirbas 2007). In addition, the fossil fuel combustion generates large amount of toxic and greenhouse gases including sulfur dioxide, carbon dioxide, and nitric oxide, leading to the environmental deterioration and global warming (John et al. 2011; Verhelst and Wallner 2009). In response to above two problems, many countries have paid much attention to explore renewable and clean alternatives for a sustainable development over the past decades (Hawkes et al. 2002; Xia et al. 2015).

Hydrogen has been widely considered as a proper fuel to solve the problems of fossil fuels crisis and environment pollution caused by fossil fuels combustion (Cai et al. 2011; Navarro et al. 2009; Wang and Wan 2009a). First, hydrogen can be used for producing electricity in conventional fuel cells (Xia et al. 2016). Additionally, hydrogen provides higher energy yield (141.9 J/kg) than some commonly used fuels, such as natural gas (50 J/kg), methane (55.7 J/kg), ethanol (29.9 J/kg), and biodiesel (37 J/kg) (Bahadar and Khan 2013; Liao et al. 2010). Most importantly, hydrogen is an environmental friendly fuel since H_2O is the only end product of its combustion (Holladay et al. 2009). Currently, more than 85% of hydrogen is generated from fossil fuels through pyrolysis, reforming, and biomass gasification (Nath and Das 2003). Water electrolysis is another hydrogen production technology and accounts for 4% of total hydrogen production (Guo et al. 2010b). All these hydrogen production techniques are unsustainable processes due to the high energy consumption. Therefore, there is an urgent need to develop an environmental friendly and a more cost-effective technology for the production of hydrogen. To this regard, producing hydrogen by dark fermentation shows great penitential in the future due to its simple operation conditions, stable hydrogen

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production and low-energy demand. More importantly, this process can use a variety of organic wastes as substrates for renewable and sustainable hydrogen production (Lee et al. 2010), which could achieve great economic and environmental benefits

Among these wastes, sewage sludge, the by-product of wastewater treatment process, has drawn extensively attentions as a low-cost substrate for fermentative hydrogen production due to its high organics content (more than 60% of dry weight), large amount and stable source (Yang et al. 2015a). Sludge fermentation contains a series of complex biochemical reactions (Wang and Yin 2017). Macromolecular organic compounds in sludge were eventually converted to volatile fatty acids (VFAs), ethanol, CO_2, and hydrogen by either facultative or strict anaerobic bacteria (Nath et al. 2004). However, the efficiency of hydrogen production from sludge fermentation was still low, which significantly restrict its economic and environmental benefits. The low hydrogen production efficiency is mainly because of the poor fermentation conditions (e.g., low C/N ratio) and complex sludge flocs restricting the utilization of organics by bacteria (Xia et al. 2016). As a result, most of previous studies have focused on how to improve the hydrogen production efficiency from sludge fermentation. Some pretreatment methods have been applied for sludge flocs disruption for better utilization of organics by microorganisms (Yang et al. 2016a). In addition, some high C/N ratio and carbohydrate-rich wastes (e.g., food waste and rice straw) were applied for co-fermentation with sludge to provide better substrate condition and dilution of inhibitory effects (Wu et al. 2016; Xie et al. 2016). In addition, a broad range of process parameters, such as temperature, pH, C/N ratio, retention time and organic loading rate (OLR), nutrients, inhibitors, and inoculum and treatment method, could significantly influence the efficiency of fermentative hydrogen production (Wang and Wan 2009a). The impacts of above-mentioned parameters on hydrogen production have also been widely investigated for optimizing process efficiency.

In order to gain insight into hydrogen production from sludge fermentation and provide some basis for future researches and applications, this chapter presents a critical review of hydrogen production using sludge as substrate through dark fermentation based on relevant publications from 2000 to 2016. Although some overviews have been published on fermentative hydrogen production from various kinds of organic wastes, including municipal waste fractions, agricultural waste, microalgae, and lignocellulosic hydrolysates (Kapdan and Kargi 2006; Guo et al. 2010b; De Gioannis et al. 2013; Nissilä et al. 2014; Xia et al. 2015).

We compiled this chapter from the following aspects: (1) principles; (2) hydrogen potential of raw sewage sludge; (3) pretreatment; (4) co-fermentation with other substrates; (4) influence factors; (5) kinetic models; (6) end products in the liquid phase; (7) two-stage process; (8) example of hydrogen production from disintegrated sludge.. Finally, concluding remarks and future perspectives were also discussed.

8.2 Potential Substrates, Microorganisms, and Enzymes

The essence of fermentative hydrogen production is degrading and converting organic matters to hydrogen by anaerobic bacteria (Hallenbeck 2009). Proteins, carbohydrates, and lipids are three main organic components of sludge, accounting for more than 80% of total volatile solids (VS) (Yang et al. 2015b). Among these organic components, carbohydrates have been widely considered as the main components for hydrogen production (Yin et al. 2014a). In addition, glycerol derived from lipids hydrolysis can also be used for producing hydrogen during sludge fermentation (Adhikari et al. 2009). However, amino acids derived from proteins hydrolysis and long-chain fatty acids derived from lipids hydrolysis are not favorable for hydrogen production during sludge fermentation (Hallenbeck 2009).

Regarding the involved microorganisms, either facultative or strict anaerobes can produce hydrogen during fermentation process (Wong et al. 2014). *Enterobacter*, *Escherichia coli*, *Klebsiella*, *Bacillus,* and *Citrobacter* are representatives of facultative anaerobes, whereas strict anaerobes contain *Clostridium*, *Ethanoligenens, Caldicellulosiruptorr, Thermotoga,* and *Desulfovibrio*. The key enzymes responsible for fermentative hydrogen production are hydrogenases (Böck et al. 2006; Meyer 2007). Hydrogenases can be divided into two main groups according to metal ions contained in the enzyme active center: Fe–Fe hydrogenases and Ni–Fe hydrogenases (Vignais and Billoud 2007). These hydrogenases contain various active sites when catalyzing relevant biochemical reactions. Generally, the Ni-Fe hydrogenases are responsible for catalyzing hydrogen oxidation, and the Fe–Fe hydrogenases are poised to proton reduction (Fontecilla-Camps et al. 2007). The catalytic activity of the Ni–Fe hydrogenases is 10–100 times higher than that of the Fe–Fe hydrogenases (Vignais et al. 2001). Most of these hydrogenases requires strict anaerobic conditions, and the activities could be decreased by 50% within 30 s under aerobic condition. The inhibition sensitivity of the Fe–Fe hydrogenases by oxygen and carbon monoxide is much higher than that of the Ni–Fe hydrogenases (Korbas et al. 2006; Steunou et al. 2008)

8.3 Pathways of Hydrogen Production from Sludge

Hydrogen production from sludge fermentation contains a serious of complex biochemical reactions (Fig. 8.1).

Macromolecule organic matters in sludge (carbohydrates, proteins, lipids) are first hydrolyzed into soluble low molecule organic substances (e.g., amino acids, sugars, glycerol and long-chain fatty acids) by hydrolytic bacteria (Yu et al. 2013b). After sludge hydrolysis, there are three possible pathways for generating hydrogen (Wang and Wan 2008a). Regarding the first pathway, sugars derived from carbohydrates hydrolysis was degraded to pyruvate through the Embden-Meyerhof-Parnas pathway. Intermediate pyruvate can be further decomposed to formate and acetyl-CoA

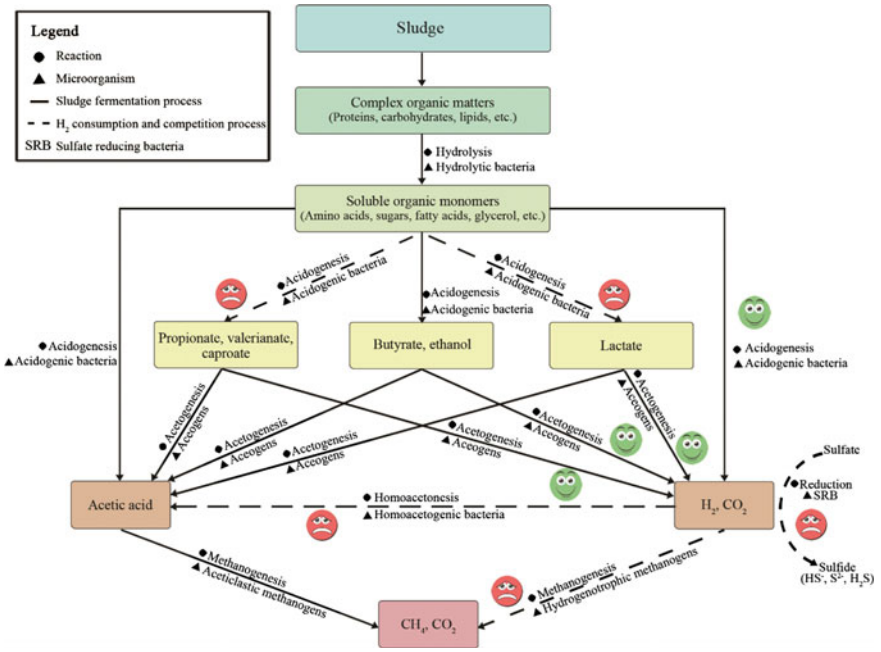

Fig. 8.1 Biochemical reactions during sludge fermentation process

by pyruvate-formate lyase, and then formate is converted to hydrogen and carbon dioxide. This metabolic pathway was notably carried out by the enteric bacteria, and the maximum hydrogen yield is 2 mol hydrogen by consuming one mole glucose. In addition, pyruvate can also be decomposed to reduced ferredoxin, carbon dioxide, and acetyl-CoA by pyruvate-ferredoxin oxidoreductase. The reduced ferredoxin is then oxidized by the Fe–Fe hydrogenase, contributing to hydrogen production. This metabolic process was notably carried out by the *Clostridia*, and the hydrogen yield is also two moles hydrogen by consuming one mole glucose. Regarding the second pathway, the NADH generated during the glycolysis process can be reoxidized to generate hydrogen, probably catalyzed by a NADH dependent Fe–Fe hydrogenase. The detailed molecular mechanisms of this pathway are still unclear. Regarding the third pathway, soluble low molecule organic substances generated from sludge hydrolysis process can be converted to ethanol and VFAs > C2 (e.g., propionic acid, butyrate, and valerate) by acidogenic bacteria. Propionic acid, butyrate, valerate, and ethanol can be used as substrates for producing acetic acid and hydrogen by the acetogens. For example, *Syntorbaterw olini* can degrade propionate to hydrogen, acetic acid, and carbon dioxide. However, this pathway is unfavorable, and can only occur under extremely low hydrogen partial pressure. Propionic acid and n-butyric acid can be degraded into acetate by acetogens only when hydrogen partial pressure is lower than 10^{-5} and 10^{-4} atm, respectively (Feng et al. 2014).

8.4 Fermentation Types

During sludge fermentation process, a serious of complex biochemical reactions causes a variety of end products in the liquid phase. Generally, sludge fermentation can be divided into four types based on the composition of end products including butyric-type, propionic-type, mixed acid-type, and ethanol-type (Wang et al. 2008). The butyric-type fermentation mainly decomposes organics to butyric acid, acetic acid, carbon dioxide, and hydrogen. The reactions can be described by the following equations:

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \tag{8.1}$$

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2CH_2COOH + 2CO_2 + 2H_2. \tag{8.2}$$

The representative microorganisms of this fermentation type includes *Clostridiums*, *C. butyricum*, *Butyriobio,* and *C. acetobutylicum*. Regarding the propionic-type fermentation, it mainly converts organics to propionic acid, acetic acid, carbon dioxide, and hydrogen. The reaction can be described by the following equation:

$$C_6H_{12}O_6 + H_2O + 3ADP + 16Pi \rightarrow CH_3CH_2COO^- + CH_3COO^- + HCO_3^- + 3H^+ + H_2 + 3ATP. \tag{8.3}$$

The representative microorganism of this fermentation type is *Propionibacterium shermanii*. Regarding the mixed acid-type fermentation, it mainly converts organics to lactate, acetic acid, ethanol, formic acid, carbon dioxide, and hydrogen. Regarding the ethanol-type fermentation, the main end products are ethanol, acetic acid, carbon dioxide, and hydrogen. The reaction can be described by the following equation:

$$C_6H_{12}O_6 + H_2O \rightarrow C_2H_5OH + CH_3COOH + 2CO_2 + 2H_2. \tag{8.4}$$

The representative microorganisms of this fermentation type include *Bacillus sp.*, *Clostridium*, *Saccharomyces*, *Fusobacterium*, *S. cerevisiae,* and *Zymomonas*. In addition, a new fermentation type has been reported by previous studies during sludge fermentation, named acetate-type fermentation. During this fermentation type, organics are mainly converted to acetate, carbon dioxide, and hydrogen, meanwhile the amount of propionate acid and butyric acid was pretty low. The detailed reaction can be described as the following equation:

$$C_6H_{12}O_6 + 2H_2O \rightarrow CH_3COOH + 2CO_2 + 4H_2. \tag{8.5}$$

However, there is not a clear boundary between acetate-type fermentation and propionic acid-type fermentation, or between acetate-type fermentation and

butyric-type fermentation in previous studies due to the almost same types of end products, casing that it is difficult to deduce the fermentation type. So, in this study, the acetate-type fermentation is defined quantitatively as follows: the acetate accounts for more than 50% of total VFAs, and while the mass ratio of butyric and propionic acid were not higher than 10% of total VFAs, respectively. Among these fermentation types, ethanol-type fermentation and butyric-type fermentation could usually achieve high hydrogen yield (Hallenbeck 2009; Wang and Wan 2009c). Furthermore, the ethanol-type fermentation is more stable than the butyric-type fermentation, especially operating at high OLR. However, the propionic-type fermentation could only produce limited hydrogen, and accumulation of propionic acid could deteriorate the fermentation system. Thus, assessing optimal fermentation type plays a significant role in enhancing hydrogen yield during sludge fermentation.

8.5 Hydrogen Consumption Pathways and Metabolic Competitors

In addition to hydrogen production pathways, simultaneous consumption pathways of hydrogen have also been observed during sludge fermentation. The main hydrogen consumers include homoacetogenic bacteria, sulfate reducing bacteria, and hydrogenotrophic methanogens. Homoacetogenic bacteria are strictly anaerobic, and can catalyze acetic acid formation from hydrogen and carbon dioxide, such as *Clostridium thermoaceticum* and *Clostridium aceticum* (Wieringa 1939; Fontaine 1942). The detailed biochemical reaction can be described as the follow equation:

$$2HCO_3^- + H^+ + 4H_2 \rightarrow CH_3COO^- + 4H_2O. \tag{8.6}$$

Regarding sulfate reducing bacteria, they can use hydrogen for sulfide formation in the presence of sulfate (Yang et al. 2016b), even at extremely low hydrogen concentration (0.02 ppm). The detailed biochemical reaction can be described as the following equation:

$$SO_4^{2-} + 4H_2 + H^+ \rightarrow HS^- + 4H_2O. \tag{8.7}$$

Regarding hydrogenotrophic methanogens, they can use hydrogen and carbon dioxide for formatting methane (Demirel and Scherer 2008). The detailed biochemical reaction can be described as the following equation: $HCO_3^- + 4\ H_2 + H^+ \rightarrow CH_4 + 3\ H_2O$. Among these hydrogen consumers, it has been observed that sulfate reducing bacteria have a thermodynamic advantage over hydrogenotrophic methanogens and homoacetogenic bacteria during sludge fermentation. In addition, other hydrogen consumption pathways have also been observed when producing propionic, valeric and caproic acid, and the detailed reactions can be described as follows (Guo et al. 2010b):

$$\begin{aligned} &C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O, \\ &C_6H_{12}O_6 + 4CO_2 + 14H_2 \rightarrow 2CH_3(CH_2)_3COOH + 10H_2O \end{aligned} \tag{8.8}$$

$$C_6H_{12}O_6 + 4H_2 \rightarrow CH_3(CH_2)_4COOH + 4H_2O \tag{8.9}$$

Some metabolic competitors of hydrogen producers were also found during sludge fermentation such as lactic acid bacteria (Noike et al. 2002). The reaction can be described as the following equation:

$$C_6H_{12}O_6 \rightarrow 2C_3H_6O_3 + 2ATP. \tag{8.10}$$

To some extent, hydrogen production efficiency can be significantly limited by these hydrogen consumers and competitors. Thus, inhibiting hydrogen consumption and competition processes could assist in improving hydrogen production efficiency.

8.6 Hydrogen Production Potential of Raw Sludge

In previous studies, simple sugars (e.g., glucose and sucrose) are commonly used as model substrates for fermentative hydrogen production (Wang and Wan 2008a; Yin et al. 2014b). Meanwhile, some kinds of solid wastes have gained much attention to be used as potential substrates for hydrogen production in recent years (Kapdan and Kargi 2006). Generally, the solid waste to be suitable for hydrogen production should be abundant, stable, cheap, and readily biodegradable. Sludge produced in wastewater treatment plants meets all above requirements. First, large amount of sludge has been generated in the world due to the increase of sewage treatment volume and ratio. For example, about 7 million tons dry sludge was generated in China in 2015 (Yang et al. 2015a). In EU-12 countries, more than 13 million tons dry sludge will be generated by the year of 2020 (Kelessidis and Stasinakis 2012). Second, the composition of sludge is stable, and sludge contains large amount of readily biodegradable organic matters (Wu et al. 2015). Finally, sludge, as the by-product of wastewater treatment process, commonly results in the extra financial burden of wastewater treatment plants (Wong et al. 2006), and thus is a kind of cheap wastes.

Using sludge as substrate for fermentative hydrogen production has been investigated since the year of 2000 (Huang et al. 2000; Cheng et al. 2000). Figure 8.2 illustrates the number of relevant articles published from 2000 to 2016 on the subject of fermentative hydrogen production from sludge. Generally, the number of article published shows the increasing trend during the past 16 years, indicating that more attention has been paid to this research subject than before. Reported hydrogen yields from raw sludge in previous studies are summarized in Table 8.1. As shown in Table 8.1, sludge from both municipal and industrial wastewater treatment process have been used as substrate for fermentative

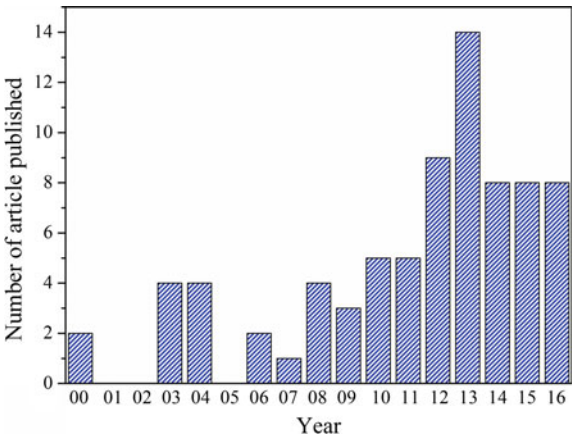

Fig. 8.2 Number of articles published on the subject of fermentative hydrogen production from sludge from 2000 to 2016 based on ISI Web of Science

hydrogen production. Industrial sludge includes bath wastewater sludge, brewery industry sludge, food processing sludge, fructose-processing sludge, molasses wastewater sludge, paperboard mill sludge, and poultry slaughterhouse sludge. The feedstock sludge types include primary sludge, waste activated sludge (WAS), mixed sludge, thickened sludge and anaerobically digested sludge (ADS). Fermentative hydrogen production from raw sludge has been studied under both batch and continuously stirred tank reactor (CSTR) tests, and has been performed under both mesophilic (30–37 °C) and thermophilic (50–55 °C) conditions. The studied initial pH values were in the range of 2.5–12, and most of studies were performed at the pH range of 5–8.

Hydrogen yields from raw sludge were expressed by a variety of indexes including mL/g-VS_{added}, mL/g-total solids $(TS)_{added}$, mL/g-chemical oxygen demand $(COD)_{added}$, mL/g-volatile suspended solids $(VSS)_{added}$, mL/L-$sludge_{added}$, mL/g-$VS_{removed}$, mL/g-$TS_{removed}$, mL/g-$COD_{removed}$, and mL/g-$VSS_{removed}$. As shown in Table 8.1, the hydrogen yield was in the range of 0–18.6 mL/g-VS_{added}, or 3.34–20 mL/g-TS_{added}, or 0.23–13.8 mL/g-COD_{added}, or 1.1–109 mL/g-VSS_{added}, or 12.98–93 mL/L-$sludge_{added}$, or 28.3–70.4 mL/g-$VS_{removed,}$ or 0.02 mL/g-$TS_{removed}$, or 5–115.7 mL/g-$COD_{removed}$, or 0.25 mL/g-$VSS_{removed}$ (Table 8.1). Clearly, raw sludge performed relatively low hydrogen yields, and several studies even observed that almost no hydrogen were generated during raw sludge fermentation (Xiao and Liu 2009; Lee et al. 2014a). These observations suggest that the fermentation of raw sludge for hydrogen production exist several difficulties. First, the sludge matrix is complex and heterogeneous, making it difficult to be hydrolyzed directly by the hydrolytic bacteria (Appels et al. 2008). Sludge matrix is mainly composed of extracellular polymeric substances and microbial cells. These extracellular polymeric substances are located outside the microbial cells. However, extracellular polymeric substances and microbial cell walls are relatively recalcitrant degraded naturally by anaerobic bacteria, leading to the rate-limiting hydrolysis step and low utilization ratio of organics in microbial cells during sludge

Table 8.1 Fermentative hydrogen potential of raw sludge

Sludge type	Inoculum	Fermentation conditions	Hydrogen yield	References
Municipal sludge				
ADS	ADS	Batch 50 °C Initial pH: 5	0.02 mL/g-$TS_{removed}$	Sato et al. (2016)
Mixed sludge	ADS	Batch 55 °C Initial pH: 5.5	28.3 mL/g-$VS_{removed}$	Tyagi et al. (2014)
WAS	ADS	Batch 35 °C Initial pH: 6	17.9 mL/g-VS_{added}	Cheng et al. (2016)
Primary sludge	ADS	Batch 37 °C Initial pH: 5.5	10 mL/g-COD_{added}	Zhou et al. (2013)
Thickened sludge	ADS	Batch 37 °C Initial pH: 8	0.25 mL/g-$VSS_{removed}$	Kim et al. (2013a)
Thickened sludge	Compost-acclimated sludge	CSTR 36.5 °C Initial pH: 6.2–6.5 SRT: 32 h ORL: 0.3 kg COD/d	13.7 mL/g-VS_{added}	Wu and Zhou (2011b)
Thickened sludge	WAS	Batch Mesophilic Initial pH: 6.8	0.13 mL/g-VS_{added}	Yu et al. (2013a)
WAS	None	Batch 36 °C Initial pH: 10	3.63 mL/g-TS_{added}	Cai et al. (2004)
WAS	None	Batch 36 °C Initial pH: 10.5	6.06 mL/g-TS_{added}	Cai et al. (2004)

(continued)

Table 8.1 (continued)

Sludge type	Inoculum	Fermentation conditions	Hydrogen yield	References
WAS	None	Batch 36 °C Initial pH: 11	8.08 mL/g-TS_{added}	Cai et al. (2004)
WAS	None	Batch 36 °C Initial pH: 11.5	7.89 mL/g-TS_{added}	Cai et al. (2004)
WAS	None	Batch 36 °C Initial pH: 12	6.6 mL/g-TS_{added}	Cai et al. (2004)
WAS	*Clostridium bifermentans*	Batch 35 °C	6.67 mL/g-COD_{added} 10.9 mL/g-TS_{added}	Wang et al. (2003)
WAS	None	Batch 35 °C Initial pH: 6.67	3.34 mL/g-TS_{added}	Jan et al. (2007)
WAS	ADS	Batch 55 °C Initial pH: 5.7	93 mL/L-$Sludge_{added}$ 12.4 mL/g-TS_{added} 18.6 mL/g-VS_{added} 5.1 mL/g-COD_{added}	Liu et al. (2013a)
WAS	WAS	Batch 30 °C Initial pH: 5. 5	12.98 mL/L-$Sludge_{added}$ 1.41 mL/g-COD_{added}	Wan et al. (2016)
WAS	None	Batch 37 °C Initial pH: 7	1.21 mL/g-VS_{added}	Xiao and Liu (2009)
WAS	None	Batch 37 °C Initial pH: 11.5	7.57 mL/g-VS_{added}	Xiao and Liu (2009)
WAS	None	Batch 37 °C Initial pH: 7	1.1 mL/g-VSS_{added} 0.56 mL/g-COD_{added}	Guo et al. (2013a)

(continued)

Table 8.1 (continued)

Sludge type	Inoculum	Fermentation conditions	Hydrogen yield	References
WAS	None	Batch 37 °C	5 mL/g-$COD_{removed}$	Kotay and Das (2009)
WAS	WAS	Batch 35 °C	13.8 mL/g-COD_{added} 20 mL/g-TS_{added}	Wang et al. (2004)
WAS	ADS	Batch 37 °C Initial pH: 5.5	7 mL/g-COD_{added} 93 mL/g-VSS_{added}	Zhou et al. (2013)
WAS	ADS	Batch 35 °C Initial pH: 5–6	0 mL/g-VS_{added}	Kim et al. (2004)
WAS	None	Batch 37 °C Initial pH: 2.5	0 mL/g-VS_{added}	Xiao and Liu (2009)
Industrial sludge				
Bath wastewater sludge	WAS	Batch 37 °C Initial pH: 5.5	2.5 mL/g-VS_{added}	Liu et al. (2013c)
Brewery industry sludge	ADS	Batch 30 °C Initial pH: 5.5	9 mL/g-VS_{added}	Assawamongkholsiri et al. (2013)
Food processing sludge	*Clostridium*	Batch 30 °C Initial pH: 6.84	7.45 mL/g-TS_{added}	Ting and Lee (2007)
Fructose-processing sludge	*Enterobactor aerogenes*	Batch 37 °C Initial pH: 5.5	0.51 mL/g-COD_{added}	Thungklin et al. (2011)
Fructose processing sludge	Hot spring sediment	Batch 37 °C Initial pH: 5.5	0.23 mL/g-COD_{added}	Thungklin et al. (2011)

(continued)

Table 8.1 (continued)

Sludge type	Inoculum	Fermentation conditions	Hydrogen yield	References
Molasses wastewater sludge	ADS	Batch 37 °C Initial pH: 5.7	0 mL/g-VS_{added}	Lee et al. (2014a)
Paperboard mill sludge	Thickened sludge	Batch 55 °C Initial pH: 6	115.7 mL/g-$COD_{removed}$ 70.4 mL/g-$VS_{removed}$	Elsamadony and Tawfik (2015)
Paperboard mill sludge	ADS	Batch 37 °C Initial pH: 5.5	2.294 mL/g-VS_{added}	Lin et al. (2013)
Paperboard mill sludge	*Clostridium thermocellum*	Batch 60 °C	24.9 mL/L-$Sludge_{added}$	Moreau et al. (2015)
Poultry slaughterhouse sludge	Hot spring sediment	Batch 37 °C Initial pH: 5.5	0.18 mL/g-COD_{added}	Thungklin et al. (2011)

SRT sludge retention time

fermentation (Carrère et al. 2010). In addition, the C/N ratio of sludge (4–8) is much lower than the suitable C/N ratio of fermentative bacteria (20–30) (Mata-Alvarez et al. 2014). Finally, sludge contains some inhibitors of fermentative bacteria, such as heavy metals, toxic organics, and dissolved sulfide (Yang et al. 2014). In order to enhance hydrogen yield from raw sludge, some pretreatment methods have been performed for disrupting the complex sludge matrix, and thus accelerating the hydrolysis step (Khanal et al. 2007). In addition, some kinds of other wastes were used to be co-fermented with sludge for improving the C/N ratio and reducing the inhibition effects of inhibitors on fermentative bacteria (Hagos et al. 2016). The enhancement effect by various pretreatment methods and the addition of co-substrates were discussed in detail in the following two sections.

8.7 Pretreatment of Sludge

8.7.1 Overview

Some pretreatment methods have been conducted for enhancing fermentative hydrogen production from sludge (Guo et al. 2008; Massanet-Nicolau et al. 2008). During the pretreatment process, the complex sludge matrix was disrupted, which caused the lysis of microbial cells. Then, recalcitrant degradable particle organics is released into the soluble phase, and converted to easily biodegradable forms, which contributed to the better utilization of intracellular organic matters by anaerobic bacteria. The main indicator for evaluating pretreatment efficiency is sludge solubilization (Yang et al. 2016a). In addition, effects of various pretreatment methods on sludge fermentation efficiency were usually evaluated by hydrogen production and organics removal (VS removal and COD removal).

Pretreatment methods for sludge disruption can be divided into four categories based on their operating mechanisms, including physical pretreatments, chemical pretreatments, biological pretreatments, and combined pretreatments. The combined pretreatment method means the combination of various pretreatment methods. Figure 8.3a illustrates the percentages of various categories of pretreatment methods. Clearly, physical pretreatment methods was the most studied group (41.6%), following by chemical (40.5%), combined (9.7%), and biological (8.2%) pretreatment methods. The physical pretreatment methods include heat, ultrasound, microwave, sterilization, and UV-light pretreatments. The chemical pretreatment methods include alkaline, acid, and oxidation pretreatments. The biological pretreatment methods include enzyme and bacteria pretreatments. The combined pretreatment methods mainly include heat-alkaline, heat-ozone, heat-ultrasound, heat-acid, alkaline-ionizing radiation, sterilization-enzyme, ultrasound-alkaline, and heat-ozone-ultrasound pretreatments. Figure 8.3b summarizes the percentages of individual pretreatment method in all reports. Clearly, alkaline (35.8%), heat (23%), and ultrasound (10.5%) are three most reported individual pretreatment methods.

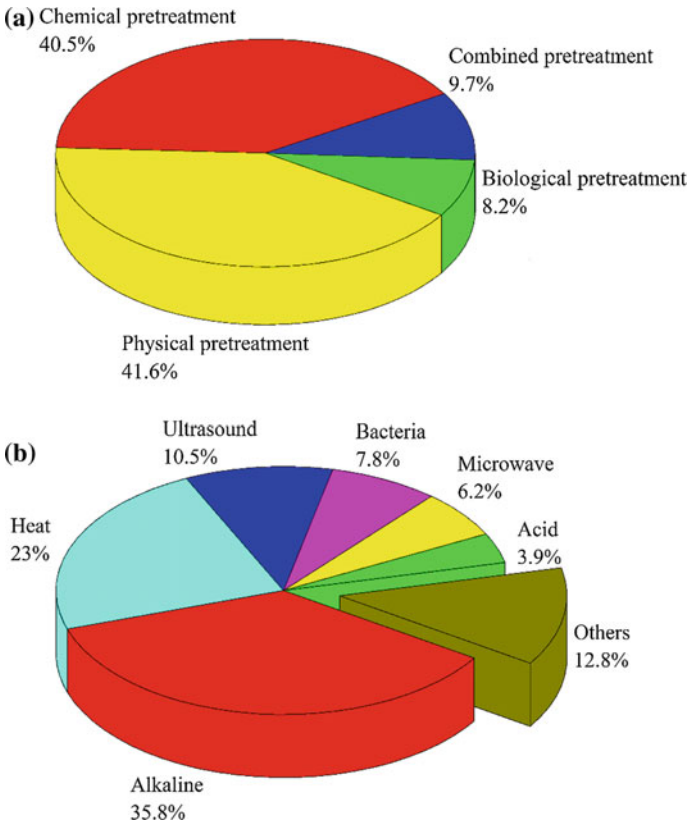

Fig. 8.3 Percentages of various categories of pretreatment methods (**a**) and individual pretreatment method (**b**) in the reviewed literature (The data in pie-chart are calculated based on the number of all relevant fermentation tests)

8.7.2 Physical Pretreatment Methods

Some physical methods have been performed to enhance fermentative hydrogen production efficiency. Table 8.2 illustrates the effects of these physical treatment methods on hydrogen production. As shown in Table 8.2, almost all physical pretreatment methods show a positive effect on hydrogen yield, however, the enhancement mechanisms by various methods are different.

8.7.2.1 Heat Pretreatment

Heat treatment was first applied as the sludge pretreatment method as early as the 1970s, which mainly aimed to enhance sludge dewaterability and anaerobic digestion efficiency (Brooks 1970). Heat pretreatment has been applied for

Table 8.2 Effects of physical pretreatment methods on fermentative hydrogen production

Substrate	Pretreatment conditions	Fermentation conditions	Results	Reference
Heat pretreatment				
WAS	121 °C 30 min	Batch 37 °C Initial pH: 6.63	Hydrogen yield achieved 12.23 mL/g-VS_{added}	Xiao and Liu (2006a)
WAS	50 °C 60 min	Batch 37 °C Initial pH: 6.65	Hydrogen yield achieved 1.17 mL/g-VS_{added}	Xiao and Liu (2006a)
WAS	100 °C 30 min	Batch 35 °C	Hydrogen yield achieved 15.3 mL/g-VSS_{added}	Guo et al. (2015)
WAS	100°C 30 min	CSTR 55 °C Initial pH: 5.4	Hydrogen yield increased 2.13 times Hydrogen content increased from 50 to 75%	Woo and Song (2010)
WAS	90 °C 10 min	Batch 55 °C Initial pH: 7	Hydrogen yield achieved 13.4 mL/g-VSS_{added}	Zheng et al. (2014a)
WAS	102 °C 30 min	Batch 37 °C Initial pH: 11	Hydrogen yield achieved 18.8 mL/g-VSS_{added}	Zhao et al. (2010)
WAS	121 °C 20 min	Batch 37 °C	Hydrogen yield increased 1.84 times	Kotay and Das (2009)
WAS	121 °C 30 min	Batch 37 °C Initial pH: 5	Hydrogen yield achieved 8.94 mL/g-COD_{added}	Wei et al. (2010)
WAS	110 °C 15 min	Batch 30 °C Initial pH: 5.5	Hydrogen yield increased 22.2%	Assawamongkholsiri et al. (2013)
WAS	100 °C 60 min	Batch 35 °C Initial pH: 6.2	Hydrogen yield achieved 14.67 mL/g-VS_{added}	Alemahdi et al. (2015)
Thickened sludge	120 °C 30 min	Batch 37 °C Initial pH: 8	Hydrogen yield increased 4 times	Kim et al. (2013a)
Thickened sludge	160 °C 30 min	Batch 35 °C	Cumulative hydrogen production achieved 3.57 mL	Li et al. (2016)
Thickened sludge	180 °C 30 min	Batch 35 °C	Cumulative hydrogen production achieved 1.5 mL	Li et al. (2016)

(continued)

Table 8.2 (continued)

Substrate	Pretreatment conditions	Fermentation conditions	Results	Reference
Ultrasound pretreatment				
Thickened sludge	35 kHz 0.6 W/mL 20 min	CSTR 36.5 °C Initial pH: 6.2–6.5 OLR: 0.3 kg COD/d	Hydrogen yield increased 2.04 times COD removal increased from 10.3 to 25.3%	Wu and Zhou (2011b)
WAS	2 W/mL 5 min	Batch 35 °C Initial pH: 6.94	Hydrogen yield achieved 5.4 mL/g-TS_{added}	Guo et al. (2008)
WAS	20 kHz 20 min	Batch 35 °C	Hydrogen yield achieved 6.2 ml/g-COD_{added}	Wang et al. (2003)
WAS	20 kHz 0.8 W/mL 30 min	Batch 37 °C Initial pH: 6.9	Hydrogen yield increased about 2.2 times Lag time decreased from 12.32 to 4 h	Xiao and Liu (2009)
WAS	20 kHz 1.4 W/mL	Batch 37 °C	Hydrogen yield increased 60%	Kotay and Das (2009)
Thickened sludge	20 kHz 30 min	Batch 37 °C Initial pH: 8	Hydrogen yield increased 6.2 times	Kim et al. (2013a)
Microwave pretreatment				
WAS	560 W 2 min	Batch 35 °C Initial pH: 8.32	Hydrogen yield achieved 18.28 mL/g-TS_{added}	Guo et al. (2008)
WAS	900 W 2 min	Batch 35 °C	Hydrogen yield achieved 14.2 mL/g-VSS_{added}	Guo et al. (2015)
WAS	600 W 2 min	Batch 37 °C	Hydrogen yield increased 66%	Kotay and Das (2009)
WAS	850 W 3 min	Batch 37 °C Initial pH: 5.5	Hydrogen yield increased from 0.23 to 7.52 mL/g-COD_{added}	Thungklin et al. (2011)
Sterilization pretreatment				
WAS	121 °C 1.2 kgf/cm^2 20 min	Batch 35 °C Initial pH: 7.38	Hydrogen yield achieved 30.4 mL/g-TS_{added}	Guo et al. (2008)
WAS	121 °C 1.2 kgf/cm^2 20 min	Batch 35 °C	Hydrogen yield achieved 13.03 mL/g-TS_{added}	Guo et al. (2013b)
UV-light pretreatment				
WAS	1.5 mW/cm^2 6 h	Batch 55 °C Initial pH: 5.7	Hydrogen yield increased 21.6%	Liu et al. (2013a)

enhancing sludge fermentative hydrogen production until recent 10 years, and the main results of these studies were summarized in Table 8.2. As shown in Table 8.2, the studied sludge types include thickened sludge and WAS. Both mesophilic (30–37 °C) and thermophilic (55 °C) fermentation were investigated, and most of studies were conducted under mesophilic conditions. The pretreatment temperature has a great variation in previous studies. Most of pretreatment temperature were performed in the range of 90–160 °C, excessively low (50 °C) and high (180 °C) temperatures were also reported (Table 8.2). In addition, the pretreatment time ranged from 10 to 66 min.

Heat pretreatment has been proved as an effective method for enhancing fermentation hydrogen production from sludge (Table 8.2). Woo and Song (2010) investigated effects of heat pretreatment on hydrogen production under thermophilic condition in a continuous system, and found that hydrogen yield was increased from 22 to 68.8 mL/g-TS_{added} by pretreatment at 100 °C for 30 min. Hydrogen content in biogas was also increased from 50 to 75%. Kotay and Das (2009) also observed that the hydrogen yield was increased by 1.84 times after treatment at 121 °C for 20 min. The enhancement mechanisms of high temperature treatment (100–180 °C) and low temperature treatment (50–100 °C) might be different. Sludge exposed to high temperature (100–180 °C) could destroy the chemical bonds of cell wall and extracellular polymeric substance, and makes the intracellular organic components accessible for anaerobic bacteria, while low temperature heat treatment (50–100 °C) could not cause disintegration of complex molecules (Carrère et al. 2010). Low temperature heat pretreatment enhanced sludge fermentation mainly through inducing macromolecules deflocculation and improving hydrolase activities during the treatment process.

The conditions of heat pretreatment, including treatment temperature and time, play a significant role on hydrogen production. As for treatment temperature, Xiao and Liu (2006a) found that the hydrogen yield was increased with the increasing treatment temperature. The hydrogen yield was 1.7, 4.85, 8.09, and 12.23 mL/g-VS_{added} at 50, 80, 100, and 121 °C, respectively. Similar results were also reported by Alemahdi et al. (2015) in the range of 75–105 °C. However, Li et al. (2016) observed that the cumulative hydrogen production showed an increasing trend in the range of 75–160 °C, but decreased when the treatment temperature was increased from 160 to 180 °C. This might be because, when the treatment temperature was below 160 °C, more organics solubilization at higher temperature contributed to higher hydrogen production. Conversely, treatment at excessively high temperature (>170 °C) could deteriorate sludge biodegradability although high organics solubilization was achieved. This is because of the occurrence of Maillard reactions, which formed melanoidins through the reactions of amino acids and carbohydrates (Dwyer et al. 2008). Melanoidins are unavailable for anaerobic bacteria, and could also increase the color of the fermentation effluent (Dwyer et al. 2008; Bougrier et al. 2008). As for the treatment time, hydrogen production showed a decreasing trend with the increase of treatment time. Assawamongkholsiri et al. (2013) found the hydrogen yield was decreased from 41 to 2 mL/g-VS_{added}, when the treatment time was increased from 15 to 60 min at

110 °C. Alemahdi et al. (2015) also observed that the hydrogen yield was decreased from 13.11 to 12.15 mL/g-VS_{added}, when the treatment time was increased from 45 to 66 min at 90 °C, while the reason is still unclear.

As whole, heat treatment is an easily operated method for enhancing hydrogen production. Besides, it also has some other advantages for the following sludge handling process including enhancing anaerobic digestion, dewatering, and disinfection. However, heat treatment requires high energy input, so the energy balance and cost analysis of this technology should be considered when performing this technology. Some other limitations of this technology include odor generation, the release of ammonia and phosphate, and fouling of the heat exchangers.

8.7.2.2 Ultrasound Pretreatment

Ultrasound has been considered as one of the most powerful technologies for sludge disintegration. The main results of ultrasound pretreatment on fermentative hydrogen production from sludge are summarized in Table 8.2. As shown in Table 8.2, the studied sludge types include thickened sludge and WAS, and only mesophilic fermentation were performed. Low frequency ultrasound (20–35 kHz) is most conducted in sludge pretreatment, and the treatment time ranged from 5 to 30 min.

Almost all previous studies reported that ultrasound pretreatment had a positive effect on fermentative hydrogen production (Table 8.2). Wu and Zhou (2011b) observed that the hydrogen yield was increased from 13.7 to 41.6 mL/g-VS_{added} by ultrasound pretreatment at ultrasonic density of 0.6 W/mL for 20 min. The COD removal efficiency was also increased from 10.3% to 25.3%. Xiao and Liu (2009) also found that hydrogen yield increased about 2.2 times by ultrasound pretreatment at ultrasonic density of 0.8 W/mL for 30 min, and the lag time of hydrogen production was decreased from 12.32 to 4 h. The main enhancement mechanisms of low frequency ultrasound is the induced cultivation effect, which result in the disruption of sludge flocs and cell membrane and the release of intercellular organics into the liquid phase. There are three main reaction ways for sludge disintegration by ultrasound treatment including hydromechanical shear forces, thermal decomposition and oxidizing effect of free radicals (·OH, ·H, ·N, and ·O) (Khanal et al. 2007). Besides, previous studies also observed that ultrasonic intensity and treatment time could significantly influence sludge disintegration efficiency (Pilli et al. 2011). Sludge solubilization efficiency usually increased with the increasing ultrasonic intensity or treatment time, because higher intensities or longer treatment time could produce higher mechanical shear forces. However, the data about effects of ultrasonic intensity and treatment time on fermentative hydrogen production is still limited.

In addition to enhance fermentative hydrogen production, ultrasound pretreatment could also enhance the following sludge anaerobic digestion, dewatering and disinfection (Pilli et al. 2011). The main advantages of this technology include easy operation, no clogging problems, no odor generation, and potential to control

reactor foaming. High energy consumption, capital, and operating costs are the major drawbacks of this technology. Thus, the energy balance and cost analysis of ultrasound pretreatment should be extensively studied when performing this technology in fermentative hydrogen production.

8.7.2.3 Microwave Pretreatment

Microwave radiation has been applied in sludge treatment in wide aspects including sludge disintegration, enhancing sludge anaerobic digestion, enhancing sludge dewatering, nutrients recovery, and sludge sanitation (Tyagi and Lo 2013). Some researchers have focused on the use of microwave radiation as a pretreatment method for enhancing sludge fermentative hydrogen production during the past few years, and the main results were summarized in Table 8.2. As shown in Table 8.2, only WAS was reported as the fermentative substrate, and fermentation tests were usually performed in the batch mode under mesophilic condition. The pretreatment power ranged from 560 to 900 W, and the treatment time was in the range of 2–3 min. Several researchers have reported that microwave radiation is an efficient technology to enhance fermentative hydrogen production. Kotay and Das (2009) reported that 66% more hydrogen yield was achieved of microwave pretreated sludge comparing with the raw sludge. Furthermore, Thungklin et al. (2011) also observed that hydrogen yield was increased from 0.23 to 7.52 mL/g-COD_{added} by microwave radiation at 850 W for 3 min. The enhancement of hydrogen production is mainly because microwave radiation breaks the sludge cells and releases intracellular organics (proteins, carbohydrates, lipids, and DNA) into the soluble phase. Some operating parameters, including power intensity, treatment temperature, and reaction time, could influence sludge solubilization efficiency, but few studies focused on the effects of above parameters on the subsequent fermentative hydrogen production.

Microwave radiation is an environmental friendly and cost-effective technology. The energy consumption of microwave radiation is less than that of conventional heating because of no heat loss by direct internal heating. The heating speed of microwave is also more rapid comparing with conventional heating. Furthermore, the process temperature of microwave radiation could be controlled precisely. The reported data on effects of this technology on sludge fermentation are still limited. In addition, the energy balance and cost analysis should also be extensively studied when performing this technology in fermentative hydrogen production.

8.7.2.4 Sterilization Pretreatment

Sterilization was usually applied to kill the pathogenic microorganisms during sludge handling process. Some researchers found that sterilization could also be used as a sludge pretreatment technology to improve fermentative hydrogen production, and the results are reported in Table 8.2. As shown in Table 8.2, WAS was

the only studied sludge type, and fermentation tests were performed in the batch mode under mesophilic condition. The pretreatments were usually performed at 121 °C and 1.2 kgf/cm^2 for 20 min. Guo et al. (2013b) reported that hydrogen yield of sterilized sludge reached 13.03 mL/g-TS$_{added}$. A higher hydrogen yield of 30.4 mL/g-TS$_{added}$ by sterilization pretreatment was also observed in Guo et al. (2008). The main advantages of this technology for sludge fermentation are that it could reduce the inhibitory effect of toxic metals and VFAs, and thus contributing to more hydrogen production. However, information about effects of sterilization on the structure of sludge flocs and sludge solublization efficiency is still limited.

8.7.2.5 UV-Light Pretreatment

UV-light radiation has been widely applied as a disinfection technology in water and wastewater treatment. Effects of UV-light radiation on fermentative hydrogen production was also reported in Liu et al. (2013a). The UV-light intensity is 1.5 mW/cm^2, and the treatment time continued 6 h. 22% higher hydrogen yield was obtained of UV-light pretreated sludge comparing with the raw sludge. UV-light radiation could induce more organics transforming into soluble phase, which made substrate to be more easily utilized by anaerobic bacteria.

8.7.3 Chemical Pretreatment Methods

The destruction of complex organics in sludge has been successfully achieved by means of adding strong alkalis, acids, and oxidants (Tyagi and Lo 2011). These chemical pretreatment methods have been developed to enhance fermentative hydrogen production, and the main results are reported in Table 8.3. As shown in Table 8.3, almost all reported chemical pretreatment methods could improve hydrogen yield. The operating principles of various methods are different, so the chemical pretreatment methods were discussed from the following three major groups (alkaline, acid, and oxidation).

8.7.3.1 Alkaline Pretreatment

Alkaline pretreatment has been a widely used method to realize sludge solubilization. NaOH, KOH, Ca $(OH)_2$, and $Mg(OH)_2$ are four main alkalis applied in sludge pretreatment, and the solubilization efficiency follows the order: $Ca(OH)_2$ and $Mg(OH)_2$ < KOH < NaOH (Carrère et al. 2010). Alkaline pretreatment has been studied for enhancing fermentative hydrogen production, and the main results are summarized in Table 8.3. As illustrated in Table 8.3, the studied sludge types include thickened sludge and WAS. Fermentation tests were usually performed in

Table 8.3 Effects of chemical pretreatment methods on fermentative hydrogen production

Substrate	Pretreatment conditions	Fermentation conditions	Results	Reference
Alkaline pretreatment				
Thickened sludge	pH = 12 24 h	Batch 36 °C Initial pH: 5	Hydrogen yield achieved 2.97 mL/g-TS_{added}	Liu et al. (2014)
Thickened sludge	pH = 9 24 h	Batch 36 °C Initial pH: 12	Hydrogen yield achieved 0.77 mL/g-TS_{added}	Liu et al. (2014)
WAS	pH = 12 24 h	Batch 36 °C Initial pH: 10	Hydrogen yield increased 3.25 times	Cai et al. (2004)
WAS	pH = 12 12 h	Batch 37 °C Initial pH: 11.3	Hydrogen yield increased from almost 0 to 11.9 mL/g-VS_{added}	Xiao and Liu (2006b)
WAS	pH = 12 12 h	Batch 37 °C Initial pH: 11.5	Hydrogen yield increased 35.5%	Xiao and Liu (2009)
WAS	pH = 10–11 24 h	Batch 37 °C	Hydrogen yield increased 64%	Kotay and Das (2009)
WAS	pH = 12 24 h	Batch 37 °C Initial pH: 11	Hydrogen yield achieved 10.32 mL/g-COD_{added}	Wei et al. (2010)
Thickened sludge	pH = 12 24 h	Batch 37 °C Initial pH: 8	Hydrogen yield increased 8.2 times	Kim et al. (2013a)
Acid pretreatment				
WAS	pH = 3 6 h	Batch 35 °C	Hydrogen yield increased 13.3%	Wang et al. (2003)
WAS	pH = 3 6 h	Batch 35 °C Initial pH: 4.72	Hydrogen yield increased 100%	Jan et al. (2007)
WAS	pH = 2 12 h	Batch 37 °C Initial pH: 7	Hydrogen yield increased 1.76 times	Xiao and Liu (2009)
WAS	pH = 3–4 24 h	Batch 37 °C	Hydrogen yield increased 44%	Kotay and Das (2009)
WAS	0.5% (w/v) HCl 24 h	Batch 30 °C Initial pH: 5.5	Hydrogen yield increased 3.56 times	Assawamongkholsiri et al. (2013)
Thickened sludge	pH = 2 24 h	Batch 37 °C Initial pH: 8	Hydrogen yield increased 2 times	Kim et al. (2013a)

(continued)

Table 8.3 (continued)

Substrate	Pretreatment conditions	Fermentation conditions	Results	Reference
Oxidation pretreatment				
WAS	Photocatalytic (3 g/L TiO_2, 350 mL/min, UV-light intensity of 1.5 mW/cm^2) 6 h	Batch 55 °C Initial pH: 5.7	Hydrogen yield increased 89.8%	Liu et al. (2013a)
WAS	Low-pressure wet oxidation (175 °C, 0.89 MPa) 30 min	Batch 36 °C Initial pH: 7	Hydrogen yield achieved 19.84 mL/g-VSS_{added}	Yin and Wang (2016b)
Thickened sludge	Wet air oxidation (161.2 °C, 661 r/min) 48 min	Batch Mesophilic Initial pH: 6.51	Hydrogen yield increased from 0.13 to 13.16 mL/g-VS_{added}	Yu et al. (2013a)

batch mode under mesophilic condition. The pretreatment pH value commonly ranged from 9 to 12, and the treatment time was in the range of 12–24 h.

Alkaline pretreatment has been proved as an efficient method for enhancing fermentative hydrogen production from different types of sludge. Xiao and Liu (2009) found that hydrogen yield from WAS was increased by 35.5% by alkaline pretreatment at pH of 12 for 12 h. Kotay and Das (2009) observed that 64% higher hydrogen yield was achieved by alkaline pretreatment at the pH value of 10–11 for 24 h. Another study performed by Kim et al. (2013a) investigated effects of the addition of alkaline on fermentative hydrogen production from thickened sludge, and hydrogen yield was increased 8.2 times at pH of 12 for 24 h. The enhancement of hydrogen yield is mainly because alkaline pretreatment could disrupt sludge flocs and cell walls, and then complex organic compounds were decomposed and transformed into the soluble phase. The increase of specific surface area and sludge solubilization contributed to easier substrate utilization of anaerobic bacteria. Alkaline could react with sludge through two ways: saponification of acetyl esters and uronic acids, and neutralization of organic acids. Some parameters, such as alkali dose and treatment time, could influence the pretreatment efficiency, sludge solubilization is usually increased with the increase of alkali dose and treatment time. However, few studies reported effects of these two parameters on hydrogen yield.

Alkaline pretreatment is an energy efficient and a simple method for enhancing fermentative hydrogen production from sewage sludge, and it could also enhance sludge anaerobic digestion and remove pathogenic microorganisms. However, as the required pH values of alkaline treatment are extremely high, sludge is required

to be re-neutralized after pretreatment to suitable condition for anaerobic bacteria. Besides, corrosion of the equipment and bad odor generation should also be taken into consideration when performing this pretreatment.

8.7.3.2 Acid Pretreatment

Acid pretreatment is another chemical method to achieve sludge solubilization, and has been studied for enhancing fermentative hydrogen production since the year of 2003. Table 8.3 illustrates the main results of adding acid on hydrogen production. The studied sludge types include thickened sludge and WAS. Fermentative hydrogen production tests were usually performed in batch mode under mesophilic condition (30–37 °C). HCl was the most commonly studied acid, perchloric acid was also applied for sludge pretreatment. The pretreatment pH value commonly ranged from 2 to 4, and the treatment time was in the range of 6–24 h.

As shown in Table 8.3, almost all studies observed a positive impact of acid pretreatment on hydrogen yield from both thickened sludge and WAS. Wang et al. (2003) reported that hydrogen yield was increased by 13.3% by acid pretreatment at pH of 3 for 6 h. Kotay and Das (2009) observed that 44% higher hydrogen yield was achieved by acid pretreatment at pH of 3–4 for 24 h. Kim et al. (2013a) found that hydrogen yield from thickened sludge was increased by about 2 times by acid pretreatment at pH of 2 for 24 h. The main enchantment mechanisms could be concluded that the addition of acid disrupted the sludge flocs and cell walls, resulting in barely degradable and particle complex organics transferring into more easily degradable forms and the soluble phase. Similar with alkaline pretreatment, effects of acidification conditions, such as acid dose and treatment time, on hydrogen production is rarely studied.

Acid pretreatment is a simple, efficient, and an energy efficient method for enhancing fermentative hydrogen production from sludge. However, inhibitory by-products (e.g., hydroxymethylfurfural and furfural) may be generated during sludge acidification process. Acid pretreatment could also cause the loss of fermentable sugar. In addition, the additional alkaline need of re-neutralization, corrosion of the equipment and odor generation are other drawbacks of acid pretreatment.

8.7.3.3 Oxidation Pretreatment

Oxidation is a promising chemical pretreatment method compared with alkaline and acid pretreatments due to no chemical addition and no chemical residues remain. Oxidation pretreatment has been applied for sludge destruction from as early as the 1950s (Tyagi and Lo). However, oxidation has been applied as a way for enhancing fermentative hydrogen production until recent 5 years, and the main results are reported in Table 8.3. The treatment methods include wet oxidation and

photocatalytic process. The studied sludge types include thickened sludge and WAS. Both mesophilic and thermophilic fermentation tests were conducted.

Almost all studies reported that various oxidation pretreatment methods could improve hydrogen yield. Yu et al. (2013a) investigated effects of wet air oxidation on fermentative hydrogen production from thickened sludge. Results showed that hydrogen yield was increased from 0.13 to 13.16 mL/g-VS_{added} by wet oxidation pretreatment compared with the control group. The enhancement principles could be concluded that wet air oxidation breaks the sludge flocs and cell walls and transforms complex organics (proteins, carbohydrates, and lipids) into more easily degradable forms. Treatment time, temperature, and stirring rate of this method could influence sludge solubilization significantly, and thus effects of above parameters on hydrogen production should be extensively studied. Besides, photocatalytic, an advanced oxidation treatment process, has also been performed for enhancing fermentative hydrogen production by Liu et al. (2013a). 89.8% higher hydrogen yield was obtained of photocatalytic pretreated sludge comparing with the raw sludge. The main mechanisms of sludge disintegration by photocatalytic pretreatment are the generation and utilization of hydroxyl radicals.

Oxidation is a kind of environment friendly sludge pretreatment methods. Oxidation pretreatment could also enhance sludge anaerobic digestion and dewatering. However, oxidation pretreatment could result in the excessive degradation of organics, which reduced the carbon source for fermentative bacteria. Additionally, corrosion of the equipment, odor generation, and high energy cost significantly limits the application of this method.

8.7.4 Biological Pretreatment Methods

Recently, biological methods have also been performed as pretreatments to improve fermentative hydrogen production from sludge, and the main results are shown in Table 8.4. The addition of specific enzymes and strains of bacteria are two main biological ways for enhancing sludge fermentation. The essence of above two ways for sludge hydrolysis is based on the added specific enzymes or enzymes secreted by added specific bacteria. As shown in Table 8.4, the studied sludge type include WAS and ADS. Fermentative hydrogen production tests were performed in batch mode under both mesophilic and thermophilic conditions. The added enzymes include bromelain and lysozyme, the added bacteria include *Pseudomonas* sp. GZ1, *Bacillus* sp. AT07-1, *Penicillium* sp. CedarWA2, *Fusarium* sp. OreYA, *Chaetomium* sp. GalleryYA, *Cunninghamella* sp. CedarWA, and *Neosartorya* sp. OreWA. The pretreatment time ranged from 4 to 20 h.

As for enzyme pretreatment, Guo et al. (2015) reported that hydrogen yield reached 12.5 mL/g-VSS_{added} by multienzymes of bromelain and lysozyme pretreatment at mixed ratio of 1:50 for 5 h. As for bacterial pretreatment, another study performed by Guo et al. (2013a) observed that hydrogen yield achieved 68.4 mL/g-VSS_{added} by adding *Bacillus* sp. AT07-1 at mixed ratio of 1:10 for 8 h.

Table 8.4 Effects of biological pretreatment methods on fermentative hydrogen production

Substrate	Pretreatment conditions	Fermentation conditions	Results	Reference
Enzyme pretreatment				
WAS	Bromelain and lysozyme (1:50) 30 °C 5 h	Batch 35 °C	Hydrogen yield achieved 12.5 mL/g-VSS_{added}	Guo et al. (2015)
Bacterial pretreatment				
WAS	*Pseudomonas* sp. GZ1 (1:50) 65 °C 12 h	Batch 35 °C	Hydrogen yield achieved 14 mL/g-VSS_{added}	Guo et al. (2015)
WAS	*Bacillus* sp. AT07-1 (1:10) 65 °C, pH 6 4 h	Batch 36 °C	Hydrogen yield achieved 28 mL/g-VSS_{added}	Guo et al. (2012)
WAS	*Bacillus* sp. AT07-1 (1:10) 65 °C, pH 6 8 h	Batch 36 °C	Hydrogen yield achieved 30.8 mL/g-VSS_{added}	Guo et al. (2012)
WAS	*Bacillus* sp. AT07-1 (1:10) 65 °C, pH 6 20 h	Batch 36 °C	Hydrogen yield achieved 26.5 mL/g-VSS_{added}	Guo et al. (2012)
WAS	*Bacillus* sp. AT07-1 (1:10) 65 °C, pH 6 8 h	Batch 35 °C Initial pH: 6.4	Hydrogen yield achieved 68.4 mL/g-VSS_{added}	Guo et al. (2013a)
ADS	4 mL *Penicillium* sp. CedarWA2	Batch 50 °C Initial pH: 5	Hydrogen yield increased 1.5 times	Sato et al. (2016)
ADS	4 mL *Fusarium* sp. OreYA	Batch 50 °C Initial pH: 5	Hydrogen yield increased 4.5 times	Sato et al. (2016)
ADS	4 mL *Chaetomium* sp. GalleryYA	Batch 50 °C Initial pH: 5	Hydrogen yield increased 7.5 times	Sato et al. (2016)
ADS	4 mL *Cunninghamella* sp. CedarWA	Batch 50 °C Initial pH: 5	Hydrogen yield increased 10 times	Sato et al. (2016)
ADS	4 mL *Neosartorya* sp. OreWA	Batch 50 °C Initial pH: 5	Hydrogen yield increased 2 times	Sato et al. (2016)

Sato et al. (2016) investigated effects of five groups of bacteria (*Fusarium* sp. OreYA, *Chaetomium* sp. GalleryYA, *Cunninghamella* sp. CedarWA, and *Neosartorya* sp. OreWA) on fermentative hydrogen production from ADS. The hydrogen yield was increased by in the range of 1.5–10 times by above five

bacteria. The main enhancement mechanisms can be concluded that the addition of hydrolase or hydrolase secreted by added bacteria solubilized suspended organics through enzyme-catalyzed reactions, which improves the biodegradation of macromolecules. The pretreatment time and addition dose could influence the sludge solubilization efficiency, and thus influence hydrogen production. Guo et al. (2012) found that hydrogen yield was increased from 28 to 30.8 mL/g-VSS_{added} when pretreatment time increased from 4 to 8 h, but was decreased to 26.5 mL/g-VSS_{added} when pretreatment time further increased to 20 h. The reported reason was that hydrogen producers were inhibited by the addition of bacteria at longer treatment time. However, effects of addition of dose on fermentative hydrogen production have been rarely studied.

Biological sludge pretreatment methods are promising and environment friendly, and can also improve the efficiency of anaerobic digestion. However, literature on this subject is still scarce compared with other types of pretreatment methods (Fig. 8.3a). In addition, high-cost restricts their application on fermentation hydrogen production from sludge.

8.7.5 Combined Pretreatment Methods

Various pretreatment methods are based on different operating mechanisms to disintegrate complex sludge flocs and solubilize particle organics. Some combined pretreatment methods have been performed to achieve a further improvement of hydrogen production and higher substrate utilization ratio. Table 8.5 illustrates effects of combined pretreatment methods on fermentative hydrogen production from sludge. The studied sludge types included WAS, primary sludge, and thickened sludge. Some physical pretreatment methods have been combined with physical, chemical and biological pretreatment methods. Among which, heat pretreatment was the most commonly studied individual method in combination with other pretreatment methods. Fermentative hydrogen production tests were performed under both mesophilic and thermophilic conditions. The reactor types included batch and CSTR.

Almost all studies reported that various combined pretreatment methods had a positive effect on hydrogen yield. Kang et al. (2012) investigated effects of alkaline-heat pretreatment on fermentative hydrogen production. The hydrogen yield was increased from 1.41 to 7.7 mL/g-COD_{added} by the combination of alkaline and heat pretreatment. Massanet-Nicolau et al. (2008) found that hydrogen yield reached 18.14 mL/g-TS_{added} from heat-enzyme pretreated thickened primary sludge. Yang et al. (2012) observed that hydrogen yield achieved 7.24 mL/g-TS_{added} after heat-ultrasound pretreatment. The enhancement of hydrogen yield by combined pretreatment methods was mainly because of the disintegration of sludge flocs and cell walls, and the increase of sludge solubilization ratio, which induced the better utilization ratio of organics in sludge by anaerobic bacteria. Some studies found that process conditions of combined

Table 8.5 Effects of combined pretreatment methods on fermentative hydrogen production

Substrate	Pretreatment method	Pretreatment conditions	Fermentation conditions	Results	Reference
WAS	Alkaline-Heat	pH = 13, 2 h; 150 °C, 30 min	Batch 30 °C Initial pH: 5.5	Hydrogen yield increased 4.6 times	Kang et al. (2012)
Primary sludge	Heat-Enzyme	70 °C, 60 min; Cellulase, 24 h	CSTR 35 °C Initial pH: 5.5	Hydrogen yield achieved 18.14 mL/g-TS_{added}	Massanet-Nicolau et al. (2008)
WAS	Bacteria-Heat	Stearothermophilus, 8 h; 90 °C, 10 min	Batch 55 °C Initial pH: 6.46	Hydrogen yield achieved 19.9 mL/g-VSS_{added}	Zheng et al. (2014a)
WAS	Heat-Ozone	90 °C, 15 min; 0.158 g O_3/g-TS, 1 h	Batch 35 °C	Hydrogen yield achieved 3.59 mL/g-TS_{added}	Yang et al. (2012)
WAS	Heat-Ultrasound	90 °C, 15 min; 20 kHz, 1.423 W/mL, 1 h	Batch 35 °C	Hydrogen yield achieved 7.24 mL/g-TS_{added}	Yang et al. (2012)
WAS	Heat-Ultrasound-Ozone	90 °C, 15 min; 1.423 W/mL, 1 h; 0.158 g O_3/g-TS,1 h	Batch 35 °C	Hydrogen yield achieved 9.28 mL/g-TS_{added}	Yang et al. (2012)
WAS	Alkaline-Ionizing radiation	pH = 12 20 kGy	Batch 36 °C Initial pH: 7	Hydrogen yield achieved 4.5 mL/g-COD_{added}	Yin and Wang (2016c)
WAS	Sterilization-Enzyme	70 °C, 1 h; 5% (v/v) Cellulase	CSTR 35 °C HRT: 48 h	Hydrogen yield achieved 32.7 mL/g-VS_{added}	Massanet-Nicolau et al. (2010)

(continued)

Table 8.5 (continued)

Substrate	Pretreatment method	Pretreatment conditions	Fermentation conditions	Results	Reference
WAS	Acid-Heat	0.5% (w/v) HCl; 110 °C, 45 min	Batch 30 °C Initial pH: 5.5	Hydrogen yield increased 1.56 times	Assawamongkholsiri et al. (2013)
Thickened sludge	Ultrasound-Alkaline	20 kHz, 15 min; pH = 12, 15 min	Batch 37 °C Initial pH: 8	Hydrogen yield increased 11.4 times	Kim et al. (2013a)
Thickened sludge	Alkaline-Ultrasound	pH = 12, 15 min; 20 kHz, 15 min	Batch 37 °C Initial pH: 8	Hydrogen yield increased 15.8 times	Kim et al. (2013a)

HRT hydraulic retention time

pretreatment methods could influence the organics solubilization, however, effects of these conditions on hydrogen production is rarely reported. In particular, Kim et al. (2013a) observed that the pretreatment order of various methods could influence the hydrogen yield from combined pretreated sludge. The hydrogen yield from alkaline-ultrasound pretreated sludge was 35.5% higher than that from ultrasound-alkaline pretreated sludge at the same treatment condition.

8.7.6 Comparison of Different Pretreatment Methods

As experimental conditions (e.g., inoculum, temperature, reactor type, pH, OLR, and sludge retention time), sludge characterizes (sludge type, sludge source, VS content, and water content) and expression units of the hydrogen yield (e.g., mL/g-VS_{added}, mL/g-COD_{added}, mL/g-TS_{added}, and mL/g-VSS_{added}) have great variation in various studies, causing that it is difficult to directly compare the results from different studies. Thus, in this chapter, the comparison of different pretreatment methods is based on the same experimental conditions and sludge characterizes, and the hydrogen yield was used as the evaluation index.

It seems that the enhancement impact on hydrogen yield by combined pretreatment method is commonly greater than that by individual pretreatment. Kim et al. (2013a) compared effects of ultrasound, alkaline, and ultrasound-alkaline pretreatments on hydrogen yield from thickened sludge. The hydrogen yield of combined ultrasound-alkaline pretreated sludge was 72.2% and 34.8% higher than that of individual ultrasound and individual alkaline pretreated sludge, respectively. Yang et al. (2012) compared the enhancement effect of heat, heat-ultrasound, and heat-ultrasound-ozone pretreatments on fermentative hydrogen production from WAS. The hydrogen yield of heat-ultrasound-ozone pretreated sludge was 28.2% higher than that of heat-ultrasound pretreated sludge, and the hydrogen yield of heat-ultrasound pretreated sludge was 5.14 times higher than that of individual heat pretreated sludge. Similar results were also obtained in Zheng et al. (2014a), which investigated effects of bacteria-heat pretreatment and individual heat pretreatment on hydrogen yield from WAS. These results might be due to the synergistic effect of various pretreatment methods.

As for individual pretreatment, there is no clear pattern in previous studies. Guo et al. (2015) studied effects of heat, microwave, bacteria and enzyme pretreatments on hydrogen production from WAS. An increase of hydrogen yield was found in the following order: heat pretreatment (100 °C, 30 min) > microwave pretreatment (900 W, 2 min) > bacteria pretreatment (mixed ratio of 1:50, 12 h) > enzyme pretreatment (mixed ratio of 1:50, 5 h). Wang et al. (2003) observed that the enhancement of hydrogen yield from WAS was in the order: acid pretreatment (pH = 3, 24 h) > sterilization pretreatment (121 °C, 1.2 kgf/cm^2, 30 min) > ultrasound pretreatment (20 kHz, 20 min). Kotay and Das (2009) studied effects of alkaline, heat, ultrasound, acid, and microwave pretreatments on fermentative

hydrogen production from WAS. The hydrogen yield was found in the following order: heat pretreated sludge > microwave pretreated sludge > alkaline pretreated sludge > ultrasound pretreated sludge > acid pretreated sludge.

8.8 Co-fermentation with Other Substrates

8.8.1 Overview

Sewage sludge is characterized by high buffer capacity and low C/N ratio. Co-fermentation of sludge and other substrates with high C/N ratio could provide more suitable growth and substrate conditions for fermentation bacteria, thus improving the efficiency of hydrogen production (Mata-Alvarez et al. 2014). There are also some other advantages, such as balanced nutrients, the dilution of toxic substances, and synergistic effects on the fermentation reactions. Besides, co-fermentation of sludge and other organic substrates could also improve the VFAs production.

Various kinds of co-fermentation substrates have been investigated for enhancing fermentative hydrogen production from sludge. These substrates can be divided into four categories based on their sources, including municipal waste fractions, crop residues, carbohydrate-rich wastewaters, pure carbohydrates, and other organic wastes. Figure 8.4 illustrates the percentages of various categories of co-fermentation substrates. As shown in Fig. 8.4, municipal waste fractions was the most studied group, accounting for 57.2% of all reports, and followed by pure carbohydrates (15.8%), carbohydrate-rich wastewaters (11.3%), crop residues (9.5%), and other organic wastes (6.2%). The main results of these co-fermentation tests are illustrated in Table 8.6, and the detail discussions are performed as following.

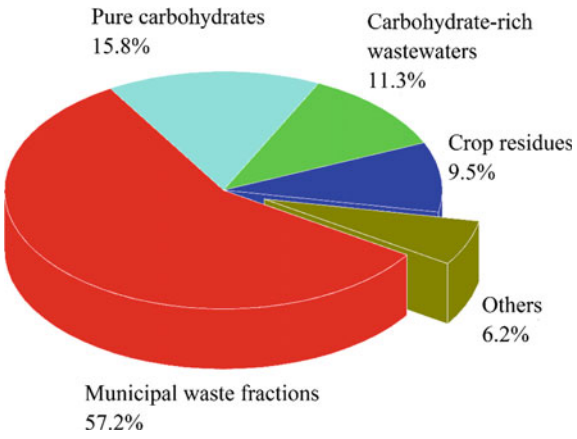

Fig. 8.4 Percentages of various categories of co-fermentation substrates in the reviewed literature (The data in pie-chart are calculated based on the number of all reported fermentation tests)

8.8.2 Municipal Waste Fractions

Municipal waste fractions, including food waste and organic fraction of municipal solid waste (OFMSW), are economical and highly biodegradable substrate for fermentative hydrogen producing bacteria. Generally, they consist of protein, starch, and lipid, cellulose, and hemicellulose. They contain 74–90% of moisture and 80–97% of VS. The C/N ratios of municipal waste fractions commonly range from 14.7 to 36.4 (De Gioannis et al. 2013). Besides, the low transport cost of municipal waste fractions make them be a suitable co-fermentation substrate for sludge. Taking into account above advantages, co-fermenting sludge with municipal waste fractions for hydrogen production have been widely studied, and the main results are illustrated in Table 8.6.

As shown in Table 8.6, the studied sludge types included mixed sludge, primary sludge, WAS, and thickened sludge. Fermentation tests were performed under both mesophilic and thermophilic conditions. The continuous stirring tank and batch reactors were all performed, and the initial pH ranged from 5 to 8. Kim et al. (2004) investigated the feasibility of hydrogen production from co-fermentation of sludge and food waste in batch reactors at 35 °C, and concluded that the maximum cumulative hydrogen production achieved 347.2 mL at a food waste/sludge ratio of 4:1 (VS basis). Meanwhile, sole sludge fermentation generated almost no hydrogen, and hydrogen production was increased with the increase of food waste addition. Zhou et al. (2013) optimized anaerobic co-fermentation of sludge and food waste for hydrogen production. The maximum hydrogen yield from co-fermentation reached 66 mL/g-COD_{added} at a sludge/food waste ratio of 1:3 (volume basis), which was 5.6 times higher than that from sole sludge fermentation. This study also found that hydrogen yield was increased with the increase of food waste addition. Cheng et al. (2016) observed that the hydrogen yield achieved 174.6 mL/g-VS_{added} at a sludge/food waste ratio of 1:3 (VS basis), which was 8.75 times higher than that from sole sludge fermentation. The addition of food waste strengthened the hydrolysis and acidogenesis process. Zahedi et al. (2013) investigated hydrogen production from thermophilic co-fermentation of WAS and OFMSW. The hydrogen yield and VS removal achieved 29 mL/g-VS_{added} and of 45% at an OFMSW/sludge ratio of 1:5 (volume basis), respectively. In particular, a pilot-scale experimental reactor was applied to evaluate the performance of co-fermentation of sludge and OFMSW (Gottardo et al. 2015), and found that the hydrogen yield reached 40 mL/g-VS_{added} at a sludge/OFMSW ratio of 1:1 (VS basis).

8.8.3 Crop Residues

Crop residues is one kind of the most cheapest and abundant organic wastes. About 200 billion tons (dry weight) of agricultural residues have been generated annually in the world (Guo et al. 2010b). Almost all kinds of agricultural crop residues could

Table 8.6 The main results of sludge co-fermentation with other substrates

Co-substrates	Sludge type	Mixed ratio (Co-substrate/sludge)	Fermentation conditions	Results	References
Municipal waste fractions					
Food waste	Mixed sludge	10:1 (COD basis)	Batch 35 °C Initial pH: 8	Hydrogen yield achieved 161 mL/g-VS_{added}	Im et al. (2012)
Food waste	WAS	1:1 (volume basis)	Batch 37 °C Initial pH: 5.5	Hydrogen yield achieved 13.8 mL/g-$VSS_{removed}$	Kim et al. (2013a)
Food waste	WAS	8.5:1.5 (VS basis)	Batch 37 °C Initial pH: 5.5	Hydrogen yield increased from 2.5 to 121.1 mL/g-VS_{added}	Liu et al. (2013c)
Food waste	WAS	–	Batch 30 °C	Hydrogen yield achieved 101.1 mL/g-VS_{added}	Sreela-or et al. (2011)
Food waste	Primary sludge	3:1 (volume basis)	Batch 37 °C Initial pH: 5.5	Hydrogen yield increased 5.6 times	Zhou et al. (2013)
Food waste	WAS	3:1 (volume basis)	Batch 37 °C Initial pH: 5.5	Hydrogen yield increased 7.86 times	Zhou et al. (2013)
Food waste	Mixed sludge	4:1 (volume basis)	Batch 37 °C Initial pH: 5.5	Hydrogen yield achieved 76 mL/g-COD_{added}	Zhou et al. (2013)
Food waste	Mixed sludge	1:1 (VS basis)	Batch 37 °C Initial pH: 5.5	Hydrogen yield increased from 2.3 to 64.48 mL/g-VS_{added}	Lin et al. (2013)
Food waste	WAS	4:1 (VS basis)	Batch 35 °C Initial pH: 5–6	Hydrogen production achieved 347.2 mL	Kim et al. (2004)

(continued)

Table 8.6 (continued)

Co-substrates	Sludge type	Mixed ratio (Co-substrate/sludge)	Fermentation conditions	Results	References
Food waste	Thickened sludge	10:1 (COD basis)	Batch 35 °C Initial pH: 8	Hydrogen yield increased 22.6 times. VS removal achieved 51%	Kim et al. (2011a)
Food waste	WAS	3:1 (VS basis)	Batch 35 °C Initial pH: 6	Hydrogen yield increased 8.75 times	Cheng et al. (2016)
OFMSW	WAS	1:1 (VS basis)	CSTR 55 °C Initial pH: 5.1 HRT: 3.3 d OLR: 18 kg $VS/m^3 \cdot d$	Hydrogen yield achieved 40 mL/g-VS_{added}	Gottardo et al. (2015)
OFMSW	Mixed sludge	1:1 (volume basis)	Batch 55 °C Initial pH: 5.5	Hydrogen yield increased 1.44 times	Tyagi et al. (2014)
OFMSW	Primary sludge	1:1 (volume basis)	Batch 55 °C Initial pH: 5.5	Hydrogen yield achieved 38 mL/g-$VS_{removed}$	Tyagi et al. (2014)
OFMSW	WAS	1:1 (volume basis)	Batch 55 °C Initial pH: 5.5	Hydrogen yield achieved 26 mL/g-$VS_{consumed}$	Tyagi et al. (2014)
OFMSW	WAS	1:5 (volume basis)	CSTR 55 °C Initial pH: 5.1 HRT: 3 d OLR: 16 kg $VS/m^3 \cdot d$	Hydrogen yield achieved 29 mL/g-VS_{added} VS removal achieved 45%	Zahedi et al. (2016)

(continued)

Table 8.6 (continued)

Co-substrates	Sludge type	Mixed ratio (Co-substrate/sludge)	Fermentation conditions	Results	References
OFMSW	WAS	1: 1 (volume basis)	Batch 55 °C Initial pH: 6	Hydrogen yield increased 88.2%	Elsamadony and Tawfik (2015)
Crop residues					
Rice straw	WAS	4:1 (VS basis)	Batch 35 °C Initial pH: 4.75	Hydrogen yield achieved 14.96 mL/g-VS_{added}	Alemahdi et al. (2015)
Rice straw	Thickened sludge	11: 1 (VS basis)	Batch 55 °C Initial pH: 7	Hydrogen yield achieved 21 mL/g-VS_{added} H_2 content achieved 60.9%	Kim et al. (2013b)
Cornstalk	WAS	2:1 (TS basis)	Batch 37 °C Initial pH: 7	Hydrogen yield increased 12.4 times	Liu et al. (2013b)
Carbohydrate-rich wastewaters					
Molasses wastewater	Mixed sludge	2:1 (volume basis)	CSTR 35 °C Initial pH: 5.5 HRT: 21 h	Hydrogen yield achieved 67.08 mL/g-VS_{added}	Lee et al. (2014a)
Molasses wastewater	Mixed sludge	10:1 (volume basis)	Batch 35 °C Initial pH: 5.7	Hydrogen yield achieved 50 mL/g $VS_{removed}$	Lee et al. (2014a)
Pure carbohydrates					
Starch	WAS	–	Batch 37 °C Initial pH: 8	Hydrogen yield increased 13.8 times TS removal increased from 16% to 38.2%	Chen et al. (2012)

(continued)

Table 8.6 (continued)

Co-substrates	Sludge type	Mixed ratio (Co-substrate/sludge)	Fermentation conditions	Results	References
Glucose	WAS	–	Batch 36 °C Initial pH: 7	Hydrogen yield achieved 25.03 mL/g-COD_{added}	Yin and Wang (2016c)
Glucose	WAS	–	Batch 36 °C Initial pH: 7	Hydrogen yield achieved 49.72 mL/g-COD_{added}	Yin and Wang (2015)
Other organic wastes					
Crude glycerol	Mixed sludge	1:100 (volume basis)	CSTR Initial pH: 5–6 HRT: 3 d OLR: 16.68 g COD/L d	Hydrogen yield achieved 230 mL/g-$VS_{removed}$	Rivero et al. (2014)
Tofu residue	Thickened sludge	–	Batch 35 °C Initial pH: 7	Hydrogen yield achieved 33.15 L/mol $hexose_{added}$	Kim et al. (2011b)
Oil palm frond juice	WAS	7:3	Batch 37 °C Initial pH: 5.8	Hydrogen yield achieved 186.7 mL/g-$glucose_{added}$	Yasin et al. (2013)

be biologically converted to hydrogen during the anaerobic fermentation process. The C/N ratio of crop residues are commonly higher than 40 (Zheng et al. 2014b). Crop residues have been applied as the co-substrates for enhancing fermentative hydrogen production from sludge. As shown in Table 8.6, the studied crop residues included rice straw and cornstalk. The studied sludge types included WAS and thickened sludge. Fermentative tests were performed under both mesophilic and thermophilic conditions. The reactors are all operated in batch mode, and the initial pH was in the range of 4.75–7.

Hydrogen production from co-fermentation of sludge and raw rice straw was investigated in Alemahdi et al. (2015). The maximum hydrogen yield achieved 14.96 mL/g-VS_{added} at a sludge/rice straw ratio of 1:4 (VS basis). The size of co-fermentation substrate could influence the metabolic pathways of anaerobic bacteria, and thus influence the hydrogen yield. The hydrogen yield from co-fermentation of sludge and large size rice straw is about 2.5 times higher than that from co-fermentation of sludge and small size rice straw. Kim et al. (2013b) studied hydrogen production from co-fermentation of raw sludge and raw rice straw, and found that hydrogen yield and hydrogen content reached 21 mL/g-VS_{added} and 60.9% at a sludge/rice straw ratio of 11:1 (VS basis). Additionally, Liu et al. (2013b) investigated the effects of adding cornstalk on fermentative hydrogen production from WAS. The maximum hydrogen yield achieved 13.4 mL/g-VS_{added} at a sludge/cornstalk ratio of 1:2 (TS basis), which is much higher than hydrogen yield from sole sludge fermentation (1 mL/g-VS_{added}). The mixed ratio of sludge and cornstalk plays a significant role in hydrogen production. The hydrogen yield was increased with the increase of cornstalk addition.

8.8.4 Carbohydrate-Rich Wastewaters

Carbohydrate-rich wastewaters are preferred substrates for fermentative hydrogen production. Among this kind of wastewaters, molasses wastewater has been co-fermented with sludge for hydrogen production. Lee et al. (2014a) evaluated the feasibility of co-fermenting WAS and molasses wastewater in a continuous stirring tank reactor under mesophilic condition. The hydrogen yield achieved 67.08 mL/g-VS_{added} at a sludge/molasses wastewater ratio of 1:2 (volume basis). In another study published by Lee et al. (2014b), which investigated effects of mixing ratios on fermentative hydrogen production in batch experiments by response surface methodology. The maximum hydrogen yield achieved over 50 mL/g-$VS_{removed,}$ when the volume mixing ratio of sludge and molasses wastewater was 1:10.

8.8.5 Pure Carbohydrates

Pure carbohydrates, such as starch, glucose, or sucrose, were the main organic components for fermentative hydrogen-producing bacteria and commonly applied as model substrates in previous studies (Yin and Wang 2016a; Wang and Wan 2008b). Starch and glucose have also been used as co-substrates for improving fermentative hydrogen production from sludge, and the main results are illustrated in Table 8.6. As shown in Table 8.6, the studied sludge type included WAS. Fermentative tests were all performed under mesophilic conditions in the batch mode. The initial pH was in the range of 7–8. Chen et al. (2012) investigated the effects of starch addition on fermentative hydrogen production from WAS. The maximum hydrogen yield achieved 118.3 mL/g-COD_{added}, which was 13.8 times higher that of sole sludge fermentation. TS removal efficiency was also enhanced from 16% to 38.2% because of the synergistic effect of starch addition. The enhancement of amylase and protease activities and more suitable fermentation pathway also contributed to higher hydrogen production and better utilization of substrate. Glucose was also used as co-substrate for improving sludge fermentation in Yin and Wang (2015) and Yin and Wang (2016c), and the hydrogen yield achieved 49.72 and 25.03 mL/g-COD_{added}, respectively.

8.8.6 Other Organic Wastes

In addition to municipal waste fractions, pure carbohydrates, carbohydrate-rich wastewaters, or crop residues, some other organic wastes have also been studied for enhancing fermentative hydrogen production from sewage sludge. As shown in Table 8.6, these co-fermentation substrates included crude glycerol, tofu residue and oil palm frond juice. The studied sludge types included mixed sludge, thickened sludge, and WAS. Most of fermentative tests were performed under mesophilic conditions. The reactor types included batch and continuous stirring tank reactors, and the initial pH was in the range of 5–7.

Crude glycerol is the by-product of the biodiesel industry. The pH value of crude glycerol is suitable for anaerobic fermentation, and can be readily utilized as carbon sources by a variety of anaerobic bacteria (Fountoulakis et al. 2010). In addition, crude glycerol could increase the C/N ratio of fermentation substrate, and has been considered as an attractive co-fermentation substrate for sludge. Rivero et al. (2014) reported that the hydrogen yield reached 230 mL/g-$VS_{removed}$ when the mixed ratio of crude glycerol and sludge was 1:100 (volume basis). Other advantages of this process include energy savings, low nutrient requirements, and the generation of stabilized fermentation effluent.

Tofu residue is the by-product of soy bean curd processing industry. It contains many kinds of organic components including cellulose, lignin, hemicellulose, and pectin. Tofu residue has also been considered as an ideal co-substrate for recovering

hydrogen from sludge fermentation. Kim et al. (2011b) investigated the utilization of thickened sludge and tofu residue for fermentative hydrogen production. The hydrogen yield reached 33.15 L/mol $hexose_{added}$. Besides, oil palm frond juice has also been used as co-substrate for improving fermentative hydrogen production from WAS due to its high content of sugars. Yasin et al. (2013) found that the hydrogen yield achieved 186.7 mL/g-$glucose_{added}$ at an oil palm frond juice/sludge ratio of 7:3.

8.9 Influence Factors

Sludge fermentation is a complex biological process with many groups of microorganisms involved. In previous studies, hydrogen yield displayed a great variation, and has been considered to be significantly influenced by numerous process factors, such as temperature, pH, agitation intensity, retention time and OLR, the presence of nutrients and inhibitors, inoculum, and applied treatment methods (Wang and Wan 2009a). Elucidating the influences of these factors on sludge fermentation is vital for optimizing hydrogen yield and substrate utilization efficiency. Besides, the effects of above-mentioned parameters are considered to be strictly interrelated, so that a change in one parameter may result in combined interactions with other process parameters. Table 8.7 illustrates the effects of various process factors on fermentative hydrogen production from sludge, and a detailed discussion of literature findings is performed in the following subsections.

8.9.1 Temperature

Temperature is a crucial factor that influence the efficiency and stability of fermentative hydrogen production process, because it determines the activities of hydrogen producing bacteria, and affects their metabolic pathway and population dynamics (Wang and Wan 2008c). In previous studies, the temperature conducted for sludge fermentation was in the range of 30–55 °C, and most of investigations were performed at mesophilic temperatures (35–37 °C). So far, there is no clear conclusion on the optimal temperature for sludge fermentation due to the variable experimental conditions and the complexity of substrate. As shown in Table 8.7, sludge usually presented higher hydrogen yield at thermophilic temperatures than that at mesophilic temperatures. Wan et al. (2016) compared the hydrogen yield from thermophilic (55 °C) and mesophilic (37 °C) fermentation of sewage sludge, and found that hydrogen yield obtained at 55 °C was 3.17 times higher than that at obtained at 37 °C. Lin et al. (2012) also observed that cumulative hydrogen production obtained at 55 °C was 1.42 times higher than that at 37 °C, and 20.4% higher than that obtained at 45 °C. The reason might be that thermophilic condition could improve the growth rate of hydrogen producing bacteria and hydrogenase

Table 8.7 Effects of various process factors on fermentative hydrogen production

Substrates	Inoculum	Studied range	Optimal value	Maximum hydrogen yield or production	Reference
Temperature					
WAS	None	37–55 °C	55 °C	80.1 mL/g-VSS_{added}	Wan et al. (2016)
WAS	ADS	35–55 °C	55 °C	19.9 mL/g-VSS_{added}	Zheng et al. (2014a)
WAS	ADS	37–55 °C	55 °C	164 mL	Kotay and Das (2009)
Initial pH					
Thickened sludge	None	2–12	5	2.97 mL/g-TS_{added}	Liu et al. (2014)
WAS	None	5–9	6	56.6 ml/g-VSS_{added}	Guo et al. (2013a)
WAS	None	4–12	10.5	16.48 mL/g-TS_{added}	Cai et al. (2004)
WAS	None	2.5–12	11	14.4 mL/g-VS_{added}	Xiao and Liu (2006b)
WAS	None	4–11	11	18.8 mL/g-VSS_{added}	Zhao et al. (2010)
WAS	WAS	5–10	5	8.94 mL/g-COD_{added}	Wei et al. (2010)
WAS	*Enterobactor aerogenes*	4–9	5.5	12.77 mL/g-COD_{added}	Thungklin et al. (2011)
WAS	ADS	6–9	7	7.81 mmol	Lin et al. (2012)
Retention time					
Thickened sludge	Compost-acclimated sludge	24–36 h (SRT)	36 h	25.2 mL/g − VS_{added}	Wu and Zhou (2011b)
Primary sludge	ADS	18–48 h (HRT)	48 h	32.7 mL/g-VS_{added}	Massanet-Nicolau et al. (2010)
OLR					
Thickened sludge	Compost-acclimated sludge	9.1–27.3 g COD/L d	27.3 g COD/L d	25.3 mL/g − VS_{added}	Wu and Zhou (2011b)
WAS	ADS	4.96–19.85 g VS/L d	8.96 g VS/L d	68.77 mL/g-TS_{added}	Woo and Song (2010)

(continued)

Table 8.7 (continued)

Substrates	Inoculum	Studied range	Optimal value	Maximum hydrogen yield or production	Reference
Paper mill sludge	Pig manure	2–5.53 g COD/L d	3 g COD/L d	620.8 mL/g-COD_{added}	Wu and Zhou (2011a)
C/N ratio					
WAS	None	21–45	38	68.4 mL/g-VSS_{added}	Guo et al. (2013a)
C/P ratio					
WAS	None	100–300	265	68.4 mL/g-VSS_{added}	Guo et al. (2013a)
Fe^{2+}					
WAS	None	20–100 mg/L	85 mg/L	68.4 mL/g-VSS_{added}	Guo et al. (2013a)
Ammonia					
WAS	WAS	175–330 mg/L	175 mg/L	7.9 mL/g-COD_{added}	Kang et al. (2012)
Agitation intensity					
Thickened sludge	Compost-acclimated sludge	0–3000 rpm	2000 rpm	29 mL/g − VS_{added}	Wu and Zhou (2011b)

activity, and suppress the activity of hydrogen consumers (Wang and Wan 2009c). Besides, the solubility of hydrogen is lower at higher temperature. However, sludge fermentation at higher temperatures could improve the energy supply and increase the difficulty of maintenance and monitoring.

8.9.2 pH

The pH value plays a significant role in the fermentative hydrogen production process, because it could directly influence the synthesis and activity of hydrogenase, spore germination, the formation of ATP, and the metabolism pathway (Yasin et al. 2011). A suitable pH could facilitate nutrients uptake, and thus sustain the growth of hydrogen producing bacteria. Additionally, it is also important for suppressing the activities of hydrogen consumers (e.g., hydrogen consuming methanogenic). As shown in Table 8.7, some researchers have investigated effects of the initial pH value on fermentative hydrogen production from sludge. The studied initial pH values were in a wide range from 2 to 12, however, the optimal pH values were inconsistent and displayed a great variation in the literature. Several studies found that the optimal initial pH values were in the range of 5–7, but other studies reported the optimal initial pH values ranged from 10.5 to 11. This difference might be due to the various substrate characteristics, inoculum characteristics, and operating conditions performed in different studies. Wei et al. (2010) found that the optimal initial pH value for alkaline pretreated sludge was 11, while the optimal initial pH value for heat pretreated sludge was 5. Besides, the optimal pH of sludge fermentation at different temperatures was also different. Lin et al. (2012) observed that, in the pH range of 6–9, the optimal pH values for hydrogen production were 9, 8, and 7 at fermentation temperatures of 37, 45, and 55 °C, respectively, indicating the interactions of different fermentation process factors.

8.9.3 Retention Time and OLR

The retention time and OLR could influence the substrate utilization efficiency, microbial population in the system as well as the metabolic pathways. Regarding retention time, the studied SRT and HRT of sludge fermentation were in the range of 24–36 h and 18 h–8 days, respectively. As shown in Table 8.7, two previous studies both found that the hydrogen yield was increased with the increasing retention time (Woo and Song 2010; Wu and Zhou 2011b). This is because that the increase of retention time increases the period for sludge hydrolysis and in turn improves the substrate conversion efficiency. Besides, lower retention time could cause the washout of active biomass, which decreased the conversion yield. Regarding OLR, the reported values were in the range of 2–27.3 g COD/L d or 4.96–24.6 g VS/L d. The results about effects of OLR on hydrogen yield were

inconsistent due to the various substrates and inoculum characterizes and OLR range studied. Wu and Zhou (2011b) found that the hydrogen yield was increased with the increase of OLR in the range of 9.1–27.3 g COD/L d under stable operation status. However, Woo and Song (2010) studied the effects of OLR (4.96–19.85 g VS/L d) on hydrogen production from WAS, and concluded that the hydrogen yield was not further increased when OLR was higher than 8.96 g VS/L d. This might be because the fermentation bacteria could not tolerate the change of operation conditions (VFA accumulation and pH change), when the OLR increased to higher than 8.96 g VS/L d. Retention time or OLR is of particular concern in the application of sludge fermentation since it determines the capital cost.

8.9.4 Agitation Intensity

Agitation intensity is another important processing factor in fermentative hydrogen production, because it could influence the contact of fermentative bacteria with substrate, and hence influence the substrate utilization efficiency, the growth rate of hydrogen producing bacteria and the metabolism pathways. The reported agitation intensities of sludge fermentation were in the range of 0–3000 rpm, and most of previous studies were performed in the range of 100–200 rpm. Wu and Zhou (2011b) investigated effects of agitation intensity on hydrogen production from thickened sludge. The hydrogen yield was increased with the increase of agitation intensity from 0 to 2000 rpm, while was decreased when agitation intensity further increased from 2000 to 3000 rpm. When the agitation intensity increased from 0 to 2000 rpm, higher agitation intensity contributed to more sufficient contact of anaerobic bacteria with organics and rapid fermentative reactions. However, when the agitation intensity was higher than 2000 rpm, hydrogen might accumulate in the liquid phase, which caused a high hydrogen pressure in the liquid phase and the decrease of hydrogen yield. This study concluded that the preferable agitation intensity for sludge fermentation is 1500–2000 rpm.

8.9.5 Nutrients and Inhibitors

Carbon, nitrogen, phosphorous and trace metal elements are essential nutrients for the growth and metabolism of microorganisms involved in sludge fermentation, and in turn influencing the hydrogen production efficiency. Carbon, nitrogen, and phosphate are crucial components for enzymes, proteins, and nucleic acids, and could influence the buffering capacity of fermentation system. Besides, some metal ions, such as Fe^{2+} and Ni^{2+}, are cofactors of hydrogenase, and essential for its formation and activity (Wang and Wan 2008b, d). An appropriate C/N ratio, C/P ratio, and metal ions concentration could stimulate the hydrogen production.

However, only few studies have investigated the effects of these nutrients on fermentative hydrogen production from sludge. Guo et al. (2013a) reported that the optimal C/N ratio, C/P ratio and Fe^{2+} concentration were 38, 265, and 85 mg/L for hydrogen production from WAS based on a systematic investigation for C/N ratio from 21 to 45, C/P ratio from 100 to 300, and Fe^{2+} concentration from 20 to 100 mg/L, respectively.

Some chemicals existed in the fermentation system could inhibit hydrogen production. Sewage sludge contains many kinds of heavy metals including zinc, cadmium, lead copper, and chromium. It have been reported that these heavy metals could cause the upset and failure of fermentation process due to the chemical binding to the enzymes and disruption of the enzyme structure (Appels et al. 2008). Free ammonia is another important inhibitor of sludge fermentation, because it can pass into bacteria cells and cause potassium deficiency and proton imbalance (Wang et al. 2009). Kang et al. (2012) found that the hydrogen yield of sludge fermentation increased 1.63 times with the decrease of ammonia concentration from 330 to 175 mg/L. Besides, high concentrations of sulfide, sodium, and potassium could also inhibit the activity of hydrogen-producing bacteria and enzymes (Yang et al. 2016b).

8.9.6 Inoculum

The inoculum type and relevant pretreatment method directly determines bacterial communities responsible for hydrogen production. Table 8.8 reported the inoculum types and relevant pretreatment methods for sludge fermentation in previous studies. Both mixed and pure cultures have been applied for sludge fermentation. The reported mixed cultures included ADS, compost-acclimated sludge, hot spring sediment, pig manure, thickened sludge, and WAS. The reported pure cultures included *Clostridium bifermentans*, *Enterobactor aerogenes*, *Enterococcurs sp.*, *Escherichia coli,* and *Pseudomonas sp.*. Mixed cultures were much more commonly applied since they would be easier to control, cheaper to operate, and capable of utilizing a variety of organic components. The comparison of hydrogen production between mixed culture and pure culture has been rarely studied in sludge fermentation. Thungklin et al. (2011) observed that the hydrogen yield from microwaved sludge seeding with *Enterobactor aerogenes* was 50% higher than that seeding with hot spring sediment.

The main disadvantage of mixed cultures was the inducing of hydrogen consumers (Das and Veziroğlu 2001). Thus, pretreatment of mixed cultures is commonly needed for enriching hydrogen producers. As shown in Table 8.8, pretreatment methods of mixed cultures included heat, alkaline, gamma radiation, infrared radiation, and the addition of chloroform. Among these pretreatment methods, heat treatment was the most reported method, and the treatment temperature and treatment time were in the ranges of 75–121 °C and 10 min–3 h, respectively. Gamma radiation and infrared radiation are emerging pretreatment

Table 8.8 Inoculum type and relevant pretreatment methods for sludge fermentation

Inoculum	Pretreatment method (conditions)	References
Mixed cultures		
ADS	Heat (120 °C, 30 min)	Liu et al. (2013a)
ADS	Heat (110 °C, 20 min)	Massanet-Nicolau et al. (2008)
ADS	Heat (100 °C, 30 min)	Woo and Song (2010)
ADS	Heat (100 °C, 20 min)	Zheng et al. (2014a)
ADS	Gamma radiation (5 kGy)	Yin and Wang (2015); Yin and Wang (2016b); Yin and Wang (2016c)
ADS	Heat (110 °C, 20 min)	Massanet-Nicolau et al. (2010)
ADS	Boiling (15 min)	Sato et al. (2016)
ADS	Heat (105 °C, 3 h)	Assawamongkholsiri et al. (2013)
ADS	Heat (90 °C, 15 min)	Kim et al. (2013a)
ADS	Heat (100 °C, 120 min)	Lin et al. (2012)
Compost-acclimated sludge	Infrared radiation (0.5–1.5 h)	Wu and Zhou (2011b)
Hot spring sediment	Heat (105 °C, 2 h)	Thungklin et al. (2011)
Pig manure	Infrared radiation (0.5–1.5 h); The addition of chloroform (0.05%, 5 mL)	Wu and Zhou (2011a)
Thickened sludge	Heat (75 °C, 10 min)	Li et al. (2016)
WAS	Heat (90 °C, 60 min)	Yu et al. (2013a)
WAS	Heat (90 °C, 15 min)	Yang et al. (2012)
WAS	Heat (121 °C, 30 min)	Wei et al. (2010)
WAS	Alkaline (pH = 12, 24 h)	Wei et al. (2010)
Pure cultures		
Clostridium bifermentans	–	Wang et al. (2013); Jan et al. (2007)
Enterobactor aerogenes	–	Thungklin et al. (2011)
Enterococcurs sp.	–	Guo et al. (2012)
Escherichia coli strain	–	Yasin et al. (2013)
Pseudomonas sp.	–	Guo et al. (2008)

technologies. Few studies compared the hydrogen production from sludge seeding with inoculums pretreated by various methods. Wei et al. (2010) reported that the heat treatment obtained higher hydrogen yield than the alkali treatment when WAS was used as the inoculum. This study also found that the heat treatment performed better on enriching acidophilic hydrogen producers, while the alkali treatment performed better on enriching basophilic hydrogen producers.

8.10 Kinetic Models

Kinetic models are useful tools for designing, analyzing and optimizing fermentation hydrogen production process (Wang and Wan 2009b). Relevant kinetic models applied in hydrogen production from sludge fermentation were summarized in Table 8.9. As shown in Table 8.9, these kinetic models include the modified Gompertz equation, response function, mass balance equation, second degree polynomial function and some other mathematical equations. Regression coefficient (R^2) is commonly used to describe the correlation between experimental results and kinetic models.

8.10.1 The Modified Gompertz Model

Some kinetic models have been applied to estimate the cumulative hydrogen production. The modified Gompertz equation (Eq. (8.11)) has been the most widely used model to describe cumulative hydrogen production over the time course (Table 8.9). The modified Gompertz equation includes three variables, namely P, $R_{m,}$ and λ, which represent the hydrogen production potential, the maximum hydrogen production rate, and the lag phase time, respectively. H represents the cumulative hydrogen production. t is the fermentation time, and e is 2.718 (Zwietering et al. 1990). As operation conditions, substrate and inoculum characterizes are various in different studies, in order to compare the experimental results in different studies, P is usually converted to specific terms, such as hydrogen production per unit mass of COD, TS, VS, VSS, and hexose. R_m is also converted to other specific terms such as maximum hydrogen production rate per unit mass of COD, VS, VSS, and TS or per unit volume of sludge and reactor (Wu and Zhou 2011b; Guo et al. 2008; Assawamongkholsiri et al. 2013; Thungklin et al. 2011; Sreela-Or et al. 2011; Elsamadony and Tawfik 2015; Kim et al. 2004; Lee et al. 2014a; Wei et al. 2010; Liu et al. 2013a). It can be seen that the modified Gompertz equation could match well with fermentative hydrogen production from different types of raw sludge (primary, secondary, mixed, and dewatered sludge) from different wastewater resources (domestic wastewater, fructose-processing wastewater, molasses wastewater, papermaking wastewater, and bath wastewater) with high regression coefficient over 0.9 (Table 8.9). Second, the modified Gompertz equation could match well with fermentative hydrogen production from sludge pretreated by various methods (sterilization, ultrasound, heat, microwave, gamma radiation, low pressure wet oxidation, alkaline, acid, thermophlic enzyme, heat-ozone, heat-ultrasound, and heat-ozone-ultrasound) with high regression coefficient over 0.95 (Table 8.9). Third, the modified Gompertz equation could match well with hydrogen production from co-fermentation of sludge and other substrates (rice straw, cornstalk, food waste, glucose, sewage, organic fraction of municipal waste, tofu residue and gelatin solid waste) with high regression

Table 8.9 Kinetic models applied in fermentative hydrogen production from sludge

Kinetics	Describing objectives	Correlation	Substrates	Inoculum	References
Modified Gompertz equation	H_2 production potential; Maximum H_2 production rate; Lag time	R^2 = 0.9947	Ultrasound pretreated sludge	Compost-acclimated sludge	Wu and Zhou (2011b)
Modified Gompertz equation	H_2 production potential; Maximum H_2 production rate; Lag time	–	Heat pretreated sludge	None	Xiao and Liu (2006a)
Modified Gompertz equation	H_2 production potential; Maximum H_2 production rate; Lag time	–	Sterilized, microwave or ultrasound pretreated sludge	Pseudomonas sp. GZ1	Guo et al. (2008)
Modified Gompertz equation	H_2 production potential; Maximum H_2 production rate; Lag time	R^2 = 0.993	Hydrothermal pretreated thickened sludge supernatant	WAS	Yu et al. (2013a)
Modified Gompertz equation	H_2 production potential; Maximum H_2 production rate; Lag time	–	Thermophlic enzyme pretreated sludge	None	Guo et al. (2013a)
Modified Gompertz equation	H_2 production potential; Maximum H_2 production rate; Lag time	R^2 of 0.991–1	Raw sludge; Alkaline pretreated sludge	None	Cai et al. (2004)
Modified Gompertz equation	H_2 production potential; Maximum H_2 production rate; Lag time	–	Raw sludge; Alkaline-heat pretreated sludge	WAS	Kang et al. (2012)
Modified Gompertz equation	H_2 production potential; Maximum H_2 production rate; Lag time	–	Raw sludge; Alkaline pretreated sludge	None	Xiao and Liu (2006b)
Modified Gompertz equation	H_2 production potential; Maximum H_2 production rate; Lag time	–	Raw sludge; Acid, alkaline, heat or ultrasound pretreated sludge	None	Xiao and Liu (2009)

(continued)

Table 8.9 (continued)

Kinetics	Describing objectives	Correlation	Substrates	Inoculum	References
Modified Gompertz equation	H_2 production potential; Maximum H_2 production rate; Lag time	R^2 of 0.9918–1	Heat, heat-ozone, heat-ultrasound or heat–ozone-ultrasound pretreated sludge	WAS	Yang et al. (2012)
Modified Gompertz equation	H_2 production potential; Maximum H_2 production rate; Lag time	R^2 = 0.989	Gamma radiated sludge; Gamma radiated sludge and glucose	ADS	Yin and Wang (2016c)
Modified Gompertz equation	H_2 production potential; Maximum H_2 production rate; Lag time	R^2 of 0.9891–0.9999	Gamma radiated sludge; Gamma radiated sludge with nutrients addition; Gamma radiated sludge and glucose with nutrients addition	ADS	Yin and Wang (2016c)
Modified Gompertz equation	H_2 production potential; Maximum H_2 production rate; Lag time	–	Alkaline or heat pretreated sludge filtrate	WAS	Wei et al. (2010)
Modified Gompertz equation	H_2 production potential; Maximum H_2 production rate; Lag time	R^2 = 0.99	Ultrasound pretreated paper mill sludge	Pig manure	Wu and Zhou (2011a)
Modified Gompertz equation	H_2 production potential; Maximum H_2 production rate; Lag time	R^2 = 0.99	Raw sludge; Acid, heat or acid-heat pretreated brewery industry sludge	Anaerobic activated sludge	Assawamongkholsiri et al. (2013)
Modified Gompertz equation	H_2 production potential; Maximum H_2 production rate; Lag time	R^2 of 0.97–0.99	Fructose-processing sludge with or without Endo nutrient; Microwave pretreated fructose-processing sludge with or without Endo nutrient	Enterobactor aerogenes	Thungklin et al. (2011)

(continued)

Table 8.9 (continued)

Kinetics	Describing objectives	Correlation	Substrates	Inoculum	References
Modified Gompertz equation	H_2 production potential; Maximum H_2 production rate; Lag time	–	Alkaline pretreated sludge; Cornstalk and alkaline pretreated sludge	WAS	Liu et al. (2013b)
Modified Gompertz equation	H_2 production potential; Maximum H_2 production rate; Lag time	–	Bath wastewater sludge; Food waste and bath wastewater sludge	Anaerobic activated sludge	Liu et al. (2013c)
Modified Gompertz equation	H_2 production potential; Maximum H_2 production rate; Lag time	–	Food waste and sludge	ADS	Sreela-Or et al. (2011)
Modified Gompertz equation	H_2 production potential; Maximum H_2 production rate; Lag time	R^2 of 0.952–0.999	Primary sludge; Secondary sludge; Food waste and secondary sludge; Food waste and primary sludge; Food waste and mixed sludge	ADS	Zhou et al. (2013)
Modified Gompertz equation	H_2 production potential; Maximum H_2 production rate; Lag time	R^2 of 0.9891–1	Alkaline-gamma radiated sludge; Glucose and alkaline-gamma radiated sludge	ADS	Yin and Wang (2016c)
Modified Gompertz equation	H_2 production potential; Maximum H_2 production rate; Lag time	R^2 of 0.989–0.992	Low-pressure wet oxidized sludge; Glucose and low-pressure wet oxidized sludge	ADS	Yin and Wang (2016b)
Modified Gompertz equation	H_2 production potential; Maximum H_2 production rate; Lag time	R^2 of 0.83–0.99	Sewage and hydrothermal pretreated dewatered sludge	WAS	Yu et al. (2014)
Modified Gompertz equation	H_2 production potential; Maximum H_2 production rate; Lag time	R^2 of 0.9377–0.9736	Sewage and hydrothermal pretreated dewatered sludge	WAS	Yu et al. (2014)

(continued)

Table 8.9 (continued)

Kinetics	Describing objectives	Correlation	Substrates	Inoculum	References
Modified Gompertz equation	H_2 production potential; Maximum H_2 production rate; Lag time	R^2 of 0.952–0.999	Paperboard mill sludge; Organic fraction of municipal waste and paperboard mill sludge; Organic fraction of municipal waste, gelatin solid waste and paperboard mill sludge	Thickened sludge	Elsamadony and Tawfik (2015)
Modified Gompertz equation	H_2 production potential; Maximum H_2 production rate; Lag time	R^2 of 0.984–0.999	Food waste and sludge	ADS	Kim et al. (2004)
Modified Gompertz equation	H_2 production potential; Maximum H_2 production rate; Lag time	$R^2 > 0.99$	Thickened sludge; Tofu residue and thickened sludge	ADS	Kim et al. (2011b)
Modified Gompertz equation	H_2 production potential; Maximum H_2 production rate; Lag time	–	Mixed sludge; Molasses wastewater and mixed sludge	ADS	Lee et al. (2014a)
Mass balance equation	Cumulative H_2 production	–	Ultrasound pretreated sludge	Compost-acclimated sludge	Wu and Zhou (2011b)
Mass balance equation	Cumulative H_2 production	–	Hydrothermal pretreated thickened sludge supernatant	WAS	Yu et al. (2013a)
Mass balance equation	Cumulative H_2 production	–	Alkaline or heat pretreated sludge filtrate	WAS	Wei et al. (2010)
Mass balance equation	Cumulative H_2 production	–	Ultrasound pretreated paper mill sludge	Pig manure	Wu and Zhou (2011a)
Mass balance equation	Cumulative H_2 production	–	Raw sludge; Heat pretreated sludge	Thickened sludge	Li et al. (2016)

(continued)

Table 8.9 (continued)

Kinetics	Describing objectives	Correlation	Substrates	Inoculum	References
Mass balance equation	Cumulative H_2 production	–	Raw sludge; Acid, heat or acid-heat pretreated brewery industry sludge	Anaerobic activated sludge	Assawamongkholsiri et al. (2013)
Mass balance equation	Cumulative H_2 production	–	Alkaline pretreated sludge; Cornstalk and alkaline pretreated sludge	WAS	Liu et al. (2013b)
Mass balance equation	Cumulative H_2 production	–	Sewage and hydrothermal pretreated dewatered sludge	WAS	Yu et al. (2014)
Mass balance equation	Cumulative H_2 production	–	Mixed sludge; Molasses wastewater and Mixed sludge	ADS	Lee et al. (2014a)
Response function	Effects of temperature, time and stirring rate on carbohydrate release	$R^2 = 0.9989$	Hydrothermal pretreated thickened sludge supernatant	WAS	Yu et al. (2013a)
Response function	Effects of C/N, C/P, pH and Fe^{2+} on the H_2 yield	$P = 0.013$ $R^2 = 0.975$	Thermophlic enzyme pretreated sludge	None	Guo et al. (2013a)
Response function	Effects of different inoculum heat treatment condition, pH, S/X ratios and co-substrate sizes on H_2 production	$P < 0.0001$ $R^2 = 0.9732.$	Rice straw and heat pretreated sludge	None	Alemahdi et al. (2015)
Response function	Effects of C/N ratio, inoculums concentration, Na_2HPO_4 concentration and Endo nutrient addition on H_2 production yield	$P < 0.0001$ $R^2 = 0.99$	Food waste and sludge	ADS	Sreela-Or et al. (2011)

(continued)

Table 8.9 (continued)

Kinetics	Describing objectives	Correlation	Substrates	Inoculum	References
2nd degree polynomial function	Effects of C/N ratio on H_2 yield	$R^2 = 0.739$	Organic fraction of municipal waste and paperboard mill sludge; Organic fraction of municipal waste, gelatin solid waste and paperboard mill sludge	Thickened sludge	Elsamadony and Tawfik (2015)
2nd degree polynomial function	Effects of Ca^{2+} concentrations on volumetric H_2 production	$R^2 = 0.7$	Organic fraction of municipal waste and paperboard mill sludge; Organic fraction of municipal waste, gelatin solid waste and paperboard mill sludge	Thickened sludge	Elsamadony and Tawfik (2015)
Full quadratic model	Effects of VS concentrations and mixed ratios on specific H_2 production potential	$R^2 = 0.9$	Sludge	ADS	Kim et al. (2004)
Mathematical equation	Contribution of substrate in different phases for H_2 production	Confidence interval of 99%	Sterilized sludge or its filtrate	Pseudomonas sp. GZ1	Guo et al. (2010a)
Exponential function	Effects of the pH drop on total ammonia production	$R^2 = 0.716$	Organic fraction of municipal waste and paperboard mill sludge; Organic fraction of municipal waste, gelatin solid waste and paperboard mill sludge	Thickened sludge	Elsamadony and Tawfik (2015)

coefficient from 0.83 to 1 (Table 8.9). Finally, the modified Gompertz equation has also been used to fit hydrogen production from sludge at different operation conditions (retention time, OLR, agitation intensities, initial pH, and temperature) with high regression coefficient of 0.83–1 (Table 8.9). Mass balance Eq. (8.12) was also used to estimate the cumulative hydrogen production from hydrothermal pretreated municipal sludge supernatant, dewatered sludge, ultrasound pretreated municipal sludge, heat pretreated municipal sludge, alkaline pretreated municipal sludge, raw brewery industry sludge, acid pretreated brewery sludge, heat pretreated brewery sludge, acid-heat pretreated brewery sludge, ultrasonic-pretreated paper mill sludge, co-substrates of cornstalk and sewage sludge, co-substrates of sewage and dewatered sludge, and co-substrates of molasses wastewater and sludge (Table 8.9).

$$\mathrm{H} = P\exp\{-exp[(\lambda - \mathrm{t})R_m e/P + 1]\} \tag{8.11}$$

$$V_{H,\mathrm{i}} = V_{H,\mathrm{i}-1} + C_{H,\mathrm{i}}\left(V_{G,\mathrm{i}} - V_{G,\mathrm{i}-1}\right) + V_H\left(C_{H,\mathrm{i}} - C_{H,\mathrm{i}-1}\right) \tag{8.12}$$

8.10.2 Response Surface Methodology

Some kinetic models have been used for optimizing various operation conditions for hydrogen production, and response functions were the most reported models. Yu et al. (2013a) used a response function (Eq. (8.13)) to describe the effects of temperature (X_1), time (X_2), and stirring rate (X_3) on carbohydrate release with a high regression coefficient of 0.9989 when sludge was pretreated by hydrothermal technology. Another response function (Eq. (8.14)) was applied to describe the effects of C/N ratio, C/P ratio, initial pH, and Fe^{2+} on the hydrogen yield from thermophlic enzyme pretreated sludge with a high regression coefficient of 0.975 (Guo et al. 2013a). Alemahdi et al. (2015) used two response functions to describe effects of inoculum heat treatment temperature (X_1) and time (X_2) on hydrogen production yield high regression coefficient of 0.9706 (Eq. (8.15)), and describe effects of substrate/inoculum ratio (X_1) and initial pH (X_2) on hydrogen production yield with high regression coefficient of 0.9732 (Eq. (8.16)), respectively. A full quadratic model (Eq. (8.17)) was also applied to analyze the effects of mixing ratios and VS concentrations on hydrogen production from co-substrates of sludge and food waste with a high regression coefficient of 0.898 (Kim et al. 2004). In addition, two second degree polynomial functions (Eq. (8.18) and (8.19)) were applied to describe the effects of C/N ratio on H_2 yield from co-substrates of paperboard mill sludge, organic fraction of municipal waste, and gelatin solid waste with a regression coefficient of 0.739, and the effects of Ca^{2+} on the volumetric H_2 production from above mixed substrates with a regression coefficient of 0.7, respectively (Elsamadony and Tawfik 2015).

$$Y = 1323.93 - 136.15X_1 - 23.18X_2 + 13.46X_3 - 163.71X_1X_2 - 57.17X_1X_3 + 0.67X_2X_3 - 236.03X_1^2 - 134.42X_2^2 - 26.32X_3^2 \tag{8.13}$$

$$Y = -201.136 + 0.004X_4^2 + 0.035X_1X_3 - 0.405X_1X_4 - 0.018X_2X_3 - 0.008X_3X_4 \tag{8.14}$$

$$\begin{aligned} HPY = & \\ & + 22.63210 - 0.62878X_1 + 0.46847X_2 + 4.70000e^{-3}X_1^2 \\ & - 1.45444e^{-3}X_2^2 2.56833e^{-3}X_1X_2 \end{aligned} \tag{8.15}$$

$$\begin{aligned} HPY = & -46.16653 + 8.02986X_1 + 17.34191X_2 - 0.84164X_1^2 \\ & - 1.68633X_2^2 - 0.12283X_1X_2 \end{aligned} \tag{8.16}$$

$$Y = -39.793 + 2.069X_1 + 48.431X_2 - 0.011X_1^2 - 8.103X_2^2 - 0.015X_1X_2 \tag{8.17}$$

$$Y = -0.179X^2 + 10.175X \tag{8.18}$$

$$Y = -0.021X^2 + 0.204X + 4.867 \tag{8.19}$$

Some kinetics models have also been applied to describe the contributions of different sludge fractions on hydrogen production. For example, a mathematical equation (Eq. 8.19) reported in Guo et al. (2010a) was applied to evaluate respective contribution of soluble phase and solid phase to hydrogen production from sterilization pretreated sludge. Where X_1 and X_2 was hydrogen yield of the supernatant and the sludge. $t_{0.01}$ was the corresponding values of t-test at a confidence level of 99% with the degree of freedom (n – 1), and $t_{0.01} = 2.86$. The result of $H > 1$ meant that the organic matters used for hydrogen production was mostly provided in the liquid phase. In addition, an exponential function (Eq. 8.20) was applied to describe the relationship between total ammonia production and pH drop with a regression coefficient of 0.716 during co-fermentation of paperboard mill sludge, organic fraction of municipal waste, and gelatin solid waste (Elsamadony and Tawfik 2015).

$$H = \sum (X_1 - X_2)/t_{0.01} n \sqrt{n \sum (X_1 - X_2)^2 - \left[\sum (X_1 - X_2)\right]^2 / n(n-1)} \tag{8.20}$$

$$Y = -1251.065e^{-1.291x} \tag{8.21}$$

8.11 End Products in the Liquid Phase

During the dark fermentation process, hydrogen production is usually accompanied with the generation of some metabolites in the liquid phase. Generally, VFAs, ethanol, and lactate are main components among these soluble metabolites (Xia et al. 2016). These metabolites could be used as important indicators for monitoring the fermentation process, and could also be used to deduce the fermentation type of hydrogen production (Elsamadony and Tawfik 2015). Table 8.10 illustrates the main end products in the liquid phases and the fermentation types when sludge was fermented alone or co-fermented with other substrates. As shown in Table 8.10, the main liquid end products included acetate, propionate, butyrate, ethanol, lactate and formate, and fermentative hydrogen production included the acetate type, the propionate type, the butyrate type, the ethanol type, the mixed acid type, the propionate-butyrate type, the propionate-ethanol type, the butyrate-ethanol type, and the propionate-butyrate-ethanol type. The ratios of above eight fermentation types in previous reports are calculated, and the results are summarized in Fig. 8.5.

As shown in Fig. 8.5, the butyrate type (44.78%), the propionate-butyrate type (24.35%) and the acetate type (8.7%) were the most three reported fermentation types. The propionate type fermentation only accounted for about 5.22% of total fermentation tests (Fig. 8.5). The hydrogen yield was closely related to the metabolites in the liquid phase (Guo et al. 2013a). Generally, Hydrogen yield usually shows positive relationship with acetate, butyrate, and ethanol production, and shows negative relationship with propionate production (Kim et al. 2004). Above results suggest that most of reported fermentation processes were suitable for hydrogen production.

Various pretreatment methods could influence the metabolites in the liquid phase, and consequently changed the sludge fermentation type (Table 8.10). Xiao and Liu (2009) found that the hydrogen production was changed from the acetate type to the propionate-butyrate type by acid, alkaline, heat, and ultrasound pretreatments. Assawamongkholsiri et al. (2013) observed that the hydrogen production was changed from the propionate-butyrate type to the butyrate type by acid and acid-heat pretreatments. This might be because various pretreatment methods could release protein and carbohydrate into the soluble phase which have been demonstrated to be closely related to VFAs production during the fermentation process (Xiao and Liu. 2009; Assawamongkholsiri et al. 2013). However, contradictory results were also obtained in previous studies (Table 8.10). Cai et al. (2004) reported that the fermentation type was not changed by alkaline pretreatment. Similar results were also found by using UV-light, photocatalysis and heat pretreatments (Liu et al. 2013a; Assawamongkholsiri et al. 2013). This difference might be due to the different sludge characterizes, inoculum characterizes, pretreatment conditions, and operation conditions in various studies. Besides, the fermentation types from sludge pretreated by various methods might be different in the same study. Guo et al. (2015) reported that hydrogen production from microwave and thermophilic bacteria pretreated sludge was the butyrate type, while

Table 8.10 The main end products in the liquid phase and fermentation types

End products in the liquid phase	Fermentation type	Fermentation conditions	Substrates	Inoculum	References
Ethanol and acetate	Ethanol type	Batch 35 °C Initial pH: 7.3	Sterilization pretreated sludge	A Pseudomonas sp. GZ1	Guo et al. (2010a)
Acetate, ethanol, lactate and formate	Mixed acid type	Batch 60 °C	Pulp and paper sludge	C. thermocellum DSMZ 1237	Moreau et al. (2015)
Acetate and butyric acid	Butyrate type	Batch 35 °C	Sterilization pretreated sludge; Ultrasound pretreated sludge	A Pseudomonas sp. GZ1	Guo et al. (2008)
Acetate	Acetate type	Batch 35 °C	Microwave pretreated sludge	A Pseudomonas sp. GZ1	Guo et al. (2008)
Ethanol and acetate	Ethanol type	Batch 35 °C Initial pH: 5–9	Thermophilic enzyme pretreated sludge	None	Guo et al. (2013a)
Acetate	Acetate type	Batch 36 °C Initial pH: 4–12	Raw sludge; Alkaline pretreated sludge	None	Cai et al. (2004)
Acetate and butyric acid	Butyrate type	Batch 35 °C	Microwave pretreated sludge; Thermophilic bacteria pretreated sludge	None	Guo et al. (2015)
Acetate, ethanol and butyric acid	Butyrate-ethanol type	Batch 35 °C	Heat pretreated sludge; Multienzyme pretreated sludge	None	Guo et al. (2015)
Acetate, propionate and butyrate	Propionate-butyrate type	Batch 55 °C Initial pH: 5.7	Raw sludge; UV-light pretreated sludge; Photocatalysis pretreated sludge	ADS	Liu et al. (2013a)

(continued)

Table 8.10 (continued)

End products in the liquid phase	Fermentation type	Fermentation conditions	Substrates	Inoculum	References
Acetate, propionate and butyrate	Propionate-butyrate type	CSTR 35 °C Initial pH: 7	Enzyme pretreated sludge	ADS	Massanet-Nicolau et al. (2008)
Acetate and propionate	Propionate type	CSTR 35 °C Initial pH: 6.5	Enzyme pretreated sludge	ADS	Massanet-Nicolau et al. (2008)
Acetate and butyric acid	Butyrate type	CSTR 35 °C Initial pH: 5–6	Enzyme pretreated sludge	ADS	Massanet-Nicolau et al. (2008)
Acetate	Acetate type	CSTR 35 °C Initial pH: 4.5	Enzyme pretreated sludge	ADS	Massanet-Nicolau et al. (2008)
Acetate, propionate and butyrate	Propionate-butyrate type	Batch 37 °C Initial pH: 6–8	Alkaline pretreated sludge	None	Xiao and Liu (2006b)
Acetate and propionate	Propionate type	Batch 37 °C Initial pH: 4–12	Alkaline pretreated sludge	None	Xiao and Liu (2006b)
Acetate and butyrate	Butyrate type	CSTR 55 °C Initial pH: 6.1 HRT: 1–5 d	Heat pretreated sludge	ADS	Woo and Song (2010)
Acetate, propionate and butyrate	Propionate-butyrate type	Batch 37 °C Initial pH: 7	Acid pretreated sludge; Alkaline pretreated sludge	None	Xiao and Liu (2009)

(continued)

Table 8.10 (continued)

End products in the liquid phase	Fermentation type	Fermentation conditions	Substrates	Inoculum	References
Acetate and butyric acid	Butyrate type	Batch 35 °C	Heat pretreated sludge; Heat-ozone pretreated sludge; Heat-ultrasound pretreated sludge	WAS	Yang et al. (2012)
Acetate, propionate and butyrate	Propionate-butyrate type	CSTR 35 °C Constant pH: 5.5 HRT: 18 h	Pasteurization-enzyme pretreated sludge	ADS	Massanet-Nicolau et al. (2010)
Acetate and butyrate	Butyrate type	CSTR 35 °C Constant pH: 5.5 HRT: 24–48 h	Pasteurization-enzyme pretreated sludge	ADS	Massanet-Nicolau et al. (2010)
Acetate, propionate and butyrate	Propionate-butyrate type	Batch 30 °C Initial pH: 5.5	Heat pretreated sludge	ADS	Assawamongkholsiri et al. (2013)
Acetate and butyrate	Butyrate type	Batch 30 °C Initial pH: 5.5	Acid-Heat pretreated sludge	ADS	Assawamongkholsiri et al. (2013)
Acetate and butyric acid	Butyrate type	Batch 36 °C Initial pH: 7	Wet oxidized sludge	ADS	Yin and Wang (2016b)
Acetate, propionate and butyrate	Propionate-butyrate type	Batch 36 °C Initial pH: 7	Wet oxidized sludge and glucose	ADS	Yin and Wang (2016b)

(continued)

Table 8.10 (continued)

End products in the liquid phase	Fermentation type	Fermentation conditions	Substrates	Inoculum	References
Acetate and propionate	Propionate type	Batch 37 °C Initial pH: 7	Alkaline pretreated sludge	WAS	Liu et al. (2013b)
Acetate	Acetate type	Batch 37 °C Initial pH: 7	Alkaline pretreated sludge and cornstalk	WAS	Liu et al. (2013b)
Acetate, propionate and butyrate	Propionate-butyrate type	CSTR Initial pH: 5–6 OLR: 7.82–17.9 g COD/m^3·d	Alkaline pretreated sludge and crude glycerol	None	Rivero et al. (2014)
Acetate and butyrate	Butyrate type	Batch 37 °C Initial pH: 5.5	Raw sludge; Raw sludge and food waste	ADS	Zhou et al. (2013)
Acetate, propionate and butyrate	Propionate-butyrate type	Batch 55 °C Initial pH: 6	Paperboard mill sludge	Thickened sludge	Elsamadony and Tawfik (2015)
Acetate and butyrate	Butyrate type	Batch 55 °C Initial pH: 6	Organic fraction of municipal waste and paperboard mill sludge; Organic fraction of municipal waste, gelatin solid waste and paperboard mill sludge	Thickened sludge	Elsamadony and Tawfik (2015)

(continued)

Table 8.10 (continued)

End products in the liquid phase	Fermentation type	Fermentation conditions	Substrates	Inoculum	References
Acetate and butyrate	Propionate-butyrate type	Batch 35 °C Initial pH: 5–6 Mixed ratio: 20:80 (VS basis)	Raw sludge and food waste	ADS	Kim et al. (2004)
Acetate, ethanol and butyrate	Propionate-butyrate-ethanol type	Batch 35 °C Initial pH: 5–6 Mixed ratio: 40:60 (VS basis)	Raw sludge and food waste	ADS	Kim et al. (2004)
Acetate and ethanol	Propionate-ethanol type	Batch 35 °C Initial pH: 5–6 Mixed ratio: 60:40 (VS basis)	Raw sludge and food waste	ADS	Kim et al. (2004)
Acetate and butyrate	Butyrate type	Batch 37 °C Initial pH: 5–10	Freeze-thermal pretreated sludge; Freeze-thermal pretreated sludge and starch	None	Chen et al. (2012)
Acetate and butyrate	Butyrate type	Batch 35 °C Initial pH: 8	Heat pretreated sludge and rice straw	None	Kim et al. (2013b)

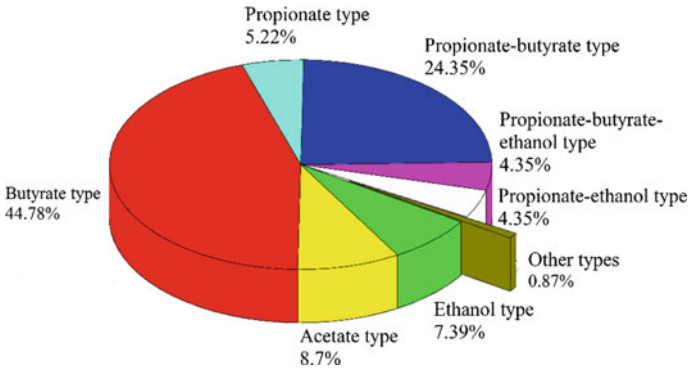

Fig. 8.5 Percentages of various fermentation types in the reviewed literature (The data in pie-chart are calculated based on the number of all reported fermentation tests)

hydrogen production from multienzyme and heat pretreated sludge was correlated with the butyrate-ethanol type. Assawamongkholsiri et al. (2013) also found that hydrogen production from acid pretreated sludge and heat pretreated sludge were the butyrate type and the propionate-butyrate type, respectively. This might be because various pretreat methods could cause the diversity of organic components distribution and microbial population during the fermentation process (Guo et al. 2015).

As for the effects of co-substrates addition on the metabolites in the liquid phase and sludge fermentation type, contradictory results were also obtained in previous studies. Most of previous studies found that the addition of co-substrates could change the fermentation type. Yin and Wang (2016c) reported that the hydrogen production from alkaline-gamma radiation pretreated sludge was changed from the butyrate to the acetate type with the addition of glucose. Liu et al. (2013b) observed that the main components of liquid end products from alkaline pretreated sludge fermentation were changed from acetate and propionate to acetate and butyrate with the addition of cornstalk, causing the shift of fermentation type from the propionate type to the butyrate type correspondingly. In addition, the mixed ratios of sludge and co-substrates could also influence the fermentation type. Kim et al. (2004) found that the sludge fermentation were the propionate-butyrate type, the propionate-butyrate-ethanol type and the propionate-ethanol type at various mixed ratios of sludge and food waste of 20:80, 40:60, and 60:40 (VS basis), respectively. The change of feedstock composition has been reported as the main reason leading to the shift of hydrogen fermentation type, since it could influence the activity of key enzymes for sludge fermentation such as the pyruvate-ferredoxin oxidoreductase (Yin and Wang. 2016a; Liu et al. 2013a). But other studies observed that the hydrogen fermentation type was not changed with the addition of food waste, molasses wastewater, and starch (Zhou et al. 2013; Lee et al. 2014a; Chen et al. 2012). These contradictory results might be because of the different sludge

characterizes, characterizes of various co-substrates, inoculums characterizes, and operation conditions in various studies.

Besides, various operation conditions showed different impacts on the fermentation type. Some parameters, including OLR, HRT, temperature, initial pH, and trace elements concentration, showed insignificant influence on the fermentation type. As for the OLR, the fermentation types from alkaline pretreated sludge and crude glycerol were all correlated with the propionate-butyrate type at various OLRs of 7.82, 15.33, 16.88, and 17.9 g COD/m^3 day (Rivero et al. 2014). As for the temperature, Wan et al. (2016) found that the hydrogen production types were both correlated with the propionate-butyrate type at 37 and 55 °C. As for the trace nutrient elements, Yin and Wang (2016c) found that the fermentation type of alkaline-gamma radiation pretreated sludge was not changed with the addition of trace nutrient elements, such as Fe^{2+}, Ni^{2+}, and Mg^{2+}. However, contradictory results were obtained on the effects of some parameters on sludge fermentation type including initial pH, hydrolytic retention time and substrate concentration. As for the initial pH, some previous studies found that it has insignificant influences on the fermentation type in the range of 4–12 (Guo et al. 2013a; Cai et al. 2004; Chen et al. 2012). However, Massanet-Nicolau et al. (2008) reported that the hydrogen production from enzyme pretreated sludge was the propionate-butyrate type, the propionate type, the butyrate type and the acetate type at the initial pH values of 7, 6.5, 5–6 to 4.5, respectively. Xiao and Liu (2006b) observed that the fermentation was the propionate-butyrate type at initial pH values of 6 and 8, but was the propionate type at initial pH values of 4, 10.5, 11, and 12. As for the HRT, Woo and Song (2010) found that the hydrogen production was all correlated with the butyrate type at hydrolytic retention times of 1, 3, and 5 days. But Massanet-Nicolau et al. (2010) found that the sludge fermentation was shifted from the propionate-butyrate type to the butyrate type with the increase of hydrolytic retention time from 18 to 24–48 h. As a whole, although many studies has been conducted for evaluating the effects of various operation parameters on hydrogen yield, studies concentrated on the effects of various operation parameters on soluble end products and fermentation type are still insufficient. Thus, more studies are needed to be conducted to verify above observations.

8.12 Two-Stage Process

8.12.1 Second Stage-Anaerobic Digestion

Although some pretreatment methods and co-substrates addition have been applied to improve hydrogen production from sludge, less than one-third of energy in sludge is finally converted to hydrogen after the fermentation process, and most of original organics still remains in the end products such as VFAs and ethanol (Xia et al. 2016). Exactly, these metabolites after hydrogen fermentation can be used as suitable substrates by the acetogen and the methanogen for methane production (Hallenbeck 2009). Thus, in order to achieve higher energy conversion efficiency,

sludge reduction and stabilization, a second stage of anaerobic digestion could be conducted to use the end products of sludge fermentation for methane production.

Table 8.11 summarizes the optimal performance of the two-stage of hydrogen and methane production processes when sludge was used as substrate. The two-stage processes using sole sludge as the substrate were all performed in the mesophilic condition (30–37 °C), and were all conducted through batch tests. The maximum hydrogen production from sole sludge was in the range of 1–2.5 mL/g-VS_{added} or 7.53–11 mL/g-TS_{added} in the first stage, and the maximum methane production was in the range of 107.9–122.1 mL/g-VS_{added} or 154.2–190.8 mL/g-TS_{added} (Table 8.11). However, only few studies reported the final organics removal after the two-stage fermentation process when sole sludge was used as the substrate. Liu et al. (2013b) found that the VS removal efficiency was 31% after the two-stage fermentation process. As for the co-fermentation of sludge and other substrates, both mesophilic and thermophilic conditions were performed in the two-stage fermentation process, and these two-stage processes were conducted under both batch and continuous conditions. Municipal waste fractions were also the most studied co-substrates for the two-stage sludge fermentation process. The maximum hydrogen production from co-fermentation of sludge and municipal waste fractions was in the range of 29–174.6 mL/g-VS_{added} in the first stage, and the maximum methane production was in the range of 264.1–353.5 mL/g-VS_{added} (Table 8.11). The final VS removal efficiency was in the range of 55.7–74.2% of the two-stage fermentation process of sludge and municipal waste fractions, which were much higher that of sole sludge fermentation. In addition, rice straw, molasses wastewater, crude glycerol, and cornstalk were also used as co-substrates in the two-stage sludge fermentation process (Kim et al. 2013b; Lee et al. 2014b; Rivero et al. 2014; Liu et al. 2013b). The corresponding maximum hydrogen yields were 21 mL/g-VS_{added}, 31 mL/g-COD_{added}, 500 mL/g-$VS_{removed}$, and 13.4 mL/g-VS_{added} in the first stage, and the corresponding maximum hydrogen yields were 266 mL/g-VS_{added}, 170 mL/g-COD_{added}, 1480 mL/g-$VS_{removed}$, and 172.6 mL/g-VS_{added} in the second stage. The final VS removal was 47.4% after the two-stage fermentation process of sludge and rice straw, and the final COD removal reached 93% after the two-stage fermentation process of sludge and glycerol.

Although few studies have been performed to compare the two-stage sludge fermentation with the conventional sludge anaerobic digestion, the two-stage of hydrogen and methane production process has some advantages over the single methane fermentation process. First, the energy production of the two-stage process is higher than that of the single methane production process from thermodynamic considerations, and the two-stage process could also achieve a better energy balance (energy output minus energy supply). Ting and Lee (2007) reported that the methane yield of the second stage process was 5 times higher than that of the single methane fermentation when sole sludge was used as the substrate. Kim et al. (2013b) found that total energy yield of the two-stage process was 59.6% higher than that of the single stage methane production process when sludge and rice straw were used as co-substrates. The final VS removal of the two-stage process was also 37.9% higher than that of the one-stage methane fermentation. Rivero et al. (2014)

Table 8.11 The optimal performance of the two-stage hydrogen and methane production fermentation

Substrates	The first stage of hydrogen production process			The second stage of methane production process				References
	Conditions	H_2 production	H_2 content	Conditions	CH_4 production	CH_4 content	Organics removal	
Sole sludge								
WAS	Batch 37 °C Initial pH: 7	1 mL/g-VS_{added}	–	Batch 37 °C	122.1 mL/g-VS_{added}	–	–	Liu et al. (2013b)
WAS	Batch 37 °C Initial pH: 5.5	2.5 mL/g-VS_{added}	–	Batch 37 °C	107.9 mL/g-VS_{added}	–	VS removal: 31%	Liu et al. (2013c)
WAS	Batch 35 °C	11 mL/g-TS_{added}	–	Batch 35 °C	154.2 mL/g-TS_{added}	–	–	Wang et al. (2003)
WAS	Batch 30 °C Initial pH: 6.84	7.53 mL/g-TS_{added}	–	Batch 30 °C	190.8 mL/g-TS_{added}	–	–	Ting and Lee (2007)
Sludge and co-substrates								
Sludge and OFMSW	CSTR 55 °C Initial pH: 5.1 HRT: 3.3 d	40 mL/g-VS_{added}	40%	CSTR 55 °C HRT: 15 d	320 mL/g-VS_{added}	67%	–	Gottardo et al. (2015)
Sludge and OFMSW	CSTR 55 °C Initial pH: HRT: 3 d	29 mL/g-VS_{added}	36%	CSTR 55 °C HRT: 16 d	287 mL/g-VS_{added}	59%	VS removal: 57%	Zahedi et al. (2016)

(continued)

Table 8.11 (continued)

Substrates	The first stage of hydrogen production process			The second stage of methane production process				References
Sludge and food waste	Batch 37 °C Initial pH: 5.5	106.4 mL/g-VS_{added}	–	Batch 37 °C	353.5 mL/g-VS_{added}	–	VS removal: 55.7%	Liu et al. (2013c)
Sludge and food waste	Batch 35 °C Initial pH: 8	161.1 mL/g-VS_{added}	–	HRT: 15 d ORL: 4.4 kg COD/m^3·d	268 mL/g-COD_{added}	–	VS removal: 74.2%	Jung et al. (2013)
Sludge and food waste	Batch 35 °C Initial pH: 6	174.6 mL/g-VS_{added}	–	Batch 35 °C	264.1 mL/g-VS_{added}	–	–	Cheng et al. (2016)
Sludge and rice straw	Batch 55°C Initial pH: 7	21 mL/g-VS_{added}	60.9%	Batch 55°C	266 mL/g-VS_{added}	75–80%	VS removal: 47.4%	Kim et al. (2013b)
Sludge and molasses wastewater	CSTR 35°C HRT: 0.9 d	31 mL/g-COD_{added}	–	UASB 35°C HRT: 3.5 d	170 mL/g-COD_{added}	–	COD removal: 77.4%	Lee et al. (2014b)
Sludge and crude glycerol	CSTR Mesophilic HRT: 3 d	500 mL/g-$VS_{removed}$	24.18%	CSTR Mesophilic HRT: 6 d	1480 mL/g-$VS_{removed}$	62.4%	COD removal: 93%	Rivero et al. (2014)
Sludge and cornstalk	Batch 37°C Initial pH: 7	13.4 mL/g-VS_{added}	–	Batch 37°C	172.6 mL/g-VS_{added}	–	–	Liu et al. (2013b)

UASB Upflow anaerobic sludge blanket

also observed that the methane yield of the second stage was 135% higher than that of the single stage methane fermentation when sludge was co-fermented with glycerol. The final VS removal achieved 88–92%, which was much higher than that of conventional sludge anaerobic digestion (30–50%). This is mainly attributed to the enhanced hydrolysis effect in the first stage, which pride more suitable substrate condition for methane production. In addition, some inhibitors (e.g., phenols) can be degraded in the first stage, which reduce the inhibition of these inhibitors on the microorganisms in the second stage. Second, the two-stage process has higher tolerance to the OLR compared with the single methane fermentation, which reduced the sludge treatment time. Rivero et al. (2014) observed that the overall co-fermentation process of sludge and glycerol (with the ORL range of 15.33–17.9 g COD/L d) was significantly accelerated by using the two-stage process compared with the single methane fermentation (with the ORL of 0.53 g COD/L d). Third, each stage can be optimized separately in the two-stage process based on the favorable conditions of various groups of microorganisms, and thus is favorable for improving biogas production.

There are also some disadvantages of the two-stage fermentation process, hydrogen producers might be inhibited by the decrease of pH due to the acids accumulation in the first stage. Thus, external alkali supplementation is commonly required to maintain the optimal pH (5.5–6.5) for hydrogen production microorganisms (Xia et al. 2016), and thus leading to the increase of total operation cost. In order to reduce the requirement of external alkali, high alkalinity effluent of the methane fermentation caused by VFAs degradation and ammonia generation might be a good alternative. Jung et al. (2013) investigated a two-stage fermentation process using sludge and food waste as co-substrates. The methane fermentation effluent was collected and recycled to the first stage, casing that the external alkali addition was reduced by 50% for pH control. However, hydrogen production in the first stage was decreased by about 15% due to the introduction of hydrogen consumers. Thus, the methane effluent may need to be pretreated by some methods before recycling to the first stage, such as membrane filtration and heat shock, to inhibit the hydrogen consumers.

8.12.2 Second Stage-Photo-Fermentation

Photo-fermentation has been applied as the second stage treatment process for further hydrogen production, since photo-fermentative bacteria could use the liquid end products of dark fermentation (e.g., VFAs) to generate hydrogen (Kraemer and Bagley 2005). Only one study reported the combination of dark and photo-fermentation of sewage sludge (Zhao et al. 2015), and observed that the total hydrogen yield of this two-stage process achieved 30 mL/g-COD_{added}. During photo-fermentation process, nitrogenase is responsible for catalyzing the reduction of H^+ to hydrogen. Zhao et al. (2015) found that high NH_4^+-N concentration of dark fermentation effluent could decrease the activity of nitrogenase. Meanwhile, the

removal of NH_4^+–N did not inhibit the growth of photosynthetic bacteria. Thus, NH_4^+–N removal is commonly required when photo-fermentation is applied as the second stage.

8.13 Sludge Solubilization by Low-Pressure Wet Oxidation for Hydrogen Production

8.13.1 Overview

Waste activated sludge is produced during the biological wastewater treatment process, about 50% organic pollutants (in terms of COD) in wastewater was transformed to sludge, and the disposal of sludge costs a lot and the public demand for the sustainable management of wastes is increasing (Baroutian et al. 2015). Sludge is mainly composed of microbial biomass, which is rich in polysaccharides and proteins, so it is a potential substrate for producing hydrogen. Thus, hydrogen production from sludge not only addresses the issue of sludge disposal but also generates clean Energy. However, most of the biodegradable organics in sludge are encapsulated within microbial cell membranes while the extracellular polymeric substances are nonbiodegradable. Furthermore, high particulate content also resulted in a low hydrolysis rate. Therefore pretreatments are necessary to increase the sludge biodegradability.

Different pretreatment methods have been studied to recover the carbon source from sludge, such as thermal/hydrothermal (Shanableh 2000; Liu et al. 2012; Bertanza et al. 2015), acid (Liu et al. 2008), base (Cai et al. 2004), aeration (Lagerkvist et al. 2015), ultrasonication (Bougrier et al. 2005), microwave (Tyagi and Lo 2013), enzymatic (Thomas et al. 1993). and ionizing irradiation (Wang and Wang 2007; Park et al. 2009), and so on.

Wet oxidation has been used for treating sludge for various purposes, such as enhancing the sludge degradation (Wang and Wang 2007; Park et al. 2009), improving the sludge dewaterability (Ni et al. 2006) and converting sludge to carbonaceous product (Peng et al. 2016). However, the high temperature of traditional wet oxidation can lead to the excessive degradation of organic matters in sludge, reducing the value of sludge as an organic source for fermentative hydrogen production (Bertanza et al. 2015). Furthermore, studies have also shown that higher temperature can lead to the formation of more recalcitrant compounds (Bougrier et al. 2007). Thus, low-pressure wet oxidation is preferred in recovering the valuable organic matters from sludge, such as phosphorus recovery and reclaiming carbon source for denitrification (Baroutian et al. 2015). However, wet oxidation has not been applied for solubilizing the sludge for fermentative hydrogen production.

The objective of this study was to investigate the effects of low-pressure wet oxidation on sludge solubilization, and examine the characteristics of hydrogen production from the solubilized sludge.

8.13.2 Sludge Characteristics

Waste activated sludge was collected from a sewage treatment plant. The collected sludge settled about 2 h to measure the physicochemical characteristics, including pH, suspended solid (SS), volatile suspended solid (VSS), total chemical oxygen demand (TCOD), soluble chemical oxygen demand (SCOD), soluble total phosphorus (STP), soluble total nitrogen (STN), polysaccharides, protein, and volatile fatty acids (VFA) (Table 8.12).

8.13.3 Sludge Solubilization Procedure

Low-pressure wet oxidation was applied as a pretreatment method to disintegrate waste activated sludge. The pretreatment process not only released organic matters encapsulated in cells, but also inactivated microorganisms present in the sludge to prevent their consumption of nutrients and hydrogen.

The low pressure wet oxidation was performed in a 500 mL stainless autoclave with a magnetic stirrer (Fig. 8.6). An external electrical furnace was used to heat the autoclave and the temperature was measured by a thermocouple. In a typical run, 400 mL (VSS = 11342 ± 480 mg/L) of raw sludge was fed into the reactor and sealed. Then, the autoclave was heated up to 175 ± 2 °C (0.89 MPa), which usually takes about 40 min, and then maintained for 30 min before the electric furnace was removed. Then, the autoclave was cooled down to room temperature.

Table 8.12 Physicochemical characteristics of the waste activated sludge

Item	Value
pH	6.7 ± 0.2
SS mg/L	16426 ± 640
VSS mg/L	11342 ± 480
TCOD mg/L	18638 ± 670
SCOD mg/L	3579.9 ± 154
Protein mg/L	32.2 ± 1.7
Polysaccharides mg/L	315.5 ± 13.8
Soluble total nitrogen mg/L	5.0 ± 0.2
Soluble total phosphorus mg/L	19.4 ± 0.8
Formic acid mg/L	199.6 ± 9.7
Acetic acid mg/L	–
Propionic acid mg/L	–
Butyric acid mg/L	168.7 ± 7.4

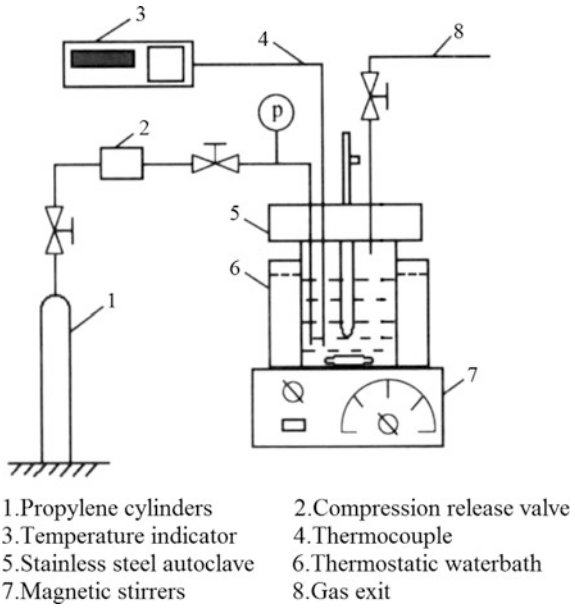

Fig. 8.6 The experimental set-up used for sludge solubilization

8.13.4 Bio-hydrogen Production and Analytical Methods

To examine the possibility of hydrogen production from low pressure wet oxidized sludge, batch experiments were conducted for hydrogen production.

The physicochemical characteristics of sludge at different states were measured by standard methods, including suspended solid (SS), volatile suspended solid (VSS), total chemical oxygen demand (TCOD), soluble chemical oxygen demand (SCOD), total phosphorus (TP), and total nitrogen (TN). The protein content was measured by the modified Lowry method using bovine serum albumin as standard. Polysaccharides content was measured by phenol sulfuric acid method using glucose as the standard. The pH value was measured by a pH meter (Model 526, Germany). The volatile fatty acids (VFA) were analyzed using an ion chromatograph (Dionex model ICS 2100) equipped with a dual-piston pump, a Dionex IonPac AS11-HC analytical column (4 × 250 mm), an IonPac AG11-HC guard column (4 × 50 mm), and a DS6 conductivity detector.

8.13.5 Sludge Dissolution by Low Pressure Wet Oxidation

Waste activated sludge was treated at 175 °C under low pressure for 30 min and the pretreated sludge showed a darker brown color than the raw sludge. Figure 8.7 shows the general physical and chemical properties of the raw sludge and sludge

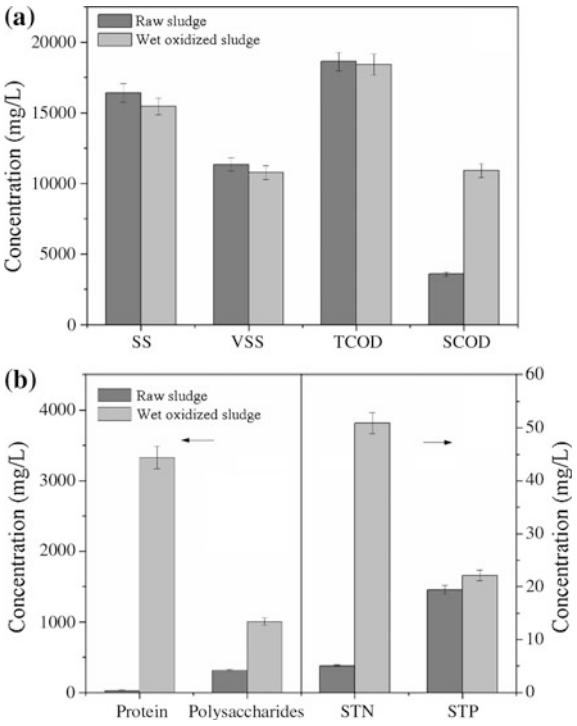

Fig. 8.7 Sludge dissolution by low-pressure wet oxidation

after wet oxidation. It can be seen from Fig. 8.7a that after wet oxidation treatment, around 5% reduction of SS, VSS, and TCOD was observed, indicating that some of the suspended matters were dissolved into the solution and some organic components were transformed into gas at high temperature. Similar results were obtained by Strong et al. (Strong et al. 2011) and Shanableh (Shanableh 2000), who used similar temperature to recover useful organic matters from waste activated sludge. Low percentage of reduction also proved that low pressure wet oxidation can preserve most organic matters in waste activated sludge for fermentative hydrogen production. Besides, SCOD showed a significant increase after treatment, which was 2 times higher than in raw sludge, indicating that low-pressure wet oxidation can disrupt the sludge floc structure and release intracellular compounds into the soluble phase effectively (Liu et al. 2008). Wet oxidation was used as an advanced and sustainable technology for sludge treatment and management, and techno-economic and environmental assessment of sewage sludge wet oxidation was studied (Bertanza et al. 2015; Slavik et al. 2015).

The main organic components used in fermentative hydrogen production include polysaccharides and protein. Thus, the disintegration of waste activated sludge leads to the nutrients recovery can be quantified by parameters, such as protein, polysaccharides, nitrogen, and phosphorus. It can be seen from Fig. 8.7b that after wet oxidation treatment, concentrations of protein and polysaccharides were

significantly improved by 102.5 and 2.2 times, respectively. Studies have shown that protein can be hardly decomposed in heat treatment (Pavlovič et al. 2013; Yin et al. 2014), thus protein was highly accumulated during the treatment process. Otherwise, as the necessary elements for microbial growth, nitrogen, and phosphorous were also released to the liquid phase, leading to the increase of STN and STP contents. In general, low-pressure wet oxidation exhibited a significant effect on nutrients recovering from waste activated sludge.

The pH increased slightly from 6.7 of raw sludge to 7.0 of treated sludge, similar phenomenon was also observed by Yin et al. (Yin et al. 2014). Studies have identified that acetic acid was highly recalcitrant within wet oxidation (Strong et al. 2011), and it can be produced as the primary VFA during hydrothermal treatment of organic wastes (He et al. 2008). However, in this study, the concentration of acetic acid in both raw sludge and treated sludge was below the detection limit. As studies have shown that the increase of acetate was usually accompanied with carbohydrate reduction in thermal treatment (Yin et al. 2014), no detection of acetic acid suggested that the low-pressure wet oxidation can prevent the transformation of polysaccharides to acetic acid.

8.13.6 Kinetic Analysis of Hydrogen Production

Hydrogen production potential of the waste activated sludge pretreated by low-pressure wet oxidation was assessed using batch tests. According to the polysaccharides concentration in the treated sludge, 1 g/L glucose was used as substrate alone to make a comparison. Furthermore, the mixture of treated sludge and 1 g/L glucose was also used as substrate to determine the effect of additional carbon source on hydrogen production from treated sludge.

As shown in Fig. 8.8, hydrogen production process of all three groups finished within 20 h. Mixture of treated sludge and glucose showed the highest cumulative hydrogen production, followed by wet oxidized sludge and glucose. Based on the data shown in Fig. 8.8, the kinetics of fermentative hydrogen production process was successfully modeled by the modified Gompertz equation, and the kinetic parameters obtained were shown in Table 8.13.

It can be seen from Table 8.13 that the cumulative hydrogen production potential of the mixture was slightly higher than the sum of cumulative hydrogen production from glucose and the wet oxidized sludge separately. Similar phenomenon was observed in our previous study applying gamma irradiated sludge as substrate (Yin and Wang 2015). Possible reason is that the addition of glucose enhanced C/N ratio of treated sludge from 0.3 to 0.6, leading to the promotion of fermentative hydrogen production. Chen et al. (Chen et al. 2012) studied the effect of carbohydrate/protein ratio on hydrogen production using waste activated sludge as substrate, and they found that hydrogen production was improved with the increasing carbohydrate/protein ratio from 0.2 to 5.0. The higher C/N ratio can result in higher hydrogen production (Lin 2004).

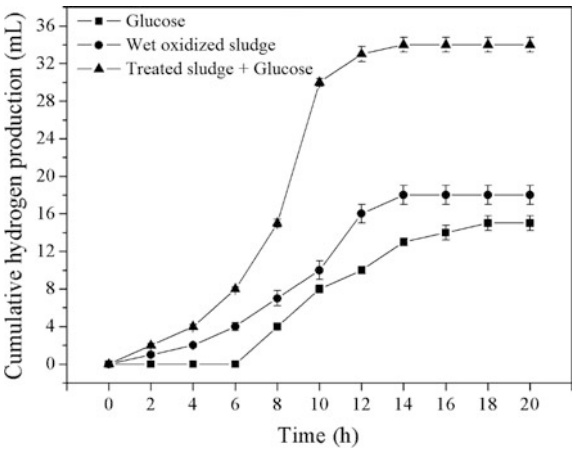

Fig. 8.8 Cumulative hydrogen production from different substrates

Table 8.13 Parameters estimated by the modified Gompertz model

Sample	Glucose	Sludge	Treated sludge + glucose
P (mL)	14.8	18.7	34.5
λ (h)	6.5	4.9	5.0
R (mL/h)	2.0	2.2	5.9
R^2	0.990	0.989	0.992
VHPR (mL/h/L)	8.2	13.4	24.6

Lag time of 6.9 h, 4.9 h, and 5.0 h were observed in groups with glucose, treated sludge, mixture of treated sludge, and glucose, respectively. The lag time was longer than our previous studies used gamma irradiation treated sludge as substrate (Yin and Wang 2016), but shorter than studies used alkaline, microwave, and sterilization treated sludge (Cai et al. 2004; Guo et al. 2008). Indicating that low-pressure wet oxidation had advantage over alkaline, microwave and sterilization in enhancing hydrogen production from waste activated sludge. Groups using sludge as substrate all showed lower lag time (λ) than that using pure glucose as substrate, indicating that hydrogen producers can better adapt to the environment with treated sludge as substrate. Possible reason is that the nutrient elements released from sludge can improve the microbial activity. Maximum hydrogen production rate (R) of 2.2 mL/h was obtained from test using the treated sludge as substrate, which was lower than 5.9 mL/h achieved from test using mixture of glucose and sludge as substrate, suggesting the insufficient carbon source from treated sludge restricted the hydrogen production rate. Volumetric hydrogen production rate (VHPR) was calculated as the production rate divided by the volume of the reactor, hydrogen production from treated sludge in this study obtained VHPR of 13.4–24.6 mL/h/L, which was higher than VHPR of 0.18–6.59 mL/h/L obtained

in study used alkaline treated sludge as substrate (Cai et al. 2004). Various studies have found the positive effect of nutrients like minerals, vitamins, and trace elements on fermentative hydrogen production (Wang and Wan 2008; Wang et al. 2009; Wang and Wan 2009; Lee et al. 2012; Taherdanak et al. 2015), concluding that the waste activated sludge was rich in various nutrients, which can be helpful for fermentative hydrogen production (Kim et al. 2011).

This phenomenon indicated that treated sludge can be a good source of nutrients for fermentative hydrogen production, which can be helpful to shorten the reaction time and enhance the cumulative hydrogen production.

8.13.7 Substrate Consumption

Substrate degradation was accompanied with the fermentative hydrogen production, and the changes of SCOD, polysaccharides, and protein concentrations in all three fermentation groups were depicted in Fig. 8.9.

It can be seen that SCOD of all groups decreased. For the group using glucose as substrate, glucose was consumed as the sole carbon source for fermentative hydrogen production, and the degradation rate of 94.1% was achieved. Besides the utilization of glucose, a small increase of protein was detected, which may be due to the metabolism and growth of hydrogen producers during the fermentation process. Similar phenomenon was also observed (Guo et al. 2008). As to the group using wet oxidized sludge as substrate, both polysaccharides and protein was decreased significantly with a degradation rate of 55.7 and 36.0%, respectively. This phenomenon was different with our previous study using gamma irradiated sludge as substrate, in which little change was observed in polysaccharides concentration after fermentation (Yin and Wang 2015). Possible reason may be that gamma irradiation treatment could cause excessive degradation of degradable polysaccharides, indicating that low-pressure wet oxidation was more preferable for degradable polysaccharides reservation during the sludge dissolution. When it came to the group using the mixture of treated sludge and glucose, polysaccharides was degraded by 24.9%, and higher protein degradation of 37.9% was obtained comparing with test using treated sludge as substrate, showing that the addition of glucose can promote the utilization of protein. This may because the more abundant degradable carbohydrate stimulated the growth of hydrogen producers, leading to both more hydrogen production and further substrate utilization. Furthermore, both test groups using treated sludge as substrate showed more protein utilization than polysaccharides, indicating that protein acted as the main carbon source for fermentation hydrogen production from waste activated sludge.

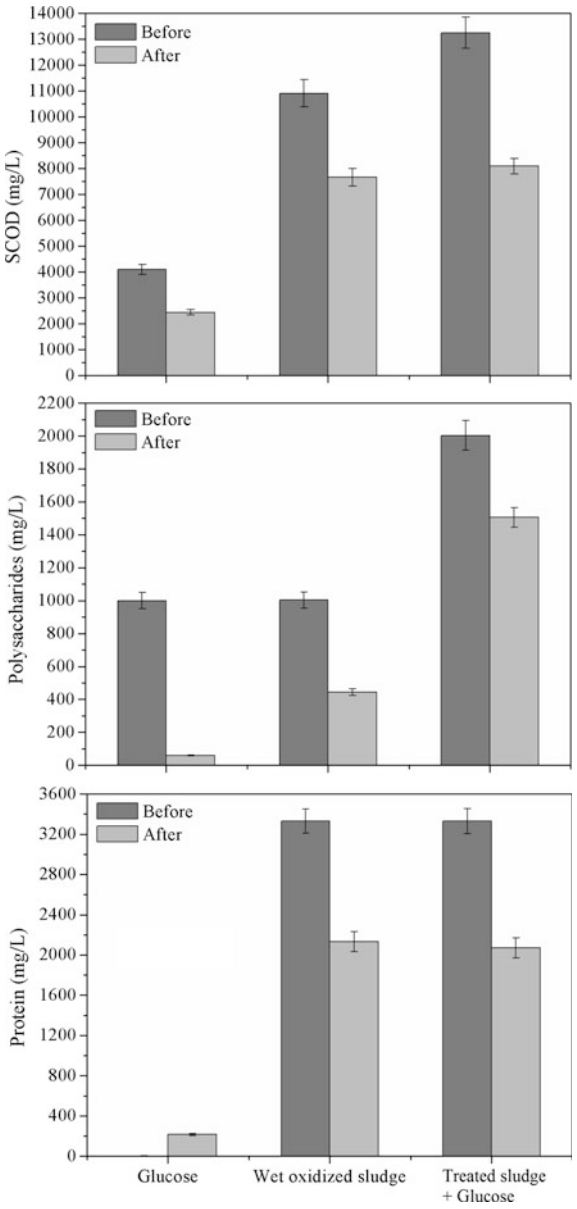

Fig. 8.9 Substrate degradation in different test groups

8.13.8 Volatile Fatty Acids Formation

Substrates consumed during the fermentation system were turned into biogas, microorganisms, and soluble metabolites. Among which, the concentrations of soluble metabolites are useful indicators for monitoring the biological hydrogen production process (Yin and Wang 2015). The major soluble metabolites formed during the fermentation process are volatile fatty acids (VFAs), and the detected VFAs in this study are shown in Fig. 8.10.

It can be seen from Fig. 8.10 that acetic acid, butyric acid and propionic acid were detected in this study. For both tests using glucose and sludge as substrate, only acetic acid and butyric acid were detected, among which acetic acid occupied 83.4 and 65.4% of total VFAs formed in two groups, respectively. Indicating that fermentation type of these two groups was dominated by acetate-type fermentation. As to the test using the mixture of sludge and glucose as substrate, acetic acid, butyric acid, and propionic acid were all formed, suggesting that it belonged to mixed acid-type fermentation. It can be seen from this phenomenon that the metabolic pathway of microorganisms was significantly affected by the composition of substrate, leading to varied fermentation types. Similar conclusion was also obtained by many other studies (Chairattanamanokorn et al. 2012; Elsamadony and Tawfik 2015; Yin and Wang 2015; Yin and Wang 2016).

Accompanied with the accumulation of VFA, pH of tests with glucose, treated sludge, mixture of treated sludge and glucose decreased from 7.0 to 5.2, 4.9, and 4.6, respectively. It has been reported that more acetic acid was formed when liquid pH was above 5.0, and the fermentation was dominated by acetate-type fermentation. When liquid pH was lower than 5.0, metabolic pathway was dominated by mixed acids fermentation (Yin and Wang 2016).

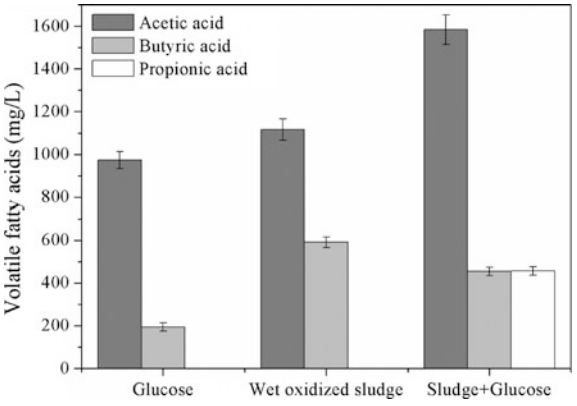

Fig. 8.10 Volatile fatty acids formation during fermentation

8.13.9 Hydrogen Yield

The hydrogen yield was estimated by dividing the amount of cumulative hydrogen production by the amount of SCOD consumed. For the test groups using treated sludge, glucose, and mixture of treated sludge and glucose as substrate, hydrogen yield was 55.4 mL/g $SCOD_{consumed}$, 90.7 mL/g $SCOD_{consumed}$, and 81.9 mL/g $SCOD_{consumed}$, respectively. A literature review showed that hydrogen yield of 15.0 mL/g COD, 11.4 mL/g COD, and 4.7 mL/g COD was achieved from sterilization, microwave, and ultrasound treated waste activated sludge (Kim et al. 2011), hydrogen yield of 1.65–10.1 mL/g COD and 4.5–15.9 mL/g COD was obtained from alkaline treated sludge (Cai et al. 2004) and boiled sludge (Wang et al. 2003). Higher hydrogen yield of 40.3 mL/g COD was obtained when combination of freezing and thawing and sterilization treatment was used (Wang et al. 2003). Indicating that hydrogen yield varied with different pretreatment methods applied to waste activated sludge. Thus, the higher hydrogen yield obtained in this study showed the advantage of low-pressure wet oxidation in treating waste activated sludge for fermentative hydrogen production.

The theoretical hydrogen production through acetate-type fermentation and butyric-type fermentation is 4 mol H_2/mol glucose and 2 mol H_2/mol glucose, respectively (Elbeshbishy et al. 2010). Thus, the acetic acid/butyric acid ratio can be a good indicator for hydrogen yield. Accordingly, we examined the correlation between hydrogen yields with the ratio of acetic acid to butyric acid (Fig. 8.11a). It can be seen that the hydrogen yield increased linearly with the increase of acetic acid/butyric acid ratio, indicating that microbial metabolism was affected by substrate composition (Wang and Wan 2008; Elbeshbishy et al. 2010; Yin and Wang 2015).

As it is known that polysaccharides can be more easily used by microorganisms, we suspect that higher hydrogen yield can be obtained from polysaccharides degradation. Thus, the polysaccharides/SCOD ratios of different substrate were examined in this study, and the relationship between hydrogen yield and polysaccharides/SCOD ratio were depicted in Fig. 8.11b. It can be seen that the highest hydrogen yield was obtained for the test using glucose as substrate, while lowest hydrogen yield was achieved for the test using treated sludge as substrate, and hydrogen yield was highly related with polysaccharides/SCOD ratio of substrate. Although studies have also showed the positive correlation between hydrogen yield and polysaccharides ratio of substrate (Chen et al. 2012), the linear relation has not been reported before. As the number of samples in this study was too small, the linear relation between hydrogen yield and polysaccharides/SCOD ratio needs to be further verified.

In summary, low-pressure wet oxidation can be used for recovering carbon source from waste activated sludge for fermentative hydrogen production. After the treatment, increase of SCOD, polysaccharides and protein concentration present in

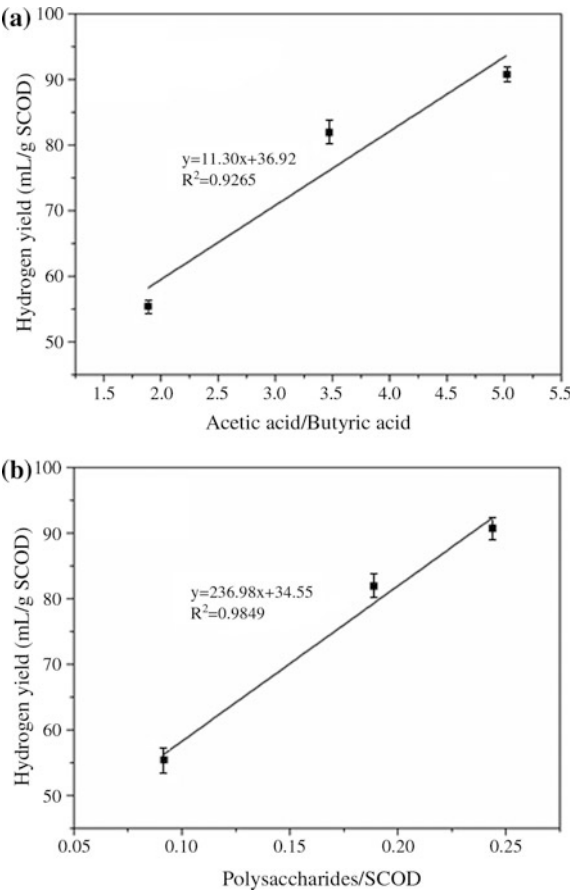

Fig. 8.11 The relationship between hydrogen yield and the ratio of acetate to butyrate (**a**) and polysaccharides to SCOD (**b**)

the liquid phase by 2.0, 2.2, and 102.5 times were obtained, respectively. Bio-hydrogen can be produced efficiently from the treated sludge and the cumulative hydrogen production can be promoted through enhancing C/N ratio in the substrate. Through comparing the hydrogen production using glucose, treated sludge, and mixture of glucose and sludge as substrate, hydrogen yield showed a linear relation with acetic acid/butyric acid ratio in soluble metabolites. Besides, hydrogen yield can be promoted through enhancing polysaccharides/SCOD ratio in substrate.

8.14 Sludge Disintegration by Radiation for Hydrogen Production

8.14.1 Overview

The main component of sludge from wastewater treatment plant is microbial biomass, and the biodegradable organics are encapsulated within microbial cell membranes (Muller et al. 1998), while the extracellular polymeric substances outside the membranes are nonbiodegradable (Muller et al. 1998). Therefore, it is necessary to disrupt the microbial cells to release the organic compounds into solution to improve the anaerobic digestion of the waste sludge and to develop the hydrogen production using sludge. Several methods can be used to treat waste sludge to improve its biodegradability, including mechanical, thermal, chemical, biological, and irradiation methods. Mechanical treatment (Bougrier et al. 2005) can solubilize the components through physically disrupting the cells, thermal treatment and microwave disintegrate the chemical bonds of the cell wall and membrane to make the cell content solubilize. Chemical treatment can hydrolyze the cell wall and membrane to release the organic matters (Kim et al. 2009). Biological method can disintegrate the sludge through the enzyme-catalyzed reactions (Guellil et al. 2001; MIAH et al. 2004). Ionizing irradiation can destroy cell structure by a number of radical species produced during water radiolysis, and these species can react with the microbial cells with high reactivity (Borrely et al. 1998; Wang and Wang 2007; Park et al. 2009). Differently pretreated activated sludge has been widely studied as substrate of hydrogen production (Alemahdi et al. 2015; Wang et al. 2015; Wan et al. 2016).

We used gamma irradiation (alone or combined with acid/alkali treatment) to disintegrate and dissolve the waste sludge, the feasibility of hydrogen production using disintegrated sludge as substrate was investigated.

8.14.2 Sludge Disintegration Procedure

Sludge was stored in 1 L sealed bottles, and divided into three groups. The pH was adjusted to 2.0, 7.0, and 12.0 with NaOH and HCl, respectively.

For gamma irradiation, different doses, i.e., 10 kGy, 20 kGy, and 30 kGy were applied at ambient temperature. Gamma irradiation was carried out by using a ^{60}Co-source in the Institute of Nuclear and New Energy Technology (INET), Tsinghua University, the radioactivity was around 1.26×10^{15} Bq. The absorbed dose was measured by using a standard Fricke dosimeter.

8.14.3 Sludge Disintegration by Radiation

The disintegration of the waste activated sludge was carried out by gamma irradiation. The effect of dose on disintegration of waste activated sludge is shown in Fig. 8.12.

It can be seen from Fig. 8.12 that there was a significant increase in the content of SCOD, polysaccharides, and protein in solution, indicating that all the pretreatment methods used in this study could destroy the cell walls and release the organic matters into the solution. In the case of disintegrated sludge without irradiation, substantial higher amount of increased SCOD, polysaccharides, and protein was observed in alkali pretreatment than acid pretreatment, suggesting that alkaline environment could lead to stronger damaging effects on cells than acidic condition. Other researchers also obtained the similar resluts (Chen et al. 2007; Liu et al. 2008; Kim et al. 2011). In the case of combining pretreatment, alkali pretreatment also showed much better performance in digesting waste sludge (Liu et al. 2008). With the increase of dose, different results were observed at different pH conditions. At neutral and alkaline conditions, the concentration of SCOD, polysaccharides and protein all increased, while almost no increase was found at acidic conditions. Assawamongkholsiri et al. (2013)found that the solubility increased with increasing heat treatment time when the combination of acid and heat was used to pretreat the

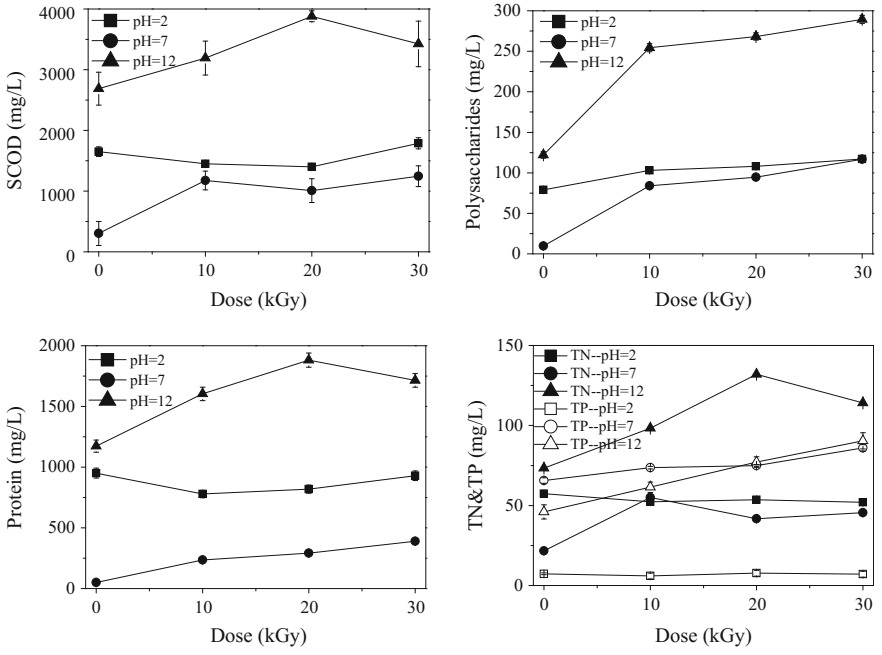

Fig. 8.12 Effect of dose on disintegration of waste activated sludge

activated sludge. Alkaline conditions showed even higher solubilizing capability for waste sludge when combing with irradiation. For example, at dose of 20 kGy pretreatment, the increment of SCOD, polysaccharides, and protein at alkaline conditions were 90, 70, and 190% higher than that at neutral conditions.

The change of TN was consistent with organic matters, while TP showed a totally different trend. Chu et al. (2011) also found that TP concentration increased with the increase of irradiation dose at neutral conditions.

The effect of pH and irradiation dose on the release of protein and polysaccharides were also shown in Fig. 8.12. The pretreatment with acid or alkali reagent resulted in greater release of protein than polysaccharides. In the absence of irradiation, the addition of acid and alkali increased protein content by 18 and 22.5 times comparing with polysaccharides by 7 and 11.5 times, respectively. On the other hand, in the neutral condition, more polysaccharides were released than protein when irradiation was used. Polysaccharides increased 10.9 times, and protein increased 6.8 times at 30 kGy irradiation.

For all 12 treated sludge samples, distinct results were obtained from alkali-irradiation disintegrated sludge. At dose of 20 kGy and pH = 12, the concentrations of SCOD, polysaccharides, and protein achieved maximum value of 3881.0 mg/L, 268.3 mg/L, and 1881.5 mg/L, the ratio of SCOD/TCOD increased from 4.2 to 54.6%. The results showed higher dissolution efficiency than the previous studies done by other researchers, who used gamma irradiation and alkaline treatment separately or combined (Cai et al. 2004; Kim et al. 2009; Chu et al. 2011). Therefore, the integration of alkali and gamma irradiation used in this study could provide quite a strong effect on breaking down the cell wall or membrane of the microorganisms, thus making the disintegrated sludge more biodegradable.

8.14.4 Hydrogen Production

Protein and polysaccharides could be used as substrates for bio-hydrogen production (Cai et al. 2004; Ozsoy et al. 2006; Guo et al. 2008). According to the amount of protein and polysaccharides generated by pretreatment, the disintegrated sludge with 20 kGy and 30 kGy at alkaline conditions were selected as substrate to further study hydrogen production. Because, the concentration of organic matters released into the solution was low, it can hardly meet the needs of hydrogen production as substrate. Therefore, besides the two sets with only disintegrated sludge as substrate (I20, I30), glucose was added into the pretreated sludge to investigate their combined impact on hydrogen production process (IG20, IG30).

Figure 8.13 shows the cumulative hydrogen production with glucose and the disintegrated sludge. According to the data shown in Fig. 8.13, the kinetic parameters can be obtained by the modified Gompertz model (Table 8.14). As shown in Fig. 8.13 and Table 8.14, the addition of glucose to the disintegrated sludge could affect the hydrogen production process.

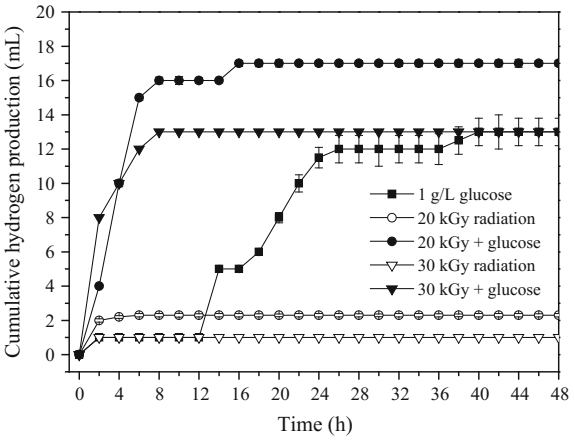

Fig. 8.13 Cumulative hydrogen productions with different substrates

Table 8.14 Parameters estimated by the Modified Gompertz model

Sample	1 g/L glucose	20 kGy irradiation	20 kGy + glucose	30 kGy irradiation	30 kGy + glucose
P (mL)	12.73	2.65	31.97	0.95	7.78
R_m (mL/h)	0.86	2.95	6.64	2.36	3.17
λ (h)	10.22	1.15	1.90	0.95	0.60
R^2	0.9868	0.9981	0.9922	0.9999	0.9367

For maximum hydrogen production potential (P), the results indicated that IG20 obtained the highest hydrogen production potential, which was far more than the sum of control and I20 sets, while the cumulative hydrogen production potential of IG30 was even lower than the control test. One possible reason may be that the high irradiation dose adopted in the pretreatment process could also degrade some biodegradable organic matters which can be used as substrate for hydrogen production, so it was not favorable for hydrogen production.

Significant higher maximum hydrogen production rates (R_m) were obtained when the disintegrated sludge and glucose were used as substrate, comparing to the control test with 1 g/L glucose as substrate (0.86 mL/h). The R_m of group IG20 was the highest (6.64 mL/h), followed by IG30 (3.17 mL/h), then I20 (2.95 mL/h), and I30 (2.36 mL/h), respectively. The high maximum hydrogen production rate may be due to the existence of various nutrients in disintegrated sludge, which may ameliorate the activity of microorganisms, thus improving the hydrogen production effectiveness (Wang and Wan 2008; Bo et al. 2009; Wang et al. 2009; Wang and Wan 2009). In addition, the disintegrated sludge could shorten the reaction time significantly: the control test reached its maximum cumulative hydrogen production within 40 h and the experimental groups finished the fermentation process within 12 h. As for the lag time, it was less than 1 h for 30 kGy pretreated sludge and 2 h

for 20 kGy pretreated sludge, much shorter than 10 h for control test. Guo et al. (2008) also obtained the similar results when they studied the hydrogen production using ultrasonication pretreated sludge. However, Cai et al. (2004) adopted alkali to pretreat sludge and used as substrate for hydrogen production, their results showed wide range of lag time from 4.8 to 77.1 h. Assawamongkholsiri et al. (2013) found that lag time was 7.4–26.8 h when using heat pretreated sludge as substrate for hydrogen production.

The high maximum hydrogen production rate of IG20 and IG30 indicated that by adding pretreated waste sludge to glucose, hydrogen production can be improved.

8.14.5 Consumption and Release of Organic Matters

The changes of SCOD, polysaccharides, and protein after hydrogen production were depicted in Fig. 8.14.

The decrease of soluble organic matters represented that they could be used as substrates for hydrogen production. For the control test, glucose was consumed to produce hydrogen. The addition of seed sludge could also enhance the disintegration of sludge, leading to the release of organic matters. Guo et al. (2008) also detected the protein and polysaccharides during fermentative hydrogen production with sludge. When it came to the tests with disintegrated sludge as substrate, protein concentration decreased sharply while little change was observed for polysaccharides, suggesting that when the disintegrated sludge was used as substrate for hydrogen production, protein could act as source of energy. Similarly, Cai et al. (Cai et al. 2004) found a greater change in the content of protein than polysaccharides during dark fermentation. Possible reason may be that the abundant bioavailable organic matters present in the disintegrated sludge. The addition of glucose could promote the use of protein and polysaccharides. It maybe attribute that the addition of glucose enriched the nutrients and further facilitated the growth of hydrogen producers, therefore more active microorganisms enhanced the utilization of nutrients and hydrogen production.

During the hydrogen production process, the consumption of organic matters was accompanied with the formation of volatile fatty acids (VFAs), as shown in Fig. 8.15.

For the control test, butyric acid was the major VFA, indicating that the hydrogen production belonged to the butyric-type fermentation. The major VFA presented in experimental sets using waste sludge as substrate was acetate acid, while the amount of butyric acid and propionate acid was pretty low, indicating that they belonged to acetate-type fermentation. In addition, the cumulative hydrogen production of these experimental sets had a strong positive correlation with acetate formation, which further confirmed that hydrogen was produced through acetate-type fermentation. This result was consistent with hydrogen production using the waste sludge pretreated by microwave, ultrasonication (Guo et al. 2008)

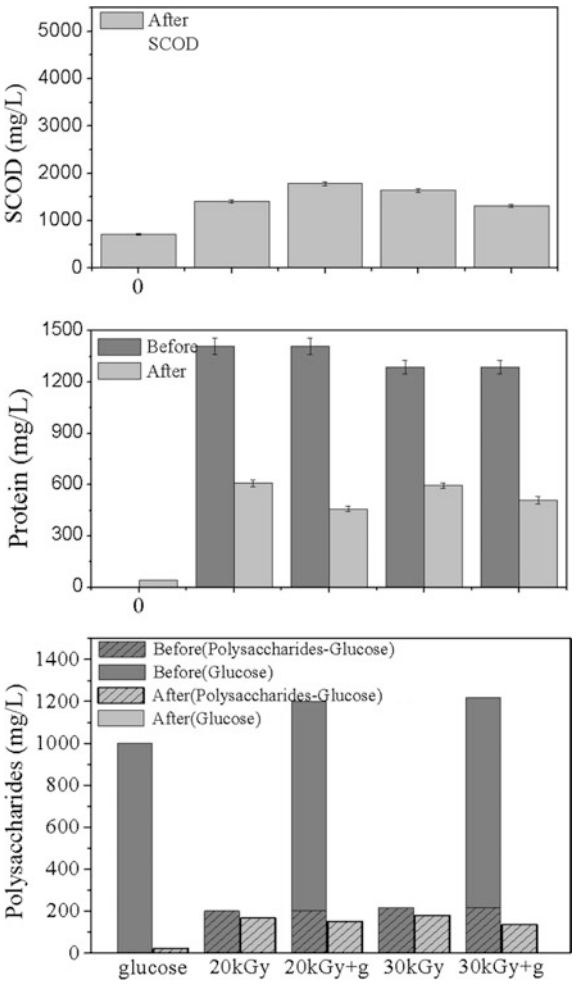

Fig. 8.14 Substrate degradation in different test groups

and alkali (Cai et al. 2004), in which acetate accounted for more than 70% of total VFAs. However, during the process of hydrogen production using the waste sludge pretreated by heat, acid, acid-heat, butyric acid was major VFA (Assawamongkholsiri et al. 2013). As it is known that acetate-type fermentation is superior to butyric-type fermentation, for its higher theoretical maximum hydrogen yield (Eqs. (8.22) and (8.23)).

$$C_6H_{12}O_6 + 2H_2O \rightarrow 4H_2 + CH_3COOH + 2CO_2 \tag{8.22}$$

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2H_2 + CH_3CH_2CH_2COOH + 2CO_2 \tag{8.23}$$

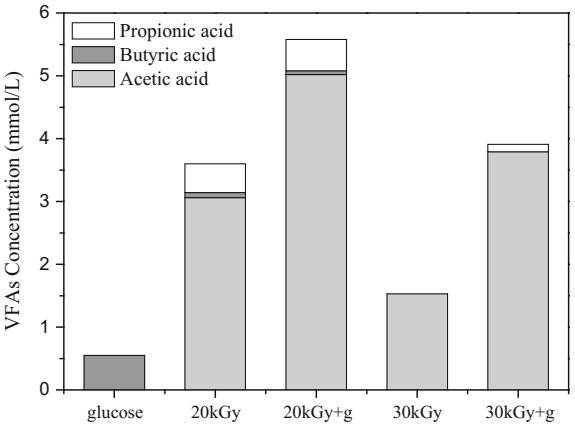

Fig. 8.15 VFAs formation during fermentation

Therefore, alkali-irradiation disintegrated sludge showed advantage over sludge treated by other methods, for its more efficient fermentation type. Furthermore, the addition of glucose to the disintegrated sludge could also lead to the shift of hydrogen production fermentation type.

Gamma irradiation or gamma irradiation combining with alkali treatment could disintegrate and dissolve the waste activated sludge effectively. The sludge pretreated at 20 kGy, 30 kGy, and pH = 12 showed the highest release of protein and polysaccharides, respectively. The alkali-irradiation method with 20 kGy dose enhanced the hydrogen production potential (P) and maximum hydrogen production rate (R_m) dramatically, and shortened the lag time (λ), and total fermentation time. The sludge disintegrated with alkali-irradiation could be used as a substrate for hydrogen production.

8.15 Concluding Remarks and Perspectives

The utilization of sludge as substrate for fermentative hydrogen production is technically feasible and shows great penitential in the future. However, this technology is still immature, and the main challenges of this technology are the low yield and rate of hydrogen production. Thus, the following aspects are needed to be intensively studied to stimulate the development of this technology, and the main efforts should still be conducted on how to improve hydrogen production efficiency.

Regarding the fermentation substrates and inoculums, most of previous reported that only carbohydrates and glycerol can be utilized by hydrogen producers for fermentative hydrogen production. However, the main organic component of sludge is protein, which accounted for about 50% of its total organics. This nature of sludge suggests that most of its organics could not be effectively used as substrates for hydrogen production, so expanding the utilization scope of substrate for

hydrogen producers could essentially improve the efficiency of fermentative hydrogen production from sludge. Additionally, low activity and growth rate of hydrogen producers restricts the hydrogen yield and production rate, so improving the activity and growth rate of hydrogen producers by some methods, such as the genetic engineering technology and low-density ultrasound stimulation, could be good ways to enhance fermentative hydrogen production from sludge. Finally, in addition to the limitations of substrate and hydrogen producers, the presence of hydrogen consumers in the mixed culture is another crucial reason leading to the low efficiency of hydrogen production. But most of previous studies paid little attention to this aspect, so the control of hydrogen consuming pathways should be intensively investigated in future researches.

Regarding the evaluation of process performance, a variety of parameters have been used for evaluating the same indicator. The use of multiple parameters make it difficult to the comparison of results obtained from different studies, restricting the proposal of suitable enhancement technologies, optimal experimental conditions, and accurate kinetic models. So the harmonization of various evaluation parameters is recommended in future researches.

Regarding the sludge pretreatment methods, various physical, chemical and biological technologies have been widely studied recent years, and have been confirmed to be efficient ways for enhancing hydrogen production. These pretreatment methods could disintegrate the complex sludge flocs and the cell wall of microorganisms, causing the release of intracellular organics. Effects of pretreatment conditions on sludge disintegration efficiency have been well understood, while effects of pretreatment conditions on hydrogen production have been rarely investigated, so more studies should also be performed to evaluate the optimum pretreatment conditions. Additionally, some nutrients (e.g., N, P) and inhibitors (e.g., heavy metals) in sludge are released during the pretreatment process (Carrère et al. 2010), effects of these released nutrients and inhibitors on hydrogen production should also be considered in future researches. Furthermore, the studied pretreatment technologies are still insufficient, more technologies should also be tried to enhance hydrogen production such as high-pressure homogenization. Combined pretreatment methods usually obtain higher hydrogen yield compared with individual pretreatment, the combination of various pretreatment methods is recommended for enhancing hydrogen production. As the essence of sludge fermentation is converting sludge organics to hydrogen, the enhancement mechanisms should be illustrated from the microorganism aspects in detail in future studies. Furthermore, although the purpose of applying sludge pretreatment is to increase hydrogen production, more attentions should also be paid to the energy balance and economic analysis of these pretreatment technologies.

Regarding co-fermentation of sludge and other substrates, it can be predicted that this technology will be more and more studied in the future due to the synergistic effects of sludge and other organic wastes. The addition of various organic wastes have been proved to enhance the hydrogen yield from sludge fermentation, so more kinds of organic wastes should be tried to co-fermentation with sludge, such as forestry waste, microalgae, and fats, oils, and greases. More attentions

should be paid to the optimal operation conditions and the enhancement mechanisms of the co-fermentation process, especially for the analysis of aspects of microorganism communities, enzyme activities, and metabolic pathways. Besides, the cost analysis of co-fermentation should also be intensively studied in future researches, stimulating the practical application of this technology. It seems that organic wastes characterized by low transportation cost are more suitable co-substrates for sludge fermentation.

Regarding the influence factors, effects of these factors on fermentative hydrogen production from sludge have been widely studied, while the optimal operation conditions are still inconsistent in previous studies. The influence mechanisms on sludge fermentation are still not clear, especially on the aspects of microorganism communities and metabolic pathways. As for the operating conditions, most of sludge fermentation reactors have been performed in batch mode, exploration of continuous high OLR reactor is crucial for the practical application of the technology. The hydrogen production results from most of previous studies where only the initial pH was adjusted, regulating the pH value in the whole fermentation process should be further investigated to achieve higher hydrogen production. Besides, oxidation redox potential is another important parameter of the fermentation process. More studies performed on this subject are recommended, and the appropriate value should also be evaluated. As for the trace metal nutrients, most of previous studies only concentrated on effects of Fe^{2+} on fermentative hydrogen production from sludge. Other metal ions, such as Ni^{2+} and Co^{2+}, could also influence the growth and activity of fermentative bacteria. Effects of these metal ions on sludge fermentation should be intensively investigated. As for the inhibitors, studies on inhibition effects of some other kinds of chemicals, such as Cu^{2+}, Na^{+}, K^{+}, sulfide, and toxicity organic compounds, on fermentative hydrogen production from sludge should be intensively performed. The fate of these heavy metals and toxicity organic compounds during sludge fermentation process should also be examined in future researches. As for the inoculum pretreatment, some technologies (e.g., heat, alkaline, acid, and ionizing radiation) have been applied for enriching hydrogen producers from various mixed cultures (e.g., sludge, pig manure, and hot spring sediment). More studies should be performed on effects of these pretreatment technologies on hydrogen producing bacteria communities and hydrogen production, and more data are needed to identify the optimal pretreatment method for various mixed cultures. Finally, substrate concentration could significantly influence the performance and cost of sludge fermentation, while few studies have been performed on this subject.

Regarding the two-stage process, two secondary processes (anaerobic digestion and photo-fermentation) are have been applied for enhancing energy conversion efficiency and sludge reduction through converting by-products of sludge fermentation for further biogas production. The technical feasibility of such two two-stage processes has been demonstrated, and the optimal operating conditions should be intensively investigated in the future. In addition, microbial electrolysis cells and microbial fuel cells have also been recommended as the second stage processes to couple with dark fermentation, which can realize further hydrogen production and

the generation of electric current, respectively (Hallenbeck 2009). However, few studies combined these two processes with dark fermentation of sewage sludge, so more studies should be performed on these subjects for more energy recovery from sludge. Finally, the energy and economic balance of above four two-stage processes should be intensively focused for their future development.

Regarding the practical application of fermentative hydrogen production from sludge, considerable efforts have been conducted during past 16 years. However, this technology seems to be still in its infant stage, and to our best knowledge there is no commercial plant yet. In future researches, more pilot-scale studies are required to stimulate the application of this technology. In addition to above-mentioned technical efforts, some management actions should also be taken from regulation and economic aspects to support the application of sludge fermentation.

8.16 Conclusions

The sewage sludge was a feasible substrate for fermentative hydrogen production. Both municipal and industrial sludge (bath wastewater sludge, brewery industry sludge, food processing sludge, fructose-processing sludge, molasses wastewater sludge, paperboard mill sludge, and poultry slaughterhouse sludge) have been studied for fermentative hydrogen production. Carbohydrates and glycerol are two main organic components in sludge for hydrogen production. However, low hydrogen production efficiency caused by complex sludge structure and low C/N ratio is the main limitation of this process. Various physical (heat, ultrasound, microwave, sterilization and UV-light), chemical (alkaline, acid, and oxidation), biological (enzyme and bacteria), and combined pretreatment technologies have been widely studied, and proved to be efficient ways for enhancing hydrogen production. Among these pretreatment methods, physical pretreatment was the most studied group (41.6%), following by chemical (40.5%), combined (9.7%), and physical (8.2%) pretreatment. Alkaline (35.8%), heat (23%), and ultrasound (10.5%) are three most reported individual pretreatment methods. The enhancement effect by combined pretreatment method is usually greater than that by individual pretreatment. Besides, the addition of co-substrates could also enhance fermentative hydrogen production from sludge. Municipal waste fractions was the most studied co-substrates, accounting for 57.2% of all reports, and followed by pure carbohydrates (15.8%), carbohydrate-rich wastewaters (11.3%), crop residues (9.5%), and other organic wastes (6.2%). Numerous factors, including temperature, pH, agitation intensity, retention time and OLR, nutrients and inhibitors, inoculum, and applied treatment methods, could influence hydrogen production from sludge, while the optimal fermentation conditions are still not concluded. In order to gain insight into the process, some kinetic models have been applied for describing sludge fermentation including the modified Gompertz equation, response function, mass balance equation, second degree polynomial function and some other mathematical

equations. The modified Gompertz equation was the most widely studied model. After sludge fermentation, the main end products include acetate, propionate, butyrate, ethanol, lactate and formate. Corresponding fermentation types include the acetate type, the propionate type, the butyrate type, the ethanol type, the mixed acid type, the propionate-butyrate type, the propionate-ethanol type, the butyrate-ethanol type, and the propionate-butyrate-ethanol type. The butyrate type (44.78%), the propionate-butyrate type (24.35%) and the acetate type (8.7%) were the most three reported fermentation types. Various pretreatment methods, the addition of co-substrates and process factors could all influence the metabolites in the liquid phase, and consequently changed the sludge fermentation type. In order to achieve higher energy conversion efficiency, second stages of anaerobic digestion and photo-fermentation have been conducted to use the end products of sludge fermentation for further biogas production.

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