CHAPTER II
LITERATURE REVIEW

2.1 Hydrogen properties and its use

2.1.1 Properties

The hydrogen atom is made up of a nucleus with positive charge and one electron. A molecule of hydrogen is made up of two hydrogen atoms and is the most basic of all molecules. At room temperature and under normal pressure, hydrogen is a colorless, odorless and non-poisonous gas which is lighter than air and helium. Hydrogen burns with a pale blue, almost invisible flame. At temperatures under –253 °C hydrogen is in a liquid state (Brady, 2000). The physical properties of hydrogen are shown in Table 2.1.

Table 2.1 The physical properties of hydrogen

Properties	Values
Atomic weight	1.008
Density (kg/m^3)	0.0899
Boiling point (K)	20.28
Critical point (K)	33.30
Freezing point (K) Molar	14.01
Molar mass (kg/mol)	0.002
Lower heating value (MJ/kg)	120.2
(MJ/Nm^3)	10.76
(kJ/mol)	241
Upper heating value (MJ/kg)	142
(MJ/Nm^3)	12.71
(kJ/mol)	285

(Kruse et al., 2002)

2.1.2 Use of hydrogen

Hydrogen is the promising energy for renewable energy system. It can be used to supply the fuel cell vehicles as well as to fuel cell power generation system (Kato et al., 2005). The use of hydrogen is an appropriate environmental solution because it does not emit the pollution such as CO_x, SO_x and NO_x like the fossil energy systems (Barreto et al., 2003). Hydrogen is first used in the transportation sector. Internal combustion engine can be fueled with pure hydrogen blended with natural gas (Momirlan and Veziroglu, 2005). Nowaday hydrogen is used in ammonia manufacture, petroleum refinement and methanol synthesis (Momirlan and Veziroglu, 2005). NASA has used liquid hydrogen to propel the space shuttle and other rocket into orbit since 1970s (Momirlan and Veziroglu, 2005).

Fuel cell is an energy conversion technology for producing electricity combining fuel (hydrogen) and oxidant (oxygen from air) gases through electrodes and across an ion conducting electrolyte (Stambouli and Traversa, 2002). The principle characteristic of a fuel cell is its ability to convert chemical energy directly into electrical energy giving much higher conversion efficiencies than any conventional thermo-mechanical system. Thus, it can extract more electricity from the same amount of fuel, operate without combustion so they are virtually pollution free and have quieter operation since there are no moving parts (Stambouli and Traversa, 2002). To convert the hydrogen fuel to electrical energy, the hydrogen fuel is fed into the anode of the fuel cell and oxygen, enters the cell through the cathode. The hydrogen, under the action of the catalyst, splits into protons (hydrogen ions) and electrons, which take different paths towards the cathode. The proton passes through the electrolyte and the electron produced can be used before reaching the cathode, to be reunited with the hydrogen and oxygen to form a pure water molecule and heat as shown in Figure 2.1 (Stambouli and Traversa, 2002). Fuel cells generate electricity via an electrochemical process combining hydrogen and oxygen. Fuel cell works similar to a battery but it does not require recharging. The cell will continue to produce electricity as long as it has a supply of fuel (Johnston et al., 2005).

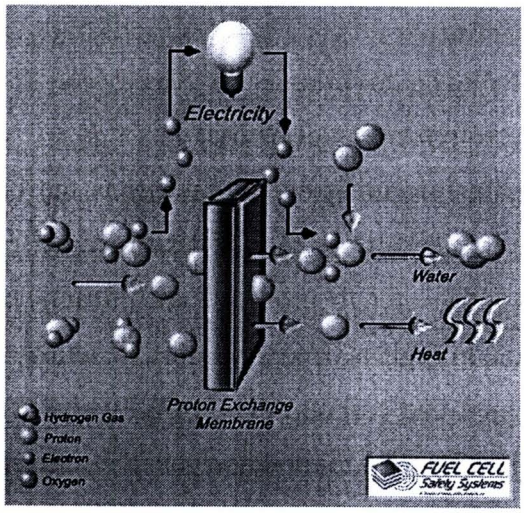


Figure 2.1 The working of hydrogen fuel cell (Johnston et al., 2005)

2.2 Hydrogen production

Hydrogen has been produced and used for industrial purposes prior this century. The total amount of hydrogen production of the world is approximately 45 million tons and over 90% comes from fossil raw materials (Kruse et al., 2002). Hydrogen can be generated mainly from fossil fuels, biomass and water by chemical or biological process (Han and Shin, 2004). However, the by-products from hydrogen production using coal, oil or natural gas as raw matterials will cause negatively impact on the environment if they are not handled in an environmentally responsible manner (Kim et al., 2004).

2.2.1 Hydrogen production from fossil raw materials and renewable energy

The following describes some of the most common techniques of producing hydrogen from hydrocarbons.

2.2.1.1 Gasification of coal

Gasification of coal is the oldest method of hydrogen production. Typically, the coal is heated up to 900 °C where it turns into a gaseous form and is then mixed with steam. It is then fed over a catalyst usually nickel. There are also other more complex methods of gasifying coal. The common factor is that they turn coal, treated with steam and oxygen at high temperatures, into H_2, CO and CO_2. However, sulfur and nitrogen compounds can be released from the raw materials and

during this hydrogen production process as the air pollution. The amounts of CO and CO_2 have to be controlled in an environmental friendly way (Soni et al., 2009).

2.2.1.2 Steam reforming of natural gas

Steam reforming of natural gas is the cheapest way to produce hydrogen, and estimate about half of the world's hydrogen production. Methane, in the natural gas, reacts with water to produce carbon monoxide and hydrogen. The carbon monoxide is put through a water–gas shift reaction where it combines with water to produce hydrogen and carbon dioxide (Williams et al., 1995). Steam, at a temperature of 700-1000 °C, is fed to methane gas in a reactor with a catalyst, at 3-25 bar pressure. In addition, the natural gas being part of the reaction process, an added 1/3 natural gas is used as energy to power the reaction. (Gaudernack, 1998).

Economically, the costs of steam reforming of natural gas are heavily dependent on the cost of the feedstock. Between 52% and 68% of the overall costs of hydrogen production can come from the cost of the natural gas (Basye and Swaminathan, 1997).

2.2.1.3 Electrolysis of water

Water electrolysis by photon exchange membrane (PEM) is considered to be a promising method of producing hydrogen through renewable energy resources such as wind and photovoltaic power (Kato et al., 2005). When hydrogen is produced by the electrolysis process, half the number of moles of oxygen is produced simultaneously as a by–product of hydrogen (Penner, 2006). If large quantities of hydrogen need to be produced on a large scale, in this situation, the by–product oxygen should be fully utilized, as oxygen is an important industrial gas used in many process such as combustion, semiconductor production and wastewater treatment (Jianwei et al., 2003). Although the hydrogen produced by renewable resource based electricity is very expensive in most cases, it is attractive because it is a very pure and clean energy carrier. In the long run, hydrogen should be produced by renewable energy resources to avoid fossil–fuel consumption and greenhouse–gas emissions (Kato et al., 2005).

2.2.1.4 Photo electrolysis

Instead of first converting sunlight to electricity and then using an electrolyzer to produce hydrogen from water, it is possible to combine these two

steps. The photovoltaic cell combines with a catalyst, which acts as an electrolyzer and splits hydrogen and oxygen directly from the surface of the cell. This quite realistically is a commercially viable means of producing hydrogen. The advantages of these systems are the elimination of cost of electrolyzers and increasing the efficiency of system. Tests performed outdoors with silicon based cells have shown an efficiency of 7.8% in natural sunlight (Holladay et al., 2009).

2.2.1.5 Thermal decomposition of water

In a thermal solar power plant with a central collector such as Solar Two, a 10 MW power plant in California, the temperatures can reach over 3,000 °C. By heating water to over 2,000 °C, it is broken down into hydrogen and oxygen. This is considered to be an interesting and inexpensive method of producing hydrogen directly from solar energy. Research is also being done on the use of catalysts to reduce the temperature for dissociation. One central problem is the separation of gases at high temperatures to avoid recombining. The efficiency factor is uncertain (Kruse et al., 2002).

2.2.1.6 Gasification of biomass

Hydrogen can also be produced by thermal gasification of biomass such as forestry by-products, straw, municipal solid waste and sewage. The amount of hydrogen in biomass is about 6-6.5 weight percent compared to almost 25% for natural gas (Kruse et al., 2002, Holladay et al., 2009). The processes involved producing hydrogen from biomass resembles the processes of production hydrogen from fossil fuel. Under high temperature, the biomass breaks down to gas. The gas consists mainly of H_2, CO and CH_4. Steam is then introduced to reform CH_4 to H_2 and CO. CO is then put through the shift process to attain a higher level of hydrogen. The by-product from this process is CO_2, but CO_2 from biomass is considered "neutral" with respect to greenhouse gas, as it does not increase the CO_2 concentration in the atmosphere. The mixed gas can also be used in fuel cells for electricity production. Compared to conventional processes for production of electric energy from biomass or waste, integrated gasification fuel cell systems are preferable. Electrical efficiency over 30% is possible for these systems (Holladay et al., 2009).

2.2.2 Biological processes for hydrogen gas production

Biological processes are carried out largely at ambient temperatures and pressures, and hence are less energy intensive than chemical or electrochemical ones. A large number of microbial species, including significantly different taxonomic and physiological types, can produce hydrogen. Among the various processes comprising the biological production of hydrogen, direct and indirect biophotolysis, fermentation, photosynthetic production and also in vitro enzymatic conversion of biomass are important (Kapdan et al., 2006). Biophotolysis involves light-driven decomposition of water in the presence of micro-algae or cyanobacteria. An attractive and even more favored possibility is the direct splitting of water to generate hydrogen by solar radiation (Benemann, 1996). This H_2O splitting can be achieved either in photochemical cells where, for example, TiO_2 is illuminated as the catalyst, or by applying photovoltaics, which indirectly utilize solar radiation for the electrolysis of water into H_2 and O_2 (Rao and Hall, 1996). Table 2.2 summarizes the relative advantages and disadvantages of some important biological hydrogen production processes. Despite its relatively lower yields of hydrogen, the fermentative route is a promising method of biohydrogenation due to its higher rate of hydrogen evolution in the absence of any light source as well as the versatility of the substrates used. Moreover fermentative organisms have high growth rate and do not suffer much from inhibitory effects of oxygen in the system, as the process is anoxygenic.

2.2.2.1 Direct Biophotolysis

Hydrogen production with high specific activity occurs in unicellular green algae (e.g. *Chlamydomonas reinhardtii*) and anaerobic fermentative bacteria (e.g.*Clostridium pasteurianum*). The reaction is catalyzed by [Fe]-hydrogenase enzymes (Viguais et al.,2001; Florin et al., 2001) which mediate the donation of high potential-energy electrons to protons (H^+), according to the reaction:

$$2H^+ + 2FD^- \rightarrow H_2 + 2FD \quad (1)$$

The enzymatic turnover rate of the [Fe]-hydrogenase is in the 6000–9000 s^{-1} range, consistent with the high specificity of the reaction. In green algae (e.g. *Chlamydomonas reinhardtii*), electrons (e^-) and protons (H^+) are extracted from water (H_2O) through photosynthesis, or from endogenous substrate through chlororespiration (Antal at al., 2003; Mus et al., 2005). The potential energy of these

electrons is elevated in the thylakoid membrane of photosynthesis, via the absorption/utilization of visible sunlight (400–700 nm region of the solar spectrum). Following this light-driven electron transport in the thylakoid membrane, the high potential-energy electrons (e^-) and protons (H^+) are combined to generate molecular H_2. The process of photosynthetic electron transport in green algae can operate with a photon conversion efficiency of 85–90% (Greenbaum, 1988) and normally generates biomass from inorganic minerals, CO_2 and H_2O. Given the high solar conversion efficiency and directness of the H_2 production process, green algae are thought to be promising in long-term efforts of photobiological H_2 production.

Previously, hydrogen evolution activity in green algae was induced upon a prior anaerobic incubation of the cells in the dark. This treatment was necessary and sufficient to remove oxygen (O_2) from the medium, as O_2 is a powerful suppressor of the [Fe]-hydrogenase gene expression (Florin et al., 2001; Wunschiers, 2001; Hoppe and Kaminski, 2002) and a potent inhibitor of the [Fe]-hydrogenase enzymatic activity (Ghirardi et. al., 1997). Following such a dark anaerobic induction, and in the course of a subsequent illumination by which to drive green algal photosynthesis, the activity of the [Fe]-hydrogenase is manifested, but it is only transient in nature (Stuart and Gaffron, 1972). It lasts from several seconds to a few minutes only. This is because photosynthetic O_2 quickly accumulates upon illumination and effectively interferes with all aspects of the cellular H_2 metabolism.

Currently, the simultaneous production of O_2 and H_2 by the photosynthetic apparatus of green algae offers a number of challenges, mainly due to the great sensitivity of the [Fe]-hydrogenase to O_2, which is evolved upon illumination of the cells (Melis et al., 2004). An additional problem, assuming that the mutual incompatibility of O_2 and H_2 coproduction is overcome, entails the separation of the two gases, a costly and technologically challenging feat. Nevertheless, a direct photosynthetic O_2 and H_2 coproduction promises the highest yields and efficiencies, approaching 13–15% (light energy to H_2), as H_2 is produced soon after the primary energy conversion event in photosynthesis, i.e., the conversion of sunlight energy into chemical energy. In support of the feasibility of this approach, it has been shown that O_2 and H_2 coproduction by green algae can be prolonged under conditions designed to actively remove O_2 from the reaction mixture (Greenbaum,

1988; Greenbaum, 1982; Greenbaum, 1983). Moreover, genetic engineering of the green algae can be applied to enhance the O_2 tolerance of the [Fe]-hydrogenase, so as to permit O_2 and H_2 coproduction (Melis et al., 2004).

Table 2.2 Comparison of important biological hydrogen production processes

Process	Type of microorganisms	Advantages	Disadvantages
Direct Biophotolysis	Green algae	Can produce H_2 directly from water and sunlight	Requires high intensity of light O_2 can be dangerous for the system
		Solar conversion nergy increased by 10 folds as compared to trees, crops	Lower photochemical efficiency
		Can produce H_2 from water	Uptake hydrogenase enzymes are to be removed to stop degradation of H_2
Indirect biophotolysis	Cyanobacteria	Has the ability to fix N_2 from atmosphere	About 30% O_2 present in gas mixture O_2 has an inhibitory effect on nitrogenase
Photo fermentation	Photosynthetic bacteria	A wide spectral light energy can be used by these bacteria	Light conversion efficiency is very low, only 1–5%
		Can use different waste materials like distillery effluents, waste etc	O_2 is a strong inhibitor of hydrogenase
Dark fermentation	Fermentative bacteria	It can produce H_2 all day long without light	Relatively lower achievable yields of H_2
		A variety of carbon sources can be used as substrates	As yields increase H_2 fermentation becomes thermodynamically unfavorable
		It produces valuable metabolites such as butyric, lactic and acetic acids as by products	Product gas mixture contains CO_2 which has to be separated
		It is anaerobic process, so there is no O_2 limitation problem	

(Nath and Das, 2004)

2.2.2.2 Indirect biophotolysis

The general reaction for hydrogen formation from water by cyanobacteria can be represented by following reactions:

$$12H_2O + 6CO_2 + \text{lightenergy} \rightarrow C_6H_{12}O_6 + 6O_2 \quad (2)$$

and

$$C_6H_{12}O_6 + 12H_2O + \text{lightenergy} \rightarrow 12H_2 + 6CO_2 \quad (3)$$

Cyanobacteria are also known as blue-green algae, cyanophyceae or cyanophytes. It is a large and diverse group of photoautotrophic microorganism. Cyanobacteria contain photosynthetic pigments, such as chl a, carotenoids and phycobiliproteins, and can perform oxygenic photosynthesis. Morphologically these organisms fall into a diverse group that includes unicellular, filamentous and colonial species. Hydrogen is produced both by hydrogenase and nitrogenase enzymes. Within the filamentous cyanobacteria, vegetative cells may develop into structurally modified and functionally specialized cells. The nutritional requirements of cyanobacteria are simple: air (N_2 and O_2), water, mineral salts and light. Hydrogen producing cyanobacteria may be either nitrogen fixing or non-nitrogen fixing. The examples of nitrogen fixing organisms are non-marine *Anabaena* sp., marine cyanobacteria *Calothrix* sp., *Oscillatoria* sp. Non-nitrogen fixing organisms are *Synechococcus* sp., *Gloebacter* sp. and *Anabaena* sp. They are found suitable for higher hydrogen evolution as compared to other cyanobacteria species (Levin et al., 2004; Pinto et al., 2002). Heterocystous filamentous *Anabaena cylindrica* is a well-known hydrogen producing cyanobacterium (Nandi and Sengupta, 1998). But, *Anabaena variabilis* has received more attention because of higher hydrogen yield (Liu et al., 2006). Hydrogen production by vegetative cells can take two routes (Liu et al., 2006):

A. Heterocystous nitrogen fixing bacteria:

$$H_2O \rightarrow \text{Photosystems (vegetative cells)} \rightarrow [CH_2O]_2 \rightarrow \text{Ferredoxin} \rightarrow \text{Nitrogenase} \quad (4)$$

$$\uparrow \qquad \downarrow \text{NADPH} \qquad \downarrow$$

$$CO_2 \text{ Recycle} \quad CO_2 \qquad H_2$$

B. Nonheterocystous nitrogen fixing bacteria:

$H_2O \rightarrow$ Photosystems (vegetative cells) $\rightarrow [CH_2O]_2 \rightarrow$ Ferredoxin $\rightarrow$ Nitrogenase (5)

$\uparrow$ $\downarrow$ NADPH or Hydrgenase

CO_2 Recycle CO_2 $\uparrow$ $\downarrow$

PS-I $\rightarrow$ ATP H_2

The growth conditions for Anabaena are simple which include nitrogen free media, illumination, CO_2 and N_2. Nitrogenase plays important role for the hydrogen generation. Activity of the nitrogenase is inhibited by oxygen. Hydrogen production takes place under anaerobic conditions. Some cultures require CO_2 during hydrogen evolution phase, although CO_2 is reported to give some inhibition effects on photo-production of H_2. Lower CO_2 concentrations (4–18% w/v) have been reported to increase cell density during growth phase, resulting in higher hydrogen evolution in the later stage. Simple sugars have been found suitable for hydrogen production. According to previous studies, more emphasis has been given to increase hydrogenase activity and bidirectional hydrogenase deficient mutants of *Anabaena* sp. to increase the rate of hydrogen production. However, the rate of hydrogen production by *Anabaena* sp. is considerably lower than that obtained by dark or photo-fermentations (Levin et al., 2004; Pinto et al., 2002; Liu et al., 2006).

With dinitrogen

$$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16 \quad (6)$$

or, without dinitrogen

$$8H^+ + 8e^- + 16ATP \rightarrow 4H_2 + 16ADP + 16Pi \quad (7)$$

2.2.2.3 Hydrogen production by photosynthetic bacteria

Photosynthetic bacteria are known as the most powerful hydrogen producers. The photobiological hydrogen evolution method provided a very simple and efficient system of solar energy conversion (Nakada et al., 1998). Photosynthetic bacteria are the most promising microbial system for the biohybrogen production (Fascetti et al., 1998). This is mainly because of their high theoretical conversion yields, and lack of oxygen evolving activity which causes oxygen inactivation problems in different biological system. In addition, they had the ability to use wide spectral light energy and consume organic substrates derived from wastes in association with wastewater treatment (Fascetti et al., 1998).

There are various kinds of photosynthetic bacteria capable of producing hydrogen and many kinds of organic substrate such as fatty acids, sugars, starches, cellulose that could be used as substrate for hydrogen production (Miyake et al., 1999). Photosynthetic bacteria do not utilize water as the starting compound for hydrogen production but use organic acids (Miyake et al., 1999) such as acetate (Barbosa et al., 2001; Boran et al., 2010), malate (Oh et al., 2004; Obeid et al., 2010), lactate (Barbosa et al., 2001; Obeid et al., 2010), butyrate (Barbosa et al., 2001), which are the main by-products of anaerobic fermentation. In addition, photo-fermentation of wastewaters including brewery (Seifert et al., 2010) and dairy wastewaters (Seifert et al., 2010) were also used as substrate for hydrogen production by photosynthetic bacteria.

Hydrogen production by purple non-sulfur bacteria is mainly due to the presence of nitrogenase under nitrogen-deficient conditions using light energy and reduced compounds (organic acids). The reaction is as follows:

$$CH_3COOH + 2H_2O + \text{light energy} \rightarrow 4H_2 + 2CO_2 \quad (8)$$

Photosynthetic bacteria have long been studied for their capacity to produce significant amounts of hydrogen (Bolton, 1996). The advantage of their use is in the versatile metabolic capabilities of these organisms and the lack of PhotosystemII (PSII), which automatically eliminates the difficulties associated with O_2 inhibition of H_2 production. Phototrophic bacteria require organic or inorganic electron source to drive their photosynthesis. They can utilize a wide range of cheap compounds. These photoheterotrophic bacteria have been found suitable to convert light energy into H_2 using organic wastes as substrate in batch processes, continuous cultures or immobilized whole cell system. The overall biochemical pathways for the photo-fermentation process can be expressed as follows (Winkler et al., 2002):

$$\begin{array}{ccccc} & \text{NADPH} & & & \\ (CH_2O)_2 & \rightarrow & \text{Ferredoxin} & \rightarrow & \text{Nitrogenase} \rightarrow H_2 \\ & \uparrow ATP & & \uparrow ATP & \end{array} \quad (9)$$

Certain photoheterotrophic bacteria within the superfamily *Rhodospirillaceae* can grow in the dark using CO as the sole carbon source to generate ATP with the simultaneous release of H_2 and CO_2. The oxidation of CO to CO_2 with the release of H_2 occurs via a water gas shift reaction as shown below:

$$CO + H_2O \rightarrow CO_2 + H_2 \quad (10)$$

2.2.2.4 Hydrogen production by dark fermentation

Dark hydrogen fermentation is a ubiquitous phenomenon under anoxic conditions (i.e., no oxygen present as an electron acceptor). When bacteria grow on organic substrates (heterotrophic growth), these substrates are degraded by oxidation to provide building blocks and metabolic energy for growth. This oxidation generates electrons which need to be disposed of to maintain electrical neutrality. In toxic environments, oxygen is reduced and water is the product. In anoxic environments, other compounds, e.g., protons, which are reduced to molecular hydrogen (H_2), need to act as electron acceptor (Levin et al., 2004). In the hydrogen fermentation process, glucose is initially converted to pyruvate by the glycolytic pathways. This is oxidized to acetyl-CoA, which can be converted to acetyl phosphate and results in the generation of ATP and the excretion of acetate. Pyruvate oxidation to acetyl-CoA requires ferredoxin (Fd) reduction. Reduced Fd is oxidized by hydrogenase which generates Fd(ox) and releases electrons to produce molecular hydrogen (Nath and Das, 2004; Nath and Das, 2005). The overall reaction of the process can be described as follows:

$$\text{Pyruvate} + \text{CoA} + 2\text{Fd (ox)} \rightarrow \text{acetyl-CoA} + CO_2 \quad (11)$$

$$2H^+ + \text{Fd (red)} \rightarrow H_2 + \text{Fd (ox)} \quad (12)$$

Anaerobic fermentation enables the mass production of hydrogen via relatively simple processes from a wide spectrum of potentially utilizable substrates, including refuse and waste products. Moreover, fermentative hydrogen production generally proceeds at a higher rate and does not rely on the availability of light sources.

The evolution of hydrogen by dark fermentation has, however, several advantages for industrial production, such as fermentative bacteria have very high evolution rate of hydrogen. Fermentative bacteria can produce hydrogen constantly through day and night from organic substrates (Kirk et al., 1985). Hydrogen is produced via an anaerobic process in which is divided into two distinct stages. The first stage is acidification in which produced hydrogen as a by-product. Hydrogen would turn to be used as an electron donor by many methanogens in the second stage of the process (Kirk et al., 1985).

2.2.3 Factors influencing fermentative hydrogen production

2.2.3.1 Temperature

Temperature, one of the most important ecological factors, influences all kinds of physiological activities of microorganism and conversion rate of fermentation product. Temperature notably influences physiological metabolism of fermentation microorganism, especially frequent temperature changes will greatly influence microorganism. Temperature also affects the maximum specific growth, substrate utilization rate and the metabolic pathway of microorganisms, resulting in a shift of by-product compositions (Lay, 2000; Li and Fang, 2007; Van Ginkel et al., 2001). It is known from hydrogen production rate response curve to temperature rise and temperature drop stimulation shown in Figure 2.2 that hydrogen production rate greatly fluctuates when temperature rises and drops. At the beginning of temperature rise, hydrogen production rate greatly drops with rising temperature, but maximum hydrogen production rate occurs at the end of temperature rise and drops back to quasi-stable state after a period of time. This oscillation curve reflects that fermentation bacteria are very sensitive to temperature, and it takes a period of adaptation time to reach a stable hydrogen production rate at a certain temperature, which is expressed as hysteresis on the curve (Li et al., 2007).

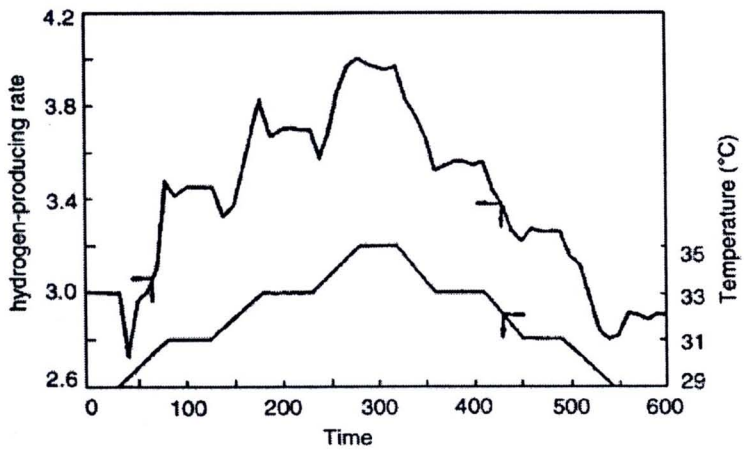


Figure 2.2 Hydrogen-producing rate response curve vs temperature rise and temperature drop (sugar as substrate). Rotating speed: 220 r/min; organic volume load: 41.2 kgCOD/m^3-d (Li et al., 2007).

Effect of temperature on hydrogen production rate is shown in Figure 2.3. There is a great difference between hydrogen production rates to reach quasi-stable state during temperature rise and temperature drop, which do not follow a route. This shows that temperature rise and temperature drop stimulation have different effects on physiological physiochemistry of microorganism. This is easy to explain according to Theory of Dissipative Structure. An organism cell is considered as a microsystem, heat feeding from outside the system (temperature rise) and releasing out of the system (temperature drop) will keep the system at different states. Within temperature limits, heat feeding from outside the system can keep the interior of cell system more orderly. Even within the same temperature limits, heat releasing out of the system will keep the interior of cell system more disorderly to cause the drop of organism metabolism rate in the system (Li et al., 2007).

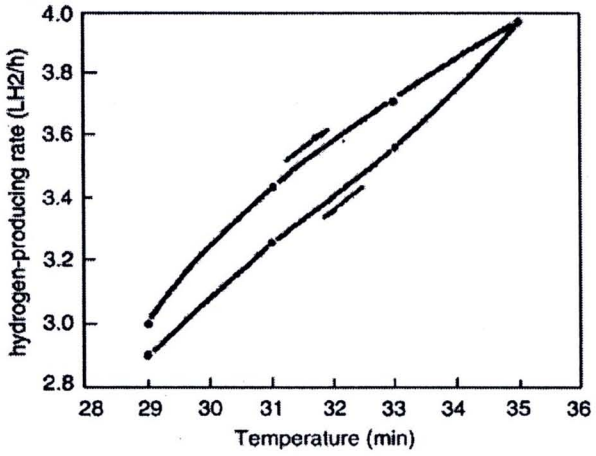


Figure 2.3 Effect of temperature on hydrogen-producing rate (Li et al., 2007).

2.2.3.2 pH

pH of the environment has great effects on vital activities of microorganism. One is that it leads to change of cell membrane charge to further influence nutritious substance ingestion of microorganism, another one is that it influences enzyme activity during metabolic process, and the other is that it changes nutritious substance supply and toxicity of harmful substances in habitat. A research shows that pH has great effects on fermentation product ingredient and is an important ecological factor to influence bacteria hydrogen producing fermentation (Table 2.3). Medium pH affects hydrogen production yield, biogas content, type of

the organic acids produced and the specific hydrogen production rate via fermentative production. The reported pH range for the maximum hydrogen yield or specific hydrogen production rate is between pH 5.0 and 6.0 (Fang and Liu, 2002; Khanal et al., 2004). However, some investigators report the optimum pH range between 6.8 and 8.0 (Collet et al., 2004; Liu and Shen, 2004) and around pH 4.5 for the thermophilic culture (Shin et al., 2004). Most of the studies indicated that final pH in anaerobic hydrogen production is around 4.0–4.8 regardless of initial pH (Liu and Shen, 2004; Morimoto et al., 2004). The decrease in pH is due to production of organic acids which depletes the buffering capacity of the medium resulting in low final pH (Khanal et al., 2004). Gradual decreases in pH inhibit hydrogen production since pH affects the activity of iron containing hydrogenase enzyme (Dabrock et al., 1992). Initial pH of 4.0–4.5 causes longer lag periods such as 20 hr (Liu and Shen, 2004; Khanal et al., 2004). High initial pH levels such as 9.0 decrease lag time; however, lower the yield of hydrogen production (Zhang et al., 2003).

Acidogens of genus Clostridia have been studied for their ability to form spores and for their potential to generate hydrogen gas (Brosseau and Zajic, 1982; Lay et al., 1999; Ueno et al., 1995). The Clostridia genus is an obligate anaerobic heterotroph that does not contain a cytochrome system (Nandi and Sengupta, 1998). This genus produces hydrogen using the activities of pyruvate-ferredoxin-oxidoreductase and hydrogenase enzymes. The activity of hydrogenase, an iron-containing enzyme is inhibited by low pH (Afschar et al., 1986; Dabrock et al., 1992; Holt et al., 1988; Ueno et al., 1996). Acid accumulation causes a sharp drop of culture pH and subsequent inhibition of bacterial hydrogen production (Oh et al. 2002, 2003; Fabiano and Perego 2002). Bacteria cannot sustain at pH values less than 5.0 thereby necessitating a way to reduce acid production or to carry out certain biochemical reactions that reduce the proton concentration on the outside of the cell proportional to the culture pH (Nath and Das, 2004). It has been reported that under the low pH ($\leq$ 4.0), the protonation of undissociate weak acid in medium which can pass through the cell membrane into cytoplasms which could inhibit the growth of microorganisms as well as their abilities to produce hydrogen (Pessoa et al., 1996). Moreover, the low or high initial pH may affect the hydrogenase activity as well as the metabolism pathway of the microorganisms (Wang and Wan, 2009). In certain

cases, the hydrogen evolution activity of hydrogenase may be suppressed in the presence of high levels of hydrogen (Kondratieva, 1983). A decrease in hydrogen concentration will favor hydrogen formation and permit bacteria to metabolize acetyl-CoA through the energy-efficient path leading to acetate and ATP production.

Table 2.3 The effect of initial pH on batch fermentative hydrogen production

Type of inoculum	Substrate	Initial pH		Optimal production value	References
		Range studied	Optimal		
C. butyricum CGS5	Sucrose	5.0-6.5	5.5	2.78 molH_2/mol sucrose	Chen et al., (2005)
Thermoanaerobacterium thermosaccharolyticum PSU-2I	Sucrose	4.0-8.5	6.2	2.53 mol H_2/mol hexose	O-thong et al., (2008b)
Municipal sewage sludge	Sucrose	5.5-8.5	7.5	2.46 mol H_2/mol sucrose	Wang et al., (2006)
Anaerobic digester sludge	Sucrose	3.0-12.0	9.0	126.9 mL H_2/g sucrose	Lee et al., (2002)
Compost	Sucrose	4.5-6.5	4.5	214 mL H_2/g COD	Khanal et al., (2004)
Municipal sewage sludge	Xylose	5.0-8.0	6.5	1.3 mol H_2/mol xylose	Lin et al., (2006)

In a typical anaerobic process, hydrogen is produced during the exponential growth phase of Clostridia (Minton and Clarke, 1989). When the population reaches the stationary growth phase, the reactions shift from a hydrogen/acid production phase to a solvent production phase. This shift occurs when the pH drops to 4.5 or below (Byung and Zeikus 1985). Apparently the build up of volatile fatty acids (VFAs) and hydrogen during the exponential growth phase induces this shift. While other researchers found that the shift occurred at pH levels above 5.7, due to enzyme synthesis or enzyme activation, which is necessary for solvent production (Gottwald and Gottschalk, 1985).

2.2.3.3 Substrate

Various substrates mainly carbohydrate and VFAs can be used for hydrogen production by fermentative bacteria. Sugars such as glucose (Yokoi et al., 1997), sucrose (Chen et al., 2005) and xylose (Li and Fang, 2007), waste water such as food wastewater (Chen et al., 2006), rice winery wastewater (Yu et al., 2002) and solids waste such as food waste (Shin et al., 2004) and cornstalk wastes (Zhang et al., 2007) were used for hydrogen production. The use of different substrate for hydrogen production by fermentative bacteria reported was summarized in Table 2.4.

Table 2.4 Hydrogen production by hydrogen-producing bacteria with different substrate.

Inocula	**Substrate**	**Mode of operation**	**Substrate (g COD/L)**		**Optimal production parameter**	**Reference**
			Range studied	Optimal		
C. 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	2.25mol/mol xylose	Lin et al., (2006)
Anaerobic sludge	Glucose	Batch	0.27-4.3	1.1	0.13mLH_2/hr	Zheng and Zeng (2008)
Digested sludge	Glucose	Batch	1.1-320	2.1	1270 mLH_2/L.g-glucose	Zhang et al., (2006)
Mixed cultures	Sucrose	Batch	1.5-44.8	7.5	38.9 mLH_2/L.g-COD	Van Ginkel et al., (2001)
C. butyricum CGS5	Sucrose	Batch	5-30	20	2.78 mol/mol sucrose	Chen et al., (2005)
C. pasteurianum CH4	Sucrose	Batch	5-40	40	2.07 mol/mol sucrose	Lo et al., (2008)
Anaerobic sludge	Starch	Batch	9.8-39.0	9.8	67 mLH_2/g starch	Zhang et al., (2003)
Anaerobic sludge	Starch	Batch	5-60	20	2.2 mol/mol hexose	Lin et al., (2008)
Anaerobic digester sludge	Food waste	Batch	0-32.3	4.6	101 mLH_2/g COD	Chen et al., (2006)
Anaerobic sludge	Food waste	Batch	3.2-10.7	6.4	1.8 mol/mol hexose	Shin et al., (2004)

Carbohydrates, mainly glucose, are the preferred carbon sources for fermentation processes, which predominantly give rise to VFAs together with hydrogen gas (Nath and Das, 2005), as follows:

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (13)$$

(glucose) (acetic acid)

$$C_6H_{12}O_6 + 2H_2O \rightarrow CH_3CH_2COOH + 2CO_2 + 2H_2 \quad (14)$$

(glucose) (butyric acid)

The end-products of glucose fermentation by anaerobic and facultative anaerobic chemoheterotrophs, e.g., Clostridia and Enteric bacteria, are produced through pyruvate. Facultative anaerobic bacteria give two mol of hydrogen per mol of glucose, whereas strictly anaerobic bacteria give four. Facultative anaerobes are less sensitive to oxygen, and are sometimes able to recover hydrogen production activity after accidental oxygen damage to them by rapidly depleting oxygen present in the broth. As a consequence, a facultative anaerobe is considered a better microorganism than a strict anaerobe to carry out fermentative hydrogen production process (Oh et al., 2002). One of the main constraints of fermentative bio-hydrogenation process is the lower yield of hydrogen, maximally 4 mol/mol glucose, compared with other processes. A yield of 2 mol H_2/mol glucose was reported for butyrate fermentation (Hewkes et al., 2002).

Clostridia are classifed as proteolytic or saccharolytic depending of types of organics they ferment. Proteolytic acidogens degrade proteins or amino acids. Saccharolytic acidogens ferment carbohydrate and are widely studied because of their ability to produce higher level of hydrogen. One of the widely studied saccharolytic Clostridia is *Clostridium butyricum* which produces butyric acid as the major fermentation product together with CO_2, acetate and H_2 (Minton and Clarke, 1989). The hydrogen fermentation reactions for sucrose as organic substrate are shown by Equations (15) and (16):

$$C_{12}H_{22}O_{11} + 5H_2O \rightarrow 4CH_3COOH + 4CO_2 + 8H_2 \uparrow, \quad (15)$$

(sucrose) (acetic acid)

$$C_{12}H_{22}O_{11} + H_2O \rightarrow 2CH_3CH_2CH_2COOH + 4CO_2 + 4H_2 \uparrow \quad (16)$$

(sucrose) (butyric acid)

This pathway is found in approximately 50% of all Clostridia that have been isolated to date. Other fermentation pathways found in sacchrolytic Clostridia are those leading to the production of propionate by *Clostridium arcticum* (Jones and Woods, 1989), succinate by *Clostridium coccoides* (Kaneuchi et al., 1976), and lactate by *Clostridium barkeri.*

Lactate and propionate can be produced either by clostridia themselves or by other bacteria competing in the mixed microflora, lowering the hydrogen yield. Clostridia such as *C. aceticum* can lower the hydrogen yield by converting H_2 and CO_2 to acetate or can convert hexose directly to acetate alone by the process of homoacetogenesis (Equations (17) and (18)) (Drake and Kusel, 2005).

$$2CO_2 + 4H_2 \rightarrow CH_3COOH + 2H_2O, \quad (17)$$

(acetic acid)

$$C_6H_{12}O_6 \rightarrow 3CH_3COOH. \quad (18)$$

(glucose) (acetic acid)

Although sugar mainly glucose and sucrose was widely used to produce hydrogen through fermentative process, hydrolysate of lignocellulosic materials were also used as substrate for producing hydrogen by Clostridia under thermophilic condition (Fangkum and Reungsang, 2010; Fangkum and Reungsang, 2011). Biological hydrogen production from lignocellulosic materials is receiving the attention due to its abundant and low/no cost. Pretreatment of lignocellulosic materials by chemical-physical method such as wet oxidation results in two fractions i.e., a hydrolysate mainly containing pentose (xylose, arabinose) and a remaining fiber presumably containing cellulose (Thomsen et al., 2006). Theoretically, when acetate is produced as a by-product, xylose and arabinose can be converted to hydrogen with a maximum yield of 3.33 mol-H_2/mol xylose and 3.33 mol-H_2/mol arabinose, respectively. If butyrate is the by-product, a lower yield of 1.67 mol-H_2/mol xylose and 1.67 mol-H_2/mol arabinose were obtained as follows:

$$C_5H_{10}O_5 + 1.67H_2O \rightarrow 1.67C_2H_3O_2^- + 1.67CO_2 + 1.67H^+ + 3.33H_2 \quad (19)$$

(pentose) (acetic acid)

$$C_5H_{10}O_5 \rightarrow 0.83C_4H_7O_2^- + 0.83H^+ + 1.67CO_2 + 1.67H_2 \quad (20)$$

(pentose) (butyric acid)

The concentrations of substrate have been considered as the important factor for hydrogen production. It has been reported that the influent organic concentration influences the distribution of metabolic products during fermentation (Yu and Fang, 2001). Therefore, previous studies have been investigated the optimum level of different substrate concentrations for hydrogen production. For example, in batch systems reported that an initial substrate concentration of 20 g COD/L for xylose (Lin and Cheng, 2006), 10.7 g COD/L for glucose (Xing et al., 2008), 9.8 g COD/L for starch (Zhang et al., 2003), 6.4 g COD/L for food waste (Shin et al., 2004) and 5.9 g COD/L for rice slurry (Fang et al., 2006) was optimum for hydrogen production. However, the previous researches demonstrated that increasing substrate concentration could increase the ability of hydrogen-producing bacteria to produce hydrogen, but substrate concentration at much higher levels could result in the hydrogen production efficiency through substrate inhibition effect (Lo et al., 2008; Van Ginkel et al., 2001). The high concentration of substrate mainly glucose was indicated the shift of metabolic pathway from acidogenic to solventogenic pathways where hydrogen was consumed to reduce the acids to alcohols (Van Ginkel et al., 2001). In the hydrogen fermentation, the glucose was simultaneously fermented by ethanol and butyrate–acetate pathway. As indicated in Equations (21) and (22), 1 mol of glucose can produce 1 mol of ethanol, and 4 mol of glucose can be converted to 3 mol of butyrate (Hwang et al., 2004).

$$C_6H_{12}O_6 + H_2O \rightarrow C_2H_5OH + CH_3COOH + 2H_2 + 2CO_2 \quad (21)$$

(glucose) (ethanol) (acetic acid)

$$4C_6H_{12}O_6 \rightarrow 2CH_3COOH + 3CH_3(CH_2)_2COOH + 8H_2 + 8CO_2 \quad (22)$$

(glucose) (acetic acid) (butyric acid)

The solventogenic pathway was reduced end-products i.e. ethanol, butanol and lactate, contain additional H atoms that are not liberated as gas (Levin et al. 2004). Therefore, alcohol production results in a correspondingly lower hydrogen yield.

2.2.3.4 Nitrogen and phasphorus

Nitrogen is very important factor for proteins, nucleic acids and enzymes which are significant as the most essential nutrient for hydrogen-producing

bacteria (Wang, Wan, 2009). An appropriate level of nitrogen is beneficial to growth of hydrogen-producing bacteria. Table 2.5 showed several reports on investigating the effect of nitrogen concentration on hydrogen fermentation. It could be seen that ammonia nitrogen was the most widely investigated nitrogen source for hydrogen production. The optimal ammonia nitrogen concentration for fermentative hydrogen production reported by Bisailon et al., (2006) was 0.01 g N/L and the reported by Salerno et al., (2006) was 7.0 g N/L. However, it has been reported that hydrogen production using organic nitrogen as the nitrogen source was higher than inorganic nitrogen sources (Ferchichi et al., 2005; Kamalaskar et al., 2010). Although a numerous researches have used inorganic nitrogen sources such as ammonium hydrogen carbonate (Logan et al., 2002; Chen et al., 2002), and ammonium chloride (Oh et al., 2003; Zhang et al., 2003) in hydrogen production media, others have shown that when ammonium chloride replaced peptone as a nitrogen source, hydrogen yield are decreased (Ueno et al., 2001). In addition, the using of organic nitrogen sources i.e. yeast extract, corn steep liquor, casamino acids, tryptone and peptone in hydrogen production could improve hydrogen yield as well as substrate consumption (Ferchichi et al., 2005). It was due to the composition of the nitrogen source in which organic nitrogen is a complex nitrogen source composed of a spectrum of peptides and free amino acids (Ferchichi et al., 2005). During the fermentation peptides and free amino acids are taken up from the medium by the cell and directly incorporated into proteins or transformed into other cellular nitrogenous constituents (Large, 1986). On the other hand, the cell spends more energy and time in amino acids and protein synthesis when inorganic nitrogen sources were used (Da Cruz et al., 2002). Among organic nitrogen sources, differences in protein and amino acid composition could have accounted for the differences in the production rates and yields (Ferchichi et al., 2005).

The nutrition values as well as buffering capacity of phosphate are important for hydrogen production. In an appropriate range, the increase phosphate concentration resulted in an increase in the capability of hydrogen-producer to produce hydrogen. However, much higher levels of phosphate concentration could decrease the hydrogen production efficiency (Bisailon et al., 2006; Lay et al., 2005).

Table 2.5 The effect of nitrogen concentration on batch fermentative hydrogen production.

Type of inoculum	Substrate	Nitrogen source	Nitrogen concentration		Optimal production value	References
			Range studied	Optimal		
Escherichai coli	Glucose	NH_4Cl	0-0.02 g N/L	0.01 g N/L	1.7 mol/mol glucose	Bisaillon et al., (2006)
Dewater and thickened sludge	Glucose	NH_4Cl	0.5-10 g N/L	7 g N/L	150 mL H_2	Salerno et al., (2006)
Grass compost	Food wastes	NH_4HCO_3	0-0.6 g N/L	0.4 g N/L	77 mL H_2/g TVS	Lay et al., (2005)
Cracked cereals	Starch	NH_4HCO_3	0.1-2 g N/L	1 g N/L	146 mL H_2/g starch	Liu and Shen (2004)
Compost	Glucose	Yeast extract	2-8% yeast extract	4% yeast extract	70 mmol	Morimoto et al., (2004)
Enterobacter aerogenes HO-39	Glucose	Polypeptone	0-5% polypepton	2% polypeptone	58 mLH_2	Yokoi et al., (1995)

The effects of C/N and C/P on fermentative hydrogen production were presented in Table 2.6. 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, respectively. It has been reported that, the concentration of phosphate at the optimum value could improve the hydrogen production because the buffering capacity can reduce the pH fluctuation caused by VFAs accumulated in the fermentation broth thus enhancing hydrogen generation and acidogenesis in the first stage of an acid-gas digestion system (Das and Veziroglu, 2001). In addition, phosphorus provided by Na_2HPO_4 is essential for the synthesis of many molecular stances such as DNA, RNA and ATP (Pan et al., 2008). However, the increasing in Na_2HPO_4 concentration at the high concentration (greater than 6.0 g/L) resulted in a decrease in hydrogen yield in which due to the negative effect of increased cytoplasmic osmotic pressure that occurs at high Na_2HPO_4 concentration (Sreela-or et al., 2011).

2.2.3.5 Microorganisms

A lot of pure cultures of bacteria have been used to produce hydrogen from various substrates. Table 2.7 summarizes a lot of studies using pure cultures for fermentative hydrogen production. As is shown in Table 2.8, *Clostridium* and *Enterobacter* were most widely used as inoculum for fermentative hydrogen production. Species of genus Clostridia are gram positive, rod shaped, strict anaerobes and endospore formers, whereas *Enterobacter* are gram negative, rod shaped, and facultative anaerobes (Li and Fang, 2007).

Furthermore, the bacteria capable of producing hydrogen widely exist in natural environments such as soil, wastewater sludge, compost and so on (Wang and Wan, 2008; Cheong and Hansen, 2006; Hu and Chen, 2007; Zhu and Beland, 2006). 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, 2007). Fermentative hydrogen 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, 2007). 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, 2008).

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, 2008). Table 2.8

summarizes several studies comparing various pretreatment methods for enriching hydrogen-producing bacteria from mixed cultures.

Table 2.6 The effect of C/N and C/P on batch fermentative hydrogen production

Type of inoculum	Substrate	C/N		C/P		Optimal production value	References
		Range studied	Optimal	Range studied	Optimal		
Wastewater activated sludge	Sucrose	40-130	47	-	-	4.8 molH_2/mol sucrose	Lin and Lay (2004)
Anaerobic sludge	Wheat powder	20-200	200	50-1000 450-650	1000	281mL H_2/g starch	Argun et al., (2008)
Anaerobic sludge	Palm oil mill effluent	45-95	74		559	6.33 L/L substrate	O-Thong et al., (2008a)

Table 2.7 The pure bacterial cultures for fermentative hydrogen production.

Organism	Substrate	Process	Max.H_2 yield	Reference
Clostridium butyricum	SCB	Batch	1.73 mol/mol hexose	Pattra et al., 2008
Clostridium butyricum	Sugarcane juice	Continuous	1.0 mol/mol hexose	Pattra et al., 2010
Clostridium acetobutylicum	Glucose	Batch	2.0 mol/mol glucose	Chin et al., 2003
Clostridium acetobutylicum	Glucose	Continuous	1.08 mol/mol glucose	Zhang et al., 2006
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 pasteurianum CH_4	Sucrose	Batch	2.07 mol/mol hexose	Lo et al., 2008
Clostridium themocellum 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. Fanp2	Glucose	Batch	0.2 mol/L medium	Pan et al., 2008
Enterobacter aerogenase HO-39	Glucose	Batch	1.0 mol/mol glucose	Yokoi et al., 1995
Enterobacter aerogenase	Starch	Batch	1.09 mol/mol starch	Fabiano and Perego, 2002
Enterobacter aerogenase E 82005	Molasses	Continuous	3.5 mol/mol sugar	Tanisho and Ishiwata, 1995
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
Escherchia coli	Glucose	Continuous	2.0 mol/mol glucose	Bisaillon et al., 2006
Thermoanaerobacterum thermosaccharolyticum KU001	Glucose	Batch	2.4 mol/mol glucose	Ueno et al., 2001
Ruminococcus albus	Glucose	Batch	2.52 mol/mol glucose	Ntaikou et al., 2008
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., 2008

As is shown in Table 2.8, there exists certain disagreement on the optimal pretreatment method for enriching hydrogen producing bacteria from mixed cultures. The possible reason for this disagreement was the difference among these studies in terms of inoculum, pretreatment method studied, specific condition of each pretreatment method and the kind of substrates. It has been reported that heat-shock was the most widely used pretreatment method for enriching hydrogen-producing bacteria from inoculum (Li and Fang, 2007). 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, 2008). Moreover, some microbial analysis methods such as PCR-DGGE have been used to determine the community structure of mixed cultures during fermentative hydrogen production (Kim and Shin, 2008; Kim et al., 2006; Shin et al., 2004). 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 (2008) reported that base pretreatment of mixed cultures would prevent the microbial population shift to non hydrogen producing acidogens, thus was beneficial for fermentative hydrogen production.

Table 2.8 The comparison of various pretreatment methods for enriching hydrogen-producing bacteria from mixed culture inoculum.

Organism	Pretreatment method	Substrate	Process	Max. H_2 yield	Reference
Digested sludge	Heat-shock	Glucose	Batch	1.8 mol/mol glucose	Wang and Wan, 2008
Cattle manure sludge	Acid	Glucose	Batch	1.0 mol/mol glucose	Cheong and Hansen, 2006
Methanogenic granules	Chloroform	Glucose	Batch	1.2 mol/mol glucose	Hu and Chen, 2007
Digested wastewater sludge	Base	Sucrose	Batch	6.12 mol/mol sucrose	Zhu and Beland, 2006
Anaerobic sludge	Sodium 2-BESA	Dairy wastewater	Batch	0.0317 mmol/g COD	Venkata Mohan et al., 2008
Activated sludge	Heat-shock	Soluble condensed fermented molasses	Continuous	2.1 mol/mol hexose	Lay et al., 2010

2.2.3.6 Reactor type

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. It has been reported that the continuous stirred tank reactor (CSTR) was widely used for continuous fermentative hydrogen production (Zhang et al., 2007; Arooj et al., 2008; Zhang et al., 2008). However, the washout of biomass may occur at shorter hydraulic retention time (HRT). 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, 2007). Biomass immobilization can be achieved through forming granules, biofilm, or gel-entrapped bioparticles (Li and Fang, 2007). For example, Zhang et al. (2007) found that the formation of granular sludge facilitated biomass concentration up to 32.2 g VSS/L and enhanced hydrogen production. 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 (Chen et al., 2008). 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. (2007) was 0.5 hr, while the optimal HRT for a CSTR using reported by Arooj et al. (2008) was 12 hr. The possible reason for this disagreement was the difference among these studies in the terms of inoculum, substrate and HRT range studied. For example, Zhang et al. (2008) 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.

2.2.3.7 Iron

Iron is an important nutrient element to form hydrogenase or other enzymes which almost all biohydrogen production needs fundamentally (Junelles et al., 1988). Furthermore, iron for bacterial metabolism is a very important trace element in mediating between hydrogenase and nicotinamide adenine

dinucleotide (NADH)-ferredoxin reductase (Zhang et al., 2005; Yang and Shen, 2006; Lee et al., 2002). A limited iron concentration in mixed cultures for producing hydrogen in continuously mode might be insufficient to maintain the microbial activity (Lee et al., 2009).

At the cellular level, some iron compounds have certain effects on the activity and number of hydrogen-producing bacteria. For example, iron shortage could influence the growth, metabolism, and hydrogen-producing ability. This suggests that adding iron may increase the specific activities of hydrogen enzyme and NADH-Fd reductase of hydrogen producing fermentation bacteria, and consequently enhance its hydrogen producing ability (Ren, 1994). Wang et al., 2009 found that the bacterium fermentation type could turn into ethanol-type fermentation from butyric acid-type fermentation by adding iron. In bacterial metabolism process, pure iron could increase the abilities of fermentation and hydrogen production of bacteria. Cui et al., (2009) and Lee et al. (2009) reported that iron also affects the fermentation pathways. Butyrate formation increased with the increasing of iron concentration while ethanol formation was favored at low iron concentration (Lee et al., 2001; Zhang et al., 2005). Reported by Lee et al. (2009) investigated the effect of iron on continuous hydrogen production. They indicated that iron sulphate concentration up to 10.9 mg/L increased hydrogenase activity and hydrogen production in a membrane bioreactor. Furthermore, the optimal iron 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. However, a higher concentration, iron may inhibit the activity hydrogen producing bacteria (Li and Fang, 2007).

2.3 Application of molecular techniques on hydrogen production research

The application of molecular techniques in hydrogen production studies were reviewed including introduction of commonly used molecular techniques, cloning-sequencing after polymerase chain reaction (PCR), denaturing gradient gel electrophoresis (DGGE), terminal-restriction fragment length polymorphism (T-RFLP), fluorescence in situ hybridization (FISH) and quantitative real-time PCR. Application of the molecular techniques in heterotrophic hydrogen production studies

are discussed in details, focusing on identification of new isolates for hydrogen production, characterization of microbial compositions in bioreactors, monitoring microbial diversity variation, visualization of microbial distribution in hydrogen-producing granular sludge, and quantification of various microbial populations. Some significant findings in recent hydrogen production studies with the application of molecular techniques are discussed, followed by a research outlook of the biohydrogen field (Li et al., 2011).

2.3.1 Molecular techniques

The starting point for the molecular methods and related procedures is the extraction of nucleic acids. The reliability of the molecular techniques depends on quality and representativeness of RNA/DNA extracted from sludge samples in the reactors. The DNA extraction process is composed of cell lysis, contamination removal, solvent extraction, precipitation and purification (Miller et al., 1999). The amount of nucleic acid (as expressed by A_{260}) and purity (as expressed by the ratios of A_{260}/A_{280} for protein contamination and A_{260}/A_{230} for salt contamination) are measured spectrophotometrically using the conventional UV–visible spectrometer or the more advanced spectrometers, such as Nanodrop. The integrity of rRNA (containing 5S, 16S and 23S) and genomic DNA could be visually checked by conducting electrophoresis using agarose gel. The most commonly used phylogenetic biomarker is 16S rRNA gene (16S rDNA) which has a huge database available (over 338, 193 of 16S rDNA sequences in GenBank as of Nov. 27, 2010).

The extracted DNA is subjected to PCR amplification using ''universal'' primers or primers designed to amplify rRNA genes from particular group of organisms. The PCR cycle takes place in three steps: denaturing, annealing and extension. The crucial part of a PCR is the selected primer set targeting genes of interest which cover special taxonomic or functional groups. These primers may be individually designed based on the alignments of relevant DNA sequences, or simply adapted from the literature. The bacteria-specific primers, such as the sets of EUB8F and UNIV1492R (Zhu et al., 2008), EUB968F and UNIV1392R (Hung et al., 2008), EUB341F and UNIV518R (Akutsu et al., 2008), and EUB357F and UNIV518R (Shin et al., 2004), were commonly used in the microbial analysis in hydrogen production studies.

The selected primer sets may target the sequences at various taxonomic levels, from domain to division, subdivision, class, family, genus, species, and even strain. The PCR products contain a mixture of multiple copies of the same segment amplified at the selected taxonomic level. The PCR products can be cloned and then sequenced to identify species. They can also be analyzed by various techniques, such as DGGE or T-RFLP, which may separate PCR products originating from different DNA sequences of various species in the sample.

2.3.1.1 Cloning and sequencing

Cloning of PCR products is to separate the PCR fragments of the same length but of different sequences. Cloning is composed of three steps: ligation, transformation and host cell reproduction. Several commercial kits are available for DNA cloning, such as the pGEM-T cloning kit and the TA cloning kit. Taking the TA cloning kit as an example, PCR products are firstly inserted into the plasmids under the action of the ligase. After ligation, the plasmids with PCR product inserts are transformed into *Escherichia coli* competent cells. Each cell carrying the plasmid with the PCR product insert forms a single white colony (called the clone) on the solid discriminative medium. Whereas, each cell carrying the plasmid without the PCR product insert forms a blue one, and each cell carrying no plasmid does not form any colony. A DNA library consists of the selected white colonies. The insert in plasmids of the white colonies may be recovered using PCR with a primer set targeting the sequence on the plasmids which locate at the two sides of the insert or the original primer set which is used to generate PCR products initially. The whole plasmid could be extracted after further cultivation of the colonies in liquid medium. The PCR product obtained using the first method, or the plasmids obtained using the second methods, will be sequenced for further identification of the sequences.

Sequencing of the full 16S rDNA (about 1540 bp) is preferred for microbial identification, especially for oligonucleotide probe design and classification of the pure culture. Once a sequence database of a clone library is established, the microbial diversity can be determined with reference to the published sequences of the pure cultures and environmental samples. The similarity analysis of DNA sequences is greatly facilitated by a number of rDNA sequence databases, such as GenBank (http://www.ncbi.nlm.nih.-gov/blast/) and the ribosomal database project

(RDP, http://rdp.cme.msu.edu/), and powerful software packages, including MEGA 2.1 (Kumar et al., 1993) and ARB (Strunk and Ludwig, 1997). By using the Blast program in GenBank, species closely related to the obtained sequences are listed in the similarity order (Altschul et al., 1990). A primary taxonomy position of the species represented by obtained sequences may also be given. More accurate taxonomy analysis of DNA sequences could be conducted by construction of phylogenetic trees.

The obtained sequence information in a clone library can be used to evaluate species diversity or richness of microbial communities in bioreactors, and to design specific probes/primers for characterization/quantification of a certain microorganism. But the cloning method is time-demanding and less applicable for analysis of a larger set of samples, such as monitoring the changes of a microbial community over time (Sanz and Köchling, 2007).

2.3.1.2 Denaturing gradient gel electrophoresis (DGGE)

PCR amplification produces DNA segments with the same size but different sequences. These segments may be separated in an acrylamide gel, but not in an agarose gel, having a linear ascending gradient of denaturants, usually urea and formamide (Muyzer et al., 1993). This so-called DGGE method is based on the differences of the electrophoresis mobility of the partially denatured double-stranded DNA fragments in the polyacrylamide gel. The universal primer for Bacteria domain 341F-518R (Fang et al., 2006a) is usually used in hydrogen production studies. After staining, DNA fragments will appear as separated bands on the gel. Each DGGE band is derived from one specific species in the original samples. Thus the band number of a DGGE profile provides a quick estimate of species richness. The intensity of a band may be used as a rough indication of the relative abundance of a species.

The separated DNA fragments may be recovered from the DGGE bands in the gel and sequenced to identify the corresponding microbial species. Combined with sequencing and similarity-based phylogenetic analysis, DGGE can give an overview of the composition of a microbial community. However, it is not easy to slice the bands and purify the carried DNA fragments in some cases. For a complex community, to get DNA sequence of a cut band often demands another

cloning-sequencing step due to co-migration or poor band separation. The separation could be optimized by changing the electrophoresis conditions, such as the denaturing gradient range, running time, voltage, etc.

Comparing patterns across the gel, especially for those including a large number of bands, is a difficult job. Different combinations of samples should be carefully designed if numerous samples are being investigated and multiple gels are required. DNA fragments of different sequences may co-migrate, and minor populations in the samples may be overlooked due to the limited detection sensitivity (Vallaeys et al., 1997). Additionally, the short sequences of the excised bands (usually 200–400 bp) are less informative for sequencing and comparing with database, and probe and primer designs, and make the resulting phylogenetic analysis less reliable than those using the full 16S rDNA sequences from cloning, especially for those novel sequences having less than 85% similarity to known sequences (Hugenholtz et al., 1998). Thus, it might need combination of DGGE with another method such as 16S rDNA sequence analysis to identify the types of bacteria. Single-stranded DNA fragments generated in PCR may appear as bands and result in an overestimation of microbial diversity in a sample (Gilbride et al., 2006). Moreover, DGGE band intensity does not always quantitatively correlate with the abundance of a specific species, as DNA copy number in PCR product depends on both the abundance of species and the ease of amplification. It is also difficult to analyze the DGGE profile with too many bands, and only the predominant species could be obtained. Lastly, the reproducibility of DGGE is relative low, compared to other fingerprint methods, such as T-RFLP.

However, DGGE method has been extensively applied to monitoring microbial diversity, investigate the composition of a microbial community and estimate relative abundance of a species due to its following advantages: (1) rapid and simple monitoring of microbial communities variation based on band patterns, (2) quick overview of the predominant populations in a sample, and (3) cost effective for a large number of samples.

2.3.1.3 Terminal-restriction fragment length polymorphism (T-RFLP)

Terminal-restriction fragment length polymorphism (T-RFLP) analysis is a fingerprinting technique based on restriction digestion of PCR products

obtained using a fluorescently labeled primer. To apply this method, one primer fluorescently labeled at 50 end enables a single species to generate a specific fluorescent terminal-restriction fragments (T-RFs) of a given size after enzymatic restrictive digestion. The segments are then separated by nondenaturing polyacrylamide gel electrophoresis or capillary electrophoresis, and distinguished by laser-induced fluorescence detection. The detailed principles of T-RFLP have been extensively described by Liu et al. (1997).

With high reproducibility, T-RFLP could be applied to conduct both quantitative and qualitative analyses of a gene (such as 16S rDNA) in a microbial community. Its advantage is the ability to detect even rare population of a sample. In addition, phylogenetic information can be inferred from the T-RF sizes of the sequences of known bacteria in the databases, including TRFMA, T-Align, PAT, and TAP. More important, T-RFLP can be standardized and be used to compare results between different researchers.

However, compared to DGGE, T-RFLP is more expensive and time-consuming, as PCR fragments need to be purified prior to enzymatic restriction digestion. Another disadvantage of T-RFLP is that the experimental fragment size may not be exactly same as the theoretical length, a 1–4 bps difference is commonly observed. As a result, T-RFLP seems to be useful in characterizing microbial communities with low-to-intermediate diversity, but may not for samples with high diversity. Additionally, the T-RFLP reproducibility may be affected by incomplete restriction digestion. Formation of pseudo TRFs, was also reported (Egert and Friedrich, 2003), resulting in overestimation of biodiversity. More information about the limitation of T-RFLP could be found in the paper of Nocker et al., (2007).

2.3.1.4 Fluorescence in situ hybridization (FISH)

FISH is a visualization technique based on microscopic examination of a given species or groups of bacteria after staining cells using specific fluorogenic oligonucleotide probes which bind RNA molecules in the cells. As a method without DNA extraction and PCR, FISH is an excellent means to overcome problems associated with PCR-based molecular methods, such as DGGE, T-RFLP, cloning and sequencing.

FISH probes are short DNA sequences (about 20 nucleotides) labeled with one or two fluorescent dyes. The cells are typically examined by epifluorescence microscope or laser scanning microscopes after staining. The specificity of the probe enables detection/identification on any desired taxonomic level, from domain down to species, depending on the probe applied.

In addition to visualization, FISH can also be used for microbial quantification in the hydrogen production reactor, basing on either the fluorescence-emitting area (Fang et al., 2005) or the number of individual cells (Fang et al., 2006c). However, the cell count method is only applicable to evenly distributed and homogeneous microbial samples. Usually, the bacterial count in a microscopic view field is required to be in the range of 30–150. Cell counts have to be performed at more ten regions in order to have statistical significance.

Compared to PCR-based molecular techniques, FISH has the following advantages: (1) fast and simple to have results in a couple of hours, (2) direct observation of uncultured microbes, (3) semiquantitative, (4) preferential/differential examination of populations, (5) structural analysis of aggregates (flocs, granular sludge, biofilms) when combined with a confocal laser scanning microscopy, (6) easy for routine application, requiring only basic knowledge of microscope, and (7) possible to measure metabolic activity of cells by analyzing the intensity of fluorescence of positive cells. However, for the mixed culture in an anaerobic reactor, strong background autofluorescence often affects the interpretation of the FISH images, subject to individual judgment and experience about the samples; the problem may partially resolved by using a standardized and automatic procedure. Other disadvantages of FISH mainly include: (1) RNA gene sequence must be known for a target microorganism if the special probe has not yet been reported yet; (2) not always possible to design an unambiguously restrictive probe for a microbial group, especially for those groups classified based on metabolic criteria; (3) difficult to optimize hybridization conditions of a newly-designed probe; and (4) hard to quantify accurately due to subjective nature involved.

Overall, FISH is a useful method in combination with either fingerprint methods (DGGE or T-RFLP) or cloning-sequencing methods to quantify

and visualize the OTUs of interest. Details of the FISH technique have been reviewed by Wagner et al. (2003).

2.3.1.5 Quantitative real-time polymerase chain reaction (qRT-PCR)

Hybridization-based techniques such as FISH, and PCR-based techniques such as DGGE and cloning-sequencing, have long been used to quantify microorganisms. Hybridization methods, which have detection limits in the order of 105 DNA/RNA copies or greater, are in general less sensitive. They can thus only be used for environmental samples of relatively high microbial concentrations. PCR-based methods, on the other hand, are capable of detecting DNA/RNA at low concentrations. However, the precision of PCR-based methods may be compromised due to a number of factors, including reagent depletion, competition of amplicons with primers, and the loss of polymerase activity as the number of amplification cycle increases (Zhang and Fang, 2006). Due to the advanced development of fluorogenic chemistry, qRT-PCR has become an emerging technique for the detection and quantification of microorganisms in the environment. Compared to the conventional hybridization- and PCR-based techniques, qRT-PCR not only has better sensitivity and reproducibility, but it is also quicker to perform and has a minimum risk of amplicon carryover contamination. Details of qRT-PCR have been reviewed by Zhang and Fang (2006).

2.3.2 Applications of molecular techniques on heterotrophic hydrogen production research

Although hydrogen production has been studied for several decades, most of related studies were conducted using dark fermentation (Li and Fang, 2007). Studies of photo fermentation were conducted mostly for suspended pure cultures with a few exceptions for mixed cultures (Li and Fang, 2009). The application of nucleic acid based techniques has thus focused on dark fermentation, with limited reports related to photo fermentation,. Some significant findings in recent hydrogen production studies with the application of molecular techniques are discussed as follows.

2.3.2.1 Identification of new isolates

Anaerobic bacteria that produce hydrogen during dark fermentation are classified into either strict or facultative anaerobes. *Clostridium*,

Ethanoligenens and *Desulfovibrio* are representatives of strict anaerobes, whereas *Enterobacter*, *Citrobacter*, *Klebsiella*, *E. coli* and *Bacillus* are facultative anaerobes (Lee et al., 2010). Among the dark fermentative bacteria, *Clostridium* and *Enterobacter* are most widely studied (Li and Fang, 2007). Photo fermentation is generally carried out by purple non-sulfur bacteria, including the fresh water species *Rhodobacter sphaeroides*, *Rhodobacter capsulatus*, *Rhodopseudomonas palustris* and *Rhodospirillum rubrum*, and the marine species *Rhodovulum* sp., *Rhodovulum sulfidophilum*, and *Rhodobacter marinus*, with the fresh water species are most widely used (Li and Fang, 2009).

Isolation of new hydrogen-producing bacteria and investigation of their hydrogen production characteristics provided important microbial species with high capacity or unique properties in hydrogen production. Previous studies shows that 16S rRNA gene sequence analysis, i.e. sequencing of the full 16S rDNA followed by phylogenetic analysis, is preferred for identification of the new isolates. Some of the new isolates were first reported to have hydrogen production ability by fermentation of organic pollutants, such as *Megasphaera elsdenii* RG1 (Ohnishi et al., 2010) and *Rubrivivax gelatinosus* L31 (Li and Fang, 2008). Some isolates demonstrated high hydrogen-producing capacity. *Klebsiella pneumoniae* ECU-15, which was isolated from anaerobic sewage sludge, showed highest hydrogen production rate of 482 mL/L-hr compared with other reported *Klebsiella* species (Niu et al., 2010). Another isolate *Enterobacter* sp. CN1 was very efficient in using xylose to produce hydrogen with a yield (2.0 ± 0.05 mol H_2/mol xylose) higher than most of other species (Long et al., 2010). Also, some new isolated species displayed unique properties in hydrogen production, such as *Pantoea agglomerans* BH-18, which was a salt-tolerant anaerobe with a wide range of initial pH (pH 5–10) in hydrogen production (Zhu et al., 2008), and *Enterobacter* sp. CN1, by which hydrogen production from xylose was superior to glucose and sucrose (Long et al., 2010).

2.3.2.2 Characterization of microbial compositions

With the development of biohydrogen research, mixed cultures have been commonly used in dark fermentation, especially in recent studies, although photo fermentation is conducted mostly using pure cultures. Besides the hydrogen-producing bacteria (HPB) in a mixed culture system for dark fermentation, hydrogen

consumers and metabolic competitors were also identified (Guo et al., 2010). Hydrogen consumers mainly include sulfate-reducing bacteria, methane-producing bacteria, and homoacetogenic bacteria. Lactic acid bacteria were often found in dark fermentation system as a competitor (Guo et al., 2010).

In order to better understand the process, the microbial community compositions were investigated and linked to the hydrogen production performance. The most commonly used method for characterization of microbial compositions in dark fermentation reactors is 16S rRNA based DGGE combined with sequencing and similarity-based phylogenetic analysis. Cloning-sequencing and T-RFLP were also used in some reports. FISH was reported to be applied for detecting the presence of specific species, such as methanogens using Archaea probe (Castelló et al., 2009). In addition, most of the predominant HPB in dark fermentation system were *Clostridium* genus under mesophilic conditions and *Thermoanaerobacterium* genus under thermophilic conditions. The predominance of *Clostridium* species is likely due to the heat shock treatment on the inoculum (Guo et al., 2010). In the hydrogen production reactors without heat pre-treatment for seed sludge, quite different microbial compositions were reported. In a hydrogen fermentation system treating food waste by microflora from leaf-litter cattle-waste compost without heat pre-treatment, *Clostridium* was absent from this system. Instead, *M. elsdenii* was the dominant HPB (Ohnishi et al., 2010). Microbial analysis results in another study without pre-treatment for seed sludge, also showed the absence of *Clostridium*, whereas *Anaerotruncus*, *Megasphaera*, and *Pectinatus* were HPB in the system (Castelló et al., 2009).

The 16S rRNA gene has been widely used as a universal molecular biomarker to characterize microbial communities. However, it is difficult to reveal the practical phylogenetic diversity of the community only based on 16S rRNA gene sequences, such as the genus *Clostridium*, which is a large and phenotypically heterogeneous bacterial group and is yet not phylogenetically well defined (Quéméneur et al., 2010). Recently, functional genes, such as Fe-hydrogenase genes have been used as specific biomarker for characterization of *Clostridium* HPB in dark fermentation bioreactors using cloning-sequencing method (Fang et al., 2006b; Huang et al., 2010). In addition to *Clostridium*, other HPB such as

Ethanoligenens, *Megasphaera*, *Syntrophomonas* and *Syntrophobacter* species were also identified and characterized using Fe-hydrogenase gene based cloning-sequencing method (Xing et al., 2008). Characterization of microbial community compositions using molecular techniques, helped to explain hydrogen production performance. The low hydrogen yields were often related to the presence of non-hydrogen producers that consumed hydrogen or competed for the substrates, such as methanogens (Castelló et al., 2009), *Bacillus racemilacticus* (Kim et al., 2006b) and *Streptococcus bovis* (Kim et al., 2008), etc. Reports of molecular techniques used for microbial composition analysis in photo fermentation were very limited. Zhang et al. (2002) found that the predominant population was *R. capsulatus* in the mixed phototrophic sludge using cloning and sequencing methods.

2.3.2.3 Monitoring microbial diversity variation

The application for molecular techniques shows that DGGE and T-RFLP are effective methods and have been extensively applied to monitor microbial diversity in hydrogen production systems. Information on microbial diversity variation gives deep insight into the function of microorganisms in hydrogen fermentation process and is very helpful to optimize operational conditions (Li et al., 2011). Microbial diversity varied with many operational conditions, such as HRT, temperature and pH, etc. Different results were reported for various microbial communities. Zhang et al. (2006) found that shortening of HRT from 50 to 6 hr reduced the microbial diversity associated with an elimination of propionate production without affecting the existence of dominant species, and increase the hydrogen yield of the glucose-feeding bioreactor. In contrast, variation of HRT had little effect on the microbial diversity for the anaerobic sludge producing hydrogen from sucrose in an upflow reactor (Li et al., 2006) and an extreme thermophilic microflora producing hydrogen from glucose at 75 °C (Yokoyama et al., 2009). Generally, microbial diversity decreased with the increase of both sludge pre-treatment temperature (Baghchehsaraee et al., 2008) and operation temperature (Karadag and Puhakka, 2010), but increased with pH (Fang and Liu, 2002; Fang et al., 2006a).

Microbial diversity also varied with seed sludge (Akutsu et al., 2008), organic loading rates (Hafez et al., 2010) and sodium concentration (Kim et al.,

2009) etc. During the operation of hydrogen production system, the diversity may also change without any condition variation because the operation of reactor itself is a microbial cultivation and selection process. Li et al. (2008) reported that *Rhodobacter* sp. was the predominant species throughout but *R. gelatinosus* and *R. sphaeroides* vanished gradually in the mixed phototrophic sludge during the operation of continuous photo fermentation reactor.

2.3.2.4 Visualization of microbial distribution in hydrogen-producing granular sludge

FISH combined with fluorescence microscopy has been applied to investigate the spatial distribution of microorganisms in the hydrogen-producing granular sludge. Since most of the studies on heterotrophic hydrogen production were conducted by suspended cultures, reports on microbial analysis for granular sludge were still very limited. In a sucrose-feeding dark fermentation bioreactor, self-forming granular sludge was formed at HRT of 0.5 hr, and *Clostridium* sp. and *Streptococcus* sp. were predominant microorganisms. FISH images showed that *Clostridium* sp. formed net-like structures where *Streptococcus* sp. aggregated to form a solid granular core inside the net, making the net structure more rigid (Hung et al., 2011). Similar spatial distribution of microorganisms was also observed in the glucose-feeding bioreactor using the same seed sludge (Hung et al., 2007).

2.3.2.5 Quantification of various microbial populations

The application of molecular techniques on quantification of microbial populations indicated that cloning-sequencing, qRT-PCR, FISH, and DGGE can be applied for quantification of various microbial populations in hydrogen-producing sludge, among which cloning-sequencing has been most extensively used so far (Li et al., 2011). Based on cloning-sequencing method, relative abundance (%) of various microbial population were investigated and the bacteria species responsible for hydrogen production in the bioreactors could be estimated, such as *Thermoanaerobacterium thermosaccharolyticum* (over 80%) in a starch-feeding thermophilic bioreactor (Akutsu et al., 2008) and thermophilic reactors of Liu et al. (2003) and Zhang et al. (2003), *Clostridium* sp. (85%) in a sucrose-degrading granular sludge (Hung et al., 2010), and *R. capsulatus* (81%) in a photo fermentation bioreactor fed with acetate, butyrate and ethanol as mixed substrates

(Fang et al., 2004; Zhang et al., 2002). Cloning-sequencing analysis also revealed the relative abundance of various HPB in a mixed culture system. Chu et al. (2010) first reported that *Clostridium* sp. strain Z6 (72%) was dominant thermophilic hydrogen-producing bacteria from food waste, whereas *T.thermosaccharolyticum* only accounted for 12% in the mixed culture.

More recently, qRT-PCR has emerged in hydrogen production studies as an effective method for quantification of microorganisms. A new group of dark fermentative bacteria was monitored quantitatively by qRT-PCR using a designed TaqMan gene probe (Li et al., 2007). The abundance of this HPB in the biomass was found to increase from 0.02% to 72% in a batch reactor treating rice slurry waste at pH 4.5 over 130 hr operation. The corresponding abundances were 4.4% at pH 5.0 and 0.01–0.02% at pH 5.5–6.5 (Li et al., 2007). A set of primers specific for Fe-hydrogenase genes of *Clostridium* species was identified and used to monitor the change of HPB in the batch reactor treating rice slurry using qRTPCR. The quantitative analysis results showed the HPB had an average generation time of 4.2 hr (Fang et al., 2006b). In a continuous photo fermentation bioreactor using acetate and butyrate as substrates, three phototrophic HPB were identified in the seed sludge. The relative abundance of these bacteria were examined by cloning-sequencing method, with the results that R*hodobacter* sp., *R. gelatinosus* and *R. sphaeroides* accounted for 42.3%, 38.5% and 7.7% respectively. Based on qRT-PCR analysis, it was found that hydrogen production rate generally increased with the amount of *Rhodobacter* sp. in the reactor, but had no clear correlations with the other two hydrogen-producing bacteria. This finding helped to confirm that *Rhodobacter* sp. was the species most likely responsible for hydrogen production in the photo bioreactor (Li et al., 2008). In addition to cloning-sequencing and qRT-PCR, microbial quantification in the hydrogen production reactor could also been analyzed by DGGE band intensity (Fang et al., 2005), and FISH basing on either the fluorescence-emitting area (Fang et al., 2005; Zhang et al., 2002) or the number of individual cells (Chu et al., 2009; Fang et al., 2006c).

2.4 Sweet sorghum

Sweet sorghum [*Sorghum bicolor* (L.) Moench] (Figure 2.4) can be classified using the biological taxonomy as:

Family Poaceae (Grass)

Genus Sorghum

Species *Sorghum bicolor*

Subspecies *Sorghum bicolor* ssp. *Bicolor*

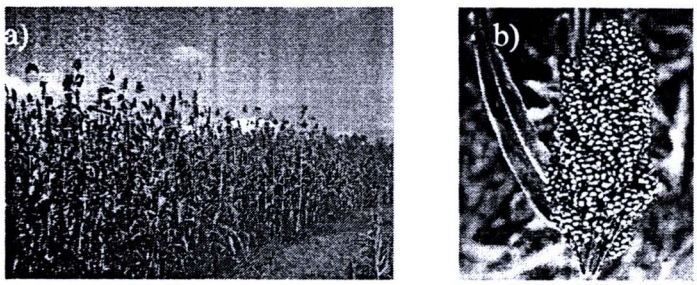


Figure 2.4 Sweet sorghum morphology (a) sweet sorghum stalk, (b) sweet sorghum seeds.

Sweet sorghum is the best multipurpose crop for simultaneous production of (i) grain from its earhead as food, (ii) sugary juice from its stalk for making syrup (Figure 2.5), jaggery or ethanol and (iii) bagasse and green foliage as an excellent fodder for animals, as biomass for gasification system, as organic fertilizer or for paper manufacturing.

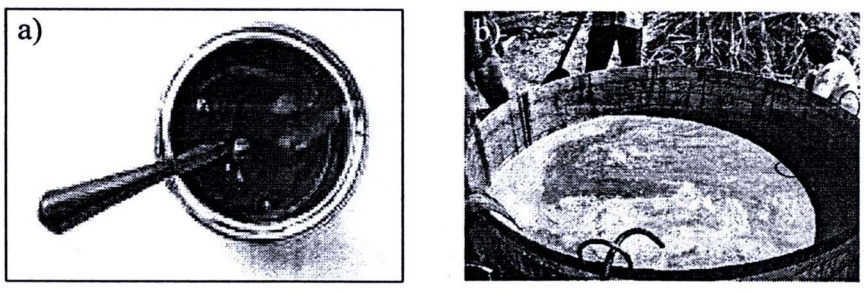


Figure 2.5 Sweet sorghum extract (a) sweet sorghum syrup, (b) sweet sorghum juice.

Moreover, sweet sorghum has a great tolerance to a wide range of climatic and soil conditions. It is a short duration crop of 110-130 days as compared to 12-18 months in sugarcane. In addition its water and fertilizer requirement is much less,

resulting in lower cost of cultivation than sugarcane. Sweet sorghum is a plant with C_4 photosynthetic pathway, so its photosynthetic rate and dry matter production in $g/m^2/day$ per unit of inputs are more than those of other sugar producing crops like sugarcane and sugar beet. These characteristics make sweet sorghum an ideal crop for syrup production. Sweet sorghum has less water and fertilizer requirements and hence lower cost of cultivation than sugarcane (Table 2.9).

Table 2.9 The comparison of sugarcane and sweet sorghum.

Crop	Sugarcane	Sweet sorghum
Duration (days)	Seasonal - 360 Pre-seasonal - 420 Adsali - 480	110-150
Fertilizer requirement N: P: K (Kg/ha)	Seasonal - 250:115:115 Preseasonal - 340:170:170 Adsali - 360:170:170	100:50:50
Amount of water required (mm)	Seasonal - 2000-2200 Preseasonal - 2500 Adsali - 3000-3500	450
Commercial cane sugar produced (T/ha-season)	9.4	2.4
Cost of cultivation of stalks (Rs./ha-season)	46,355	23,245

(Nimbkar et al., 2006)

Sweet sorghum can be an additional or an alternative raw material to sugarcane. In most situations, it will be a supplement rather than a substitute for sugarcane. Sweet sorghum extracts containing with soluble carbohydrate i.e. glucose and sucrose, insoluble carbohydrate i.e. cellulose and hemicelluloses. In general, biomass from energy crops, such as sweet sorghum, can be used as raw material for biohydrogen production. Sweet sorghum stalks mainly consist of sucrose that amounts up to 55% of dry matter and of glucose (3.2% of dry matter) which contained cellulose (12.4%) and hemicellulose (10.2%) (Antonopoulou et al., 2008). Sweet sorgum juice also

contained several nutrients in the sweet sorghum juice composition (Table 2.10). To date, ethanol and methane are among the best-known microbial products produced from sweet sorghum (Laopaiboon et al., 2009; Antonopoulou et al., 2008). Specifically, the energy yield from ethanol obtained from the above referenced studies ranged between 6500–8900 kJ/kg dry and 1400–2700 kJ/kg fresh sorghum biomass, respectively (assuming that the energy yield from ethanol is 26500 kJ/kg).

Table 2.10 Nutrient composition in sweet sorghum juice

Composition	Content (ppm)
Ammonium nitrogen	21.4
Ammonium nitrate	4.4
Phosphorus	20
Potassium	1790
Sodium	170
Sulfur	120
Calcium	166
Magnesium	194
Iron	2
Manganese	3
Copper	0.3
Zinc	1.4

(Laopaiboon et al., 2009)

2.5 Hydrogen production from sweet sorghum

Sweet sorghum biomass is rich in readily fermentable sugars and thus it can be considered as an excellent raw material for fermentative hydrogen production. Overall, out of many ''new crops'' that are currently investigated as potential raw materials for energy and industry, sweet sorghum seems to be the most promising one (Dalianis et al.,1996; Gosse, 1996).

Sweet sorghum juice is used for bioethanol production, and was recently tested for biogas and hydrogen production (Antonopoulou et al., 2008, Claassen et al., 2004). The feasibility of biohydrogen production from sweet sorghum has been established

(Antonopoulou et al., 2007; Ntaikou et al., 2008). These studies have demonstrated that sweet sorghum extract can be used for hydrogen and biogas production in a two-stage process where the hydrogenogenic effluent can be used for biogas production without any additional treatment. *Ruminococcus albus* is one of the outstanding candidates for hydrogen production (Ntaikou et al., 2008), and in chemostat cultures at 37 °C, significant amount of hydrogen has been generated from sorghum stalks, an aqueous extract of sorghum containing the free sugars, and the sorghum residues after the sugar extraction process. The hydrogen yield from the aqueous extract of sorghum was the same as that from glucose batch experiments, i.e. approximately 2.5 mol H_2/mol glucose. Sweet sorghum juice supported the growth and hydrogen production of the extreme thermophile *Caldicellulosiruptor saccharolyticus* for about 60 hr with an average production rate of 10 mmol H_2 /L-hr (0.24 mol H_2/L-d) during the first 16 hr and a maximal production rate of 21 mmol H_2 /L-hr (0.5 mol H_2/L-d) at 10 hr after the start of fermentation (Claassen et al., 2004).

2.6 Experimental design methods for biohydrogen production

2.6.1 One-factor-at-a-time design

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 substrates, nutrients, operational conditions and so on. Therefore 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 (Li and Fang, 2007).

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. For example, Kim et al. (2006) 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). 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 cannot 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 (Kim et al., 2006). 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 and temperature and dilution rate on hydrogen productivity using one-factor-at-a-time design, with around 30 runs of experiments being conducted.

2.6.2 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 (Kuehl, 2000). 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 (Montgomery, 2005). In this review, the models are expressed based on coded factor levels. Such models can be expressed based on actual factor levels when necessary.

2.6.2.1 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. For example, Chou et al. (2008) 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. Two second-order polynomial models were constructed to describe the effects of the two factors on hydrogen yield and specific hydrogen production rate. The optimal condition for hydrogen fermentation were pH of 6.0 and stirring speed of 120 rpm. 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. 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. 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 (Luftig and Jordan, 1998).

2.6.2.2 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 (Luftig and Jordan, 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 (Pan et al., 2008; Argrun et al., 2008; Karlsson et al., 2008; Zhao et al., 2008).

2.6.2.3 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). Taguchi design was used to produce hydrogen with the effects of 13 nutrient concentrations by Lin and Lay (2005). 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).

2.6.2.4 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, has been extensively used to screen important factors for further investigation (Kennedy and Krouse, 1999). 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 (Kuehl, 2000). 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 (Equation 23) is usually used to describe the effects of various factors on it based on the experimental results from a Plackett–Burman design.

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i \chi_i \tag{23}$$

Where y is the response, β_0 is the constant and 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) and (Weuster-Botz, 2000). Pan et al. (2008) used Plackett–Burman design to study the effects of 8 factors on fermentative hydrogen production. The 3 main factors (glucose, phosphate buffer and vitamin solution) were significantly effects on the specific hydrogen production potential.

2.6.2.5 Steepest ascent method

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 (Equation 23) 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 2.6 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 (Kuehl, 2000). 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. It was indicates that the region of optimal response is in the neighborhood of that condition (Kuehl, 2000).

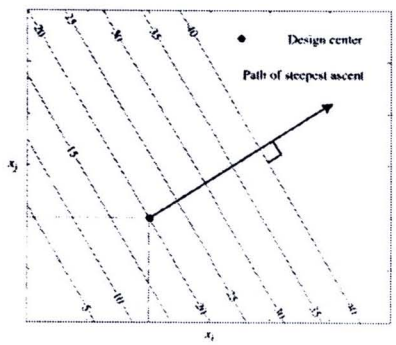


Figure 2.6 Contour plot of a response and the path of steepest ascent (Wang and Wan, 2009).

2.6.2.6 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 (Kuehl, 2000). 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. Results 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. In addition, 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 (O-Thong et al. 2008).

Box–Behnken design is a three-level fractional factorial design developed by Box and Behnken (1960). 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. Results 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 (Pan et al., 2008). 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. O-Thong et al. (2008) studied the effects of 3 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 (Equation 24) 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.

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i \chi_i + \sum_{i=1}^{k} \beta_{ii} \chi_i^2 + \sum_{i<j} \beta_{ij} \chi_i \chi_j \tag{24}$$

Where y is the response, β_0 is the constant and 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 Figure 2.7 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.

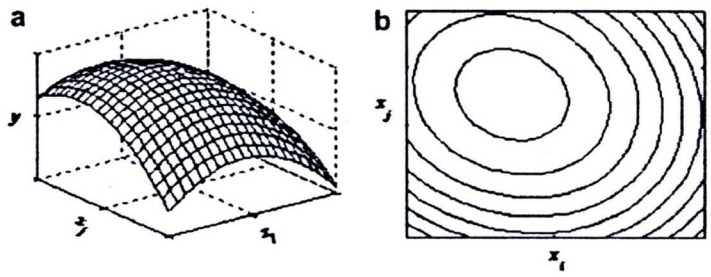


Figure 2.7 Surface plot (a) and contour plot, (b) for a response (Wang and Wan, 2009).

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. (2008) 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).

2.6.2.7 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 (Montgomery, 2005). 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 (Equation 25), which is the geometric mean of all the individual desirability functions.

$$D = \left(d_1 \times d_2 \times \ldots\ldots \times d_m\right)^{1/m} \tag{25}$$

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.

2.6.2.8 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. 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 (Wang and Wan, 2009).

2.7 Anaerobic sequencing batch reactor

Anaerobic sequencing batch reactor (ASBR) systems have benefits to alternative conventional flow systems for the biological treatment of both domestic and industrial wastewater (Ying-Chih et al., 2007). There are some advantages and disadvantages in ASBR process (Table 2.11). ASBR process consisted of five steps for operation which are filling, reaction, settling, decanting and idle (Figure 2.8).

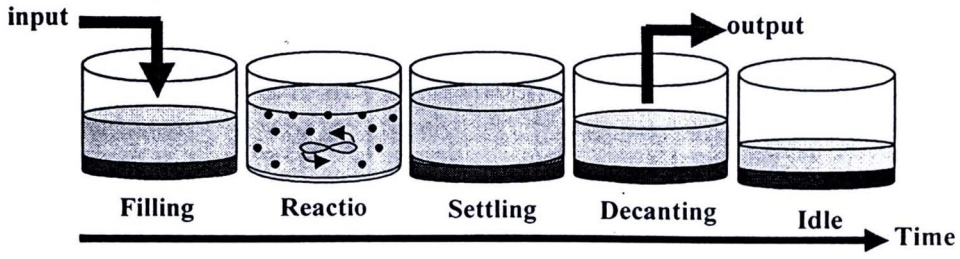


Figure 2.8 ASBR operational steps

Table 2.11 Advantage and disadvantages in ASBR process

Advantage	Disadvantage
- Equalization, primary clarification, biological treatment, and secondary clarification can be achieved in a single reactor vessel. - Operating flexibility and control. - Minimal footprint. - Potential capital cost savings by eliminating clarifiers and other equipment. - No short circuit, as in the case of fixed-bed continuous systems. - High efficiency for both COD removal and gas production. - No primary and secondary settles. - Flexible control.	- A higher level of sophistication is required especially for larger systems, of timing units and controls. - Higher level of maintenance associated with more sophisticated controls, automated switches, and automated valves. - Potential of discharging floating or settled sludge during the decanting phase with some ASBR configurations. - Potential plugging of aeration devices during selected operating cycles, depending on the aeration system used by the manufacturer. - Potential requirement for equalization after the ASBR, depending on the downstream processes.

(Kim, 2011)

2.8 Hydrogen production using ASBR

Anaerobic sequencing batch rector (ASBR) process has become the reactor option for producing hydrogen due to its advantages. Distinct advantages of ASBR when compared to CSTR system including high biomass concentration, a high degree of process flexibility, no requirement to apply a separate clarifier (Dague et al., 1992). Moreover, ASBR system is efficient in operating control, without primary or secondary settling, with high organic removal efficiency and simple operation (Ratusznei et al., 2000). The ASBR cycle is composed of four steps which were filling, reacting, settling and decanting steps (Sung and Dague, 1995). Fresh substrate is fed to the bioreactor in each filling phase, followed by the reaction phase. A completely mixed condition is only reached during the reaction phase (Ying-Chih et al., 2007). It has been reported that using ASBR with different operational parameter

such as HRT, substrate, seed obtained different hydrogen production efficiency (Table 2.12).

Table 2.12 Comparison on hydrogen production using ASBR operation

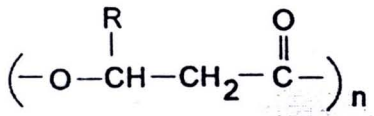
HRT (hr)	Reactor	Substrate	Seed	H_2 yield (mol H_2/mol substrate)	H_2 production rate (mol H_2/L-d)	Reference
6-12	ASBR	Sucrose	Non-pretreated microflora	0.7-2.6 mol H_2/mol sucrose	0.07-0.33	Lin and Jo, 2003
4-18	ASBR	Starch	Heat-treated sludge	0.06-0.51 mol H_2/mol hexose	0.04-0.18	Arooj et al., 2008a
24-42	ASBR	Food waste	Heat-treated sludge	0.18-1.12 mol H_2/mol hexose	0.01-0.12	Kim et al., 2006
4-12	ASBR	Sucrose	Acid-enriched sewage sludge	0.73-2.5 mol H_2/mol sucrose	0.07-0.33	Lin and Chau, 2004

ASBR has been used to produce hydrogen from various kinds of substrates such as dairy wastewater and chemical wastewater with the maximum hydrogen production rate of 0.0245 and 0.0089 mmol H_2/min-g COD, respectively (Venkata Mohan et al., 2007a, b). Venkata Mohan et al., (2007a) used ASBR to investigate the effect of organic loading rate (OLR) on hydrogen production from dairy wastewater. It was found that the seed sludge pretreatment was required to improve the hydrogen production from dairy wastewater while the hydrogen ability was found to be dependent on the OLR applied (Venkata Mohan et al., 2007a). ASBR was used for hydrogen production from plam oil mill effluent (POME) (O-Thong et al., 2007). Results indicated that nutrient supplementation improved simultaneous hydrogen production and pollution reduction from POME using thermophilic fermentation. In addition, nutrient supplementation was increased the bacterial diversity and amount of hydrogen-producing bacteria possibly leading to increase the hydrogen production process (O-Thong et al., 2007). Investigations on important parameters for ASBR

operation including solid retention time (SRT), organic loading rate (OLR), cyclic duration and hydraulic retention time (HRT) have been reported. Most of these, HRT is one of the important parameters that determines the economics of the hydrogen production process. The lower HRT attributed to a smaller reactor, cost reduction and therefore enhanced the productivity of the hydrogen production process (Kim et al., 2006). Lin and Chou, (2004) reported that ASBR operated under short HRT (< 8 hr) indicated the better hydrogen production from sucrose by mixed cultures.

2.9 Polyhydroxyalkanoates (PHAs) production from wastewater

Polyhydroxyalkanoates (PHAs) are a family of biopolyesters synthesized and accumulated by many bacteria as carbon storage materials (Anderson and Dawes, 1990). They are natural biopolymers synthesized and decomposed via microbial metabolisms, but can be melted and molded like petrochemical thermoplastics. PHAs have attracted academic and industrial attention because of their potential use as biodegradable thermoplastics. Figure 2.9 shows the general chemical structure of PHAs.



n = 1 R = hydrogen : Poly (3-hydroxypropionate),

n = 1 R = methyl : Poly (3-hydroxybutyrate)

n = 1 R = ethyl : Poly (3-hydroxyvalerate)

n = 2 R = hydrogen : Poly (4-hydroxybutyrate)

n = 3 R = hydrogen : Poly (5-hydroxyvalerate)

Figure 2.9 The general chemical structure of polyhydroxyalkanoates (PHAs) (Lee, 1995).

Many PHAs are formed from precursors or structurally related substrates (Rehm and Steinbuchel, 1999). The hydroxyalkanoate monomers that are supplied via native cell metabolism are usually 3-hydroxyalkanoates (3HAs) and almost all 3HAs are in

R configuration due to the stereo-specificity of enzymes involved in PHAs biosynthesis (Yu, 2007).

PHAs have been produced from hydrogenogenic effluent by mixed cultures (Ntaikou et al., 2009; Venkata Mohan et al., 2010). Many bacteria are capable of producing PHAs by polymerizing fatty acids and polymers are stored into specific granules of their cells under unfavorable conditions (Kessler et al., 2001). There are several types of biopolyesters, some of which have similar properties to polyvinylchloride and polystyrene and thus are characterized as bioplastic. Under certain environmental conditions the accumulated bioplastic can reach up to 80% of the bacterial cell dry mass (Beak Kim and Lenz, 2001). Consequently, the viability of the overall process depends on using highly productive microorganisms, together with low-cost substrates (Shilpi and Srivastava, 2005), such as acidified olive oil mill wastewater (OMW) (Ntaikou et al., 2009). It was found that PHAs were accumulated under nitrogen limitation by mixed cultures of *Pseudomonas* sp. and produced maximum PHAs of 8.94% (w/w) (Ntaikou et al., 2009). Even though, PHAs production by mixed microbial cultures (Rodgers and Wu, 2010) is more energy-efficient than that involving pure culture (Kek et al., 2008; Lee et al., 2008), but the PHAs content was not high (Serafim et al., 2008) whereas high PHAs content was observed in pure culture (Li et al., 2007).

The Gram-negative, facultative bacterium *Cupriavidus* sp. (formerly *Ralstonia eutropha*) can utilize VFAs (acetic, propionic, and butyric acids) as its sole carbon and energy source for growth and PHAs synthesis (Du and Yu, 2002; Chakraborty et al., 2009). *Cupriavidus* sp. could accumulate PHAs under nitrogen-limited or unbalanced conditions (Wang et al., 2007). Organic wastewater containing with high VFAs, such as effluent from fermentation processes have been used for the production of PHAs (Kristi and Oliver, 2009). PHAs could be continuously produced from filtered fermented food wastes with the maximum content of 87% by *C. necator* (Hafuka et al, 2011). Wang et al., (2010) investigated PHAs production and VFAs consumption by *C. necator*. It was found that the cell growth, activities and production of PHAs of *C. necator* were significantly affected by the initial VFAs concentration (Wang et al., 2010).