CHAPTER II
LITERATURE REVIEW

2.1 Bio-refinery

A bio-refinery is a facility that integrates biomass conversion processes and equipment to produce fuels, power, and chemicals from biomass. The bio-refinery concept is analogous to today's petroleum refineries, which produce multiple fuels and products from petroleum. Industrial bio-refineries have been identified as the most promising route to the creation of a new domestic biobased industry (Kaparaju et al., 2009). Renewable energy deriving from solar, wind, and biomass sources has great potential for growth to meet our future energy needs. Fuels such as ethanol, methane, and hydrogen are characterized as bio-fuels because they can be produced by the activity of microorganisms. Bio-fuel production is best evaluated in the context of a bio-refinery (Figure 2). In a bio-refinery, agricultural feedstocks and by-products are processed through a series of biological, chemical, and physical processes to recover bio-fuels, biomaterials, nutraceuticals, polymers, and specialty chemical compounds (Walker, 2005). This concept can be compared to a petroleum refinery in which oil is processed to produce fuels, plastics, and petrochemicals. The recoverable products in a bio-refinery range from basic food ingredients to complex pharmaceutical compounds and from simple building materials to complex industrial composites and polymers (Gravitis, Suzuki, 1999; Walker et al., 1999).

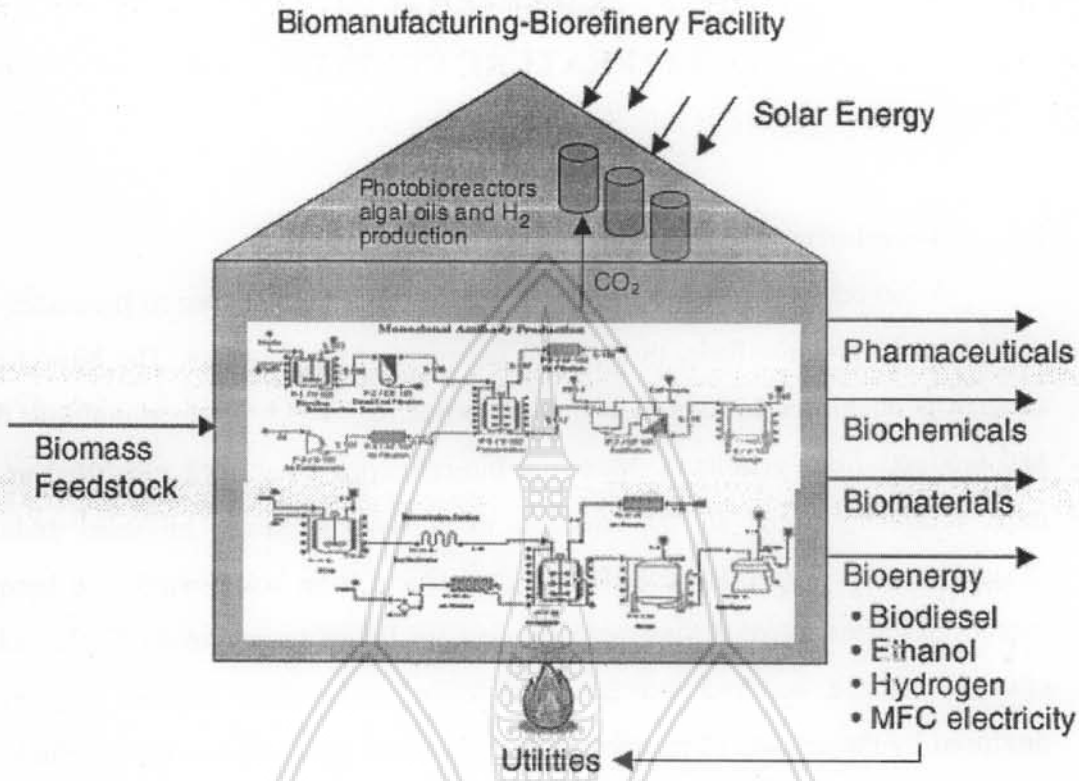

Figure 2 Integrated biorefinery showing example bioprocesses of monoclonal antibody and ethanol production (Walker, 2005).

2.2 Sugarcane

Sugarcane is one of the important industrial crops in Thailand. It can be cultivated in all parts of Thailand, except in Southern Thailand, with the cultivation area of more than 960,000 ha; approximately 48 million tons of sugarcane is produced per year (Office of the Cane and Sugar Board, 2006). Sugarcane juice is mainly used for producing sugar. However, from the report of Office of the Cane and Sugar Board (Thailand) (2006), sugar production from sugarcane every year is greater than sugar consumption. Therefore, this research was designed to investigate an alternative way of sugarcane product value-added in renewable energy production.

Sugarcane juice mainly consisted of sucrose of approximately 200 g/L and also fructose and glucose (Pattra et al., 2008). Sucrose has been reported as a substrate for producing hydrogen by various types of microorganisms such as *C. pasteurianum* (Lin, Lay, 2004) and *C. butyricum* CGS5 (Chen et al., 2005) with the yield of 4.80

and 2.78 mol hydrogen/mol sucrose, respectively. Sugarcane bagasse (SCB) is a waste left after sugarcane extraction process. Since the bagasse accounted for approximately 25% of sugarcane mass, about 12 million tons of SCB is produced annually. The most common use for SCB is the energy production by combustion (Neureiter et al., 2002) which can cause environmental problem from the emissions of CO_2. In addition, SCB can be used also to produce chemical compounds such as furfural or hydroxymethylfurfural (Almazan et al., 2001), paper paste (Carachi et al., 1996) or ethanol (Laser et al., 2002).

SCB consists of three main fractions i.e., cellulose, hemicellulose and lignin. It contains ca. 30-35% of hemicellulose (Gamez et al., 2005). Dilute acid treatment of hemicellulose fraction in SCB yields a solution containing mainly glucose and xylose (Aguilar et al., 2002) with small amount of arabinose (Gamez et al., 2005). Since bonds in cellulose are stronger than in hemicellulose, therefore a solid waste formed by cellulose and lignin is obtained in the dilute acid hydrolysis of SCB (Aguilar et al., 2002). Glucose and xylose were reported as a substrate for producing hydrogen by various types of microorganisms such as *C. acetobutylicum* (Chin et al., 2003) and *C. butyricum* (Yokoi et al., 1997) with the yield of 2.0 and 2.3 mol H_2/mol glucose, respectively. Due to its composition, hydrolysate of SCB is a very attractive raw material for the production of hydrogen.

2.3 Hydrogen Properties and Its Use

2.3.1 Properties

Hydrogen is the most abundant chemical element, constituting in roughly 75% of the universe's elemental mass. 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 (Kofstad, 1992; Brady, 2000). The physical properties of hydrogen were shown in Table 1.

Table 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

2.3.2 Hydrogen in use

Hydrogen is one of 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 (Wang, Wan, 2009). The use of hydrogen is an environmental friendly 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 firstly used in the transportation sector. Internal combustion engine can be fueled with pure hydrogen blended with natural gas (Momirlan, Veziroglu, 2005). Nowadays hydrogen is used in ammonia manufacture, petroleum refinery and methanol synthesis (Holladay et al., 2009).

Fuel cell is energy conversional technologies that produce electricity combining with fuel (hydrogen) and oxidant (oxygen from air) gases through electrodes and across an ion conducting electrolyte (Shekhawat el al., 2009). The principle characteristic of a fuel cell is its ability to convert chemical energy directly to electrical energy and provide higher conversion efficiencies than any conventional thermo-mechanical system. Thus, it can extract more electricity from the same amount of fuel, operate without combustion resulting in virtual pollution free and have quieter operation since there are no moving parts (Shekhawat el al., 2009). To convert the hydrogen fuel to electrical energy, the hydrogen fuel is fed into the anode

of the fuel cell and oxygen is entered 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 (Stambouli, Traversa, 2002). Fuel cell works similar to a battery but it does not require recharging. The cell will continuously produce electricity as long as it has a supply of fuel (Johnston et al., 2005).

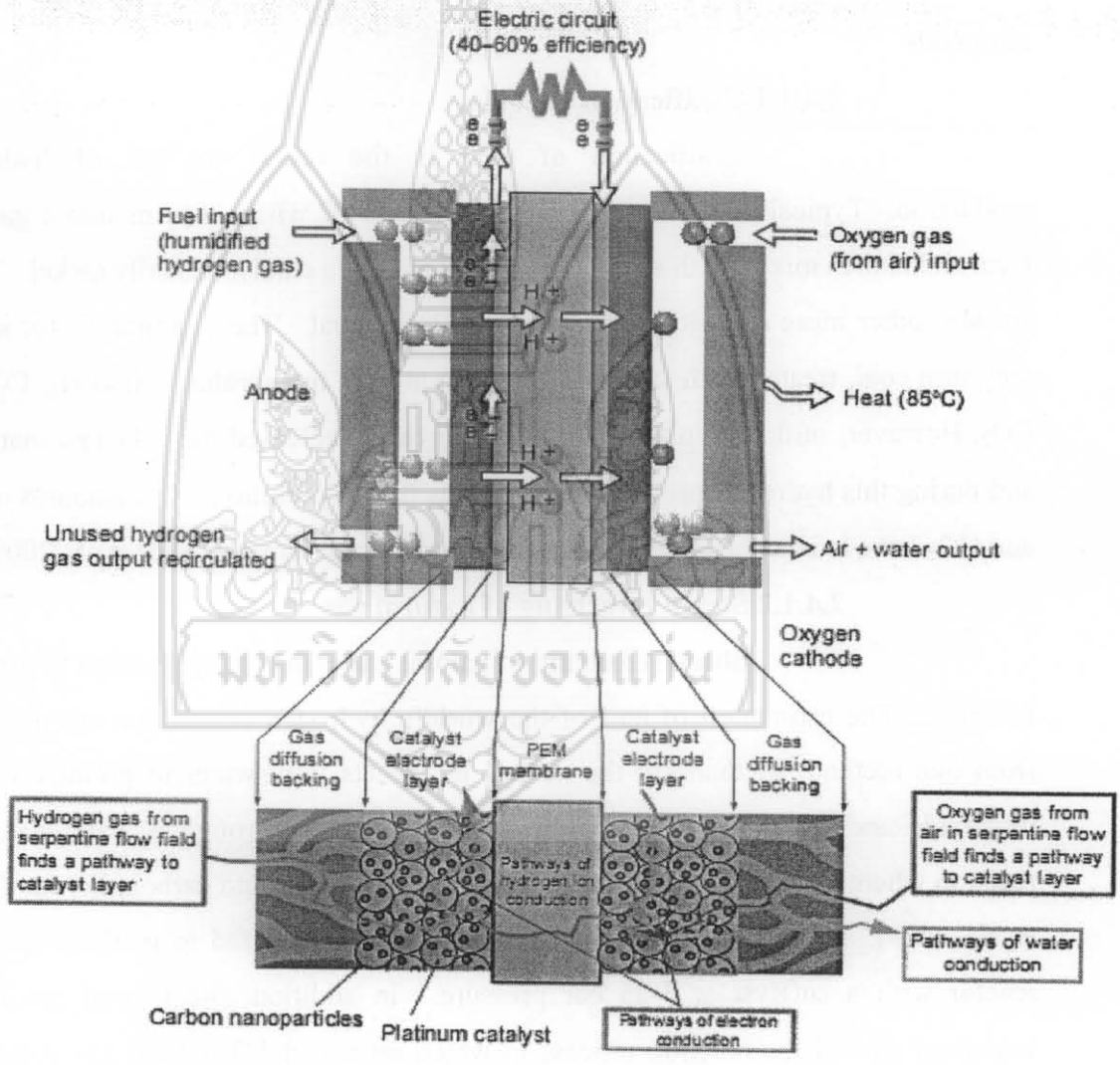

Figure 3 Schematic working of Fuel cell (Jacobson, 2007).

2.4 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, 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).

2.4.1 Hydrogen production from fossil and renewable resources as raw materials

2.4.1.1 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).

2.4.1.2 Steam reforming of natural gas

Steam reforming of natural gas is the cheapest ways to produce hydrogen. The estimation of half of the world's hydrogen production are produced from this method. 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 (Yang et al., 2009). 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 is the important part of the reaction process in which an added 1/3 natural gas is used as energy to control 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, Swaminathan, 1997).

2.4.1.3 Electrolysis of water

Water splitting in its simplest form uses an electrical current passing through two electrodes to break water into hydrogen and oxygen (Holladay et al., 2009). If large quantities of hydrogen need to be produced on a large scale, the by–product, oxygen, should be fully utilized such as for 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 term, hydrogen should be produced by renewable energy resources to avoid fossil–fuel consumption and greenhouse-gas emission (Kato et al., 2005).

2.4.1.4 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 be 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).

2.4.1.5 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 becomes an interesting and inexpensive method of producing hydrogen directly from solar energy. Research is also being undertaken on the use of catalysts to reduce the temperature for dissociation. The central problems are the separation of gases at high temperature to avoid recombining and the efficiency factor is uncertain (Holladay et al., 2009).

2.4.1.6 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). Gasification reactors have been developed to produce methanol from biomass. Several of these can be used in hydrogen production. Especially those that use air instead of oxygen are economically feasible (Ogden, Nitsch, 1993).

2.4.2 Biological hydrogen production

Bio-hydrogen is defined as a hydrogen produced from renewable resources such as water, organic wastes or biomass, either biologically or photobiologically (Das, Veziroglu, 2008). It is one of the approaches to create a safe and efficient energy production system (Miyake et al., 1998). It is a low capital cost due to the extended periods of viable cells. Microorganisms are capable of producing hydrogen via either fermentation (Taguchi et al., 1996; Yokoi et al., 1997) or photosynthesis (Lichtl et al., 1997; Matsunaga et al., 2000).

The biological species involved in hydrogen production are green algae and various species of hetrocystous cyanobacteria, non-hetrocystous cyanobacteria, photosynthetic bacteria and fermentative bacteria which are tabulated in Table 2. In addition, a good review of research work on biological hydrogen generation from the renewable sources can be found in the report of Das, Veziroglu (2008).

Table 2 Hydrogen-evolving microorganisms

Classification	Microorganisms	References
Green algae	*Scenedesmus obliquus*	Winkler et al. (2002)
	Chlamydomonas reinhardii	Winkler et al. (2002)
	Chlamydomonas moewusii	Winkler et al. (2002)
Cyanobacteria	*Anabaena variabilis*	Liu et al. (2006)
heterocystous	*Anabaena cylindrira*	Neil et al. (1976)
non heterocystous	*Oscillotoria Miami BG7*	Philips, Mitsui (1983)
Photosynthetic	*Rhodobacter sphaeroides*	Kars et al. (2006)
bacteria	*Rhodobacter capsulatus*	Ozturk et al. (2006)
	Rhodopseudomonas palustris	Chen et al. (2007)
	Rhodospirillum rubrum	Younesi et al. (2008)
Fermentative bacteria	*Enterobacter aerogenes*	Fabiano, Pergo (2002)
	Enterobacter cloacae IIT-BT 08	Kumar, Das (2000)
	Clostridium butyricum	Fang et al. (2006)
	Citrobacter sp. Y19	Oh et al. (2003)
	Bacillus coagulans	Kotay, Das (2007)
	Clostridium acetobutylicim ATCC824	Oh et al. (2003)

(Das, Veziroglu, 2008)

2.4.2.1 Hydrogen production by photosynthetic microorganism

1) Hydrogen production by green algae and cyanobacteria

Green algae and cyanobacteria (blue-green algae) are photosynthetic microorganisms which have demonstrated a capability for light-induced hydrogen evolution (Das, Veziroglu, 2008). They directly convert water to hydrogen and oxygen by using light as an energy source as following reaction;

$$H_2O \longrightarrow H_2 + \tfrac{1}{2} O_2 \qquad (2.1)$$

The reaction requires only water and sunlight. However, naturally the organisms that carry out this process showed rather low efficiency of hydrogen production due to the complicated reaction system which remains to overcome the large free energy (+ 242 kJ/mol hydrogen) (Miyake, 1998). For future application, many improvements are required.

Previous work reported several applications of biological hydrogen photoproduction by green algae and cyanobacteria. For examples, green algae could produce hydrogen under light anaerobic conditions, as first observed by Gaffron, Rubin (1992). The marine blue-green alga *Oscillatoria* sp. strain Miami BG7 was observed as a light-induced hydrogen production species (Kumazawa, Mitsui, 1981; Mitsui et al., 1985) and a freshwater alga, *Chlamydomona reinhardtii*, showed the stable O_2/H_2 production system in and alternating light/dark cycle (Miura et al., 1982). Fouchard et al. (2008) reported that *C. reinhardtii* could produce maximum hydrogen production rate (2.5 mL/L/h) from sulfur deprived medium under constant agitation at 300 rpm and continuous illumination at 110 mmol photons /m^2/s.

2) Hydrogen production by photosynthetic bacteria

Photosynthetic bacteria are known as the most powerful hydrogen producers. The photobiological hydrogen evolution ethod provided a very simple and efficient system of solar energy conversion (Holladay et al., 2009). Photosynthetic bacteria are the most promising microbial system for the biohydrogen production (Fascetti et al., 1998) 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). Hydrogen gas production capabilities of some purple photosynthetic bacteria such as *Rhodobacter spheroids* (Federov, 1998; Eroglu, 1999; Zhu et al., 1999; Koku, 2002), *Rhodobacter capsulatus* (Ooshima et al., 1998; He et al., 2005; Fang, Zhang, 2005), *Rhodopseudomonas palustris* (Barbosa, 2001; Oh et al., 2004) and *Rhodospirillum bubrum* (Zurrer, Bachofen, 1982) have been investigated to some extent.

Photosynthetic bacteria can also use organic acids as the starting compound for hydrogen production (Miyake et al., 1999) such as acetic acid (Barbosa et al., 2001), malic acid (Tabanoglu et al., 2002; Oh et al., 2004), lactic acid (Mao et al., 1986; Sasikala et al., 1990) and butyric acid (Mao et al., 1986) which are the main by-products of anaerobic fermentation. An equation as below shows the production of hydrogen from lactate by photosynthetic bacteria (Miyake et al., 1999).

$$C_3H_6O_3 + 3H_2O \longrightarrow 6H_2 + 3CO_2 \quad (2.2)$$

In the end of this reaction, free energy is obtained at about +8.5 kJ/mol hydrogen. In case of photosynthetic bacteria, less light energy is required to produce hydrogen when starting from organic substances compared to algal hydrolysis (Miyake et al., 1999). Anaerobic fermentation provided low molecular weight organic acids which were then converted to hydrogen by photosynthetic bacteria in anaerobic-light condition (Hillmer et al., 1997). Bacteria species of *Rhodopseudomonas* could produce hydrogen from acetate in the rate of 25 ml H_2/l.h under light intensity of 680 μmol photon/m^2.s (Barbosa et al., 2001). Otsuki et al. (1998) reported that hydrogen could be produced from mixture of acetate, propionate and butyrate by photosynthetic bacteria with a light efficiency of 0.31%. Similarly, photohydrogen production by *R. capsulatus* using acetate and butyrate as carbon sources was studied. The maximum hydrogen production yield obtained at around 2.5 mol H_2/mol acetate and 3.7 mol H_2/mol butyrate at the initial pH of 8.0 and 9.0, respectively (Fang et al,. 2005). Oh et al. (2004) indicated that *R. palustris* P4 produced hydrogen from glucose in the combinations of dark and light fermentation by repeated dark fermentation techniques. Chen, Chang (2006) enhanced phototropic hydrogen production by using solid-carrier to assist fermentation. An internal optical-fiber illumination using acetate concentration of 1000 mg-COD/L was used as the sole carbon substrate. The results showed that addition of clay and silica gel was effective in promoting hydrogen production resulting in 67.2-50.9% and 37.2-32.5% increase in hydrogen production rate and hydrogen yield, respectively, while the hydrogen yield and hydrogen production rate from batch operation by free culture were 43.8 ml/l/h and 3.63 mol H_2/mol, respectively. In addition, Gadhamshetty et al. (2008) found that the optimum light intensity for maximum hydrogen yield from malate by *Rhodobacter sphaeroids* was 150-250 W/m^2.

2.4.2.2 Hydrogen production by dark fermentation

Hydrogen production by fermentation of composite organics has been received attention due to high evolution rate of hydrogen produced, and ability to emit hydrogen throughout the day and night (Holladay et al., 2009). Fermentative bacteria producing hydrogen in the dark may be cultivated in pure

culture or occur in uncharacterized mixed cultures selected from natural sources such as anaerobically digested sewage sludge or soil (Das, Veziroglu, 2008). Biohydrogen production by dark fermentative bacteria is an attractive process in which the biowaste can be used as substrates facilitating both bioremediation and energy recovery (Kotay, Das, 2007). Microbial hydrogen production by dark fermentation is carried out via glycolysis or Embden-Meyerhoff-Parnas pathway in which organic materials are converted to hydrogen, carbon dioxide, organic acids and solvent (Das, Veziroglu, 2008).

The evolution of hydrogen by fermentation has several advantages for industrial production such as high evolution rate of hydrogen. Fermentative bacteria can produce hydrogen constantly through day and night from organic substrates (Holladay et al., 2009). Hydrogen is produced via an anaerobic process which is divided into two distinct stages. The first stage is acidification which produced hydrogen as a by-product. Hydrogen would turn to be an electron donor by many methanogens in the second stage of the process (Kirk et al., 1985).

1) Hydrogen production by pure-culture microorganism

The majority of microbial hydrogen production in dark fermentation was driven by the anaerobic metabolism of pyruvate, formed during the catabolism of various substrates (Hallenbeck, Benemann, 2002; Das, Veziroglu, 2008). The dark fermentative bacteria grown on carbohydrate-rich substrates give organic fermentation end products, hydrogen and carbon dioxide. Hydrogen producing bacteria including *Enterobacter* sp. (Fabiano, Perego, 2002) and *Clostridium* sp. (Taguchi et al., 1992) have been found to produce hydrogen in the dark fermentation process. Some species of *Enterobacter* and *Clostridial* are able to degrade soluble starch without pretreatment step (Yokoi et al., 1997; Kumar, Das, 2001). Among the hydrogen producing strain, *C. butyricum* has been most extensively studied.

The amount of hydrogen that can be produced per mole of glucose as a substrate was found to be almost 2-3 moles of hydrogen (Hallenbeck, Benemann, 2002). Hydrogen production rate produced, by *C. butyricum* and *E. aerogenes* were 7.3 and 17 mmol/g-dry cell/h, respectively (Karube et al., 1976; Tanisho, Ishiwata, 1994). In addition, *Clostridium* sp. offered potential for converting cellulosic waste materials to hydrogen gas (Levin et al., 2004).

Some hydrogen production studies have been conducted on mixed cultures of anaerobic bacterial. Hussy et al. (2005) studied the continuous fermentative hydrogen production from sucrose and sugar beet. Hydrogen yield for refined sucrose and pulped sugar beet by anaerobic digester sludge were 1±0.1 and 0.9±0.2 mol/mol hexose, respectively. The maximum hydrogen yield in batch test from 20-g/L soluble starch by anaerobic mixed bacteria was 274 ml/g starch at pH 7 and 35°C (Yang et al., 2006). Fang et al. (2006) showed that pH 4.5 and temperature of 37°C was the most effective conditions to produce hydrogen from rice slurry by anaerobic mixed culture. Anaerobic digester sludge was used as seed after a 100 °C heat treatment for 30 min. Results showed that sludge had a maximum specific hydrogen production rate of 2.1 L/(g-VSS d) and hydrogen yield of 346 mL/g-carbohydrate. In addition, continuous production of hydrogen from the anaerobic acidogenesis of a high-strength rice winery wastewater by a mixed bacteria flora was demonstrated by Yu et al. (2002). An optimum hydrogen production rate of 9.33 L H_2/g VSS d was achieved at an HRT of 2 h, COD of 34 g/L, pH 5.5 and 55°C. The hydrogen yield was in the range of 1.37-2.14 mol/mol-hexose. The microbial diversity of dark fermentative hydrogen production were tabulated in Table 2.3.

2) The Fermentative bacteria strain *Clostridium butyricum*

Clostridium butyricum (Figure 4) is a generally gram positive, rod-shaped, strict anaerobes and endospore formers (Kapdan, Kargi, 2006). The biochemical pathway of *C. butyricum* (Figure 5), it utilized for the conversion of carbohydrates such as glucose, xylose and sucrose to hydrogen, carbon dioxide, fatty acids and solvents (Andreesen et al., 1989). The carbon flow acetyl- CoA

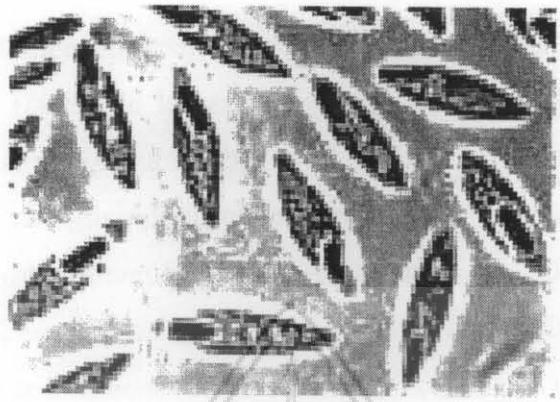

Figure 4 *Clostridium butyricum* morphology (Microbiology, 2008).

through the main branches of the pathway leading to the formation of acids and solvents is shown in Figure 5. Hydrogen was generated in acids phase and then alcohol such as ethanol and butanol were generated in solvent phase (Figure 5).

Most of the studies using pure cultures of these bacteria for fermentative hydrogen production were conducted in batch mode and used glucose as substrate. However, it is more desirable to produce hydrogen from organic wastes using pure cultures in continuous mode because continuous fermentative hydrogen production from organic wastes is more feasible for industrialization to realize the goal of waste reduction and energy production. As shown in the Table 3, *C. bytyricum* was most widely used as inoculum for fermentative hydrogen production.

3) Hydrogen production by immobilized cell

Immobilized whole cell technique represents an efficient approach to bio-catalyze several biochemical reactions with the advantage of improving cell survival and possibility to be reused and used in continuous process (Kumar, Das, 2001). Support materials for immobilization systems should be screened for optimum performance. Characteristics of support material are (i) high surface area, for optimum microbial development, (ii) low bulk density for the easiest and cheapest carrier operation, (iii) high void fraction to the limit pressure drop and clogging problems, (iv) low cost (Nicrolella et al., 2000). Lignocellulosic materials such as coconut coir presenting abundantly in agro-residues are of an interest to use as support material for cell immobilized in hydrogen production. It is because they are

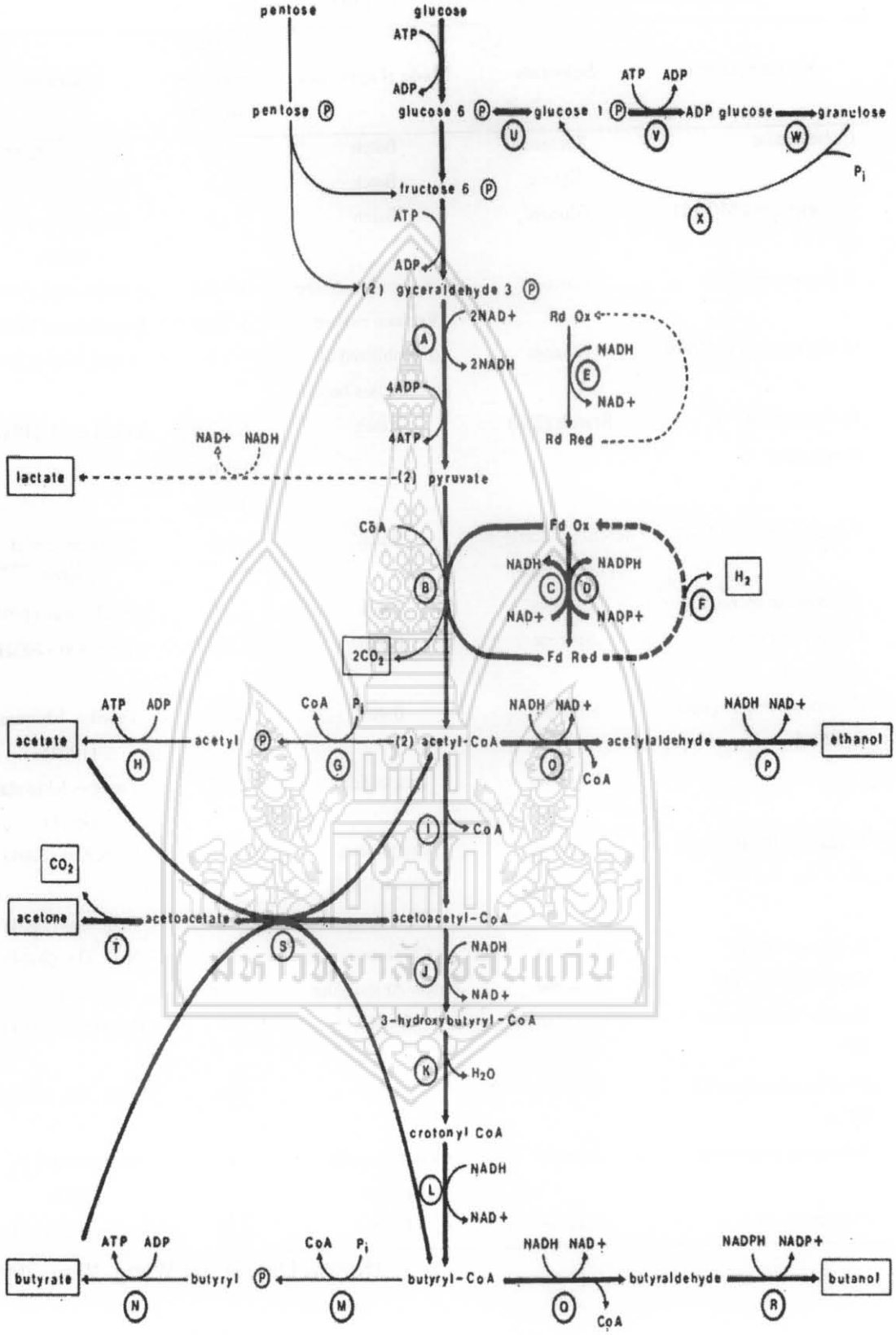

Figure 5 Biochemical pathway in *Clostridium butyricum* (Saint-Amans et al., 2001).

Table 3 Fermentative hydrogen production by pure culture

Microorganism	Substrate	Mode of operation	H_2 yield (mol/mol sugar)	Reference
C. butyricum	Sucrose	Batch	2.78	Chen et al. (2005)
	Xylose	Batch	2.1	Taguchi et al. (1994)
C. butyricum LMG 121 3tl	Glucose	Batch	1.5	Heyndrickx et al. (1986)
C. butyricum SC-E1	Glucose	Non-vacuum culture	1.4-2.0	Kataoka et al. (1997)
		Vacuum culture	1.3-2.2	Kataoka et al. (1997)
C. butyricum IFO 13949	Glucose	Immobilized on porous glass beads	1.9	Yokoi et al. (1997)
C. butyricum + *E. aerogenes*	Starch (2%)	Batch	2.5-2.6 mol/mol glucose	Yokoi et al. (1998)
C. paraputrificum M-21	GlcNAc(N-acetyl-d-glucosamine)	Batch	2.5	Evvyernie et al. (2000)
Clostridium sp. no. 2	Glucose	Batch	2	Taguchi et al. (1994)
Clostridium sp. + *Bacillus* sp.	Sucrose	Batch	1.5291	Sung et al. (2002)
Enterobacter aerogenes E. 82005	Molasses	Batch	1.24	Tanisho, Ishiwata (1994)
	Molasses	Suspended	0.81	Tanisho, Ishiwata (1994)
E. cloacae IIT-BT 08 wt	Glucose	Continuous (immobilized)	2.2	Nath, Das (2004)
	Sucrose	Continuous	3.0	Nath, Das (2004)
E. cloacae DM11	Glucose	Continuous	3.8	Nath, Das (2004)
Citrobacter sp. Y19	Glucose	Batch, Ar sparging	2.49	Oh et al. (2003)
Bacillus licheniformis JK1	Wheat slurries	Batch	1.5	Kalia et al. (2001)
Bacillus coagulans IIT-BT S1	Glucose	Batch	2.28	Kotay, Das (2007)
Thermotoga maritime	Sucrose	Contimuous N_2 sparged	4	Woodward et al. (2002)
T. neapolitana	Glucose	Batch	0.53	Suellen et al. (2001)

(Kotay, Das, 2007; Wang, Wan, 2009)

low cost environmental friendly materials without possessing the disposal problems after finished the fermentation process (Kumar, Das, 2001). Several researchers reported the finding on hydrogen production using immobilized whole cells as shown in Table 4.

Table 4 Comparison of hydrogen yield by various types of microorganism when sugar was used as a carbon source

Microorganism	Substrate	Support material	Fermentation process	H_2 yield ($molH_2/mol$ substrate)	Reference
Escherichia coli	Glucose	Flatbed agar plates	Batch	1.2	Ishikawaa et al. (2008)
Activated sludge	Sucrose	Polymethyl methacrylate	Batch	2.25	Wu et al. (2007)
E. areogenes HO-39	Glucose	Porous glass beads	Continuous fermentation	0.73	Yokoi et al. (1997)
Anaerobic sludge (*Clostridium* species dominate)	Glucose	Activated carbon	Continuous fermentation	0.87	Wu et al. (2008)
Anaerobic sludge (*Clostridium* species dominate)	Glucose	Granular sludge	Continuous fermentation	1.57	Wu et al. (2008)
C. tyrobutyricum JM1	Sucrose (hexose unit)	Polyurethane foam	Continuous fermentation	1.79	Jo et al. (2008a)
Anaerobic sludge	Sucrose (hexose unit)	Polyethylene-octane elastomer	Continuous fermentation (Fluidized-bed)	0.64	Wu et al. (2007)
Anaerobic sludge	Sucrose	Polyethylene-octane elastomer	Continuous fermentation (Packed-bed)	0.44	
Activated sludge		polymethyl methacrylate	Continuous fermentation	2.0	

4) Hydrogen production by mixed culture

The bacteria capable of producing hydrogen widely exist in natural environment such as soil, wastewater sludge and compost (Wang, Wan, 2009). Thus these materials can be used as inoculum for fermentative hydrogen production. Fermentative hydrogen production processes using mixed cultures are more practical than those using pure cultures because the former are simpler to operate, easier to control and may have a broader source of feedstock (Li, 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. 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 bacterial (Wang, Wan, 2009). The pretreatment methods reported for enriching hydrogen producing bacteria fr n mixed cultures mainly include heat-shock, acid, base, aeration, freeing and thawing, choloroform, sodium 2-bromoethanesulfonate or 2-bromoethanesulfonic acid and iodopropane (Wang, Wan, 2008). Table 5 summarizes a several studies comparing various pretreatment methods for enriching hydrogen-producing bacteria from mixed cultures.

Table 5 Various pretreatment methods for enriching hydrogen-producing bacteria from mixed culture inoculum in batch hydrogen fermentation

Inoculum	Pretreatment methods	Substrates	Hydrogen yield	References
Digested sludge	Heat-shock	Glucose	1.8 mol/mol glucose	Wang, Wan (2008)
Cattle manure sludge	Acid	Glucose	1.0 mol/mol glucose	Cheong, Hansen (2006)
Methnogenic granules	Chloroform	Glucose	1.2 mol/mol Glucose	Hu, Chen (2007)
Digested wastewater sludge	Base	Sucrose	6.12 mol/mol sucrose	Zhu, Beland (2006)
Anaerobic sludge	Sodium 2-bromoethaesulfonate	Dairy wastewater	0.03 mmol/g-COD	Mohan et al. (2008)

2.4.3 Enzyme Involved in Hydrogen Production

Two enzymes especially critical for hydrogen production are nitrogenase and hydrogenase.

2.4.3.1 Nitrogenase

Hydrogen production is associated mainly with the action of nitrogenase. This enzyme catalyzes hydrogen in the absence of molecular nitrogen as follow:

$$2H^+ + 2e^- + 4ATP \longrightarrow H_2 + 4ADP + 4P_i \qquad (2.3)$$

Efficient operation of nitrogenase requires large amounts of ATP and reducing power. For this reason, synthesis and activity of this enzyme are subject to strict regulatory controls.

The primary inhibitor/repressor of nitrogenase is oxygen, which irreversible destroys this enzyme. Ammonium, the salts of which frequently used as nitrogen sources in the cultivation of photosynthetic purple non-sulfur bacteria is the second inhibitor of concern. It represses the synthesis of nitrogenase and inhibits nitrogenase activity (Gest et al., 1962; Jones, Monty, 1979). Concentration as low as 20 μM have been found to rapidly inhibits existing nitrogenase activity in *R. sphaeroides*. However, the inhibition is reversible and nitrogenase can recover its activity once ammonium is consumed or removed. It has been found for *R. capsulatus* grow on lactate and glutamate that, if lactate is exhausted earlier than glutamate, net formation of ammonia occurs, eventually leading to inhibition of nitrogenase (Hillmer, Gest, 1997).

In the presence of N_2, the nitrogen fixation reaction dominates and hydrogen production is insignificant (Sasikala et al., 1990).

Nitrogenase synthesis is strongly stimulated by light, resulting in a corresponding increase in nitrogenase activity as observed in continuous culture of *R. capsulatus* (Jouanneau et al., 1985). It is also worth noting that a diurnal pattern of illumination (alternating periods of light and dark) results in a more stable nitrogenase activity (Meyer et al., 1978).

2.4.3.2 Hydrogenase

A hydrogenase is an enzyme that catalyses the reversible oxidation of molecular hydrogen (H_2). Hydrogenases play a vital role in anaerobic metabolism (Adams, 1990). Hydrogen uptake (H_2 oxidation, equation 1) is coupled to the reduction of electron acceptors such as oxygen, nitrate, sulfate, carbon-dioxide, and fumarate, whereas proton reduction (H_2 evolution, equation 2) is essential in pyruvate fermentation and in the disposal of excess electrons. Both low-molecular weight compounds and proteins such as ferredoxins, cytochrome C_3, and cytochrome C_6 can act as physiological electron donors (D) or acceptors (A) for hydrogenases (Chen, Blanchard, 1978).

$$H_2 + A_{ox} \rightarrow 2H+ + A_{red} \quad (1)$$

$$2H+ + D_{red} \rightarrow H_2 + D_{ox} \quad (2)$$

Hydrogenases can be classified into three groups with respect to the metals present in the active sites the only [Fe] hydrogenases, the [NiFe] hydrogenases and the [NiSeFe] hydrogenases. These enzymes present important differences in terms of specific activity and bidirectionality (Fauque et al., 1988). The environmental factors could be effected the activity of the enzyme in the hydrogen production process. Two factors i.e., pH and iron concentration have a direct influence on the activity of the enzyme. Several reports observed that the hydrogen production initiation was carried out only after pH decreased to approximately 5.5. Studies found that the hydrogenases activity measured in whole cells from acid-producing cultures maintained at pH 5.8 was about 2.2 times greater than that at pH 4.5 (Andersch et al., 1983). In general, hydrogenase activity is low in cells maintained at pH<5.2 (George, Chen, 1983). Iron concentration seems to have an effect on hydrogenase activity since this enzyme consists of a binuclear iron site bound to a (4Fe-4S) cluster (Vignais et al., 2001). Lee et al. (2001) studied the effect of the Fe concentration in the external environment on the hydrogen production using sucrose solution and the mixed microorganism from a soybean-meal silo. The result shown that maximum specific hydrogen production rate was 24 mL/g VSS at 4000 mg

$FeCl_2$/L (1760 mg Fe^{2+}). On the other hand, Lay et al. (2005) found a much smaller optimal Fe^{2+} concentration (132 mg Fe^{2+}/L) for hydrogen producing composts using solid food wastes as a substrate. These result depict that iron limitation could limit the hydrogenase activity along hydrogen evolution.

Hydrogenase is inhibited primarily by carbon monoxide and oxygen, though the resistance to the latter is considerably higher than nitrogenase. Hydrogenases are nickel enzymes, and limitation of nickel may attenuate the synthesis of this enzyme (Fissler et al., 1994). The presence of ethylenediaminetetraacetic acid (EDTA) is also known to inhibit hydrogenase activity (Kern et al., 1994, Fissler et al., 1994). The comparative properties of nitrogenase and hydrogenase are given in Table 6.

Table 6 The comparative properties of nitrogenase and hydrogenase

Properties	**Nitrogenase**	**Hydrogenase**
Substrates	ATP, H^+ or N_2, electrons	H_2
Products	H_2 or NH_4^+	ATP, H^+, electrons
Number of proteins	Two (Mo-Fe and Fe)	One
Metal components	Mo, Fe	Ni, Fe, S
Optimum temperature	30 °C (*A. Vinelandii*)	55 °C (*R. rubrum*)
		70 °C (*R. capsulatus*)
Optimum pH	7.1-7.3 (*A. Vinelandii*)	6.5-7.5 (*R. sulfidophilus*)
Inhibitor, repressors	N_2 (of H_2 production only), NH_4^+,	CO, EDTA, O_2, presence of
	O_2, high N to C ratio	organic compounds
Stimulators	Light	H_2 (*R. sphaeroides*)
		Absence of
		organic compounds

(Koku et al., 2002)

2.4.4 Factors Affecting Hydrogen Production

2.4.4.1 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, 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 with the different purposes. The use of different substrate for hydrogen production by fermentative bacteria reported was summarized in Table 7. Glucose and sucrose were most widely used substrate for hydrogen production. However, a few studied have begun to use organic wastes as substrate for hydrogen production (Wang, Wan, 2009). The concentrations of substrate have been considered as the important factor for hydrogen production. 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). Furthermore, the disagreement on the optimal substrate concentration for fermentative hydrogen production could be found, for example, the optimal sucrose concentration for fermentative hydrogen production reported by Van Ginkel et al. (2001) was 7.5 g-COD/L, while that reported by Lo et al. (2008) was 40 g-COD/L. The possible reason for this disagreement was the difference among inoculum used in these studies. Some complex substrates could not be used for fermentative hydrogen production due to their complex structures, however, they can be easily used by hydrogen-producing bacteria to produce hydrogen after pretreated by some methods. Zhang et al. (2007) reported that the hydrogen yield from cornstalk wastes after acidicfication pretreatment was much larger than that from cornstalk waste without any pretreatment (Zhang et al., 2007).

2.4.4.2 Nitrogen and phosphate

Nitrogen is very important factor for proteins, nucleic acids and enzymes which are significance 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 2.8 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.

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). The effects of C/N and C/P on fermentative hydrogen production were presented in Table 2.9. 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.

Table 7 Hydrogen production by hydrogen-producing bacteria with different substrates

Inocula	Substrate	Mode of operation	Substrate concentration (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	2.25molH_2/mol xylose	Lin et al. (2006)
Anaerobic sludge	Glucose	Batch	0.27-4.3	1.1	0.13mL H_2/h	Zheng, 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 (2001)
C. butyricum CGS5	Sucrose	Batch	5-30	20	2.78 molH_2/mol sucrose	Chen et al. (2005)
C. pasteurianum CH4	Sucrose	Batch	5-40	40	2.07 molH_2/mol sucrose	Lo et al. (2008)
Anaerobic sludge	Starch	Batch	9.8-39.0	9.8	67 mL/g starch	Zhang et al. (2003)
Anaerobic sludge	Starch	Batch	5-60	20	2.2 mol/mol hexose	Lin et al. (2008a)
Anaerobic digester sludge	Food waste	Batch	0-32.3	4.6	101 ml/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)

(Wang, Wan, 2009)

Table 8 Effects 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 H_2/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, Shen (2004)
Compost	Glucose	Yeast extract	2-8% yeast extract	4% yeast extract	70 mmol H_2	Morimoto et al. (2004)
Enterobacter aerogenes HO-39	Glucose	Polypeptone	0-5% polypepton	2% polypepton	58 mLH_2	Yokoi et al. (1995)

(Wang, Wan, 2009)

Table 9 Effects 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, Lay (2004)
Anaerobic sludge	Wheat powder	20-200	200	50-1000	1000	281mL H_2/g starch	Argun et al. (2008)
Anaerobic sludge	Palm oil mill effluent	45-95	74	450-650	559	6.33 L/L substrate	O-Thong et al. (2008a)

2.4.4.3 pH

pH is one of the most important factors influencing the ability of hydrogen-producing bacteria to produce hydrogen. It has been reported to affect the hydrogenase activity and the metabolism pathway. It has been demonstrated that in an appropriate range, increasing pH could increase the ability of hydrogen-producing bacteria to produce hydrogen during fermentative. Too high or too low pH can result in a low hydrogen production in which the activity of hydrogenase could be inhibited depend on the species of seed inoculum used (Nigam, 2000). Chen et al. (2005) reported that at a higher pH cell growth was more efficient than hydrogen production. At a weak acid condition (an initial pH of 5.5-6.5), *Clostridia* species can be activated to extrude the express proton from cytoplasm to facilitate the resumption of the cell growth as well as producing hydrogen which normally exhibited at the exponential growth phase of this species (Ferchichi et al., 2005). Chen et al. (2005) reported that pH in the range of 5.5-6.0 was an optimum pH for hydrogen production by *C. butyricum*. In addition, the optimum pH range of 5.0-5.7 to produce hydrogen from glucose by anaerobic fermentative bacteria with the maximum hydrogen evolution efficiency was also reported (Zoetemeyer et al., 1982). At a very low pH concentration, a protonation of undissociates weak acids in the medium can pass through the cell membrane into cytoplasms. This can cause the loss of glycolytic enzyme activities and structural damaging of cell membrane, DNA and protein which could slow down (Ferchichi et al., 2005) or inhibit the growth of the microorganisms as well as inhibit the ability to produce hydrogen (Chen et al., 2005). The researches investigating the effect of pH on hydrogen production were shown in Table 10.

2.4.4.4 Temperature

Temperature is another important factor that influence on hydrogen evolving activity. It has been demonstrated that in an appropriate range, increasing temperature could increase the ability of hydrogen-producing bacteria to produce hydrogen during fermentative hydrogen production. However, temperature at much higher levels could decrease it with increasing levels (Wang, Wan, 2008). Table 2.11 show the effect of temperature on fermentative hydrogen production. From this Table 2.11, there exists certain agreement on the optimal temperature for fermentative hydrogen production. For example, the optimal temperature for fermentative

hydrogen production reported by Wang et al. (2005) was 35.1, while that reported by Mu et al. (2006) was 35.5. The possible reason for this agreement was the same among these studies in the terms of inoculum, substrate, reactor type and temperature range studied.

Table 10 Effects 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-21	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)

(Wang, Wan, 2009)

2.5 Hydrogen production from lignocelluloses material

2.5.1 Lignocellulosic materials

The use of renewable energy derived from the crop avoids many of the environmentally detrimental aspects of petroleum-based fossil fuels such as the greenhouse effect which led to a global warming. However, renewable energy has its own environmental costs including ecological damages caused by nitrogen and phosphorus fertilizers, pesticides, and soil erosion. In addition, as demand for both food and energy rise in the coming decades, the conflicts of using crop for food or energy production could be occurred. These reasons draw the interest of the researchers to use lignocellulosic materials which are the most abundant and widely

spread renewable biomass in the world as a feed stock for hydrogen production. The typical percentages of dry weight are 35–50% cellulose, 20–35% hemicellulose and 5–30% lignin (Lynd et al., 2002; Bhat, Bhat, 1997). Cellulose is a linear polymer of b-1,4-linked D-glucopyranose monomers. Cellulose fibers are assembled by a large

Table 11 Effects of temperature on batch fermentative hydrogen production

Type of inoculum	Substrate	Temperature (^{O}C)		Optimal production value	References
		Range studied	Optimal		
Anaerobic sludge	Sucrose	25-45	35.1	3.7 molH_2/mol sucrose	Wang et al. (2005)
Anaerobic sludge Cow dung	Sucrose	25-45	35.5	252 mL H_2/g sucrose	Mu et al. (2006)
Cow waste slurry Anaerobic sludge	Cow dung	37-75	60	743 mL H_2/kg cow dung	Yokoyama et al. (2007)
Anaerobic sludge	Cow waste slurry	37-85	60	392 mLH_2/L slurry	Valdez-Vazquez et al. (2005)
	Glucose	25-55	40	275.1 mL H_2/g glucose	
	Glucose	33-41	41	1.67 mol H_2/mol glucose	Mu et al. (2006)

(Wang, Wan, 2009)

amount of intra- and intermolecular hydrogen bonds (Zhbankov, 1992). This makes cellulose insoluble in water and most organic solvents in which the use of lingo-cellulosic materials for hydrogen production require pretreatment steps (Swatloski et al., 2002). Lignin is an irregular polymer of several monoaromates and forms a three-dimensional network in which cellulose and hemicellulose fibers are embedded.

Wooden biomass exhibits high lignin contents (Huber et al., 2006). Due to this complex structure, lignocellulose has a remarkable resistance against chemicals and microbial attacks (Zhu et al., 2006) and makes it recalcitrant against its hydrolysis which is the central barrier for its widespread utilization (Lynd et al., 2002). Several pretreatments for lignocelluloses were developed (Mosier et al., 2005a; Wyman et al.,

2005). After a mechanical comminution hydrothermal or chemical pretreatments are applied, the hydrolysate obtained could be used for hydrogen production. Hydrothermal processes can be steam explosion (Cara et al., 2008) or hot water treatment (Mosier et al., 2005b). Chemical processes include dilute-acid treatment (Lloyd, Wyman, 2005), alkali treatment (Kaar, Holtzapple, 2000), organosolv process using organic solvents (Holtzapple, Humphrey, 1984), ammonia fiber explosion ("AFEX") (Teymouri et al., 2005), ammonia recycle percolation (Kim, Lee, 2005), and ozonolysis (Vidal, Molinier, 1988).

Sugarcane is a one of lignocelluloses material type. Sugarcane bagasse (SCB) is a waste left after sugarcane extraction process. Dilute acid treatment of hemicellulose fraction in SCB yields a solution containing mainly glucose and xylose (Aguilar et al., 2002) with small amount of arabinose (Gamez et al., 2005). Table 12 show comparison of sugar compositions and inhibitor concentration obtained from different acid hydrolysis conditions of sugarcane bagasse. It could be seen that sugar compositions from acid hydrolysis of sugarcane bagasse were different depending on terms of liquid to solid ratio, hydrolysis period and temperature range studied.

Table 12 Comparison of sugar compositions and inhibitor concentration obtained from different acid hydrolysis conditions of SCB.

Acid	Liquid to solid ratio (g/g)	Temp (°C), time (min)	Concentration (g/L)					Reference
			Glucose	Xylose	Arabinose	Acetic acid	Furfural	
6% HNO_3	10	122, 9.3	2.87	18.60	2.04	0.90	1.32	Rodriguez-Chong (2004)
4% H_3PO_4	8	122, 300	3.00	17.60	2.60	4.00	1.20	Gamez et al. (2005)
2.5% HCl	10	140, 30	5.84	21.50	2.95	1.89	5.45	Chandel et al. (2007)
2% H_2SO_4	10	122, 24	3.00	21.60	NA	3.65	0.50	Aguilar et al. (2002)
7% H_2SO_4	5	125, 120	4.50	47.2	6.2	10.7	0.83	Nigam (2000)

NA: not applicable

2.5.2 Hydrogen production from lignocellulosic materials

Hydrogen could be produced from pretreated lignocellulosic materials using fermentative microorganisms in both pure and mixed cultures. Theoretically, 1 g of cellulose will yield 567 ml H_2 (Lui et al, 2003). The efficiency of fermentative H_2 production from the enzymatically hydrolyzed rice husk was examined with hydrogen-producing pure bacterial isolates at an initial reducing sugar concentration of 0.36 g l^{-1}. *C. butyricum* CGS5 exhibited efficient H_2 production from the rice husk hydrolysates with a cumulative H_2 production and H_2 yield of 88.1 ml l^{-1} and 19.15 mmol H_2/g reducing sugar or 17.24 mmol H_2/g cellulose, respectively (Lo et al., 2009). Fernandes et al. (2009) investigated the effects of different thermo-chemical pre-treatment methods were determined on the biodegradability and hydrolysis rate of lignocellulosic biomass. Hydrogen production by simultaneous saccharification and fermentation from steam-exploded corn straw (SECS) using *C. butyricum* AS1.209 was studied by Li, Chen (2007). The maximum specific hydrogen production rate and maximal hydrogen yield were 126 ml/g VSS d and 68 ml/g SECS, respectively (Li, Chen, 2007). Corn stalk was bio-pretreated with 7.5 g/kg of microbe additives at 25 °C with saccharification period of 15 days under the anaerobic condition. The hydrogen fermentation of hydrolysate from pretreated con stalk by *Clostridium* sp. form panda manure at pH 5.5, 36 °C and substrate concentration of 15 g/L gave the maximum H_2-production yield and rate of 176 ml H_2/g-TS and 18 ml H_2/g-TS h^{-1}, respectively, with the hydrogen content of 44.3–57.2% (v/v) (Fan et al., 2008). A co-culture of *C. acetobutylicum* X9 and *Enethanoligenens harbinense* B49 in microcrystalline cellulose for hydrogen production was investigated. Results showed that *E. harbinense* B49 could produce hydrogen efficiently from monosaccharides but not cellulose and the bioaugmentation of both strains improved hydrogen production from microcrystalline cellulose from 755 ml H_2/L-medium to 1,810 ml H_2/L-medium (Ren et al., 2008). *Thermoanaerobacterium* produced hydrogen that peaked at 7.56 mg H_2 g^{-1} cellulose from a 5 g cellulose l^{-1} suspension maintained at pH 6.5 and 55 °C (Wang et al.,2008) *Clostridium* sp. strain no. 2, isolated from termite, could produce hydrogen from oat spelts xylan with the yield of 18.6 mmol per gram substrate (Taguchi et al., 1995). This *Clostridium* strain was then used to produce hydrogen from hydrolyzed cellulose. During 81 h stationary culture in an aqueous two

phase system, the bacterium consumed 0.92 mmol glucose/h and produced 4.1 mmol H_2/h (Taguchi et al., 1996). *C. thermocellum* was used for hydrogen production using delignified wood fibers and substrate. The hydrogen yield of 1.6 mol H_2/mol glucose could be achieved in which the bacterial cell has to adhere to the cellulose to efficient hydrolysis of cellulosic materials (Bayer et al., 1983). Hydrogen production using heat treated anaerobic sludge as inoculum was studied by Lay (2001). Results indicated that an increase in cellulose concentration from 12.5 g/L to 50 g/L resulted in a lower yield from 2.18 mmol/g cellulose to 0.42 mmol/g cellulose, respectively. The higher temperature resulted in a higher conversion of cellulose to hydrogen, which was from 43 ml H_2/g cellulose at 37 °C to 69 ml H_2/g cellulose at 55 °C. The maximum hydrogen yield was obtained at 102 ml H_2/g cellulose at pH 6.5 and 55 °C (Lay, 2001). The relationship of reducing sugar in cellulose hydrolysate and hydrogen produced in a sequencing bacterial hydrolysis and dark fermentation was reported by Lo et al. (2008). Lignocellulosic materials were initially degraded by *Cellulomonas* sp. rich sludge, and the reducing sugar-rich hydrolysate was readily used by pure *Clostridium* sp. for hydrogen production. With an initial reducing sugar concentration of 0.8 g/L, the hydrogen production and yield were approximately 23.8 ml H_2/L and 1.21 mmol H_2/g-reducing sugar (1.09 mmol H_2/g cellulose), respectively (Lo et al., 2008). Acid pretreatment of lignocellulosic materials by using diluted HCl could increase hydrogen production approximately 10 to 136-fold in comparison to hydrogen production from raw waste (Fan et al., 2006; Zhang et al., 2007). Table 13 showed the recent hydrogen production from hydrolysate of lignocellulosic material.

Table 13 Hydrogen production from hydrolysate lignocelluloses

Lignocellulosic treatment	Seed inoculum	Substrate	Hydrogen yield	Reference
0.2% HCl	Cow dung composts	Cornstalk	149.69 mL H_2/g TVS	Zhang et al. (2007)
Adjusted pH 3 with H_2SO_4	*Rhodobacter spp.* *C. butyricum*	Ground Wheat	1.23 mol H_2/mol glucose	Kapan et al. (2008)
0.5% H_2SO_4		Sugarcane bagasse	1.73 mol H_2/mol sugar	Pattra et al. (2008)
2% HCl	Cow dung composts	Wheat straw	68.1 mL H_2/g TVS	Fan et al. (2006)
Augmentation with *C. paraputrificum*	Anaerobic sludge from UASB reactor	Corn stalk	2.61 mol (63.7 l) hydrogen/ kg corn stalk	Lu et al. (2009)

2.6 Continuously stirred tank reactors (CSTR) for bio-hydrogen production

Large-scale hydrogen production requires continuous mode of operation for practical engineering reasons. Continuously stirred tank reactors are known as a well mixed reactor to enhance homogenous mass transfer of substrate cell of the inoculum (Wang, Wan, 2009). The CSTR are easily visualized as vessels or tanks that are stirred to achieve uniformity throughout the tank. It is composed of a reactor and a mixer such as a stirrer, a turbine wing or a propeller as shown in Figure 6. The advantages of CSTR include good temperature control, simplicity of construction, low operating (labor) cost and easy to clean and maintain.

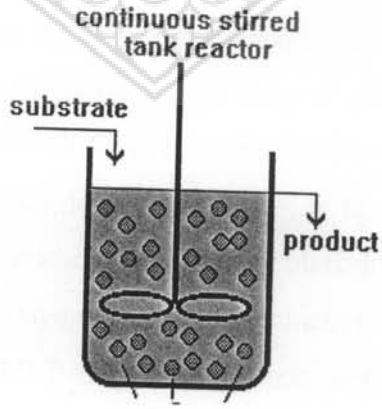

Figure 6 Continuous stirred tank reactor (Arooj et al., 2008).

Tables 14 showed the studies using CSTR for continuous hydrogen production. Hydraulic retention time (HRT) is the one of important factor for hydrogen fermentation in CSTR. Too long HRT results in the accumulation of product which could decrease the efficiency of hydrogen production by product inhibition effect. In another way, too short HRT can result in the higher organic loading rate over the range of CSTR capacity which can cause the decrease in the performance of CSTR on hydrogen production. In addition the shorter HRT directly causes a greater dilution rate which might result in a wash-out of the seed from the reactor (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 h, while the optimal HRT for a CSTR using reported by Arooj et al. (2008) was 12 h. The possible reason for this disagreement was the difference among these studied in the terms of inoculum, substrate and HRT range studied.

2.7 Recovery of hydrogenogenic effluent for production of fuels

Throughout the successful process of hydrogen production from sugarcane juice and SCB, large amounts of organic wastewater were generated. Wastewater contained residual organic matter such as butyric and acetic acids and residual sugar, which were valuable as substrates for methane and ethanol productions.

2.7.1 Methane production

Anaerobic digestion is a biological process known for energy recovery, especially methane from wastewater. Not only are a recover of biogas but also volume reduction and waste stabilization are considered as its advantages (Wang et al., 2008). A two-stage anaerobic digestion process for producing hydrogen and methane from organic material has been reported (Cooney et al., 2007; Liu et al., 2006; Ting, Lee, 2007; Ueno et al., 2007; Xie et al., 2008; Zhu et al., 2008). In the first stage, the acidogenic bacteria convert substrate to hydrogen, carbon dioxide and Volatile Fatty Acids (VFAs). Then the acetogenic and methanogens convert VFAs to mainly carbondioxide and methane in the second stage Figure 7 (Metcalf, Eddy, 2003).

Table 14 CSTR used for biohydrogen production

Inoculum	Substrate	HRT (h)		Optimal hydrogen value	Reference
		Range studied	Optimal		
Municipal sewage sludge	Glucose	0.5–2	0.5	Maximum hydrogen yield (1.81 mol/mol glucose)	Zhang et al. (2007)
C. butyricum strain SC-E1	Glucose	24-4	8	2.2 mol H_2/mol hexose	Kataoka et al. (1997)
Anaerobic sludge	Glucose	2–12	4	Maximum hydrogen production rate (115.68 mmol/d)	Gavala et al. (2006)
Municipal sewage sludge	Sucrose	2–12	4	Maximum hydrogen yield (4.70 mol/mol sucrose)	Chen et al. (2008)
Municipal sewage sludge	Sucrose	2–13.3	8	Maximum hydrogen yield (4.52 mol/mol sucrose)	Chen, Lin (2003)
Municipal sewage sludge	Fructose	2–8	8	Maximum hydrogen yield (1.68 mol/mol hexose)	Lee et al. (2007)
Anaerobic sludge	Starch	2–12	12	Maximum hydrogen yield (1.5 mol/mol hexose)	Lin et al. (2008a)
Anaerobically digested sludge	Glucose	6–12	10	Maximum hydrogen yield (1.95 mol/mol glucose)	Zhang et al. (2006)
Anaerobic sludge	Glucose	4–12	10	Maximum hydrogen yield (1.63 mol/mol glucose)	Wu et al. (2008a)
Municipal sewage sludge	Xylose	4–12	12	Maximum hydrogen yield (1.63 mol/mol xylose)	Wu et al. (2008b)
Municipal sewage sludge	Glucose	4–12	12	Maximum hydrogen yield (1.36 mol/mol hexose)	Lee et al. (2007)
Municipal sewage sludge	Sucrose	2–12	12	Maximum hydrogen yield (1.60 mol/mol hexose)	Lee et al. (2007)
Anaerobic digester sludge	Starch	4–18	12	Maximum hydrogen yield (0.92 mol/mol glucose)	Arooj et al. (2008)
Municipal sewage sludge	Xylose	2-6	6	Maximum hydrogen yield (0.8 mol/mol xylose)	Wu et al. (2008b)

(Wang, Wan, 2009)

In order to effectively produce methane, there is a need to study the factors affecting methane production process e.g. substrate concentration, temperature, pH, and metal ion (Wang et al., 2008). High concentration of VFAs was reported to inhibit methane production from VFAs by mixed anaerobic microorganisms (Siegert, Banks, 2005). The growth rate of methanogen can be greatly reduced at the pH value of lower than 6.6 (Mosey, Fernandes, 1989). An excessively alkaline pH can lead to disintegration of microbial granules and subsequent failure of the digestion process (Sandberg, Ahring, 1992). The optimum pH range for anaerobic digestion producing methane is 6.8-7.2 (Ward et al., 2008). Buffer capacity is often referred to an alkalinity in anaerobic digestion system, which is the equilibrium of carbon dioxide and bicarbonate ions providing a resistance to significant and rapid changes in pH of the system. Buffer capacity is proportional to the concentration of bicarbonate, therefore, $NaHCO_3$ has been wildly used to create buffer system during anaerobic digestion process (Guwy et al., 1997). Speece (1996) found that alkalinity to COD concentration ratio (w/w) of 1.2-1.6 was required for sufficiently maintained the pH to be approximately 6.6 in anaerobic digestion of carbohydrate waste to produce methane. Tables 15 shows factors affecting methane production from hydrogenogenic effluent i.e., effluent from hydrogen production.

Table 15 Factors affecting methane production

Inoculum	Reactor type	Factor			Optimal production value	Reference
Anaerobic granular sludge	Batch	Effluent from biohydrogen process (7.77 gVS/L)	Ratio of inoculum to substrate (2.81)	Calcium concentration (380.82 mg/L)	565.76 mL CH_4/g VS_{add}	Wang et al. (2008)
Digested sludge	continuous	Effluent from biohydrogen process (5.0 gVS/L)	HRT (90 h) OLR (2.94 kg VS/m^3.day)	pH 7	225 L/kg TS	Zhu et al. (2008)
Anaerobic granular sludge	continuous	Effluent from biohydrogen process		Solid retention time (RST) (26.67 day)	0.546 m^3 CH_4/kg VS	Wang et al. (2009)

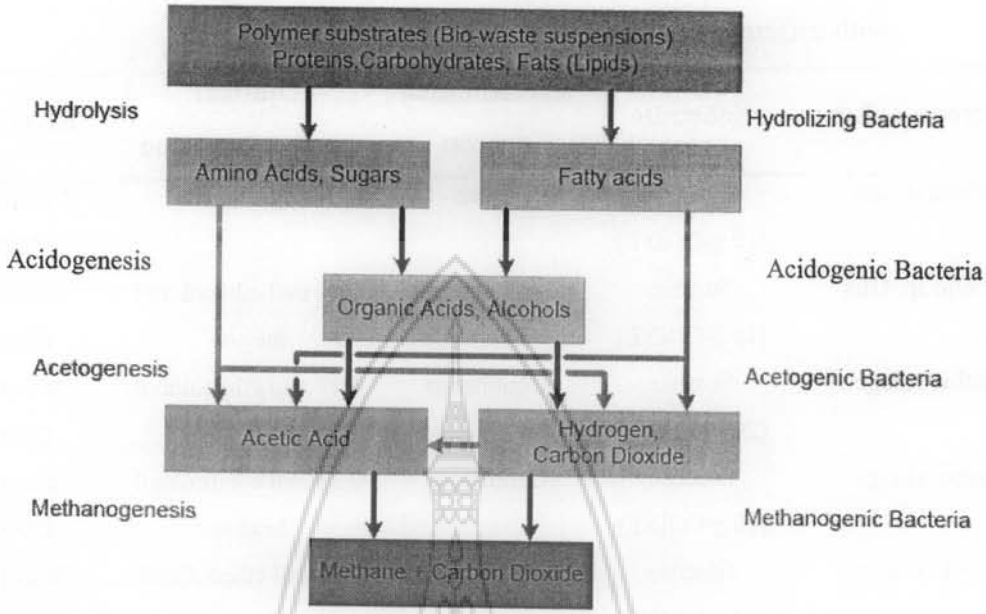

Figure 7 Anaerobic digestion process (Metcalf, Eddy, 2003).

2.7.2 Ethanol production

Ethanol is one of alternative fuels. The use of ethanol produced from biomass offers significant greenhouse gas benefits (Niven, 2005). In anaerobic fermentation, some of obligate anaerobes and facultative anaerobes under anaerobic condition, as a acidification, are able to convert carbohydrates to volatile fatty acids (VFAs) such as acetic (HAc), propionic (HPr) and butyric (HBu) acids together with a gas phase containing carbondioxide and hydrogen (Metcalf, Eddy, 2003). These bacteria can further produce ethanol from a reduction of the carboxylic group of a VFAs produced (Steinbusch et al., 2008). The conventional method for bioethanol production depends on the use of raw materials containing high levels of fermentable sugar (Steinbusch et al., 2008). Therefore, ethanol is also being a promising alternative fuels for the present energy crisis. Table 16 shows comparison of ethanol production in various types of microorganism with different substrate.

Table 16 Comparison of ethanol production by various types of microorganisms with different substrate under anaerobic condition

Microorganism	Substrate	Fermentation process	Optimal production value	Reference
Cow dung sludge	Xylose (20 g-COD/L)	Batch	4.4 g/L ethanol	Lin, Hung (2008)
Klebsiella sp. HE1	Sucrose (10 g-COD/L)	Batch	0.81 mol ethanol/mol sucrose	Wu et al. (2008)
Anaerobic sludge	Sucrose (20 g-COD/L)	Continuous	0.49 mol ethanol/mol hexose	Wu et al. (2007)
Anaerobic sludge	Glucose (20 g-COD/L)	Continuous	0.33 mol ethanol/mol hexose	Wu et al. (2007)
Anaerobic sludge	Glucose (20 g-COD/L)	Continuous	0.65mol ethanol/mol hexose	Wu et al. (2007)
Anaerobic sludge	VFAs (50mM VFAs)	Batch	0.9 mmol ethanol /L/day	Steinbusch et al. (2008)

2.8 Microbial Community of bio hydrogen producer

Fermentative hydrogen production is affected by many parameters such as pH, hydraulic retention time, temperature and feedstock concentration as well as the nature of the microbial community. Molecular ecology technology, such as 16 rRNA gene cloning and sequencing (Amann, 1995), Fluorescence *In Situ* Hybridization (FISH) (Amann, 1995) and Denaturing Gradient Gel Electrophoresis (DGGE) (Muyzer et al., 1993; Muyzer, Smalla, 1998) are nowadays the most powerful tools available to assess the community of hydrogen producer.

Hydrogen producing bacteria can be classified into 4 major groups: strictly anaerobes, facultative anaerobes, aerobes and photosynthetic bacteria (Nandi, Sengupta, 1998). *Clostridium* species are capable of producing hydrogen, including *C. butylicum* (Kataoka et al., 1997; Karube et al., 1982), *C. acetobutylicum* (Esteso et al., 1996), *C. kluyveri* (Benemann et al., 1973), *C. roseum* (Fang et al., 2006), *C. histolyticum* (Zhang et al., 2003) and *C. pasteurianum* (McTavish, 1998). Some species are known as acidophilic bacteria. It has been reported that *C. acetobutylicum* and *C. butylicum* can grow at pH range 4.0-4.3. Acidophilic *Enterobacter aerogenes* strain HO-39 capable of producing hydrogen at the pH 4.0 has been investigated by

Yokoi et al. (1997). Lin, Lay (2005) determined the microflora hydrogen activity for hydrogen fermentation from sucrose using completely stirred tank bioreactor. Hydrogen gas content, hydrogen productivity and hydrogen rate were HRT dependent and their values ranged 38.7-45.9%, 0.9-3.5 mol H_2/mol sucrose and 263-408 mmol H_2/L day, respectively, with HRT of 4 h having peak hydrogen production. The biomass activity was also HRT-dependent with each gram of biomass producing 65-145 mmol H_2/day. The DGGE analysis shows that the microbial species shifted during the HRT-reduction operation but *C. ramosum* was dominant. Fang et al. (2005) investigated the feasibility of hydrogen production from acetate and butyrate by a mixed phototrophic culture. The microbial community of the phototropic sludge was analyzed using DGGE profiles of 16S rDNA fragments and FISH-method. Results showed that phototrophic hydrogen producing sludges comprised of predominant species resembling *Rhodobacter capsulatus* with over 80% relative abundance. Recently, Lin et al. (2006) investigated 16s rDNA gene for monitoring bacterial community shift and bacterial identification on fermentative hydrogen production from xylose using natural mixed culture. The bacterial community of the incubated sewage sludge comprised mainly of *Clostridium* species, *Klebsiella* sp. and *Streptococus* sp. Shin et al. (2004) investigated and identified the hydrogen producing microorganism from food waste in anaerobic mesophilic and thermophilic acidogenesis. Hydrogen producing microorganisms of *Thermoanaerobacterium thermosaccharolytium* and *Desulfotomaculum geothermicum* were detected from the thermophilic acidogenic culture while *Thermotogales* strain and *Bacillus* species were obtained from the mesophilic acidogenic culture by PCR-DGGE analysis.

The hydrogen producing bacteria isolated from anaerobically digested sludge under anaerobic condition using 16S rDNA analysis were identified as *Clostridium* sp., *Bacillus* sp., *Enterobacter* sp., *Escherichia Coli*, *Rhodoseudomonas palustris*, *Rhodobacter sphaeroides*, *Desulfovibrio vulgaris* and *D. desulfuricans*. The result found that *Bacillus coagulans* strain IIT-BT was the most suitable candidate for fermentative hydrogen production due to its highest capacity of produce hydrogen (Kotay, Das, 2007). Recently, Fang et al. (2006) showed hydrogen production from rice slurry that was found most effective at pH 4.5, 37°C treating a slurry containing 5.5 g-carbohydrate/L. An anaerobic sludge was used as seed after a 100°C heat

treatment for 30 mins. Based on the 16S rDNA analysis, the PCR-amplified products were then screened using DGGE to investigate the microbial population variations and the results showed that *Clostridium* sp. was comprised mainly in this heat treated sludge. In addition, Lin et al. (2008a) reported that hydrogen production from starch with anaerobic sludge as seed inoculums had *C. butyricum, C. pasteurianum, Klebshilla pneumoniae, Streptococcus* sp. and *Pseudomonas* sp. as dominant species.

2.9 Response Surface Methodology (RSM) and Central Composite Design (CCD)

To facilitate the study on the interactive effect, a statistical experiment design techniques using the response surface methodology (RSM) is widely employed (Wang et al., 2005; Pan et al., 2008). These techniques provide statistical models which helpful for understanding the interactions between the parameters at varying levels and calculating the optimal level of each parameter for a response target (Wang et al., 2008). An improvement of product yield, a reduction in process variability, a closer confirmation of the output response and a reduction in the experimental time and overall costs are the results of the statistical approach (Reddy et al., 2003; Franeis et al., 2003).

2.9.1 Response Surface Methodology (RSM)

Response surface methodology (RSM) was developed by Box and collaborators in the 50s (Montgomery, 2001). This term was originated from the graphical perspective generated after fitness of the mathematical model, and its use has been widely adopted in texts on chemometrics. RSM consists of a group of mathematical and statistical techniques that are based on the fit of empirical models to the experimental data obtained in relation to experimental design. Toward this objective, linear or square polynomial functions are employed to describe the system studied and, consequently, to explore (modeling and displacing) experimental conditions until its optimization (Montgomery, 2001). Some stages in the application of RSM as an optimization technique are as follows: (i) the selection of independent variables of major effects on the system through screening studies and the delimitation of the experimental region, according to the objective of the study and the experience of the researcher; (ii) the choice of the experimental design and

carrying out the experiments according to the selected experimental matrix; (iii) the mathematic–statistical treatment of the obtained experimental data through the fit of a polynomial function; (iv) the evaluation of the model's fitness; (v) the verification of the necessity and possibility of performing a displacement in direction to the optimal region; and (vi) obtaining the optimum values for each studied variable (Montgomery, 2001).

2.9.2 Central Composite Design (CCD)

The central composite design was presented by Box and Wilson (Montgomery, 2001). This design consists of the following parts: (i) a full factorial or fractional factorial design; (ii) an additional design, often a star design in which experimental points are at a distance α from its center; and (iii) a central point. Figure 2.7a and 2.7b illustrates the full central composite design for optimization of two and three variables. Full uniformly routable central composite designs present the following characteristics: (i) require an experiment number according to N= k2 +2k+cp, where k is the factor number and (cp) is the replicate number of the central point; (ii) α -values depend on the number of variables and can be calculated by α =2(k−p)/4. For two, three, and four variables, they are, respectively, 1.41, 1.68, and 2.00; (3) all factors are studied in five levels (−α, −1, 0, +1, + α) (Montgomery, 2001).

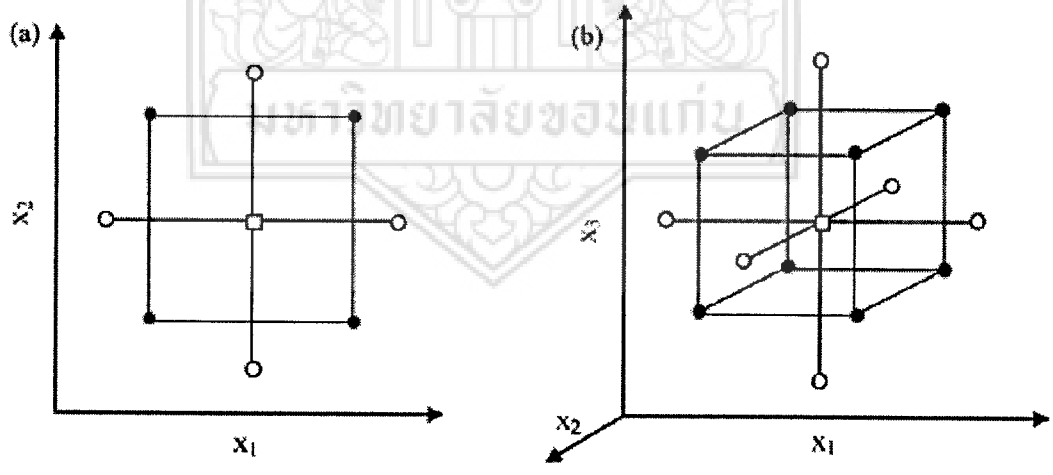

Figure 8 Central composite designs for the optimization of: (a) two variables (α= 1.41) and (b) three variables (α = 1.68) (Montgomery, 2001). (●) Points of factorial design, (○) axial points and (⊓) central point.

Figure 8a and b shows representations of central composite designs for two- and three-variable optimization, respectively. Table 17a and b presents the coded values of the experimental matrices for the application of these designs. Many applications of the central composite design in the optimization of analytical procedures can be found in the Table 18. However, the main factors on biogas production were substrate concentration, initial pH of substrate and mineral nutrient solution.

Table 17 Experimental matrices for central composite designs: (a) two variables and (b) three variables

(a)			(b)			
	X1	X2		X1	X2	X3
Factorial design	-1	-1	Factorial design	-1	-1	-1
	1	-1		1	-1	-1
	-1	1		-1	1	-1
	1	1		1	1	-1
Axial points	-α	0		-1	-1	1
	α	0		1	-1	1
	0	- α		-1	1	1
	0	α		1	1	1
Central point	0	0	Axial points	- α	0	0
				α	0	0
				0	- α	0
				0	A	0
				0	0	- α
				0	0	α
			Central point	0	0	0

2.9.3 RSM for optimizing hydrogen, ethanol and methane production

Many factors such as temperatures, initial pH and substrate concentrations can influence the fermentative hydrogen production, because they can affect the activity of some essential enzymes such as hydrogenases for fermentative hydrogen production. Thus, RSM with various designs was used to optimize the hydrogen production to obtain the appropriate values of these three factors with the

advantage of time-saving. The interactive effect among the variables and minimize the error in determining the effect of parameters can be depicted, thus it is a better method to optimize the fermentative hydrogen production process (O-Thong et al., 2008; Agun et al., 2008). The RSM with the Box–Behnken design achieved a high hydrogen production rate of 5.1 L H_2 /g dry cell.h at the glucose concentration of 102.08 mM, 35 °C and pH 6.5, and also found that all three factors had significant influences on the specific hydrogen production rate of *C. tyrobutyricum* JM1 (Jo et al., 2008b). The temperature, initial pH and glucose concentration on fermentative hydrogen production by mixed cultures from a primary anaerobic digester in batch tests was optimized using RSM with a central composite design (CCD) (Wang, Wan, 2008). Results indicated that the three factors had impact on hydrogen production individually and interactively. The maximum hydrogen yield of 289.8 mL/g glucose was obtained at 38.6 °C, the initial pH of 7.2 and the glucose concentration of 23.9 g/L. The maximum hydrogen production rate of 28.2 mL/h was estimated at 37.8 °C, the initial pH of 7.2 and the glucose concentration of 27.6 g/L (Wang, Wan, 2008). The effects of glucose concentration, temperature and pH on the hydrogen production by *E. aerogenes* were investigated in batch fermentation. Box–Behnken design and RSM and were used to determine the optimum condition to produce hydrogen. The hydrogen production rate was investigated by simultaneously changing the three independent variables in which all variables had significant influences on the hydrogen production rate. The optimum condition of glucose concentration 118.06 mM, 38 °C and pH 6.13 gave the maximum hydrogen production rate of 425.8 ml H_2/g dry cell.h (Jo et al., 2008c). Optimum pH, temperature and substrate concentration on the biohydrogen production from sucrose by mixed anaerobic cultures were optimized using RSM with CCD. Experimental results indicated that these three factors had individually significant influenced on specific hydrogen production potential and the maximum hydrogen production rate. There was a significant interaction on hydrogen production potential and maximum hydrogen production rate while substrate concentration and pH were slightly interdependent. A maximum hydrogen production potential of 252 mL H_2/g sucrose was predicted under the optimum conditions of pH 5.5, 34.8 °C and sucrose concentration of 24.8 g/L, while a maximum hydrogen production rate of

1511 mL H_2/h was estimated under the optimum conditions of pH 5.5, 35.5 °C and sucrose concentration of 25.4 g/L (Mu et al., 2006).

The medium compositions could be optimized for a certain purpose by using RSM. Based on the Plackett–Burman design, the most critical for nutrient salts for hydrogen production by *Ethanoligenens harbinense* B49 were Fe^{2+} and Mg^{2+}. The Box–Behnken design was then applied to optimize the combination of the selected factors and the sole carbon source, glucose, and achieved the estimated maximum hydrogen yield of 2.21 mol/mol glucose when the concentrations of glucose, Fe^{2+} and Mg^{2+} were 14.57 g/L, 177.28 mg/L and 691.98 mg/L, respectively. The confirming test at the optimum condition obtained by RSM gave the maximum hydrogen yield of 2.20 mol/mol glucose, in which the practicability of this optimum strategy could be verified (Gou et al., 2009). RSM with CCD was conducted to optimize the hydrogen yield together w: · POME degradation. The results indicated that the presence of 257 mg Fe^{2+}/L, a C/N ratio of 74 and a C/P ratio of 559 were optimal for simultaneous hydrogen production and COD removal. The predicted maximum simultaneous hydrogen production and COD removal were 6.5 L H_2/L-POME and 58%, respectively. In a confirmation experiment at the optimum conditions, a hydrogen production and COD removal efficiency were 63± 0.142 H_2/L-POME and 55±1.5%, respectively. The total carbohydrate conversion was 92±2.7%. The hydrogen production rate reached 25.9 mmol H_2/L.d and increased by 60% in comparison to the use of raw POME (O-Thong et al., 2008).

RSM is also can be used to optimize the production of target product in the reactor system. Hydrogen production from sucrose in a granule-based upflow anaerobic sludge blanket (UASB) reactor was optimized by using RSM with CCD. Experimental results show that the hydrogen yield was individually dependent on influent sucrose concentration and HRT ($P<0.05$), while their interactive effect on the hydrogen yield was not significant ($p \geq 0.05$). The maximum hydrogen yield of 1.62 mol-H_2/mol-hexose was obtained under the optimum conditions of 14.5 g/L influent sucrose concentration and 16.4 h-HRT (Zhao et al., 2008).

There have some research on the optimization of ethanol and methane production by using RSM. Based on the 2^3 factorial CCD, RSM was used to optimize the conditions of enzymatic saccharification and ethanol fermentation using food

waste. The optimum conditions for enzymatic saccharification were pH of 5.20, reaction temperature of 46.3 °C and enzyme concentration of 0.16% (v/v) and for ethanol fermentation were pH of 6.85, fermentation temperature of 35.3 °C and fermentation time of 14 h. The maximum concentration of reducing sugar and ethanol under the optimum conditions were 117.0 g reducing sugar/L and 57.6 g EtOH/L, respectively. Experimental results were in close agreement with model prediction with 120.1 g reducing sugar/L and 57.5 g EtOH/L, respectively (Kim et al., 2008). The optimization of the conditions for simultaneous saccharification and fermentation for ethanol production from kitchen garbage in open and close fermentation was also investigated. Maximum ethanol concentration of 33.05 g/L was obtained with the optimum fermentation time of 67.60 h, 35 °C and pH of 4.18. The linear effect of pH, cross-effect between time and pH, square effects of time and pH showed trivial influence on ethanol production. The conformation experiment under the optimum conditions agreed well with the model predictions with the ethanol concentration of with 34.21 and 33.05 g/L for open and close fermentation, respectively (Wang et al., 2008).

RSM with CCD was employed to study the individual effects and interactive effects of substrate concentration, ratio of inoculum to substrate and Ca^{2+} concentration on methane production from the hydrogenogenic effluent, effluent from hydrogen fermentation, by anaerobic granular sludge from UASB (Xing et al., 2008). The results showed that the effects of substrate concentration, Ca^{2+} concentration and ratio of inoculum to substrate were individually statistically significant ($p<0.05$), however, the interactive effect of substrate concentration and Ca^{2+} concentration, ratio of inoculum to substrate and were found to be insignificant ($p\geq0.05$). The verification experiment confirmed that the RSM was useful to optimize the methane yield from hydrogenogenic effluent with the yield of 557.23 ml CH_4/g VSS added which was not much different from the estimated value obtained from RSM (565.76 ml CH_4/g VSS) (Xing et al., 2008). The examples of biogas production optimization using response surface methodology (RSM) are shown in Table 19

Table 18 Optimization factors for biogas production using Response Surface Methodology (RSM)

Substrate	Microorganism	Optimum factor	Optimal production value	Reference
Glucose	*Clostridium* sp. Fanp2	23.75 g/L glucose, 0.159 M phosphate buffer and 13.3 mL/L vitamin solution at 36±1 °C	175.4 mL H_2/g substrate	Pan et al. (2008)
Sucrose	Mixed anaerobic cultures	24.8 g/L sucrose, pH 5.5 at 34.8°C 25 g/L total sugar, initial pH 4.78 and 1.45 g/L $FeSO_4$ at room temperature (30-32 °C)	84.0 mL H_2/g substrate	Mu et al. (2006)
Sweet sorghum syrup	Heat-treated sludge	Effluent from biohydrogen process (7.77 g VS/L), Ratio of inoculum to substrate (2.81), Calcium concentration (380.82 mg/L)	275.9 mL H_2/g substrate	Sarapirom, Reungsang (2008)
Hydrogenogenic effluent	Anaerobic granular sludge		565.76 mL CH_4/g VS_{add}	Wang et al. (2005)