CHAPTER II

LITERATURE REVIEW

2.1 Literature Review

The fossil fuel accounts for the majority of anthropogenic greenhouse gas emissions released into the atmosphere. Transportation sector and electric power plants are significant contributors to the global air quality problems. Many experts have indicated that if highly populated countries like China, India and Indonesia were to adopt energy consumption patterns similar to those of the United States or Europe, world energy supplies would have to increase enormously to meet demand. These will eventual lead to the future depletion of limited fossil energy resources. Research into new energy sources that would replace fossil fuels started 40 years ago, and is finally starting to yield tangible results. Hydrogen is expected as the energy for future fuel. The combination of hydrogen and fuel cell technologies can generate electricity and thermal energy, with only water as a by- product, resulting in a global no net carbon-emission. Hydrogen, although the most abundant element in the universe, it is naturally exist in small quantities, is not sufficient to end-use application for hydrogen fuel cells. It must be produced from other compounds such as fossil fuel, biomass or wastes. The method of hydrogen production can be roughly categorized as (1) thermochemical, (2) electrochemical and (3) biological processes. The former two methods are known as intensive energy consumption whereas the biological hydrogen production, so called biohydrogen, requires less energy, which makes them the most attractive with regard to both cost saving and preserving the environment.

The fuel cell is an energy converter which permits the production of electric power from fuel and oxygen. Fuel cells utilize the chemical energy of fuel such as hydrogen to produce electricity, and only heat and water are emitted as the by-product. Fuel cells a characterized by their electrolyte, operating temperature and level of hydrogen purity required. The following table summarizes the characteristics of various fuel cell types.

Table 2-1 Fuel cell

Type	Electrolyte	Operating Temperature	Sensitivities to Hydrogen Purity
Proton Exchange Membrane (PEM)	Solid organic polymer polyperfluorosulfonic Acid	60 – 100 °C	High sensitivities to impurities, must have < 1 0 ppm CO
Alkaline	Aqueous solution of potassium hydroxide soaked in a matrix	90 – 100 °C	High sensitivity to carbon dioxide
Phosphoric Acid	Liquid phosphoric acid soaked in a matrix	175 – 200 °C	Sensitive to CO
Molten Carbonate	Liquid solution or lithium, sodium and/or potassium carbonates, soaked in a matrix	600–1000 °C	Low sensitivity to CO, Hydrogen/carbon monoxide mixtures can be used. CO_2 is required
Solid Oxide	Solid zirconium oxide to which a small amount of ytriam is added	600–1000 °C	Low sensitivity to CO, Hydrogen/ carbon dioxide/ methane mixtures can be used

2.2 Biological hydrogen production

Different microorganisms participate in the biological hydrogen generation system such as green algae, cyanobacteria (or blue-green algae), photosynthetic bacteria and fermentative bacteria, which are tabulated in Table 2-2, along with their potential in hydrogen generation and size of bioreactor required to power PEM fuel cells (Table 2-3). About 50 years ago Gaffron *et al.* discovered that the eukaryotic unicellular green algae, *Scenedesmus obliquus*, is able to evolve molecular hydrogen by means of a hydrogenase in the light under anaerobic condition (Gaffron and Rubin,

1992). This is called direct biophotolysis. It is possible to maintain the direct biophotolysis processes are the paths followed by cyanobacteria. In this system, photosynthesis (O_2 evolution and CO_2 fixation) and N_2-fixation (thus H_2 production) are either spatially or temporally separated from each other. The spatial separation is achieved by differentiation of two cell type "vegetative" cells, which carry out normal photosynthesis and provide the nitrogen fixing "heterocysts" with the reductant (carbohydrate) required by nitrogenase. In the heterocyst, nitrogenase is protected from O_2 by a heavy cell wall, that reduces O_2 diffusion, and by high rates of respiration, absorbing an residual oxygen (Weare and Benemann, 1874). Importance of hydrogenase present in fermentative bacteria for H_2 generation has been pointed out by several researchers (Woodward *et al.*, 1996; Baron *et al.,* 1997)

Table 2-2 Overview of biological hydrogen production processes

Process	General reaction	Microorganisms
Direct biophotolysis	$2H_2O \xrightarrow{\text{light energy}} 2H_2 + O_2$	Green algae
Indirect biophotolysis	$12H_2O + 6CO_2 \xrightarrow{\text{light energy}} C_6H_{12}O_6 + 6O_2$ $C_6H_{12}O_6 + 12H_2O \xrightarrow{\text{light energy}} 12H_2 + 6CO_2$	Blue-green algae (cyanobacteria)
Photo-fermentation	$CH_3COOH + 2H_2O \xrightarrow{\text{light energy}} 4H_2 + 2CO_2$	Purple non-sulphur bacteria
Water-gas shift reaction	$CO + H_2O \longrightarrow CO_2 + H_2$	Photoheterophic bacteria, Purple non-sulphur bacteria
High-yield dark fermentation	$C_6H_{12}O_6 + 6H_2O \longrightarrow 12H_2 + 6CO_2$	Fermentative bacteria

Table 2-3 Comparison of H_2 production rates from various biohydrogen system and size of bioreactor required to power PEM fuel cells

System	H_2 production rate	Size of bioreactor (Hussy *et al.*) required to power a fuel cell of:			
	m molH_2/L.h	1.0 kW	1.5 kW	2.5 kW	5.0 kW
Direct photolysis	0.07	$3.41x10^5$	$5.12 x10^5$	$8.56 x10^5$	$1.71x10^6$
Indirect photolysis	0.355	$6.73 x10^4$	$1.01 x10^5$	$1.69 x10^5$	$3.37x10^5$
Photo-fermentation	0.16	$1.49 x10^5$	$2.24 x10^5$	$3.74 x10^5$	$7.58x10^5$
Water-Gas-Shift of CO	0.96	$2.49 x10^2$	$3.74 x10^2$	$6.24 x10^2$	$1.25x10^3$
Dark fermentation min.	121.0	$1.98 x10^2$	$2.97 x10^2$	$4.95 x10^2$	$9.89x10^2$
Dark fermentation max.	8.0	$2.91 x10^3$	$4.38 x10^3$	$7.31 x10^3$	$1.46x10^4$

Source: Levin *et al.*, 2004

2.3 Dark fermentation

Dark fermentation is the common anaerobic fermentation which similar to the anaerobic digestion process. The term "dark" let us known that this fermentation need no light energy in the metabolic pathway. The theoretical maximum H_2 yield of 12 mol/mol glucose fermented has been expected to gain by dark fermentation (see Table 2-2). In practice yields are always lower, as some organic substants are inclined to be converted to cell biomass and end products like acetic and/or butyric acid, not H_2 according to thermodynamic reason. That is why the second-stage is required to convert these end-products to improve amount of H_2 nearby to the maximum value or to other forms of energy, for example, anaerobic digestion to methane, photo-fermentation or microbial fuel cell (Hawkes *et al.*, 2007). Table 2-4 summarizes literature studies on sequential and combined dark and photo-fermentation processes.

Table 2-4 Yields and production rates of bio-hydrogen by the sequential and combined dark-photo fermentations

Fermentation type	Organisms	Carbon source	Organic acid	Total H_2 yield Mol/mol glucose	Specific H_2 Production Rate (SHPR)
Sequential dark-photo fermentation	C. *buytricum*, E. *aerogenes*, *Rhodobacter* sp. M-19	Sweet potato starch residue	Acetic, butyric, lactic	7	
	C. *buytricum*, E. *aerogenes*, *Rhotobacter* sp. M-19	Starch manufacturing wastes	Acetic, butyric, lactic	7.2	
	Lactobacillus amylovorus, R. *marinum* A-501	Algal biomass (D. *tertiolecta*)	Lactic acid		2.47 mmol/L culture h
	Mixed anaerobic culture, R. *sphaeroides* RV	Solid waste	Lactic acid		~ 110 mL/g DW h
Combined dark-photo fermentation	C. *buytricum*, *Rhodobacter* sp. M-19	Starch	-	6.6	
	Lactobacilus amylovorus, R. *marinum* A-501	Algal biomass (D. *tertiolecta*)	Lactic acid		1.55 mmol/ L culture h
	V. *fluvialis*, R. *Marinum* A-501	Algal biomass (C. *reindhartii*)	Lactic acid		1.18 mmol/L culture h

Source: (Kapdan and Kargi, 2006)

2.3.1 Biochemistry of dark fermentation

As previously mentioned, dark fermentation has the same principal of anaerobic digestion process. A wide variety of microbial groups can survive by utilizing free energy (ATP) derived from glucose oxidation in the cytosol. The pathway of glucose consumption starts with their oxidation via glycolysis pathway to pyruvate, which is then oxidized by either pyruvate: formate lyase (PFL) or pyruvate: ferredoxin oxidoreductase (PFOR) to acetyl – CoA, reduced ferredoxin ($Fd_{red.}$) is further oxidized and converted to H_2, CO_2 and various intermediate products such a acetate, butyrate, lactate or ethanol, dependent on liberation of microbial enzyme. Under anaerobic or anoxic circumstance, the enteric bacteria (e.g., *Enterobacter aerogenes*) can derive both of H_2 and ATP from formate oxidation, while the strictly anaerobes (e.g., *Clostridium sp.*) derived from $Fd_{red.}$ (Vijayaraghavan *et al.*, 2004).

According to Reddy (2007), the redox reaction in living organisms are essentially energy transfers. Often they occur together, "linked", in what are referred to as oxidation/reduction reactions. Reduction is the gain of an electron. Sometimes we also have H^+ ions along for the ride, so reduction also becomes the gain of H. Oxidation is the loss of an electron and hydrogen (Figure 2-1). In oxidation/reductions one chemical is oxidized, and its electron are passed (like a hot potato) to another chemical.

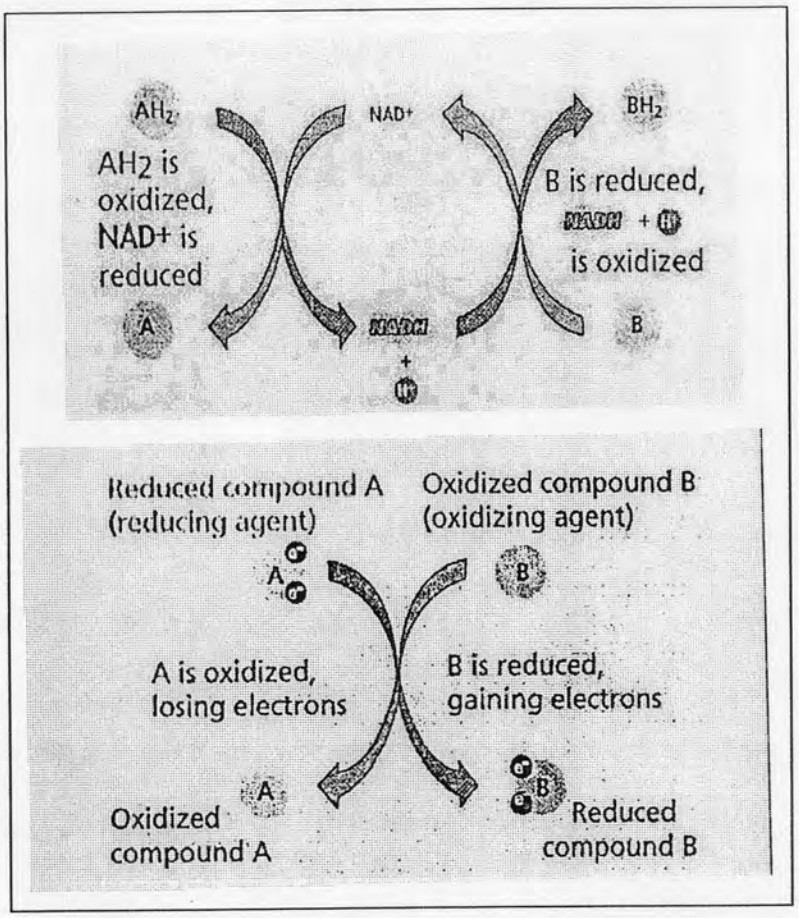

Figure 2-1 Passage of electrons from compound A to compound B. When A loses its electrons it is oxidized; when B gains the electrons it is reduced.

This formation of H_2 was claimed to be of the anaerobic machanism to maintain the redox balance in cells (Vrige and Claassen, 2003)

The example of H_2 formation in strictly anaerobes is shown in Figure 2-2.

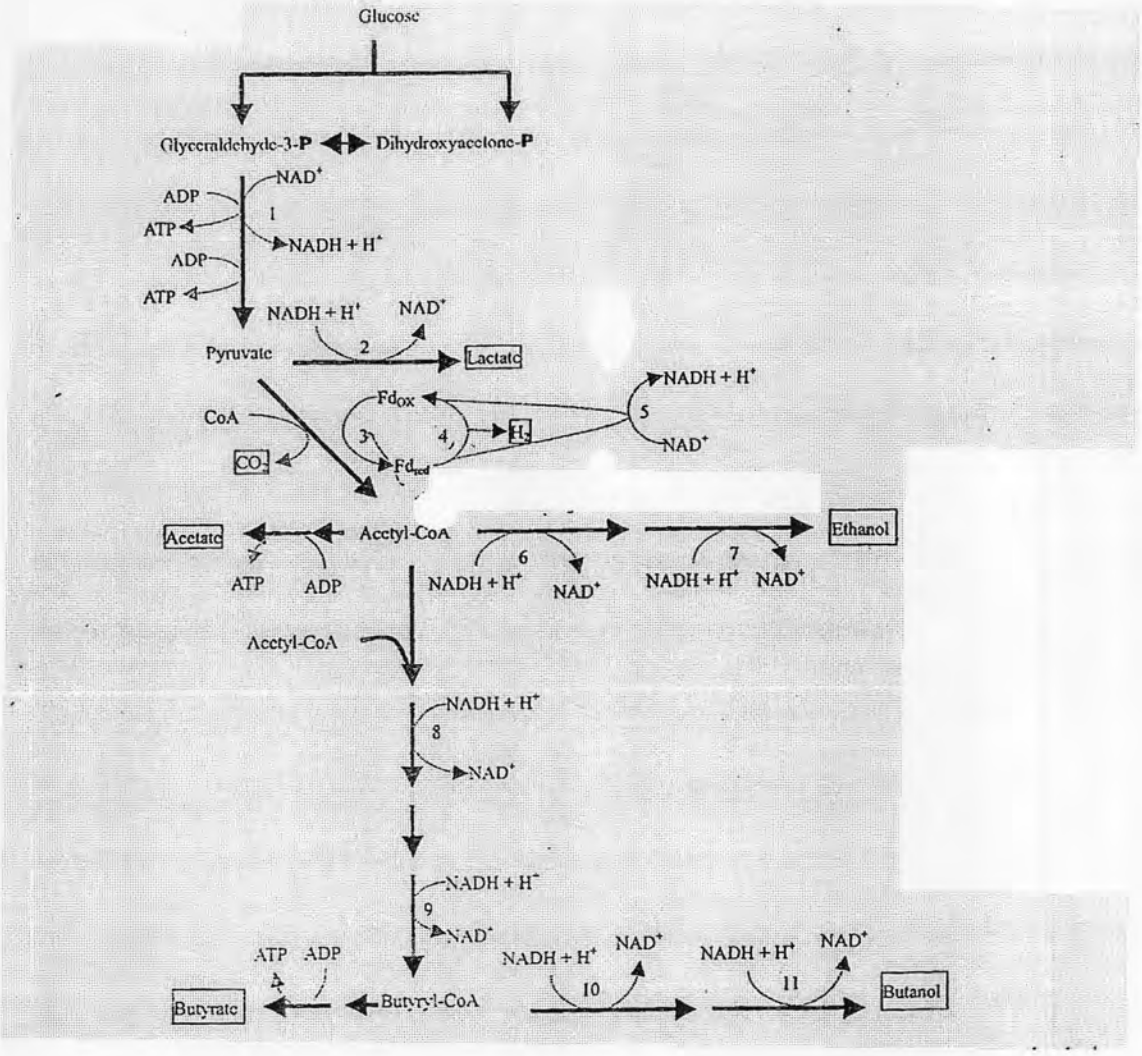

Figure 2-2 Illustrates the fermentative pathway of glucose via glycolysis by strictly anaerobic of *C. pasteurianum* species. Carbon (thick arrows) and electron (thin arrow) flow in *C. pasteurianum* during glucose fermentation. Enzymes involved in oxidation-reduction reactions are numbered as follows: 1, Glyceraldehydes-3-phosphate dehydrogenase; 2, lactate dehydrogenase; 3, pyruvate-ferredoxin oxidoreductase; 4, hydrogenase; 5, NADH-ferredoxin oxidoreductase; 6, acetaldehyde dehydrogenase; 7, ethanol dehydrogenase; 8, β- hydroxybutyryl-CoA dehydrogenase; 9, butyryl Co-A dehydrogenase; 10, butyraldehyde dehydrogenase; 11, butanol dehydrogenase. P, phosphate (Dabrock *et al.*,1992).

It can be described that during the oxidative decaboxylation of pyruvate, abundant of excess electron are released from the metabolism which always referred to as reducing equivalents: reduced nicotinamide adenine dinucleotide (NADH) (Figure 2-3) and reduced ferredoxin (Fd_{red}) are disposed by microbial oxidation in the form of molecular hydrogen via the reaction: $2H^+ + 2e^- \longleftrightarrow H_2$

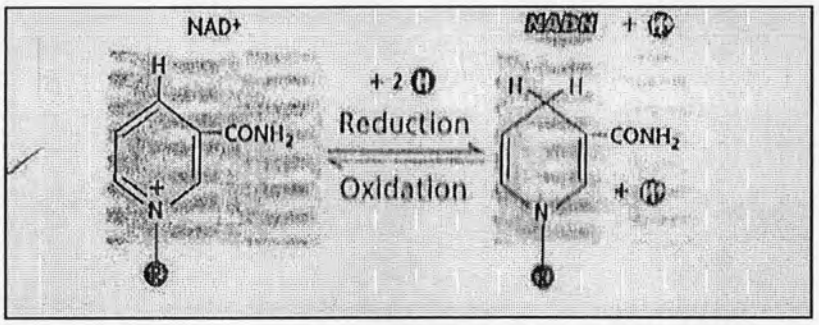

Figure 2-3 Nicotinamide Adenine Dinucleotide (NADH)

These reactions are driven by the catalytic enzymes such as PFOR, NADH: ferrodoxin oxidoreduction including hydrogenase, which can be formed commonly in H_2 producing anaerobes (Simon and Albracht, 2003; Hallenbeck and Benemaan, 2005). Therefore hydrogen production is the mean by which bacteria lose excess electrons (Mizuno *et al.*, 2000) Confirmation by Hawkes *et al.* (2007), that the transformation of electron from Fd_{red} or NADH to proton (H^+) to regenerate the oxidized form of Fd_{ox} or NAD^+ in the anaerobic metabolism is the way to enable the process proceeds to generate free energy (ATP) in the organisms.

2.3.2 H_2 producing microorganism

There are a large number of microorganisms that can produce biological H_2 production by fermenting various kind of organic substrates (chemoheterotrophs). Among these, strictly and facultative anaerobes are the most efficient H_2 producers such as Clostridia, Bacillus and Enterobacter.

The different results of H_2 production from various kinds of microbial species can be categorized into 3 groups as follow:

1. Strictly anaerobes
2. Facultative anaerobes
3. Mixed culture

1) Strictly anaerobes

1.1 Clostridium

Clostridiums are obligate anaerobes and spore forming organisms. Pure cultured organisms are known to produce the higher H_2 yield from a wide range of carbohydrate rich sources, cellulose and hemi cellulose. In batch growth, Clostridia can produce the highest H_2 production during the lag or exponential growth phase until entering the stationary phase, H_2 production terminated, as a result of the metabolic shift from H_2/ VFAs production pathway to the formation pathway of alcohol.

Among the Clostridium sp., C. *pasteurianum*, C. *butyricum,* and C. *beijierinskii* are formed to give high H_2 production while C. *propionicum* gives low (Levin *et al.*, 2004).

1.2 Bacillus

The other pure strain known to produce H_2 from carbohydrate is species of *Bacillus Licheniformis* (Kalia *et al.*, 1994). Clostridium and Bacillus groups are characterized by the formation of spores.

1.3 Thermophiles

Many anaerobic thermophiles (40-65 °C), extream thermophiles (65-80 °C) and hyperthermophiles (>80 °C) are known to produce H_2 from carbohydrates (Goorissem *et al.*, 2001) such as species of Anaerocellum, Spirocheta, Thermotoga and Thermoanaerobacter.

2) Facultative anaerobes

Facultative anaerobes have long been known as oxygen resistant organisms and can survive either with oxygen or without oxygen. These bacteria have the ability to recover anaerobic condition immediately after accidentally leakage of oxygen penetrated in the reactors. Indeed the facultative anaerobic bacteria have a respiratory system that uses preferentially oxygen (Muller, 2003) as an electron acceptor. The oxygen is reduced and become water as the products. But in the absent of oxygen, the other compound such as proton, NO_3, SO_4, etc, are need to act as the alternative electron accepters. These are finally converted to the end product of H_2, N_2 and H_2S (Veigi and Classen, 2003).

2.1 Enterobacter

The dominant species of E. *aerogenes* and E. *cloacae* are able to produce H_2 and energy from formate pathway via glucose formulation of biomass (Podesta *et al.*, 1997). The H_2 production by this strain itself is less susceptible to high H partial pressure as well as a small amount of oxygen in the process (Tanisho *et al.*, 2005). In

general, H_2 conversion yield from glucose is observed in lower amounts compared to that of genus Clostridium.

2.2 Aerobes

In addition, the aerobic bacteria involved in the H_2 fermentation e.g., *Aeromonas spp., Pseudomonas spp., Vibrio spp.*, are also recently reported (Oh *et al.*, 2003).

3) Mixed culture

Although pure culture of Clostridium and Facultative anaerobes have been represented as the strong H_2 producers and resistant to oxygen, respectively. So far mixed culture has been proven to be more effective for H_2 production compared to pure culture, particularly mixed culture rich in Clostridia species (Noike and Mizuno, 2000). Some advantages of using mixed cultured over pure culture are due to (1) the stability and higher yield of H_2 production (Lin *et al.*, 2006) (2) easily operating because of its similarity to a well-understood anaerobic digestion. process (3) the saving costs on hygienic process and plenty of complicated regulatory equipments required by pure culture (Reith *et al.*, 2003) and (4) more diversity of effective H_2 producers are taken from natural sources e.g., digester sludge, animal waste or soil.

However, the recovery of methanogens has still been found in the prolong period, and yet their mechanism has not been clearly known well. It still needs more information in this field, likewise the further works on a large – scale experiments with real wastes/wastewater are also needed much more.

2.3.3 H_2 yield

In general, the theoretical H_2 fermentation is dependent on (1) substrate (2) pathway of fermentation, and (3) end products. For example, when using pure culture, the H_2 production from the sole glucose metabolized is 4 mol/mol glucose via acetate pathway and 2 mol/mol glucose via butyrate pathway as the following:-

$$C_6H_{12}O_6 + 2H_2O \longrightarrow 2CH_3COOH + 2CO_2 + \mathbf{4H_2}$$

$$C_6H_{12}O_6 \longrightarrow CH_3CH_2CH_2COOH + 2CO_2 + \mathbf{2H_2}$$

Currently, the practical H_2 studies have been favored to be conducted by using mixed culture as inoculum in the modified A. D process[1], from which H_2 is generated in the two steps of hydrolysis and acidogenesis (see Appendix B) and creating a mixture of acetic acid and butyric acid as the end product as follow:-

$$C_6H_{12}O_6 + 2H_2O \longrightarrow 2CH_3COOH + 3CH_3CH_2CH_2COOH + 8CO_2 + \mathbf{10H_2}$$

Thus, the stoichiometric amount of H_2 is equal to 2.5 mol/mol of glucose fermentation. Afterward the metabolic pathway of glucose fermentation is shift away from acetate/butyrate production to the pathway of reduced products like ethanol, butanol or lactic acid, as a result of microbial enzyme activities. These reduced compounds are able to lower H_2 yield due to the addition of H atom required in their molecules.

Besides methanogens and sulfate reducing bacteria (SRB) containing in mixed culture known as H_2 consumers, lactic acid bacteria was also reported by Noike

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[1] modified A.D means the anaerobic digestion process which has no methanogens presence due to their activities are repressed.

(2002) that it is capable to form intermediate product, so called bacteriocrine, inhibiting H_2 producing bacteria.

$$C_6H_{12}O_6 \xrightarrow[\text{or } \textit{Enterococcus durans}]{\textit{Lactobacillus paracasai}} 2CH_3CHOHCOOH$$

Clostridia such as *C. aceticum*, including acidogens can lower the H_2 yield by converting $H_2 - CO_2$ to acetate or converting glucose exclusively to acetate. This process known as homoacetogenesis is according to the following:-

$$2CO_2 + 4H_2 \longrightarrow CH_3COOH + 2\,H_2O$$

$$C_6H_{12}O_6 \longrightarrow 3CH_3COOH$$

And therefore, the increasing of acetate concentration in the metabolic pathway need not cause to improve the rate of H_2 production (Kim *et al.*, 2006). Hussy *et al.*, 2003 observed a double increase in acetate production not linked to an increased H_2 yield.

The maximum theoretical H_2 yield from fermentation of carbohydrates is known as 4 mol H_2/mol hexose or glucose converted, with acetic acid as the fermentation end product. In practice H_2 production is lower, as some carbohydrate is converted to bacterial cells, and end products other than acetic acid, e.g. butyric acid, are formed. The fermentation H_2 producing process is influenced by many factors to favour H_2 producing performance; these include heat treating the inoculum to select for spore- forming clostridia. The optimizing environmental conditions such as nutrients, pH, temperature, hydraulic retention time (HRT), reducing H_2 partial pressure etc., are also taken into account to affect the H_2 production.

2.4 Factor affecting H_2 fermentation

2.4.1 Heat treatment of inoculum

Heat treatment method, sometimes called heat-shock technique is commonly used to inactivate or eliminate non-spore forming microorganism and to select H_2 producing bacteria. This technique has long been used for industrial solvent production for example acetone-butanol production, to select spores of Clostridial strains. Namely, when mixed communities of microorganism expose to unfavorable environmental conditions such as high temperature, lack of carbon or nitrogen sources or contact to toxic substances etc. H_2 producing organisms can be survived by forming endospores whereas non-spore forming bacteria (e.g. methanogens) is eliminated. When favorable condition return, the spores germinate and become vegetative cells (Doyle, 1989). Besides, heat treatment can inhibit non-spore forming bacteria, it can promote spore germination (Doyle *et al.*, 1989 and Hui *et al.*, 1994)

Several studies have used heat treatment of the inoculum as seed to H_2 production reactor as shown in Table 2-5. However, the different temperature of heat treatment had an effect on the lag phase of bacteria. The higher temperature made the longer lag periods (Krongthamchat and Dararat, 2006). The lag phase may be explained by the fact that spores need time to germinate and vegetative cell needs time to grow (after germination). Thus, in continuous operation, HRTs longer than the lag period would be employed (Doungmanee *et al.*, 2002). In addition heat treatment can prevent growth of non-spore forming propionate producers which convert glucose alone without forming hydrogen (Cohen *et al.*, 1985).

Table 2-5 Heat treatment protocol from various researchers

Inocula	Heat treatment method	References
Anaerobic digested sludge	Boiled / 15 min	Lay *et al.*, 2000
Activated sludge	Pasteurized at 80°C / 20 min	Cohen *et al.*, 1985
Compost, soils	Baking at 104 °C / 2 hr.	Van Ginkel *et al.*, 2001
Anaerobic digested sludge	Boiled at 70 °C / 20 min	Doungmanee *et al.*, 2002
Anaerobic digested sludge	Boiled / 15 min	Han and Shin, 2004
Anaerobic digested sludge	Boiled at 90 °C / 10 min	Kim *et al.*, 2006
Anaerobic digested sludge	Boiled at 100 °C / 45 min	Feng *et al.*, 2003
Anaerobic digested sludge	100 °C / 45 min	Chang and Lin, 2004

2.4.2 Effect of pH

The control of pH is a crutial factor to H_2 production due to the effect of pH on the hydrogenase activity and on the metabolic pathway (Dabrock *et al.*, 1992). Besides, pH control is also important to suppress hydrogen consumers and to obtain an enriched culture of hydrogen producing clostridia (Khanal *et al.*, 2004). The reported pH range for the maximum H_2 yield and specific H_2 production rate is between pH 5.0 and 6.0 and pH below 5.0 inhibited fermentative H_2 bacteria (Chen *et al.*, 2002; Okamoto *et al.*, 2000; Fang and Liu, 2002). Medium pH affects H_2 production yield, biogas content, type of the organic acids produced and the specific H_2 production rate. However, some investigator report the optimum pH range between 6.8 – 8.0 (Liu and Shen, 2004; Zhang *et al.*, 2003; Lay, 2001) and around pH 4.5 for the thermophilic culture (Kim, 2003). Ginkle *et al.*, (2001) demonstrated that the initial pH, between 4.5-6.5, did not have as profound effect on both hydrogen production rate and hydrogen production potential. To confirm these results, a series of batch tests conducted using two different substrates as shown in Figure 2-4 and Figure 2-5.

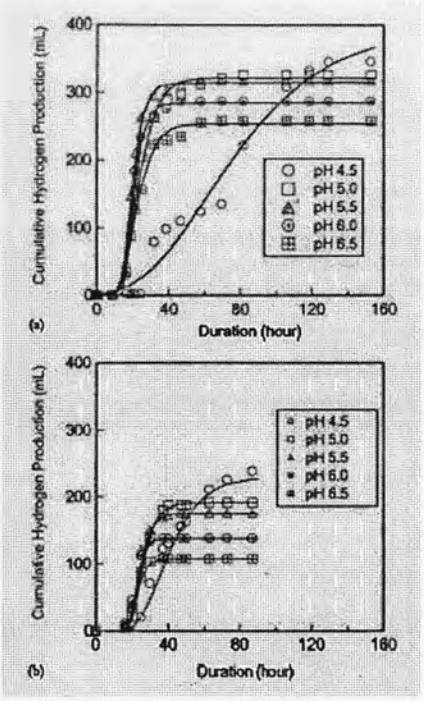

Figure 2-4 Cumulative H_2 production at different initial pH (a) sucrose and (b) starch

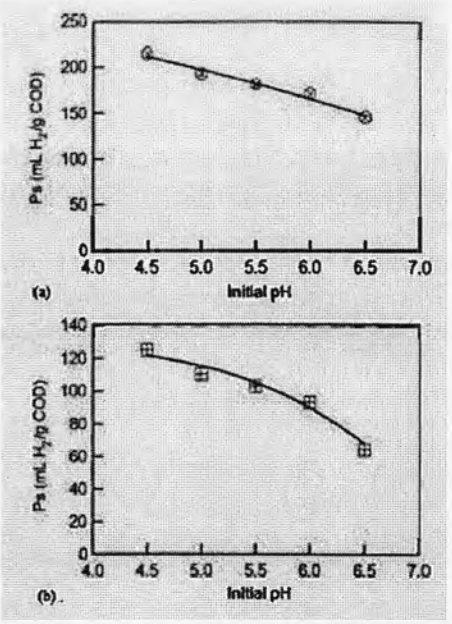

Figure 2-5 Specific H_2 production potential at different initial pH (a) sucrose and (b) starch

The trend showed no different in hydrogen production at initial pH above 5.0, particularly at pH of 5.0 and 5.5 (Khanal *et al.*, 2004). Most of the studies indicated that final pH in anaerobic H_2 production is around 4.0 – 4.8 regardless of initial pH (Morimoto *et al.*, 2004; Liu *et al.*, 2003; Zhang *et al.*, 2003; Liu and Shen, 2004). The decrease in pH is due to production of organic acids which deplete the buffering capacity of the medium resulting in low final pH (Yu *et al.*, 2002). Therefore, control of pH at the optimum level is required to maintain a high rate of hydrogen production process.

2.4.3 Effect of Temperature

Microbial growth and product formation are the result of a complex series of chemical reactions. Like all chemical reactions, they are influenced by temperature. Growth may be described by:

$$\frac{dX}{dt} = \mu X - k_d X \quad or \quad \frac{1}{X}\frac{dX}{dt} = \mu - k_d$$

Where k_d is a specific death rate. Thus the observed specific growth rate $(1/X)\ dX/dt$ is a balance of growth and death. Usually microorganisms are grown when $\mu > k_d$ and k_d can be neglected. However, since both μ and k_d are likely to be temperature-dependent they will both be considered here.

Three typical growth temperature curves are illustrated in Figure 2-6 These curves correspond to psychrophilic, mesophilic, and thermophilic growth. Although there are individual exceptions, most microorganisms will only grow over a temperature range of 20-30 °C. Furthermore, most micro organism fall into one of these patterns. Those with a temperature for maximum growth rate below 20 °C are psychrophilic, those around 30-35 °C are mesophilic, and those above 50°C are thermophilic.

The curves are alike in shape. As temperature is increased towards the optimal growth temperature, the growth rate approximately doubles over a 10 °C range. Above the optimum growth temperature, growth temperature, growth rate declines rapidly with increasing temperature.

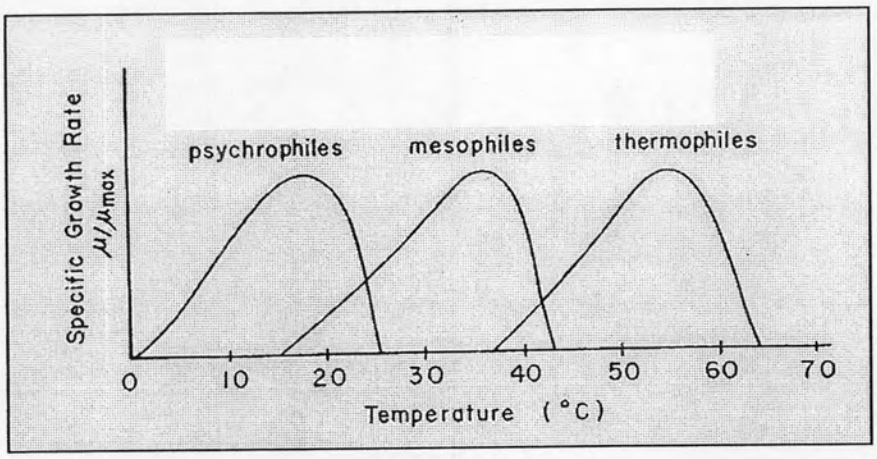

Figure 2-6 Effect of temperature on growth

Product formation by microorganisms is also dependent on temperature in a manner similar to growth. However, the temperature optimum for growth and product formation are not necessarily the same and must be examined separately. Thus a compromise may be necessary.

2.4.4 Hydraulic retention time (HRT)

The resident time of the feed in the digester before it is replaced with new material is theoretically calculated by diving the effective digester volume (V) by the rate of material input as follows:

$$HRT = \frac{V}{Q}$$

Retention time, sometimes called as dilution rate (D), is equal to the reciprocal of the microbial growth rate ($1/\mu$) when it is long enough to allow the maintenance of the microbial population. There is a minimum HRT below which efficient fermentation ceases, due to the wash-out of the microbial population. The HRT could be used as a tool to select microbial population whose growth rates are able to catch up with the mechanical dilution created by continuous volumetric flow rate (Tijhuis *et al.*, 1994). That shorter retention time led to reduction of propionate production in anaerobic H_2 production by mixed cultures has been reported (Lin and Chang, 2004; Wang *et al.*, 2003). Cha and Noike (1997) noticed that propionate production was present at HRT's of 24 h and 48h but absent at a HRT of 12h in a chemostat test using starch as the substrate. According to Hussy (2003), it was found that intermediate reduction of propionate production was observed in a continuous H_2 fermentation from wheat starch as HRT was shortened to 12h.

In continuous fermentations higher dilution rates are used to wash-out the slow growing methanogens and selected for acid-producing bacteria. HRT also has a pronounced effect on metabolic balance Clostridium producer VFAs and H_2 in the

exponential growth phase, and rapid alcohol production occurs in late growth phase (Lang, 2000)

As suggesting by Chen *et al.*, the high maximum specific growth rate (μ_m) reported in the literature (see Table 2-6) including their study imply that the H_2 bioreactor could be operated at a shorter HRT and give the better performance of H_2 production (Chen *et al.*, 2006). At HRT as short as 4 hour, the highest H_2 production of neary 7 l/d was achieved in the CSTR (Majizat *et al.*, 1997).

Table 2- 6 Summary of source kinetics of H_2 producing bacteria

Test Type	Culture	Temp (°C)	Substrate	μ_{max} (h^{-1})	Ks (gCOD/L)	Yx/s gVSS/ g substrate	Reference
Continuous	Citrobacter	37	glucose	0.220	NA	0.114	Brossau and Zajic, 1982
Continuous	Granular Sludge	35	sucrose	0.172	0.068	0.1	Chen *et al.*, 2001
Continuous	Mixed	37	glucose	0.333	NA	0.25	Horiuchi *et al.*, 2002
Batch	*Enterobacter clvacae*	37	glucose	0.568	3.914	0.08	Kumar *et al.*, 2000
Batch	Caldicellu losiruptor sacchrolyticus	70	sucrose	0.130	0.801	NA	Van Niel *et al.*, 2003
Batch	Mixed	35	glucose	NA	NA	0.625	Mojizat A. *et al.*, 1997
Continuous	Mixed	36	glucose	NA	NA	0.21	Fang *et al.*, 2002
Continuous	Mixed	-	sucrose	NA	NA	0.08	Kim *et al.*, 2006
Continuous	Granular Sludge	26	sucrose	NA	NA	0.2	Liu and Fang, 2002
Continuous	Granular Sludge	-	sucrose	0.172	NA	0.1	Chang and Lin, 2004
Continuous	Granular Sludge	-	sucrose	NA	NA	0.334	Yu and Mu, 2006

2.4.5 Solid Retention Time (SRT)

The relationship between microorganisms growth rate and SRT is given by: $1/\mu$ = SRT, where μ is the specific biological growth rate (d^{-1}). To prevent the washout of slowly-growing anaerobic bacteria, a minimum SRT must be maintained so that microorganism growth rate exceeds the washout rate determined by HRT. These conditions require large reactor volumes with their associated capital costs.

New anaerobic technologies have incorporated changes which allow the HRT and SRT to be varied independently. Typically, the solids in the reactor effluent are allowed to settle and are recycled back to the reactor compartment. This modification, called high-rate anaerobic process, allows long SRTs to be maintained even with large hydraulic (short HRT) throughput and, as a result, the size of the reactor and associated cost have been reduced significantly. Recycling the solids allows a faster development of a biological population, thereby reducing start-up time. (Christensen and Eblen, 1984).

2.4.6 Organic loading Rate

Loading rate can be defined in various terms as hydraulic loading, which is controlled by influent flow rate (or hydraulic retention time; HRT) and organic loading, which uses organic matter input to the system (BOD or COD) as a controller. Another parameter, solid loading, employs the amount of influent volatile solids (VS) to control the unit function. Thereby, organic loading rate is varied with hydraulic retention, volatile solids and COD concentration of substrate (according to the following equation), but also affect by temperature.

$$OrganicLoading = \frac{(VSorBODorCOD) * Q}{V}$$

Where Q is the rate of influent and V is the effective volume the digester.

Shock loading is one of the key process parameters in CSRT performance. It is conducted by either increasing influent flow rate or feed concentration or both. Several studies on continuous biohydrogen reactor reported in the literature (see Table 2-7) shows that reactor operated at high organic loading rate (OLR) presented a better performance of H_2 conversion efficiency in terms of H_2 production rate and H_2 yield. Chen *et al.* also suggested that the operation of H_2 bioreactor required a high influent substrate concentration or high organic loading rate (Chen *et al.*, 2006).

Table 2-7 OLR bacterial biomass retained, H_2 production rate and H_2 yield in various reactor types

Reactor Type	Substrate	OLR ($gl^{-1}d^{-1}$)	VSS (gl^{-1})	Volumetric H_2 prod. Rate ($l\,l^{-1}d^{-1}$)	Specific H_2 prod. Rate ($l\,g\,VSS^{-1}h^{-1}$)	H_2 yield (mol mol^{-1} hexose)
CSTR	Beer processing waste yeast	NG	2.3	0.045	0.019	NA
CSTR	Beer manufacturing waste	70 (as COD)	NG	0.129	NG	NA
CSTR	Sucrose	80	5.7	0.5	0.088	1.15
CSTR	Glucose	80 (as COD)	4.59	0.8	0.093	2.06
Sequencing batch	Sucrose	82	4.4-7.5	0.42	0.065	0.94
Leaching bed	Food waste	11.9 (as VS)	NA	0.15	NA	NA
Immobilised sludge (gel) repeat batch	Sucrose	NA	1 (initial)	NG	0.24-6.08	NG
Biofilm (Activated carbon)	Sucrose	427	15	1.32	0.088	0.57
Biofilm (activated carbon)	Sucrose	854	NG	7.4	NG	1.59
CSTR	Glucose	72	2.2	0.146	0.066	0.4
CSTR + membrane			5.8 (TSS)	0.187	0.032(TSS)	
Granules	Sucrose	49	20	0.54	0.029	2.2
Granules	Sucrose	60	5.0	0.28	0.056	0.86
AC seeded granules	Sucrose	427	15	2.7	0.173	1.2

Source: Hawkes *et al.*, 2007
NA-not applicable, NG-not given, TSS-total suspended solids.

2.4.7 Metabolic shift

The generation of hydrogen by fermentative bacteria also accompanies the formation of organic acids as metabolic products, but these anaerobes are incapable of further breaking down the acids. Acid accumulation causes a sharp drop of culture pH and subsequent inhibition of bacterial hydrogen production (Oh *et al.*, 2002; 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. The use of an aciduric facultative anaerobic with a lower pH limit for H_2 production that is as low as possible in order to reduce alkali consumption might be an option (Fabiano and Perego, 2002; Fang and Liu, 2002). Another approach to improve the hydrogen yield is to block the formation of these acids, through redirection of metabolic pathways (Mahyudin *et al.*, 1997; Kumar *et al.*, 2001). Disposal of excess reducing equivalents generated during fermentation is one of the major bottlenecks in facultative anaerobes that produce hydrogen (Lay *et al.*, 1999). These excess reducing equivalents could be disposes of via proton reduction, facilitated by hydrogenase and electron carriers, leading to the formation of hydrogen in organisms such as *Enterobacter aerogens* and *E. cloacae*. Taking this into account, it is not unrealistic to consider the production of bacterial hydrogen as a device for the disposal of electrons released in metabolic oxidations through the activity of hydrogenases, that in effect catalyze the reaction (Adams and Stiefel, 1998; Hallenbeck and Benemann, 2002).

2.4.8 H_2 partial pressure and gas sparging

The conversion of acetate to hydrogen according to the reaction

$$CH_3COOH + 2\ H_2O \longrightarrow H_2 + CO_2$$

In thermodynamically unfavorable at moderate temperature ($\triangle G = +104.6$ kJ mol^{-1}) is strongly determined by the hydrogen partial pressure (Classen *et al.*, 1999). Hydrogen evolution pathways are sensitive to H_2 concentrations and are subject to end-product inhibition. As hydrogen concentrations increase, H_2 synthesis decreases and metabolic pathways shift towards production of more reduced substrates, such as lactate, ethanol, acetone, butanol or alanine (Levin *et al.*, 2004). Continuous hydrogen synthesis requires a P_{H2} of < 50 kPa at 60°C, < 20 kPa at 7°C and < 2 kPa at 98°C (Van Niel *et al.*, 2003). Gas sparging has been found to be a useful technique to reduce hydrogen partial pressure in the liquid phase for enhancement of its yield. In a study by Mizuno *et al.* (2000), it was observed that the specific hydrogen production rate increased from 1.446 ml hydrogen min^{-1} g^{-1} biomass to 3.131 ml hydrogen min^{-1} g^{-1} biomass under nitrogen sparging contitions. With N_2 sparging at a flow rate approximately 15 times the hydrogen rate, the hydrogen yield was 1.43 mol H_2 (mol glucose)$^{-1}$. This shows an increase in hydrogen yield with nitrogen sparging of about 50%. A report by Tanisho *et al.* (1998) revealed that sparging with argon results in an increase of residual NADH, which might be expected to increase hydrogen production, although H_2 production was not actually measured. The yields were mainly estimated by calculating the amount of residual NADH. A hollow fiber/silicone rubber membrane effectively reduced biogas partial pressure in a dark fermentation system, resulting in a 10% improvement in the rate of hydrogen production and a 15% increase in H_2 yield (Liang *et al.*, 2002). In fermentation with mixed anaerobic cultures, the accumulation of hydrogen is normally balanced by

rapid hydrogen consumption by methanogens, resulting in little net hydrogen accumulation in the system (Mahyudin *et al.*, 1997). In order to obtain high concentrations of hydrogen, a system must be designed that both removes hydrogen before leads to repression of its productions and prevents interspecies hydrogen transfer leading to methanogenesis (Mahyudin *et al.*, 1997 and Tanisho *et al.*, 1998).

Availability of CO_2 also determines the yield of hydrogen-because cells synthesize succinate and formate via CO_2, pyruvate and NADH-via the hexose monophosphate pathway (Das and Veziroglu, 2001). NADH results from fermentative conversion of glucose to pyruvate, and re-oxidation of NADH evolves hydrogen. Thus, removing CO_2 efficiently from the culture medium prevents consumption of NADH and increases the yield of hydrogen. Several attemps to remove CO_2 have been made either by inert gas sparging to drive out hydrogen and carbon-dioxide from the reactor or by employing other membrane-base processes. Not only inert gas like Ar but also hydrogen was effective in the removal of CO_2. The method is also economical because a production plant does not need to separate the mixed gas if the produced hydrogen is used as the removing gas (Tanisho *et al.*, 1998). The yield increased from 0.52 up to 1.58. mol H_2 (mol glucose)$^{-1}$ by the combined effects of CO_2 removal and conditions of sufficient nitrogen source (Tanisho *et al.*, 1998).

The technical and economic feasibility of several gas stripping and membrane-absorption technologies for removal of hydrogen from a gas mixture has been studied by Van Groenestijn *et al.*, (2002). They selected four different techniques, namely absorption of H_2 in Pd or $NaNi_3$, stripping of H_2 by boiling, stripping of H_2 with recirculating gas and stripping H_2 by evaporation, for the study of economic recovery of heat from fuel-cell hot exhaust gas. To compensate for the heat loss by water

evaporation, a part of the water and heat is recovered after gas compression. The remaining is recovered from stream produced by the fuel cell in which the H_2 gas is converted into electricity (Groenestijn *et al.*, 2002). Table 2-8 provides and overview of the technologies for hydrogen removal from a reaction system.

Table 2-8 Different methods of hydrogen removal

Methods	Advantages	Disadvantages	References
N_2 sparging	50% increase in H_2 yield compared to no sparging	Sparging gas should be free of CO, othersiwise it could inhibit hydrogenase	Mizuno *et al.*, 2000
N_2 / Ar sparging	Simultaneous lowering of H_2 partial pressure	Too much sparging dilutes H_2 and creates problems in separation	Hawkes *et al.*, 2002
Sparging with fuel cell exhaust gas	Under investigation	Feasibility not yet reported	Hawkes *et al.*, 2002
Hollow fiber submerged silicone membrane	Effectively reduces biogas partial pressure in the reactor	Reduced efficiency due to biofilm build-up	Liang *et al.*, 2002
Steam stripping by evaporation at a large surface	Separation of steam from H_2 is much easier. (by condensation)	May lead to cooling of fermentation broth in thermoreactor	Van Groenestijn *et al.*, 2002
Strippping H_2 with recirculation gas	Can be carried out in reactor having either liquid or gas as continuous phase	High energy consumption. Problem of creation of gas-liquid interface	Van Groenestijn *et al.*, 2002
Pd-Ag membrane reactor	High hydrogen selectivity. 85-90% of H_2 can be removed	CO concentration affects membrane performance	Nielsen *et al.*, 2001
Synthetic polyvinyl-trimethylsilane membrane	High H_2 selectivity	-	Teplyakov *et al.*, 2002

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2.4.9 Reactor configuration

Substantial increases in hydrogen yields can also be achieved through optimum design of the bioreactor used for fermentation. Fixed-bed bioreactors containing a consortium of mesophilic bacteria enhance the rate of hydrogen production to a greater extent (Chang *et al.*, 2002) than reported for other approaches (121 m mol H_2 $l^{-1}h$). This remarkable rate of hydrogen production was achieved using activated carbon as a support matrix which allowed retention of the H_2 producing bacteria within bioreactor. In another method, hydrogen was produced in a high rate bioreactor in the presence of hyperthermophilic bacteria, which form a biofilm within an anaerobic tricking filter containing packing materials with a very high surface area (Groenestijn *et al.*, 2002). This resulted in the continuous flow of liquid-suspended biomass substrate through the filter, so that the biomass substrate, H_2-producing bacteria and the resulting gas phase were in close proximity. In addition, low hydrogen and CO_2 partial pressures are maintained by steam stripping of H_2 from the reactor. The energy required to run this process is at least four times lower than the combustion value of the H_2 gas produced in such reactors (Groenestijn *et al.*, 2002). Gas hold-up poses another major problem in tubular bioreactors with immobilized whole cells. To mitigate the problem, tapered and rhomboidal bioreactors can be used to improve performance in terms of both the rate of hydrogen production and gas hold-up. The latter was found to be reduced by 67% using a rhomboid bioreactor compared with a tubular bioreactor. The maximum hydrogen production rate achieved was 75.6 mmol l^{-1} at a dilution rate of 0.93 h^{-1} and recirculation ratio of 6.4 (Kumar and Das, 2001). The substrate conversion efficiency was increased by 15% under these conditions compared with the system with no recycling.

2.4.10 Nutrient requirement

N and P, nutrients known to be significant for efficient hydrogen production include S and Fe, significant components in hydrogen-producing hydrogenases. For most laboratory studies with pure substrates, nutrient concentrations were presumably well in excess to ensure carbon limitation, though few papers actually state this. The simple salts medium chosen by Zoetemeyer *et al.*, was said to guarantee excess of all essential nutrients other than glucose and nearby allow glucose-limited growth of the bacteria. Most laboratory work to date seeking to optimize nutrient addition has used batch cultures. More information is needed on minimum amounts of nutrients needed for continuous operation.

Although some clostridia are nitrogen fixing, laboratory studies with defined carbohydrates all use a nitrogen source and many report yields of more than 1 molH_2mol^{-1} hexose with addition of mineral nutrients only. Whilst Ren *et al.* report hydrogen production from sugar or corn starch with addition only of N and P salts, most studies have used more complex mineral salts solutions. From several reports in the literature (Mizuno *et al.*, 2000; Lin and Chang, 1999; Zoetemeyer *et al.*, 1982; Fang and Liu., 2002), COD: P ratios show an even wider variation from 73: 1 to 970: 1. All other hydrogen yields are between 1.4 and 2.1 mol mol^{-1} hexose. Thus reasonable hydrogen yields were obtained despite the wide variety of nutrients added.

Apart from a N and P source, K, Mg and Fe are common to all hydrogen bioreactor including trace elements, Ca, B, Mo, Zn, Co, Cu, Mn or I. As the technology is applied to a greater range of substrates, studies of nutrient requirement will become more necessary for process optimization and cost-effectiveness. The minimum amounts of N, P, S, Fe and trace elements needed for efficient H_2

production in continuous reactors at various conditions, e.g. pH and substrate concentrations, must be determined (Hawkes *et al.*, 2007).

2.4.11 Substrate

Carbohydrate, mainly glucose, are the performed carbon sources for fermentation process which predominantly give rise to acetic and butyric acid with H_2 (Classen *et al.*, 1999). Substrate concentratins used in continuous H_2 production with mixed microflora in CSTRs have until recently been commonly around 10g/l carbohydrate. Higher substrate concentrations should allow more energy-efficient operation buy product inhibition is likely to set the upper limit. With glucose concentrations between 2.5 and 10g COD/l and HRTs of 1-10 h, increased OLR decreased H_2 yield from 2.8 to 1.7 mol H_2/mol glucose at 30°C and pH 5.5 (Ginkel and Logan, 2005). This effect was mostly related to increased glucose concentration rather than changes in HRT and attributed to H_2 inhibition. However, Kim *et al.*(2006), investigating the effect of a range of sucrose concentrations (10-60 g/l as COD) in a CSTR at pH 5.4, 12 HRT, found the hydrogen yield at hexose concentration below 20g COD/l decreased. Above 35g COD/l, overload occurred, with the maximum molar H_2 yield of 1.22 mol/mol hexose consumed at an inlet sucrose concentration of 30g $CODl^{-1}$. A decrease performance in term of molar H_2 yield at 40g/l was noted by van Ginkel and Logan (2005) when comparing operation of a CSTR on 10, 20, 30, and 40 g/l glucose at 2.5 and 10 HRTs. Kyazze *et al.* (2006) examined continuous operation at pH 5.2, 12h HRT on 10-50 g/l sucrose. Stable operation was obtained with influent sucrose concentration up to 40g/l sucrose within 5 days from start-up. At $50gl^{-1}$ the system was less able to maintain steady state buy sparging with N_2 improved stability. The H_2 yield decreased from 1.7 ± 0.2 mol H_2/mol hexose added at 10g/l sucrose to 0.8 ± 0.1 mol H_2/mol hexose added at 50g/l.

Thus the upper limit of substrate concentration for sucrose or glucose without marked decrease in molar hydrogen yield may be around 30g/l.

2.5 Fundamental of Anaerobic Microbial Kinetics

The microbial kinetics need for improving and optimizing anaerobic biological process depends on dynamic of substrate utilization and growth of microorganisms. Effective design and operation requires a well-understanding of the biological reaction occuring and the basic principles governing the microbial growth rate including the environmental conditions, whether physical, chemical or biological factors that affect the system. Commonly anaerobic bacteria, about 30-50% of the degradable soluble substrate is converted to bacterial cell and about 50-70% is derived as the energy for life (Karot, 1996). The relation between substrate utilization rate and microbial growth in a batch reactor can be modeled with the following expansion for soluble substrates.

$$ds/dt = \frac{kSX}{K_s + S} \qquad (2\text{-}1)$$

Where ds/dt = substrate utilization rate , mg/l

k = maximum specific substrate utilization rate, g substrate/g microorganism ·d

X = biomass (microorganism) concentration, mg/l

K_s = half-velocity coefficient, mg/l

S = growth limiting substrate concentration, mg/l

For substrate removed, this equation has been referred to as the Michaelis-Menten equation and is also of the form proposed by Monod for the specific growth rate of bacteria in which the limiting substrate in available to microbial biomass in a dissolved form (Metcalf and Eddy, 2004). The biomass growth with the rate of substrate utilization can be described by the equation.

$$dx/dt = Y\frac{ds}{dt} - k_d X \qquad (2\text{-}2)$$

By divided by X for both side of the equation

$$(dx/dt)/X = Y\left(\frac{ds}{dt}\right)\cdot\frac{1}{X} - k_d \qquad (2\text{-}3)$$

Where dx/dt = net biomass production rate, mg/l·d

Y = synthesis yield coefficient, g biomass/g substrate

K_d = endogeneous decay coefficient, time^{-1}

The inverse of the term on the left-hand side Equation 2-3 in defined as the solids retention time (SRT) or sludge age or mean cell residence time (θ_c). By definition the SRT is the solids in the system divided by the mass of solids removed per day. The term $\frac{1}{SRT}$ is also related to μ, the specific biomass growth rate, as given by Equation 2-4:

$$\frac{1}{SRT} = \mu \qquad (2\text{-}4)$$

Using the above definition of SRT and substituting ds/dt from Equation 2-1 for the right-hand side of the equation, the Equation 2-3 can be rewritten as

$$\frac{1}{SRT} = \frac{YkS}{K_s + S} - K_d \qquad (2\text{-}5)$$

The solids retention time (SRT) is a fundamental design and operating for all anaerobic process. In general, SRT value should greater than the critical SRT value for effective treatment performance. The critical SRT value or so-called the minimum solids retention time, SRT min, is the residence time at which the cells are washed out from the system faster than they can reproduced. The SRT can be calculated using Equation 2-5, in which $S=S_0$. When wash-out occurs, the influent concentration S_0 is equal to the efficient waste concentration (S), and S_0 is much greater than K_s, so that

$$\frac{1}{SRT_{\min}} = Y_k - K_d \qquad (2\text{-}6)$$

or

$$\frac{1}{SRT_{\min}} = \mu_{\max} - K_d \qquad (2\text{-}7)$$

For the completing mix anaerobic digestion, the reactor SRT may be in the range of 15 to 30 days to provide sufficient safety factors for operation and process stability (Parkin and Owen, 1986)

2.6 Measurement of kinetic coefficients

Two basic modes of operation of the bacteria culture are commonly used for measurement of kinetic and growth coefficients: continuous culture (chemostat) and batch culture tests. The use of continuous culture versus batch culture tests for measurement of kinetic parameters has been the subject of controversy among researchers. Some investigators suggested that continuous-culture tests provide more "realistic" data because the tests are conducted under conditions more closely approaching operating treatment processes. Other researchers recommend that batch tests are equally suitable and simpler for measurement of kinetics parameter (Daratana, 2001)

Batch feed experiments are the simple and most rapid method for the determination of Method Kinetic parameters (K, K_s, Y and K_d). Unfortunately, no single batch feed method exists for the determination of all four growth kinetics parameters. A batch culture is transition, non-steady state system in which the culture causes the substrate concentration to decrease from an initial high to an extreme low after a period of incubation. Therefore, steady-state assumptions do not apply, and the substrate utilization and growth equation (Equation 2-1 and 2-2) must be simultaneously solved for *S* and *X*. To circumvent this problem, previous researchers have used the general approach of assuming that the decay coefficient, K_d, in Equation 2-8

$$\frac{dX}{dt} = Y * \left(\frac{-dS}{dt} \right) \qquad (2\text{-}8)$$

which may be integrated to give

$$X = X_0 + Y * (S_0 - S) \qquad (2\text{-}9)$$

Where X_0 and S_0 are the initial microorganism and substrate concentration respectively.

Combining (2-8) and (2-9) gives:

$$\frac{-dS}{dt} = \frac{k * S[X_0 + Y * (S_0 - S)]}{K_s + S} \qquad (2\text{-}10)$$

Integration of Eq. (2-10) between the limits of S_0 to S gives:

$$t = \frac{1}{k}\frac{K_s}{X_0 Y_g S_0} + \frac{1}{Y_g}\ln X_0 + Y_g(S_0 - S) + \frac{K_s}{X_0 + Y_g S_0}\ln\frac{S_0}{X_0 S} - \frac{1}{Y_g}\ln(X_0) \qquad (2\text{-}11)$$

Equation 2-11 which describes the shape of the substrate utilization curve, cannot be explicitly solved for S. Hence its solution must be approximated by numerical methods. Previous investigators have employed various non-linear least squares techniques to estimate the kinetic parameters by fitting Equation 2-11 to batch experimental data including Smith *et al.* (1998).

The main advantage of this batch method is that the kinetic parameters can be obtained from the measurement of the change in substrate concentration with time. The batch method allows much quicker determination of kinetic values than the continuous feed method. Consequently, the kinetics of a given reaction can be measured by under a number of different environmental conditions within a reasonable time period. Also of importance, the bacterial dominance is not likely to change over the period of the measurement. The disadvantages of the batch technique are that the initial substrate concentration needs to be near the K_s value and the active microorganism concentration should be sufficiently low so that there is a sufficient

deflection in the substrate utilization rate at, or near, K_s , so that K_s can be accurately determined .

2.7 Molecular Technology for Microbial Diversity and community

In the wake of "the great plate count anomaly" (Staley and Konopka, 1985) and the discovery that the majority of environmental microorganisms are effectively "unculturable" a variety of culture-independent methods have been developed and routinely used to analyze microbial community composition. Culture independent methods employ direct extraction of nucleic acids from environmental samples. It often involves the amplification of DNA or cDNA from RNA extracted from environmental samples by PCR and the subsequent analysis of the the diversity of the amplified molecules (community fingerprinting) (Ludwig and Schleifer, 1994). Alternatively, the amplified products may be cloned and sequenced to identify and enumerate bacterial species present in the sample. Also, the direct extraction of nucleic acids from environmental samples accounts for the very large proportion of microorganisms that are not readily cultured in the laboratory, but that be responsible for the majority of the biodegradation activity of.

The ribosomal RNA (rRNA) approach is becoming a widely used method for studying the microbial community structure of natural and man-made environments in a truly cultivation-independent way. Ribosomal RNA genes have particular advantages as a molecular marker in molecular methods. Firstly, all living cells contain ribosomes, which are part of the cells apparatus for translating deoxyribonucleic acid (DNA) into protein. This rRNA is a dominant cellular macromolecule. Most bacterial cells have somewhere between 103 and 105 ribosomes

(Rheims *et al.*, 1996). This natural amplification results in excellent sensitivities of hybridization assays. Secondly, the cellular RNA content varies depending on the general metabolic activity or growth rate of a given species (Olesen *et al.*, 1986). Thirdly, rRNA are excellent molecules for discerning evolutionary relationships among bacteria because RNA molecules contain conserved and variable regions which make it possible to find general as well as specific target sites for probes. These regions are used for identification purposes. A practical reason for using rRNA is the public availability of large databases. They have enough sequence information to be used as a phylogenetic marker (Ludwig and Schleifer, 1994).

DNA based techniques have been developed, which allow the identification of single bacterial species in sample material without the cultivation of the organisms (Armann *et al.*, 1995; Ludwig *et al.*, 1994; Muyzer *et al.*, 1993; Ward *et al.*, 1990). Most of the experiments which have been carried out in this field so far are based on ribosomal sequences are presented in all organisms and contained variable and highly conserved regions which allow distinguish between organisms on all phylogenetic levels. In addition, a lot of data exist in the databases (Maidak *et al.*, 1999), which can be used to compare the DNA-sequences of unknown microorganisms and allow a phylogenetic identification.

To identify bacteria in sample material, ribosomal sequences are analyzed by transcribing ribosomal RNA into cDNA, which can then be cloned (Ward *et al.*, 1990). Alternatively, extracted DNA can be used as a template to amplify ribosomal gene fragments with primers for universal sequences by PCR. The PCR amplified fragments can be cloned as well. The result of both strategies is a clone library, containing ribosomal sequences as inserts. By sequencing individual inserts and comparing the obtained sequences with sequences present in databases, it is possible

to identify the phylogenetic position of the corresponding bacteria without their cultivation (Woese, 1987; Ludwig and Schleifer, 1994).

Genetic analysis can add value to H_2 producing technology if the species identified have well characterized optimum operating conditions which can be implemented to improve H_2 production. As molecular techniques development, information on species abundance and on proteomics indicating change in optimization studies (Hawkes *et al.*, 2007).

2.8 Denaturing gradient gel electrophoresis (DGGE)

Denaturing gradient gel electrophoresis (DGGE) is the method if choice for is a gel electrophoresis method that separates genes of the same size that differ in base sequence. The technique employs a gradient of a DNA denaturant, such as mixture of urea and formamide. When a double-stranded DNA fragment moving through the gel reaches a region containing sufficient denaturant, the strands begin to "melt" at which point migration stops. Differences in melting properties are to a large degree controlled by differences in base sequence. Thus, the different bands observed in a DGGE gel are different forms of a given gene that vary, sometimes only very slightly, in their sequences (Muyzer *et al.*, 1993; Lapara *et al.*, 2000).

The denaturant concentration at which a DNA duplex melts is influenced by two factors: a) hydrogen bonds between complementary base pairs GC rich regions melts at higher concentration than AT rich regions; and b) attraction between neighboring bases of the same strand ["stacking"]. Thus, a DNA molecule may have several melting domains with characteristic melting denaturant concentrations determined by the nucleotide sequence (Muyzer *et al.*, 1993).

DGGE exploits the fact that identical DNA molecules which differing only one nucleotide, require different melting denaturant concentrations. When separated by electrophoresis through a gradient of increasing denaturant concentrations, the mobility of the molecule is retarded at the concentration at which the DNA strands of low melt domain dissociate. The branched structure of the single stranded of the molecule becomes entangled in the gel matrix and no further movement occurs. Complete strand separation is prevented by the presence of a high melting domain, which is artificially created at one end of the molecule by incorporation of a GC clamp. This is accomplished during PCR amplification using a PCR primer with a 5' tail consisting of 40 GC (Sheffeld *et al.*, 1989).

Once DGGE has been preformed, individual bands can be excised and sequence. Using 16S rRNA genes, for example, DGGE analysis yield a detailed picture of the number of phylotypes (distinct 16S rRNA genes) present in a habitat. By sequencing these bands, the actual species present in the community can be determined by comparison of the sequences with those of known species available from appropriate database. Using other genes, such as metabolic genes, information can be obtained in the same way according to the number of different types of organisms present in the community that contain the specific gene (Ferris and Ward, 1997; Kowalechuk *et al.*, 1997; Ovreas *et al.*, 1997).

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