CHAPTER II
LITERATURE REVIEW

2.1 Hydrogen properties and applications

In 1766, British scientist Henry Cavendish discovered hydrogen from reaction of Zinc metal (Zn) and hydrochloric acid (HCl). After that, he tested applying a spark to hydrogen resulting to formation of water (H_2O). From this experiment, scientist realized that water is made of hydrogen (H) and oxygen (O), and Antoine Lavoisier (French) named this new element after Greek words "hydro" (means water) and "genes" (means forming). (Demirbas, 2009)

Hydrogen (H) is the simplest and lightest element in the world with atomic weight1.008 (IUPAC, 2011). It normally contains of one proton, one electron, and one neutron which also called "protium". However, there are two isotopes, "deuterium", two-neutron hydrogen (^2H), and radioactive "tritium", three-neutron hydrogen (^3H) (Demirbas, 2009). 99.985% of naturally occurring hydrogen is protium, then deuterium with 0.015% in nature, and then tritium. Hydrogen is a high reactive atom thus it mostly exists as a combination with itself called hydrogen molecule (H_2) or with other elements, and most abundant form of hydrogen on earth is a combination of H and O as H_2O or water (Rajeshwar et al., 2008). Due to its high reactivity, hydrogen is used in various chemical industries, for example reaction with nitrogen (N) to form ammonia (NH_3) (Eq 2.1), reaction with reactive metals to produce metal hydrides (Eq2.2), reaction with metal oxide to extract pure metal in metallurgy (Eq 2.3).

$$N_2 + 3H_2 \rightarrow 2NH_3 \qquad \text{(Eq 2.1)}$$

$$2Na + H_2 \rightarrow 2NaH \qquad \text{(Eq 2.2)}$$

$$CuO + H_2 \rightarrow Cu + H_2O \qquad \text{(Eq 2.3)}$$

At room temperature and atmospheric pressure, hydrogen molecule is in a gas phase, because of its low boiling point about -253 °C and melting point

approximately -259 °C. (Averaged from Demirbas, 2009; Kreith and Goswami, 2007; Rajeshwar et al., 2008) It is a colorless, odorless, tasteless, and nontoxic gas, (Demirbas, 2009; Leon 2008; Kreith and Goswami, 2007; Rajeshwar et al., 2008), and composes of two types of molecular spin mixed in nature; ortho- (o-) and para-hydrogen (p-hydrogen). In o-hydrogen, both H atoms spin in the same direction, whereas p-hydrogen spins anti-parallel to each other, as shows in Figure 2.1. At room temperature and atmospheric pressure, 75% of molecular hydrogen exists as ortho-hydrogen while 25% is para-hydrogen, because p-hydrogen is unstable at this environmental condition. Nevertheless, para-hydrogen becomes more stable when temperature decreasing, and may reach 100% p-hydrogen when transform into liquid phase.

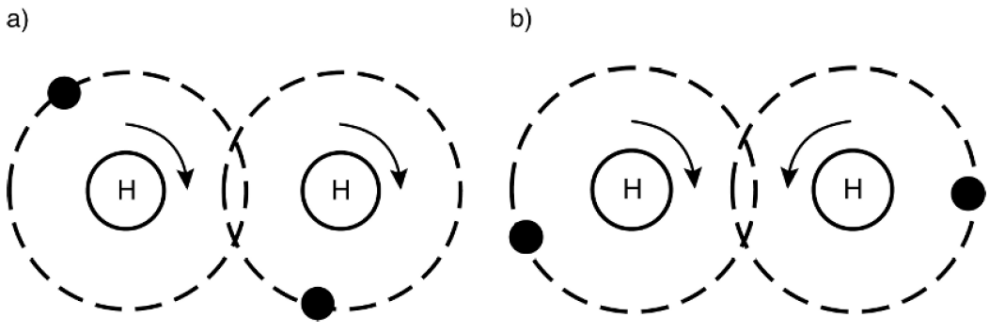


Figure 2.1 molecular spin of Hydrogen a) ortho-hydrogen (o-hydrogen), spin of each hydrogen atom spinning in the same direction b) para-hydrogen (p-hydrogen), spin of each hydrogen atom spinning in the opposite direction (Rajeshwar et al., 2008)

As mentioned, adding a spark to hydrogen in air makes it combusts and gives water. Similarly, blending of hydrogen with an oxidant (regularly air) in the appearance of any ignition source, such as sparks from electrical devices; metal welding; open flame; lightning; can causes hydrogen fires and explosion. The higher heating value (HHV) of hydrogen is 141.9 kJ/g, and the lower heating value (LHV) of hydrogen is 119.9 kJ/g (Demirbas, 2009; Rajeshwar et al., 2008). However, hydrogen gas is the lightest gas. It is approximately 14 times lighter than air (density of hydrogen is 0.0838 kg/m^3whereas density of air is 1.225 kg/m^3) (Rajeshwar et al., 2008). This promotes hydrogen gas to float higher, and confines its horizontal

expansion. Consequently, the flame horizontal spreading is limited. (Rajeshwar et al., 2008) Table 2.1 summarizes some important properties of hydrogen.

Hydrogen has good properties as a motor fuel. Vezirocjlu and Barbir (1992) reported that hydrogen is convenient fuel for transportation because it is easily converted into various forms of energy, environmental friendly, safe to use, has high utilization efficiency compared to conventional fuels. Besides, in the 1920s, Rudolf Erren (German engineer) converted the internal combustion engines of automobiles to use hydrogen or hydrogen mixtures. Moreover, hydrogen has highest energy content per unit mass comparing to other conventional fuels hence since 1958 to present, liquid hydrogen is used to drive rockets and produce electric currents and water in spaceships through fuel cell because of its high energy providing at low weight (Demirbas, 2009, Rajeshwar et al., 2008).

Table 2.1 Properties of hydrogen (IUPAC, 2011; Demirbas, 2009; Kreith and Goswami, 2007; Rajeshwar et al., 2008)

Properties	Values
Atomic weight	1.008
Boiling point	minus (-) 253°C
Melting point	minus (-) 259°C
Density	0.0838 kg/m^3
Higher heating value (HHV)	141.9 kJ/g
Lower heating value (LHV)	119.9 kJ/g

Accordingly, hydrogen is considered as a promising energy carries because (a) when burn it in air, there is no pollution emission. The main product from this process is water and heat, no carbon monoxide (CO), carbon dioxide (CO_2), soot or any hydrocarbons. (b) Hydrogen is a non-toxic gas. (c) It can be produced from water which is the most abundant on the earth crust as well as other renewable sources such as biomass. (d) Hydrogen can be stored in a large amount for long period. (e) Transmission of energy into hydrogen form is more cost-effective than using electric wire system for long distance of energy transportation. (f) Except the energy aspect,

hydrogen plays its role in chemical industries, food industries, electronics industry, and also alloys and metal industries.

2.2 Hydrogen production

Hydrogen is not a primary source of energy but an energy carrier (Demirbas, 2009; Rajeshwar et al., 2008; Grimes et al., 2008). It has to be produced, and needs energy to extract it from any hydrogen sources. (Rajeshwar et al., 2008) There are three main processes of hydrogen production (a) thermal process (steam reforming, water splitting, pyrolysis and gasification) (b) electrochemical process (electrolysis and photo-electrochemical) (c) biological process (bio-photolysis and fermentation with microorganisms) (Demirbas, 2009).

Table 2.2 Main hydrogen production processes and substrate resources (Demirbas, 2009)

Method	Process	Feedstock
Thermal	Steam reforming	Natural gas
	Water splitting	Water
	Pyrolysis	Biomass
	Gasification	Coal, biomass
Electrochemical	Electrolysis	Water
	Photo-electrochemical	Water
Biological	Bio-photolysis	Water and algae
	Fermentation with microbes	Biomass or organic wastewater

2.2.1 Steam reforming

Steam reforming is the process to produce or reform hydrogen from natural gas by reacting with steam at high temperature (700K or higher) and pressure (1.5 atm or higher). (Demirbas, 2009; Grimes et al., 2008) Natural gas (a mixture of light weight hydrocarbons gas) will react with steam, resulting to partial oxidation of hydrocarbon and forming carbon monoxide (CO) and H_2 (Eq 2.4) which commonly

called synthesis gas or syngas. Since methane (CH_4) is the most abundant component in natural gas, steam reforming can also mean steam methane reforming.(Eq 2.5) Furthermore, hydrogen can supplementary be produced from CO through water-gas shift reaction (Eq 2.6) which CO reacts with water generating carbon dioxide (CO_2) and H_2.

$$C_nH_m + nH_2O \rightarrow nCO + (n+m/2)H_2 \quad \text{(Eq 2.4)}$$

$$CH_4 + H_2O \rightarrow CO + 3H_2 \quad \text{(Eq 2.5)}$$

$$CO + H_2O \rightarrow CO_2 + H_2 \quad \text{(Eq 2.6)}$$

2.2.2 Water splitting

Water splitting is the thermal process to dissociate water molecule (H_2O) into hydrogen and oxygen (Eq 2.7). The process was thermally operated by heating H_2O to temperature around 2500K making liquid water transforms into its gas phase and bond breaking into hydrogen and oxygen. Hydrogen was separated from equilibrium mixture.

$$2H_2O(l) \xrightarrow{heat} 2H_2(g) + O_2(g) \quad \text{(Eq 2.7)}$$

Due to high temperature of heat required, water splitting is also driven by thermochemical force. Hydrogen in water can be extracted by reacting with chemical in multistep at lower required temperature (below 1140K). Five principal reactions are shown in Eq 2.8-2.12 (Demirbas, 2009). The forming intermediates can be used repeatedly.

$$2H_2O+SO_2+I_2+4NH_3 \rightarrow 2NH_4I+(NH_4)_2SO_4 \quad \text{(Eq 2.8)}$$

$$2NH_4I \rightarrow 2NH_3+H_2+I_2 \quad \text{(Eq 2.9)}$$

$$(NH_4)_2SO_4+Na_2S_2O_7+H_2O+2NH_3 \quad \text{(Eq 2.10)}$$

$$Na_2S_2O_7 \rightarrow SO_3+Na_2SO_4 \quad \text{(Eq 2.11)}$$

$$SO_3 \rightarrow SO_2+0.5O_2 \quad \text{(Eq 2.12)}$$

2.2.3 Pyrolysis

Pyrolysis is the process which can transform biomass and generate solid product (charcoal), aqueous product (oil and tar), and various gaseous products (H_2, CH_4, CO, CO_2). Biomass was heat at 650-800 K and 0.1-0.5 MPa in an absence of air. Eq 2.13 represents general equation of pyrolysis of biomass (Ni et al., 2006). By-products (methane) can also be reformed by steam reforming to produce more hydrogen.

$$\text{Biomass} \xrightarrow{\text{heat}} H_2 + CH_4 + CO + CO_2 + \text{charcoal} + \text{tar} + \text{oil} \qquad \text{(Eq 2.13)}$$

2.2.4 Gasification

Gasification is the thermal process aimed to generate gaseous product from biomass by partial oxidation. However, other than gaseous products can be formed such as charcoal. Unlike pyrolysis, gasification can be performed in presence of air. Biomass is partially oxidized at temperature above 1000K. Eq 2.14 illustrates general equation of gasification of biomass (Ni et al., 2006).

$$\text{Biomass} + \text{steam} \xrightarrow{\text{heat}} H_2 + CH_4 + CO + CO_2 + \text{Others} \qquad \text{(Eq 2.15)}$$

2.2.5 Electrolysis of water

Electrolysis of water is the process to separate water into hydrogen and oxygen by using electrical power. The electricity is passed through water. Water then receives electrons and reduction occurs resulting to hydrogen evolution at cathode while oxygen generates at anode. Reduction, oxidation, and overall reaction equation is written as in Eq 2.16, Eq 2.17, and Eq 2.18, respectively.

$$4H_2O + 4\bar{e} \rightarrow 2H_2 + 4OH^- \qquad \text{(Eq 2.16)}$$

$$4OH^- \rightarrow 4\bar{e} + 2H_2O + O_2 \qquad \text{(Eq 2.17)}$$

$$2H_2O \rightarrow 2H_2 + O_2 \qquad \text{(Eq 2.18)}$$

2.2.6 Photo-electrochemical

Moreover, electrolysis can also be combined solar power by replacing one of two electrodes in electrolysis cell with photovoltaic semiconductor material. This is called photo-electrochemical hydrogen production. The photovoltaic cell will adsorb an incident light and transform into electrical power for electrolysis of water.

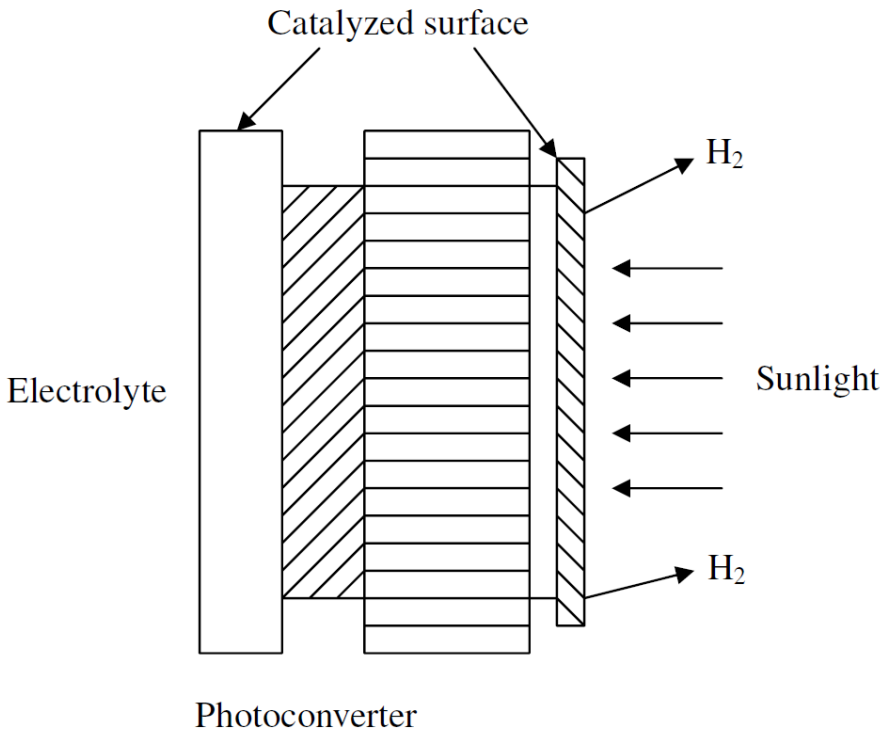


Figure 2.2 Schematic image of photo-electrochemical hydrogen production. The sunlight attacks to the photo-sensitive material which can convert solar energy into electrons. The electricity produced is used in electrolysis of water (Demirbas, 2009)

2.2.7 Bio-photolysis

Biophotolysis is a biological process using solar energy to convert water into hydrogen through photosynthesis of microalgae such as green algae and Cyanobacteria. (Karthic and Shiny, 2012; Hallenbeck and Benemann, 2002, Ni et al., 2006) Hydrogen which comes from biological process is called biohydrogen. There are two main processes, direct and indirect bio-photolysis.

2.2.7.1 Direct bio-photolysis

In direct bio-photolysis, water is directly converted into hydrogen and oxygen. Firstly, photosystem II (PSII) in microalgae absorbs photons with a wavelength shorter than 680 nm. Solar energy makes dissociation of water into oxygen (O_2), proton (H^+) and electrons. Electrons, then, transfer through series of electron carriers until reaching photosystem I (PSI). PSI absorbs photons with wavelength shorter than 700 nm which resulting increasing energy of transferred electron. Activated electrons reduce ferredoxin (Fd) into its reduced form (Fd_{red}). Fd_{red} is utilized by enzyme hydrogenase (H_2-ase) then generate hydrogen molecule (H_2) (David et al., 2004; Yu and Takahashi 2007). Eq 2.19 shows the overall reaction equation (Ni et al., 2006; Hallenbeck and Benemann, 2002; David et al., 2004).

$$2H_2O \xrightarrow{light} 2H_2 + O_2 \qquad \text{(Eq 2.19)}$$

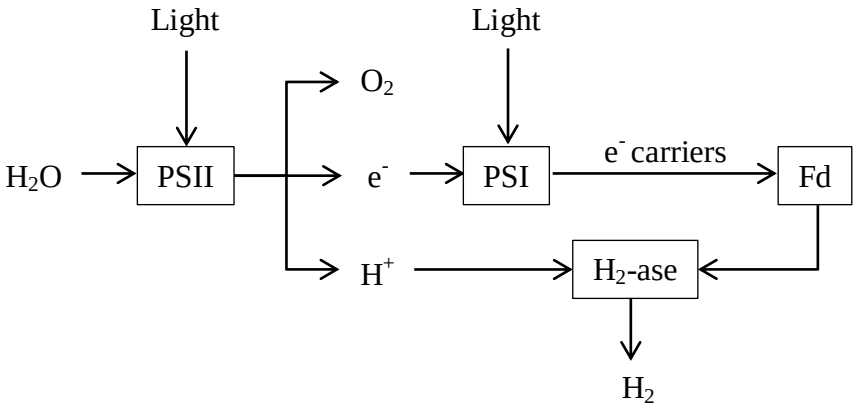


Figure 2.3 Direct biophotolysis process. Using light activate biochemical process in order to convert water into hydrogen (Hallenbeck and Benemann, 2002**)**

2.2.7.2 Indirect biophotolysis

In indirect biophotolysis, water is not directly converted into hydrogen and oxygen. It is firstly transformed through photosynthesis of microalgae into carbohydrate which reserved as endogenous stored energy. Then stored carbohydrates are released by fermenting in a dark condition, and processed to form hydrogen with helping of hydrogenase enzyme (Yu and Takahashi, 2007, Karthic and

Shiny, 2012). The process can be categorized into two stages based on presence of oxygen. The first stage is able to perform in the presence of oxygen while the second is not due to sensitivity of hydrogenase enzyme to oxygen. Each stage contains two steps hence there are four steps related in indirect biophotolysis (a) production of biomass (carbohydrates) through photosynthesis (b) accumulation of biomass which further used as cell materials (c) dark fermentation of biomass generating 4 moles of H_2 and 2 moles of acetate and (d) conversion of acetate into hydrogen (Hallenbeck and Benemann, 2002; Ni et al., 2006). The overall reaction is illustrated in Eq 2.20 and Eq 2.21 (Ni et al., 2006).

$$12H_2O + 6CO_2 \xrightarrow{\text{light}} C_6H_{12}O_6 + 6O_2 \qquad \text{(Eq 2.20)}$$

$$C_6H_{12}O_6 + 12H_2O \rightarrow 12H_2 + 6CO_2 \qquad \text{(Eq 2.21)}$$

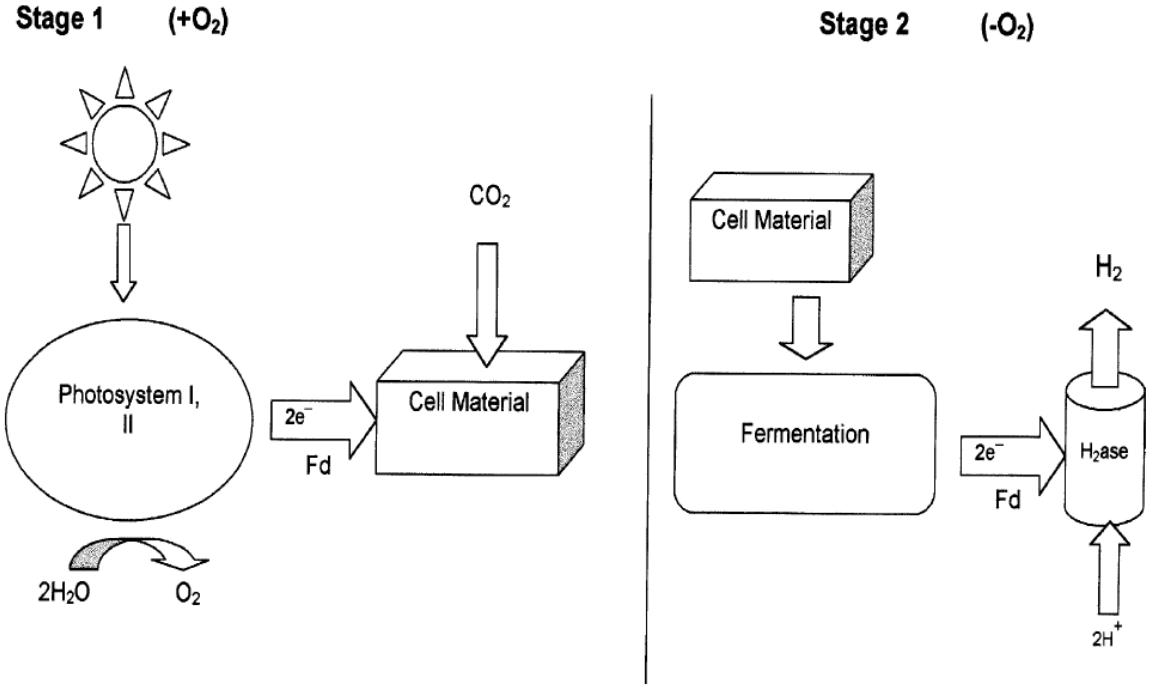


Figure 2.4 Indirect biophotolysis process. Using light activate biochemical process in order to indirectly convert water into biomass and then hydrogen (Hallenbeck and Benemann, 2002**)**

2.2.8 Fermentation with microorganisms

Not only bio-photolysis process, biohydrogen is able to produce by fermentation of biomass or organic wastewater with microbes. Organic substrates, mostly carbohydrates, are processed through series of biochemical reaction, and then converted into hydrogen gas. There are two main processes for hydrogen production by fermentation with microorganisms, photo-fermentation and dark fermentation.

2.2.8.1 Photo-fermentation

Photo-fermentation is fermentation of organic substrates with photosynthetic microbes driven by light energy to produce hydrogen. Photosynthetic microbes can generate hydrogen through their nitrogenase enzyme (N_2-ase) system. N_2-ase system is normally functioned to transform nitrogen (N_2) into ammonia (NH_3), but is able to evolve H_2 in an absence of N_2 gas environment (Das and Veziroğlu, 2001). Figure 2.5 shows diagram of the photo-fermentation process.

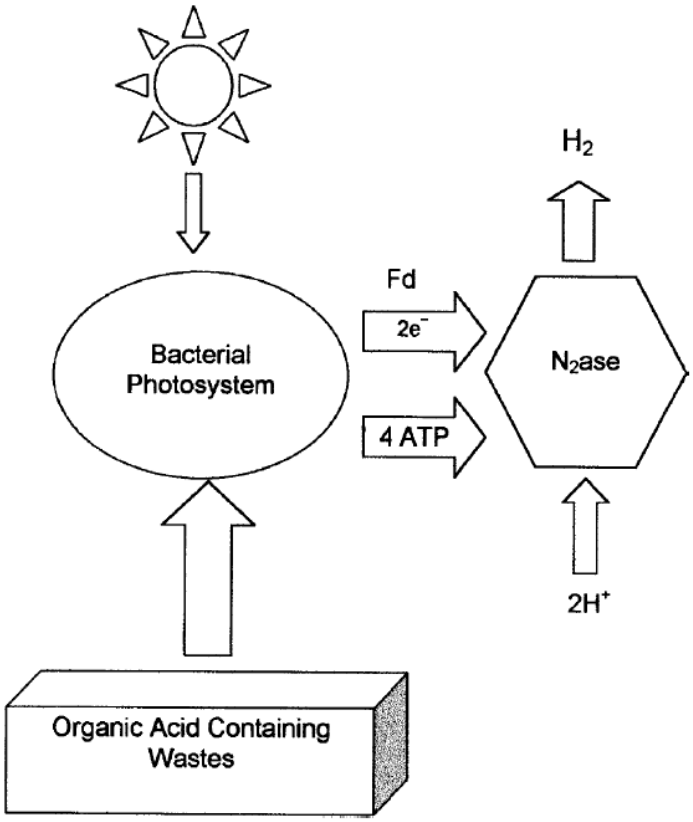


Figure 2.5 Photo-fermentation process, using photosynthesis to convert organic compounds and produce hydrogen (Hallenbeck and Benemann, 2002**)**

2.2.8.2 Dark fermentation

Unlike photo-fermentation, dark fermentation does not require light energy to impulse the process. Microbes used for dark fermentation does not need to be photosynthetic microbes, but should be anaerobic or facultative bacteria, since the process is performed in dark and oxygen absence condition. Therefore, variety of microbial strain is able to perform dark fermentation. Also, mixed cultures can be utilized.

Dark fermentation contains series of biochemical reactions (Figure 2.6). Organic substrates, glucose as a model, are transformed into pyruvate via glycolysis. Then co-enzyme A (CoA) reacts with pyruvate and generates acetyl-CoA. There are two possible pathways to produce acetyl-CoA, pyruvate ferredoxin oxidoreductase (PFOR) and pyruvate-formate lyase (PFL). Eq 2.22 and 2.23 illustrate the former and latter reaction equation, respectively (Hallenbeck and Benemann, 2002; Ntaikou et al., 2010).

$$\text{Pyruvate} + \text{CoA} + 2\text{Fd}_{ox} \xrightarrow{\text{enzyme}} \text{Acetyl-CoA} + \text{CO}_2 + \text{Fd}_{red} \qquad \text{(Eq 2.22)}$$

$$\text{Pyruvate} + \text{CoA} \xrightarrow{\text{enzyme}} \text{Acetyl-CoA} + \text{HCOOH} \qquad \text{(Eq 2.23)}$$

The pathway of acetyl-CoA evolution is the factor that can predict whether the theoretical hydrogen yield will be 4 or 2 moles of H_2 per mole of consumed glucose. When acetyl-CoA is produced by Eq 2.22, it can further transform into either acetate or butyrate (Uyeda and Rabinowitz, 1971).

In case of acetate formation, re-oxidation of 2 moles of ferredoxin (Fd) will generate 2 moles of hydrogen, and more 2 moles of hydrogen was produced from reduction of 2 moles of reduced form of nicotinamide adenine dinucleotide (NADH). Consequently, total hydrogen yield is 4 moles of hydrogen per mole of consumed glucose. Overall reaction when obtaining acetate is shown in Eq 2.24.

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \qquad \text{(Eq 2.24)}$$

In case of butyrate formation, re-oxidation of 2 moles of ferredoxin (Fd) will evolve 2 moles of hydrogen like in acetate pathway, but NADH is used for oxidation of acetoacetyl-CoA to butyrate, thus there is no more 2 moles of hydrogen produced. Thereby, total hydrogen yield for butyrate pathway is 2 moles of hydrogen per mole of consumed glucose (Ntaikou et al., 2010). Indeed, total reaction when obtaining butyrate is displayed in Eq 2.25.

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad \text{(Eq 2.25)}$$

When acetyl-CoA is produced by Eq 2.23, it can be converted into formate (HCOOH) which can further generate 2 moles of hydrogen as displayed in Eq 2.27 (Knappe et al., 1974; Kim et al., 2010). Accordingly, another procedure to produce 4 moles of hydrogen per mole of consumed glucose is these two steps (i) reduction of 2 moles of NADH in acetate formation (giving 2 moles of hydrogen) (Eq 2.26) and (ii) breakdown of HCOOH molecule (giving another 2 moles of hydrogen) (Eq 2.27) (Ntaikou et al., 2010).

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2HCOOH + 2H_2 \quad \text{(Eq 2.26)}$$

$$2HCOOH \rightarrow 2CO_2 + 2H_2 \quad \text{(Eq 2.27)}$$

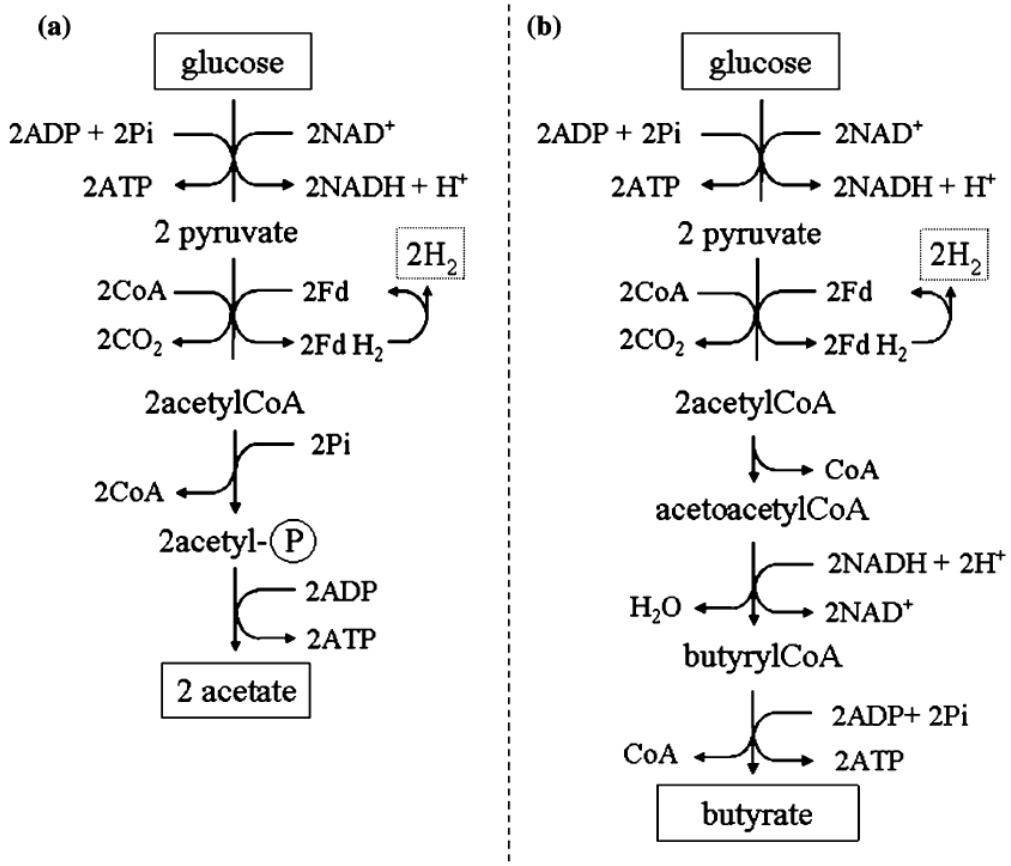


Figure 2.6 (a) Acetate and (b) butyrate pathway for hydrogen production via dark fermentation (Ntaikou et al., 2010)

2.3 Factors affecting biohydrogen production by fermentation

2.3.1 Bacterial culture: pure or mixed culture

Pure cultures are mostly used for theoretical studies of fermentative hydrogen production. According to Wang and Wan (2009), it was concluded that pure cultures are mostly operated in batch reactor with glucose and other saccharides, for instance of these studies, Chin et al. (2003); Lo et al. (2008); Pan et al. (2008); Yokoi et al. (1995); Ogino et al. (2005); Jo et al. (2008). However, for practical usage, the fermentation may have to run in non-sterile conditions, thus mixed cultures may answer this requirement. There is no need to screen for pure species and sterilize for usage, making mixed cultures is easier to operate. Moreover, hydrogen-producing

mixed cultures are able to find from various sources, for example, wastewater sludge, municipal sewage, compost and soil (Wang and Wan, 2009; Show et al., 2011).

2.3.1.1 Hydrogen-producing bacteria

There are many microbes reported generating hydrogen through the dark fermentation. Table 2.3 is some genus frequently reported.

Table 2.3 Example of hydrogen-producing microorganisms

Genus	Reported preferable environment	Example of reported hydrogen-producing species	Studies
Clostridium	pH: 5.5-8.5 Temperature: mesophilic, thermophilic range	*C. buytricum* *C. thermolacticum* *C. pasteurianum* *C. bifermentants* *C. thermocellum*	Tian et al., 2015; Ortigueira et al., 2015; Patel et al., 2015; Kapdan and Kargi, 2006.
Bacillus	pH: 6.0-7.0 Temperature: mesophilic, thermophilic range	*B. cereus* *B. subtilis* *B. licheniformis* *B. badius* *B. tequilensis*	Sinha and Pandey, 2014; Bala-Amutha, and Murugesan, 2012; Goud et al., 2012;
Escherichia	pH: 6.0-7.0 Temperature: mesophilic	*E. coli*	Bakonyi et al., 2011; Redwood et al., 2008; Yoshida et al., 2005;
Enterobacter	pH: 5.0-7.5 Temperature: mesophilic range	*E. cloacae* *E. aerogenes*	Mishra and Das, 2014; Sridevi et al., 2014; Reungsang et al., 2013.

2.3.2 Type of substrate

Any organic substrate rich in carbohydrates, fats and proteins is theoretically possible to use as substrate for biohydrogen production. However, Lay et al. (2003) compared biohydrogen production from carbohydrate-rich waste (rice and potato), fat-rich waste (fat meat and chicken skin), and protein-rich waste (egg and

lean meat). The result illustrated that ability of hydrogen production from carbohydrate-rich waste was approximately 20 times higher than the others. Consequently, carbohydrates, carbohydrate-rich waste and carbohydrate-rich biomass are concerned as the most suitable substrates for biohydrogen production. There are two main types of substrate that has been used for fermentative hydrogen production, based on their chemical composition and source (a) pure carbohydrate and (2) carbohydrate-rich solid wastes/wastewater.

2.3.2.1 Pure carbohydrate

Pure carbohydrate is 100% carbohydrate hence it must be suitable for fermentative hydrogen production. Carbohydrate in form of, especially, simple sugars such as glucose and sucrose, are mostly studied because they are easily degraded and spend short period of fermentation time for hydrogen production. They have been widely utilized as a model substrate. However, the cost of pure carbohydrate sources is high thus for industrial production they may not economically suitable raw material (Kapdan and Kargi, 2006). Moreover, they do not answer the goal of waste conversion and fuel reproduction.

2.3.2.2 Carbohydrate-rich industrial solid waste/wastewater

Carbohydrate containing substrates are able to obtain from various places, but solid waste and wastewater from agriculture and agricultural or food industries are gaining interest because it meets the concept of waste treatment as energy production (Karthic and Shiny, 2012). Besides, they are cost-effective, easy to find, and biodegradable according to criteria of feedstock selection (Ntaikou et al., 2010; Kapdan and Kargi, 2006).

Various carbohydrate-rich has been reported using for biohydrogen production. Lignocellulosic wastes, for example corn stalk, wheat straw, sugar cane, bagasse, and more, are abundant and almost zero cost substrate. Nevertheless, they contain complex structure carbohydrates such as cellulose, so they may not be readily degraded, and may require pretreatment methods prior to the fermentation (Wang and Wan, 2009; Show et al., 2011). Zhang et al. (2007a) compared fermentative hydrogen production between pretreated cornstalk waste and non-pretreated one, and found that hydrogen yield from pretreated cornstalk waste is larger than the non-pretreated Industrial solid waste or wastewater from agricultural or

food industry consist high carbohydrate contents in form of starch, cellulose, and/or sugars.

Industrial waste and wastewater are considered as promising substrate for biohydrogen production both in terms of environment and economic. They are renewable source, helping pollution control, and low cost (Ntaikou et al., 2010). Moreover, they contain high composition of degradable carbohydrate and low concentration of inhibitors of microbiological activity.

2.3.3 Concentration of substrate

Nonetheless, appropriate concentration of substrate is still no agreement. This is probably because studied concentration range in individual work is different. Chen et al. (2005) researched on a variation of sucrose concentration from 5-30 g COD/L on fermentative hydrogen production using *Clostridium butyricum* CGS5, and found that an optimal concentration for hydrogen generation is 20 g COD/L, while Lo et al. (2008) utilized concentration range of sucrose 5-40 g COD/L operated with *Clostridium pasteurianum* CH4, and reported 40 g COD/L of sucrose is a suitable concentration. Moreover, it has been reported that in a suitable range of substrate concentration increasing of substrate concentration could improve ability of hydrogen-producing bacteria to generate hydrogen gas, but extensive substrate concentration could approach a stable production or decrease hydrogen evolution (Wang and Wan, 2009; Lo et al., 2008, Kim et al., 2006; Wang and Wan, 2008a; Antonopoulou et al., 2011).

2.3.4 Temperature

Temperature is one of the most significant factors. Microbes contain ability to produce hydrogen in a temperature range of 15-85°C (Kanai et al., 2005) which 15-30°C is referred to ambient temperature range, 30-40°C is referred to mesophilic temperature range, and 50-60°C is referred to thermophilic temperature range (Wang and Wan, 2009; Li and Fang, 2007). According to Li and Fang (2007), although there are differences in substrate, seed sludge, temperature used, and other environmental conditions, hydrogen yield and generation rate in ambient temperature range is lower than the other ranges. Therefore, many studies conducted fermentative

hydrogen fermentation at mesophilic and thermophilic temperature. Furthermore, temperature also affects the metabolic pathway, influencing to difference of by-products composition and hydrogen production. Valdez-Vazquez et al. (2005) reported that at 37°C butyrate was more favorable produced, but at 55°C, acetate was more favorable produced. Nevertheless, Yu et al. (2002) reported that acetate was preferable formed at 20°C while at 55°C a production of acetate declined. Thus, an optimal temperature is stilled difference in each research.

2.3.5 pH

pH is one of the most essential considered factors influencing hydrogen production yield. It directly affects to microorganism community and activity, hydrogenase activity (Li and Fang, 2007; Show et al., 2011, Guo et al., 2010; Valdez-Vanquez and Poggi-Varaldo, 2009). pH in a range of 5-7 is probably preferred for activity of hydrogenase enzyme, and may suppress activity of hydrogen-consuming enzyme such as methanogens (Phelps and Zeikus, 1984). Besides, pH also affect metabolism pathway (Li and Fang, 2007; Guo et al., 2010; Show et al., 2011; Valdez-Vanquez and Poggi-Varaldo, 2009; Wang and Wan, 2009). At low pH butyrate and acetate pathway is more preferable, whereas at higher pH is organic alcohol formation is more favorable (Guo et al., 2010). However, an optimal pH for fermentation of hydrogen production still does not conclude due to differences in cultures, substrate and pH range that were studied (Li and Fang, 2007; Wang and Wan, 2009).

2.3.6 Iron (Fe) metal ions

Iron (Fe) is an important composition in hydrogenase enzyme which responsible for hydrogen generation through fermentation process. There are three types of hydrogenase enzyme presently known, [NiFe]-hydrogenase; [FeFe]-hydrogenase; and [Fe]-hydrogenase. The former two are most widely found in microbes, while [Fe]-hydrogenase is discovered in some methanogens (Show et al., 2011). Consequently, trace amount of iron ion can improve fermentative hydrogen production. Lee et al. (2001) tested an effect of iron concentration added in the form of $FeCl_2$ on fermentative hydrogen production from sucrose with mixed cultures. The studied iron concentration ranged from 0 to 4000 mg $FeCl_2$/L. The maximum

hydrogen production yield (131.9 mL/g sucrose) was generated at 800 mg $FeCl_2$/L condition, whereas the minimum hydrogen production yield (2.0 mL/g sucrose) is obtained at 0 mg $FeCl_2$/L or no ferrous ion environment. However, optimal concentration of iron ion showed contradictory, possibly due to differences in microorganisms, substrate, and iron concentration range (Wang and Wan, 2009; Li and Fang, 2007).

2.4 Starch processing wastewater

Starch manufacturing wastewater contains high carbohydrate content, COD and total solid (TS) (O-Thong et al., 2011). Starch is originated from various plants such as cassava roots, corns, wheat, rice, sticky rice, potato, and so on. Fresh plants were processed through series step then obtain starch or flour as an ended product. To illustrate, production of tapioca starch begins by weighing fresh cassava roots to approximate percentage of starch. Then the roots were removed any impurity in a rotary screener, and sent to peeling-and-cleansing device to clean and take of their cover. After that, fresh clean cassava is processed to remove protein and fiber, and dried by hot air dryer. Finally, starch is packed to deliver to markets. (Thai Tapioca Starch Association, 2004) Other than starch, wastewater also release from the process. Sangyoka et al. (2007) reported that 1 kg of fresh cassava roots may release 5-7 L of wastewater.

Starch containing wastewater is easier to hydrolyze into glucose and maltose, and processed biohydrogen (Kapdan and Kargi, 2006). After hexose formation, as mentioned in Topic 2.2, hexose will pass through series of biochemical reaction and convert into hydrogen gas.

2.5 Immobilization of microorganisms

Immobilization of microbes may be defined as the physical localization of intact cells to a region or space with the preservation of some catalytic activity (Karel et al, 1985). Biofilm is formed when cells were immobilized on supporting materials.

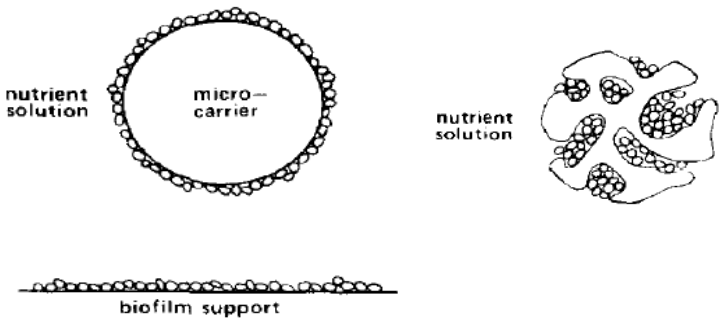
Cell immobilization provide possibility of increasing volumetric productivity, increasing product concentration in output stream, and decreasing substrate remaining in the outlet stream, helping acclimatization of microbes, decreasing lag phase of bacterial cultivation (Cheng et al., 2006; Prieto et al., 2002), and increasing density of consortia (Wu et al., 2003). Moreover, in continuous reactor, cell immobilization also allows product generation without cell washing out (Szentgyörgyi et al., 2010; Valdez-Vazquez and Poggi-Varaldo, 2009, Karel et al., 1985).

2.5.1 Methods of cell immobilization

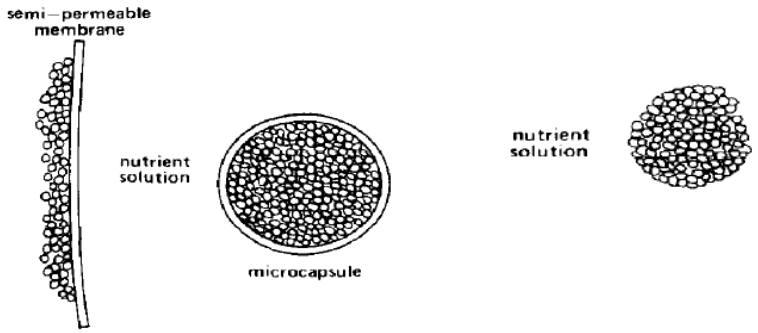
Immobilized methods can be categorized into four processes based on the physical mechanism of immobilization (a) attachment or adsorption of cell to a surface (b) entrapment of cell in a porous materials (c) containment of cell behind a barrier and (d) self-aggregation. Figure 2.7 illustrates schematic image of various methods of immobilization.

2.5.1.1 Attachment or adsorption of cell to a surface

When cells are attached to the surface with any type of binding (physical or chemical), this immobilization process is grouped into surface-attached method. Adsorption of cell to matrix surface can be found everywhere in nature, although human body, for example, dental plaque. Immobilization by adsorption method is popular because it is simple. Moreover, it is widely applied for a wastewater treatment process in many industries (Henze and Harremoës, 1983). A schematic of cell adsorption is displayed in Figure 2.7 (a).



(a) (b)



(c) (d)

Figure 2.7 Method of cell immobilization (a) attachment or adsorption of cell to a surface (b) entrapment of cell in a porous materials (c) containment of cell behind a barrier and (d) self-aggregation (Karel et al., 1985)

2.5.1.2 Entrapment of cell in porous materials

This process is typically done with cross-linked polymer such as alginate and polyacrylamide (Kumar et al, 1995). There are two methods of entrapment. The first is synthesis of supporting around the cells inside the reaction chamber, and the second is self-diffusion of cells into the pore area of supporting. In the latter process, cells were limited their mobility because of existence of the other cells, then were entrapped inside the porous matrix. A schematic of entrapment is showed in Figure 2.7 (b).

2.5.1.3 Containment of cell behind a barrier

There are two methods of containment similar to entrapment (a) cell diffusing then locked inside the barrier or (b) the barrier was formed later surrounding the cells. However, containment of cell can be immobilized on non-porous supporting materials functioned as barrier. The barrier can be a simple liquid/liquid interface between two immiscible fluids, and can be a spherical-shaped (Mohan and Li, 1975) or planar boundary (Matson et al., 1985). This method is useful in the system which cell and/or product separation from effluent is required. The boundary allows diffusion of nutrients into the cells and diffusion of produced products out of the cells while maintain the cells inside the barrier. (Karel et al., 1985) Furthermore, containment of cell behind a barrier has been applied to immobilized

mammalian cells for use as a manufactured pancreas (Chick et al., 1977; Sun et al., 1980). A schematic of containment of cell behind a barrier is illustrated in Figure 2.7 (c).

2.5.1.4 Self-aggregation

Cells can be naturally aggregate as showed in Figure 2.7 (d). The cells performing self-aggregation contains molds which naturally form pellets in culture. However some microorganisms do not have molds so pellet formation does not naturally occur. The artificial flocculating or cross-linking agents may be added to induce the process (Tenney and Verhoff, 1973). Microbial aggregation has been reported to use in wastewater treatment (Lian et al., 2008) sewage treatment (Yu et al., 2009) and potentially biodiesel production (Lee et al., 2009a).

2.5.2 Biofilm formation and structure

When microbes are immobilized on some surface, they may form a biofilm. Thus those supporting material can be also called biofilm carrier. Figure 2.8 displays a schematic image of biofilm formation process. Different genetic and environmental conditions will affect to biofilm formation. To illustrate, flagella and cilia allow the cell to move and bind to the supporting surface. To sustain the attachment of cell and the surface, cell membrane proteins or called adhesins play an important role. When no adhesion or its activity is forbidden, biofilm formation does not occur (Pratt and Kolter, 1998; Watnick and Kolter, 1999). Furthermore, differences in environmental conditions such as pH, temperature, nutrients and more, say different signals to biofilm forming. Hommais et al. (2002) studied effect of pH on biofilm formation of *Vibrio cholerae*, and reported that when pH lowers than 7 the ability of biofilm formation is decreased.

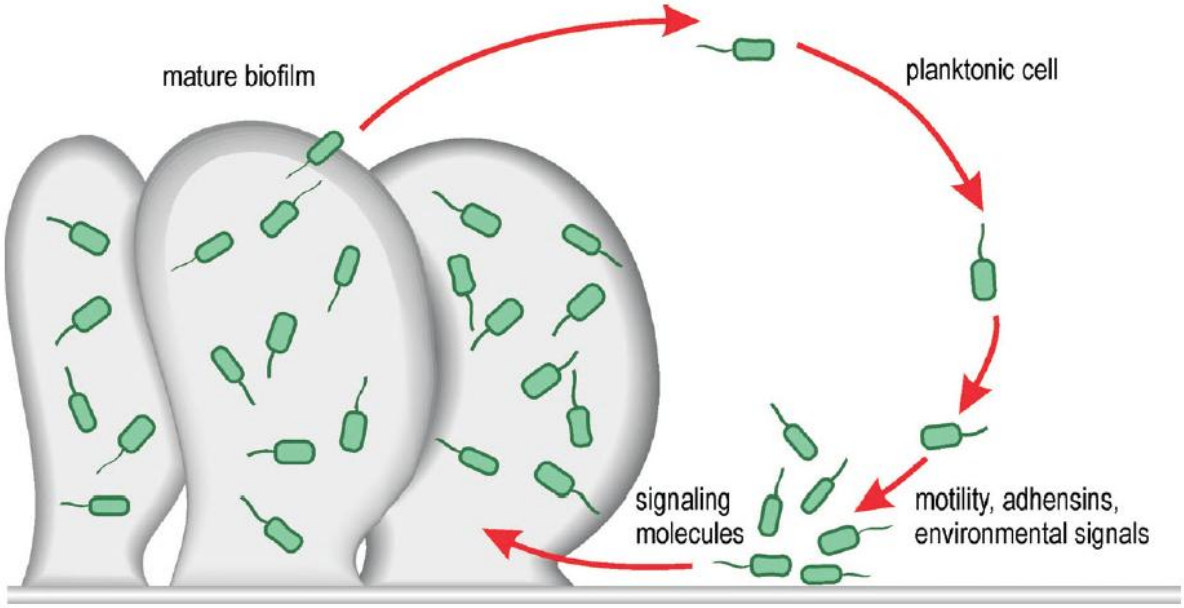


Figure 2.8 Biofilm formation (Maric and Vranes, 2007)

Basic unit of biofilm is micro colonies which is an individual community of bacterial cells submerged in extracellular polysaccharide matrix (EPS). EPS is functioned as cell protector from various negative environment conditions such as changing of pH. Micro colonies are mostly found in mushroom-shaped (Figure2.9) or rod-like shaped, and may include one or more type of bacteria (Maric and Vranes, 2007). Nevertheless, Structure of biofilm can change depending on amount of nutrients and hydrodynamics. Stoodley et al. (1999) reported modification of biofilm structure at various glucose concentration and type of hydrodynamic flow (laminar and turbulent flow). They found that when glucose concentration is high, micro colonies grew faster and biofilm thickness was thicker than low concentration of glucose. In addition, in laminar flow the micro colonies became round unlike in turbulent flow.

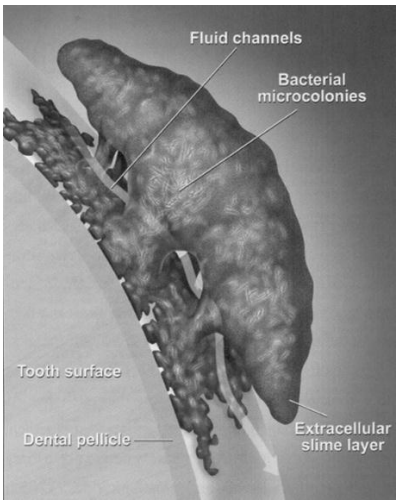


Figure 2.9 Example of mushroom-shaped microcolonies of dental plaque biofilm (Nield-Gehrig, 2013)

2.6 Supporting materials for immobilization

There are two main categories of supporting material that has been used in biohydrogen production, synthetic or inorganic materials and biological or natural materials.

2.6.1 Synthetic or inorganic materials

Various synthetic or inorganic materials has been reported and widely used to apply in biohydrogen production such as glass beads (Zhang et al., 2006), Brick dust (Kumar et al., 1995), expanded clay (Amorim et al., 2009; Barros et al., 2010), polystyrene (Barros et al., 2010; Barros and Silva, 2012), alginate (Kumar et al., 1995; Wu et al., 2003), activated carbon (Zhang et al., 2007b), polyethylene tetra phthalate (Barros and Silva, 2012), polyvinyl-alcohol (Szentgyörgyi et al., 2010).

According to Chang et al. (2002), expanded clay and activated carbon provided better immobilization of cell than loofa sponge which is natural materials, and was further examined for feasibility in helping biohydrogen production.

2.6.2 Biological supporting materials (BM)

Because synthetic materials may possess disposal problems, environmental friendly biological materials are still concerned (Kumar and Das, 2001). Biological materials may collect from some part of plants and/or animals.

2.6.2.1 Biological materials from plants

Biological materials from plants that have been reported to use as immobilized supporting materials in biohydrogen production, for example, loofa sponge (Chang et al., 2002), rice straw, bagasse, coir (Kumar and Das, 2001). Plant cell walls are composed of cellulose (mainly), hemicellulose, and lignin. Cellulose is a linear polysaccharide consisting of D-glucose units (Figure 2.10). Cellulose chains are arranged into micro fibrils which forming both crystalline and amorphous region. Bundles of cellulose micro fibrils form macro fibrils, and are surrounded in an amorphous matrix of hemicellulose and lignin (Meyers et al. 2008)

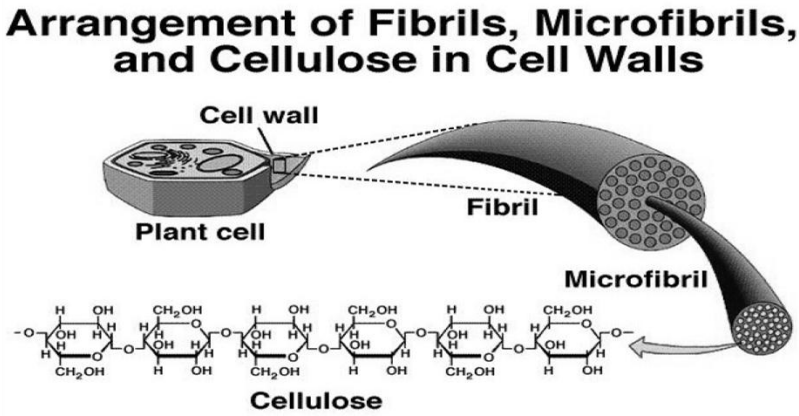


Figure 2.10 Cellulose polymer chain (Mounika and Ravindra, 2015)

Hemicellulose is a branched polysaccharide containing various sugars, typically, L-arabinose, D-galactose, D-glucose, D-mannose, and D-xylose, and may contain other components such as acetic, glucuronic, and ferulic acids (Mussatto and Teixeira, 2010). Finally, lignin, a complex polymer of phenolic acids (Wanitwattanarumlug et al., 2012) linked in a three-dimensional structure (Mussatto and Teixeira, 2010). Figure 2.11 presents schematic image of plant cell wall structure which cellulose is embed in hemicellulose and lignin. An example of BM from plants is shown in Figure 2.12, loofa sponge which is porous fiber network.

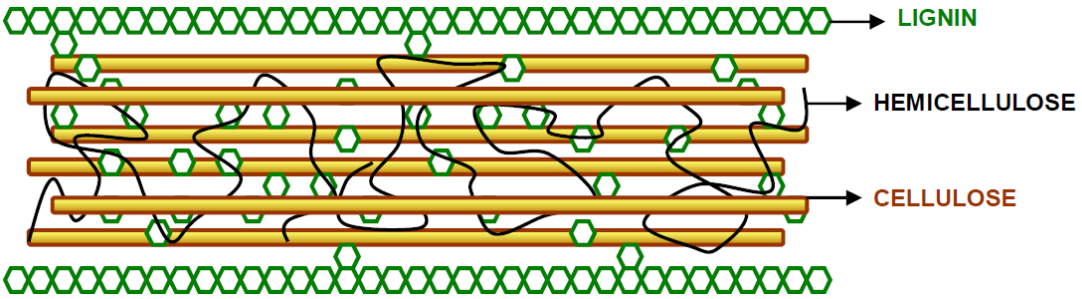


Figure 2.11 Schematic image of plant cell wall structure which cellulose is embed in hemicellulose and lignin (Mussatto and Teixeira, 2010)

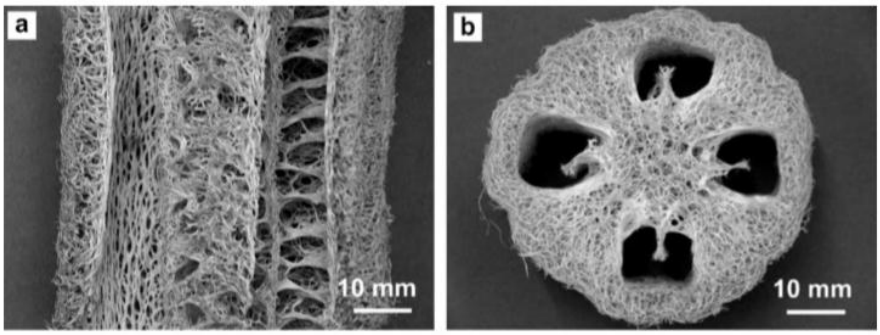


Figure 2.12 SEM image of loofa sponge (a) vertical cross-section (b) Top-view

2.6.2.2 Biological materials from animals

Biological materials from animals are barely reported for its usage in biohydrogen production. However, there are many reports of using BM from animal in material science. Based on main chemical components, BMs from animal in this study are classified into calcium carbonate ($CaCO_3$)-based, chitin-based, and protein based materials.

$CaCO_3$-based material used in this study is shells. Figure 2.13 shows shell of bivalve (the right one) of *Nemocardium peramibilis*. Its surface is rough and contains holes.

Chitin is long chain polysaccharide of N-acetyl-D-glucosamine (Figure 2.14) which can be found in some algae, fungus walls, and arthropods exoskeleton. Chitin-based material in this study is crab exoskeleton. Its chitin has highly crystalline structure. Chitin nanofibers are surrounded by protein, then rods of chitin-protein hybrid polymer is arranged in twisted plywood structure or called

Bouligand structure (Meyers et al., 2008; Shinsuke et al., 2011). Figure 2.15 shows Schematic picture of microstructure of crab shell.

Silkworm cocoon is one of example of protein-based biomaterials. It is mainly made of two different proteins, fibroin ($C_{15}H_{26}N_6O_6$) and sericin ($C_{15}H_{23}N_5O_8$) (Zhao et al., 2005). Surface of cocoon is a fiber network and has pore (Figure 2.16)

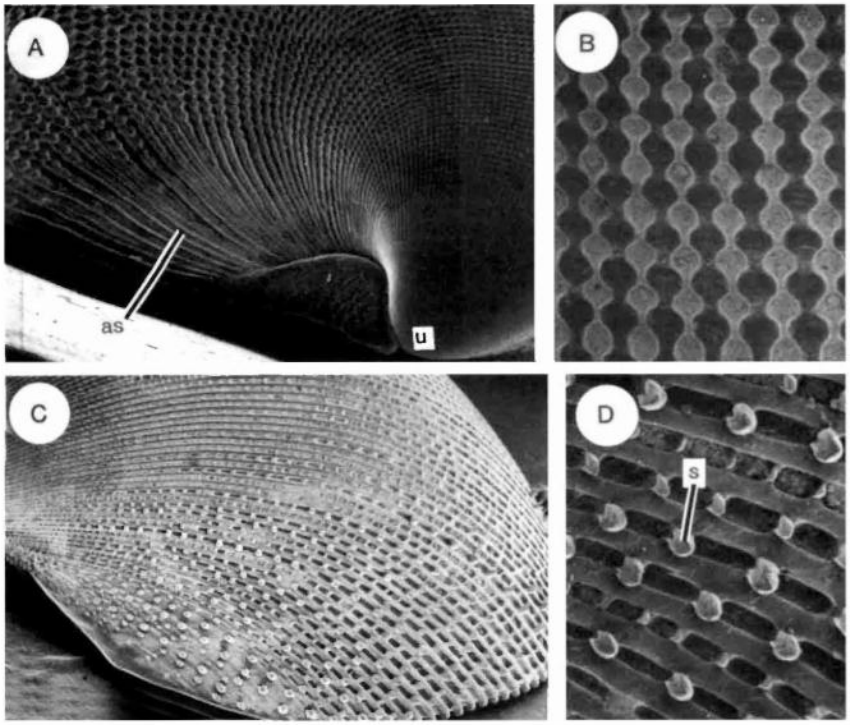


Figure 2.13 Surface of right valve of *Nemocardium peramibilis* A) Dorsal view of anterior slope, absence of radial ribs, and presence of concentric sculpture confluent with concentric sculpture on central slope B) Detail of central slope C) Posterior slope, presence of spines and ladder-like cross-stripe D) Detail of posterior slope, showing spines. (Schneider et al., 1995)

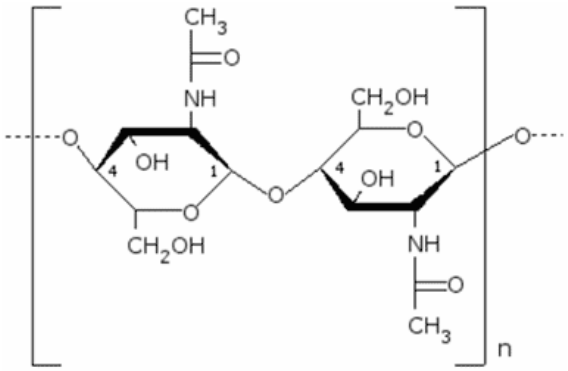


Figure 2.14 Chitin chain polymer (Dharmananda S., 2005)

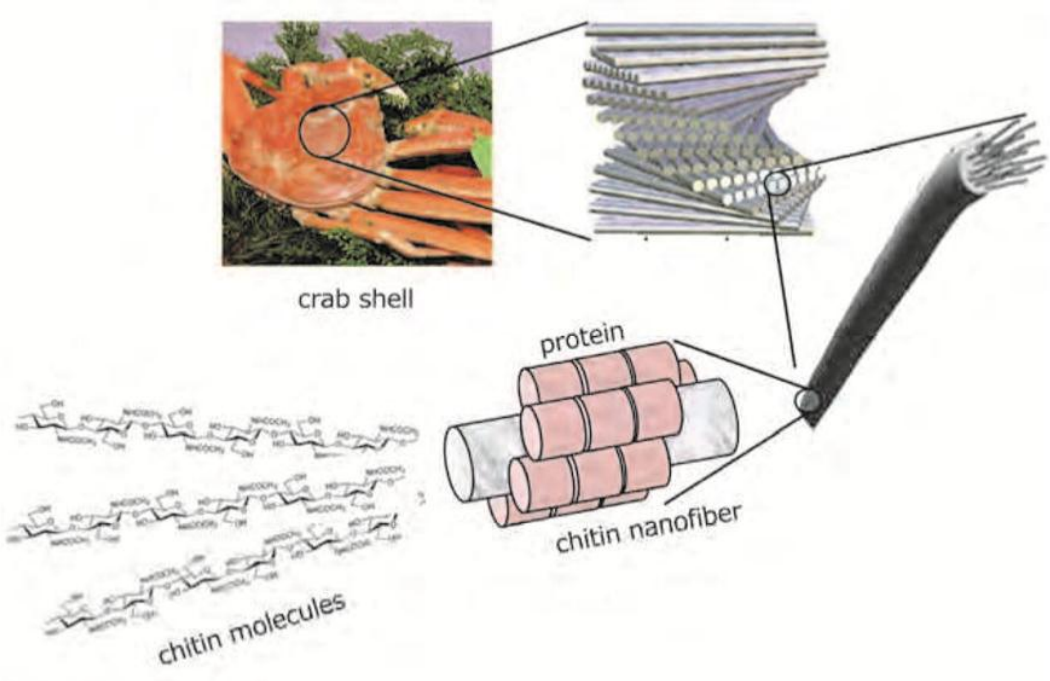


Figure 2.15 Schematic picture of the exoskeleton structure of crab shell (Shinsuke et al., 2011)

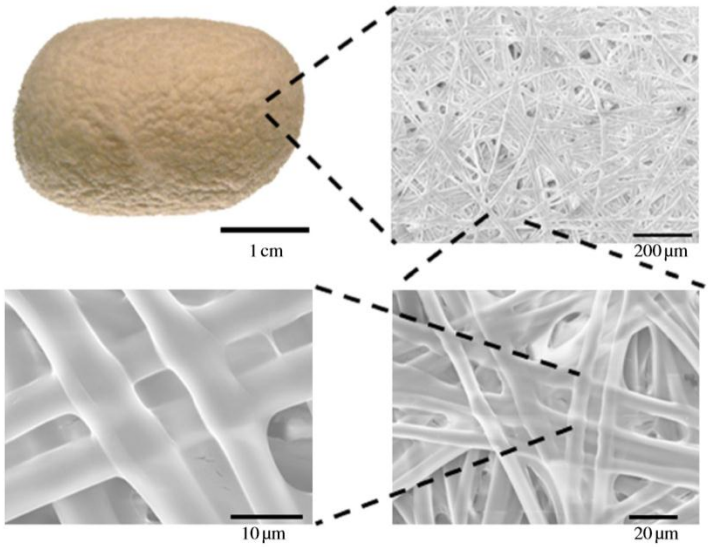


Figure 2.16 Image of the morphology of a *Bombyx mori* cocoon. (Chen et al., 2012)

2.7 Fluorescence *in situ* hybridization (FISH)

Fluorescence *in situ* hybridization is a technique used to quantitative or qualitative analysis of microbes by detecting nucleic acid sequences in the cells. The sequences of an interested genus or specie are specified by fluorescently labeled probe containing complementary sequences.

2.7.1 Brief history

In situ hybridization (ISH) was introduced by two independent research group, Pardue and Gall (1969) and John et al. (1969). ISH allowed us to determine inside cells without altering the cells morphology. The probe in early ISH was labeled by radioactive substances. Later, fluorescent labels were developed and steadily replaced the original isotropic one. There are many advantages of fluorescent labels over the radioactive labels including stability, resolution, speed, cost-effective, ease of use and safety. In addition, it allows labeling different dyes thus able to analyze several targets within a single run (Moter and Gobel, 2000). FISH was firstly applied with bacteriology in 1989. DeLong et al. (1989) used 16s rRNA-targeted fluorescent probes for the determination of single whole bacterial cells. Since then, this technique became a routine for molecular analysis of microbial community (Kubota et al., 2006).

2.7.2 16s rRNA genes-targeted probe

Evolutionary relationships of any life-forms could be examined by correlating a stable part in the genetic code (Woese et al, 1987). Ribosome or ribosomal RNA (rRNA) are necessary for existence of all organisms and are highly conserved in both prokaryotes and eukaryotes (Gurtler and Stanisich, 1996). It contains 2 subunits; small and large (Figure 2.17). These subunits contain various polypeptides and rRNA species. The large subunit consists of 23s and 5s rRNA molecule while the small subunit includes a 16s rRNA molecule (Voet and Voet, 2004). The most frequently targeted in FISH is 16s rRNA genes, also designed as 16s rDNA, due to genetic stability, function as a domain structure, high copied quantity, and highly conserved. Dubnau et al. (1965) reported a conservation of the 16s rRNA gene sequence relationship in *Bacillus* spp, and after the pioneering works of Woese (1987), 16s rRNA gene are widely targeted to identify microbial identification and taxonomy.

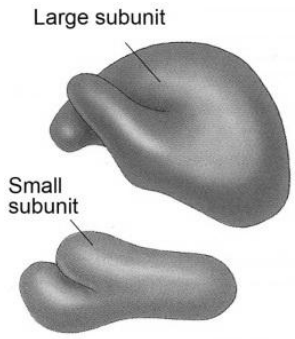


Figure 2.17 Ribosonal RNA (rRNA) structure (The Pennsylvania State University, 2015)

However, rRNA-targeted FISH had a limitation. That was low fluorescence intensity which caused from, for example, low rRNA content, impermeability of cell walls, and, especially, Low probe hybridization efficiency. Yilmaz and Noguera (2004) and Yilmaz et al. (2006) studied affinity of probe to the target gene. The affinity was described as the overall Gibbs free energy change ($\Delta G^{\circ}_{overall}$) and was studied utilizing a model consisting of the DNA-RNA (probe-rRNA hybrid), DNA-DNA (probe self-folding), and RNA-RNA (rRNA self-folding).

The result was found that more than 90% of rRNA-targeted probes obtained moderate to high brightness after hybridization equilibrium.

2.7.3 Procedure

The general procedure conclude these following steps (Figure 2.17) (a) fixation of sample to protect the nucleic acid sequences from a degradation by some endogenous enzyme (Moter and Gobel, 2000) and help infiltration of the fluorescence probe (b) the sample is spotted on the glass slide which is pretreated with coating agent, e.g. gelatin, for better attachment of the sample to the glass slide (c) the sample is hybridized with an appropriate fluorescent probe (d) the unbound probe is wash out by suitable washing solution (e) then waiting for the hybridization of the target sequence and the probe (f) visual the hybridization under dark humid chamber to analysis the results.

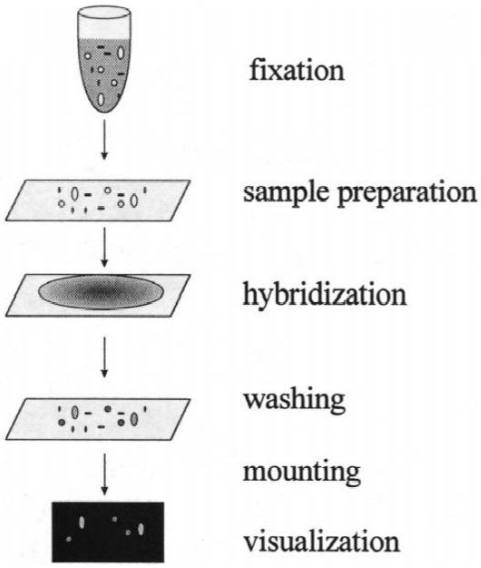


Figure 2.18 General procedures for FISH technique (Moter and Gobel, 2000)

2.7.4 Applications

FISH has been used in medicine, for example, detection of pathogens and infections within tissues and/re organs (Kempf et al., 2000; Jansen et al., 2000; Jensen et al., 2000). Moreover, it has been applied to identify bacterial diversity in various

natural environments such as aquatic habitats (Lolbet-Brossa et al., 1998; Ravenschlag et al., 1999) and wastewater treatment (Kim and Ivanov, 2000; Jang and Kim, 2011).

2.8 Related researches

Sen and Sutra (2012) reported that heat treatment on the seed sludge showed the highest biohydrogen production from sago-starch powder thus this treatment was selected to further use in the next experiment with sago-starch wastewater. Then they observed the effect of starch concentration, pH, inoculum size and nutrients (PY and Fe ions) on the fermentation operated under 30 °C. They obtained the optimal condition at 0.5% substrate, pH 7, 10% inoculum size and 0.1% PY. The maximum hydrogen yield and rate was 412.6 mL/g starch and 78.6 mL/L·h, respectively.

O-thong et al. (2011) examined possibility to produce biohydrogen from cassava starch processing wastewater via fermentation by using three thermophilic mixed cultures which collected from a hot spring placed in the Southern part of Thailand. The fermentation was operated in batch at working temperature in range of 53-68°C and pH 5.5. Three mixed cultures were called as PK, SW, and PR, and each generates maximum hydrogen yields of 287, 264, and 232 mL H_2/g starch in the wastewater, severally. From phylogenetic analysis, thermophilic mixed cultures were likely *Thermoanaerobacterium saccharolyticum, Thermoanaerobacterium thermosaccharolyticum, Anoxybacillus, Geobacillus and Clostridium*, and also observed that fermentation of cassava starch processed through acid-ethanol and acetic-lactic acid pathways.

Chu et al. (2009) combined 16S rRNA-based fluorescence in-situ hybridization (FISH) to quantitative analysis of hydrogen-producing microorganisms in hydrogen fermentation of wastewater containing glucose. Quantitative investigation especially focused on genus *Clostridium*. The results showed positive correlation between the cells quantity and hydrogen yield of the respective sludge. The numbers of hydrogen-producing bacteria were gradually deducted when HRT of the fermentation rose. Therefore, percentage of the hydrogen-producing bacteria to total

bacteria is an advantage parameter which should considered for development of fermentative hydrogen production process.

Yang and Shen (2006) investigated effect of ferrous ions (Fe^{2+}) concentration on hydrogen production from fermentation of soluble starch with mixed microorganism at 35°C and various pH (7.0 and 8.0). Ferrous ions were added in the form of $FeSO_4$. When $FeSO_4$ of 150 mg/L, both pH value obtained maximum hydrogen yield of 296.2 mL/g Starch for pH 7.0 and 279.9 mL/g Starch for pH 8.0. The minimum hydrogen yield was found when no ferrous ion added at both pH. Moreover, inhibition of iron did not occur although ferrous ions concentration increased to 4000 mg/L. In contrast, the fermentation process was accelerated resulting to lag phase and fermentation ending time were abbreviated.

Zhang et al. (2003) researched effect of pH and starch concentration on conversion of synthetic wastewater containing starch into hydrogen through fermentation of mixed culture obtained from a sucrose-containing wastewater treatment reactor. The fermentation was processed on 37 and 55°C to compare feasibility for hydrogen production. At 55°C, lag phase was longer, but could turn larger starch into hydrogen, and was selected to further study of an effect of pH (4.0-9.0). The maximum hydrogen yield (92mL/g of starch) was found at pH 6.0. Starch concentration in wastewater was varied from 9.2 to 36.6 g/L. The maximum hydrogen production yield was found at starch concentration 9.2 g/L, and the yield decreased when starch concentration increased. Decline of production outcome could be ensued from rising of total acids and/or alcohols formation which may suppress generation of hydrogen, and the researcher also reported that total acids and/or alcohols production directly varied to starch concentration.

Chang et al. (2002) studied immobilization on several porous supporting materials (loofa sponge, expanded clay, and activated carbon), and its performance of hydrogen production from sucrose operated in fixed-bed bioreactor. Seed sludge was collected from Municipal Sewage Treatment Plant in Taiwan. Among supporters, loofa sponge provided inefficiency for cell immobilization. Expanded clay and activated carbon showed better capability for microbial immobilization, and were selected to further experiment and pack in the fixed-bed reactors for continuous fermentative hydrogen production. A hydraulic retention time (HRT) was varied from

0.5 to 5 h. Expanded clay operated maximum hydrogen production rate of 0.0965 L/h biomass at hydraulic retention time (HRT) equaled to 2 hours. Activated carbon performed better maximum hydrogen generation rate of 1.32 L/h at the same HRT.

Kumar and Das (2001) researched on effect of several lignocellulosic materials and difference of bioreactor configuration on continuous hydrogen production. Lignocellulosic materials were coir, rice straw, and bagasse. Due to holding up of gas in packed bed reactor, various bioreactor shapes (tabular, tapered, rhomboid shape) were considered to solve this problem, and tapered and rhomboid shape provided improvement of reducing gas hold-up. Compare to tabular shape, rhomboid shape decreased gas hold-up better by 67%, and generated maximum hydrogen production rate of 71 mmol/L·h. Three lignocellulosic materials were immobilized by *Enterobacter cloacae* pure cultures. Among materials used, coir showed the best in terms of cell immobilization (0.44 g dry cell/g dry carrier), packing density (100 g/liter reactor volume), cell loading (44 g dry cell/liter reactor volume) and hydrogen production rate (62 mmol/L·h).

Lee et al. (2001) studied effect of iron concentration on hydrogen production from fermentation of sucrose with mixed consortia. Iron concentration was varied from 0 to 4000 mg $FeCl_2$/L. The maximum hydrogen yield of 131.9 mL/g sucrose was obtained at concentration of iron equaled to 800 mg $FeCl_2$/L which is much higher than the condition that no $FeCl_2$ added (2.0 mL/g sucrose). At 0 mg $FeCl_2$/L, acetate was the main product (260.6 mg/g sucrose) following by butyrate (32.9 mg/g sucrose). Variety of metabolites formation increased when iron concentration increase. At iron concentration of 800mg $FeCl_2$/L, there were acetate (134.9 mg/g sucrose), butyrate (131.7 mg/g sucrose), ethanol (103.2 mg/g sucrose), butanol (67.6 mg/g sucrose), propionate (15.5 mg/g sucrose), and propanol 2.3 mg/g sucrose).