CHAPTER 2 LITERATURE REVIEWS

Biohydrogen is regarded as an attractive future clean energy carrier due to its high energy content and environmental-friendly conversion. There are many attempts to improve the performance of biohydrogen process. As the biohydrogen relied on the biological process, many unknown parameters could be modified to achieve the maximum rate of hydrogen production. Consequently, the pathways of reactions involved the biohydrogen process are another key parameter that is useful for enhancing the performance of biohydrogen process. Therefore, the biohydrogen process is being dynamically developed. The fundamentals and applications of biohydrogen are presented in the research hypothesis.

2.1 Biohydrogen Process

Biohydrogen is a sustainable energy resource due to its potentially higher efficiency of conversion to usable power, non-polluting nature and high energy density. Hydrogen is one of the alternative fuels and it is recognised as a promising future energy carrier. It is considered a clean fuel since it is free from greenhouse gas emission (e.g. carbondioxide, and nitrous oxide) and the toxic gaseous during combustion [13]. The biological processes of hydrogen production fundamentally depend upon the presence of hydrogen producing enzyme. These enzymes can catalyse the chemical reaction in the metabolism pathway as: $2H^+ + 2e^- \leftrightarrow H_2$. A survey of all presently known enzymes capable of hydrogen evolution shows that they often contain the complex metallo-clusters as active sites [14].

Two types of enzymes can catalyse the reduction of protons to H_2, namely nitrogenase and hydrogenase. Both enzymes are metalloproteins containing Fe/S clusters. The [MoFe]-nitrogenase, with a MoFe cofactor at its active site, is the most widespread and best studied of the nitrogenases. The hydrogenase enzymes are more diversity and these can be distributed into two classes of distinct phylogenetic origin as the [NiFe] hydrogenases and the [FeFe] hydrogenases. The hydrogenases enzymes usually contain ion of Ni coupled with one Fe ion or two Fe ions, respectively. The Ni and Fe ions are form the ligand by cysteine residues, at their active site. The nitrogenase complex catalytic enzymes can reduce or denitrogenise the nitrogen compounds to ammonia,

using electrons from low potential reducer [15]. Biohydrogen can be produced by both light and dark fermentation processes. Dark fermentation processes can provide a high hydrogen yield with a reasonable production cost [16, 17]. Biohydrogen can be generated via different pathways which can be broadly categorised into two distinct groups, viz. light-dependent and dark fermentative processes. Light-depended processes include photolysis and photo fermentation, whereas dark fermentation is the major light independent process [18].

2.1.1 Direct photolysis

This method is similar to the processes found in plants and algal photosynthesis. In this process solar energy is directly converted to hydrogen. This is an attractive process since solar energy is used to convert a readily available substrate, water, to oxygen and hydrogen. However, only under special conditions hydrogen production is possible by this method since Fe-hydrogenase activity is extremely oxygen sensitive [14].

The process uses the photosynthetic capability of green algae and cyanobacteria to split water by the directly absorbed light energy and concomitant transfer of electrons to a hydrogenase or a nitrogenase for H_2 production. In order to collect enough energy, large bioreactor surface area is needed, which increases the cost for production of hydrogen fuel. Photosynthetic energy conversion efficiency can be as high as 10%. This value is close to the practical maximum value because the overall process is limited primarily by the efficiency of the dark reaction, but it is not disturbed by the light energy capture process. A great proportion of the absorbed photon energy is wasted by the photosynthetic apparatus and dissipated as heat or fluorescence. Conversion efficiency at 10%has been demonstrated under low-light and low oxygen partial pressure conditions [20]. Heterocystous cyanobacteria offer a possible solution of the problem. Cyanobacterial H_2 evolution takes place in the anaerobic environment of the heterocysts by the nitrogenase enzyme complex [21]. The amount of H_2 evolved during nitrogen fixation may be similar to that of the hydrogenases but the process requires a lot of chemical energy, rendering the system economically unfeasible at the moment. The ATP synthesised by photophosphorylation may not always be the limiting factor, nevertheless, nitrogenase based H_2 evolution can reach significant level due to the high copy numbers of these enzymes [19]. The direct biophotolysis reaction can be written as follows.

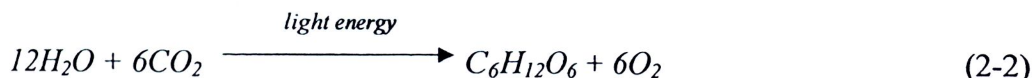
$$2H_2O_2 \xrightarrow{light\ energy} 2H_2+O_2 \qquad (2\text{-}1)$$

2.1.2 Indirect biophotolysis

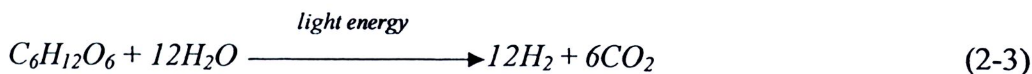
Indirect biophotolysis has the advantage of separating in time the O_2 and H_2 evolution steps. It involves a photosynthetic biomass production step and an anaerobic dark fermentation of the biomass to produce H_2. If the oxygen and hydrogen evolution steps can be separated in time, the apparently inherent O_2 sensitivity of the H_2 evolving process is circumvented. Several models to achieve indirect biophotolysis have been developed. These systems utilise algae in most cases and intend to exploit their capability to produce high biomass yield per surface area photoautotrophically, to be able to carry out dark fermentation as well as photoheterotrophic growth. Detailed feasibility study of a concept [22] has been presented that includes production of algal biomass, which is rich in easily fermentable storage carbohydrates in open ponds, and subsequent anaerobic fermentation of the stored carbohydrates to yield 4 mole of $H_2$and 2 mole of acetate from each mole of glucose. In the photofermentative, the photoconversion of the acetates theoretically gives 8 mole of H_2 per 2 mole of acetate. The process is estimated to be economically, but it may be unrealistic due to the high production cost of the photobioreactors. A significant bioenergetic limitation is the ultimate use of nitrogenase as H_2 producing catalyst and nitrogenases are notorious for their extremely high chemical energy demand. The basic process can be supplemented with anaerobic digestion, i.e., biogas production, from the waste biomass and separation of H_2 and CO_2 with a subsequent recycling of CO_2 to the algal growth ponds. The indirect biophotolysis production process has been reported which uses the green algae *Chlamydomonas reinhardtii* [23].

In the indirect biophotolysis process, problems of sensitivity of the hydrogen evolving process are potentially circumvented by separating temporally and/or spatially oxygen evolution and hydrogen evolution. Thus indirect biophotolysis processes involve separation of the H_2 and O_2 evolution reactions into separate stages, coupled through CO_2 fixation/evolution. Cyanobacteria may have the unique characteristics of using CO_2 in the air as a carbon source and solar energy as an energy source. The cells can take up CO_2 firstly to produce cellular substances, which are subsequently used for hydrogen

production [14]. The general reaction for hydrogen formation can be presented by following reactions:



$$12H_2O + 6CO_2 \xrightarrow{\text{light energy}} C_6H_{12}O_6 + 6O_2 \quad (2\text{-}2)$$



$$C_6H_{12}O_6 + 12H_2O \xrightarrow{\text{light energy}} 12H_2 + 6CO_2 \quad (2\text{-}3)$$

2.1.3 Dark fermentation

Fermentative processes can use biomass ultimately and the $H_2$is obtained without a photosynthetic solar energy conversion system. Much is presently known about the molecular biology of the H_2 producing enzymes [24] and metabolic engineering to direct fermentation to H_2 production is scientifically feasible [25]. Dark microbial H_2 production is driven by the anaerobic metabolism of the key intermediate, pyruvate. The complete oxidation of glucose would yield a stoichiometry of 12 mole H_2 per mole of glucose but in this case no energy is gained to support growth and metabolism of the producing organism [26]. Under carefully chosen conditions the thermophiles can produce H_2up to 60–80% of the theoretical maximum, demonstrating that higher hydrogen yields can be reached by extremophiles than using mesophilic anaerobes [27]. The oxidative pentose phosphate pathway, as an alternative metabolic route exists for example in microalgae, which can produce stoichiometric amount of H_2 from glucose. However, this pathway is usually not functional for energetic reasons. The potential benefit of an in vitro system using the pentose phosphate pathway has been demonstrated [28]. The reactions can be written as follows.

End product: acetic acid

$$C_6H_{12}O_6 + 2H_2O \longrightarrow 2CH_3COOH + 4H_2 + 2CO_2 \quad (2\text{-}4)$$

End product: butyric acid

$$C_6H_{12}O_6 + 2H_2O \longrightarrow CH_3CH_2CH_2COOH + 2H_2 + 2CO_2 \quad (2\text{-}5)$$

2.1.4 Photofermentation

Photofermentation, or photoheterotrophic hydrogen production, is a mechanism found among a diverse group of photosynthetic bacteria and they can well characterized as purple nonsulfur bacteria PNSB. This biomechanism is primarily mediated by the enzyme nitrogenase. Similarly to cyanobacteria, PNSB may bidirectionally uptake hydrogenases that may contribute to hydrogen cycling within cells. PNSB provides an advantage over cyanobacteria and algae because they exist solely under anaerobic conditions and do not have to separate their growth phase from the H_2 generating activities. Photohetertrophs can obtain energy from sunlight, oxidising organic compounds and generating the electron potential, which are needed to drive H_2 production. By utilising energy from the sun to drive thermodynamically unfavorable reactions, PNSB can potentially divert 100% of electrons from an organic substrate to H_2 production [29]. This is a considerable advantage over fermentative bacteria, which can only divert a theoretical 1/3 of electrons (in practice about 15%) from highcarbohydrate waste streams for H_2 production [30]. Photoheterotrophs typically utilise the smaller organic acids that are often produced but they are not metabolised, during dark fermentation. Thus, waste streams from photofermentation contain the few byproducts such as the organic compounds, which are fully reduced to the forms of H_2 and CO_2.

Photosynthetic bacteria have long been studied for their capacity to produce significant amounts of hydrogen [31]. The advantage of their use is in the versatile metabolic capabilities of these organisms and the lack of Photosystem II, which automatically eliminates the difficulties associated with O_2 inhibition of H_2 production. Phototrophic bacteria require organic or inorganic electron source to drive their photosynthesis. This is a small disadvantage because they can utilise a wide range of cheap compounds. The significant disadvantages in these systems are dealing with the use of the nitrogenase enzyme as H_2 generation catalyst, the requirement for elaborate and expensive anaerobic photobioreactors covering large areas, and the low photosynthetic efficiencies. The slow and highly energy demanding nitrogenase enzyme can be replaced by the newly discovered bidirectional NiFe hydrogenases in purple sulphur phototrophic bacteria. Photosynthetic bacteria can offer the advantage of combining organic waste disposal problems with the production of the clean fuel H_2. In a proposed integrated system dark fermentation and photofermentation are combined in order to

achieve maximal conversion of the substrate to biohydrogen [27].The reaction is presented as follows.

$$C_6H_{12}O_6 + 12H_2O \xrightarrow{\text{light energy}} 12H_2 + 6CO_2 \quad (2\text{-}6)$$

2.2 Principles of dark fermentation

Dark fermentation under anaerobic conditions seems to be the most favorable among the bioproduction processes. The fermentation can be carried out at higher rates and lower cost using various organic substrates and wastewaters [32]. Dark fermentation uses primarily anaerobic bacteria, although some algae are also used, on carbohydrate rich substrates and they can be grown without the need of light energy [5].

Hydrogen is a key compound in the metabolism of many anaerobic, as well as a few aerobic, microorganisms. Many organisms have the capacity to use this energy-rich molecule when it is available in the environment and derive electrons from its oxidation to drive energy generation. In the absence of external electron acceptors, some organisms dispose of excess electrons generated during metabolism by reducing protons to hydrogen. In both cases, key enzymes are hydrogenases. There are two main types of hydrogenases which are phylogenetically distinct and containing different active sites where the relevant chemistry occurs; Ni–Fe hydrogenases and [FeFe] hydrogenases. The occurrence, enzymology and maturation of both Ni–Fe and [FeFe] hydrogenases have been extensively studied [33]. In general, NiFehydrogenases are poised to catalyse hydrogen oxidation, although exceptions are known (e.g. Hyd3 of the enteric bacteria is responsible for hydrogen evolution), and [FeFe] hydrogenases are extremely active in proton reduction, although there are exceptions (e.g. the periplasmic [FeFe] hydrogenases of sulphate reducing bacteria). Depending upon the organism, hydrogen evolution may be catalysed by a soluble [FeFe] hydrogenase (e.g. *Clostridia*) or a special class of membrane bound NiFehydrogenase, Escherchia (e.g. *Escherchia coli*). The Echhydrogenases, found in a variety of organisms, are interesting since they are capable to be coupled with hydrogen evolution, generating a membrane proton gradient [34].

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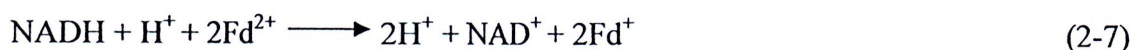
2.2.1 Mechanisms

In dark fermentation, microbes anaerobically break down carbohydrate-rich substrates into organic acids and alcohols, releasing H_2 and CO_2 in the process. The substrate for dark fermentation can often be supplied by several wastewater streams, performing the dual functions of treatment and energy production. A variety of microbes can be used, and the consortium that develops in such a reactor can affect the distribution of byproducts, including the makeup of biogas. Typically, seeded with a mixture of undefined microorganisms and the other strains in the fermentor are selected based upon the feedstock delivered to the bioreactor and the reactor conditions (residence time, temperature, etc.). Hydrogen-producing sludges are often dominated by *Clostridium* species [35]. Members of this genus develop heat resistant spores, and sludge samples are heat-treated to remove methanogens or other bacteria that interfere with biohydrogen production by utilising the H_2 being produced. This is one advantage of dark fermentation systems, as communities of microorganisms are generally more stable and adaptable to changes in environment and feedstock than pure cultures [36], making them better suited for continuous operations and waste stream applications. Pure cultures are more ideally suited for specific tasks and metabolic engineering. Fermentative biohydrogen production is characterised by high production rates but low substrate efficiencies. The low hydrogen yield stems from the fact that carbohydrates are not fully metabolised, leading to incomplete conversion to H_2 and CO_2 and the generation of byproducts, such as organic acids, in the waste stream. The biogas produced from dark fermentation contains much lower fractions of H_2 than ones are achievable in biophotolysis or photofermentation systems, so a separation step is needed to recoverH_2. The redox potential needed for electron carriers for hydrogen reduction are governed from ferredoxin and NADH, however they can only be maintained when the partial pressure of H_2 is less than 0.3 atm and 6×10^{-4}atm, respectively [30]. Thus, continuous flushing of nitrogen gas is needed to maintainH_2 production. Utilisation of waste streams presents challenges for managing infiltration of competing microorganisms from nonsterilised feedstocks by pretreatment or maintaining specific environmental conditions to favour H_2- producing consortia. In addition, identifying consortia or specific strains to metabolise particular feedstocks is a challenge. Carbohydrates are the preferred substrate for hydrogen producing fermentations. Glucose yield different amount of hydrogen depending on the fermentation pathway and endproduct(s). In strictly anaerobic bacteria, a theoretical maximum of 4 moles of

hydrogen per mole of glucose is obtained. The facultative anaerobes like *Escherichia coli* can produce maximum 2 moles of hydrogen per mole of glucose. In laboratory experiments, hydrogen production rate of the order of 77 mmol/h-L has been achieved [37].

2.2.2 Dark fermentation pathway

Dark fermentative hydrogen production is a ubiquitous phenomenon under anoxic or anaerobic conditions. Many bacteria use reduction of protons to hydrogen via hydrogenases as a means of oxidising the carriers reduced during fermentation, which allows the carriers to recycle and maintain electrical neutrality. So a continuous supply of adenosine triphosphate (ATP) can be generated by substrate-level phosphorylation [38]. Molecular hydrogen formation is generally followed two routes in the presence of specific coenzymes, i.e., either by formic acid decomposition pathway or by the re-oxidization of nicotinamide adenine dinucleotide (NADH) pathway. The reactions involving this process are presented as follows.



$$NADH + H^{+} + 2Fd^{2+} \longrightarrow 2H^{+} + NAD^{+} + 2Fd^{+} \quad (2\text{-}7)$$

$$2Fd^{+} + 2H^{+} \xrightarrow{\text{hydrogenase}} 2Fd^{2+} + H_2 \quad (2\text{-}8)$$

The Embden–Meyerhof or glycolytic pathway is applied to convert glucose into pyruvate associated with the conversion of NADH from NAD+ via anaerobic glycolysis, which could be presented by:

$$C_6H_{12}O_6 + 2NAD^{+} \longrightarrow 2CH_3COCOOH + 2NADH + 2H^{+} \quad (2\text{-}9)$$

Hydrogen is generated through the reoxidisation of NADH by some specific microorganisms under acidogenic conditions in the presence of ferredoxin oxidoreductase and hydrogenase [39]. This metabolic route is present in some species of *Clostridium*. The obligate anaerobic *Clostridia* may lack of a typical cytochrome system and they amy obtain energy by substrate-level phosphorylation during fermentation. Oxidising carbohydrates generates electrons which need to be disposed of to maintain electrical neutrality. For the saccharolytic *Clostridia*, hydrogen evolution via a hydrogenase is a major route through which the cells dispose excess electrons produced

from the oxidative breakdown of carbohydrates [40]. NADH-ferredoxin reductase functions are primarily accounted as an electron carrier and these involved in pyruvate oxidation to acetyl-CoA and carbon dioxides as well as proton reduction to molecular hydrogen. *Clostridia* can break down pyruvate to acetyl-CoA to produce 2 mole of NADH and 2 mole of reduced ferredoxin.

Four moles of hydrogen per mole of glucose is achieved, which is the theoretical maximum yield of dark hydrogen fermentation, if all of the substrate would be converted to acetic acid. Once, the substrate is converted to butyric acid, this H_2production ratio becomes two moles of hydrogen per more of glucose, since 2 mole of NADH has been consumed during the conversion of intermediate products. The available hydrogen from glucose fermentation is determined by the ratio of butyrate/acetate produced during fermentation. As mentioned earlier, no NADH is used as a reductant for alcohol production. However, the disposal of electrons via pyruvate-ferredoxin oxidoreductase or NADH-ferredoxin oxidoreductase and hydrogenase might be affected by the corresponding NADH and acetyl-CoA levels as well as environmental conditions. As a result, the oxidation–reduction state has to be balanced through the NADH consumption to form some reduced compounds, i.e., lactate, ethanol and butanol, resulting in a lowered hydrogen yield [18].

2.3 Production Rates of Biohydrogen Systems

Hydrogen production rates for several biohydrogen systems are given in Table 2.1 [16]. The unit of hydrogen production is standardised into mmol H_2 per L of culture per hour (mmol H_2/L-h). Light dependent biohydrogen systems such as direct biophotolysis, indirect biophotolysis, and photo fermentation all have rate of hydrogen synthesis lower than 1 mmol H_2/L-h, while only dark fermentation system produce hydrogen at rates above 1 mmol H_2 /L-h. Dark fermentation systems utilised undefined consortia of mesophilic bacteria have the hydrogen synthesis rates of 64.5 and 121.0 mmol H_2/L-h, respectively.

Table 2.1 Rates of hydrogen synthesis [16]

System	Hydrogen synthesis rate (mmol H_2/L-h)	Reference
Direct biophotolysis	0.07	[41]
indirect biophotolysis	0.355	[42]
Photo-fermentation	0.16	[43]
Dark-fermentations		
mesophilic, pure strain [a]	21.0	[44]
mesophilic, undefined [b]	64.5	[45]
mesophilic, undefined	121.0	[46]
thermophilic , undefined	8.2	[46]
extreme thermophilic, pure strain [c]	8.4	[47]

Note: [a] is *Clostridium* species #2. [b] is a consortium of unknown microorganisms cultured from a natural substrate and selected by the bioreactor culture conditions. c is *Caldicellulosiruptor saccharolyticus*.

2.4 Microbial Colony

Fermentative hydrogen production can be carried out via a wide range of microbes, with quite diverse requirements in terms of substrate preference, pH and temperatures [48]. Those parameters do not only determine the growth of the microorganisms, but also have a crucial role on the metabolic path that the microorganisms may follow, affecting the final observed hydrogen yield from the whole process. Hydrogen production can be achieved either through mixed acidogenic microbial cultures, derived from natural environments such as soil, wastewater sludge, and compost, or through pure cultures of selected hydrogen producing bacteria. Such bacteria can be mesophilic (25–40°C), thermophilic (40–65°C), extreme thermophilic (65–80 °C), or even hyperthermophilic (>80°C). In any case, the final selection of the type of culture to be used (mixed, pure, co-culture) as well as the specific microorganism, has to be based on the specific requirements of each possess [49].

2.4.1 Mixed Cultures

In general, for a fullscale application the selection of mixed cultures is considered to be favourable, at least from an engineering standpoint. This is due to the fact that the control and operation of the process is facilitated without medium sterilisation to reduce the overall cost, whereas it also allows for a broader choice of feedstock

selection [50]. The mixed consortia can be derived from a variety of different natural sources, such as sewage sludge, anaerobically digested sludge, acclimated sludge, compost, animal manure and soil or even from the indigenous microbes found in certain wastes [51].

By subjecting seed cultures to high temperatures, only the sporeforming acidogen can survive after the thermal shocking process, whereas the methanogenic non sporeforming bacteria is killed. The applied temperatures to heat shock have been varied, at 75°C [52], 100°C [53] or even 121°C [54], and the duration of heating process has been varied between 15 min and 2 hours [54]. Apart from the heat treatment, an acid/base treatment of the seed has also been applied as a possible way for selection of hydrogen producing bacteria [55]. The principle of this method is to maintain the microbial seed for a prolonged period of time at very acidic or very basic conditions, which can eventually remove the methanogens since they cannot survive in such extreme pH environment.

2.4.2 Pure Cultures of Wild Strains

Many researchers have focused on the use of pure cultures of specified hydrogen producing species. The main arguments for their advantageous use are the selectivity of substrates, the easiest manipulation of the metabolism by altering growth conditions, the higher hydrogen yields can be achieved because of an effect of the reduction of undesired byproducts. Besides, the process can be well repeatability. On the other hand, the pure cultures are quite sensitive and they are easily contaminated by the other unwanted species. In most cases, the aseptic conditions may be employed, which significantly increases the overall cost of the process.

There is a wide range of microbes that are capable of producing hydrogen via dark fermentation. This includes strict anaerobes (*Clostridia*, methylotrophs, rumen bacteria, methanogenic bacteria, archaea), facultative anaerobes (*E. coli*, *Enterobacter*, *Citrobacter*), and even aerobes (*Alcaligenes*, *Bacillus*). Among the hydrogenproducing bacteria, *Clostridium sp.* and *Enterobacter*, are the most widely studied. Species of genus *Clostridium* such as *C. butyricum* [56], *C. acetobutyricum* and *C. beijerinckii* [57] and *C. thermolacticum* [58] are examples of strict anaerobic and spore forming microbes, generating hydrogen gas during the exponential growth phase. In parallel,

facultative anaerobes such as *E. coli* and species of genus *Enterobacter*, such as *E. aerogenes* [59] and *E. cloacae* [37] have also been used for hydrogen production.

2.4.3 Ambient and Mesophilic Strains

Hydrogen is consumed by the consortia as it is produced mainly by methanogenic archaea, acetogenic bacteria and sulfate reducing bacteria. Diverse microbes capable of hydrogen production are distributed across a wide variety of bacterial groups. Research studies on anaerobic microbes have been intensively developed in recent years, and some new or effective bacterial species and strains for dark hydrogen fermentation have been isolated and recognised [60]. In general, the isolated and identified mesophiles are mainly affiliated with two genera: facultative *Enterobactericeae* [61] and strictly anaerobic *Clostridiaceae* [58], whereas the most numbers of thermophiles are belonged to genus *Thermoanaerobacterium* [62]. Among the fermentative anaerobes, *Clostridia* have been well known and studied extensively. It is not for the hydrogen production capability, but also the role in the industrial solvent production from various carbohydrates.

2.5. Major Enzymes

There are three fundamentally different hydrogen producing and metabolising enzymes found in algae and cyanobacteria: (1) the reversible or classical hydrogenases, (2) the membrane-bound uptake hydrogenases, and (3) the nitrogenase enzymes.

2.5.1. Reversible or Classical Hydrogenases

The oxidized ferredoxin or other low redox electron carriers, governed from both natural and artificial process, can bring a readily reversible reaction in the metabolic process. The hydrogen evolution reaction in green algae, was firstly described in 1942 [63], it was stimulated by a reversible hydrogenase.

2.5.2. Membrane Bound Uptake Hydrogenases

The membrane bound uptake hydrogenases can take up hydrogen at low partial pressures, reducing a relatively high-potential electron acceptor (at the level of the NAD=NADH couple, or even FAD=FADH). However, this ezymes can produce a little or no measurable hydrogen [1].

2.5.3. Nitrogenase Enzymes

The nitrogenase enzymes can normally reduce N_2 to ammonia and they can evolve hydrogen, particularly in the absence of N_2 gas. Among the algae species, only the blue-green algae (cyanobacteria) can generate these enzymes. Hydrogen evolution by these enzymes is an irreversible reaction coupled to the hydrolysis of at least four $ATP=H_2$ produced, making this low efficiency reaction energy. Microalgae can generate one, two or all three types of these enzymes, which are active simultaneously. The activities of enzyme can be changed by large factors, however it can influence the growth condition with the relatively minor impact. Furthermore, several forms of each of the enzymes are known, distinguished primarily by the metal content (e.g., Ni, Fe for hydrogenase and Mo, V or Fe for nitrogenase).The enzyme sometimes presents simultaneously in the same organism. This can lead to some difficulty in the interpretation of any net hydrogen evolution or uptake processes [64].

2.6 Types of Feedstocks

Theoretically, any organic substrate rich in carbohydrates, fats and proteins could be considered as possible substrate for biohydrogen production. As reported by numerous studies, carbohydrates are the main source of hydrogen during fermentative processes, so wastes and biomass enriched in sugars and/or complex carbohydrates can be accounted to be the most suitable feedstocks for biohydrogen generation [5]. The major criteria that have to be met for the selection of substrates suitable for fermentative biohydrogen are the availability, cost, carbohydrate content and biodegradability. Simple sugars such as glucose, sucrose and lactose are readily biodegradable, so too these substrates are the suited substrates for hydrogen production [65]. However, pure carbohydrate sources are expensive raw materials for real scale hydrogen production. The pure subtracted can only be viable whenever the highly demand of renewable energy and affordable cost [66]. Different types of feedstocks have been reported so far for the description of biomass and wastes that have been used for hydrogen productions. The classification criteria used are often either the chemical composition of the major present substrate [56] or the origin of the biomass and waste [67].

2.6.1. Use of Simple Sugars.

Glucose is an easily biodegradable carbon source, which can be observed in most of the food industrial effluents and abundantly from agricultural wastes. Theoretically bioconversion of 1 mole of glucose yields 12 mole of hydrogen gas (H_2). According to reaction stoichiometry, bioconversion of 1 mole of glucose into acetate yields 4 mole H_2/mole glucose, but only 2 mole H_2/mole glucose is formed when butyrate is the end product. The highest hydrogen yield obtained from glucose is around 2.0–2.4 mole H_2/mole glucose [68]. Production of butyrate can bring the lower H_2 yield than the production of acetate. This may be one of the reasons for deviations between the measurable and the theoretical yield. Even the case of highly concentrated glucose feeding, more than 95% glucose is degraded, the yield is less than 1.7 mole H_2/mole glucose [69].

Batch and continuous hydrogen gas production from sucrose has been widely focused. The study of Chen (2003) had obtained a yield of 4.52 mole H_2/mole sucrose in a CSTR with hydraulic residence time at 8 hours [70]. The H_2 yield is higher than the other reports, which are 3.47 mole H_2/mole sucrose in CSTR [71] and 1.5 mole H_2/mole sucrose in UASB [72] under the same HRT. However, the H_2yield from glucose substrateis only 0.91 mole H_2/mole glucose under the same operating conditions in CSTR [73]. Optimisation of C/N ratio at 47 can provide the highly efficient conversion of sucrose to hydrogen gas with a yield of 4.8 mole H_2/mole sucrose [74]. Similarly, cumulative hydrogen production from 1.5 g sucrose was 300 mL while it was only 140 mL from 10 mL concentrated starch solution (150 g/L). The hydrogen content in the biogas reached a maximum value of about40% [75]. *Enterobacter cloacae* ITT-BY 08 can produce 6 mole H_2/mole sucrose, which is the highest yield among the other carbon sources [60]. The maximum hydrogen yield from lactose substrates is of 3 mole H_2/mole lactose, although theoretical yield is 8 mole H_2/molelactose [58]. These results indicate that the higher hydrogen yields can be obtained from sucrose compared to other simple sugars. However, the yield per mole of hexose remains almost the same for all types of the disaccharides.

2.6.2. Use of Starch Containing Wastes

Starch containing materials are abundant and they have the great potential to be used as a carbohydrate source for hydrogen production. According to the stoichiometry, a maximum of 553 mL hydrogen gas is produced from one gram of starch with acetate as a by-product [76]. However, the yield may be lower than the theoretical value because of utilisation of substrate for cell synthesis. The maximum specific hydrogen production rate was 237 mL H_2/g VSS-d, when 24 g/L of food grade corn starch was used by *C. pasteurianum* [77]. The specific yield was 480mL H_2/gVSS-d with 4.6 g/L starch was consumed by a mixed culture at temperature of 37 °C. Even though the thermophilic condition at 55 °C was employed to the *Thermoanaerobacterium,* the H_2 production rate was not boosted, yielding 365mL H_2/g VSS-d with [76]. The experiment of Yokoi (2001) suggested that the dried sweet potato starch residue could be supplied to the mixed culture of *C. butyricum*and *E. aerogenes* to yield H_2. Hydrogen production rate in 300 hr batch operations was at 2.7 mole H_2/mol glucose when a 2.0% of starch residue containing wastewater was fed [59].

2.7 Heat Shock Treatment

Some microbial species such as *Bacillus* and *Clostridium* have the capacity to sporulate when environmental conditions become hostile such as heat shock, changes in nutrients status and the presence of deleterious chemicals [78]. The spores are metabolically dormant and resistant to heat, radiation, desiccation, pH extremes and toxic chemicals [79]. Under anaerobic environments, the main spore forming microbes are consist several genera of acidogenic bacteria. This fact has been applied to eliminate or kill the non spore forming microbes, mainly methanogens, by means of a heat treatment with inoculums typically at approximately 100 °C for 15–120 minutes. This treatment simultaneously selects spores of acidogenic bacteria that can germinate, producing H_2under the favorable growth conditions. Optimal conditions for obtaining high populations of H_2producing microbes seem to be treated at 80 °C for 3 hours at 3% TS. It is proven that the higher the initial TS, the longer time of heat treatment [80]. The transition of dormant spores to actively vegetative forms can be divided into three phases: activation, germination and outgrowth (Figure 2.1). Spore activation is usually achieved by heating spores in aqueous suspension [81]. In this way, it is possible to conclude that heat treatment (also called heatshock pretreatment, HSP) can effectively

eliminate vegetative cells and activate the presence of spores in the inoculum. Later, germination is initiated by interaction of the spore with specific germinants, the most frequent nutrient germinant being L-alanine. Finally, outgrowth leads to the formation of a new vegetative cell [78]. In the literature, there are a wide number of reports have described these three phases with species of Bacillus spores, but very rare proportion with H_2-producing bacteria such as *Clostridium* [82]. Few studied focused on the most favourable conditions for spore germination along with the H_2 production, in spite of its significance for fermentative H_2 production processes.

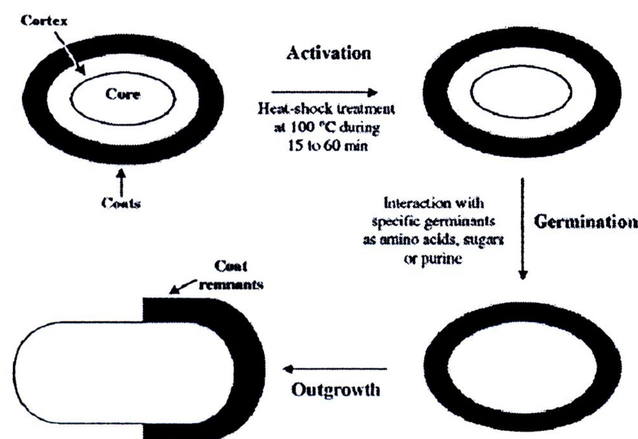


Figure 2.1 Transition of dormant spores to active vegetative forms [79]

2.8 Types of Reactors

Reactor configuration is considered to be crucial for the overall performance of fermentative hydrogen production. It influences the microenvironment such as the prevailing microbial population, the established hydrodynamic behaviour and the contact between substrate and consortia [83]. The most commonly used reactor type is the mixed (continuously stirred) suspended growth reactor. In general, mixed reactors for fermentative hydrogen production can be operated in either batch or continuous mode. Batch mode fermentative hydrogen production has been shown to be more suitable for research purposes [84], but the industrially feasible process would most likely have to be performed on a continuous or at least semicontinuous (fed or sequencing batch) basis.

Many works reflect that batch hydrogen production gives higher hydrogen yields than continuous operation [85]. This can be attributed to the substantially different microenvironment of the microbes in a batch reactor (time-varying and consequently higher substrate concentrations in the early part of the batch), leading to a different metabolic flux than in the constantly low substrate microenvironment in a CSTR. A batch bioreactor has the disadvantage of requiring long times of operation and starting up processes. An interesting alternative operation of a mixed bioreactor is the sequencing batch operation. One cycle is comprised of "feed", "react", "settle", "decant" phases, so the bioreactor is in essence operating in a semicontinuous mode. The optimisation of a mixed bioreactor operation requires appropriate modelling. Relatively few studies have been presented so far on modeling fermentative hydrogen production [86].

Sequencing batch reactor (SBR), either aerobic or anaerobic, is generally reputed for more biomass retention. The inherent capacity to compensate for variable loading conditions in biological systems for wastewater treatment is limited by a relatively slow adaptation of the mass of active bacteria in the system. SBR processes offer distinct advantages when compared with continuous processes, including a high degree of process flexibility and no requirement for a separate clarifier. In SBR, feed injection, reaction, settling, and decanting occur in a reactor sequentially. SBR can decouple SRT from HRT and maintain higher biomass concentration than continuously stirred tank reactor (CSTR). As settling, liquid/ solid in SBR can be self deposited, a separator or clarifier may not be needed. The reactor configuration is also suitable for treating organic solid waste, unlikely other high-rate configurations, such as upflow anaerobic sludge blanket (UASB) reactors. With its intrinsic flexibility, the modern SBR is widely spread and it is subject to extensive research to better comprehend and exploit the advantages. The perplexing abundance of possible design choices in the SBR makes it becomes an important task to develop a unified design basis [87].

2.9 Optimisation of Bioprocess Parameters

Many influencing factors have been studied to optimise the bioprocess. There are type of organism/organisms, pH, substrate loading (OLR – organic loading rate), type of reactor/growth conditions (batch, sequencing batch, continuous; CSTR, UASB, etc.),

and type of substrate (pure carbohydrate, various waste streams). Pure cultures growing on defined substrates are best for examining limiting factors and the effects of genetic manipulations whereas mixed cultures are probably more appropriate for fermentation of complex substrates. With pure cultures, fermentation patterns (and products) can be controlled by the choice of organism, but with mixed cultures, metabolic pathways have to be manipulated through bioprocess parameters [36]. Dealing with the complex nature of the substrates, and the often unknown composition of the mixed culture, these are very dependent upon inoculums source. This is difficult to compare one study with another. An idea of the variability possible is given by a recent study which examined substrate conversion by a mixed culture of four different pure monosaccharide and found that productivity, conversion efficiencies and even products varied greatly [88].

There is a great deal of interest in this area at present and the results of many studies are appearing in the literatures. Several researches showed that there are many factors which affect bioprocess [89, 90, 91]. pH control is important as high fermentation rates lead to strong acidification due to the production of organic acids. This can affect both product distribution and even cell viability [92]. A variety of OLRs have been tested. Although the results are highly variable given the different substrates used, it is obviously seen that high substrate concentrations are to be preferred from an operational standpoint since they potentially lead to high volumetric production rates. The effect of OLR, at least with mixed cultures, on hydrogen yields is somewhat contradictory with no facile explanation for the disparity in these results [93]. With pure cultures, hydrogen yields are favoured at low substrate [carbon] concentrations whereas hydrogen productivity is favoured at high substrate [carbon] concentrations [94]. Recent studies with mixed cultures also generally support this idea, although the relationship seems more complex [95].

In terms of ease of operation, CSTR-type reactors are probably to be preferred, and are useful for model studies since they assure maximum mixing and homogeneity. However, in practical terms, it would appear that some kind of fixed bed or granular-type reactor would be best adapted since they allow the achievement of very high cell densities and permit the reactor to be run at HRTs that are independent of the bacterial growth rate [96]. Thus, very high volumetric rates of production can be achieved with these types of reactors, often ten times or more that of CSTRs. Various types of

substrates have been used with many studies characterising conversion from pure sugars. In practical terms, it is proved that these substrates, or any food derivative substrate, may not be available, and useful conversions may be those that utilise various waste streams, generating energy while at the same time carrying out necessary waste treatment. Carbohydrate rich waste streams should be targeted, and hydrogen fermentations are favoured by relatively high C/N [97]. With pure cultures nitrogen limitation produces maximum yields [94]. Some very nitrogen poor substrates may require amendment with a more nitrogen rich waste to achieve better hydrogen production [98].

The key parameter to maintain the intracellular acid concentration is the pH of extracellular environment. The optimum pH for hydrogen production is in the range of 5–7 [99], with the optimum at pH 5.5 [100]. The effect of the undissociated form of acetic or butyric acid is greater at low pH ($pH < 4.5$) which causes the inhibition on cell growth. It is too optimist to predict the optimum pH of hydrogen production by using mixed culture inocula due to the uncertainty of the presence of microbes. Fang et al. (2006) [101] demonstrated a study on acidophilic hydrogen production from rice slurry. The hydrogen production was the most effective at pH of 4.5. Microbial profiling showed that the uncultured microbes were basically *Clostridium sp.* Without the pH controlling, pH of the system may drop due to organic acid accumulation. Thus, under certain low pH, methane evolution can be shut off, obtaining H_2/CO_2in the bioprocess [102]. Many methanogen species can grow up in the other pH, which is apart a relatively narrow pH range (6–8). Some acidophilic species such as *Methanobacterium espanolae*, grow up at the pH between 5.6 and 6.2, but they are unable to grow and they may produce methane at pH 4.7 showed that inhibition of methanogenesis activity is necessary for avoiding methane formation from evolved H_2 when an anaerobic inoculums is used [103]. The acidic pH (around 5.0) is effective for inhibiting methanogenesis activity and obtaining an inoculum enriched in H_2 producers. The acclimatisation times that reveal time to get a methanogen free inoculum. The yield of H_2 and organic acids are variable among different work and fall in a range between 3 and 30 days [104]. In order to enhance H_2 production, inoculums enrichment relevance at low pH was established by inoculum acid enrichment during 80 hours, producing the 330 times more H_2 compared with the control (without enrichment).

Environmental factors such as pH and HRT may affect the metabolic balance, affecting the growth phase in batch culture. *Clostridia* produce VFA and H_2 in the exponential growth phase and rapid alcohol production occurs in late growth phase [105]. Lay et al. (1999) had set up the experiment with a MSW mixture and boiled sewage sludge inoculums [4]. Results showed that H_2 and VFA were produced after 2.2 days of lag period, followed by 3.2 days and 4 by propanol/butanol production as the H_2 production decreased. Similar alcohol production after the peak of hydrogen/VFA production was found in batch studies using heat-treated sludge inoculum and microcrystalline cellulose [106].

In hydrogen fermentation processes, nitrogen, phosphate and other inorganic trace minerals are necessary supplements for carbohydrate based feedstocks in order to obtain optimal cell cultivation and hydrogen production. Previous study indicated that organic nitrogen seems to be more favourable for hydrogen evolution compared with inorganic one [59]. Phosphate is one of the important inorganic nutrients required for optimal hydrogen production [107]. Excess phosphate may favour VFAs and hydrogen production over solvent production, so phosphate supplementation may be needed with carbohydrate-rich feeds. Lin and Lay (2004b) [108] examined the effect of trace elements including of Mg, Na, Zn, Fe, K, I, Co, NH_4^+, Mn, Ni, Cu, Mo and Ca on hydrogen production by *C. pasteurianum*-predominant culture. The elements of Mg, Na, Zn and Fe are important supplements and they propose an optimum nutrient formulation containing $MgCl_2 \cdot 6H_2O$ 120 mg/L, NaCl 1000 mg/L, $ZnCl_2$ 0.5 mg/L and $FeSO_4 \cdot 7H_2O$ 3 mg/L for the optimal hydrogen yield. However, the effect of NH_4^+ and a lower value of optimum iron concentration are negligible [109].

Metal ions at cellular level have effects on cell growth as enzyme cofactor, transport processes and dehydrogenases. Lin and Lay (2004b) [108] summarised the research which had been carried out on nutrient supplementation for hydrogen production. Taguchi orthogonal array was applied to find out the most significant nutrients. Magnesium, sodium, zinc and iron were the most important trace metals that affected hydrogen production with magnesium the most significant factor. Hydrogen production reached maximum at 3.52 mole H_2/ mole sucrose when $MgCl_2$ and NaCl concentrations were 120 and 1000 mg/L, respectively. Magnesium ion is an important cofactor that

activates almost 10 enzymes including hexokinase, phosphofructokinase and phosphoglycerate kinase during glycolysis process [110].

2.10 Exploitation of Byproducts

The major products in hydrogen production by anaerobic dark fermentation of carbohydrates are acetic, butyric and propionic acids. Formation of lactic acid was observed when lactose and molasses (sucrose) were used as the substrates [111]. pH also affects the type of organic acids produced. More butyric acid is produced at pH 4.0–6.0. Concentration of acetate and butyrate could be almost equal when pH is 6.5–7.0 [68]. Ethanol production was observed depending on the environmental conditions. Methane was not detected in most of the hydrogen production studies because of elimination of methane producers by heat digestion of sludge [74, 111]. However, long retention times may cause methane formation by the mesophilic cultures. Methane production was also observed when sewage sludge was used as the substrate [112].

Hydrogen production rate may be modeled with the biomathematical model. The experimental data are fitted to the model in order to examine the parameters. These calculated figures are important to describe the performance of hydrogen producing microbe. The details of biomathematical model are provided in the following section.

2.11 Biomathematical Model

The modified Gompertz equation is introduced to determine the progress of hydrogen production process. The biomathematics equation is given as follows [113].

$$H_t = H_{max} \cdot \exp\left\{-\exp\left[\frac{R \cdot e}{H_{max}}(\lambda - t) + 1\right]\right\} \qquad (2\text{-}10)$$

where H_t is the cumulative volume of H_2 production (mL), R is the maximum H_2 producing rate (mL/h), H_{max} is H_2 producing potential (mL), λ is the lag time (h) and t is the incubation time (h). The e represents Euler's number that is 2.71828.

The modified Gompertz equation can be adopted to model the production of VFAs in the hydrolysis of cellulose chain of starch. The equation is given as follows [113].

$$P_t = P_{\max} \cdot \exp\left\{-\exp\left[\frac{R_P \cdot e}{P_{\max}}(\lambda - t) + 1\right]\right\} \tag{2-11}$$

where P_t is the cumulative VFAs production (mM), R_p is the maximum VFA producing rate (mM/h) and $P_{\max}$ is VFA producing potential (mM).

The H_2 production rate relies on the limiting substrates (starch or glucose) concentration, the direct fermentation or anaerobic fermentation are simulated using Monod-type kinetic model. The equation is given as followed [113].

$$v_{H_2} = \frac{v_{\max,H_2} C_{glucose/starch}}{K_s + C_{glucose/starch}} \tag{2-12}$$

where v_{H_2} is the volumetric H_2 yield from hydrogen producing and/or direct fermentative microbe (mLH_2/h), $v_{\max,H_2}$ is the maximum H_2 production rate (mLH_2/L-h), K_s is half saturation constant (gCOD/L) and $C_{glucose/starch}$ is the glucose or starch concentration (gCOD/L).

2.12 Summary

Biological hydrogen production is a viable alternative to the aforementioned methods for hydrogen gas production. In accordance with sustainable development and waste minimization issues, biohydrogen gas production from renewable sources, also known as "green technology" has received considerable attention in recent years. The major criteria for the selection of waste materials to be used in biohydrogen production are the availability, cost, carbohydrate content and biodegradability. Simple sugars such as glucose are readily biodegradable and preferred substrates for hydrogen production. However, pure carbohydrate sources are expensive raw materials for hydrogen production. Starch and cellulose containing renewable resources such as biomass and agricultural wastes constitute inexpensive and easily fermentable raw materials for biohydrogen production. Most of the biohydrogen production studies by dark fermentation of carbohydrate rich raw materials were realisedby batch operation. The major pathway begins since the hydrogen producing microbes generate the enzyme to

hydrolyse glucose and then glucose is consumed as carbon source. Information from laboratory-scale suggests operating at 30 °C, at a pH around 5.5, and for simple substrates. To be successfully yielded the high hydrogen gas production rate, the numbers of biomass and substrate must be balance as well as optimum condition must be maintained.