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Bio-hydrogen production by dark fermentation from organic wastes and residues

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Ph.D. Thesis
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Department of Environmental Engineering
Technical University of Denmark

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from Organic Wastes and Residues

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The thesis will be available as a pdf-file for downloading from the homepage of
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Preface

This Ph.D. thesis is the result of a research project carried out at the Department of Environmental Engineering, Technical University of Denmark (DTU), during the period from October 2004 to February 2008. Professor Irini Angelidaki was the main supervisor. Dr. Raymond Zeng and Dr. Booki Min were the co-supervisors.

The hydrogen generation at extreme-thermophilic temperature (70°C) from mixed culture by using household solid waste as substrate, and hydrogen from cattle manure by bio-electrochemical process in CSTR reactor were the first time demonstrated in this Ph.d project.

The thesis is organized in two parts. The first part is a dissertation providing background for understanding the important aspects of the anaerobic methanogenesis process and the dark hydrogen fermentation process. The second part consists of the following papers.

Paper I: Liu DW, Liu DP, Zeng RJ, Angelidaki I. 2006. Hydrogen and methane production from household solid waste in the two-stage fermentation process. 'Water Research' 40(11):2230-2236.

Paper II: Liu D, Zeng RJ, Angelidaki I. 2008. Effects of pH and hydraulic retention time on hydrogen production versus methanogenesis during anaerobic fermentation of organic household solid waste under extreme-thermophilic temperature ($70°$C). 'Biotechnology and Bioengineering'. Accepted.

Paper III: Liu D, Zeng RJ, Angelidaki I. 2008. Enrichment and adaptation of extreme-thermophilic ($70°$C) hydrogen producing bacteria to organic household solid waste by repeated batch cultivations, 'Internatioanl Journal of Hydrogen Energy'. Submitted.

Paper IV: Liu D, Booki Min, Angelidaki I. 2008. Bio-Hydrogen Production from Organic Household at extreme-thermophilic temperature (70°C) – Influence of pH and Acetate Concentration, 'Biotechnology and Bioengineering'. Submitted.

Paper V: Liu D, Ellegaard L and Irini Angelidaki，Bio-electrochemical system applied in anaerobic CSTR reactor for biohydrogen production from cattle

manure as substrate , Manuscript, going to be submitted to 'Environmental Science & Technology'.

Paper VI: Christiansen Trine Løbner, Liu D, Liu D, Cirauqui B, Batstone Damien J and Angelidaki Irini. Bio-hydrogen production by anaerobic fermentation of waste in book: Anaerobic Digestion, 10th World Congress, 29th August - 2 September 2004, Montreal, Proceedings, pages: 2216-2219, 2004, NRC & IWA, Montreal. Poster Presentation.

Paper VII: Liu DW, Liu DP, Zeng Raymond Jianxiong and Angelidaki Irini. Hydrogen and methane production from household solid waste in the two-stage fermentation process. part of: 4th International Symposium on Anaerobic Digestion of Solid Waste, Copenhagen, August 31 - September 2, 2005, Volume 2 - Poster Presentations (ISBN:) , pages: 93-100, 2005, BioCentrum-DTU, Kgs. Lyngby., Oral Presentation

Paper VIII: Liu DW, Zeng Raymond Jianxiong and Angelidaki Irini. 2007. Enrichment and adaptation of extreme-thermophilic (70°C) H2 producing bacteria to organic household solid waste by repeated batch cultivations.part of: Bioenergy for our future, 11th IWA world congress on anaerobic digestion (AD11) held in Brisbane, Australia 23-27 September 2007, 2007, Brisbane, Oral Presentation

The papers are not included in this www-version but may be obtained from the Library at the Department of Environmental Engineering, Miljøvej, Building 113, Technical University of Denmark, DK-2800 Kgs. Lyngby (library@env.dtu.dk).

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Abstract

The demand for improvement of the hydrogen production by dark hydrogen fermentation is increasing. However there exists no full scale bio-hydrogen plant due to it is not economically reasonable. A two-stage process which combines bio-hydrogen and bio-methane production is one possible solution for enhancing the efficiency of the dark fermentation process. In the two-stage bio-hydrogen and bio-methane process with household solid waste (HSW) as substrate operated at 37^oC, a hydrogen yield of 43mL/gVSadded could be achieved. Moreover, the methane production in two-stage process was 21% higher than in tradition one-stage methanogenesis process due to it improved the hydrolysis of the HSW, which was proved to be the main obstacle of anaerobic digestion of HSW. Sparging with the methane produced from the methanogenesis stage could increase the bio-hydrogen production by 88%. Production of hydrogen from complex organic wastes, like household solid waste (HSW), mixed cultures fermentation was more applicable as pure cultures can be easily contaminated by the wastes without sterilization. During fermentative hydrogen production by mixed cultures, loss of hydrogen through interspecies transfer primarily to methane, needs to be prevented. pH, hydraulic retention time (HRT) and temperature seem to be the most applicable methods to prevent methanogenesis in an industrial-scale system. However short HRT could not be applied as a method for producing hydrogen from complex material such as HSW and manure, due to poor hydrolysis of the relatively slowly hydrolysis step of the complex organic materials. The mixed culture obtained from thermophilic temperature could not be used at extreme-thermophilic temperatures directly and needed to be adapted. Unadapted inoculum was leading to the lactate accumulation and resulted in low hydrogen production. Repeated batch cultivation was used as an effective method to adapt and enrich bio-hydrogen producing mixed cultures at extreme-thermophilic temperatures that could ferment HSW with high hydrogen yield and without significant lag phase. After adaptation, hydrogen was produced directly in the HSW feedstock (10 g-VS/L) with the maximum yield of 101.7 ± 9.1 mL H_2/gVS_{added}. The lag phase was reduced to a couple of hours. Furthermore, the enrichment cultures could successfully assist fast start up of biohydrogen reactors, while the process failed with unadapted cultures at extreme-thermophilic temperature. The pH optimum and inhibition effect of the acetate concentration under extreme-thermophilic mixed culture was investigated by using HSW as substrate. The highest hydrogen production was found at a natural pH (pH 7). The acetate was proved to be the inhibitor even at natural pH. Intial inhibition of the biohydrogen process was found at acetate concentration of 50mM, while at acetate concentration of 200mM, the hydrogen production (36mL/gVSadded) was 6 times lower than at 5-25mM acetate (250mL/gVSadded).

Electricity application (3.5V) in an anaerobic manure CSTR reactor resulted in high hydrogen production (200mL/gVSadded) under thermophilic conditions. The electricity energy input was 6113kJ (1.70 kwh)/m^3 H$_2$, while the energy content in the hydrogen (122kJ/g H$_2$) was 10800 kJ (3.03 kwh)/m^3 H$_2$, which corresponds to 1.78 times higher energy output than the electricity energy input to the system. Water electrolysis and/or organic matter electrolysis was exluded as the reason for the hydrogen production from bio-electrochemical system. Hydrogen could be obtained by applying a voltage of 3.5V directly in the reactor liquid phase. At 2.5V-3.0V voltage methane was produced, while when the voltage was increased to 3.5V, methanogenesis disappeared and instead high hydrogen production was detected. The reason for hydrogen production was attributed to inhibition of methanogens due to electricity application.

Resume

Der er stigende opmærksomhed omkring biohydrogen. Ved hydrogen fermentering kan kun en lille del af det organiske materiale eller COD i affald omdannes til hydrogen. Der findes endnu ingen full-skala bio-hydrogen anlæg, eftersom effektive rentable teknologier ikke er udviklet endnu. En to-trins proces der kombinerer bio-hydrogen og bio-metan produktionen er en attraktiv mulighed til at øge det totale energi-udbytte af fermentering af organisk materiale. I en to-trins proces, med bio-hydrogen som første trin og bio-methan som andet trin, kunne der opnås 43mL-H2/gVSadded ved 37°C fra husholdningsaffald (HHA) som substrat. Derudover var metanproduktionen i en to-trins proces 21 % højere sammenlignet med en traditionel et-trins proces. Grunden til det større methan-udbytte ved et to-trins proces var den forbedrede hydrolyse af HHA. Gennemblæsning af methan gennem hydrogen reaktoren kunne forøge bio-hydrogen produktionen med 88 % i en to-trins proces i forhold til et-trins proces. Hydrogen produktionen kunne forøges ved at skifte temperaturen fra mesofil til ekstrem-termofil. Hydrogen produktion ved ekstrem-termofile temperaturer med blandede kulturer blev demonstreret for første gang. Der blev etableret en hydrogen producerende berigelseskultur ved 70˚C. Denne kultur blev startet fra termofilt podemateriale, som beviser at ekstrem-termofile bakterier findes under termofile forhold. Denne kultur blev adapteret til høje koncentrationer af HHA ved gentagen batch dyrkning. Ved denne proces kunne kulturen adapteres til HHA og udviste en høj hydrogen udbytte (250 ml/gVS) hvilket var ca. 6 gange højere end det udbytte der var opnået under mesofile forhold. Tilsætning af brint-berigelseskulturen til podematerialet ved opstart af brint kontinuerte reaktorer viste sig at kunne være gavnlig for en hurtig proces opstart og med højt brint effektivitet. Uden berigelseskulturer fejlede processen, på trods af gentagen genpodning.

Optimale procesforhold for brint producerende processer blev bestemt. pH optimum af brintproducerende kulturer var 7.0 og acetat var hæmmende for brintproduktionen. Initial hæmning af brintprocessen viste ved acetatkoncentration på 25mM, hvorimod processen blev total hæmmet ved en acetatkoncentration på 200mM.

En ny proces blev opfundet, hvor anvendelse af strøm (3.5V) hæmmede methanproduktion totalt til fordel for brint produktion. Det blev vist for første gang at ved at anvende 3.5 V strøm, høj brint produktion (200mL/gVSadded) kunne opnås fra gylle.

Table of Content

1. Aim of the study

1.1. Background

Bio-hydrogen and bio-methane will play important roles for future energy economy as clean, CO_2 neutral and environmental friendly energy.

Among the hydrogen production methods, the most promising and environmentally friendly method seems to be dark fermentation from organic wastes as it combines the hydrogen generation with waste treatment (Benemann 1996).

Anaerobic digestion process includes hydrolysis/acidogenesis and methanogenesis. As shown in Figure 1, hydrolysis and acidogenesis produce hydrogen gas and organic acids, which can be further used to produce methane in methanogenesis. The hydrogen production step requires 1-2 days hydraulic retention time (HRT) and methane production step requires longer HRT (12-20 days). If hydrogen gas is not harvested and further used for methane production, it is called one-stage fermentation process. Otherwise it is called two-stage fermentation process.
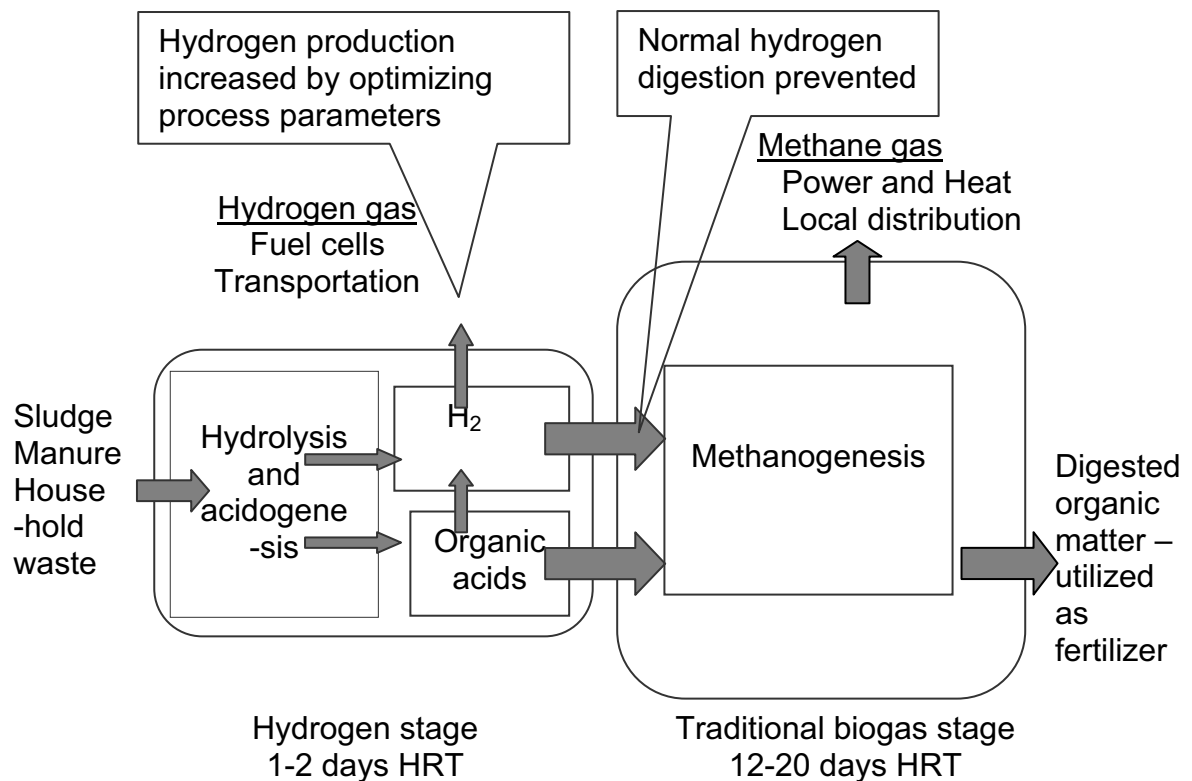
Figure 1. Principle diagram of two-stage process for hydrogen and methane production

In an anaerobic fermentation process, the hydrogen synthesis pathways are severely affected by environmental factors, such as pH, temperature and HRT (Chang et al., 2002). It has widely been accepted in bio-hydrogen research that pH is one of the key factors affecting the hydrogen production. Hydrogen synthesis pathways are sensitive to pH and are subject to end-products (Craven 1988). Dark hydrogen fermentation reactions can be operated at a temperature range from mesophilic (25-40°C) to hyper-thermophilic (>80°C). Up to now, most of dark fermentation experiments are conducted at 35-37°C, and the possible advantages of operating out of mesophilic range are not completely clear (Levin et al., 2004). HRT is also an important parameter for dark fermentation process. In continuously stirred tank reactor (CSTR) system, short HRTs were used to wash out the slow growing methanogens and select for the acid producing bacteria (Chen et al., 2001), while too short HRT could lead to bad hydrolysis of organic wastes (Han and Shin 2004).

In terms of substrates, current hydrogen studies mainly focus on household solid waste or pure substrate, like glucose. Other organic wastes, like manure and energy crops were only demonstrated successfully for methane production. One difficulty is that these waste types contain ligninocellulosic material. Lignin is non-biodegradable and strongly hampers the utilization of cellulose and hemicellulose under anaerobic conditions. That's because the bonding in lignocellulose resists mobilization and chemically degraded lignin is often inhibitory to microbial growth (Reith et al., 2003a).

1.2. Objectives of the current Ph.D Project

● **Evaluate an innovative two-stage process for combining biohydrogen and biomethane production**

It has been proved that a two-stage process combining biohydrogen and biomethane production is a rational configuration because it provides the preferred environments for acidogenic hydrogenesis and methanogenesis (Han et al., 2005). The objective of this part of research is to use organic household waste to evaluate a two-stage process combining hydrogen and methane production and to compare it with the traditional one-stage process, and to investigate the key factors for this two-stage technology.

● **Investigation of effects of environmental parameters in dark fermentation process**

For dark hydrogen fermentation, the most important environmental parameters are pH, hydrogen partial pressure, temperature, and HRT (Nath et al., 2006). These parameters have strong effects on the selection of synthesis pathways. The objective of this part of research is to evaluate the individual effect of pH and HRT and to find out how these parameters influened the hydrogen synthesis pathways. CSTR reactors with HSW as substrate were operated under extreme-thermophilic temperature (70^0C) with HRT range from 1 day to 6 days and pH range from 4.5 to 7, respectively.

- **Enrichment of the mixed culture for extreme-thermophilic hydrogen production**

  Hydrogen can be produced from pure cultures and mixed cultures. It is believed that for future industrial applications the use of mixed cultures for hydrogen production from organic wastes might have more advantages because pure cultures can easily be contaminated with hydrogen consuming bacteria. However, for dark fermentation of organic household waste at 70^oC, the mixed culture is still not available. The objective of this part of research was to enrich the extreme-thermophilic mixed culture isolated from digested manure with thermophilic methanogenesis CSTR reactor, and to adapt it to high solid content of organic household waste via repeated batch cultivations. Batch and CSTR reactors were operated at 70^oC with mixed cultures. The batch experiment started with low concentration of household waste together with BA medium, and then the household waste concentration was increased gradually until no BA medium was added. CSTRs were used to test the hydrogen production in continuous system.

- **Investigation of dark fermentation from different feedstock for bio-hydrogen production**

  To apply the dark hydrogen fermentation process in practice, more waste types and residues should be invesgated than glucose and household waste. The objective of this part of research is to investigate the possibility of using manure as the feedstock for dark hydrogen fermentation to identify the problems for dark fermentation of this waste type.

2．Introduction

Household waste includes domestic waste, bulky waste, and garden waste. In Denmark, household covers 20% of total waste generation: 2.8 million ton household waste is generated per year (James and James 2002). The main treatment methods for household waste are incineration, composting, landfill, and anaerobic digestion (De Baere 2000). Anaerobic digestion is a treatment method that converts the waste in anaerobic reactor to biogas (McCarty 1981). Household waste is a good material for anaerobic digestion. All the garden waste and 40% to 45% of domestic waste are organic materials (James and James 2002).

Due to the improvement of collection and separation system at household level, more and more household waste is treated in biogas plants (Angelidaki and Ellegaard 2002).

2.1. Anaerobic digestion

Anaerobic digestion, a technology that has traditionally been viewed as symble of 'energy-from-waste' - can provide a range of benefits in addition to the valuable renewable energy from the biogas, such as waste treatment, and reduce pollution, odors and disease (Angelidaki 2002). Furthermore, it could contribute to recycle the nutrients back to the soil (thereby reducing the requirements for artificial fertilizers); improve soil quality by recycling the organic matter as humus, thus preserving fragile topsoil, sanitization of the compost, reducing the spread of soil-borne pathogens and weeds (James and James 2002).
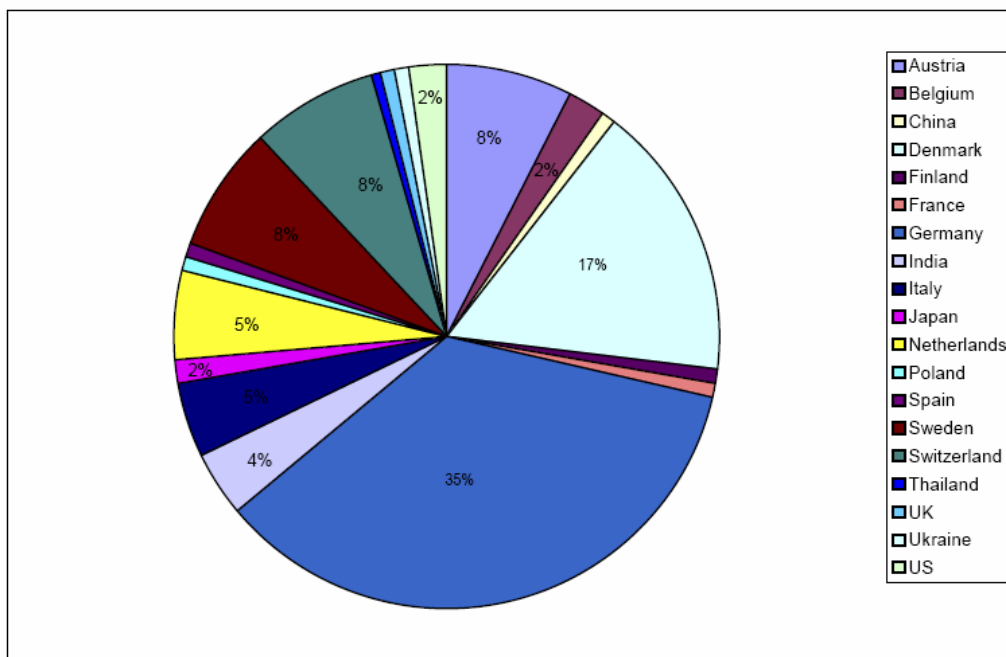
Figure 2 .   Worldwide distribution of biogas Plants (Adapted from www.biogasworks.com)

Most of the anaerobic biogas plants were operating in Europe (91%), with some in Asia (7%) and a few in the US (2%). Germany had 35% of all AD plants, followed by Denmark (17%) and Sweden and Switzerland and Austria (8%) (Bolzonella et al., 2003). In 1996, both Germany and Denmark pledged to double their biogas production by the year 2000 and triple it by the year 2005. Figure 2 shows the worldwide distribution of biogas plant in 1998.
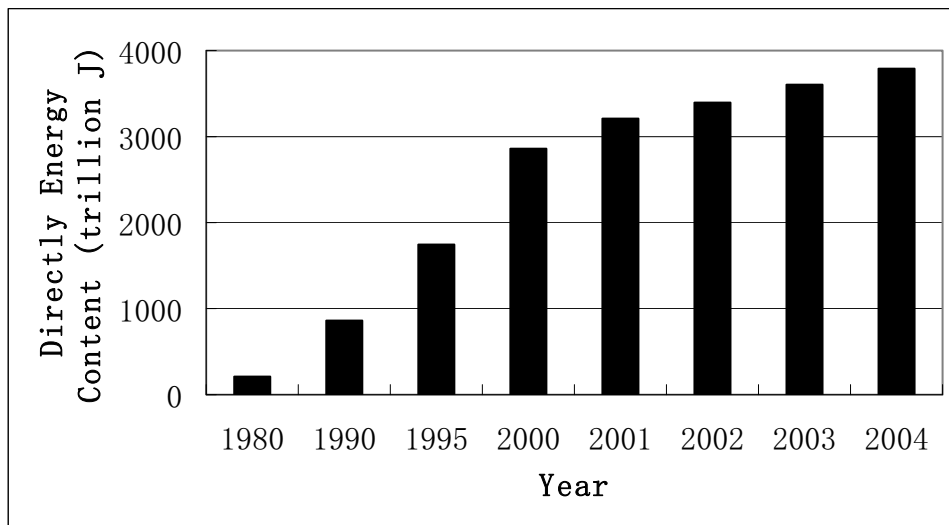
Figure 3. Energy production from biogas in Denmark Data from: (Olsen 2005)

In Denmark, the first biogas plant for treatment of organic waste and energy generation was built in the 1980s. In 2001, there were 1.2million tons of manure and 300,000 tones of organic waste treated in the biogas plants (Angelidaki and Ellegaard 2002). The total biogas production has been increased 4-5 times from 1990 to 2004 (Olsen 2005), as illustrated in Figure 3. There are 20 large-scale centralized plants and more than 80 farm scale biogas plants in operation in Denmark (Kanokwan 2006). It has been proven that it is possible to build and operate large biogas installations which are economically sustainable with co-digestion of organic waste and animal manure as substrates. It is therefore expected that the use of biogas will rise (Stærkind 2005).

2.1.1. The three-stage model of anaerobic digestion

The general model for degradation of organic material (polymeric substances like carbohydrates, protein, and fats) under anaerobic conditions operates principally with three main groups of bacteria which together convert the organic material to methane,

carbon dioxide and water (Batstone et al., 2002). The fermentative bacteria (group I, figure 4) hydrolyze the polymers to soluble oligomers and monomers by action of extracellular enzymes. After that the dissolved products are taken up by the bacteria and fermented, forming acetate and other short-chain fatty acids, alcohols, hydrogen and carbon dioxide which are released into the environment (Angelidaki et al., 2002). Figure 4 illustrates the mechanisms of how the microorganisms transform organic material into biogas in the stages of hydrolysis, fermentation and methanogenesis.
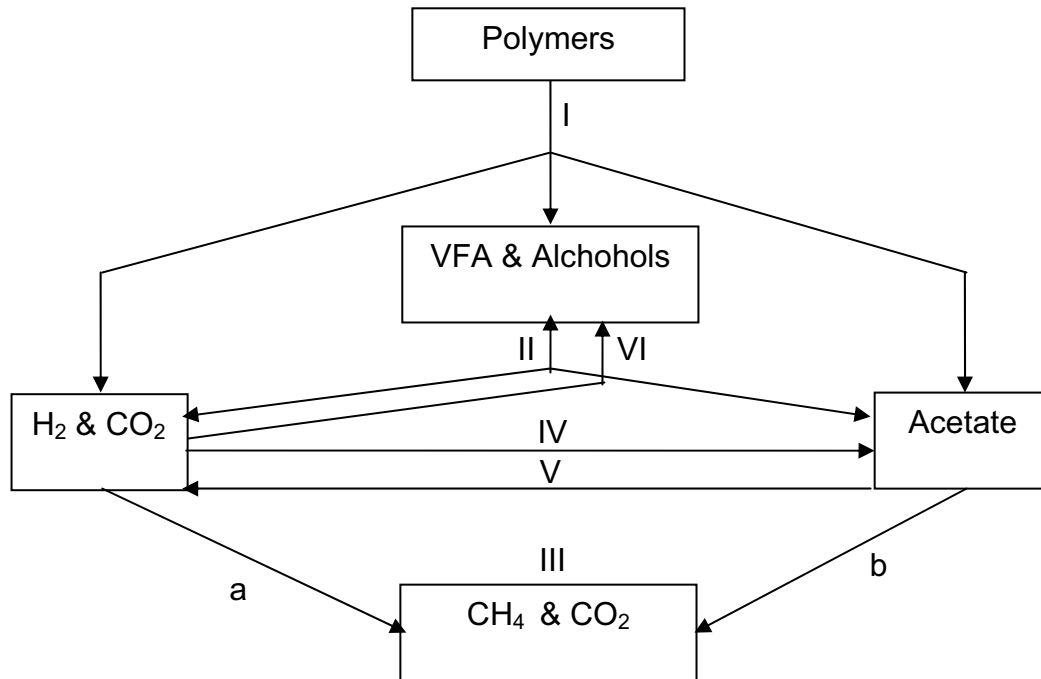
Ⅰ. Hydrolytic and fermentative bacteria
Ⅱ. Hydrogen-producing acetogenic bacteria
Ⅲ. Methanogetic bacteria
    a. hydrogen utilizers
    b. aceticlastics
Ⅳ. Homoacetogetic Bacteria
Ⅴ. Acetate oxidizing bacteria
Ⅵ. Acid synthesizing bacteria
Figure 4. Carbon flow in methanogenic environments (Angelidaki et al., 2002)

2.2．Dark hydrogen fermentation

Our energy requirements are almost totally provided by carbon-containing fossil sources, such as oil, coal and nature gas (Reith et al., 2003a). At present approximately 85% of

the world's energy requirement is provided by fossil sources, 7% by nuclear energy and 8% by renewable sources, primarily through the use of burning wood as a fuel and hydropower (Watts 2002). But they cause serious environmental problems during combustion, such as acid rain, CO_2 emission and climate changes. Moreover, oil, coal and nature gas are finite resources, the consumption of them is much faster than their forming. After the oil crisis of 1970s, there has been an increase in effort to develop a renewable energy resource (Okamoto et al., 2000). The recent increase in oil and nature gas prices also make the current economy toward alternative energy resources (Van Ginkel et al., 2001).

Hydrogen gas has been deemed the fuel of the future, and it is believed that a hydrogen fuel based economy would be less pollution than a fossil fuel based economy (Fyfe 1999; Lenssen and Flavin 1996; Levin et al., 2004). Hydrogen as an energy carrier has been proved to be one of the best fuels for transportation, the most versatile, the most efficient and also one of the safest fuel (Barbir et al., 1990; Dickinson and Cicerone 1986; Liang 2003; Veziroglu and Barbir 1992). The combustion of hydrogen produces only water vapor without CO, CO_2, hydrocarbons or fine particles, and since it can be produced without causing any environmental problems, hydrogen as a future fuel has been drawing more and more attention (Bockris 1972; Valdez-Vazquez et al., 2005b; Yamin 2006; Yamin et al., 2000).

Hydrogen has significant advantages as an energy resource:

➢ The combustion of hydrogen only produces water vapor, which is a non-greenhouse gas. It does not cause environmental and atmosphere pollution (Armor 2005).
➢ The combustion of hydrogen in automobiles is 50% more efficient than gasoline. Hydrogen battery is deemed as future supply for automobiles (Reith et al., 2003a).
➢ Hydrogen gas has a high energy yield of 122 kJ/g, and this yield is 2.75-fold greater than that from hydrocarbon fuels on mass level (Ramachandran and Menon 1998).
➢ The conversion efficiency of hydrogen to electricity could be doubled using fuel cell instead of gas turbine (Reith et al., 2003a).
➢ Hydrogen can be easily stored as a metal hydride (Dong et al., 2007).
➢ The transmission of hydrogen through natural gas pipelines would be more efficient than the transmission of electricity down power lines (Kloeppel and Rogerson 1991).

At present, hydrogen gas is mainly produced from natural gas reforming, and alternative route of hydrogen productions that are cost effective and pollution free are still demanded (Das and Veziroglu 2001). There are many methods which can generate hydrogen, such as, water electrolysis, thermo-chemical processing, photo-chemical

processing, photo-catalytic processing, and photo-electro-chemical processing (Momirlan and Veziroglu 1999; Momirlan and Veziroglu 2002). The two methods for hydrogen production from microorganisms are photo synthetic and dark hydrogen fermentation. The photo-fermentation produces hydrogen by photosynthetic microorganisms, such as algae, protists and photosynthetic bacteria (Ike et al., 1997; Melis and Happe 2001). The dark hydrogen fermentation is carried out by fermentative hydrogen-producing microorganisms, such as facultative anaerobes and obligate anaerobes (Joyner et al., 1977; Nandi and Sengupta 1998). The most promoting method for hydrogen production seems to be dark hydrogen fermentation method (Benemann 1996). In anaerobic conditions, hydrogen is produced during the breakdown of organic compounds by the microorganisms. When organic compounds are the only carbon and energy source for providing metabolic energy, the process is called dark hydrogen fermentation (Liang 2003). The hydrogen fermentative process has the advantage of high bio-hydrogen production rate (Van Ginkel et al., 2001). Studies on microbial hydrogen production have been conducted mostly by pure cultures (Asada et al., 2000; Evvyernie et al., 2001; Fabiano and Perego 2002; Thompson et al., 2006; Yoshida et al., 2006), and under mesophilic to thermophilic temperature ranges (Valdez-Vazquez et al., 2005a; Van Ginkel et al., 2001; Wongtanet et al., 2007). Dark hydrogen fermentation can use wastewater (Alzate-Gaviria et al., 2007; Ke et al., 2005; Li and Fang 2007; Ueno et al., 1996) or solid waste (Gomez et al., 2006; Lay et al., 2003; Shin and Youn 2005; Sparling et al., 1997; Valdez-Vazquez et al., 2006; Valdez-Vazquez et al., 2005b) as substrate by mixed cultures (Liang 2003).

2.2.1. Biochemical reactions of dark hydrogen fermentation

Dark hydrogen fermentation processes produce a mixed gas which mainly contains hydrogen and carbon dioxide, but may also contain methane, carbon monoxide, and hydrogen sulfide depending on different system and feedstock (Datar et al., 2004; Hussy et al., 2003; Kotsopoulos et al., 2006; Najafpour et al., 2004; Temudo et al., 2007).

As presented in equation 1, the complete oxidation of glucose to hydrogen and carbon dioxide yields a maximum of 12 mole hydrogen per mole of glucose. However, there are no metabolic energy is obtained in this case. Currently this high yield reaction is not reported in fermentative systems.

$C_6H_{12}O_6 + 6\ H_2O \rightarrow 12H_2 + 6CO_2$ $\quad\quad \Delta G_0 = +3.2$ kJ $\quad\quad\quad\quad$ (*Equation 1*)
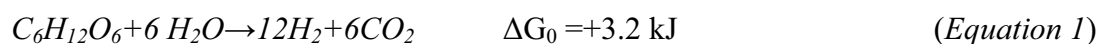
Glucose, isomers of hexoses, or polymers in the form of starch or cellulose, yields different amount of hydrogen per mole of glucose, depending on the fermentation pathway and end-products (Levin et al., 2004).

9

The available hydrogen production from glucose is determined by the butyrate/acetate ratio (Reith et al., 2003a). When acetic acid is the end-product, a theoretical maximum of 4 moles hydrogen per mole glucose is obtained (Eq. 2):

$C_6H_{12}O_6+2H_2O{\rightarrow}4H_2+2CH_3COOH$ *(acetate)* $+2CO_2$ ΔG_0= -206kJ   *(Equation 2)*
And when butyrate is the end-product, a theoretical maximum of 2 moles hydrogen per mole glucose is produced (Eq.3).

$C_6H_{12}O_6+2H_2O{\rightarrow}2H_2+CH_3CH_2CH_2COOH$ *(butyrate)* $+2CO_2$ ΔG_0=-254kJ (*Equation 3*)
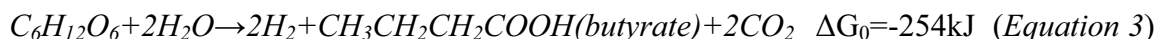
Thus, the highest theoretical yields of hydrogen are associated with acetate as the fermentation end-product. In practice, high hydrogen yields are associated with a mixture of acetate and butyrate fermentation products, and low H_2 yields are associated with propionate and reduced end-products such as alcohols and lactic acid (Levin et al., 2004).

2.2.2. Hydrogen-producing species bacteria

It has been reported that the hydrogen producing bacteria include strict anaerobes (*Clostridia*, methylotrophs, rumen bacteria, methanogenic bacteria, archaea), facultative anaerobes (*Escherichia coli*, *Enterobacter*, *Citrobacter*), and some aerobes (*Alcaligenes, Bacillus*) (Holt et al., 1994; Liang 2003; Nandi and Sengupta 1998).

However, the knowledge for hydrogen producing bacteria is still limited. Table 1 lists the bacteria which have been published so far.

Table 1. Hydrogen producing species bacteria , adapted from Reith et al. (2003a).

Microorganism	Temperature (^{o}C)	Substrate	Reference
Clostridium sp. no 2	36	glucose, xylose	(Taguchi et al., 1994; Taguchi et al., 1995; Taguchi et al., 1996)
C. paraputrificum M-21	37	GlcNAc1	(Evvyernie et al., 2001; Evvyernie et al., 2000)
C. butyricum LMG1213tl	36	glucose	(Heyndrickx et al., 1986)
Thermotoga neapolitana	55	glucose	(Sakai et al., 2005)
Pyrococcus furiosu	98	glucose	(Nakashimada et al., 1999)

Thermotoga maritima	80	glucose	(Schroder et al., 1994)
Thermotoga elfii	65	glucose	(de Vrije et al., 2002; Ravot et al., 1995; van Niel et al., 2002)
Clostridium thermocellum	70	glucose, sucrose	(Yokoyama et al., 2007b)
Caldanaerobacter subterraneus	70	glucose, sucrose	(Yokoyama et al., 2007b)
Caldicellulosiruptor	70	sucrose	(de Vrije et al., 2007; Gibbs et al., 2000; Huang et al., 1998a; Kadar et al., 2004; van Niel et al., 2002; van Niel et al., 2003)
saccharolyticus	72.5	glucose	*(de Vrije et al., 2007; Kadar et al., 2004; van Niel et al., 2002; van Niel et al., 2003)*
Enterobacter	35	glucose	*(Lu et al., 2007; Nath et al., 2006; Sen and Das 2005; Shin et al., 2007; Thompson et al., 2008)*
E. aerogenes	38	glucose	(Tanisho and Ishiwata 1994; Tanisho et al., 1987; Tanisho et al., 1983)
E. cloacae IIT-BT 08 wt	36	glucose, sucrose	(Kumar and Das 2000; Kumar and Das 2001; Kumar et al., 2001; Tanisho et al., 1987)
E. cloacae IIT-BT 08 m DM11	36	glucose	(Kumar and Das 2001; Kumar et al., 2001)
E. aerogenes E.82005	38	molasses	(Tanisho and Ishiwata 1994)
E. aerogenes HU-101 wt	37	glucose	(Rachman et al., 1998)
C. butyricum IFO13949 +	37	Starch	(Yokoi et al., 2002; Yokoi et al., 1998; Yokoi et al., 2001)

3. Parameters affecting dark hydrogen fermentation

Hydrogen fermentation has been extensively studied   because it has the potential for providing sustainable and renewable energy for the future. It has been reported that the temperature, pH, HRT, hydrogen/carbon dioxide partial pressure, volatile fatty acids and inorganic content are the main parameters that affect the anaerobic hydrogen fermentation process.

3.1. Temperature

The temperature affects the   hydrogen producing bacteria activities and hydrogen production rate (Nath et al., 2006; van Groenestijn et al., 2002). Dark hydrogen fermentation reactions can be operated at different temperatures: mesophilic (25-40°C), thermophilic (40-65°C), extreme thermophilic (65-80°C) or hyperthermophilic (>80°C) (Levin et al., 2004). Up to now, most of dark fermentation experiments are conducted at 35-55°C. The extreme thermophilic process provides a number of advantages compared with the mesophilic and thermophilic. Firstly, the hydrogen production is much higher at extreme-thermophilic conditions than at mesophilic and thermophilic conditions. It has been reported that extreme-thermophilic anaerobic hydrogen fermentation can achieve more hydrogen production and higher hydrogen production rates   than mesophilic hydrogen fermentation (van Groenestijn et al., 2002). It has been reported that at exteme-thermophilic condition (70°C), hydrogen yield reached the theoretical maximum of 4 mole hydrogen per mole glucose, where the ones at mesophilic and thermophilic conditions were normally less than 2 mole hydrogen per mole glucose (van Niel et al., 2002). Secondly, it has much better pathogenic destruction for digested residues performed at high temperatures(Sahlstrom 2003). Thirdly, it minimizes the contamination by hydrogen consumers such as methanogens, solventogens. Hallenbeck (2005) reported that at high fermentation temperature it was thermodynamically favorable for a hydrogen-producing reaction as the high temperature resulted in the increase in the entropy term, and made dark hydrogen fermentation more energetic while the hydrogen utilization processes were negatively affected with temperature increase (Amend and Shock 2001; Conrad and Wetter 1990). Extreme thermophilic bacteria show a better tolerance to high hydrogen partial pressures which will cause a metabolic shift to non-hydrogen producing pathways, such as solvent production (van Niel et al., 2003).

At mesophilic conditions, Lay et al. (2003) reported a hydrogen production of 50mL/gVS$_{added}$ from HSW batch fermentation. Okamoto et al. (2000) found a hydrogen production of 19.3-96.0 mL/gVS$_{added}$ from individual HSW fraction such as rice and carrot by mesophilic batch cultivation. Valdez-Vazquez et al. (2005a) reported that 95

mL-H_2/gVS$_{added}$ a was achieved respectively under semi-continuous CSTR. Our research found a hydrogen production of 43mL-hydrogen /gVS$_{added}$ from mesophilic HSW fermentation (Paper I).

We found that a hydrogen production of 100-250 mL-hydrogen /gVS$_{added}$ could be obtained at extreme-thermophilic conditions with proper parameter control, which was much higher than the ones in literatures (Paper III, IV).

3.2. pH

pH level has an effect on enzyme activity in microorganisms, since each enzyme is active only in a specific pH range and has maximum activity at its optimal pH (Lay et al., 1997). It has been accepted in hydrogen research that pH is one of the key factors affecting the hydrogen production. Hydrogen fermentation pathways are sensitive to pH and are subject to end-products (Craven 1988). Many studies have been conducted to produce hydrogen from solid wastes. Results indicated that the control of pH was crucial to hydrogen production. It has been reported that under unoptimal pH, the hydrogen fermentation process shifted to solvent production (Temudo et al., 2007), or prolonged the lag phase (Cheng et al., 2002b; Liang 2003). The lactate production was always observed together with sudden change of environment parameters, such as pH, HRT, and temperature, which indicated the culture was not adapted to the new environment conditions (Demirel and Yenigun 2004; Han and Shin 2004; Liu et al., 2008a; Temudo et al., 2007).

In our research on mesophilic hydrogen dark fermentation, it has been found that the optimal pH is around 5.0-5.5 (Liu et al., 2006), as shown in figure 5. The pH optimum around 5.5 was also reported by most of the other researchers for hydrogen production using HSW as substrate (Alzate-Gaviria et al., 2007; Gomez et al., 2006; Lay et al., 1999; Shin and Youn 2005). Moreover, at unoptimum pH, a fermetnation pathway changeing from acetate pathway to butyrate pathways was detected and thus decreased the hydrogen production (Paper III).

Hydrogen Production VS pH (after 12 hours)
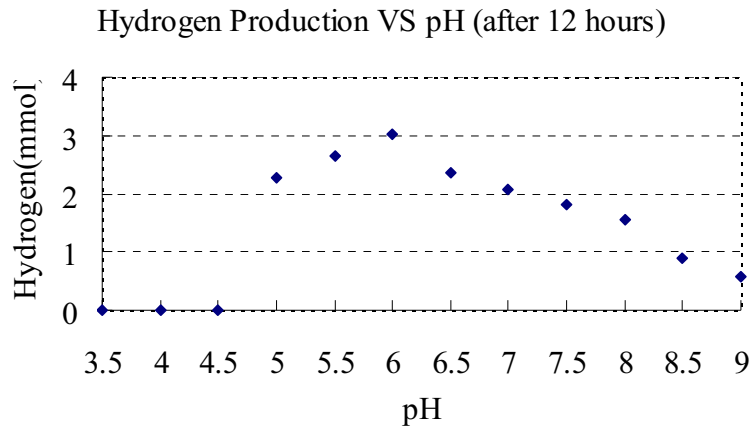
Figure 5. Hydrogen production at different pH at mesophilic temperature (37°), adapted from Paper I.

However, for hydrogen fermentation at extreme-thermophilic temperatures, all the publication used a pH at 6.5-7.5. van Niel et al. (2002) used pure culture of *Caldicellulosiruptor saccharolyticus and Thermotoga elfii* for dark hydrogen fermentation of sucrose and glucose under 70^oC. The pH was maintained at 7 and 7.4 throughout the experiment. Schröder et al. (1994) used pure culture of *Thermotoga maritime* with glucose as the substrate under 80^oC and controlled the pH at 6.5. Kadar et al. (2004) reported hydrogen production by paper sludge hydrolysate with pure culture *Caldicellulosiruptor saccharolyticus* under pH maintained at 7.2. These indicate most of the extreme-thermophilic hydrogen producing bacteria prefer an optimum of naturalized pH. The recent research with the extreme-thermophilic mixed culture adapted from manure also reported with the pH optimum at 7 (Yokoyama et al., 2007a).
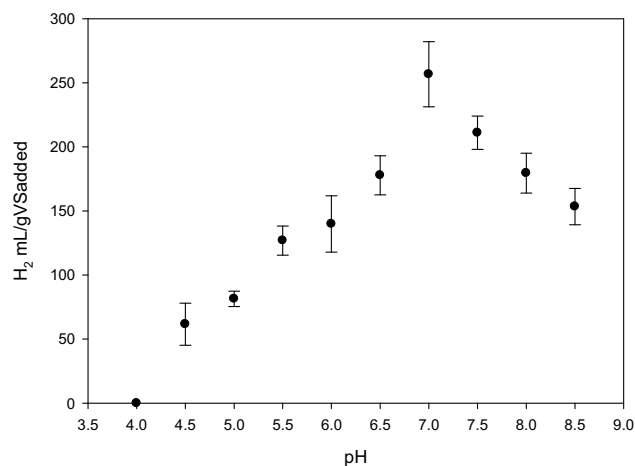
Figure 6. pH optimum test at extreme-thermophilic temperature (70°), adapted from paper IV.

15

Also, in our research, we found extreme-thermophilic hydrogen producing mixed culture adapted from manure and cultivated with HSW as substrate also had a pH optimum at 7, as shown in figure 6.

3.3. HRT

HRT is also an important parameter for dark fermentation process. In a CSTR system, short HRTs are used to wash out the slow growing methanogens and select for the acid producing bacteria (Chen et al., 2001), while too high dilution rate could lead to bad hydrolysis of organic wastes (Han and Shin 2004). In a CSTR system, Kim et al. (2004) reported that short HRT (< 3 days) would favor hydrogen production as methanogens require more than approx. 3 days HRT before they were washed out from a CSTR reactor. Normally, in an anaerobic process, pH and HRT are coupled parameters: short HRT results in low pH. Both pH and HRT have been demonstrated as the effective ways to separate hydrogen producing bacteria and hydrogen consuming archaea at mesophilic and thermophilic conditions (Oh et al., 2004). However, effects of pH and HRT are interrelated that no dedicated research has isolated the effect of these two parameters separately.
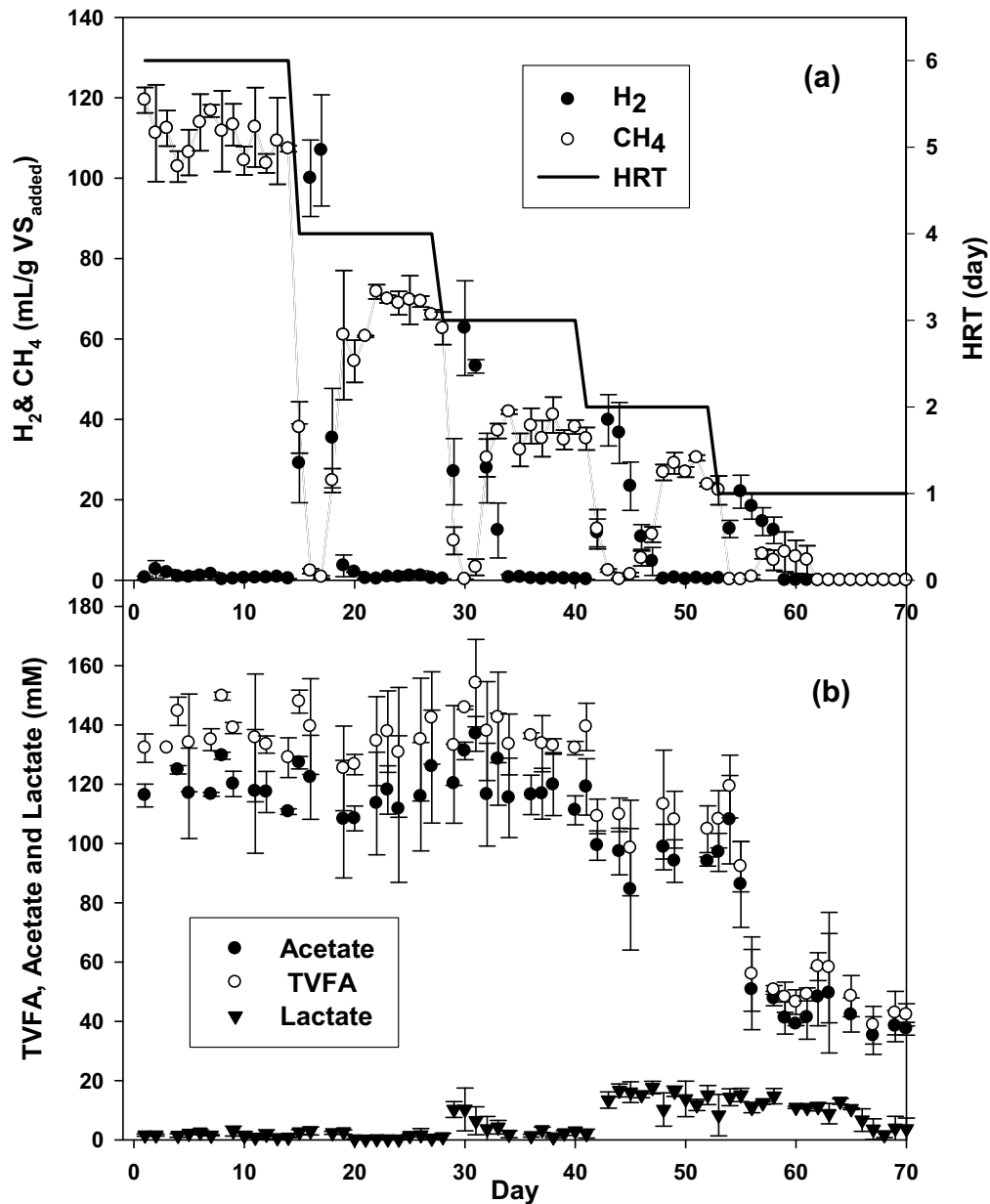
Figure 7. HRT effect with pH controlled at 7 under extreme-thermophilic temperature (70°), adapted from paper II

For HSW fermentation at extreme-thermophilic temperatures, HRT should not be less than 2 days. Otherwise it resulted in bad hydrolysis and washout the bacteria, as shown in figure 7. It also indicated at naturalized pH, even HRT was as short as 2 days, the methanogens could still grow and utilize hydrogen as substrate (Paper II).

3.4. Hydrogen and carbon dioxide partial pressure

The accumulation of hydrogen and carbon dioxide can lead to repression of its production and formation of more reduced products, respectively.

3.4.1. Hydrogen partial pressure

The hydrogen concentration in the liquid phase, related to hydrogen partial pressure, is one of the key factors affecting the hydrogen production (Hawkes et al., 2002). The partial pressure of H_2 (pH_2) is an extremely important factor especially for continuous H_2 synthesis (Hawkes et al., 2007). Hydrogen synthesis pathways are sensitive to H_2 concentrations and are subject to end-product inhibition. As H_2 concentrations increase, H_2 synthesis decreases and metabolic pathways shift to production of more reduced substrates such as lactate, ethanol, acetone, butanol, or alanine. As the temperature increases, however, conditions that favor hydrogen formation reactions are less affected by H_2 concentration (Tamagnini et al., 2002). Continuous H_2 synthesis requires pH_2 of 50 kPa at 60°C (Lee and Zinder 1988), 20 kPa at 70°C (van Niel et al., 2002), and 2 kPa at 98°C under standard conditons (Adams 1990; Levin et al., 2004).

3.4.2. Carbon dioxide partial pressure

In case of carbon dioxide, high CO_2 concentration can favor the production of fumarate or succinate, which contributes to consume electrons, and therefore decrease hydrogen production (Tanisho et al., 1998). It has been reported that the removal of CO_2 can improve the hydrogen production in dark fermentation (Tanisho et al., 1998). After CO_2 was removed, the hydrogen production was doubled. Furthermore, when removing the CO_2 from the liquid with sparging of argon gas and hydrogen gas, they also found, compared to hydrogen partial pressure, the CO_2 partial pressure had higher inhibition effect to the dark fermentation process.

In current research, CH_4 gas was used as the sparging gas to remove the hydrogen and carbon dioxide from the liquid. As illustrated in Figure 8, gas sparging resulted in significant increase of the hydrogen production (88%). Mizuno et al. (2000) reported that hydrogen production was increased 68% after sparging with N_2. This phenomenon could be directly explained by the decrease of hydrogen partial pressure and CO_2 concentration (paper I).
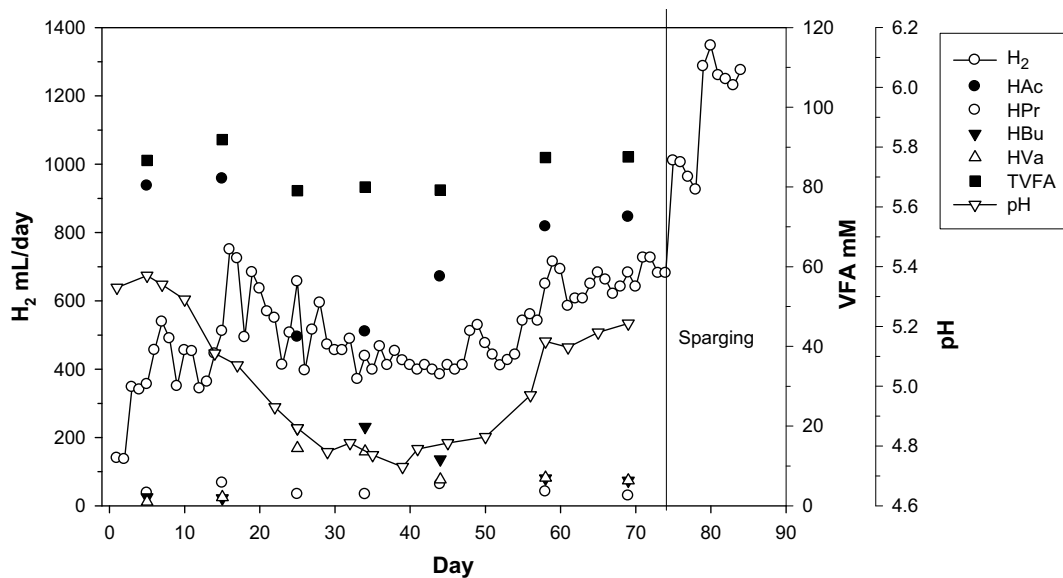
Figure 8. Sparging in the liquid phase effect on hydrogen production at mesophilic temperature (37°), paper I

3.5. Organic acid concentration

It has been reported that high concentration of the organic acids result in a collapse of the pH gradient across the membrane and cause the total inhibition of all metabolic functions in the cell (Jones and Woods 1986). It has been claimed that both the total acetate or butyrate acid concentration and the undissociated form of these acids can inhibit the dark hydrogen fermentation process (Jones and Woods 1986; Van Ginkel and Logan 2005; van Niel et al., 2003).

A near-complete H_2 production inhibition was observed by Van Ginkel and Logan (2005) with added acetic acid to give undissociated acid concentrations in the reactor of 63 mM, which occured at pH 5.5 and 165mM acetate addition. They reported that the fermentation pathway changing from organic acid and hydrogen to solvent was not detected. It also has been reported that the total acetate concentration is a strong inhibitor to hydrogen fermentation process. van Niel et al. (2003) reported that undissociated acetate concentration didn't seriously inhibit the hydrogen production at pH 6.5 and 7.2 under 70°C by pure culture of *Caldicellulosiruptor saccharolyticus*, and the total acetate concentration was the main inhibitor for extreme-thermophilic hydrogen fermentation. Huang et al. (1998b) utilized *coltridium formicoaceticum* to ferment fructose at pH 7.6 and temperature under 37°C. They found the total acetate concentration but not undissociated acetate concentration had a noncompetitive inhibition effect for hydrogen fermentation. Nakashimada et al. (1999) found that the hydrogen fermentation was completely inhibited by the total acetate concentration of more than 25mM at pH 6.5 by a hyper-thermophilic hydrogen producing bacteria

19

Pyrococcus furiosus. They further continuously sparged the reactor with N_2 gas, and found the inhibition was caused by acetate concentration but not hydrogen partial pressure.

In our research, as shown in figure 9, the acetate concentration started to inhibit hydrogen fermentation at more than 50mM. At a acetate concentration of 200mM, the hydrogen production ($36mL/gVS_{added}$) was 7 time lower than at 5-25mM acetate ($250mL/gVS_{added}$), and moreover, the lag phase was more than 100 hours，as described in paper IV.
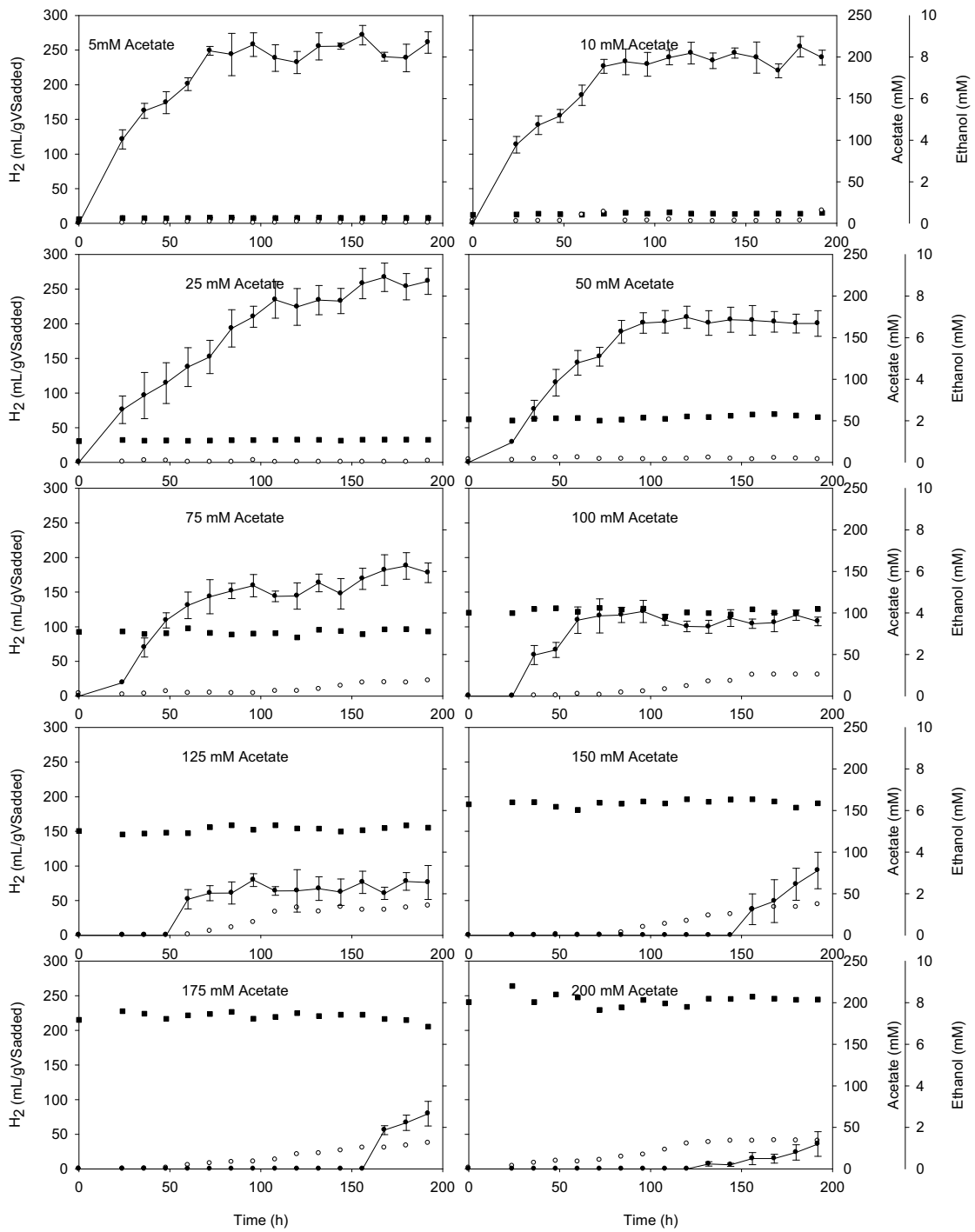
Figure 9. Inhibition on hydrogen fermentation by acetic acid addition on extreme-thermophilic hydrogen fermentation, -●- hydrogn, ○ ethanol, and ■ acetate, Paper IV

3.6. Inorganic elements

Recent research indicates elements such as iron and nitrogen, and compounds such as carbonate and phosphate can affect the hydrogen production in dark fermentation process as well.

3.6.1. Iron concentration

Hydrogenases are important enzymes as they directly involved in the hydrogen production during hydrogen fermentation process. It has been reported that by increasing iron concentration, the hydrogen production increases significantly (Lee et al., 2001).

In the process of fermentative hydrogen production, Fd, an iron–sulfur protein, functions primarily as an electron carrier and is involved in pyruvate oxidation to acetyl-CoA and CO_2 and in proton reduction to molecular H_2 (Lee et al., 2001). Vanacova et al. (2001) demonstrated that iron could induce metabolic change and be involved in the expression of both Fe–S and non-Fe–S proteins operating in hydrogenase. Therefore, the authors presumed that the addition of iron had some effects on the growth of fermentative organisms and the rate of hydrogen production.

3.6.2. C/N ratio

The carbon/nitrogen (C/N) ratio is also important for dark fermentation process stability (Tanisho et al., 1998). It has been reported that proper C/N ratio can increase the hydrogen production in mesophilic hydrogen fermentation from sewage sludge (Lin and Lay 2004). They found at the C/N ration of 47, the hydrogen production was 5 times higher than the one at C/N ratio 40.

4. Dark hydrogen fermentation in a two-Stage process for combination of bio-hydrogen and bio-methane production

The two-stage process has traditionally been used for methane production (Vollmer 1985). The argument of using two-stage process was to separate hydrolysis/acidogenesis and methanogenesis and optimize each process separately, leading to a larger overall reaction rate and biogas yield (Blonskaja et al., 2003; Mataalvarez et al., 1993). Furthermore, a better pathogenic destruction is achieved by a two-stage process, which combines a short hydrolysis stage performing at thermophilic or hyper-thermophilic temperatures and methane stage at thermophilic or mesophilic temperatures (Bendixen 1994). However, the two-stage systems have not won inpass as it adds the complexity and as a consequence increasing investment and operational costs. Furthermore, the effect of increasing biogas production has not been accepted broadly, as separation of the two processes i.e. hydrolysis/acidogenesis and methanogenesis negatively affects syntrophic association and prevents interspecies hydrogen transfer between acidogenens/acetogens and methanogens(Reith et al., 2003a). Currently, 90% of full-scale biogas plants in Europe rely on one-stage process due to the lower cost comparing to two-stage process (Choi et al., 1997; De Baere 2000). It is clear that this two-stage process technology remains unproven in the field. However, the first stage can be used as an independent hydrogen production unit but not as a precoursor/pretreatment for the methanogenic reactor. This kind of two-stage process has been reported to achieve enhanced stability and higher loading capacities for the methanogenesis process compared with the traditional one stage process. Furthermore, two-stage process, it achieved greater process efficiencies overall (Ke et al., 2005).

Hawkes et al. (2007) reviewed the recent publications on two-stage hydrogen-methane process and found most of them reported a higher total efficiency on waste treatment and energy recovery than the traditional one stage process. In a two-stage hydrogen-methane fermentation process with household solid waste as substrate at mesophilic temperature in our study, HRT was controlled at 2 and 15 days in hydrogen stage and methane stage, respectively. It was found that the hydrogen production was 43 mL-H_2/gVS_{added}, and the methane production was 500 mL CH_4/g VS_{added}. The methane production was 21% higher than the one in one-stage process (in paper I). Similarly, a two-stage hydrogen-methane process developed by Sapporo Breweries Ltd. together with Shimadzu Corp. and Hiroshima University successfully produced H_2 and CH_4 from bread waste, and achieved 10% more methane production compared to traditional one-stage process (Greenbiz.com 2006). The Energy Technology Research Institute of the National Institute of Advanced Industrial Science and Technology in Japan operated a semi-pilot scale two-sage hydrogen-methane plant using kitchen waste, paper waste and food waste. When an overall HRT was reduced from 25 to 15 days, the

decomposition of organic wastes was increased from 60–65% to 80% and energy recovery increased from 40–46% to 55% in comparison to traditional one-stage methane fermentation (AIST 2005). These proved the two-stage process could achieve not only hydrogen production but also higher methane production by enhancing the hydrolysis in the hydrogen stage.

5. Repeated batch cultivations for extreme-thermophilic hydrogen producing mixed cultures

Up to now, studies concerning extreme-thermophilic hydrogen fermentation mainly focused on pure cultures isolated from extreme environments, such as deep-sea volcanoes and hot springs (van Niel et al., 2002). These pure cultures normally have special requirement on the medium for growth. For an example, the culture achieved from deep-sea volcanoes needs high NaCl concentration and the culture achieved from hot springs needs high sulfur concentration for growth (Schroder et al., 1994; van Niel et al., 2002). However, for a technologically feasible process, stable, mixed cultures easily obtainable from natural sources able to operate on non-sterile feedstock is required (Hawkes et al., 2002). In most cases the mixed culture inocula need to be enriched and adapted from inocula obtained from thermophilic environments before applying to extreme-thermophilic dark hydrogen fermentation, as extreme-thermophilic inocula are often not available.

Repeated batch cultivation is a well-known method for enhancing the productivity of microbial cultures (Radmann et al., 2007). In repeated batch cultivations, the batch reactor is initially filled with the inoculum together with the cultivation medium and incubated under specific conditions. After a certain period, a specific volume of the culture is removed and replaced with an equal amount of fresh medium. Consequently a part of cultivation medium is kept in reactor as starting inoculum (Radmann et al., 2007). Repeated batch culture provides an excellent condition for control the nutrients feed rate to optimize the productivity (Giridhar and Srivastava 2001). Weigand (1981) reported that the repeated batch cultivation obtained the highest productivity increase comparing to fed batch and continuous cultivation methods. Furthermore, this method has operational advantages, such as avoiding variation in the inoculum and thus maintaining the microorganism at high growth rates (Fabregas et al., 1996).

The repeated batch method has been reported that it can improve dark hydrogen fermentation and increase hydrogen production. Yokoi et al. (2002) reported that they improved the hydrogen production from starch-manufacturing wastes by a mixed culture of *C. butyricum* and *E. aerogenes* under 35°C by repeated batch method. Their result showed the hydrogen production increase along with the repeated transfer and increase 70% after 5 successful transfers. Kawagoshi et al. (2005) used 6 kinds of inocula, which were from waste sludge, soil from watermelon field and lake sediment, for hydrogen dark fermentation from glucose. They found the repeated batch method was effective both on achieving higher hydrogen production and acclimation of the hydrogen generating bacteria.

Repeated batch cultivation was also proved as an efficient method that can overcome the acetate inhibition, which was one of the main obstacles for dark hydrogen fermentation. It has been reported that the pure culture of *Thermotoga neapolitana* can tolerate 4 times higher acetate concentration after repeated batch cultivation under $55^{o}C$ (Sakai et al., 2005). Similar result has been reported by Nakashimad (1999) , who found *Pyrococcus furiosus* can tolerate 2.7 times higher acetate concentration after it was acclimated and adapted by repeated batch cultivation under $98^{o}C$. Moreover, they also reported the hydrogen was enhanced by repeated batch cultivation.

The mixed culture hydrogen fermentation at temperature over $60^{o}C$ was just started. There were only few publications on it (Kotsopoulos et al., 2006; Liu et al., 2008a; Yokoyama et al., 2007a; Yokoyama et al., 2007b; Zheng H et al., 2008). Yokoyama et al., (2007b) cultivated the extreme-thermophilic hydrogen producing bacteria from cow manure by repeated batch cultivation and successfully produced a hydrogen production of 2.65mole-H_2/mole glucose, which was the highest hydrogen production reported from mixed cultures from glucose, as shown in table 2. This indicated the repeated batch cultivation was a useful method for cultivating and adapting the extreme-thermophilic hydrogen producing bacteria.

Table 2. Comparison of hydrogen production from different mixed cultures (adapted from Yokoyama et al.(2007a)

Inoculum	Substrate	Fermentation Temperature (°C)	Hydrogen Yield (H2 Mol/Mol-Hexose)	Molar Ratio Of Produced (Butyrate/Acetate)	Reference
Cow	Glucose	75	2.65	0.14	(Yokoyama et al., 2007b)
Cow	Cellobiose	75	2.68	0.14	(Yokoyama et al., 2007b)
Sludge from Thermophilic Methanogenic Reactor	Glucose	70	2.47	0.62	(Kotsopoulos et al., 2006)
Compost of Sewage Sludge	Sugary Wastewater	60	2.59	1.27	(Ueno et al., 1996)
Sludge from Thermophilic Acidogenic	Food Waste	55	1.80	1.07	(Shin and Youn 2005)

Reactor					
Heat-Pretreated Sewage Sludge	Sucrose	40	1.93	1.53	(Wu et al., 2006)
Acclimated Sewage Sludge	Glucose	36	1.89	0.82	(Fang et al., 2002b)
Fermented Soybean-Meal	Bean Curd Manufacturing Waste	35	2.54	0.76	(Noike and Mizuno 2000)
Heat-Pretreated Sludge of Anacrobic Digester	Wheat Starch Co-Product	30	1.87	1.0	(Hussy et al., 2003)
Aggregated Granules from Sewage Sludge	Sucrose	26	1.93	0.73	(Fang et al., 2002a)

It is worth to mention that the highest hydrogen production in table 2 was achieved after 6-7 repeated transfers during repeated batch cultivation (Yokoyama et al., 2007a).

In our research, it was found that during the cultivation of extreme-thermophilic hydrogen producing mixed culture at the HSW concentration of 1g-VS/L , the hydrogen production was increased along with the repeated transfers and also the lag phase was significiently reduced, as shown in figure 10 (Paper III).
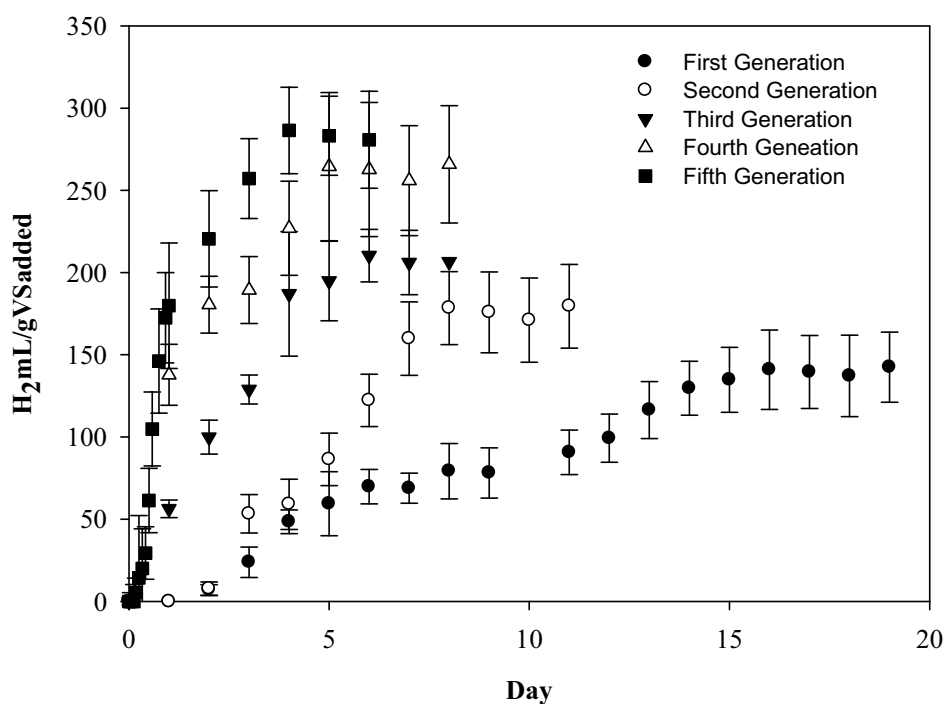
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Figure 10. Hydrogen production profiles of 1 g-VS/L HSW cultivation in consecutive 5 generations. The error bars are standard deviations (Paper III).

The same phebomenon was found during 2g-VS/L to 10g/L HSW cultivation. The hydrogen production from the 1st and last genetation of repeated batch cultivation for each HSW concentration was listed in table 3.

Table 3. Summary of hydrogen yields with standard deviation in the 1st and final generation during cultivation of 1-10 g-VS/L (Paper III).

HSW	Hydrogen yield (mL H_2/gVS$_{added}$)		Transfer times	Time consumed
gVS/L	1st generation	Last generation		Month
1	84.3±12.7	169.5±11.8	5	1
2	64.2±16.2	125.1±13.1	5	1
3	57.1±11.4	108.2±14.8	5	1
4	50.5±8.8	104.3±11.3	5	2
6	48.0±7.6	105.2±5.9	7	2
10	33.9±18.3	101.7±9.1	15	4

6. Electrochemically assisted biohydrogen production from cattle manure in anaerobic membraneless CSTR reactor

It is believed that in industrial applications the use of mixed cultures for hydrogen production from organic wastes might be more advantageous because pure cultures can easily be contaminated with H_2 consuming bacteria, like methanogens (Reith et al., 2003a). In the research on dark fermentation process, inhibition of methanogens is required and necessary for hydrogen production.

Three methods have been reported to inhibit methanogens, which are heat shock, pH control, and *2-Bromoethanesulfonic* (BES) acid control. Most bio-hydrogen researchers heated their inoculum at $100^{o}C$ or over $100^{o}C$ before their experiments (Chang and Lin 2004; Oh et al., 2003; Okamoto et al., 2000). The theory of heat shock method is based on that heat shock treatment can inactivate hydrogenotrophic bacteria and harvest anaerobic spore-forming bacteria such as *Clostridium*. The pH control method is base on inhibiting/inactivating the methanogens in a low pH environment (Chang et al., 2002; Oh et al., 2003). BES ($C_2H_4BrO_3SNa$) is introduced as a specific methanogen inhibitor. However, it does not work well in practice. Many research reported the failure to inhibit methanogens by using this chemical (REF). That is probably due to the added BES concentration is far from the requirements of real situations. Different BES concentrations have been reported to inhibit methanogens, which range from 0.01 mM to 6 mM (Le Van et al., 1998; Nollet et al., 1997).

The combination of pH and HRT control is the most popular method for preventing hydrogen consumption from methanogens. Especially for dark fermentation from feedstocks such as household waste, wastewater, and other feedstocks with high microbial content, avoiding the contamination by methanogens in the system is a challenge. Effective biohydrogen production from wastewater could be achieved by HRT of approx. 12 hours which was enough to ensure effective washout of methanogens (Chen et al., 2001; Ueno et al., 1996). Although a relative short HRT was effective to suppress methanogensis for hydrogen production from wastewater fermentation systems, it was not enough from more complex substrates, such as household solid waste or manure, because they contained higher content of slowly degradable organic matter such as lignocellulosic material. In such systems a combination of low pH and short HRT was necessary for preventing methanogenesis. A combination of HRT 2-3 days and pH lower than 6 could secure biohydrogen production from HSW (Alzate-Gaviria et al., 2007; Gomez et al., 2006; Lay et al., 1999; Shin and Youn 2005). The relatively higher HRT (2-3 days) needed for HSW compared to hydrogen production from wastewater (12-24 hours) was due to slow hydrolysis rate of the complex material contained in HSW (Liu et al., 2008a). pH control alone, even at

pH as low as 4.5, was often not enough to suppress methanogensis when the HRT is long enough. For an example, Kim et al. (2004) reported that the methanogens could not be inhibited with pH 4.5 at 9days HRT by glucose fermentation under $37^{o}C$ in semi-CSTR reactors.

It is estimated that 26 million tons of animal manures are generated every year in Denmark alone (Holm-Nilsen and Seadi 2007). 5% of them (approx. 1.3 million tonnes) was treated in 20 centralised and 60 farm-scale biogas plants (Angelidaki and Ellegaard 2002), and produced 0.91 PJ electricity by incineration of the methane gas produced from manure. The total electricity potential from animal manure is more than 25 PJ per year in Denmark along (Holm-Nilsen and Seadi 2007).

Biohydrogen production from manure has been difficult, and no successful biohydrogen production from manure has been reported until now. Both pH and HRT control method are difficult to apply for manure. Manure has a strong buffer capacity due to its high ammonia content (Angelidaki and Ahring 1993b). Attempt to produce biohydrogen from manure by controlling the pH required addition of unrealistically high amounts of hydrochloric acid (Zhu et al., 2007). As much as 1 mL 37% HCL addition was needed to decease the pH from 7.8 to 5 for 1 ml raw manure (Personal Experience)(Zhu et al., 2007). Decreasing the HRT caused poor hydrolysis due to the high content of biofibers (lignocellulosic material). Hill and Bolte (2000) reported that anaerobic digestion of pig manure in mesophilic CSTR was failed at 1day HRT for three replicate tests. They further found the process failure was not caused by the ammonia or VFAs inhibition, but was the bacteria washout due to short HRT.

Bio-electrochemical hydrogen production has recently received increasing attention (Cheng and Logan 2007; Rabaey et al., 2007; Rozendal et al., 2006). Complete oxidization of 1 mole glucose could stoichiometrically result in 12 moles hydrogen, according to equation 1.

However this is not practically achievable through dark hydrogen fermentation due to thermodynamic constrains (Eq.1). The theoretical thermodynamically possible hydrogen yield with maximum hydrogen yield of 4 mole hydrogen per mole glucose is obtained with acetate as the only byproduct (Eq. 2).

The further oxidation of acetate is thermodynamically unfavourable under standard conditions (Eq. 4).

$$CH_3COOH + 2H_2O \rightarrow 4H_2 + 2CO_2 \qquad \Delta G_0 = +104.6 \text{ kJ} \qquad (Equation\ 4)$$

Acetate oxidization to biohydrogen has been reported by photosynthetic process, where light is used as the extra energy for the metabolism (Barbosa et al., 2001). In bio-electrochemical systems, electricity provides the extra energy needed for the dark fermentation process to make acetate oxidization to hydrogen thermodynamically possible (Rabaey et al., 2007). Liu et al. (2005) reported that hydrogen could be obtained from acetate when 250mV voltage was applied in a microbial fuel cell (MFC). At 850mV, 2.9mole-H_2/mole acetate could be generated. Similarly, Rozendal et al., (2006) reported that 2 mole-H_2/mole acetate was obtained in an MFC assisted with 500mV voltage supply. Conclusively, supply of electrical voltage/power in an anaerobic reactor shall assist hydrogen production from manure.

A hydrogen production of 200mL/gVS_{added} or 400mL/gVS removal was achieved, as shown in figure 11, paper V. The methane productions of applied voltage of 2.5V, and 3.0V were 19.3± 2.1mL/gVSadded, 29.4± 4.5mL/gVSadded respectively. The methane production of these reactors was slightly higher than the control reactor, where no electricity was applied, which was 17.8± 1.9mL/gVSadded(Fig. 11).
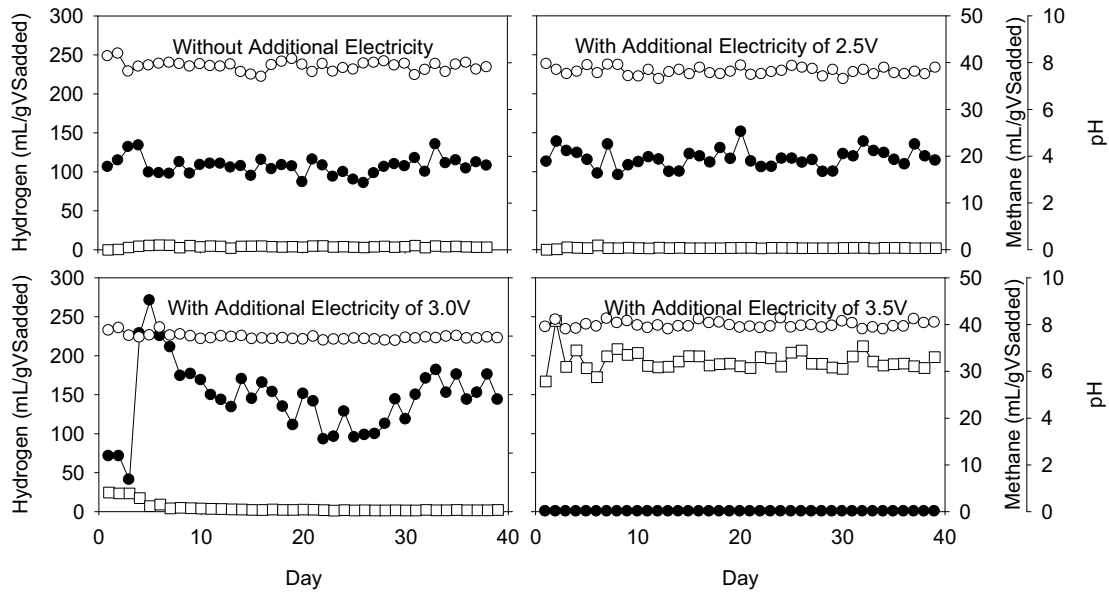

Figure 11. Hydrogen methane and pH from high strength cattle manure (3.7% VS) at different voltage addition and control -○-pH, -●- methane, and -□- hydrogen, paper V

The electricity energy input used in our study was 1.70 kWh/m^3 H_2, which is much lower than the typical energy consumption for water electrolysis of 4.5-6kwh/m^3 H_2 (Liu et al., 2005). Comparing the energy input as electricity to the electrogenic system, to the energy content of the produced hydrogen assuming that energy yield of hydrogen is 122 kJ/g-H_2, corresponding to 3.03 kWh/m^3 H_2, we found that the energy content of

the produced biohydrogen was 1.78 times higher than the electricity energy input to the system (Paper V).

7. Conclusions and future plan

A two-stage hydrogen-methane process from household solid waste was demonstrated working successfully. This process produced 43 mL H_2/g VS_{added}, and 500 mL CH_4/g VS_{added}. The methane production was 21% higher than the one in one-stage process. Sparging with methane in hydrogen production stage increased hydrogen production by 88%. It was demonstrated that HRT alone could not be used to wash out the methanogens at pH 7 at extreme-thermophilic conditions. At 3days HRT, pH 5.5 was enough to inhibit the methanogens completely and produce hydrogen. It was found that repeated batch cultivation was a very useful method to adapt and cultivate the cultures to enhance the hydrogen production and reduce the lag phase. After adaptation, hydrogen was produced directly in the HSW feedstock (10 gVS/L) with the maximum yield of 101.7±9.1 mL H_2/gVS_{added}. The lag phase was reduced to a couple of hours. pH was proved to be the key factor for dark hydrogen fermentation. The pH optimum was different at different fermentation temperatures. The optimum pH for hydrogen fermentation from HSW at 37^oC was found to be between pH 5 to 5.5. At extreme-thermophilic temperature, the optimum pH was found to be 7. Acetate was proved to be the inhibitor for dark hydrogen fermentation under 70^oC. Hydrogen fermentation by bio-electrochemical with anaerobic digestion in CSTR has been investigated with cattle manure as substrate under 55^oC. The result indicates that hydrogen can be obtained at an applied voltage of 3.5V. The hydrogen production was 193.5±13.6 mL/gVS_{added} or 400.6±28.1mL/gVSremoval respectively. The electricity energy using in the current study was 1.70kwh/m^3 H_2. The energy yield (122 J/g-H_2) from the hydrogen obtained in the current study was 1.78 times higher than the electricity energy input to the system.

The future plan is that the research of bio-electrochemical system with anaerobic reactor for hydrogen production is going to be continued. The bio-electrochemical system with membrane and without membrane is going to be compared. Moreover, acetate oxidization in bio-electrochemical system in membraneless CSTR reactor is going to be investigated.

8. References

Adams MWW. 1990. The Metabolism of Hydrogen by Extremely Thermophilic, Sulfur-Dependent Bacteria. Fems Microbiology Reviews 75(2-3):219-237.

AIST. 2005. AIST World first biogas plant to recover H2 and methane quickly from kitchen waste. http://www.aist.go.jp/aist_e/latest_research/2004/20040728/20040728.html.

Alzate-Gaviria LM, Sebastian PJ, Perez-Hernandez A, Eapen D. 2007. Comparison of two anaerobic systems for hydrogen production from the organic fraction of municipal solid waste and synthetic wastewater. International Journal of Hydrogen Energy 32(15):3141-3146.

Amend JP, Shock EL. 2001. Energetics of overall metabolic reactions of thermophilic and hyperthermophilic Archaea and Bacteria. Fems Microbiology Reviews 25(2):175-243.

Angelidaki I. 2002. Anaerobic Digestion. Course 12133, Environment Biotechnology.

Angelidaki I, Ahring BK. 1993. Thermophilic anaerobic-digestion of livestockwaste - the effect of ammonia. Applied Microbiology and Biotechnology 38(4):560-564.

Angelidaki I, Ellegaard L. 2002. Anaerobic digestion in Denmark: Past, present and future. Anaerobic digestion for sustainability in waste (water) treatment and reuse.(Proceedings of 7th FAO/SREN-Workshop, 19-22 May 2002, Moscow, Russia).

Armor JN. 2005. Catalysis and the hydrogen economy. Catalysis Letters 101(3-4):131-135.

Asada Y, Koike Y, Schnackenberg J, Miyake M, Uemura I, Miyake J. 2000. Heterologous expression of clostridial hydrogenase in the cyanobacterium Synechococcus PCC7942. Biochimica Et Biophysica Acta-Gene Structure and Expression 1490(3):269-278.

Barbir F, Veziroglu TN, Plass HJ. 1990. Environmental-Damage Due to Fossil-Fuels Use. International Journal of Hydrogen Energy 15(10):739-749.

Barbosa MJ, Rocha JMS, Tramper J, Wijffels RH. 2001. Acetate as a carbon source for hydrogen production by photosynthetic bacteria. Journal of Biotechnology 85(1):25-33.

Batstone DJ, Keller J, Angelidaki I, Kalyuzhnyi SV, Pavlostathis SG, Rozzi A, Sanders WTM, Siegrist H, Vavilin VA. 2002. The IWA Anaerobic Digestion Model No 1 (ADM1). Water Science and Technology 45(10):65-73.

Bendixen HJ. 1994. Safeguards against Pathogens in Danish Biogas Plants. Water Science and Technology 30(12):171-180.

Benemann J. 1996. Hydrogen biotechnology: Progress and prospects. Nature Biotechnology 14(9):1101-1103.

Blonskaja V, Menert A, Vilu R. 2003. Use of two-stage anaerobic treatment for distillery waste. Advances in Environmental Research 7(3):671-678.

Bockris JOM. 1972. Hydrogen Economy. Science 176(4041):1323-&.

Bolzonella D, Battistoni P, Mata-Alvarez J, Cecchi F. 2003. Anaerobic digestion of organic solid wastes: process behaviour in transient conditions. Water Science and Technology 48(4):1-8.

Chang JS, Lee KS, Lin PJ. 2002. Biohydrogen production with fixed-bed bioreactors. International Journal of Hydrogen Energy 27(11-12):1167-1174.

Chen CC, Lin CY, Chang JS. 2001. Kinetics of hydrogen production with continuous anaerobic cultures utilizing sucrose as the limiting substrate. Applied Microbiology and Biotechnology 57(1-2):56-64.

Cheng S, Logan BE. 2007. Sustainable and efficient biohydrogen production via electrohydrogenesis. Proceedings of the National Academy of Sciences of the United States of America 104(47):18871-18873.

Cheng SS, Chang SM, Chen ST. 2002. Effects of volatile fatty acids on a thermophilic anaerobic hydrogen fermentation process degrading peptone. Water Science and Technology 46(4-5):209-214.

Choi HB, Hwang KY, Shin EB. 1997. Effects on anaerobic digestion of sewage sludge pretreatment. Water Science and Technology 35(10):207-211.

Conrad R, Wetter B. 1990. Influence of Temperature on Energetics of Hydrogen Metabolism in Homoacetogenic, Methanogenic, and Other Anaerobic-Bacteria. Archives of Microbiology 155(1):94-98.

Craven SE. 1988. Increased Sporulation of Clostridium-Perfringens in a Medium Prepared with the Prereduced Anaerobically Sterilized Technique or with Carbon-Dioxide or Carbonate. Journal of Food Protection 51(9):700-706.

Das D, Veziroglu TN. 2001. Hydrogen production by biological processes: a survey of literature. International Journal of Hydrogen Energy 26(1):13-28.

Datar RP, Shenkman RM, Cateni BG, Huhnke RL, Lewis RS. 2004. Fermentation of biomass-generated producer gas to ethanol. Biotechnology and Bioengineering 86(5):587-594.

De Baere L. 2000. Anaerobic digestion of solid waste: state-of-the-art. Water Science and Technology 41(3):283-290.

de Vrije T, de Haas GG, Tan GB, Keijsers ERP, Claassen PAM. 2002. Pretreatment of Miscanthus for hydrogen production by Thermotoga elfii. International Journal of Hydrogen Energy 27(11-12):1381-1390.

de Vrije T, Mars AE, Budde MAW, Lai MH, Dijkema C, de Waard P, Claassen PAM. 2007. Glycolytic pathway and hydrogen yield studies of the extreme thermophile Caldicellulosiruptor saccharolyticus. Applied Microbiology and Biotechnology 74(6):1358-1367.

Demirel B, Yenigun O. 2004. Anaerobic acidogenesis of dairy wastewater: the effects of variations in hydraulic retention time with no pH control. Journal of Chemical Technology and Biotechnology 79(7):755-760.

Dickinson RE, Cicerone RJ. 1986. Future Global Warming from Atmospheric Trace Gases. Nature 319(6049):109-115.

Dong GX, Wu BR, Zhu L, Du J. 2007. Microstructure and electrochemical properties of low-temperature hydrogen storage alloy used in Ni/MH batteries. Transactions of Nonferrous Metals Society of China 17:S941-S944.

Evvyernie D, Morimoto K, Karita S, Kimura T, Sakka K, Ohmiya K. 2001. Conversion of chitinous wastes to hydrogen gas by Clostridium paraputrificum M-21. Journal of Bioscience and Bioengineering 91(4):339-343.

Evvyernie D, Yamazaki S, Morimoto K, Karita S, Kimura T, Sakka K, Ohmiya K. 2000. Identification and characterization of Clostridium paraputrificum M-21, a chitinolytic, mesophilic and hydrogen-producing bacterium. Journal of Bioscience and Bioengineering 89(6):596-601.

Fabiano B, Perego P. 2002. Thermodynamic study and optimization of hydrogen production by Enterobacter aerogenes. International Journal of Hydrogen Energy 27(2):149-156.

Fabregas J, Patino M, Morales ED, Cordero B, Otero A. 1996. Optimal renewal rate and nutrient concentration for the production of the marine microalga Phaeodactylum tricornutum in semicontinuous cultures. Applied and Environmental Microbiology 62(1):266-268.

Fang HHP, Liu H, Zhang T. 2002a. Characterization of a hydrogen-producing granular sludge. Biotechnology and Bioengineering 78(1):44-52.

Fang HHP, Zhang T, Liu H. 2002b. Microbial diversity of a mesophilic hydrogen-producing sludge. Applied Microbiology and Biotechnology 58(1):112-118.

Fyfe WS. 1999. Clean energy for 10 billion humans in the 21st century: is it possible? International Journal of Coal Geology 40(2-3):85-90.

Gibbs MD, Reeves RA, Farrington GK, Anderson P, Williams DP, Bergquist PL. 2000. Multidomain and multifunctional glycosyl hydrolases from the extreme thermophile Caldicellulosiruptor isolate Tok7B.1. Current Microbiology 40(5):333-340.

Giridhar R, Srivastava AK. 2001. Repeated fed-batch sorbose fermentation by Gluconobacter oxydans. Chemical and Biochemical Engineering Quarterly 15(3):127-129.

Gomez X, Moran A, Cuetos MJ, Sanchez ME. 2006. The production of hydrogen by dark fermentation of municipal solid wastes and slaughterhouse waste: A two-phase process. Journal of Power Sources 157(2):727-732.

Greenbiz.com. 2006. Japanese research group generates hydrogen from bread waste. http://www.greenbiz.com/news/news_third.cfm?NewsID=27619.

Hallenbeck PC. 2005. Fundamentals of the fermentative production of hydrogen. Water Science and Technology 52(1-2):21-29.

Han SK, Kim SH, Kim HW, Shin HS. 2005. Pilot-scale two-stage process: a combination of acidogenic hydrogenesis and methanogenesis. Water Science and Technology 52(1-2):131-138.

Han SK, Shin HS. 2004. Biohydrogen production by anaerobic fermentation of food waste. International Journal of Hydrogen Energy 29(6):569-577.

Hawkes FR, Dinsdale R, Hawkes DL, Hussy I. 2002. Sustainable fermentative hydrogen production: challenges for process optimisation. International Journal of Hydrogen Energy 27(11-12):1339-1347.

Hawkes FR, Hussy I, Kyazze G, Dinsdale R, Hawkes DL. 2007. Continuous dark fermentative hydrogen production by mesophilic microflora: Principles and progress. International Journal of Hydrogen Energy 32(2):172-184.

Heyndrickx M, Vansteenbeeck A, Devos P, Deley J. 1986. Hydrogen Gas-Production from Continuous Fermentation of Glucose in a Minimal Medium with Clostridium-Butyricum Lmg-1213t1. Systematic and Applied Microbiology 8(3):239-244.

Hill DT, Bolte JP. 2000. Methane production from low solid concentration liquid swine waste using conventional anaerobic fermentation. Bioresource Technology 74(3):241-247.

Holm-Nilsen JB, Seadi AL. 2007. Biogas in Denmark-Country up-date 2007. http://www.iea-biogas.net/Dokumente/countryreports/07/report_danemark_07.pdf.

Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST. 1994. Bergeys Manual of Determinative Bacteriology, 9th ed. Williams & Willkins, Baltimore.

Huang CY, Patel BK, Mah RA, Baresi L. 1998a. Caldicellulosiruptor owensensis sp. nov., an anaerobic, extremely thermophilic, xylanolytic bacterium. International Journal of Systematic Bacteriology 48:91-97.

Huang YL, Mann K, Novak JM, Yang ST. 1998b. Acetic acid production from fructose by Clostridium formicoaceticum immobilized in a fibrous-bed bioreactor. Biotechnology Progress 14(5):800-806.

Hussy I, Hawkes FR, Dinsdale R, Hawkes DL. 2003. Continuous fermentative hydrogen production from a wheat starch co-product by mixed microflora. Biotechnology and Bioengineering 84(6):619-626.

Ike A, Toda N, Tsuji N, Hirata K, Miyamoto K. 1997. Hydrogen photoproduction from CO2-fixing microalgal biomass: Application of halotolerant photosynthetic bacteria. Journal of Fermentation and Bioengineering 84(6):606-609.

James, James. 2002. International Directory of Solid Waste Management: The ISWA Yearbook.

Jones DT, Woods DR. 1986. Acetone-Butanol Fermentation Revisited. Microbiological Reviews 50(4):484-524.

Joyner AE, Winter WT, Godbout DM. 1977. Studies on Some Characteristics of Hydrogen Production by Cell-Free-Extracts of Rumen Anaerobic Bacteria. Canadian Journal of Microbiology 23(3):346-353.

Kadar Z, De Vrijek T, van Noorden GE, Budde MAW, Szengyel Z, Reczey K, Claassen PAM. 2004. Yields from glucose, xylose, and paper sludge hydrolysate during hydrogen production by the extreme thermophile Caldicellulosiruptor saccharolyticus. Applied Biochemistry and Biotechnology 113-16:497-508.

Kanokwan B. 2006. Online monitoring and control of the biogas process. Phd Thesis Institute of Environment & Resouces, Technical University of Denmark.

Kawagoshi Y, Hino N, Fujimoto A, Nakao M, Fujita Y, Sugimura S, Furukawa K. 2005. Effect of inoculum conditioning on hydrogen fermentation and pH effect on bacterial community relevant to hydrogen production. Journal of Bioscience and Bioengineering 100(5):524-530.

Ke SZ, Shi Z, Fang HHP. 2005. Applications of two-phase anaerobic degradation in industrial wastewater treatment. International Journal of Environment and Pollution 23(1):65-80.

Kim IS, Hwang MH, Jang NJ, Hyun SH, Lee ST. 2004. Effect of low pH on the activity of hydrogen utilizing methanogen in bio-hydrogen process. International Journal of Hydrogen Energy 29(11):1133-1140.

Kloeppel J, Rogerson S. 1991. The Hydrogen Economy. Electronics World & Wireless World 97(1666):668-671.

Kotsopoulos TA, Zeng RJ, Angelidaki I. 2006. Biohydrogen production in granular up-flow anaerobic sludge blanket (UASB) reactors with mixed cultures under hyper-thermophilic temperature (70 degrees C). Biotechnology and Bioengineering 94(2):296-302.

Kumar N, Das D. 2000. Enhancement of hydrogen production by Enterobacter cloacae IIT-BT 08. Process Biochemistry 35(6):589-593.

Kumar N, Das D. 2001. Continuous hydrogen production by immobilized Enterobacter cloacae IIT-BT 08 using lignocellulosic materials as solid matrices. Enzyme and Microbial Technology 29(4-5):280-287.

Kumar N, Ghosh A, Das D. 2001. Redirection of biochemical pathways for the enhancement of H-2 production by Enterobacter cloacae. Biotechnology Letters 23(7):537-541.

Lay JJ, Fan KS, Chang J, Ku CH. 2003. Influence of chemical nature of organic wastes on their conversion to hydrogen by heat-shock digested sludge. International Journal of Hydrogen Energy 28(12):1361-1367.

Lay JJ, Lee YJ, Noike T. 1999. Feasibility of biological hydrogen production from organic fraction of municipal solid waste. Water Research 33(11):2579-2586.

Lay JJ, Li YY, Noike T. 1997. Influences of pH and moisture content on the methane production in high-solids sludge digestion. Water Research 31(6):1518-1524.

Lee MJ, Zinder SH. 1988. Hydrogen Partial Pressures in a Thermophilic Acetate-Oxidizing Methanogenic Coculture. Applied and Environmental Microbiology 54(6):1457-1461.

Lee YJ, Miyahara T, Noike T. 2001. Effect of iron concentration on hydrogen fermentation. Bioresource Technology 80(3):227-231.

Lenssen N, Flavin C. 1996. Sustainable energy for tomorrow's world - The case for an optimistic view of the future. Energy Policy 24(9):769-781.

Levin DB, Pitt L, Love M. 2004. Biohydrogen production: prospects and limitations to practical application. International Journal of Hydrogen Energy 29(2):173-185.

Li CL, Fang HHP. 2007. Fermentative hydrogen production from wastewater and solid wastes by mixed cultures. Critical Reviews in Environmental Science and Technology 37(1):1-39.

Liang T-M. 2003. Application of membrane separation on anaerobic hydrogen producing process. Phd Thesis Department of environmental engineering, national Cheng Kung University.

Lin CY, Lay CH. 2004. Carbon/nitrogen-ratio effect on fermentative hydrogen production by mixed microflora. International Journal of Hydrogen Energy 29(1):41-45.

Liu D, Zeng RJ, Angelidaki I. 2008. Effects of pH and hydraulic retention time on hydrogen production versus methanogenesis during anaerobic fermentation of organic household solid waste under extreme-thermophilic temperature (70°C). Biotechnology and Bioengineering Accepted.

Liu DW, Liu DP, Zeng RJ, Angelidaki I. 2006. Hydrogen and methane production from household solid waste in the two-stage fermentation process. Water Research 40(11):2230-2236.

Liu H, Grot S, Logan BE. 2005. Electrochemically assisted microbial production of hydrogen from acetate. Environmental Science & Technology 39(11):4317-4320.

Lu WY, Wen JP, Chen Y, Sun B, Jia XQ, Liu MH, Caiyin Q. 2007. Synergistic effect of Candida maltosa HY-35 and Enterobacter aerogenes W-23 on hydrogen production. International Journal of Hydrogen Energy 32(8):1059-1066.

Mataalvarez J, Mtzviturtia A, Llabresluengo P, Cecchi F. 1993. Kinetic and Performance Study of a Batch 2-Phase Anaerobic-Digestion of Fruit and Vegetable Wastes. Biomass & Bioenergy 5(6):481-488.

McCarty P, L. 1981. One Hundred Years of Anaerobic Treatment in Anaerobic Digestion 1981. ed. Hughes et al. Anaerobic Digestion 1981. Elsevier Biomedical Press B. V., pp. 3--21.

Melis A, Happe T. 2001. Hydrogen production. Green algae as a source of energy. Plant Physiology 127(3):740-748.

Mizuno O, Dinsdale R, Hawkes FR, Hawkes DL, Noike T. 2000. Enhancement of hydrogen production from glucose by nitrogen gas sparging. Bioresource Technology 73(1):59-65.

Momirlan M, Veziroglu T. 1999. Recent directions of world hydrogen production. Renewable & Sustainable Energy Reviews 3(2-3):219-231.

Momirlan M, Veziroglu TN. 2002. Current status of hydrogen energy. Renewable & Sustainable Energy Reviews 6(1-2):141-179.

Najafpour G, Younesi H, Mohamed AR. 2004. Effect of organic substrate on hydrogen production from synthesis gas using Rhodospirillum rubrum, in batch culture. Biochemical Engineering Journal 21(2):123-130.

Nakashimada Y, Nakae K, Nishio N. 1999. Inhibitory effect of acetic acid on growth of hyperthermophilic archaeon Pyrococcus furiosus. Journal of Bioscience and Bioengineering 87(2):155-160.

Nandi R, Sengupta S. 1998. Microbial production of hydrogen: An overview. Critical Reviews in Microbiology 24(1):61-84.

Nath K, Kumar A, Das D. 2006. Effect of some environmental parameters on fermentative hydrogen production by Enterobacter cloacae DM11. Canadian Journal of Microbiology 52(6):525-532.

Noike T, Mizuno O. 2000. Hydrogen fermentation of organic municipal wastes. Water Science and Technology 42(12):155-162.

Oh YK, Kim SH, Kim MS, Park S. 2004. Thermophilic biohydrogen production from glucose with trickling biofilter. Biotechnology And Bioengineering 88(6):690-698.

Okamoto M, Miyahara T, Mizuno O, Noike T. 2000. Biological hydrogen potential of materials characteristic of the organic fraction of municipal solid wastes. Water Science and Technology 41(3):25-32.

Olsen T. 2005. Danish Energy Authority, Energy Statistics 2004. The Danish Ministry of Transport and Energy. Frederiksholms 27, DK 1220, Copenhagen K, Denmark.

Rabaey K, Rodriguez J, Blackall LL, Keller J, Gross P, Batstone D, Verstraete W, Nealson KH. 2007. Microbial ecology meets electrochemistry: electricity-driven and driving communities. Isme Journal 1(1):9-18.

Rachman MA, Nakashimada Y, Kakizono T, Nishio N. 1998. Hydrogen production with high yield and high evolution rate by self-flocculated cells of Enterobacter aerogenes in a packed-bed reactor. Applied Microbiology and Biotechnology 49(4):450-454.

Radmann EM, Reinehr CO, Costa JAV. 2007. Optimization of the repeated batch cultivation of microalga Spirulina platensis in open raceway ponds. Aquaculture 265(1-4):118-126.

Ramachandran R, Menon RK. 1998. An overview of industrial uses of hydrogen. International Journal of Hydrogen Energy 23(7):593-598.

Ravot G, Magot M, Fardeau ML, Patel BKC, Prensier G, Egan A, Garcia JL, Ollivier B. 1995. Thermotoga Elfii Sp-Nov, a Novel Thermophilic Bacterium from an African Oil-Producing Well. International Journal of Systematic Bacteriology 45(2):308-314.

Reith J, Wijffels R, Barteb H. 2003. Bio-methane & Bio-hydrogen: Status and perspectives of biological methane and hydrogen production http://gasunie.eldoc.ub.rug.nl/root/2003/3339875/.

Rozendal RA, Hamelers HVM, Euverink GJW, Metz SJ, Buisman CJN. 2006. Principle and perspectives of hydrogen production through biocatalyzed electrolysis. International Journal of Hydrogen Energy 31(12):1632-1640.

Sahlstrom L. 2003. A review of survival of pathogenic bacteria in organic waste used in biogas plants. Bioresource Technology 87(2):161-166.

Sakai S, Nakashimada Y, Inokuma K, Kita M, Okada H, Nishio N. 2005. Acetate and ethanol production from H-2 and CO2 by Moorella sp using a repeated batch culture. Journal of Bioscience and Bioengineering 99(3):252-258.

Schröder C, Selig M, Schonheit P. 1994. Glucose Fermentation to Acetate, Co2 and H-2 in the Anaerobic Hyperthermophilic Eubacterium Thermotoga-Maritima - Involvement of the Embden-Meyerhof Pathway. Archives of Microbiology 161(6):460-470.

Schroder C, Selig M, Schonheit P. 1994. Glucose Fermentation to Acetate, Co2 and H-2 in the Anaerobic Hyperthermophilic Eubacterium Thermotoga-Maritima - Involvement of the Embden-Meyerhof Pathway. Archives of Microbiology 161(6):460-470.

Sen D, Das D. 2005. Multiple parameter optimization for the maximization of hydrogen production by Enterobacter cloacae DM11. Journal of Scientific & Industrial Research 64(12):984-990.

Shin HS, Youn JH. 2005. Conversion of food waste into hydrogen by thermophilic acidogenesis. Biodegradation 16(1):33-44.

Shin JH, Yoon JH, Ahn EK, Kim MS, Sim SJ, Park TH. 2007. Fermentative hydrogen production by the newly isolated Enterobacter asburiae SNU-1. International Journal of Hydrogen Energy 32(2):192-199.

Sparling R, Risbey D, PoggiVaraldo HM. 1997. Hydrogen production from inhibited anaerobic composters. International Journal of Hydrogen Energy 22(6):563-566.

Stærkind K. 2005. Perspectives to 2025 and Draft action plan for the future electricity infrastructure. The Danish Ministry of Transport and Energy, Frederiksholms 27, DK 1220, Copenhagen K, Denmark.

Taguchi F, Mizukami N, Hasegawa K, Saitotaki T. 1994. Microbial Conversion of Arabinose and Xylose to Hydrogen by a Newly Isolated Clostridium Sp No-2. Canadian Journal of Microbiology 40(3):228-233.

Taguchi F, Mizukami N, Taki TS, Hasegawa K. 1995. Hydrogen-Production from Continuous Fermentation of Xylose During Growth of Clostridium Sp Strain No-2. Canadian Journal of Microbiology 41(6):536-540.

Taguchi F, Yamada K, Hasegawa K, TakiSaito T, Hara K. 1996. Continuous hydrogen production by Clostridium sp strain no 2 from cellulose hydrolysate in an aqueous two-phase system. Journal of Fermentation and Bioengineering 82(1):80-83.

Tamagnini P, Axelsson R, Lindberg P, Oxelfelt F, Wunschiers R, Lindblad P. 2002. Hydrogenases and hydrogen metabolism of cyanobacteria. Microbiology and Molecular Biology Reviews 66(1):1-+.

Tanisho S, Ishiwata Y. 1994. Continuous Hydrogen-Production from Molasses by the Bacterium Enterobacter-Aerogenes. International Journal of Hydrogen Energy 19(10):807-812.

Tanisho S, Kuromoto M, Kadokura N. 1998. Effect of CO2 removal on hydrogen production by fermentation. International Journal of Hydrogen Energy 23(7):559-563.

Tanisho S, Suzuki Y, Wakao N. 1987. Fermentative Hydrogen Evolution by Enterobacter-Aerogenes Strain E-82005. International Journal of Hydrogen Energy 12(9):623-627.

Tanisho S, Wakao N, Kosako Y. 1983. Biological Hydrogen-Production by Enterobacter-Aerogenes. Journal of Chemical Engineering of Japan 16(6):529-530.

Temudo MF, Kleerebezem R, van Loosdrecht M. 2007. Influence of the pH on (open) mixed culture fermentation of glucose: A chemostat study. Biotechnology and Bioengineering 98(1):69-79.

Thompson LJ, Gray V, Lindsay D, von Holy A. 2006. Carbon : nitrogen : phosphorus ratios influence biofilm formation by Enterobacter cloacae and Citrobacter freundii. Journal of Applied Microbiology 101(5):1105-1113.

Thompson LJ, Gray VM, Kalala B, Lindsay D, Reynolds K, von Holy A. 2008. Biohydrogen production by Enterobacter cloacae and Citrobacter freundii in carrier induced granules. Biotechnology Letters 30(2):271-274.

Ueno Y, Otsuka S, Morimoto M. 1996. Hydrogen production from industrial wastewater by anaerobic microflora in chemostat culture. Journal of Fermentation and Bioengineering 82(2):194-197.

Valdez-Vazquez I, Rios-Leal E, Carmona-Martinez A, Munoz-Paez KM, Poggi-Varaldo HM. 2006. Improvement of biohydrogen production from solid wastes by intermittent venting and gas flushing of batch reactors headspace. Environmental Science & Technology 40(10):3409-3415.

Valdez-Vazquez I, Rios-Leal E, Esparza-Garcia F, Cecchi F, Poggi-Varaldo HA. 2005a. Semi-continuous solid substrate anaerobic reactors for H-2 production from organic waste: Mesophilic versus thermophilic regime. International Journal of Hydrogen Energy 30(13-14):1383-1391.

Valdez-Vazquez I, Sparling R, Risbey D, Rinderknecht-Seijas N, Poggi-Varaldo HM. 2005b. Hydrogen generation via anaerobic fermentation of paper mill wastes. Bioresource Technology 96(17):1907-1913.

Van Ginkel S, Logan BE. 2005. Inhibition of biohydrogen production by undissociated acetic and butyric acids. Environmental Science & Technology 39(23):9351-9356.

Van Ginkel S, Sung SW, Lay JJ. 2001. Biohydrogen production as a function of pH and substrate concentration. Environmental Science & Technology 35(24):4726-4730.

van Groenestijn JW, Hazewinkel JHO, Nienoord M, Bussmann PJT. 2002. Energy aspects of biological hydrogen production in high rate bioreactors operated in the thermophilic temperature range. International Journal of Hydrogen Energy 27(11-12):1141-1147.

van Niel EWJ, Budde MAW, de Haas GG, van der Wal FJ, Claasen PAM, Stams AJM. 2002. Distinctive properties of high hydrogen producing extreme thermophiles, Caldicellulosiruptor saccharolyticus and Thermotoga elfii. International Journal of Hydrogen Energy 27(11-12):1391-1398.

van Niel EWJ, Claassen PAM, Stams AJM. 2003. Substrate and product inhibition of hydrogen production by the extreme thermophile, Caldicellulosiruptor saccharolyticus. Biotechnology and Bioengineering 81(3):255-262.
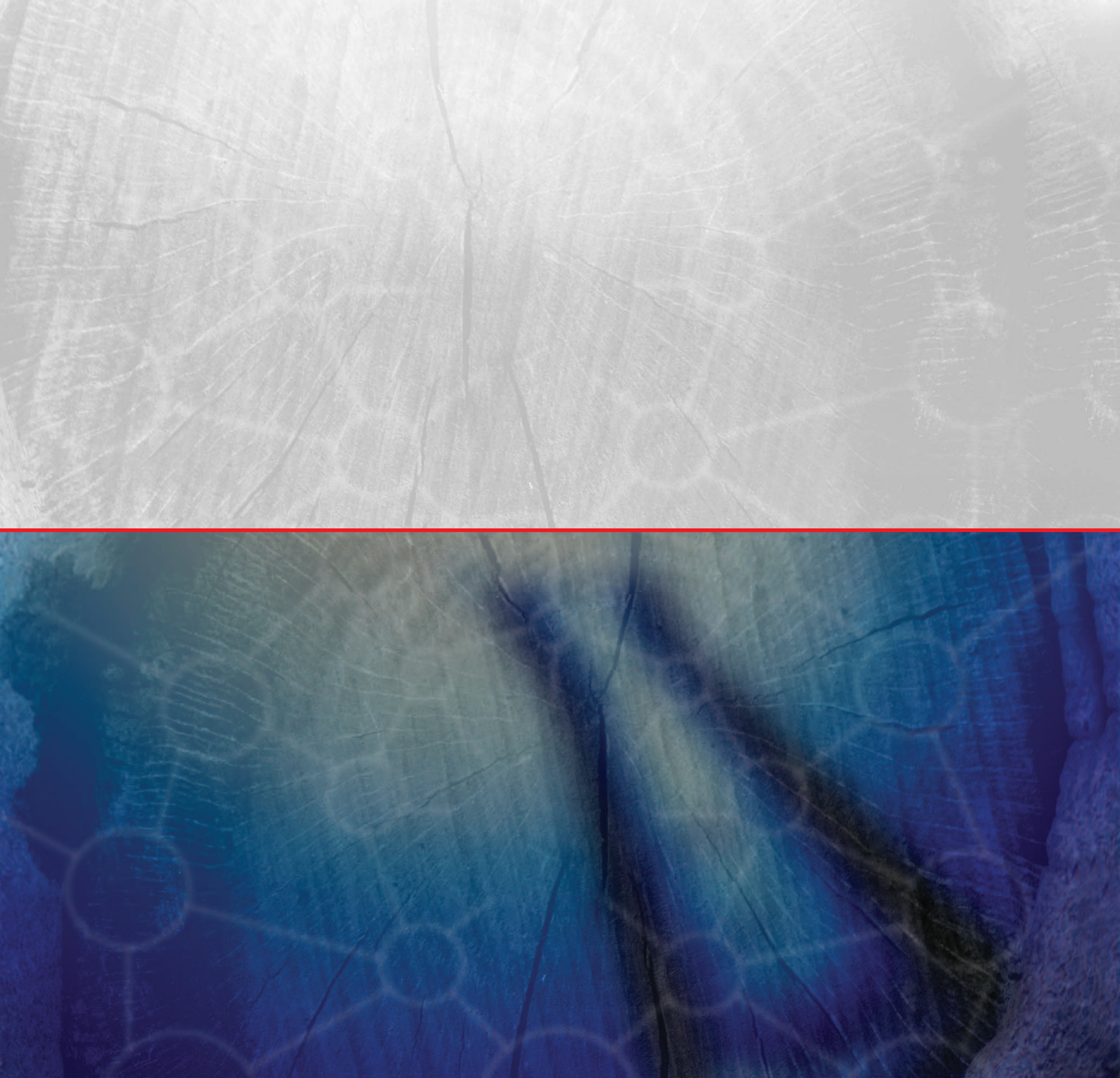
Vanacova S, Rasoloson D, Razga J, Hrdy I, Kulda J, Tachezy J. 2001. Iron-induced changes in pyruvate metabolism of Tritrichomonas foetus and involvement of iron in expression of hydrogenosomal proteins. Microbiology-Uk 147:53-62.

Veziroglu TN, Barbir F. 1992. Hydrogen - the Wonder Fuel. International Journal of Hydrogen Energy 17(6):391-404.

Vollmer H. 1985. 2-Stage Biological Treatment of Slaughterhouse Effluent. Fleischwirtschaft 65(11):1310-&.

Watts P. 2002. Energy Needs,Choices and Possibilities. Shall International http://www.shell.com/static/media-en/downloads/51852.pdf.

Weigand WA. 1981. Maximum Cell Productivity by Repeated Fed-Batch Culture for Constant Yield Case. Biotechnology and Bioengineering 23(2):249-266.

Wongtanet J, Sang BI, Lee SM, Pak D. 2007. Biohydrogen production by fermentative process in continuous stirred-tank reactor. International Journal of Green Energy 4(4):385-395.

Wu SY, Hung CH, Lin CN, Chen HW, Lee AS, Chang JS. 2006. Fermentative hydrogen production and bacterial community structure in high-rate anaerobic bioreactors containing silicone-immobilized and self-flocculated sludge. Biotechnology and Bioengineering 93(5):934-946.

Yamin JAA. 2006. Comparative study using hydrogen and gasoline as fuels: Combustion duration effect. International Journal of Energy Research 30(14):1175-1187.

Yamin JAA, Gupta HN, Bansal BB, Srivastava ON. 2000. Effect of combustion duration on the performance and emission characteristics of a spark ignition engine using hydrogen as a fuel. International Journal of Hydrogen Energy 25(6):581-589.

Yokoi H, Maki R, Hirose J, Hayashi S. 2002. Microbial production of hydrogen from starch-manufacturing wastes. Biomass & Bioenergy 22(5):389-395.

Yokoi H, Mori S, Hirose J, Hayashi S, Takasaki Y. 1998. H-2 production from starch by a mixed culture of Clostridium butyricum and Rhodobacter sp. M-19. Biotechnology Letters 20(9):895-899.

Yokoi H, Saitsu A, Uchida H, Hirose J, Hayashi S, Takasaki Y. 2001. Microbial hydrogen production from sweet potato starch residue. Journal of Bioscience and Bioengineering 91(1):58-63.

Yokoyama H, Moriya N, Ohmori H, Waki M, Ogino A, Tanaka Y. 2007a. Community analysis of hydrogen-producing extreme thermophilic anaerobic microflora enriched from cow manure with five substrates. Applied Microbiology and Biotechnology 77(1):213-222.

Yokoyama H, Waki M, Moriya N, Yasuda T, Tanaka Y, Haga K. 2007b. Effect of fermentation temperature on hydrogen production from cow waste slurry by using anaerobic microflora within the slurry. Applied Microbiology and Biotechnology 74(2):474-483.

Yoshida A, Nishimura T, Kawaguchi H, Inui M, Yukawa H. 2006. Enhanced hydrogen production from glucose using ldh- and frd-inactivated Escherichia coli strains. Applied Microbiology and Biotechnology 73(1):67-72.

Zheng H, Zeng RJ, I. A. 2008. Biohydrogen production from glucose in upflow biofilm reactors with plastic carriers under extreme-thermophilic conditions (70°C). . Biotechnology & Bioengineering Accepted.

Zhu J, Wu X, Miller C, Yu F, Chen P, Ruan R. 2007. Biohydrogen production through fermentation using liquid swine manure as substrate. Journal of Environmental Science and Health Part B-Pesticides Food Contaminants and Agricultural Wastes 42(4):393-401.

Paper I: Liu DW, Liu DP, Zeng RJ, Angelidaki I. 2006. Hydrogen and methane production from household solid waste in the two-stage fermentation process. 'Water Research' 40(11):2230-2236.

Paper II: Liu D, Zeng RJ, Angelidaki I. 2008. Effects of pH and hydraulic retention time on hydrogen production versus methanogenesis during anaerobic fermentation of organic household solid waste under extreme-thermophilic temperature (70°C). 'Biotechnology and Bioengineering'. Accepted.

Paper III: Liu D, Zeng RJ, Angelidaki I. 2008. Enrichment and adaptation of extreme-thermophilic (70°C) hydrogen producing bacteria to organic household solid waste by repeated batch cultivations, 'Internatioanl Journal of Hydrogen Energy'. Submitted.

Paper IV: Liu D, Booki Min, Angelidaki I. 2008. Bio-Hydrogen Production from Organic Household at extreme-thermophilic temperature (70°C) – Influence of pH and Acetate Concentration, 'Biotechnology and Bioengineering'. Submitted.

Paper V: Liu D, Ellegaard L and Irini Angelidaki，Bio-electrochemical system applied in anaerobic CSTR reactor for biohydrogen production from cattle manure as substrate，Manuscript, going to be submitted to 'Environmental Science & Technology'.

Paper VI: Christiansen Trine Løbner, Liu D, Liu D, Cirauqui B, Batstone Damien J and Angelidaki Irini. Bio-hydrogen production by anaerobic fermentation of waste in book: Anaerobic Digestion, 10th World Congress, 29th August - 2 September 2004, Montreal, Proceedings, pages: 2216-2219, 2004, NRC & IWA, Montreal. Poster Presentation.

Paper VII: Liu DW, Liu DP, Zeng Raymond Jianxiong and Angelidaki Irini. Hydrogen and methane production from household solid waste in the two-stage fermentation process. part of: 4th International Symposium on Anaerobic Digestion of Solid Waste, Copenhagen, August 31 - September 2, 2005, Volume 2 - Poster Presentations (ISBN:) , pages: 93-100, 2005, BioCentrum-DTU, Kgs. Lyngby., Oral Presentation

Paper VIII: Liu DW, Zeng Raymond Jianxiong and Angelidaki Irini. 2007. Enrichment and adaptation of extreme-thermophilic (70°C) H2 producing bacteria to organic household solid waste by repeated batch cultivations.part of:

Bioenergy for our future, 11th IWA world congress on anaerobic digestion (AD11) held in Brisbane, Australia 23-27 September 2007, 2007, Brisbane, Oral Presentation