Development of anaerobic immobilized-cell processes for biohydrogen production

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DEVELOPMENT OF ANAEROBIC IMMOBILIZED-CELL PROCESSES FOR BIOHYDROGEN PRODUCTION

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**SCHOOL OF CIVIL AND ENVIRONMENTAL ENGINEERING
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2008**

Development of Anaerobic Immobilized-Cell Processes for Biohydrogen Production

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SUMMARY

Self-formation of granules or biofilms has been noted in anaerobic biohydrogen reactors, yet few underlying principles describing the phenomenon have been drawn. A major drawback of the cell immobilization is the long startup period, which generally requires a few months for the development of hydrogen-producing granules or biofilms. The present research attempted to accelerate the development of cell immobilization and to lay a rational foundation describing the mechanisms involved by examining physicochemical, microbiological, and biological characteristics of the hydrogen-producing cultures.

An approach to rapidly initiate granulation of hydrogen-producing sludge was developed. To induce microbial immobilization, the acclimated sludge was subjected to an acid incubation for 24 h by shifting the culture pH from 5.5 to 2.0. The present study demonstrated that it was able to achieve rapid formation of hydrogen-producing granules within 3-5 days in a CSTR and an AFBR through acidifying microbial culture. However, applying acid incubation to the hydrogen-producing culture did not shorten the time required by biofilm attachment on granular activated carbon (GAC) support carriers. GAC-supported biofilm was able to spontaneously form within 36 h without the acid incubation. Lowering incubation pH resulted in improvement in bacterial surface physicochemical properties favoring microbial agglutination and immobilization, including reduced electrostatic repulsion, improved hydrophobic surface, more extracellular polymers (ECP) secretion and a higher ratio of extracellular proteins/polysaccharides. By comparing the formation of biofilms and granules, it is concluded that initial nuclei are important for microbial immobilization of hydrogen-producing bacteria and the acid incubation substantially promoted the development of microbial nuclei and granules.

Phylogenetic analysis of the microbial population in the granules and biofilms showed that only distinct bacterial strain existed in the respective microbial

community, which exhibited a high similarity (98%) to the species of *Clostridium pasteurianum*. Because bacterial species *C. pasteurianum*-like was also found abundant in hydrogen-producing granules in previous studies, it was believed that *C. pasteurianum* might be the crucial species in the hydrogen-producing community to form granules and biofilms. The strong agglutination property of *C. pasteurianum* might be attributed to its high productivity of ECP which would serve as a bio-glue to facilitate cell-to-cell or cell-to-substratum interaction. As abundant proteins were noted in the granule cores indicated by the confocal laser scanning microscope images, it can be derived that rapid formation of the hydrogen-producing granules could be due to the establishment of precursor protein-rich microbial nuclei.

All the reactors were operated at a pH of 5.5 ± 0.2 and a temperature of 37°C for fermentative hydrogen production using glucose as the limiting substrate. Formation of granular and biofilm sludge substantially enhanced biomass retention, and hydrogen production rate was highly related to the reactor biomass retention. In contrast to a maximum hydrogen production rate of $0.32 \text{ L/L}\cdot\text{h}$ obtained in the CSTR with suspended sludge, a 10-fold and a more than 20-fold increase in hydrogen production rate while keeping comparable hydrogen yields ranged from $1.66\text{--}1.81 \text{ mol-H}_2/\text{mol-glucose}$ were achieved in the granule-based CSTR and granule-based or biofilm-based AFBRs, respectively. Although a close performance in hydrogen production was demonstrated by granular sludge and biofilm sludge in the AFBRs, substantial washout of biofilms occurred and reactor biomass was replaced completely after the operation of 50 days. It is concluded that a column-type reactor system with granular sludge may be the process of choice for continuous hydrogen fermentation.

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ACRONYMS

AA	Acetic acid
AFBR	Anaerobic fluidized bed reactor
ATP	Adenosine triphosphate
BA	Butyric acid
<i>C.</i>	<i>Clostridium</i>
CIGSB	Carrier-induced granular sludge bed
CLSM	Confocal laser scanning microscope
CoA	Coenzyme A (-Mercaptoethylamine + Pantothenic acid + ADP with 3'-phosphate group)
COD	Chemical oxygen demand (mg/L)
CSTR	Continuous stirred tank reactor
D	Dilution rate (h^{-1})
$d_{0.1}$	Volume (10%) particle diameter
$d_{0.2}$	Volume (20%) particle diameter
$d_{0.5}$	Volume (50%) particle diameter or mean particle diameter
$d_{0.9}$	Volume (90%) particle diameter
DGGE	Denaturing gradient gel electrophoresis
DLVO	Derjaguin, Landau, Verwey and Overbeek
DNA	Deoxyribonucleic acid
DS	Dry solids
<i>E.</i>	<i>Enterobacter; Escherichia</i>
ECp	Extracellular polymers
EGSB	Expanded granular sludge bed reactor
Eq.	Equation
EtOH	Ethanol
Fd^+	Ferredoxin (reduced form)
Fd^{2+}	Ferredoxin (oxidised form)
FITC	Fluorescein isothiocyanate
GAC	Granular activated carbon

h	Hour
HA	Hexanoic acid
HPR	Hydrogen production rate (L/L·h)
HRT	Hydraulic retention time (h)
k_d	Endogenous decay coefficient (/h)
L	Liter
MBR	Membrane bioreactor
mM	milli-molar
MSD	Mass selective detector
NADH	Nicotinamide adenine dinucleotide
NAD ⁺	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NCBI	National Centre for Biotechnology Information
NJ	Neighbor-jointing
OFMSW	Organic fraction of municipal solid wastes
OLR	Organic loading rate (g-glucose/L·h)
PA	Propionic acid
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
rDNA	Ribosomal deoxyribonucleic acid
S	Substrate concentration in reactor (g/L)
S_0	Influent substrate concentration in feed (g/L)
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscope
SHPR	Specific hydrogen production rate (mmol-H ₂ /g-VSS·h)
SS	Suspended solids
t	Time (h)
TCD	Thermal conductivity detector
UASB	Upflow anaerobic sludge blanket
V	Specific substrate utilization rate (g-glucose/g-VSS·h)
VA	Valeric acid
VFA	Volatile fatty acids

VS	Volatile solids
VSS	Volatile suspended solids
w/v	weight/volume (%)
WAP	Water absorbing polymer
X	Biomass concentration (g-VSS/L)
Y	True yield coefficient (g-VSS/g-glucose)
$Y_{X/S}$	Yield coefficient (-)
μ	Specific growth rate (/h)
μ_{max}	Maximum specific growth rate (/h)
Φ	diameter (mm)

CHAPTER 1 INTRODUCTION

1.1 Background

The whole economy, particularly in the developed countries, currently depends on fossil fuels; but at the same time, some associated problems with fossil fuels, this is, their sustainability, environmental pollution and global warming have emerged and even made a strong impact on our lives. These concerns have forced the developed countries in the world to search for alternative or new energy sources. Hydrogen is a promising alternative to conventional fossil fuels because it has the potential to eliminate all of the problems that the fossil fuels create, and hydrogen itself is lightweight and abundant. Hydrogen may release energy explosively in heat engines or generate electricity quietly in fuel cells while producing water as its only byproduct. Hydrogen is also the raw material for the synthesis of ammonia, alcohols, and aldehydes, and for the hydrogenation of various petroleum and edible oils, coal, and shale oil ([Fang et al. 2002b](#)).

However, a major doubt on hydrogen as a clean energy alternative is how it is produced. This is largely owing to the fact that most of the hydrogen at present is generated from fossil fuels by thermochemical processes, such as hydrocarbon reforming, coal gasification and partial oxidation of heavier hydrocarbons ([Das and Veziroglu 2001](#); [Levin et al. 2004](#)). Biological hydrogen production by microorganisms has attracted worldwide attention instead, due to its potential to become an inexhaustible, low-cost and renewable source of clean energy. Studies on biohydrogen production have been focused on biophotolysis of water using algae and *cyanobacteria*, photo-decomposition of organic compounds by photosynthetic bacteria and dark fermentation from organic compounds with anaerobes. Among these processes, anaerobic (dark) hydrogen fermentation seems to be more favorable, since hydrogen is yielded at a high rate and various organic waste and wastewater enriched with carbohydrates as the substrate results in low cost for producing hydrogen.

Although positive features of anaerobic hydrogen fermentation technology, such as high production rate, low energy demand, easy operation and sustainability, have been demonstrated in laboratory studies, this technology is yet to compete with those commercial hydrogen production processes from fossil fuels in terms of cost, efficiency and reliability. To enhance the volumetric production rate of hydrogen is thus a major challenge to anaerobic hydrogen fermentation process. Previous studies indicated that hydrogen production rate is highly related to the reactor biomass retention (Chang et al. 2002; Fang et al. 2002a; Wu et al. 2006; Yu and Mu 2006). Such being the case, immobilized-cell systems have become the process of choice for continuous hydrogen production since they are more capable of maintaining higher biomass concentration and being operated at high dilution rates (D) without biomass washout (Chang and Lin 2004; Wu et al. 2006). Biomass immobilization can be achieved through forming granules (Fang et al. 2002a), biofilms (Yokoi et al. 1997a) or gel-entrapped bioparticles (Wu et al. 2003), which have been employed with different reactor systems such as continuous stirred tank reactor (CSTR) (Fang et al. 2002a; Liu and Fang 2003), upflow anaerobic sludge blanket (UASB) reactor (Chang and Lin 2004; Mu and Yu 2006) and fixed-bed reactor (Chang et al. 2002; Rachman et al. 1998).

It is generally believed that spontaneous biofilm formation or granulation of hydrogen-producing bacteria can occur with reduced HRT in upflow column reactors or CSTRs (Fang et al. 2002a; Oh et al. 2004; Yu and Mu 2006). A long startup period of a few months, however, is needed for complete development of those bioparticles (Fang et al. 2002a; Yu and Mu 2006). Furthermore, the formation mechanisms of hydrogen-producing granules or biofilms remain rather limited. Understanding the cell immobilization process is highly relevant for improving reactor design and performance which indicate a technical and economic advantage.

1.2 Objectives

The primary aim of this research was to develop a high-efficient and high-rate biosystem for continuous hydrogen fermentation. For this purpose the following objectives were set for the study:

1. To develop a rapid initiation approach to the immobilization of hydrogen-producing sludge;
2. To study formation mechanisms of hydrogen-producing granules and biofilms;
3. To develop a high-rate hydrogen-producing biosystem.

1.3 Scope and Organization of the Thesis

The scope of this work includes development of a rapid initiation approach to cell immobilization of hydrogen-producing culture, immobilization mechanism investigation and performance evaluation on those developed cell-immobilized biosystems. All the tests were conducted in laboratory-scale bioreactors wherein acclimated digested sludge was inoculated and synthetic glucose wastewater was fed as the limiting substrate. The thesis structure is as follows:

Chapter 2 is a detailed literature review and discussion about anaerobic hydrogen production processes in terms of metabolic mechanisms, pathways and the influence of environmental conditions, and some important factors affecting microbial granulation. **Chapter 3** describes a hydraulic retention time (HRT)-related procedure used for the acclimation of seed sludge in a CSTR. The acclimated sludge would be used as the seed sludge for granulation and biofilm formation. An approach of acid incubation to rapidly initiate granulation of hydrogen-producing sludge developed in a CSTR is demonstrated in **Chapter 4**. In **Chapter 5**, applicability of the acid incubation approach developed in the CSTR for microbial granulation is validated in column-shaped reactors. The effectiveness of the approach on biofilm formation on support medium is also evaluated in this chapter. Biological and microbiological roles in the cell immobilization of hydrogen-producing culture were elucidated by examining the characteristics of

granules and biofilms in **Chapter 6**. **Chapter 7** evaluates the hydrogen production performance of different biohydrogen systems developed in this research and concludes the optimum biosystem favorable continuous hydrogen fermentation. Finally, some most important conclusions and suggested future work evolving along the lines developed in this present study are outlined in **Chapter 8**.

CHAPTER 2 LITERATURE REVIEW

2.1 Introduction

In order to obtain a clear understanding of the state-of-the-art of anaerobic hydrogen production globally, a literature review is carried out and presented in this chapter. This part reviews the literature in two areas that are related to the present research. It is, correspondingly, divided into two subsections. Section 2.2 reviews anaerobic hydrogen production technologies, including hydrogen-producing microorganisms, metabolic pathways, important operating conditions and biohydrogen systems. Section 2.3 presents microbial granulation technologies. Some important factors affecting granulation are also touched upon. Finally, a summary is drawn and described in Section 2.4.

2.2 Anaerobic Biohydrogen Production Process

Anaerobic fermentation process has been well known since production of methane, a useful energy carrier, is associated with the degradation of organic pollutants. The anaerobic fermentation process can be divided into two distinct stages: acidification and methane production. Each stage is carried out by specific microorganisms through syntrophic interactions. Hydrogen is produced in the first stage as an intermediate which in turn is used as an electron donor by many methanogens at the second stage of the process. It might be feasible to harvest hydrogen at the acidification stage of anaerobic fermentation, leaving the remaining acidification products for further methanogenic treatment. In fact, hydrogen is utilized by the consortia as it is produced, mainly by methanogenic archaea, acetogenic bacteria and sulphate-reducing bacteria. Therefore, it is possible that only acidogens are left to produce hydrogen gas, CO₂ and volatile fat acids (VFAs) if the final stage or methanogenesis and other hydrogen-consuming biochemical reactions are inhibited. Such a purpose is able to achieve through regulating biohydrogen cultures at a low pH, and/or short HRT conditions ([Kim et al. 2005](#); [Mizuno et al. 2000](#)) or through

inactivating hydrogen consumers by heat treatment (Lay 2001; Logan et al. 2002) and chemical inhibitors (Sparling et al. 1997; Wang et al. 2003b).

Various organic wastes and wastewaters enriched with carbohydrates as the substrate results in low cost for producing hydrogen. Nevertheless, fermentation efficiency of hydrogen production is highly related to microbial species compositions and culture conditions. Metabolic pathways involved in the hydrogen fermentation and those important factors affecting hydrogen production are reviewed in the following sections.

2.2.1 Hydrogen-Producing Bacteria

A wide variety of bacteria are capable of hydrogen production, which are distributed across 10 of the 35 bacterial groups (Fang et al. 2002b). Nandi and Sengupta (1998) reviewed microorganisms known to produce hydrogen prior to 1998. Such bacteria include strict anaerobes (*Clostridia*, *Methylotherophs*, methanogenic bacteria, Rumen bacteria, archaea), facultative anaerobes (*Escherichia coli* and *Enterobacter*), aerobes (*Alcaligenes* and *Bacillus*), photosynthetic bacteria, and *Cyanobacteria* (Nandi and Sengupta 1998). Research studies on anaerobically fermentative organisms have been intensively developed in recent years, and some new or efficient bacterial species and strains for dark hydrogen fermentation have been isolated and identified. Pertinent development is reviewed in this section. In general, the isolated and identified mesophiles are mainly affiliated with two genera: facultative *Enterobacteriaceae* (Kumar and Das 2000; Penfold et al. 2003; Tanisho and Ishiwata 1995; Yokoi et al. 1995) and strictly anaerobic *Clostridiaceae* (Collet et al. 2004; Evvyernie et al. 2001; Wang et al. 2003a), whereas most thermophiles belong to genus *Thermoanaerobacterium* (Ahn et al. 2005; Ueno et al. 2001; Zhang et al. 2003). In addition, aerobes such as *Bacillus* (Kalia et al. 1994; Kumar et al. 1995; Shin et al. 2004), *Aeromonas* species, *Pseudomonas* species and *Vibrio* species (Oh et al. 2003b) have been cultivated or isolated, but their hydrogen yields are generally less than 1.2 mol-H₂/mol-glucose under anaerobic conditions.

Since facultative anaerobes can be grown more easily than obligate ones, attempts have already been made to work with pure cultures of facultative anaerobes of genus *Enterobacteriaceae*. Tanisho and Ishiwata (1995) isolated and characterized as *E. aerogenes* strain E. 82005, and some other bacterial strains such as *E. aerogenes* strain HO 39 (1995), *E. aerogenes* HU-101 strain AY2 (Rachman et al. 1997) and *E. cloacae* IIT-BT 08 (Kumar and Das 2001; Kumar et al. 2000) were also harvested by different researchers. Hydrogen yields of *Escherichia* species as obtained in a pure culture of *E. coli* NCIMB 11943 are in a range of 0.2-1.8 mol-H₂/mol-hexose while fermenting glucose or starch hydrolysate (Perego et al. 1998), whereas the hydrogen yields demonstrated by the pure cultures of *Enterobacter* species are much higher, ranging from 1.1 mol-H₂/mol-hexose to ca. 3.0 mol-H₂/mol-hexose (Fabiano and Perego 2002; Kumar and Das 2000; Palazzi et al. 2000; Rachman et al. 1997). The hydrogen production capabilities of pure cultures of microorganisms are tabulated in Table 2-1.

Recently, Oh and his group (2003c; 2002) have successfully isolated two new microorganisms producing H₂ from carbon monoxide and water, *Rhodopseudomonas palustris* P4 and *Citrobacter* sp. Y19, both affiliated with genus *Enterobacteriaceae*. *Rps. palustris* P4, isolated from a sludge digester, is a facultative anaerobe and can grow in a photoautotrophic or chemoheterotrophic manner. *Citrobacter* sp. Y19 isolated from an anaerobic wastewater sludge digester, is also a facultative anaerobe. The authors pointed out that hydrogen production is observed only under anaerobic conditions for both strains. *Rps. palustris* P4 and *Citrobacter* sp. Y1 can produce hydrogen from the sugars under a wide range of pH (5-9) and temperature (25-40°C), giving the maximum hydrogen yields of 2.8 mmol-H₂/mmol-glucose (Oh et al. 2002) and 2.5 mol-H₂/mol-glucose (Oh et al. 2003c), respectively. A thermophilic hydrogen-producing bacterial strain *Klebsiella oxytoca* HP1 which belongs to genus *Enterobacteriaceae* was isolated from a 65°C hot spring (Minnan et al. 2005). Its hydrogen production activity increases with temperature increasing from 25°C to 35°C in batch culture on media with glucose, achieving the maximum hydrogen yield of 1.0 mol-H₂/mol-glucose.

Table 2-1. Microbial yield and production rate by pure cultures of dark fermentative microorganisms.

Genus classification	Substrate	Culture operation	Temperature (°C)	Hydrogen yield (mol-H ₂ /mol-hexose)	Hydrogen production rate (L/L·h)	References
Facultative anaerobes						
<i>E. coli</i> NCIMB 11943	Starch hydrolysate	Batch	37	1.8	0.33	(Perego et al. 1998)
<i>E. aerogenes</i> NCIMB 10102	Starch hydrolysate	Batch	40	1.1	0.24	(Perego et al. 1998)
<i>E. cloacae</i> IIT-BT08	Glucose	Batch	36	2.2	0.45	(Kumar and Das 2000)
<i>E. cloacae</i> IIT-BT08	Sucrose	Batch	36	3.0	0.66	(Kumar and Das 2000)
<i>E. cloacae</i> IIT-BT08	Cellobiose	Batch	36	2.7	0.65	(Kumar and Das 2000)
<i>E. cloacae</i> IIT-BT08	L-Arabinose	Batch	36	1.5	0.36	(Kumar and Das 2000)
<i>E. cloacae</i> IIT-BT08	Fructose	Batch	36	1.6	0.44	(Kumar and Das 2000)
<i>E. cloacae</i> IIT-BT08	Maltose	Batch	36	1.4	0.18	(Kumar and Das 2000)
<i>E. cloacae</i> IIT-BT08	Potato starch	Batch	36	-	0.20	(Kumar and Das 2000)
<i>E. cloacae</i> IIT-BT08	C.M. cellulose	Batch	36	-	0.09	(Kumar and Das 2000)
<i>E. aerogenes</i> HU-101 AY2	Glucose	Batch	37	1.2	-	(Rachman et al. 1997)
<i>E. aerogenes</i> DM11	Glucose	Batch	36	3.80	-	(Kumar et al. 2001)
<i>E. coli</i> BL-21	Glucose	Continuous	37	3.12	2.18	(Chittibabu et al. 2006)
<i>Klebsiella oxytoca</i> HP1	Sucrose	Continuous	38	1.30	0.35	(Minnan et al. 2005)
<i>Rhodospseudomonas palustris</i> P4	Glucose	Batch	37	2.76	-	(Oh et al. 2002)
<i>Citrobacter</i> sp. Y19	Glucose	Batch	36	2.49	-	(Oh et al. 2003c)
Strict anaerobes						
<i>C. butyricum</i> SC E1	Glucose	Continuous	30	2.3	-	(Kataoka et al. 1997)
<i>C. butyricum</i> IFO13949	Glucose	Continuous	36	2.3	1.15	(Yokoi et al. 1997a)
<i>C. thermolacticum</i> DSM 2910	Lactose	Continuous	58	1.5	0.06	(Collet et al. 2004)
<i>C. beijerinchi</i> AM21B	Glucose	Batch	36	2.0	0.66	(Taguchi et al. 1992)
<i>C. beijerinchi</i> AM21B	Starch	Batch	36	1.8	0.41	(Taguchi et al. 1992)
<i>C. paraputrificum</i> M-21	N-acetyl-v-glucosamine (GlcNAc)	Batch	45	2.4 ^a	-	(Evvyernie et al. 2001)
<i>C. paraputrificum</i> M-21	Ball-milled raw shrimp and lobster shells	Batch	45	1.5 ^a	0.21	(Evvyernie et al. 2001)
<i>C. paraputrificum</i> M-21		Batch	45	1.2 ^a	0.06	(Evvyernie et al. 2001)

Genus classification	Substrate	Culture operation	Temperature (°C)	Hydrogen yield (mol-H ₂ /mol-hexose)	Hydrogen production rate (L/L·h)	References
<i>C. paraputrificum</i> M-21	Acid/alkali treated raw shrimp and lobster shells	Batch	45	1.3 ^a	0.1	(Evvyernie et al. 2001)
<i>C. paraputrificum</i> M-21	Corn fiber	Batch	45	1.1	-	(Evvyernie et al. 2001)
<i>C. paraputrificum</i> M-21	Cellobiose	Batch	45	1.4	-	(Evvyernie et al. 2001)
<i>C. paraputrificum</i> M-21	Glucose	Batch	45	1.1	-	(Evvyernie et al. 2001)
<i>C. bifermentans</i>	Wastewater sludge	Batch	35	2.1 ^b	-	(Wang et al. 2003a)
<i>C. butyricum</i> CGS5	Sucrose	Batch	37	1.39	0.21	(Chen et al. 2005)
<i>C. acetobutylicum</i> ATCC 824	Glucose	Continuous	30	1.08	0.22	(Zhang et al. 2006)
<i>C. saccharoperbutylacetonicum</i> ATCC 27021	Lactose	Batch	30	1.41	0.12	(Ferchichi et al. 2005b)
<i>C. saccharoperbutylacetonicum</i> ATCC 27021	Sucrose	Batch	30	1.42	0.20	(Ferchichi et al. 2005b)
<i>C. saccharoperbutylacetonicum</i> ATCC 27021	Maltose	Batch	30	1.39	0.12	(Ferchichi et al. 2005b)
<i>C. saccharoperbutylacetonicum</i> ATCC 27021	Glucose	Batch	30	1.37	0.16	(Ferchichi et al. 2005b)
<i>C. saccharoperbutylacetonicum</i> ATCC 27021	Fructose	Batch	30	1.20	0.14	(Ferchichi et al. 2005b)
<i>C. saccharoperbutylacetonicum</i> ATCC 27021	Cheese whey	Batch	30	1.35	0.14	(Ferchichi et al. 2005a)
(Hyper) Thermophiles <i>Thermococcus kodakaraensis</i> KOD1	Starch	Continuous	85	3.33	0.21	(Kanai et al. 2005)
<i>Thermotoga maritima</i>	Glucose	Batch	80	4.00	-	(Schröder et al. 1994)
<i>Caldicellulosiruptor saccharolyticus</i>	paper sludge hydrolysate	Batch	70	3.84	0.12	(Kadar et al. 2004)
<i>Caldicellulosiruptor saccharolyticus</i>	Sucrose	Batch	70	3.33	0.20	(Van Niel et al. 2002)
<i>Thermotoga elfi</i>	Glucose	Batch	65	3.33	-	(Van Niel et al. 2002)
<i>Thermotoga neapolitana</i>	Glucose/soluble starch	Batch	70	-	0.01	(Van Ooteghem et al.

Genus classification	Substrate	Culture operation	Temperature (°C)	Hydrogen yield (mol-H ₂ /mol-hexose)	Hydrogen production rate (L/L·h)	References
Aerobes						
<i>Bacillus licheniformis</i> Jk1	wheat slurries	Continuous	38-40	0.20	-	2002; Van Ooteghem et al. 2004)
Co-cultures						
<i>C. butyricum</i> (strain IFO13949) and <i>E. aerogenes</i> (strain HO-39)	Starch	Continuous	36	2.6	1.3	(Kalia et al. 1994)
<i>C. butyricum</i> and <i>E. aerogenes</i>	Starch residue	Batch		1.7		(Yokoi et al. 2001)
<i>C. butyricum</i> and <i>E. aerogenes</i> HO-39	starch + corn steep	Continuous	37	2.7	-	(Yokoi et al. 2002)
<i>C. butyricum</i> IFO13949 and <i>E. aerogenes</i> HO-39	Starch residue	Batch	37	1.7	-	(Yokoi et al. 2001)

^amol/mol-GlcNAc; ^bmmol H₂/g-chemical oxygen demand (COD).

Among the fermentative anaerobes, *Clostridia* have been well known and studied extensively, not for their hydrogen production capability but for their role in the industrial solvent production from various carbohydrates. Some species such as *C. acetobutylicum* (Andersch et al. 1983; Holt et al. 1984; Kim et al. 1984) and *C. beijerinckii* (Chen and Blaschek 1999) were previously used as the industrial solvent producers. In general, in a batch growth of *Clostridia* predominantly acetate-butyrate fermentation with hydrogen being produced occurs at neutral pH, whilst solvents are formed in substantial quantities when the pH of the medium falls to a certain extent. Recently, use of *Clostridia* for hydrogen production attracts much attention. The hydrogen yields of the pure cultures belonging to genus *Clostridiaceae* have been examined, including *C. paraputrificum* (Evvyernie et al. 2001), *C. lentocellum* (Ravinder et al. 2000), *C. thermosuccinogenes* (Sridhar and Eiteman 2001), *C. bifermentans* (Wang et al. 2003a), *C. thermolacticum* (Collet et al. 2004), *C. butyricum* (Chen et al. 2005), *C. saccharoperbutylacetonicum* (Ferchichi et al. 2005b), *C. acetobutylicum* (Zhang et al. 2006) and *C. pasteurianum* (Dabrock et al. 1992). The optimum hydrogen yields observed of each species vary between 1.1 mol-H₂/mol-hexose and 2.6 mol-H₂/mol-hexose, dependent on the organism per se as well as environmental conditions (Table 2-1).

Use of undefined mixed cultures in non-sterile conditions would be more attractive for anaerobic hydrogen production, since the pure culture is easily contaminated by other bacteria including hydrogen consumers such as methane producing bacteria and sulfate-reducing bacteria (Adams et al. 1980; Ueno et al. 1995). There is a risk of hydrogen consumers coexisting in the mixed hydrogen-producing cultures. Several types of anaerobic bacteria are able to use hydrogen as a source of energy by coupling its oxidation to the reduction of a variety of electron acceptors. This group also includes facultative anaerobes such as *E. coli* (subsection IIB), which is capable of hydrogen-dependent fumarate reduction, and *Paracoccus denitrificans*, which, although classified as an aerobic hydrogen bacterium (subsection IID), uses either O₂ or nitrate as a terminal electron acceptor during respiratory hydrogen oxidation. All these reactions are characterized by the uptake of hydrogen via a hydrogenase (Adams et al. 1980).

The inhibition of hydrogen consumers present in the mixed cultures is essential for net hydrogen production and for further scale-up and industrial application. *Clostridium* may form endospores, which can be considered “survival mechanisms” developed by these organisms when unfavorable environmental conditions are encountered (e.g., high temperature, desiccation, lack of carbon or nitrogen source, and chemical toxicity) (Sung et al. 2002). Because of this characteristics of *Clostridium species*, heat pretreatment is a common and feasible method to select clostridial spore-formers from natural environments such as activated sludge, anaerobic digested producers, soil and animal bodies (Iyer et al. 2004; Monmoto et al. 2004; Ueno et al. 1995; Wu et al. 2002). Generally, the selected inoculum is heated at 70-80 °C for 1~2 h or baked and boiling at 105 °C for few of minutes (Lay 2000; Van Ginkel et al. 2001) to inhibit or kill hydrogen-consuming microbes, which has shown the potential to enable successful start-up of continuous laboratory-scale and higher hydrogen production of batch mode. It has been found that a 30 min period of heat treatment at 50°C is adequate to inactivate the growth of lactic acid bacteria (*Lactobacillus paracasei*) (Noike et al. 2002). In addition, incubating mixed cultures at acidic or alkaline condition could also inhibit hydrogen consumers and enhance overall hydrogen production capability of the mixed cultures (Lee et al. 2004; Lin et al. 2006).

Thermoanaerobacterium species are found to be most popular thermophiles in thermophilic hydrogen fermentation (Ahn et al. 2005; Zhang et al. 2003). Ueno et al. (2001) demonstrated that most of the isolates belonged to the cluster of the thermophilic *Clostridium/Bacillus* subphylum of low G+C gram-positive bacteria, but the most dominant bands showed a high sequence similarity to *T. thermosaccharolyticum* in a thermophilic culture fed with cellulose. A similar microbial composition was reported by Ahn et al. (2005), when fermenting synthetic glucose wastewater with a thermophilic culture in the trickling biofilter reactors. Zhang et al. (2003) summarized several species of *Thermoanaerobacterium* known for their hydrogen producing characteristics, including *T. thermosaccharolyticum*, *T. polysaccharolyticum*, *T. zae*, *T.*

lactoethylicum and *T. aotearoense*. Species of *Thermoanaerobacterium* have been isolated from thermal volcanic spring, hot spring, and a high temperature acidic leachate of a waste pile from a canning factory, and have the optimal growth conditions of 55-70°C and pH 5.2-7.8 (Zhang et al. 2003).

Besides *T. thermosaccharolyticum*, in a thermophilic culture fermenting food wastes, it was found that *Desulfotomaculum geothermicum* was present as one of main hydrogen-producing bacterial strains (Shin et al. 2004). Some thermophilic species of *Clostridia* are also detected in thermophilic hydrogen-producing cultures. Thermophilic cellulolytic bacteria, *C. thermocellum* and *C. cellulosi* are detected by Ueno et al. (2001) with cellulose powder as hydrogen-producing substrate. Working with a culture of *C. thermolaiticum* fed with lactose, Collet et al. (2004) obtained a hydrogen yield of 1.5 mol-H₂/mol-hexose.

Hydrogen yields by mixed thermophilic cultures are reported in a range of 0.7-3.2 mol-H₂/mol-hexose (Liu et al. 2003a; Monmoto et al. 2004; Shin et al. 2004; Ueno et al. 1995; Ueno et al. 1996; Valdez-Vazquez et al. 2005). The highest yield was obtained from organic solid wastes (60% fruit/vegetable waste and 40% office paper waste, based on dried weight) by a mixed culture at 55°C (Valdez-Vazquez et al. 2005), whereas the lowest one was achieved on fermenting starch-containing wastewater at 55°C (Zhang et al. 2003). Recently, several reports on microbial hydrogen production using hyperthermophiles have been reported. For example, van Niel et al. (2002) obtained a hydrogen yield of 3.33 mol-H₂/mol-hexose by using a pure culture of extremophiles either *Caldicellulosiruptor saccharolyticus* on sucrose (70°C) or *Thermotoga elfi* on glucose (65°C), and the same yield was reported by Kanai et al. (2005) from starch fermentation with hyper-thermophilic archaeon, *Thermococcus kodakaraensis* KOD1 at 85°C. Moreover, it is stressed that a yield of 4 mol-H₂/mol-hexose, the maximal theoretical value, was reported by Schröder et al. (1994) with a batch eubacterial culture of *Thermotoga maritima* on glucose at 80°C. However, the authors pointed out that both microbial growth of the culture and glucose utilization are low. In addition, comparing with mesophilic cultures, hyperthermophiles exhibit much lower production rates of hydrogen, generally

ranging from 0.01 to 0.2 L/L·h (Table 2-1), which is largely attributed to the slow-growing characteristics of hyperthermophiles.

For a purpose of comparison and summarization, operating temperature of hydrogen producing cultures can be divided into four regimes: ambient (15-30°C), mesophilic (30-39°C) and thermophilic (50-64°C) and hyper-thermophilic (>65°C). As shown in (Table 2-1), hydrogen yields are comparable at mesophilic and thermophilic temperatures, but lower at the ambient temperature. However, hyper-thermophilic cultures seem to possess a superior ability to generate more hydrogen (Kadar et al. 2004; Kanai et al. 2005; Schröder et al. 1994; Van Niel et al. 2002), and the highest hydrogen yields reported in the world are close to the theoretical maximum of 4.0 mol-H₂/mol-glucose which was achieved by using extreme thermophiles (Schröder et al. 1994).

2.2.2 Hydrogen Production Pathways

Anaerobic hydrogen production is a ubiquitous phenomenon under anoxic or anaerobic conditions. Many bacteria use the reduction of protons to hydrogen via hydrogenases as a means of oxidizing the carriers reduced during fermentation, which is required to allow the carriers to recycle and maintain electrical neutrality so that a continuous supply of adenosine triphosphate (ATP) can be generated by substrate-level phosphorylation (Adams et al. 1980). Molecular hydrogen formation is generally followed two routes in the presence of specific coenzymes, i.e. either by formic acid decomposition pathway or by the re-oxidization of nicotinamide adenine dinucleotide (NADH) pathway [Eqs. (2.1) and (2.2)].

The Embden-Meyerhof or glycolytic pathway is undoubtedly the most common route for glucose degradation to pyruvate, which is found in all major of microorganisms and functions in the presence or absence of oxygen. In this pathway, glucose is converted into pyruvate associated with the conversion of

NADH from NAD^+ via anaerobic glycolysis, which could be represented by Eq.(2.3):

Hydrogen is generated through the re-oxidization of NADH by some specific microorganisms under acidogenic conditions in the presence of ferredoxin oxidoreductase and hydrogenase (Dabrock et al. 1992; Tanisho et al. 1998). This metabolic route is present in some *species* of *Clostridium* as shown in Figure 2-1. The obligate anaerobic *Clostridia* lack a typical cytochrome system and obtain energy by substrate-level phosphorylation during fermentation. Oxidizing carbohydrates generates electrons which need to be disposed of to maintain electrical neutrality. For the saccharolytic *Clostridia*, hydrogen evolution via a hydrogenase is a major route through which the cells dispose excess electrons produced from the oxidative breakdown of carbohydrates (Chen and Mortenson 1974). NADH-ferredoxin reductase functions primarily as an electron carrier and is involved in pyruvate oxidation to acetyl-CoA and carbon dioxides as well as proton reduction to molecular hydrogen (Kim et al. 1984; Schonheit et al. 1979). *Clostridia* break down pyruvate to acetyl-CoA to produce 2 moles of NADH and 2 moles of reduced ferredoxin. However, NADH produced during the conversion of glucose to acetyl-CoA can be used for the formation of butyrate, and even alcohols such as ethanol and butanol. The available hydrogen from glucose fermentation is determined by the ratio of butyrate/acetate produced during fermentation.

Figure 2-1. Some common microbial pathways involved in anaerobic hydrogen fermentation (Prescott et al. 2002).

Four moles of hydrogen per mole of glucose is achieved, which is the theoretical maximum yield of hydrogen fermentation if all of the substrate would be converted to acetic acid. If all the substrate would be converted to butyric acid, this value is two moles of hydrogen per mole of glucose, since 2 moles of NADH has been consumed during the conversion of intermediate products. In the cases mentioned above, no NADH is used as a reductant for alcohol production. However, the disposal of electrons via pyruvate-ferredoxin oxidoreductase or NADH-ferredoxin oxidoreductase and hydrogenase might be affected by the corresponding NADH and acetyl-CoA levels as well as environmental conditions. As a result, the

oxidation-reduction state has to be balanced through the NADH consumption to form some reduced compounds, i.e. lactate, ethanol and butanol, resulting in a lowered hydrogen yield.

In the anaerobic *Clostridia* formate is not a major intermediate in the breakdown of pyruvate, although such organisms possess formate dehydrogenase (Adams et al. 1980). In contrast, the facultative anaerobes, especially members of the family *Enterobacteriaceae*, can metabolize pyruvate to formic acid and other products in a process sometimes called the formic acid fermentation. As with the *Clostridia* these organisms have to dispose of excess reductant produced during fermentation, and this is accomplished by the eventual production of hydrogen. Pyruvate is first converted into formic acid by pyruvate-formate lyase with the production of acetyl-CoA, and energy is conserved by the formation of ATP via acetyl phosphate. Under anaerobic condition and in the absence of suitable electron acceptors, the formic acid will be further degraded into hydrogen and carbon dioxides via formic hydrogenlyase [Eqs. (2.4) and (2.5)].

It is noted that NADH produced during anaerobic glycolysis is rarely used for hydrogen production by the bacteria of genus *Enterobacteriaceae*, due to the absence of specific coenzymes such as ferredoxin oxidoreductase. Nevertheless, NADH must still be oxidized back to NAD^+ , otherwise anaerobic glycolysis will cease. Many microorganisms solve this problem by slowing or stopping pyruvate dehydrogenase activity and using pyruvate or one of its derivatives as an electron and hydrogen acceptor for the re-oxidation of NADH in a fermentation process. As a result, the oxidation-reduction state has to be balanced through the NADH consumption to form a large amount of mixed acids and alcohols, most of which are the hydrogen-containing reduced products, are accompanied with the formation of formate. There are two types of formic acid fermentation. Mixed-acid fermentation results in the excretion of ethanol and a complex mixture of acids, particularly acetic, lactic, succinic and formic acids. This pattern is seen in *Escherichia*, *Salmonella*, *Proteus*, and other genera. The second type, butanediol fermentation, is

characteristics of *Enterobacter*, *Serratia*, *Erwinia*, and some species of *Bacillus*. Pyruvate is converted to acetoin, which is then reduced to 2, 3-butanediol with NADH. At the same time, ethanol is also produced, together with smaller amount of the acids found in mixed acid fermentation. Production of these reduced compounds limits hydrogen release in a gas form and results in a lowered hydrogen yield which is generally less than 2 mol-H₂/mol-glucose by facultative anaerobes compared with anaerobic *Clostridia*.

Theoretical H₂ yield of 4 mol-H₂/mol-glucose should be achieved with acetate as fermentation end product. In practice, high H₂ yields are usually associated with butyrate production, while low yields associated with the production of propionate and reduced end products (e.g. alcohols, lactic acid). As demonstrated in [Figure 2-1](#), there are a wide spectrum of metabolic pathways involved in the anaerobic carbohydrate fermentation, which are closely dependent on microorganisms involved and environmental conditions. In order to yield as much hydrogen as possible, a niche favorable hydrogen evolution has to be created by regulating microbial metabolism away from formation of alcohols and reduced acids (e.g., lactate) and towards production of acetate and butyrate. Those factors affecting hydrogen production, such as operating conditions, feedstock and reactor configuration will be systematically reviewed and extensively discussed in the following sections.

2.2.3 Factors Affecting Hydrogen Production

In this section, the operating conditions that affect the performance of anaerobic hydrogen fermentation are investigated. They include culture pH, HRT, hydrogen partial pressure, inorganic nutrients, temperature, substrate type, and substrate and product concentrations.

2.2.3.1 Culture pH

Medium pH has been recognized to be one of the most important environmental factors affecting metabolic pathways involved in the anaerobic hydrogen

fermentation and thus the yield of hydrogen. The major products in hydrogen production by anaerobic fermentation of carbohydrates are acetate, butyrate, propionate, and ethanol, which are strongly dependent on culture pH. Hwang et al. (2004) reported that the main products are butyrate at pH 4.0-4.5, but ethanol at pH 4.5-6.0, and propionate at pH 5.0-6.0. Innac et al. (1996) and Yu et al. (2002; 2003b) also pointed out that pH 5.0-6.0 is favourable for the production of propionate, and a pH of around 5.0 or below 5.0 is recommended for the prevention of propionate accumulation. However, Yu et al. (2002; 2003b) reported that both acetate and butyrate increase with pH from 4.0 to 6.5, and production of solvents is dominated at a pH lower than 5.0. In addition, it has been reported that *C. tyrobutyricum* is able to switch from butyrate formation towards lactate production and high lactate accumulation occurs at pH < 6.0 (Zhu and Yang 2004). This finding is similar to those found in *C. acetobutylicum* (Bahl et al. 1986) and *C. pasteurianum* (Dabrock et al. 1992) fermentation in the low-iron media. These results indicate that hydrogen fermentation undergoes complex and pH-dependent multi-branched pathways. An optimum pH for hydrogen production must be a point or range of maximizing the formation of hydrogen-associated products such as acetate and butyrate, and minimizing hydrogen-consuming and non-hydrogen-associated reactions such as the production of propionate, lactate and other reduced alcohols.

A variety of studies have been conducted to investigate the effect of pH on hydrogen production in batch or continuous experiments. Comparative studies with respect to the effect of pH on continuous hydrogen production revealed that the optimum pH range to achieve the maximum hydrogen yield or specific hydrogen production rate was found between 5.2 and 6.0 in the most of studies using pure or mixed cultures of bacteria (Fang and Liu 2002; Hussy et al. 2003; Kumar and Das 2000; Lin and Chang 1999; Oh et al. 2004; Yu et al. 2002). In those studies using batch cultures, medium pH is mostly uncontrolled and thus it varies dynamically due to the production of organic acids. A much wider initial pH range, from 3.0 to 12.0 could be applied to the batch cultures compared to the continuous ones. The pH optima reported are so conflicting that they varied from 4.5 to 9.0, dependently

on substrate type and concentration, medium buffering capability, inoculated organisms per se and other operating conditions (Cai et al. 2004; Ferchichi et al. 2005a; Lay et al. 1999; Zhang et al. 2003). The optimum operating pH for the batch hydrogen production has been suggested to be 5.5-5.7 by some researchers using experimental or calculating models (Lay 2000; Van Ginkel et al. 2001; Zheng and Yu 2004b).

2.2.3.2 Hydraulic Retention Time

In general, it is known that classic methanogenic activity might be inhibited by a weakly acidic pH of 4.5~6.5 (Sparling et al. 1997). Fang and Liu (2002) reported that the biogas produced is free of methane at a pH of 5.5 or lower as the continuous hydrogen-producing culture is operated at a HRT of 6 h. A lower pH condition however, could not always inhibit the activities of methanogens present in the mixed hydrogen-producing cultures. Hwang et al. (2004) noted that hydrogenotrophic methanogens is still active at pHs of 4.0-6.0 and HRT of 3 days in semi-continuous reactor. Such methanogenesis at the same HRT condition was able to be inhibited completely only at a pH below 4.0. Kim et al. (2004a) also reported the hydrogenotrophic methanogenic activity at a pH of 4.5 and a HRT of 9 days. These finding indicate that the hydrogen was converted to methane at longer HRT conditions via Eq.(2.6):

The HRT could be used as a tool to select microbial populations whose growth rates are able to catch up with the mechanical dilution created by continuous volumetric flow (Tijhuis et al. 1994). The typical specific growth rate (μ) of methane-producing bacteria is about 0.0167-0.02 h⁻¹, much lower than that of hydrogen-producing bacteria of about 0.172 h⁻¹ (Chen et al. 2001). Therefore, hydrogen-producing bacteria are likely retained, whereas methane-producing bacteria are washed out in continuous hydrogen production process by HRT regulation. Chen et al. (2001) found that the methane concentration was considerable (0.0011–0.0058 mol/L) at low dilution rates ($D=0.002$ – 0.0167 1/h), but was hardly measured for $D>0.075$ 1/h, indicating negligible methanogenic activity at high dilution rates. This

means that HRT is capable of inhibiting or terminating methanogenesis in hydrogen production by anaerobic fermentation.

Although higher dilution rates seem to lead to larger hydrogen production rates, the D value should not be allowed to exceed the critical value ($\mu_{\max}$), to avoid reactor failure due to washout of suspended H_2 -producing bacteria. It has been reported that chemostat cultures may be subject to system upset when the HRT is shortened to less than 6 h. In the suspended-culture hydrogen production systems, a HRT of 6-8 h is recommended for the simple carbohydrates such as synthetic glucose and sucrose wastewater fermentation, whereas a relative longer HRT may be required by the fermentation of complicated organic materials. Lin and Chang (1999) obtained the highest yield of hydrogen production from glucose at a HRT of 6 h, while Hussy et al. (2003) and Ueno et al. (1996) found that the optimum HRTs for starch fermentation and sugar wastewaters are around 13.7 h and 12 h, respectively.

A much shorter HRT can be used in the cell-immobilized systems. In general, the HRTs used in the cell-immobilized processes range from 0.25 h to 24 h, and optimum HRTs favorable hydrogen production are found to be between 2 h and 0.25 h for the granule-based CSTR and granule/biofilm-based upflow column-shaped reactors. A HRT of 0.5 h is proven to be most optimal for hydrogen production from glucose or sucrose synthetic wastewaters in the granular sludge bed reactor (Lee et al. 2004) and granular sludge-based CSTR (Wu et al. 2006), whilst a HRT of 2 h is in favor of the sucrose fermentation in an anaerobic fluidized bed reactor (AFBR) (Wu et al. 2003).

A shorter HRT operation could avoid the accumulation of organic acids produced during hydrogen fermentation in the reactors. Han and Shin (2004) found that substantial VFAs were accumulated during the hydrogen production from food wastes at a low D of 2.1 d^{-1} which resulted in a lowered hydrogen production. In contrast, a D of 4.5 d^{-1} was found favorable for washout of excess VFAs and thus a higher hydrogen yield is associated.

2.2.3.3 Hydrogen Partial Pressure

The dissolved hydrogen concentration in the liquid phase, calculated from the hydrogen partial pressure, is one of the key factors affecting microbial pathways involved in fermentative hydrogen production. As stated previously, hydrogen production is regulated by the relative concentrations of NADH and NAD⁺ in which bacteria re-oxidise the reduced ferredoxin and hydrogen-carrying coenzymes. By assuming a constant internal cell pH, the ratio of the two concentrations could be related to hydrogen partial pressure and hydrogen production is less favourable as the hydrogen partial pressure rises (Tiwari et al. 2006).

Increase of the partial pressure of hydrogen in the headspace during the fermentation leads to a decrease in hydrogen production. A higher proportion of hydrogen gas in the biogas might push the production of propionate (Hussy et al. 2003; Vavilin et al. 1995). As shown in Eq. (2.7), Vavilin et al. (1995) gave the overall equation for the production of propionate from glucose, thus the production of propionate should be avoided, due to its consumption of hydrogen. Simultaneously, the authors proposed that the limiting substrate for butyrate production is glucose, while the limiting substrate for propionate production is H₂, and the two groups of organisms producing these end products are in balance in the microbial consortium producing H₂ (Vavilin et al. 1995). Such a conception is strongly supported experimentally by Kleerebezem and Stems (2000) who stated that the accumulation of propionate is affected only by the hydrogen partial pressure but butyrate is accumulated due to two factors, the hydrogen partial pressure and the pH. However, the formation of propionic acid is still under argument. McCarty and Mosey (1991) hypothesized a model for anaerobic acidogenesis that propionate-producing bacteria are unaffected by hydrogen partial pressure and grow fast at neutral pH and high substrate. Inanc and his team (1996) found that the production of propionic acid is affected by low pH and is independent of the hydrogen partial pressure, thus a pH of around 5 is recommended for the prevention of propionate accumulation.

The hydrogen-producing cultures could switch to alcohol production and hence produce much less hydrogen when hydrogen partial pressure increases to a certain level in the reactor headspace (Doremus and Linden 1985; Kim et al. 2004a). Kleerebezem and Stams (2000) found that hydrogenase may be suppressed in the presence of high levels of hydrogen which results in that the H^+/H_2 redox potential is lowered and the flow of electrons from reduced ferredoxin to molecular hydrogen is inhibited. Khanal et al. (2004) further demonstrated that the electron flow would be shifted to the generation of NADPH via the action of the appropriate ferredoxin oxidoreductase, resulting in an increase in the production of butanol and ethanol or higher molecular weight acids such as butyric and propionic acids. On the contrary, the decrease in hydrogen concentration will favour hydrogen production and permit bacteria to metabolise acetyl-CoA through the energy-efficient path leading to acetate and ATP production.

In addition, Oh et al. (2003a) found that a high hydrogen pressure is thermodynamically favorable for the production of ethanol from acetic acid via Eq. (2.8) and hydrogen at a pH of 6.2 in the batch tests. This is a reversible reaction, and at a hydrogen partial pressure of 4 Pa, acetic acid production from ethanol becomes thermodynamically possible.

Thus, it is important to remove excess hydrogen from the system to maintain hydrogen production. Many strategies of removal or separating excess hydrogen gas have been developed to avoid the negative effect of the hydrogen accumulation in the gas phase. Mizuno et al. (2000) found an increase in the hydrogen yield from 0.85 to 1.43 mol- H_2 /mol-hexose when the reactor is sparged with nitrogen at 15 times hydrogen produced rate. Hussy et al. (2003) also reported a 1.5 times enhanced hydrogen yield from 1.3 to 1.9 mol- H_2 /mol-hexose by sparging the reactor with nitrogen to reduce hydrogen in the off-gas from 50% to 7%.

Liang et al. (2002) efficiently reduced hydrogen partial pressure by a hollow fiber membrane installed in hydrogen-producing bioreactor and demonstrated a 15%

increase in hydrogen yield and hydrogen production rate. Hydrogen partial pressure is also related to stirring regime. Lay (2000) stated that an increase in agitation speed of a continuous reactor mixed by a magnetic stirrer from 100 to 700 rev/min more than doubled the daily rate of hydrogen production from starch.

2.2.3.4 Nutrients

In microbial hydrogen fermentation processes, nitrogen, phosphate and other inorganic trace minerals are necessary supplements for carbohydrate-based feedstocks in order to obtain optimal cell cultivation and hydrogen production.

Previous study indicated that organic nitrogen seems to be more favourable for hydrogen evolution compared with inorganic one (Ueno et al. 2001; Yokoi et al. 2001). Yokoi et al. (2001) obtained the highest level of H₂ production from sweet potato starch residue by a repeated batch culture containing *C. butyricum* IFO13949 when 1.0% polypepton was added as nitrogen source. In contrast, addition of urea, (NH₄)₂SO₄ or NH₄Cl resulted in the absence of hydrogen evolution by the same culture. In addition, Cheng et al. (2002) pointed out that peptone is not only a nitrogen source but also a pH buffer since products derived from peptone can neutralize the organic acids produced in fermentative hydrogen production. However, the expensive cost of peptone might limit its application in hydrogen production. Inorganic ammonium is the most popular alternative. Lin and Lay (2004b) investigated the effect of carbon/nitrogen (C/N) ratio ranging from 40 to 130 on biological hydrogen production with sucrose and NH₄HCO₃ as the respective carbon and nitrogen sources in batch cultures. Experimental results indicated that the hydrogen production ability of the anaerobes (dominated by *C. pasteurianum*) is closely related to the influent C/N ratio. A C/N ratio of 47 is recommended, at which the hydrogen yield and hydrogen production rate reach the respective maximum values of 4.8 mol-H₂/mol-sucrose and 270 mmol H₂/L·day. In the same study, the authors further pointed out that nitrogen limitation might cause the shift of metabolic pathways towards alcohol production (Lin and Lay 2004b), which is in agreement with a previous study (Kanchanatawee and Maddox 1990). However, excess supplement of ammonia would result in a decrease in hydrogen

yield. Working with a continuous mixed microbial culture at a pH of 5.5 and a HRT of 10 h, Salerno et al. (2006) found that hydrogen yield decreased progressively from 1.9 to 1.1 mol-H₂/mol-hexose when culture ammonia concentration from 0.8 to 7.8 g-N/L.

Phosphate is one of the important inorganic nutrients required for optimal hydrogen production (Lin and Lay 2004a). Excess phosphate may favor VFAs and hydrogen production over solvent production, so phosphate supplementation may be needed with carbohydrate-rich feeds. Phosphate is proved to be a suitable and reliable growth-limiting factor to ensure a high substrate turnover with a high yield of solvents for some species of *Clostridia* (Bahl et al. 1986; Girbal et al. 1995; Kanchanatawee and Maddox 1990). Hawkes et al. (2002) compared a varying concentration of phosphate used in different laboratory-scale studies and recommended an optimum carbon/phosphate ratio of 130:1 for anaerobic hydrogen fermentation.

Iron is an important component of the enzymes participating in fermentative hydrogen production from carbohydrates. Ferredoxin from *C. pasteurianum* is an iron-sulfur protein with a molecular weight of 6000 containing 8 non-heme iron and 8 acid-labile sulfur atoms. This protein functions primarily as an electron carrier and is involved in pyruvate oxidation to acetyl-CoA and carbon dioxides as well as proton reduction to molecular hydrogen (Schonheit et al. 1979). Besides ferredoxin *C. pasteurianum* is known to contain the following iron proteins related to hydrogen production: pyruvate synthase (6 Fe per mol) (Uyeda and Rabinowicz 1971) and hydrogenase (12 Fe per mol) (Chen and Mortenson 1974). Except for ferredoxin none of these proteins can be replaced by non-iron proteins. *C. pasteurianum* can thus grow normally only if enough iron is available for the synthesis of these iron proteins (Schonheit et al. 1979).

There have been some studies on how the external iron concentration affects fermentation by anaerobic non-photosynthetic bacteria, and it has been reported that the in vivo activity of the hydrogenase decreases with iron depletion (Junelles et al.,

1988; Dabrock et al., 1992; Peguin and Soucaille, 1995). For example, with a batch culture fed with glucose, iron limitation (0.2 mg/L) affects carbon and electron flow in addition to hydrogenase of *C. acetobutylicum*, leading to a 40% decrease in hydrogenase specific activity (Junelles et al. 1988). Either lactic acid or butanol is found to be the major fermentation product, depending on the pH, in the iron-limited chemostat with *C. acetobutylicum* (Bahl et al. 1986). In addition to influencing the electron flow, the authors postulated that iron limitation creates a bottleneck for carbon flow, presumably at the pyruvate-ferredoxin oxidoreductase step. At neutral, electrons are thus more readily disposed of via reduction of pyruvate to lactate than via synthesis of the electron acceptors needed for acetate and butyrate formation (Bahl et al. 1986). Iron limitation might cause pyruvate conversion to lactate production, rather acetyl-CoA conversion, due to the lowered iron-contained enzymes. Dabrock et al. (1992) reported that iron concentrations of less than 0.56 mg/L are growth limiting for *C. pasteurianum* and cause significant lactate production in batch experiments on glucose, although iron limitation did not cause a decrease of H₂ production in continuous culture. Lee et al. (2001) obtained the maximum hydrogen yield and maximum hydrogen production rate as the concentrations of FeCl₂ are 800 mg/L and 4000 mg/L in batch experiments, respectively. Reducing FeCl₂ concentration results in a production of ethanol and butanol.

In addition to iron, some other inorganic elements such as magnesium and zinc are also essential for the synthesis of various anaerobic microorganisms, many of which are believed to be related to the bacterial enzyme cofactors, transport processes and dehydrogenase (Lin and Lay 2004c). Lin and Lay (2004c) examined the effect of trace elements including Mg, Na, Zn, Fe, K, I, Co, NH₄⁺-N, Mn, Ni, Cu, Mo and Ca on hydrogen production by a *C. pasteurianum*-predominant culture. The authors found elements Mg, Na, Zn and Fe being important supplements and proposed an optimum nutrient formulation containing 120 mg/L MgCl₂·6H₂O, 1000 mg/L NaCl, 0.5 ZnCl₂ and 3 mg/L FeSO₄·7H₂O in 1 L culture for the optimal hydrogen yield. However, less effect of NH₄⁺-N and a lower value of optimum iron concentration

indicated here are argued with those values recommended in other studies (Lee et al. 2001; Lin and Lay 2004b).

2.2.3.5 Temperature

Microbes are capable of producing hydrogen in a temperature range of 15°C to 85°C (Kanai et al. 2005; Lin and Chang 2004), but most of laboratory-scale studies were conducted under mesophilic conditions as stated by Li and Fang (2007) in a recent review that about 73% studies were carried out with mesophilic cultures. Lin and Chang (2004) studied the hydrogen production capability of a mixed culture under varying temperatures from 15 to 34°C and found that hydrogen yield and specific hydrogen production rate increased with temperature, achieving respective maximum values of 359 mmol/L·day and 1.42 mol-H₂/mol-glucose at 30-34°C and 28-32°C, respectively. However, both values obtained at the ambient temperatures are much lower compared with the corresponding values of 574 mmol/L·day and 1.70 mol-H₂/mol-glucose at a consistent temperature of 35 ± 1°C. Minnan et al. (2005) and Oh et al. (2003c) also reported that increasing temperatures from 25°C to 35-36°C improved the hydrogen production activity.

Zhang and Shen (2006) pointed out that increasing the culture temperature favoured the production of hydrogen by a mixed culture when it was in a range of 25-40°C, but the temperature went on increasing to 45°C, the hydrogen production was almost inhibited. On the contrary, Yu et al. (2002) found that specific hydrogen production rate increased with temperature from 20°C to 55°C, and optimum hydrogen production rate of 9.33 L-H₂/g-VSS·day and hydrogen yield of 1.9 mol-H₂/mol-glucose were achieved at an a temperature of 55°C. Valdez-Vazquez (2005) reported that the hydrogen yield obtained from a mixture of organic solid wastes by thermophilic culture at 55°C is noticeably superior to the corresponding yield of the mesophilic culture at 37°C (3.2 VS 1.5 mol-H₂/mol-hexose). These experimental results, together with the results obtained by mesophilic cultures in other studies (Fabiano and Perego 2002; Iyer et al. 2004; Lee et al. 2006; Wu et al. 2005) seem to indicate that, although hydrogen-producing bacteria are able to perform at ambient temperature conditions, increasing temperature in the mesophilic regime always

improves the hydrogen production, while further increasing culture temperature beyond mesophilic range may cause a decrease in hydrogen production, presumably dependent on physiological characteristics of microbial cultures.

In spite of numerous studies on thermophilic hydrogen fermentation, few studies were conducted to optimize the temperature conditions for the thermophiles. There are only two such studies available in literature. In a thermophilic culture fed with glucose, Oh et al. (2004) studied the effect of temperature (55-64°C) on hydrogen production and found that a temperature of 60°C was most favourable for hydrogen production by anaerobic sludge. Morimoto et al. (2004) reported that changing temperature in a thermophilic regime gave rise to different effects on hydrogen production capability of different microflora. Hydrogen yields were achieved the peaks at 50°C by the sludge compost, while at 60°C by the palm oil mill effluent sludge and CREST compost.

Temperature has an important influence on microbial activity. In a pure culture of *Citrobacter* sp. Y19, Jung et al. (2002) found that a temperature of 30-40°C was favourable for cell growth and specific hydrogen production activity increased up to 50°C and then decreased rapidly as culture temperature further increased. Yokoi et al. (1995) reported that hydrogen yield and hydrogen production rate of a pure culture of *E. aerogenes* Strain HO-39 increased with an increase in temperature from 30 to 38°C, but decreased sharply at temperatures higher than 40°C and hydrogen was hardly produced at 43°C.

Changing temperature may cause a shift of microbial population of the mixed cultures. Iyer et al. (2004) found that the steady-state hydrogen production rate increased slightly to 463 mL/h and yield was 1.8 mol-H₂/mol-glucose when the temperature of the 10-h HRT reactor was increased from 30°C to 37°C. The authors found that RISA fingerprints at 37°C from the 10-h HRT bioreactor, comparing to 30°C, exhibited a clear shift from populations related to *C. acidisoli* to populations related to *C. acetobutylicum*. Shin et al. (2004) reported that hydrogen-producing organisms of *Thermoanaerobacterium thermosaccharolyticum* and

Desulfotomaculum geothermicum were detected from the thermophilic acidogenic culture, while *Thermotogales* strain and *Bacillus* species were found predominant in the mesophilic acidogenic culture by polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis (DGGE) analysis. In that study, butyrate was the main acid product in the thermophilic culture, while butyrate, acetate and propionate were found predominant with equivalent levels in the mesophilic culture, resulting in enhanced hydrogen production obtained from the thermophilic acidogenic culture compared with the mesophilic culture.

The change in microbial hydrogen production with culture temperature is generally accompanied with a shift of metabolic pathways and product distribution. Yu et al. (2002) reported that acetate was the dominant product of a mixed culture at 20°C, but a low level of acetate was detected at 55°C with increasing production of butyrate, propionate and ethanol. Zhang and Shen (2006) studied the effect of temperature on hydrogen production by mixed bacteria and found that a pronounced production of propionate was detected as the main soluble product at a low ambient temperature and ethanol production varied significantly from 550 mg/L to 360 mg/L in a temperature range of 20-40°C, while the production of total VFAs decreased significantly at 45°C. As for the mixed cultures, it is likely that some hydrogen-producing species survive during temperature change and become predominant in some cases, while microbial activities are inhibited completely by a temperature change in other cases.

2.2.3.6 Feedstocks

It has been reported that biological hydrogen can be produced from a wide spectrum of carbohydrates, but most of studies have been limited to using food products, such as pure monosaccharides (glucose) (Horiuchi et al. 2001), disaccharides (sucrose) (Lee et al. 2003; Yu and Mu 2006) and polysaccharides (starch, cellulose and hemicellulose) (Czernik et al. 1999; Van Ginkel et al. 2001; Yokoi et al. 1998; Zhang et al. 2003). Simple carbohydrates such as xylose, lactose, maltose (Ferchichi et al. 2005b) and cellobiose (Kumar and Das 2000) have also been used as feedstocks for hydrogen production. Glucose and sucrose are easily degraded and

thus a short period of fermentation time or process HRT are required in batch and continuous hydrogen production experiments, respectively. On the contrary, it takes a relatively longer fermentation time to degrade starch and cellulose, since they have to be hydrolyzed into monosaccharides before used for hydrogen production. The maximum hydrogen yields obtained from these pure carbohydrates vary from 2.40 mol-H₂/mol-hexose using cellulose (Ueno et al. 1995) to 3.33 mol-H₂/mol-hexose from starch (Kanai et al. 2005) and glucose or sucrose (Van Niel et al. 2002), indicating that these carbohydrates are suitable as feedstocks for dark hydrogen fermentation.

Recently, considerable attention of research activity on fermentative hydrogen-production has been focused on the conversion of biomass reproducible resources to hydrogen by microbes (Fan et al. 2006b; Han and Shin 2004; Shin et al. 2004; Wu and Lin 2004). The renewable biomass is mainly derived from food-related wastes, i.e. food industry wastes/wastewaters and food residues in municipal solid wastes. Various studies have been conducted to investigate the feasibility of biological hydrogen recovery from the processing byproducts, including rice and wheat bran (Noike and Mizuno 2000), wheat bran (Noike and Mizuno 2000), bean curd manufacturing waste (Mizuno et al. 2000), noodle manufacturing waste (Mizuno et al. 2000), apple processing and potato processing wastewater (Van Ginkel et al. 2005); cheese whey (Ferchichi et al. 2005a), sugar factory wastewater (Ueno et al. 1996), rice winery wastewater (Yu et al. 2002; Yu et al. 2003a), molasses (Tanisho and Ishiwata 1995), sugar beet wastewater (Hussy et al. 2005), dehydrated brewery mixture (Fan and Chen 2004), starch manufacturing wastes (Yokoi et al. 2002; Yokoi et al. 2001). The hydrogen yields obtained from the food processing wastes or wastewaters range from 0.68 mol-H₂/mol-hexose to 2.70 mol-H₂/mol-hexose, which are comparable to those from pure carbohydrates. Of these yields reported, the highest yield was demonstrated by Yokoi et al. (2002) while fermenting sweet potato starch residue with a mixed continuous culture, whereas the lowest one was demonstrated by Fan and Chen (2004) in a mixed batch culture fed with dehydrated brewery solid waste.

Organic fraction of municipal solid wastes (OFMSW) can be considered as one of important feedstocks for hydrogen production. On average, almost 50% of the municipal solid wastes of underdeveloped countries consist of a fermentable and biodegradable fraction (Valdez-Vazquez et al. 2005). Because of their high carbohydrate and protein contents, food wastes constitute a major OFMSW. The OFMSW had significant potential of biological hydrogen production, but heavily dependent on composition of the OFMSW. Among the OFMSW, Okamoto et al. (2000) reported that hydrogen production potentials of individual carbohydrates varied from 26.3-61.7 mL/g-VS (cabbage), 44.9-70.7 mL/g-VS (carrot) to 19.3-96.0 mL/g-VS (rice), and carbohydrates produced the most hydrogen compared with proteins (egg: 2.60-7.07 mL/g-VS; lean meat: 2.47-7.68 mL/g-VS) or lipids (chicken skin: 3.56-10.2 mL/g-VS; fat: 4.41-11.14 mL/g-VS). Latterly, a similar observation reported by Lay et al. (2003) supported the fact that hydrogen production potential of carbohydrates-rich high-solid organic wastes (HSOW) (rice and potato) is approximately 20 times larger than those of fat-rich HSOW (fat meat and chicken skin) and protein-rich HSOW (egg and lean meat). This allows the suggestions that most carbohydrates-rich materials are a suitable feedstock for dark hydrogen fermentation, while proteins- and lipids-rich substance is probably less suitable or even unsuited.

Wasted sludge produced during wastewater treatment processes is mainly composed of microorganism bodies, and its organic substance composition is therefore different from carbohydrate-rich substrates such as glucose or starch. Cai et al. (2004) suggested that proteins are the largest constituent of the waste activated sludge, which account for about 32-41% of total dry solid, while Kim et al. (2004b) indicated a much higher proportion of protein in the sewage sludge (0.20 g carbohydrate-COD/g-VS and 0.73 g protein-COD/g-VS). Lower hydrogen yields from such wastewater sludge would be expected, due to a large protein proportion. In fact, Cai et al. (2004) reported a hydrogen yield of aerobic activated sludge at 9.1 mL-H₂/g-dry solids (DS) and found that hydrogen yield could be enhanced to 16.6 mL-H₂/g-DS as the raw sludge was subject to the alkaline treatment. Wang et al. (2003a) obtained a hydrogen yield of 20.2 mL-H₂/g-DS, equivalent to 0.6 mmol-

H₂/g-COD of the inoculum and sludge while fermenting wastewater sludge with *C. bifermentan*. Pretreatment of the original sludge through freezing and thawing, and sterilization markedly increased the hydrogen yield from 20.2 to 49.3-71.7 mL-H₂/g-DS (Wang et al. 2003a). However, the enhanced yields are little higher than those obtained from proteins-rich OFMSW, and comparable to the reported yields from some kinds of carbohydrate-rich OFMSW (Lay et al. 2003; Okamoto et al. 2000).

2.2.3.7 Substrate and Soluble Product Concentrations

Continuous hydrogen production performs at a wide range of substrate concentration from 2.5 to 40 g-hexose/L (Van Ginkel and Logan 2005b; Wu et al. 2006), but most of all has been commonly operated at a substrate concentration of 10-20 g-hexose/L. The reported effect of substrate concentration on hydrogen production, however, has been a point of debate. In studies concerning the effect of substrate concentration, Kim and his co-workers (2006b) found hydrogen yield to increase with increasing glucose concentration from 10 g/L to 35 g/L at a HRT of 12 h, whereas Kyazze et al. (2006) examined continuous hydrogen production at 12 h HRT on 10-50 g/L sucrose and found that the hydrogen yield decreased from 1.7 ± 0.2 mol-H₂/mol-hexose added at 10 g/L-sucrose to 0.8 ± 0.1 mol-H₂/mol-hexose added at 50 g/L. A decrease in hydrogen yield could occur at HRTs of 2.5 h and 10 h when the CSTR feeding strength increased from 10 g-glucose/L to 40 g-glucose/L (Van Ginkel and Logan 2005a). These studies indicate that besides substrate concentration other operating conditions such as HRT and composition of microbial cultures also affect continuous hydrogen production.

With a high feeding sucrose concentration of up to 30-40 g COD/L the granular sludge-based continuously stirred anaerobic bioreactor produced hydrogen more efficiently at a short HRT of 0.5 h, with the highest volumetric rate of 15 L/h/L and an optimal yield of ca. 3.5 mol-H₂/mol-sucrose (Wu et al. 2006). Only a slight decrease in hydrogen yield was reported by Oh et al. (2004) in a biofilm reactor and Yu et al. (2002) in an UASB reactor when the influent substrate concentration increased from 6.9 to 27.4 g-glucose/L and 14 to 36 g-COD/L, respectively. It was

noted that inhibitory concentration of substrate on acidogenic bacteria was estimated to be about 5-6 g-glucose/L in the suspended cultures (Majizat et al. 1997; Van Den Heuvel and Beefink 1988). These results imply that a more concentrated substrate is allowed in the cell-immobilized reactors, and microorganisms in the biofilm and granule biomass matrix might react more actively under a stressful substrate condition in comparison to the biomass in the form of suspension.

A high substrate concentration might induce metabolic pathways of microbial culture from hydrogen production to solvent production. It has been known that *C. pasteurianum* does not grow well at low pH and is a classic VFA and hydrogen producers, but its metabolism of glucose can be shifted away from hydrogen production and towards solvent production by a high initial glucose concentration (12.5% w/v) (Dabrock et al. 1992). However, Wu and Lin (2004) reported that the maximum hydrogen productivity of an unknown batch microbial culture reached 47.1 mmol-H₂/g COD_{re} at a substrate concentration of 40 g COD/L and pH 6.0. By analyzing product distribution, the authors pointed out that lowered hydrogen production resulted from the substrate inhibition, rather than product inhibition or metabolic shifts since butyrate was the main soluble product (41.0–62.5% of total VFA and alcohol) and its concentration increased gradually from 89 mM to 193 mM when the substrate concentrations increased from 10 to 160 g COD/L, while alcohols, predominated by ethanol remained a consistent level of 4–7% of the total concentrations of VFA and alcohol (Wu and Lin 2004). In fact, in a study by Zheng and Yu (2005), it was found that a substantial decrease in hydrogen production occurred by product inhibition only when the mixed batch microbial culture (pH=6.0) was supplemented from the outset with large amounts of butyrate of up to 25.08 g/L (285 mM).

2.2.4 Reactor Type and Operation

Many fundamental studies are conducted in batch hydrogen-producing reactors, while continuous hydrogen production is much preferred with respect to a

viewpoint of engineering. Besides CSTR which is the most commonly used reactor type, some high-rate hydrogen producing bioreactors such as biofilm-based or granule-based systems are developed for the continuous hydrogen production.

2.2.4.1 Batch and Semi-Continuous Processes

Many experiments were designed and conducted in batch mode to examine the characteristics of hydrogen-producing bacteria and optimize the culture operating conditions in the laboratory-scale studies (Han and Shin 2004; Kapdan and Kargi 2006; Lay et al. 2005; Li and Fang 2007; Van Ginkel et al. 2001; Wu and Lin 2004; Zhang et al. 2005; Zheng and Yu 2004a; Zhu and Beland 2006). In batch hydrogen fermentation, however, the microbial cultures perform inefficiently, leading to lower hydrogen production rates (0.06-0.66 L/L·h) (Table 2-1). Enhancing hydrogen production efficiency, stability and sustainability is thus a major challenge to batch hydrogen systems. To produce hydrogen continuously, a semi-continuous hydrogen production pattern was developed.

Han and Shin (2004) developed a semi-continuous mode for anaerobic hydrogen production from food waste. In their study, pretreated seed sludge and food waste are loaded into an anaerobic leaching-bed reactor, and then dilution water is continuously delivered to the reactor by a peristaltic pump at different dilution rates. The microbial reaction is assumed to reach the end as biogas production ceases, which generally needs around 7 days. Experimental results indicate that dilution rate is an important environmental factor during food waste fermentation. Appropriate control of dilution rate could enhance hydrogen fermentation efficiency by the improved degradation of slowly degradable matters. Also, dilution rate control might delay the shift of predominant metabolic flow from hydrogen- and acid-forming pathway to a route of solvent production. Valdez-Vazquez et al. (2005) examined hydrogen production from municipal solid wastes in another semi-continuous pattern. The reactors which are fed with substrate twice a week in a draw-and fill mode in an anaerobic chamber are operated continuously at 35°C and 55°C. More than 40 days of operation demonstrated that hydrogen is able to be produced steadily in such a semi-continuous biohydrogen system. Reactor

efficiency is unable to be evaluated since the hydrogen production rates are not mentioned in these two reports.

2.2.4.2 Continuous Suspended Sludge Processes

A CSTR is frequently used for continuous hydrogen production (Chen and Lin 2003; Majizat et al. 1997; Yu et al. 2003a). In such a system, hydrogen-producing bacteria are well suspended in the mixed liquor and less suffered from the mass transfer resistance. Because of its intrinsic structure and operating pattern, the CSTR is unable to maintain high levels of fermentative biomass which is commonly reported in a range of 1-4 g-VSS/L, dependent on the operating HRT. Washout of CSTR biomass easily occurs at shorter HRTs, and thus the hydrogen production rates are considerably restricted. The highest hydrogen production rate of CSTR culture was reported to be 1.12 L/L·h while fermenting sucrose with a mixed hydrogen-producing culture (Chen and Lin 2003).

2.2.4.3 Immobilized-Cell Processes

Immobilized-cell systems have become popular alternatives to suspended-cell systems in continuous operations since they are more capable of maintaining higher biomass concentration and being operated at high dilution rates without biomass washout. Biomass immobilization can be achieved through forming granules, biofilm or gel-entrapped bioparticles. Many researchers immobilized pure or mixed cultures of hydrogen-producing bacteria by gel entrapment in a form of bio-gel, such as *C. butyricum* IFO13949 in agar gel (Yokoi et al. 1997a), *E. aerogenes* strain HO-39 in k-carrageenan, calcium alginate or agar gel (Yokoi et al. 1997b) and sewage sludge in calcium alginate beads, or alginate bead with adding activated carbon powder, polyurethane and acrylic latex/silicone (Wu et al. 2002), and sewage sludge and activated carbon powder fixed by ethylene-vinyl acetate copolymer (Wu et al. 2005). The peaks of hydrogen production rate obtained by continuous gel-immobilized sludge range from 0.090 L/L·h (Yokoi et al. 1997b) in a chemostat with stirring to 0.93 L/L·h (Wu et al. 2003) in a fluidized bed reactor.

Fixed- or packed-bed reactor is operated under the conditions with a lesser extent of hydraulic turbulence, thus its immobilized cultures usually encounter mass transfer resistance which would result in lowered rates of substrate conversion and hydrogen production. Kumar and Das (2001) investigated hydrogen production by *E. cloacae* attaching on coir in packed bed reactors with different configurations, i.e. tubular, tapered or rhomboid shape at a HRT of 1.08 h. The comparative results indicate that the rhomboid bioreactor with convergent-divergent configuration has a maximum hydrogen production rate of 1.60 L/L·h as compared with 1.46 L/L·h in tapered reactor and 1.40 L/L·h in tubular reactor. The enhancement in hydrogen production rate could be attributed to higher turbulence created by reactor geometry favourable mass transfer and reduced gas hold-up. In addition, Rachman et al. (1998) found that high H₂ molar yield could not be maintained consistently in a packed-bed reactor, although pH in the effluent was controlled at more than 6.0, because pH gradient distribution along the reactor column results in a heterogeneous distribution of microbial activity. In order to overcome the mass transfer resistance and pH heterogeneous distribution, they recommended that fluidized bed or expended bed reactor system with recirculation flow are more appropriate for further enhancing the H₂ production rate and yield. In fact, it has been noted that the mass transfer resistance in a packed-bed reactor can be alleviated by increasing slurry recycle ratio. Kumar and Das (2001) observed that hydrogen production and substrate conversion rates of a packed-bed reactor both increased with the recycling ratio, and obtained the maximum hydrogen production rate of 1.69 L/L·h at a recirculation ratio of 6.4. The reactor performance of some cell-immobilized systems is summarized in Table 2-2.

UASB reactor system has been applied in hydrogen production due to its potential of high biomass concentration and treatment efficiency. Yu and Mu (2006) studied hydrogen production from synthetic sucrose wastewater in an UASB reactor with granular sludge operated at 38°C and a pH of 4.4±0.1 for over three years. Hydrogen yield was found to be in a range of 0.49-1.44 mol-H₂/mol-glucose. The results indicate that hydrogen production rate increased with increasing substrate

concentration from 5.33 to 28.07 g-COD/L, but decreased with increasing HRT from 3 to 30 h.

Recently several other high-rate hydrogen-producing systems based on granule techniques have been developed. Packing of a small quantity of carrier matrices at the bottom of the upflow reactor significantly stimulated sludge granulation that can be accomplished within 100 h in a carrier-induced granular sludge bed (CIGSB) bioreactor (Lee et al. 2004). The granular sludge was rapidly formed in CIGSB supported with activated carbon and reached a maximal concentration of 26 g/L at a HRT of 0.5 h. The reactor achieved an optimal volumetric hydrogen production rate similar to 7.3 L/L·h (7.15 mol/L·d) and a maximal hydrogen yield of 3.03 mol-H₂/mol-sucrose, when it was operated at a HRT of 0.5 h with an influent sucrose concentration of 20 g COD/L. In a granule-based CSTR, biomass concentration reached the maximum of 35.4 g-VSS/L which in turns resulted in a significant increase in hydrogen production rate up to 15 L/L·h (Wu et al. 2006).

The cell immobilization processes, espically those granule-based processes, however, have some limitations. A major drawback is the long startup period, which generally requires a few months for complete development of granules (Fang et al. 2002a; Yu and Mu 2006). During the startup of a UASB hydrogen-producing reactor, Mu and Yu (2006) found that small granules was formed with diameters of 400-500 µm at the reactor bottom after 140 days of operation and developed rapidly to 2.0 mm after 60 more days. In that study, although UASB reactor reached steady-state conditions, in terms of hydrogen production and substrate degradation after 5 months of startup operation, development and accumulation of the mature and stable granular sludge were completed beyond 8-month of operation. A similar observation was reported by Chang and Lin (2004) working with a UASB reactor, who noted that it took 39 days to achieve constant gas production at a HRT of 24 h and granules become visible after 120 days of reactor operation. A much longer period of around 180 days, however, was required for the further development of granules.

Table 2-2. Comparison on the hydrogen production performance of immobilized-culture processes.

Process	Culture immobilization method	Optimal HRT ^a (h)	HPR ^b (L/L·h)	SHPR ^c (mmol/g-VSS·h)	Biomass (g-VSS/L)
Column reactor (Yokoi et al. 1997a)	Gel Entrapment	3	0.37	-	-
AFBR (Wu et al. 2003)	Gel Entrapment	2	0.93	13.75	3.0
Packed-bed (Kumar and Das 2001)	Biofilm on ligocellulosic agroresidues	1.08	1.85	1.87	44
Fixed bed (Chang et al. 2002)	Biofilm on activated carbon	1	1.32	3.73	15.8
Trickling biofilter (Oh et al. 2004)	Biofilm on fibrous polymeric material	4	1.07	1.99	24
Packed-bed (Lee et al. 2003)	Biofilm on GAC	0.5	7.41	12.82	25.8
Packed-bed (Palazzi et al. 2000)	Biofilm on glass beads	10	0.25	3.70	3.02
UASB (Chang and Lin 2004)	Granule	8	0.28	2.47	5.06
UASB (Yu and Mu 2006)	granule	3	0.20	-	-
Packed-bed (Rachman et al. 1998)	Granule	1.5	1.42	3.72	17
CSTR (Fang et al. 2002a)	Granule	6	0.54	1.30	20
CSTR (Wu et al. 2006)	Granule and gel-entrapped bead	0.5	15.09	19.27	35.84
Column reactor (Lee et al. 2004)	Granule	0.5	7.33	12.53	26.1

^a on the basis of hydrogen production rate. ^b hydrogen production rate. ^c specific hydrogen production rate.

Understanding the granulation process is highly relevant for improving reactor design and performance. The formation and mechanisms of conventional granulation of anaerobic sludge in UASB reactor have been well documented. However, literature on initiating granulation of a hydrogen-producing culture and the formation mechanisms involved is somewhat limited. Reviewing on the formation mechanisms and affecting factors of methanogenic granule development seems to be helpful to understand microbial granulation of hydrogen-producing cultures, which is described in the following sections.

2.3 Microbial Granulation Technologies

In this section, the process of anaerobic granulation and factors affecting granulation are reviewed.

2.3.1 Granulation Process

Microbial adhesion or granulation, i.e., a cell attaches to a surface or another cell, is the starting point of anaerobic granulation process. This process thus can be defined in terms of energy involved in the interaction of cell to cell and is governed by the surface physicochemical characteristics of microbial cells. Anaerobic granulation has been demonstrated extensively. Schmidt and Arhing (1996) proposed that granulation process is a dynamic process comprising four steps:

1. Transport of cells to the surface of an uncolonized inert material or other cells (in the following called the substratum);
2. Initial reversible adsorption to the substratum by physicochemical forces;
3. Irreversible adhesion of the cells to the substratum by microbial appendages and /or polymers attaching the cell to the substratum; and
4. Multiplication of the cells and development of the granules.

In a thermodynamic sense, microbial sludge stability is governed by a charge balance among several repulsive forces which include electrostatic, salivation (hydration) and steric forces, and attractive forces including van der Waals, short range hydrogen bonds, and electrostatic forces (Erdinler et al. 2001). The

mechanisms of transport could be diffusion (Brownian motion), advective transport by fluid flow, gas flotation, sedimentation, or the mobility of cells. In a UASB system, mobility is not likely to be dominant. Alternatively, the initial adsorption is more likely to be achieved through a collision between a microbial cell and the substratum. Microbial adsorption behavior can be approximately explained by the DLVO theory. This theory, presented by Derjaguin, Landau, Verwey and Overbeek, with an original aim of explaining colloid stability has been well accepted in describing the adhesion behavior of microbial cells (Hulshoff Pol et al. 2004; Schmidt and Ahring 1996; Tay et al. 2006). The DLVO theory considers the overall Gibbs free energy as a function of the sum of attractive and repulsive forces over a distance between the cells. Since bacteria have negatively charged surface under normal pH conditions, the electrostatic repulsion dominates or is balanced by a weak attraction so that microbial cells are located a certain distance from each other (Step 2). At this stage, microbial adsorption is usually a reversible physicochemical process but can lead to immobilization of the bacteria. Irreversible adhesion of the bacteria can occur due to specific bacterial characteristics such as appendages, cell surface structure or polymers (Hulshoff Pol et al. 2004). When the bacterium is adhered, colonization has started. The immobilized cells start to divide within the extracellular polymers (ECP) matrix so that the progeny cells are trapped within the granule. This results in the formation of microcolonies of identical cells. The granulation process depends on cell division and recruitment of new bacteria from the liquid phase. The granular matrix can also contain trapped extraneous macromolecules. The organization of the bacteria in the granules can ease the transfer of substrates and products. The arrangement may depend on the local hydrophobicity, local presence of polymers or cell geometry.

2.3.2 Factors Enhancing Granulation

Anaerobic granulation has been investigated extensively to understand the mechanisms involved in the granulation process. Most important factors which can promote the granulation of microbial cells are discussed below.

2.3.2.1 Initial Nuclei

Nuclei can be microbial aggregates present in the sludge, and inert organic or inorganic particles together with the sludge and feed. This could be, e.g., precipitates or straw in the sludge (Schmidt and Ahring 1996). The inert nuclei model for anaerobic granulation had been proposed by several researchers and widely accepted so far to understand microbial granulation process of methanogens (Chen and Lun 1993; Lettinga et al. 1980; Morgan et al. 1991; Vanderhaegen et al. 1992; Wiegant 1998). In this model, one of the contributing factors to the development of granules from suspended sludge is the presence of inert nuclei or bio-aggregates for microbial attachment. The attachment of cells to these particles has been recognized as the initial step for granulation. Since the second step was the formation of a dense and thick biofilm on the cluster of the inert carriers, this step could be considered as biofilm formation. In other words, once the initial nuclei were formed and a proper operating routine was followed, subsequent granulation was inevitable (Hulshoff Pol et al. 2004). Several studies had provided evidence to support the inert nuclei in enhancing anaerobic sludge granulation in the UASB reactor (Hulshoff Pol 1989; Wirtz and Dague 1996; Yu et al. 1999). These inert particles might consist of zeolite, hydro-anthracite, water adsorbing polymer, GAC or powered activated carbon with diameters ranging from 40 μm to 400 μm .

Hulshoff Pol (1989) demonstrated the importance of inert support particles in the granulation process. When the particles (40-100 μm) were removed from the inoculated sewage sludge, granulation was not observed within the period of time required for granulation of dispersed sludge with no removal of inert particles in the seed sludge. He also found that the startup speed and granulation rate were accelerated via adding hydro-anthracite particles with a diameter of 100 μm (Hulshoff Pol 1989). Imai et al. (1997) noted that the startup of UASB reactors were able to be enhanced while adding water absorbing polymer (WAP) particles into anaerobic digester sludge in a lab-scale study. The WAP is a pulverulent resin with particle size ranging from 100 to 200 μm , which swells in water and exhibits a complex network.

There is evidence that anaerobic granules could be developed even without the addition of any inert particles (Liu et al. 2003b). In this case, the initial nuclei are able to be achieved via self-flocculation of dispersed cells into microbial aggregates. Some specific microorganisms are believed to play important roles in the methanogenic granulation process.

Wiegant (1998) observed that steady granules could be developed from the precursors mainly containing filamentous *Methanosaeta* in UASB reactors. Initially, *Methanosaeta* form very small aggregates via bridging between microbial cells or microflocs, or attaching to finely dispersed matter. Cellular multiplication and entrapment of non-attached bacteria lead to the growth of the precursors to form granules. This phenomenon is further investigated by Macleod and his co-workers (1990) who proposed a three-layered structure model for anaerobic granulation. In that model, the granulation started with *Methanosaeta* aggregates which might act as nucleation centres necessary for the initiation of granule development, and then acetogens (syntrophs) and hydrogenotrophic methanogens were predominant species in the middle layer, and finally acidogens and hydrogenotrophic methanogens colonized on the surface. Similar observation on *Methanosaeta*-rich nucleus was also found in the anaerobic granules by other researchers (Chen and Lun 1993; Morgan et al. 1991). These authors support the idea that *Methanosaeta* plays an important role in granule strength by forming a network that stabilizes the overall structure but also emphasise the role of extracellular polymers and cell walls.

Another main genus present in methanogenesis, *Methanosarcina* are also found be responsible for the nucleus formation (Bochem et al. 1982; Wu et al. 1996). *Methanosarcina* granules develop due to its capacity to secrete much more ECP and form clumps of bacteria. The clumps can reach macroscopic dimensions and other species, such as *Methanosaeta* tended to inhabit in the *Methanosarcina* clumps. However, *Methanosarcina* aggregation only occurs at a high acetate concentration (> 200 mg/L), and the growth of *Methanosarcina* aggregation will stop when the acetate concentration decreases (Grotenhuis et al. 1988). Although it is possible to use *Methanosarcina* aggregation as a nucleus, *Methanosarcina* predominant

granules are usually of less practical significance, since this type of granules are very easily washed out of the reactor (Wiegant and Deman 1986).

Vanderhaegen et al. (1992) suggest that anaerobic granulation is promoted when acidogens form sufficient biomass and polymers at a high concentration of sugar substrate, which act as nucleation centres for subsequently association of other microorganisms. However, they also observed the multi-layered structure stated by MacLeod et al. (1990). It would be very interesting to know the conversion process of the original acidogens-rich nucleus into *Methanosaeta*-predominant one. In addition, recent work showed that UASB granules had large dark non-staining centres, in which neither archaeal nor bacterial signals could be found (Rocheleau et al. 1999). In fact, the non-staining centre in the UASB granules might be formed as a result of the accumulation of metabolically inactive, decaying biomass and inorganic materials (Liu et al. 2003b).

2.3.2.2 Extracellular Polymers

Bacterial ECP is defined as polysaccharide containing structures of bacterial origin lying outside the integral elements of the outer membrane of Gram-negative cells and the peptidoglycan of Gram-positive cells (Schmidt and Ahring 1996). ECP is sticky materials secreted by cells, which either exists on the microbial aggregate, such as bioflocs, biofilm and granules in a binding form or dissolves in the bulk environment in a form of macromolecules, colloids and slimes (Sponza 2003). ECP is made of organic debris, phages, lysed cells, and other organic material excreted by the microbial cells. It contains polymers of polysaccharides, proteins, lipids, phenols, and nucleic acids (Schmidt and Ahring 1996). ECP content of granules is between 0.6 and 20% of the volatile suspended solid content, which consists mainly of protein and polysaccharides, typically in a ratio of 2:1 to 6:1 (Schmidt and Ahring 1996). It should be pointed out that polysaccharide is the only one of these components that is synthesized extracellularly for a specific function, while proteins, lipids, and nucleic acids exist in the extracellular polymer network due to excretion of intracellular polymers or as a result of cell lysis (Liu et al. 2004b).

There is strong evidence that ECP are highly involved in microbial adhesion behaviour and granulation process (Guibaud et al. 2003; Jia et al. 1996; Liu et al. 2001; Schmidt and Ahring 1994; Sponza 2003; Tay et al. 2006). The roles of ECP playing in the formation of granules are attributed to any one or a combination of the following mechanisms (Jia et al. 1996; Liu and Fang 2002; Liu et al. 2003b; Liu et al. 2004b; Sallis and Uyanik 2003; Zhou et al. 2006). First, because of the polymeric structure and colloidal property, the ECP provides an extensive surface area per unit volume for microorganism binding, entrapping and adhering. Second, ECP shows an affinity to divalent cations such as Ca^{2+} and Mg^{2+} (Guibaud et al. 2005; Liu et al. 2003b). Production of ECP could change the surface negative charge of the bacteria by adsorbing divalent cations, and therefore bridge two neighbour cells physically to each other as well as with other inert particulate matters. Finally, ECP composition determines floc structure and stability by balancing hydrophobic and hydrophilic interactions (Sponza 2003). Floc surface charge and hydrophobicity/ hydrophilicity are the sum effect of these exopolymer interactions, which are important properties in regulating the agglomeration of bioflocs to form granules.

2.3.2.3 Hydrophobicity

Hydrophobicity and hydrophilicity are usually used to describe a molecule or a structure having the feature of being rejected from an aqueous medium (i.e., hydrophobicity), or being positively attracted (i.e., hydrophilicity) (Liu et al. 2004a). Hydrophobicity of bacterial surface is an important factor affecting adhesion behavior of cells. Hydration interaction becomes significant at surface separations of 2-5 nm, depending on the nature of bacterial surface. Numerous researchers have described bacterial adhesion in terms of surface free energy (Liu et al. 2003b; Vanloosdrecht et al. 1987). Surface free energy was calculated from the contact angle of a drop of water or other liquid on a give surface or on a closed layer of bacteria. Although the measurement of contact angle does not indicate the thermodynamic quantity of solid surface free energy, the contact angle is a relative measurement of the hydrophobicity of the surface which in most cases shows a inverse correlation with the surface free energy after making some

nonthermodynamic assumptions (Vanloosdrecht et al. 1987). This means that increasing hydrophobicity might lead to a decrease in the surface free energy, which in turn promotes cell-to-cell interaction and further serves as driving force for cell self-separation from liquid phase (Liu et al. 2003b). Experimentally, it is reported that the hydrophobicity of bacterial surface plays a crucial role in initiating anaerobic granulation (Jorand et al. 1994; Liu et al. 2004a; Tay et al. 2000; Vanloosdrecht et al. 1987).

It is generally believed that that proteins and amino acids are the hydrophobic components of ECP, while polysaccharides are hydrophilic. The high hydrophobicity of microorganism is usually associated with the presence of fibrillar structure on cell surface and specific cell wall proteins (Liu et al. 2004a). Fibrils may attach to the surface of receptors. Cell wall proteins may work in two ways. One is that exposed hydrophobic proteins directly bind to extracellular matrix proteins. Alternatively, cell surface hydrophobicity may mediate attachment by facilitating and maintaining specific receptor–ligand interactions. There is evidence suggesting that the hydrophobicity of cellular surface is influenced by the ratio of proteins to polysaccharides in the ECP, rather total quantity of proteins and polysaccharides (Houghton and Quarmby 1999; Jorand et al. 1998; Liao et al. 2001). Jorand et al. (1998) found that the hydrophobic fraction of ECP was made up only of proteins and not carbohydrates. Their findings indicate the hydrophobic ECP participating in the organization of microbial flocs.

Cell surface hydrophobicity may also be related to other surface properties except ECP, such as surface charges. Previous studies indicates that there is a strong inverse correlation between the surface charge and hydrophobicity of sludge (Liao et al. 2001; Sponza 2003). This is explained since the surface charge is related to the ionizable groups present on sludge surfaces; it increases the polar interactions of ECP with water molecules. Therefore, the more charged the sludge surfaces, the lower the hydrophobicity. However, the authors point out that this correlation, in relation to other studies, sometimes is weak and likely owing to the influence of environmental factors.

2.3.2.4 Multi-Valent Cations and Cationic Polymers

Microbial cells have negatively charged surfaces under usual pH conditions. Adding multi-valent cations, such as Ca^{2+} , Mg^{2+} and Al^{3+} could expedite anaerobic granulation process through improving cell attractive forces (Liu et al. 2003b; Schmidt and Ahring 1996; Tiwari et al. 2006). First, according to DLVO theory, cations may directly neutralize the negatively charged cell surface and result in a reduction in electrostatic repulsion between bacteria. Second, intercellular van der Waals attractive forces would strengthen due to the condensed diffusive double-layers of microbial cells. In addition, the multi-valent cations may promote cell adhesion through bridging ECP of two neighbor cells, which would promote the formation of microbial aggregates, and then granules. Most of the Co^{2+} , Fe^{2+} , Ca^{2+} and Mg^{2+} were found in extracted ECP, and high affinity between affinity between ECP and Ca^{2+} had been observed (Schmidt and Ahring 1993; Schmidt et al. 1992; Yu et al. 2000; Yu et al. 2001a; Yu et al. 2001b). They might bind to the polymer and play important roles in linking ECP that is usually negatively charged.

Cationic polymers have been successfully applied to expedite the startup of UASB reactors. Show and his group demonstrated positive effects of “AA 184 H” cationic polymer on anaerobic granulation and UASB reactor startup (Show et al. 2004b; Wang et al. 2004). This is largely attributed to the facts that cationic polymer may form bridges between negatively charged bacterial cells through electrostatic charge attraction and/or reduce the electrostatic repulsion between cells by neutralizing cellular surface negative charges.

2.3.2.5 Operating Conditions

The most common bioreactor type used for anaerobic granulation is the upflow column-shaped reactors, such as UASB and expanded granular sludge bed reactor (EGSB), while the CSTR is rarely reported (Tay et al. 2006). Vanderhaegen et al. (1992) cultivated anaerobic granules in a CSTR, and found that granular sludge disappeared within three weeks when the reactors were incubated statically instead

of being shaken. The hydrodynamic shear force appeared to be necessary for maintaining the integrity of granular sludge, and anaerobic granulation may not be reactor type-dependent, but related to the way by which the reactor is operated. It should be pointed out that hydrodynamic flow patterns in UASB and CSTR are quite different. In CSTR, the bacteria move in a stochastic way, i.e. there is no regular liquid upflow pattern. Alternatively, a good phase separation is able to be achieved in an UASB reactor due to plug flow pattern, depending on the liquid upflow velocity.

It has been a general observation that anaerobic granulation proceeds well at relatively high liquid upflow velocity, while absence of granulation at weak hydrodynamic shear force has been reported (Alphenaar et al. 1993; Tay et al. 2006). Alphenaar et al. (1993) observed that the granulation process in UASB reactor was favored by the combination of high liquid upflow velocity and short hydraulic retention time (HRT). So far, the effect of liquid upflow velocity on anaerobic granulation has been most usually explained by the selection pressure theory (Hulshoff Pol et al. 1988). The selection pressure may result from environmental conditions (e.g. temperature), process operation conditions (e.g. HRT, upflow velocity, wastewater type, organic loading rate and pH), type of reactor and seed sludge. A long HRT accompanied with a low liquid upflow velocity may allow dispersed bacterial growth and be less favorable for microbe granulation, and a short HRT combined with a high liquid upflow velocity could cause washout of non competent bacteria in granulation, and in turn promote sludge granulation. It is obvious that in UASB reactor liquid upflow velocity and HRT are interrelated, and can serve as selection pressure on microbial ecology.

Research attempts have been given to develop strategy for speed-up of granulation process by controlling hydrodynamic conditions in UASB reactor. Noyola and Moreno (1994) conducted a series of experiments to investigate the effect of liquid upflow velocities for a rapid formation of granules through a purely physical aggregation due to the hydraulic stress applied to the anaerobic flocculant sludge with increasing the upflow velocities. Experiments showed that flocculant anaerobic

sludge could be converted to a relatively active anaerobic granular sludge by enhancing agglomeration with only hydraulic stress in a very short time less than 8 h, and the settleability of those anaerobic granules in terms of SVI and sludge settling velocity were significantly improved as the liquid upflow velocity increased. The increased settleability of granules in turn reduced washout of sludge from 46% to 2%. Similarly, Arcand *et al.* (1994) also reported that the liquid upflow velocity had a significant positive effect on mean granule size, but its effect on the specific washout rate of the smaller particles was little. It is most likely that relatively high upflow velocity combined with a short HRT seem to be in favor of fast formation and production of anaerobic granular sludge. However, for a successful start-up and stable operation of UASB reactors, the reactor HRT would not be below a critical value, namely the minimum HRT.

2.4 Summary

Microbial hydrogen production has been demonstrated by many fermentative anaerobes from a variety of carbohydrate-rich wastes. *Clostridia*-rich mixed cultures are believed to be the most promising hydrogen-producing bio-catalyzers used in practical scale hydrogen production. Hydrogen yield is an important parameter that is used to measure microbial conversion efficiency of hydrogen from the substrates. The maximum theoretical yield of hydrogen is 4 moles per mole hexose associated with acetate as the fermentation end-product. In practice, however, hydrogen yields are associated with a mixture of acetate, propionate, butyrate, lactate and alcohol, which depends on the metabolic pathways involved in the anaerobic hydrogen fermentation. High hydrogen yields are related to acetate and butyrate fermentation products, while low hydrogen yields are coupled with propionate and reduced end products such as ethanol and lactate. The pathways are greatly influenced by the operating conditions. System with a low pH of 5.0-6.0, low hydrogen partial pressure, short HRT, moderate strength carbohydrates, mesophilic or thermophilic regime, and necessary supplements of inorganic elements such as, P and Fe, is favorable for sustainable hydrogen production.

The volumetric hydrogen production rate is another important index in assessing the hydrogen-producing system performance. Immobilized-cell systems such as biofilm-based and granule-based processes showed a superior performance in terms of hydrogen production rate compared to the suspended-cell systems due to enhanced biomass retention. Several extremely high hydrogen production rates have been demonstrated by granular-sludge-based systems. It appears that granulation technology may be the best choice for fermentative hydrogen production.

However, the granular sludge processes have some limitations. A major drawback is the long startup period, which generally requires a few months for the development of compact and stable hydrogen-producing granules. Understanding the granulation process is highly relevant for improving the reactor design and system performance, and a rapid granulation and startup could render a cost competitive operation.

The formation and mechanisms of conventional granulation of anaerobic sludge have been well documented. Microbial granulation of methanogens could be speeded up by regulating physicochemical, biological and microbiological characteristics of anaerobic sludge and environmental parameters such as hydrodynamic conditions and substrate types. However, literature on initiating granulation of a hydrogen-producing culture and formation mechanisms is limited. In this respect, the present study attempted several strategies for initiating rapid formation of hydrogen-producing granules. A better understanding of the granulation mechanisms of hydrogen-producing cultures with respect to biological, microbiological, physicochemical of microbial culture and macro-environmental conditions would be essential.

CHAPTER 3 ACCLIMATION OF HYDROGEN-PRODUCING CULTURE

3.1 Introduction

CSTR processes have been widely used for fundamental studies of continuous anaerobic hydrogen production due to its well-mixing characteristics to allow hydrogen-producing bacteria to overcome mass transfer resistance (Majizat et al. 1997; Yu et al. 2003a). Nevertheless, many factors are contributing to the continuous production of hydrogen by the mixed cultures in a CSTR, including system pH (Fang and Liu 2002), hydraulic retention time (HRT) (Fan et al. 2006a), temperature (Lin and Chang 2004), carbon source (Hussy et al. 2003) and its strength (Kim et al. 2006b), metabolic pathways involved (Hartmanis and Gatenbeck 1984), and microbial diversity (Kim et al. 2006a). HRT is one of the important control parameters affecting continuous production of hydrogen, since HRT control can avoid the hydrogen utilization by hydrogen-consumers, such as the methanogens (Chen et al. 2001). However, the reported optimum HRTs for hydrogen production are rather inconsistent and varies from 8 h for sucrose (Chen and Lin 2003) and 12 h for glucose (Lin and Chang 1999) to 18-24 h for brewery wastewater (Fan et al. 2006a; Yu et al. 2003a).

The objective of the present study was to acclimate a mixed culture in a CSTR for hydrogen production by controlling culture HRT. The evolution of microbial community was monitored through PCR-DGGE techniques.

3.2 Materials and Methods

3.2.1 Seed Sludge

Anaerobically digested sludge obtained from a wastewater treatment plant in Singapore was used as inoculum. The collected sludge was sieved using a 425 μm mesh, and the filtrate was stored in a 4°C fridge. The seed sludge was heat treated at

98°C for 2 h and acid treatment at a pH of 2 for 24 h before inoculation. Initial concentration of volatile suspended solids (VSS) in the reactors was determined to be about 3500 mg/L.

3.2.2 Medium Composition

The synthetic wastewater mainly consisting of glucose (10 g/L) was used as feed, which also contained the following nutrients (mg/L): 200 NH_4Cl , 200 peptone, 200 KH_2PO_4 , 30 $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 20 $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 10 $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 5 $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 6 $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 5 $\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$, 5 $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, and 5 $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$.

3.2.3 Experimental Setup

A glass CSTR (Biostat B, B. Braun Biotech, Melsungen, Germany) was used in the present study as shown in [Figure 3-1](#). It had a working volume of 6 liters with an internal diameter of 160 mm and a height of 345 mm. The CSTR was covered with aluminum foil to avoid sun light and stirred at a constant rate of 280 rpm using two disc impellers, each having 6 flat blades ($\Phi = 64$ mm). The pH of mixed liquid in the reactor was controlled at 5.5 ± 0.05 with 4 M NaOH and 4 M HCl by automatic titration. The reactor was operated at a constant temperature of 37 °C through a water jacket. The feed stored in a 4°C fridge was pumped into the reactor by a peristaltic pump (Masterflex L/S, Cole-Parmer, USA).

After inoculation, 10 gram glucose was added to the reactor. The reactor was flushed with industrial argon gas for 10 min to create an anaerobic environment before the start-up of reactor. The reactor was initially operated for 1 day in batch mode without additional feed supply in order to avoid washout of inactivated biomass, and then converted to continuous operation. The HRTs were controlled in a shortening mode from 50 h to 8 h to start up the reactor. After reaching steady-state stage, the reactor was operated at HRTs of 8 h, 6 h, 8 h, 10 h and 12 h. The reactor was operated for at least one week at each HRT for data collection. Steady-state conditions were considered to be reached when the variations of products along with biomass concentration were less than 10%.

Figure 3-1. Schematics of anaerobic continuous stirred tank reactor system.

3.2.4 Analytical Methods

The biogas flow produced in the reactor was measured by a wet gas meter (Ritter TG 05, Germany). The biogas composition was analyzed by a Micro-Gas Chromatograph (Varian 4900, USA) equipped with a thermal conductivity detector. Hydrogen was analyzed using a Molsieve 5A Plot column with argon as carrier gas at 60°C; Methane and carbon dioxide were analyzed using a Propac Q column with helium as carrier gas at 60°C.

Aqueous samples were filtered through a 0.45 μm membrane before analysis. Volatile fatty acids (VFAs) and alcohol were determined by a gas chromatograph (Agilent 6890, USA) equipped with a mass selective detector (220°C), injector (200°C) and a 30m $\times$ 0.25 μm HP-FFAP fused-silica capillary column. The temperature of the oven was initially maintained at 60°C for 4 min, increased to 168°C at a ramp of 6 °C/min and lastly heated to 200°C at 10°C/min and maintained for 2 min. Helium was used as the carrier gas. Glucose concentration

was determined by the phenol-sulfuric acid method (Dubois et al. 1956). Measurements of suspended solids (SS) and volatile suspended solids (VSS) were performed according to the Standard Methods (APHA 1998).

The microbial populations in hydrogen-producing community at each HRT condition were examined and compared by analyzing the DGGE profiles of 16S rDNA fragments. DNA in the sludge samples was extracted, and then the 16S rDNA fragments were amplified by PCR, and separated by DGGE. For DNA extraction, a 100-200 mg sludge sample (wet weight) was mixed with 50 μ L 20% sodium dodecyl sulfate, 600 mL glass bead with a diameter of 0.1 mm and 500 μ L saturated phenol in a 2 mL tube and treated in a bead beater at 5000 oscillations/s for 3 min. Subsequently, DNA extraction was carried out according to the protocols described previously (Watanabe et al. 1998a; Watanabe et al. 1998b). The primers 357FGC (5' - CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG -3') and 517R (5'-ATT ACC GCG GCT GCT GG-3') were used for PCR amplification. A touchdown thermal profile technique (Watanabe et al. 1998a) using 2 min for activation and 10 min for extension was performed. The DGGE was performed following the method described by Moyer and his co-workers (1993). Electrophoresis was conducted in a 1X TAE buffer solution at 85 V and 60 $^{\circ}$ C for 12 h.

3.3 Results and Discussion

3.3.1 HRT-Dependent Performance

The effect of HRT on hydrogen production was investigated in a completely mixed reactor at a consistent pH and temperature of 5.5 and 37 $^{\circ}$ C, respectively. The reactor mixing condition was ensured by keeping the variation of VSS concentrations to less than 5% at three different reactor heights, i.e., at 50, 170 and 280 mm from the reactor bottom.

The initial HRT was set at 50 h for reactor start-up. To accelerate the acclimation process of the mixed culture, HRT was shortened gradually once the glucose

conversion rate reached 95% (Figure 3-2a and b). A dynamic increase in biogas production was observed during the start-up stage (Figure 1c). This is probably attributed to the fact that the microorganisms are adapting to the new environment resulted from the HRT shifting. Steady gas production was not observed until day 19, at a HRT of 8 h. After steady-state conditions were reached, the HRT was shortened to 6 h, and then increased in an increment of 2 h to 12 h. Five steady-state conditions, marked as Runs 1- 5, were at least maintained for one week at each HRT before changing the HRT. (Figure 3-2 and Table 3-1). The biogas produced consisted of hydrogen and carbon dioxide and was free of methane throughout the study. The result thus lends support for the effectiveness of pretreatment protocol on inactivating methanogens. Hydrogen content increased from initially 49% to 67% at day 5 and thereafter leveled off (Figure 3-2d). Hydrogen gas accounted for about 61% of total biogas evolved in Run 1 and increased to 64% in the following runs (Table 3-2). This value is much higher than that of other studies (34-53%) (Chang et al. 2002; Lin and Chang 2004; Lin and Chang 1999) with the exception of Ueno and his team (1996) who obtained the same level.

Table 3-1. Reactor performance at steady-state stage.

Run No.	Operating time (day)	HRT (h)	VSS (mg/L)	Glucose conversion rate (%) ^a	H ₂ yield (mol-H ₂ /mol-glucose)	H ₂ production rate (L/L·day)
1	20-25	8	910.83±39.06 ^b	91.63±0.20	1.62±0.03	6.08±0.19
2	26-38	6	847.50±26.86	77.82±0.38	1.88±0.03	7.77±0.13
3	39-49	8	897.64±25.17	90.46±0.30	1.93±0.02	6.92±0.03
4	50-58	10	843.33±65.60	96.24±0.52	1.95±0.03	6.11±0.07
5	59-68	12	1013.33±95.57	99.44±0.07	1.91±0.02	4.95±0.03

^a of 10 g/L in the influent;

^b mean value ± standard deviation

As summarized in Table 3-1, a hydrogen yield of 1.6 mol-H₂/mol-glucose was obtained in Run 1, at which the reactor was operated at HRT 8 h. Hydrogen yield increased by about 0.3 mol-H₂/mol-glucose when the HRT was shortened to 6 h in

Run 2. However, a relatively consistent hydrogen yield of approximately 1.9 mol- H_2 /mol-glucose was obtained as the HRTs were increased to 8 h, 10 h and 12 h in Runs 3, 4, and 5, respectively. The hydrogen production rate reached the maximum of 7.8 L/L·day at a HRT of 6 h, and it decreased, but not in a linear trend, with increasing HRT, whereby glucose conversion rate increased from 77.8% at 6 h HRT to 99.4% at 12 h HRT. It is interesting to note that an increase of 0.8 L/ L·day in hydrogen production rate was obtained in Run 3 as compared to Run 1, both at 8 h HRT, as a consequence of the increased hydrogen yield.

Figure 3-2. Variations in (a) HRT, (b) glucose conversion rate, (c) biogas production rate, (d) biogas contents, and (e) soluble metabolites over time (AA = acetic acid; PA = propionic acid; BA = butyric acid; EtOH = ethanol).

Table 3-2. Soluble and gaseous metabolites obtained at steady-state stage.

Run No.	HRT (h)	Soluble metabolites							Gaseous metabolites			
		Total VFAs and alcohol (mmol/L)	AA ^a (%) ^b	PA ^a (%)	BA ^a (%)	VA ^a (%)	HA ^a (%)	EtOH ^a (%)	AA+BA (%)	BA/AA (-)	H ₂ (%)	CO ₂ (%)
1	8	51.98±6.23 ^c	26.30±5.77	8.92±0.81	56.01±4.37	0.66±0.14	0.82±0.13	7.30±3.30	82.30±3.63	2.13±0.15	60.90±1.22	39.10±1.22
2	6	47.71±5.60	29.65±5.18	0.34±0.11	62.22±4.23	0.07±0.19	0.90±0.30	6.82±1.19	91.87±1.53	2.10±0.51	64.78±0.75	35.22±0.75
3	8	48.71±3.34	26.98±2.22	0.27±0.02	65.88±2.39	0.00±0.00	0.78±0.05	6.10±1.01	92.85±1.06	2.44±0.28	63.03±0.95	36.97±0.95
4	10	57.15±5.20	29.02±5.39	0.24±0.28	62.81±6.01	0.26±0.25	0.76±0.10	6.91±1.16	91.83±1.40	2.16±0.60	64.75±1.54	35.25±1.54
5	12	63.60±3.13	29.17±2.62	0.14±0.10	64.90±2.78	0.41±0.02	0.73±0.08	4.64±0.36	94.07±0.43	2.22±0.35	63.66±0.77	36.34±0.77

^aAA = acetic acid; PA = propionic acid; BA = butyric acid; VA = valeric acid; HA = hexanoic acid; EtOH = ethanol;^b of total products (mol/100 mol);^c mean value ± standard deviation

The biomass concentration retained in the reactor was in a range of 843-1013 mg/L, but not in correlation with the HRT (Table 3-1). As only 77.8% of glucose was utilized at a HRT of 6 h it is not meaningful to shorten HRT to less than 6 h to further enhance the hydrogen production rate.

3.3.2 Soluble Metabolites

Figure 3-2e reveals the production of main VFAs and alcohol associated with the HRT. Butyric, acetic and propionic acids and ethanol were the main metabolites at the start-up, but butyric and acetic acids increased significantly and became the dominant metabolites after reaching steady-state stage. Butyric and acetic acids accounted for approximately 82-94% (molar percentage) of total metabolites, while relatively consistent percentage of ethanol (4.6-7.3%) was obtained at the steady-state stage, regardless of the variation of HRT. Notably, considerable production of propionic acid was observed during the culture acclimation and first 8 h HRT (Run 1) periods, but it decreased to 0-0.18 mmol/L immediately after the HRT was transited from 8 h to 6 h (Figure 3-2e). It appears that these propionate producers are highly sensitive to a low HRT and 6 h whereby might be a critical HRT to remove propionate producers from the system, which is supported by the durable absence of propionic acid during the HRT of 8 -12 h in Runs 3-5. Consequently, a higher hydrogen yield would be expected as aforementioned, because propionate production involves consumption of both organic substrate and hydrogen which is produced in other pathways according to the theoretical equation [Eq.(2.7)].

The HRT could be used as a tool to select microbial populations whose growth rates are able to catch up with the mechanical dilution created by continuous volumetric flow (Tijhuis et al. 1994). That shorter retention time led to reduction of propionate production in anaerobic hydrogen production by mixed cultures has been reported (Cha and Noike 1997; Hussy et al. 2003; Lin and Chang 2004). Cha and Noike (1997) noticed that propionate production was present at HRTs of 24 h and 48 h but absent at a HRT of 12 h in a chemostat test using starch as the substrate. Hussy and his co-workers (Hussy et al. 2003) found that immediate reduction of propionate

production was observed in a continuous hydrogen fermentation from wheat starch as HRT was shortened to 12 h. The propionate level decreased significantly as the HRT was shortened to 6 h in the study by Lin and Chang (2004). These results seem to provide evidence that the amount of propionate producers in the mixed cultures is influenced by the HRT. However, it was also noted that the propionate concentration did not vary markedly in a HRT range of 4-24 h (Chen and Lin 2003; Yu et al. 2003a), and even decreased with increasing HRT (Dinopoulou et al. 1988). The hydraulic dilution effects in those studies appear rather inconsistent along with the present study. This is presumably due to the influence of other parameters such as substrate, inoculum and pretreatment protocol used. Nevertheless, the retention time did show an effect of hydraulic selection on the mixed culture in the present study.

Moreover, the ratio of butyric acid and acetic acid (BA/AA ratio) has been considered as a crucial indicator for evaluating the efficiency of hydrogen-producing cultures (Chen et al. 2001; Nandi and Sengupta 1998). The ratios of butyrate and acetate obtained at steady-state conditions are summarized in Table 3-2, showing BA/AA ratios of 2.1-2.4, which are very close to the values reported by other researchers (Chen et al. 2001; Kim et al. 2004a). This corresponds to a maximal theoretical hydrogen yield of 2.4 mol-H₂/mol-glucose according to the fact that 4 and 2 moles of hydrogen are generated from 1 mole of glucose, when acetate and butyrate are produced, respectively. Since part of the glucose is shunted to the formation of ethanol and some other acids, i.e., valeric and hexanoic acids, which do not contribute to hydrogen production, a hydrogen yield of 1.95 mol-H₂/mol-glucose obtained in the present study is reasonable and consistent with that (1.70 - 2.45 mol-H₂/mol-glucose) obtained in other studies using mixed cultures as the hydrogen producers (Chen and Lin 2003; Fang and Liu 2002; Lin and Chang 1999; Van Ginkel et al. 2001).

3.3.3 Microbial Diversity

The diversity of microbial communities at different HRTs were analyzed and compared by PCR-DGGE techniques, with the DGGE profiles of 16S rDNA gene fragments, as shown in Figure 3. Each band on the DGGE profile corresponded to a gene fragment of unique 16S rDNA sequences and accordingly represented a specific species in the microbial community. The intensity of a band represents the relative abundance of the corresponding microbial species (Fang and Liu 2002; Zhang and Fang 2001).

Figure 3-3. Variation of DGGE profiles with HRTs (See [Table 3-3](#) for the sampling times and HRTs associated with the lanes).

The DGGE profiles clearly show a shift of microbial population with HRTs ([Figure 3-3](#)). An apparently intense band, representing the dominant species on each lane was observed at each HRT after the reactor was started up as compared to the seed sludge pretreated. The dominant species (the brightest band in Lanes 2-4), however, were still shifting until the HRT was shortened to 12 h during the start-up stage,

which could have contributed to a varying increase of hydrogen production rate as observed. Thereafter, the dominant species (Band B) in the microbial community did not vary much. Moreover, the diversity of microbial communities decreased with the HRT shortening to 6 h as Bands A and C disappeared or faded, but after 15 days of acclimation it stabilized in Run 2 to Run 5, regardless of the HRTs extended. This seems to be evidences with respect to microbiological aspect to support the washout effect of HRT on propionate producers. Comparing Lanes 5 and 6, whose sludge samples were taken at days 16 and 24, respectively during the first 8 h HRT period no variation of microbial population can be seen, indicating that the microbial population did not change by the acclimation process itself but by the HRT shifts applied during this period.

Table 3-3. Information of sludge samples for DGGE.

Lane No.	HRT (h)	Sampling time (day)
1	Seed ^a	0
2	50	2
3	24	6
4	12	10
5	8	16
6	8	24
7	6	34
8	8	47
9	12	64

^aSeed sludge pretreated by heat and acid incubation

In summary, shortening of the HRT to 6 h was able to reduce the diversity of microbial population associated with an elimination of propionate production without affecting the existence of dominant species, which was the presumable reason for the observed increase in hydrogen yield. On the other hand, steady microbial population and hydrogen yield observed as the HRT increased from 6 h to 12 h (Runs 2-5) indicate, that hydrogen yield representing the capability of microorganisms to convert carbohydrate into hydrogen gas is independent on the

HRT. As a result, the hydrogen yield could be considered as a function of the microbial populations in the culture, but the HRT affects the microbial community to a certain extent, and in turn shows an impact on hydrogen yield. This might explain the view of HRT-dependent hydrogen yield obtained by some other researchers in the similar systems (Fan et al. 2006a; Lin and Chang 2004; Lin and Chang 1999). In Chen and Lin's study (2003), for example, it is likely that the increased hydrogen yield from 0.57 mol-H₂/mol-glucose to 1.70 mol-H₂/mol-glucose with a shortened HRT from 2 days to 0.5 day resulted from the contributions of HRT-selected microbial population.

3. 4 Conclusions

Microbial population in a CSTR culture shifted with the reduction in HRT until 6 h a stable microbial population was established. The increased hydrogen yield, from 1.6 mol-H₂/mol-glucose to 1.9 mol-H₂/mol-glucose, after transition from 8 h to 6 h HRT is caused by a washout of the propionate producing population during the 6 h HRT period. The hydrogen composition and yield then stabilized at about 64% and 1.9 mol-H₂/mol-glucose, respectively during the subsequent 8-12 h HRT period.

CHAPTER 4 RAPID GRANULATION OF HYDROGEN-PRODUCING BACTERIA IN A CSTR

4.1 Introduction

UASB process has been studied extensively and applied successfully for anaerobic conversion of organic pollutants in wastewater into methane gas. The formation of granular sludge enhances the reactor biomass and eventually promotes the reactor efficiency in organic pollutant removal and methane production. Nevertheless, the application of granular sludge processes for anaerobic hydrogen production only began in recent years, and is still developing but showing its potential in enhancing hydrogen production rate as compared to the suspended sludge systems (Kotsopoulos et al. 2006; Liu and Fang 2003). However, the granular sludge processes have some limitations. A major drawback is the long startup period, which generally requires a few months for the development of hydrogen-producing granules (Chang and Lin 2004; Fang et al. 2002a; Mu and Yu 2006; Mu et al. 2006; Yu and Mu 2006).

Understanding the granulation process is highly relevant for improving reactor design and performance. To achieve the purpose, the mechanisms for granulation should be understood fully. Due to absence of initial cores for cell attachment, microbial granulation is preceded with a self-adhesion or aggregation of microbial cells (Tay et al. 2006). This process thus can be defined in terms of energy involved in the interaction of cell to cell and is governed by the surface physicochemical characteristics of microbial cells. In a thermodynamic sense, microbial sludge stability is governed by a charge balance among several repulsive forces which include electrostatic, salvation (hydration) and steric forces, and attractive forces including van der Waals, short range hydrogen bonds, and electrostatic forces (Erdincler et al. 2001). Some physicochemical models therefore have been proposed, e.g. secondary minimum adhesion model (Liu et al. 2002; Liu et al. 2003b), ECP bonding model (Schmidt and Ahring 1994), inert nuclei model

(Lettinga et al. 1980) and ion-bonding model (Mahoney et al. 1987). The granulation of microbial cells is a complicated process, in which biological, microbiological and hydrodynamic factors are also involved other than physicochemical forces. For example, different models and hypotheses based on aforementioned factors have been proposed for anaerobic granulation, including structural models, proton translocation-dehydration theory, cellular automaton model and cell-to-cell communication model (Liu et al. 2002; Liu et al. 2003b; Tay et al. 2006).

The formation and mechanisms of conventional granulation of anaerobic sludge in UASB reactor have been well documented (Liu et al. 2002; Liu et al. 2003b; Schmidt and Ahring 1996). However, literature on initiating granulation of a hydrogen-producing culture and the formation mechanisms involved is somewhat limited. In the present study, an approach to accelerate microbial granulation of hydrogen-producing culture was developed in an anaerobic CSTR. Factors affecting microbial granulation with respect to surface physicochemical characteristics of cells and the reactor performance were examined.

4.2 Materials and Methods

4.2.1 Experimental Approach

A CSTR fermenter with a working volume of 6 L (160 mm diameter; 345 mm high) was used as shown in Figure 3-1. Detailed description on the reactors configuration and operation can be found in Chapter 3. The seed sludge was taken from a local wastewater treatment plant and acclimated with glucose (10 g-glucose/L) by stepwise HRT reduction from 50 h to 6 h in the CSTR at pH 5.5 for more than 2 months as described previously (Chapter 3). The concentration of acclimated culture was estimated to be 1.0 g-VSS/L. The medium used for hydrogen fermentation contained 10 g glucose per liter as the sole carbon source and sufficient amount of nutrients whose composition had been described in Chapter 3.

Two series of runs were conducted to assess the effect of acid incubation on microbial granulation. Firstly, the reactor culture was operated as a control run in a normal manner whereby its pH was kept consistently at 5.5 throughout the test. The detailed procedures of reactor startup and operation had been described in [Chapter 3](#). In the control run, the culture was operated with HRTs of 12 h, 10 h, 8 h, 6 h, 3 h and 2 h, each lasting two weeks at steady-state conditions except the shortest one due to the complete washout of sludge. In the acid incubation run, the seed sludge reloaded was reactivated by operating the reactor at a HRT of 12 h and pH of 5.5. After reaching steady-state conditions, the reactor culture was subject to a 24-h period of acid incubation by adjusting culture pH from 5.5 to 2.0. No substrate was introduced during the period of acid incubation. After that the culture was returned to the normal pH condition (pH 5.5) and run with HRTs of 12 h, 6 h, 2 h, 1 h and 0.5 h. The reactor culture was mixed continuously using two disc impellers, each having 6 flat blades ($\Phi = 64$ mm) at 280 rpm during the operation of control run and the first 360 h of acid incubation run. The stirring rate of acid incubation run was then reduced to 160 rpm after one-week modification on reactor mode to allow build-up of granular sludge. An overview of the experimental conditions of acid incubation run is given in [Table 4-1](#).

Table 4-1. Operation parameters of anaerobic CSTR for microbial granulation.

Operation time (h or day)	Mixed liquor pH (-)	HRT (h)	Feed conc. (g glucose/L)	Stirring rate (rpm)
-0 h	5.5	12	10	280
0-24 h	2.0	- ^a	-	280
24-89 h	5.5	12	10	280
89-92 h	5.5	6	10	280
92-116 h	5.5	2	10	280
116-342 h	5.5	2	10	280
One-week modification on reactor operation mode				
1-10 day	5.5	2	10	160
11-18 day	5.5	1	10	160
19-27 day	5.5	0.5	10	160

^a no feed flow was supplied.

4.2.3 Analytical Methods

The analytical protocols for gas composition, glucose concentration and VSS can be found in [Chapter 3](#).

Zeta potentials were measured by a Zeta Potential Analyzer (ZetaPals, Brookhaven Instruments, USA) and analyzed by a software of BIC Pals Zeta Potential analyzer. The concentration of wet biomass used for zeta potentials analysis was maintained at about 0.02% on a wet-weight basis via dilution with MiliQ water at a pH of 2 and 5.5, respectively. The particle size used as a parameter for measuring zeta potential was determined by the Zeta Pals Particle Sizing software. Measurements were carried out in triplicate.

Ten mL slurry samples were centrifuged at 10,000 rpm twice for 10 min each to separate the cells and supernatant, which were collected for attached and dissolved ECP measurements, respectively. After the separation, the tubes containing the cell pellets were resuspended with another 10 mL MiliQ water. Attached ECP was extracted according to formaldehyde-NaOH method ([Liu and Fang 2002](#)), while dissolved ECP polysaccharides were isolated using ethanol extraction method ([Bonet et al. 1993](#)) and other dissolved contents were measurable directly. The ECP was analyzed for total carbohydrates, proteins and DNA, which are the dominant components typically found in extracted ECP ([Fang et al. 2002a](#); [Frolund et al. 1996](#); [Liao et al. 2001](#)). Carbohydrate, protein, and DNA concentrations were determined using glucose, bovine serum albumin, and DNA type VIII sodium salt from *E. Coli* strain B (Sigma) as the respective standards according to the protocols described previously ([Aquino and Stuckey 2003](#)). The total ECP is the sum of the attached ECP and dissolved ECP.

Hydrophobicity of sludge was evaluated by water contact angle measurements using Contact angle system (OCA 20, dataphysics instruments GmbH, Germany). The sludge samples were prepared as described by Liao and his co-workers ([2001](#))

with modifications that the cellulosic membrane was fixed onto the surface of rubber stopper by double-faced adhesive tape rather than solidified agar plate, and the fixed samples were dried for 3 h at room temperature before contact angle measurements.

The size of sludge flocs or granules was measured by either a laser particle size analysis system (Malvern Mastersizer HYDRO2000SM, Malvern Instruments Ltd., UK) or an image analysis system (Quantimet 500 Image Analyser; Leica Cambridge Instruments, Cambridge, UK) when the granule size was beyond the Mastersizer's measurable limitation (0.05–2000 μm). Microbial observation was conducted by using either a digital microscopy (Keyence VH-8000; VH-Z450, Japan) or the image analysis system. The microbial compositions of granules were observed qualitatively with a scanning electron microscope (SEM) (Stereoscan 420; Leica Cambridge Instruments, UK). Detailed protocols of sample preparation and SEM observation were reported previously by Tay et al. (2001a).

4.3 Results and Discussion

4.3.1 General Observations

4.3.1.1 Control Run

To evaluate the effect of acid incubation on granulation, a control run was conducted using an anaerobic CSTR with acclimated hydrogen-producing culture at a pH of 5.5 and 37°C. The reactor performed stably as the HRT was shortened step-wise from 12 h to 3 h, but experienced serious upset at a HRT of 2 h due to the complete washout of hydrogen-producing sludge. In a well-mixed reactor, the specific maximum growth rate of hydrogen-producing culture is close to the washout dilution rate ($D = 1/\text{HRT}$), which was found to be 0.5 1/h in the present study. No granules were noted throughout the operation for more than 2 months. Hydrogen production performance of suspended sludge was not presented here, but will be discussed in [Chapter 7](#).

4.3.1.2 Acid Incubation Run

Acid incubation is normally applied to improve hydrogen-producing ability of seed sludge. This is achieved due to the reason that *Clostridium* species which are the dominant bacteria for hydrogen fermentation are able to survive under low pH condition, whereas hydrogen-consumers are inhibited after the acid treatment (Chen et al. 2002; Lee et al. 2004). The suspended hydrogen-producing culture that was initially run at a HRT of 12 h and pH 5.5 was subject to an acid incubation by adjusting the culture pH to 2.0 in an anaerobic CSTR. After 24 h of acid incubation under famine condition, the reactor culture was resumed to a normal operating pH of 5.5 and HRTs of 12 h, 6 h and 2 h sequentially (Table 4-1).

Figure 4-1. Microscopic images of culture (a) at 0 h, (b) at 0.17 h, (c) 24 h, and (d) at 101 h operation.

The microbial aggregation of predominantly rod-shaped bacteria initially present in suspension (Figure 4-1a) took place immediately after the acid incubation (Figure 4-1b). The process of microbial aggregation was expressed qualitatively and

quantitatively by microscopic observation (Figure 4-1b-d) or bioparticle size monitoring (Figure 4-2). The results showed that the floc size increased significantly with time and the flocs eventually developed into granules. Onset of granulation is adopted here as the formation of bioparticles having diameter larger than 500 μm (Show et al. 2004b). As depicted in Figure 4-2, granules had formed beyond 114 h of operation.

Figure 4-2. Evolution of bioparticle size distribution (Curve 1: at 0 h operation, Curve 2: at 20 h operation, Curve 3: at 51 h operation, Curve 4: at 114 h operation, and Curve 5: at 148 h operation).

Figure 4-3 depicts the evolution of bioparticle size in terms of volume weighted mean diameter in the first 148 h. The average diameter of bioparticles increased slightly from 18.60 μm to 29.06 μm in the initial 20 h; and then increased significantly from 152.55 μm to 761.57 μm from 101 h to 148 h. This suggests that the granulation process from microbial aggregates took place rapidly. It was noted that the average diameter (Sauter mean diameter) of granule at 148 h was determined to be around 1.7 mm using the image analyzer system. Figure 4-4 illustrates the images of hydrogen-producing granules sampled at 148 h and day 10. Compared to young granules which exhibited a rough surface and irregular shape (Figure 4-4a and b), mature granules were predominantly, egg-shaped with smooth surface (Figure 4-4c). The granules were composed of rod-shaped bacteria surrounded with a large amount of extracellular polymers under SEM observation (Figure 4-4d).

Figure 4-3. Time-course profile of bioparticle diameter in the first 148 h of operation.

Previous studies reviewed that formation of hydrogen-producing granules was usually accomplished in 2 to 4.5 months (Chang and Lin 2004; Fang et al. 2002a; Huang et al. 2004; Yu and Mu 2006). In the study by Fang and his team (2002a) using a similar reactor as the present study, granules appeared after 15 days of operation and steady operation was achieved after 60 days. In contrast, hydrogen-producing granules were noted at 114 h of operation due to the acid incubation in the present study. These findings seem to suggest that acid incubation plays a crucial role in initiating and accelerating the granulation of hydrogen-producing culture. The experimental results of the present study suggested that microbial granulation is a consequence of cellular response to the low pH environment which leads to the changes of surface characteristics of cells. To better understand the mechanisms involved in acid-induced microbial granulation, further examination on zeta potential, ECP components and hydrophobicity of bacteria were carried out.

Figure 4-4. Images of hydrogen-producing granules (a) microscopic photograph at 148 h, 10x; (b) SEM photograph at 148 h, 30x; (c) SEM photograph at day 10, 30x; (d) SEM photograph at 148 h, 3000x.

4.3.2 Effect of Acid Incubation on Surface Properties of Microbial Cells

4.3.2.1 Zeta Potential

Zeta potential is defined as the difference in the electrical charge between the stern layer of ions surrounding the particle and the charge of the bulk of the suspended fluid surrounding this particle. As shown in [Figure 4-5](#), the zeta potential of the untreated culture was determined to be -11.6 ± 1.1 mV. The zeta potential increased immediately to -3.6 mV as the culture pH decreased from 5.5 to 2.0 and stabilized at -3.5 ± 0.4 mV during the entire period of acid incubation. The zeta potential resumed to its normal value at around -11.8 mV when the culture pH increased to

5.5. The results indicated that the zeta potential of hydrogen-producing bacteria is closely related to the culture pH.

Figure 4-5. Zeta potential (Error bars $\pm$ SD based on averages measured in three samples).

It has been generally known that the zeta potential of charged particles is affected by not only the charge carried on the particle surface but also the environmental conditions in which the particles are present. As demonstrated in previous studies (Lin et al. 2003; Perez et al. 1998), the zeta potential of pure cultures increased significantly with decreasing culture pH. Thus the sharp increase in zeta potential at pH 2.0 in the present study was likely resulted from the lowered culture pH environment. One possible explanation for the increase in the zeta potential is neutralization or overlaying of positive charges, i.e. H^+ from the acid incubation to the negative charges laying on the cell surface. Moreover, the zeta potential of microbial cells could be influenced by adsorbing the charged ECP (Liu et al. 2003b; Zhou et al. 2006). Comparing with the charges carried by microbial cells, relatively fewer negative charges are carried by ECP, and even positive charges could be carried by amino groups of extracellular proteins (Liao et al. 2001; Tsuneda et al. 2003). Acid incubation caused an increase in ECP quantity, especially extracellular

protein proportion (Figure 4-6). However, the zeta potential of dissolved ECP was determined to be -5.5 ± 0.3 mV at a pH of 2.0, suggesting that absorbing ECP contributes negatively to the zeta potential of biomass. Consequently, the increased zeta potential of biomass at pH 2.0 is mainly attributed to the lowered culture pH.

4.3.2.2 Extracellular Polymers

Acid incubation led to the variations in ECP quantity and components as shown in Figure 4-6. The ECP are consisted mainly of polysaccharides, proteins and DNA which can serve as the construction materials for microbial granulation (Liu et al. 2004b). The total quantities of ECP, in the attached and dissolved forms increased significantly to peak at 4 h and then stabilized by the end of acid incubation. The presence of DNA indicated that the intracellular substances were stimulated to excrete actively or leaked passively as a result of the acid incubation. The released DNA was detected initially in both the attached and dissolved forms, but it dissolved almost completely into the bulk solution after 4 h of incubation, suggesting that the ECP DNA had not participated in microbial aggregation. The total increments of protein and polysaccharide during the acid incubation were estimated to be about 27.5 mg/L and 18.0 mg/L, respectively. While almost all of the protein was attached on the microbial cells, only about 67% of the increased polysaccharides were found on the cellular surface. This led to an increased ratio of proteins to polysaccharides of microbial cells from 0.2 to 0.5-0.8.

After acid incubation, the ratio of proteins to polysaccharides resumed to the initial value of 0.2, suggesting that the similar ECP components are excreted by the dispersed and aggregated cells. However, the ECP quantity of hydrogen-producing culture increased again with the process of microbial granulation. This might be explained by considering the fact that in contrast to the dispersed cells, the excreted ECP which composed mainly polysaccharides and proteins of aggregated cells is inclined to build up as a part of structural components due to the relatively inefficient mass transfer.

Figure 4-6. Variation of extracellular polymers (a) total ECP components, (b) attached ECP components and protein/polysaccharide ratio, and (c) dissolved ECP components during the process of microbial granulation.

4.3.2.3 Hydrophobicity

ECP composition could affect the hydrophobic properties of cellular surface. The surface hydrophobicity of biomass is expressed by contact angle measured of the sludge samples. The contact angle of sludge was estimated at about 31° at a pH of 5.5 as depicted in Figure 4-7. The hydrogen-producing bacteria showed hydrophilic characteristics, which is consistent with the reported observation that most acidogens exhibit hydrophilic surface with a contact angle less than 45° (Daffonchio et al. 1995). The cell surface contact angle increased immediately with the reduction in culture pH, but it did not recover as the culture pH did, indicating that culture pH is not a direct factor affecting the cell hydrophobicity. Instead, the cell hydrophobicity was highly correlated to the protein/polysaccharide ratio, but independent of the total ECP quantity as shown in Figure 4-6b and Figure 4-7. These findings are in substantial agreement with the previous studies which showed that the hydrophobicity of cellular surface was influenced by ECP composition rather than ECP quantity (Houghton and Quarmby 1999; Jorand et al. 1994; Liao et al. 2001). Bonet et al. (1993) reported that the cell surface hydrophobicity is inversely correlated to the amount of polysaccharide composition, but positively to the protein proportion.

The cell hydrophobicity appeared to be independent on the change of cellular zeta potential (Figure 4-5 and Figure 4-7). It should be noted that a strongly inverse correlation between the surface charge and contact angle of sludge has been reported (Liao et al. 2001; Sponza 2003). In their studies, the activated sludge with more surface charges might absorb more ECP by electrostatic interaction which would increase the polar interactions of ECP with water molecules, and consequently increased the hydrophilic characteristics of sludge. In the present study, however, the acid incubation resulted in the increased ECP quantity and less charges of the hydrogen-producing culture simultaneously. This indicated that the ECP quantity was not correlated positively to the surface charge numbers. One possible reason for the discrepancy could be the difference between the experimental conditions or culture properties.

Figure 4-7. Contact angle of culture (Error bars $\pm$ SD based on averages measured in three samples).

4.3.3 Triggering Forces for Microbial Granulation

The fact that initiating microbial aggregation occurred with the reduction in surface charges of microbial cells indicates that microbial adsorption behavior can be approximately explained by the DLVO theory. This theory, presented by Derjaguin, Landau, Verwey and Overbeek, with an original aim of explaining colloid stability has been well accepted in describing the adhesion behavior of microbial cells (Hulshoff Pol et al. 2004; Schmidt and Ahring 1996; Tay et al. 2006). The DLVO theory considers the overall Gibbs free energy as a function of the sum of attractive and repulsive forces over a distance between the cells. Since bacteria have negatively charged surface under normal pH conditions, the electrostatic repulsion dominates or is balanced by a weak attraction so that microbial cells are located a certain distance from each other (secondary minimum). The abrupt increase in zeta potential at a pH of 2.0 could whittle down the electrostatic repulsion between microbial cells. As a result, the van der Waals forces are predominant. Consequently, irreversible adhesion of microbial cells might occur with the reduction in electrostatic repulsion when bacterium reaches the primary minimum.

Figure 4-8. Time-course profile of culture concentration, redox potential and hydrogen production rate.

Studies have been reported that microbial aggregation could be initiated and accelerated via different approaches to altering bacterial surface charges. Perez and his team (1998) reported that the auto-agglutination of the pure cells increased as the zeta potential changed from -39 mV at pH 7 to -7 mV at pH 2. Reducing electrical repulsion between cell and solid surface could improve the microbial adhesion performance (Bonet et al. 1993; Tsuneda et al. 2003). Moreover, microbial granulation is accelerated by introducing multi-valence positive ions and cationic polymers to reduce electrostatic repulsion between negatively charged bacteria (Mahoney et al. 1987; Show et al. 2004a; Show et al. 2004b; Wang et al. 2004).

When a cell approaches another one at a certain distance (primary minimum), short-range interaction forces like hydrogen bonds or hydrophobic interaction would become crucial. Previous studies indicated that there is a strong correlation between microbial auto-aggregation and cell surface hydrophobicity (Baldi et al. 1999; Del Re et al. 2000; Kos et al. 2003; Liu et al. 2003c; Liu et al. 2004a; Tay et al. 2000). Improved hydrophobicity of bacteria is conducive to creating a favorable

environment for the contact of bacterium-to-bacterium or impelling bacteria out of aqueous phase environments to aggregate. Consequently, improved hydrophobicity of cell surface observed in the present study was likely to contribute to microbial aggregation, along with the reduced surface charges, while the ECP could serve as the construction materials for microbial aggregation by polymeric interactions with microbial cells (Erdinçler et al. 2001; Schmidt and Ahring 1996; Tsuneda et al. 2003).

Figure 4-9. Time-course profile of anaerobic CSTR performance in terms of reactor biomass concentration, hydrogen content (H₂%), and biogas production rate.

Although the role of ECP bonding in initiating microbial aggregation remains unclear, associated increases in ECP production with the development of granules indicate that the function of ECP is more likely to maintain structural integrity of microbial granules (Figure 4-6b). The electrostatic repulsion between the cells increased to original level once the acid incubation ceased. At the same time, hydrophobic interactions between the microbial cells became weak. Both of these factors would drive aggregated cells apart again. Nevertheless, no disaggregation was observed throughout the study, suggesting that the irreversible adhesion previously established could be maintained by ECP bonding. In fact, it was found

that hydrogen-producing bacteria in the granules were surrounded with a large amount of ECP as illustrated in [Figure 4-4d](#). Notably, besides the microbial surface physicochemical properties, the hydrodynamic, biological and other forces may also influence or contribute to microbial adhesion and aggregation. For example, appropriate operating hydraulic retention time provided the hydraulic selection pressures favorable for aerobic granulation ([Pan et al. 2004](#)), although these are not discussed in the present study.

4.3.4 Build-up of Granular Sludge

The biomass concentration decreased in the first 76 h, from 850 mg-VSS/L initially to 90 mg-VSS/L ([Figure 4-8](#)). This indicated that the acid incubation resulted in the lowered microbial activity of hydrogen-producing bacteria, and their growth rate could not catch up with the mechanical dilution rate employed. An apparent increase in biomass concentration took place up to 76 h, subsequently the redox potential began to recover ([Figure 4-8](#)). With further recovery in redox potential, the biomass concentration increased rapidly to 628 mg-VSS/L at 90 h. It seems that redox potential could be considered as an index to evaluate the microbial activity and to manipulate the HRT of reactors. To ensure the successful development of granules by inhibiting the multiplication of dispersed cells, a shorter HRT of 2 h, corresponding to a dilution rate of 0.5 1/h at which washout of dispersed biomass occurred in the control run was employed at 93 h. The microscopic observation at 101 h ([Figure 4-1d](#)) illustrated that microbial aggregates dominated over dispersed cells, indicating that the multiplication of dispersed cells had been inhibited successfully at a HRT of 2 h by either hydraulic washout or adherence to microbial aggregates.

Although the accumulated biomass quantity decreased significantly after the HRT transition from 6 h to 2 h, it recovered rapidly and peaked at 3362 mg-VSS/L at 179 h. Nevertheless, the biomass concentration decreased promptly due to substantial washout of granular sludge and evenly fluctuated at approximately 1000 mg-VSS/L since 300 h. Biogas production was observed after 44 h, which consisted of

hydrogen and carbon dioxide. Hydrogen accounted for 58-64% of the total biogas produced with the remaining consisted of carbon dioxide. The hydrogen production was highly correlated to the retained biomass quantity after 44 h, and the production rate varied with the reactor biomass and reached the maximum of 0.5 L/L·h at the highest biomass concentration of 3362 mg-VSS /L (Figure 4-8).

In the first 360 h, the culture was mixed at 280 rpm by two disc impellers which were located at heights of 20 and 180 mm above the reactor bottom. The well-mixed operation of the culture caused a severe washout of granular sludge. To minimize washout of biomass in the granular systems, different attempts to maintain the biomass were taken. These included lowering the top impeller closer to the bottom impeller, installation of a three-phase separator, and reduction of the stirring rate to 160 rpm. As a result, influent substrate and alkali could be mixed thoroughly, and a consistent layer of granular sludge was maintained.

4.3.5 Reactor Performance - Hydrogen Production

The performance of anaerobic CSTR granular system was evaluated as the HRT was shortened from 2 h to 0.5 h at a feed glucose concentration of 10 g/L as shown in Figure 4-9 and Table 4-2. Granular sludge built up substantially from 1.64 g/L to 30.43 g/L in the initial 5 days, and subsequently stabilized at 29.5 g/L in the period of 2 h HRT. The granular sludge formed a consistent blanket occupying a space of about 2.8-3.0 L. Biogas production increased with the biomass quantity initially, which stabilized by day 3 albeit further increase of the biomass. With the reduction in HRT from 2 h to 0.5 h, the overall biomass content of the granular sludge blanket increased slightly from 29.5 to 32.2 g/L. Hydrogen composition increased with the reduction in HRT, accounting for 53.0-63.5% of the total biogas. The remaining biogas composition consisted of carbon dioxide. As a result, an increase in hydrogen production rate from 0.94 L-H₂/L·h to 3.20 L-H₂/L·h and hydrogen yield from 1.39 mol-H₂/mol-glucose to 1.81 mol-H₂/mol-glucose was recorded. However, glucose conversion rate decreased significantly as the HRT was shortened to 0.5 h, where only 65% glucose was utilized.

Table 4-2. Granule-based CSTR performance at steady-state conditions.

HRT (h)	Reactor VSS (mg/L) ^a	Glucose conversion rate (%) ^b	H ₂ (%)	H ₂ yield (mol-H ₂ /mol- glucose)	H ₂ production rate (L/L·h)
2	29.76±0.51 ^c	99.36±0.12	52.96±0.46	1.39±0.05	0.94±0.03
1	29.86±0.61	90.32±2.79	60.79±0.61	1.76±0.04	2.15±0.05
0.5	32.06±0.40	65.04±3.32	63.45±0.57	1.81±0.03	3.20±0.06

^a biomass concentration of the granular sludge blanket.^b ten grams per litre in the influent.^c mean value ± standard deviation

The granular anaerobic CSTR can be operated at an extremely low HRT of 0.5 h, while a relatively high biomass concentration of up to 32.0 g/L was retained. The ability to retain a large amount of biomass in the form of granular sludge allowed the superior hydrogen production rate to be accomplished in the granule-based CSTR system. Comparing with the reported hydrogen production (0.15-2.36 L/L·h) (Chang et al. 2002; Kumar and Das 2001; Oh et al. 2004; Rachman et al. 1998; Wu et al. 2003), with an exception of the carrier-induced granular sludge bed process whose hydrogen production rate reached 7.3 L/L·h (Lee et al. 2004), the hydrogen production rate achieved in the present study is far more superior.

4.4 Conclusions

An approach for initiating rapid formation of hydrogen-producing granules was demonstrated with an anaerobic CSTR. Compared with the absence of granules in the control run, applying acid incubation on the culture demonstrated an ability to initiate microbial granulation of sludge within 114 h of operation. The granulation period was much shorter than that normally required of 2-4.5 months reported in other studies. Lowering the system pH could result in improvement in surface physicochemical properties of the culture favoring microbial granulation. The improved zeta potential, hydrophobicity and extracellular protein better facilitated the interactions between microbial cells which accelerated granule development.

Formation of granular sludge significantly increased the reactor biomass concentration to as much as 32.0 g-VSS/L, which in turn increased the hydrogen production rate by up to 3.20 L-H₂/L·h with a hydrogen yield of 1.81 mol-H₂/mol-glucose.

From the experimental results, it can be concluded that acid incubation is an effective means to initiate and accelerate microbial granulation of hydrogen-producing culture, leading to enhanced performance of an anaerobic continuous stirred tank reactor. Further assessment on applicability of the acid incubation approach for microbial granulation is recommended in other biohydrogen production systems.

CHAPTER 5 RAPID FORMATION OF GRANULES AND BIOFILM IN UPFLOW COLUMN REACTORS

5.1 Introduction

A rapid approach of acid incubation to initiating the formation of hydrogen-producing granules was successfully developed in our laboratory ([Chapter 4](#)). In the study, hydrogen-producing granules were formed rapidly in an anaerobic continuous stirred tank reactor within around 5 days as seed culture was subjected to a 24-h period of acid incubation at pH 2.0. Obviously, the culture operating conditions of a CSTR were quite different to upflow column-shaped reactors, such as UASB reactor, fixed-bed reactor and fluidized bed reactor, which were all more popularly used in the cell-immobilized processes. However, applicability of the acid incubation approach for microbial granulation in these column-shaped reactors remains unclear. Furthermore, the effectiveness of the approach on biofilm formation on support medium also requires validation. The lack of such information has deterred the rapid granulation technology developed in the CSTR process to be developed into a practical means for microbial immobilization in other hydrogen-producing systems.

As part of our continuing program of investigation on rapid immobilization of microbial cells, a study was designed to further understand the role of acid incubation in stimulating microbial granulation and biofilm formation in upflow column-shaped reactors which were operated in a fluidization pattern. Granular activated carbon (GAC) was used as a support medium for biofilm growth. Changes of surface physicochemical characteristics of microorganisms during the acid incubation were monitored and the resulting effect on microbial immobilization was evaluated. Also, hydrogen production by granule-based reactor and biofilm-based reactor was assessed and compared with other cell-immobilized reactors in literature. It is hopeful that findings derived from the present study could further

extend the acid incubation approach to the entire field of cell-immobilized techniques used for biohydrogen production.

5.2 Materials and Methods

5.2.1 Experimental Approach

Four identical column reactors, each mounted with a three-phase separator were operated as AFBRs (Figure 5-1). The reactor consisted of a glass tubular section of 40 mm internal diameter and 500 mm height with a conic bottom. An upper section of 80 mm internal diameter and 150 mm length was mounted to minimize carry over of suspended particles into the effluent which also served as a gas holder. A 40 mm height fixed-bed of grass beads was located at the conic bottom, serving as a distributor for incoming liquid. Each reactor had a total volume of 1.4 L inclusive of reaction-zone volume of 0.6 L. The column has four sampling ports located at 50, 120, 250 and 400 mm above the reactor bottom. These ports were used to extract liquid and bioparticle samples along the reactor. The mixed slurry was recycled through a recycling pump (peristaltic pump) connecting the outlet located at settling section and feed inlet at a constant recycling rate of 200 mL/min. Culture pH in the reactor was controlled at 5.5 ± 0.2 by automatic titration using respective peristaltic pumps connecting to an integrated controller with 4M NaOH and 4M HCl. The reactors were operated at a consistent temperature of 37°C maintained by a heating blanket. Biogas flow was measured using water displacement method, and biogas volume was calibrated to a temperature of 25°C and pressure of 1 atm conditions.

GAC was used as the support medium for biofilm attachment, and its physical characteristics were offered by the supplier as follows: size range, 500 – 800 μm ; surface area, 1100-1250 m^2/g ; bulk density, 440-480 g/L ; and real density, 1450 g/L (Kimic Chemitech, Singapore). Seventy gram of GAC was loaded for each reactor, occupying a static bed height of 130 mm. The seed sludge was taken from a local wastewater treatment plant and acclimated with glucose (10 g-glucose/L) by stepwise HRT reduction from 50 h to 6 h in the CSTR at pH 5.5 for more than 2 months as described previously (Chapter 3). The acclimated sludge had a

concentration of 6.1 g-VSS/L. The medium used for hydrogen fermentation contained 10 g glucose per liter as the sole carbon source and sufficient amount of nutrients whose composition had been described in [Chapter 3](#).

Figure 5-1. Schematic diagram of the anaerobic fluidized bed reactor system.

The acclimated anaerobic culture was inoculated into four reactors (1 L, each) designated R1, R2, R3 and R4, with R3 and R4 filled with GAC as support medium. The reactors were topped up with tap water and flushed with industrial argon gas for 10 mins to create an anaerobic environment before the startup of reactors. To evaluate the influence of acidification, the microbial culture in R2 and R3 was subject to an acid incubation for 24 h by shifting the culture pH from 5.5 to 2.0. No substrate was introduced during the period of acidification to all the reactors. The culture was returned to normal pH (pH 5.5) subsequently and operated

at HRTs of 12 h, 2 h, 1 h, 0.5 h and 0.25 h in series. An overview of the experimental conditions is shown in [Table 5-1](#). The HRT was calculated on the basis of the total reactor volume, while the organic loading rate (OLR) was computed from the concentration of glucose loaded to the reactor per unit HRT. Pseudo steady-state conditions at each HRT level were considered attained when biogas production and glucose conversion rate fluctuated within 5% for 24 consecutive HRT cycles. Evaluation of the system performance was carried out during the pseudo steady-state conditions.

Table 5-1. Operating parameters of AFBRs.

Operating time (h)	R1 (control) No GAC	R2 No GAC	R3 with GAC	R4 with GAC
0-24	- ^a	Acid incubation ^a	Acid incubation ^a	- ^a
24-57	12 h ^b	12 h	12 h	12 h
58-121	2 h	2 h	2 h	2 h
122-168	1 h	1 h	1 h	1 h
169-193	1 h	0.5 h	0.5 h	0.5 h
194-367	1 h	0.25 h	0.25 h	0.25 h
368-531	0.25 h	0.25 h	0.25 h	0.25 h

^ano substrate was fed

^boperating HRT

5.2.3 Analytical Methods

The analytical protocols for gas composition, glucose concentration and VSS can be found in [Chapter 3](#), and measurement of culture characteristics, including zeta potential, contact angle and ECP composition, sludge flocs or bioparticles size and microbial species composition was performed according to the procedures described in [Chapter 4](#).

5.3 Results and Discussion

5.3.1 General Observation

All AFBRs were inoculated with acclimated hydrogen-producing sludge and started up under similar operating conditions. The operating parameters are tabulated in [Table 5-1](#). To ensure successful development of immobilized cells rather than overgrowth of individual cells, the operating HRT was shortened to 2 h after being operated for 57 h for all AFBRs. Upon shortening of the HRT, the microbial activity was affected but was resumed rapidly as evidenced by a prompt reduction in redox potential to -200 mV. This HRT corresponded to a dilution rate of 0.5 1/h which is much larger than the maximum specific growth rate of hydrogen-producing bacteria.

[Figure 5-2](#) depicts the distribution of bioparticle size in terms of volume percentage diameters ranging from $d_{0.1}$ to $d_{0.9}$ in the first 175 h in R1 and R2 (d_a , volume percentage diameter; a , those particles with a diameter less than d_a accounts for $(a/10)\%$ of the sum of particle volume). A substantial increase in particle size with the acid incubation was noted in R2 compared with R1 whose culture was free of the acid incubation. Mean particle diameter ($d_{0.5}$) in R2 was estimated to be 60.6 μm during the acid incubation, which was much larger in contrast to the corresponding value of 26.8 μm obtained in R1. This suggested that microbial aggregation occurred immediately after the acid incubation. Such rapid aggregation of microbial cells was noted and recorded microscopically in a previous chapter ([Chapter 4](#)). Particle size increased significantly after 57 h of operation when the HRT was shortened from 12 h to 2 h, but was generally less than 300 μm in the first 330 h (ca. 14 day) in R1, while the particles had a mean size of 60 μm in the first 78 h in R2 and then increased so promptly that the diameter of some particles (10% in volume) had exceeded 1200 μm at 92 h. The mean diameter of particles in R2 increased from 43.27 μm at 78 h to 698.75 μm at 140 h. Onset of granulation is subjectively defined as the formation of bioparticles having diameter larger than 500 μm based on a study reported by Show and his co-workers ([2004b](#)). Some granules were able to be detected at 346 h in R1 and stable granular sludge layer

was established until around 400 h. In contrast, a rapid accumulation of granular sludge was noted after 92 h of operation in R2 due to the multiplication of granular sludge and washout of smaller suspended particles. The granular sludge layer increased significantly to a height of 14 cm at 120 h and reached the set maximum height of 48 cm at 153 h. In addition, morphology of granules cultivated in R1 and R2 was rather different (Figure 5-3). Compared to a spherical shape and smooth surface of granules induced by the acid incubation in R2, granules developed in R1 exhibited irregular shape and were flocky in appearance. Although granules were accomplished in both R1 and R2, the time for granulation required in R2 was significantly reduced by nearly three-fold compared with R1.

Figure 5-2. Time-course profile of bioparticle diameter (a) in R1 and (b) R2.

It is worth noting that no granules were formed in the CSTR without acidification on the microbial culture as presented in [Chapter 4](#). Shortening HRT to 2 h resulted in a complete washout of microbial culture in the CSTR. Successful development of granules in R1 was presumably attributed to the column-shaped configuration of the reactor which might be in favor of biomass retention. Although the HRT was shortened to 2 h at which washout of suspended sludge occurred in the CSTR, a large amount of biomass, with a concentration of around 3 g-VSS/L was able to maintain in the AFBRs. Unlike CSTR, a continuous selection of sludge flocs occurred in the column-shaped reactors due to the hydraulic hoisting, this is, light and dispersed sludge would be washed out, while heavier components would remain in the system. In that case, sludge accumulation might improve cell-to-cell contact and interaction and eventually promote the development of flocs in size, due to the gravitational compression. In the column-shaped reactor, the selection pressure is generally created by upflow liquid flow pattern ([Liu et al. 2003b](#)). By dint of the hydraulic stress, methanogenic granules were successfully developed in an UASB reactor through a purely physical aggregation of anaerobic sludge ([Noyola and Moreno 1994](#)).

Figure 5-3. Images of initial granules developed in (a) R1 at 336h, 10x; and (b) R2 at 120 h, 10x.

Biofilm formation was deemed to occur as the support carrier was completely surrounded with continuous biofilms ([Tijhuis et al. 1994](#)). It was observed that continuous biofilms were first appearing on the surface of some carriers, and such

biofilm-covered particles increased gradually in quantity which resulted in an increase in expanded bed height. As a result, the change of expanded bed height was monitored and considered as an index to estimate the development of biofilms.

Figure 5-4. Variation of expanded bed height in R3 and R4.

Figure 5-4 depicts the variation of expanded bed height in R3 and R4. A substantial increase in bed height occurred after 77 h in R3, whereas GAC bed layer expanded rapidly in R4 after the feed was supplied continuously, indicating that microbial colonization might take place promptly on the support media under normal conditions. Considering the starting time of granulation in R2 (Figure 5-2b), the results indicated that a period of 77-78 h was likely required to activate hydrogen-producing bacteria from the acid incubation. By visual inspection, continuous biofilms were noted at around 30 h in R4. Figure 5-5 illustrates several SEM images of biofilm particles sampled from the top layer of expanded bed in R3 and R4 at 36 h. While continuous biofilms had formed in R4, no biofilm was observed visually in R3 at that time. A close examination (3000 times magnification) revealed that individual cells had attached on the surfaces as well as within the cavities of GAC. It should be realized that microbial colonization might not proceed within the first 78 h due to the acid incubation, and thus those attached cells at 36 h was likely to achieve as a result of the physical adsorption. It is interesting to find that the

expanded bed reached the largest set height of 48 cm in R3 and R4 simultaneously after 104 h of operation. The effective time of biofilm development to an expansion height of 48 cm was estimated to be 26 h in R3 if an inactive period of 78 h was deducted, which was much shorter than 104 h required by R4. These findings indicate that acid incubation accelerated the microbial colonization of attached hydrogen-producing bacteria once their microbial activity was resumed.

Figure 5-5. SEM images of biofilms developed at 36 h in R3: (a) overview and (b) cells attaching on the surface of GAC, and in R4: (c) overview and (d) cells attaching on the surface of GAC.

5.3.2 Factors Affecting Cell Immobilization

5.3.2.1 Roles of Initial Nuclei

The physical and structural properties of particle-supported biofilms and granules are similar, as are their hydrodynamic, mass transfer and reaction characteristics (Nicoletta et al. 2000b; Schmidt and Ahring 1996). Granular sludge can be

characterized as a spherical biofilm and similarities between biofilm formation and the granulation process exist. Biofilm formation has been investigated intensively, but the granulation process has not been fully understood. Biofilm formation seems to be a mere phenomenon of an increase of biofilm thickness as a result of microbial multiplication on the support carriers, once physical attachment of cells to those nuclei had been established. Because of the absence of support carriers, establishment of such nuclei appears to be crucial to the granulation. Several researchers proposed an inert nuclei model for anaerobic granulation which had been widely accepted so far to understand microbial granulation of methanogens (Hulshoff Pol et al. 2004; Liu et al. 2003b; Schmidt and Ahring 1996; Tay et al. 2006). In this model, one of the contributing factors to the development of granules from suspended sludge is the presence of nuclei or bio-carriers for microbial attachment. The attachment of cells to these particles has been recognized as the initial step for granulation. Since the second step was the formation of a dense and thick biofilm on the cluster of the inert carriers, this step could be considered as biofilm formation. In other words, once the initial nuclei were formed and a proper operating routine was observed, granulation would be ensued. Several studies had provided evidence to support the inert nuclei in enhancing anaerobic sludge granulation in the UASB reactor (Hulshoff Pol et al. 2004; Show et al. 2004b; Wang et al. 2004). These inert particles could be in the forms of zeolite, hydro-anthracite, water adsorbing polymer, GAC or powdered activated carbon with diameters ranging from 100 μm to 400 μm .

The experimental results obtained from the present study are consistent with the reported findings. Adding GAC that served as an inert nucleus resulted in a prompt microbial colonization and biofilm development. GAC has been known as a hydrophobic sorbent favorable for cell attachment. On the other hand, acidogens are a kind of fast-growing organisms, and the maximum specific growth rate of the present culture was found to be close to 0.5 h^{-1} . As a consequence, hydrogen-producing biofilms formed rapidly as observed in R4, while the development of hydrogen-producing granules was retarded substantially in R1, due to no inert nuclei available. Acid incubation significantly promoted the establishment of initial

nuclei. According to the size criteria of inert particles (100–400 μm) (Hulshoff Pol et al. 2004; Show et al. 2004b; Wang et al. 2004), microbial nuclei were able to form within 10 mins after the acid incubation as observed that at least 10% (in volume) flocs had a diameter larger than 200 μm (Figure 5-2b). Such microbial nuclei were not possessed in R1 until 72 h (Figure 5-2a). In contrast to a period of 92 h required for the granulation in R2, it took a much longer time for the development of granules from these microbial nuclei in R1. Obviously, the acid incubation not only accelerated the formation of microbial nuclei, but also created a favorable niche for further development of microbial nuclei into granules.

5.3.2.2 Cell Surface Physicochemical Properties

Microbial immobilization is preceded with a microbial cell attachment to a surface or another cell (Schmidt and Ahring 1996; Tay et al. 2006). This process thus can be defined in terms of energy involved in the interaction of cell to support or cell and is governed by the surface physicochemical characteristics of microbial cells and support media. In a thermodynamic sense, microbial sludge stability is governed by a charge balance among several repulsive forces which include electrostatic, salivation (hydration) and steric forces, and attractive forces including van der Waals, short range hydrogen bonds, and electrostatic forces (Erdinçler et al. 2001). The initial adsorption is usually regulated by local physicochemical forces between cell and cell or between cell and support medium, and can be described, as a first approximation, by the well-known DLVO theory (Hulshoff Pol et al. 2004; Schmidt and Ahring 1996; Tay et al. 2006).

As shown in Figure 5-6, zeta potential of the culture was closely related to the culture pH. The culture zeta potential stabilized at -25.37 ± 1.60 mV at a pH of 5.5 while a substantial increase in zeta potential was found to be 11.90 ± 0.54 mV as the culture pH was reduced to 2.0. One possible explanation for the increase in the zeta potential with reduction in pH is neutralization or overlaying of positive charges, i.e. H^+ from the acid incubation to the negative charges laying on the cell surface. It was noted that, despite a same trend of the culture zeta potential increasing with reduction in pH, the values of culture zeta potential were different with the previous

Chapter (Chapter 4) wherein the culture zeta potentials were determined to be -11.6 mV and -3.5 mV at pHs of 5.5 and 2.0, respectively. The most plausible factor resulting in an inconsistent observation would appear to be the difference in particle size. The particles used for zeta potential measurement had an average diameter of 1.8 μm and 3.1 μm in the previous and present studies, respectively (measured by the Zeta potential analyzer). The GAC used in the present study had a measured zeta potential of 2.7 mV at a pH of 5.5. However, the zeta potential of GAC was found to increase significantly to 76.1 mV at a pH of 2.0.

Figure 5-6. Variation of the zeta potential of microbial culture during and after the acid incubation.

According to the DLVO theory, acid incubation resulted in a decrease in electrostatic repulsion between microbial cells due to a relatively small amount of charges carried on the surface of cells. As a result, the van der Waals forces are predominant, which could result in greater adsorption by allowing microbial interaction of cell to cell and hence irreversible adhesion. In the analysis of surface charges, cells and GAC both having much larger positive surface potentials at a pH of 2, exhibited much greater electrostatic repulsion and underwent profound inhibition of cell adhesion to GAC by strong electrostatic repulsive forces. In contrast, since the measurement of zeta potentials showed that microbial cells and GAC carried negative surface charges and positive charges, respectively at a pH of 5.5, the cell adhesion to GAC was more favorable, because of opposite charge adsorption. These adsorbed cells colonized on the surface of GAC and developed into continuous biofilms rapidly as illustrated in [Figure 5-5](#).

Figure 5-7. Production of extracellular polymers (solid symbol) and variation of proteins/polysaccharides ratio (open symbol).

It was noted that the irreversible aggregation established between cells during the acid incubation did not break with the termination of acid incubation, although the

electrostatic repulsive forces present in the aggregates was resumed, and even larger than the original level due to the shortened distance between cells in the microbial aggregates. This indicates that there were some other forces maintaining the irreversible adsorption. Previous studies showed that microbial granulation is a process associated with the production and accumulation of ECP (Liu et al. 2004b; Zhou et al. 2006). The ECP excreted can mediate both cohesion and adhesion of cells and play a crucial role in maintaining structural integrity of microbial matrix. It had been reported that ECP composition could affect the hydrophobic properties of cellular surface which also affected microbial immobilization. Examination of ECP and associated changes in hydrophobicity was therefore conducted during the acid incubation and thereafter.

ECP variation with the cell immobilization process is depicted in Figure 5-7. The ECP are consisted mainly of polysaccharides and proteins which contribute to microbial adhesion (Liu et al. 2004b). The changes of environmental pH caused an enhanced production of ECP, especially proteins. Compared with the stabilized ECP production and composition in R1, ECP content increased substantially with the acid incubation, from 66 mg/g-VSS to 136 mg/g-VSS in R2, accompanying with an increased ratio of proteins to polysaccharides from 0.2 to 0.5.

These findings imply that changes in ECP contents and composition observed during the acid incubation might be a physiological response of microbial cells to survive the stressful culture conditions. However, this phenomenon had been reported in a previous study that the increased ECP, presumably resulted from the passive leakage of intracellular substance at least partially as DNA, was detectable in the bulk solution and around the surface of microbial cells as described in Chapter 4.

A concomitant increase in ECP production was observed with the initiation of microbial colonization in all reactors. For example, ECP content stepped up rapidly from 136 mg/g-VSS at 80 h to 258 mg/g-VSS at 139 h in R2 and at the same time microbial aggregates were developed into granules. These findings suggest that, in

contrast to dispersed cells, ECP produced by adhered cells was inclined to build up as a part of structural components of granules or biofilms, due to the blocking of product transfer. Compared to a consistent production ratio of proteins and polysaccharides throughout the studies in R1 and R4, the ratio resumed to be an original level of 0.2 in R2 and R3 after 130 h of the operation, suggesting that microbial cells subjecting to the acidification had secreted ECP just like normal cells and that similar ECP components were excreted by the dispersed and aggregated cells.

Figure 5-8. Variation of the ratio of proteins to polysaccharides and contact angle.

The measurement of contact angle, which represents the hydrophobic properties of microbial cells, showed that the cellular hydrophobicity was in good agreement with the evolution of ECP composition. [Figure 5-8](#) demonstrates typical time-course profiles of contact angle and protein/polysaccharide ratio of sludge cultivated in R1 and R2. The contact angle increased from 31° to 38°, corresponding to the increase in protein/polysaccharide ratios from 0.2 to 0.5, and then resumed to 31° with the reduction in protein/polysaccharide ratio. It is likely that cell hydrophobicity was not influenced directly by the culture pH, but was a

function of the ECP composition. These findings correspond to the previous studies which showed that hydrophobicity of cellular surface was inversely correlated to the polysaccharide content, but positively to the protein proportion (Bonet et al. 1993; Houghton and Quarmby 1999; Jorand et al. 1994; Liao et al. 2001).

Based on the current knowledge built on methanogen granulation, short-range interaction forces like polymer bonding or hydrophobic interaction would become crucial when a cell approaches another one at a certain distance (primary minimum). ECP in aggregates is hypothesized to bridge two neighboring cells physically to each other as well as with other inert particles, and settle out as aggregates. Acid incubation stimulated the production of ECP serving as a bio-glue to facilitate cell-to-cell interaction and further strengthen aggregate structure through formation of a polymeric matrix. Microscopic observation provided visual evidence that ECP was present around the surface of hydrogen-producing granules during the initial stage of granulation in a CSTR (Figure 4-4d). Improved proportion of proteins in ECP resulted in a more hydrophobic environment where irreversible adhesion of microbial cells might occur. Theoretically, increasing the hydrophobicity of cell surfaces would cause a corresponding decrease in the excess Gibbs energy of the surface, which in turn promotes cell-to-cell interaction and further serves as a driving force for cell self-separation from liquid phase (Liu et al. 2003b). The experimental results obtained from the present study are consistent with the reported findings.

Like biofilm development, granules are formed following a dynamic process wherein microbial attachment, detachment and growth involved (Nicolella et al. 2000b; Tijhuis et al. 1994). Enhanced cell ECP production and hydrophobic property may improve and strengthen inter-cell structural affiliation. As illustrated in Figure 5-3a, granules had a loose structure and flocky appearance in R1. Cell detachment might prefer to occur on such type of granules compared with compact and dense granules developed in R2. In fact, a relatively longer period for granule and biofilm development had been observed in R1 and R4 due to the absence of acidification stimulation. This substantiated the contribution of the ECP and

hydrophobic properties of microbial cells to microbial granulation process and biofilm development.

5.3.4 Reactor Performance

In cell-immobilized processes, reactor start-up period, organic loading capability and corresponding substrate conversion rate are the most important indices for reactor performance evaluation. In a specific case of biohydrogen production, hydrogen production efficiency is also a crucial consideration. As demonstrated earlier, biofilm-particles and acid-induced granules were noted in a very short period, generally less than 92 h. To further evaluate reactor performance, biomass accumulation with cell immobilization and concomitant hydrogen production as well as glucose loading and utilization were presented in [Figure 5-9](#). Influent glucose concentration was maintained at 10 g/L in all reactors throughout the study, whereas HRT was shortened step by step from 2 h to 0.25 h, corresponding to an OLR of 5-40 g-glucose/L·h. After reaching steady-state conditions (as evidenced by consistent biogas production and more than 90% glucose conversion rate), the reactor organic loading was increased through step shortening the HRT.

Sodium hydroxide was supplemented automatically in the present systems to control the culture pH at a consistent level. A high loading of up to 40 g-glucose/L·h required dosing of a large amount of concentrated NaOH to neutralize organic acid produced during the hydrogen fermentation. However, system upset occurred, since sodium hydroxide added was unable to dilute rapidly and distribute evenly throughout the reactor at a higher OLR. Sodium bicarbonate, as an alternative to strong alkali, was supplemented into feed solution with a concentration of 5 g/L since 197 h. Thereafter, less NaOH was required for culture pH adjustment and the reactors could perform stably. Immobilized-cell bed layer was controlled at a height of 48 cm and superfluous biomass was removed manually to prevent system upset due to the biomass accumulation in the settling zone.

Figure 5-9. Reactor performances in terms of biogas production rate (a), hydrogen content (b), glucose conversion rate (c), and biomass retention (d).

Table 5-2. Hydrogen production performance of granule-based and biofilm-based reactors.

HRT (h)	Glucose conversion rate (%)		Hydrogen content (%)		H ₂ yield (mol/mol)		H ₂ production rate (L/L·h)	
	Granule ^a	Biofilm ^b	Granule	Biofilm	Granule	Biofilm	Granule	Biofilm
2	99.28±0.27	98.14±2.00	55.55±0.46	53.79±1.36	1.44±0.11	1.37±0.19	0.97±0.07	0.91±0.10
1	99.00±0.73	99.26±0.19	62.51±0.93	60.05±1.44	1.64±0.08	1.69±0.05	2.20±0.10	2.28±0.06
0.5	99.30±0.31	99.13±0.04	64.09±1.55	63.65±1.25	1.83±0.09	1.72±0.13	4.93±0.25	4.62±0.36
0.25	77.38±1.53	80.71±0.80	42.72±0.72	41.98±0.50	1.66±0.04	1.71±0.03	6.98±0.30	7.49±0.20

^aData (average value ± standard deviation) at HRTs of 2-0.5 h were derived from R2 only, while data at a HRT of 0.25 h from R1 and R2.^bData (average value ± standard deviation) were derived from R3 and R4.

Biogas production was noted along with the continuous feeding in R1 and R4, while biogas production was retarded for about 24 h in R2 and R3, due to the inhibition of acidification on microbial activity (Figure 5-9a). Biogas production increased substantially after the HRT was shortened from 12 h to 2 h at 72 h and stabilized at around 102-120 h in all reactors except R1, in which consistent biogas production was not observed until the formation of granular sludge at 346 h. After steady-state conditions were reached, granule-based reactor and biofilm-based reactor indicated almost similar performance in hydrogen production at the same HRT condition. The biogas consisted of hydrogen and carbon dioxide throughout the study in all reactors. Hydrogen content increased slightly with the reduction in HRT from 2 h to 0.25 h in all the reactors, accounting for 53-64% of the total biogas in the first of 197 h (Figure 5-9b). A significant decrease in hydrogen content, from $63.80 \pm 0.75\%$ to $42.35 \pm 0.66\%$ was found after adding NaHCO_3 as alkaline buffer as the reactors were operated at a HRT of 0.25 h. As tabulated in Table 5-2, the hydrogen yields increased in all reactors as the HRT were shortened from 2 h to 0.5 h, but decreased slightly with the further reduction in HRT, achieving the maximum values of 1.83 and 1.72 mol- H_2 /mol-glucose for the granular sludge and biofilm sludge, respectively. Hydrogen production rates increased substantially with decreasing HRT, and reached the respective peaks of 6.98 and 7.49 L- H_2 /L·h at the shortest HRT of 0.25 h in granule-based reactors and biofilm-based reactors. Shortening HRT to 0.25 h, however, caused a substantial decrease in glucose conversion rate to a level of around 80%, which was much lower in contrast to nearly 99% glucose conversion rate at HRT ranging from 2 h to 0.5 h (Figure 5-9c).

A major benefit of cell immobilization is the enhancement of biomass retention. As presented in Figure 5-9d, the mean biomass concentration was computed from the quantity of immobilized biomass over the reactor volume (1.4 L). Biomass retention increased significantly since the appearance of biofilms and granules, and reached a consistent level of 34-38 g-VSS/L at around 170 h in all reactors except R1 wherein stable biomass retention was not obtained after 400 h of operation. The ability to retain a large amount of immobilized biomass allowed the superior hydrogen production rate to be accomplished in those column-shaped reactors. Lee et al.

(2004) obtained a hydrogen production rate of 7.3 L/L·h in a carrier-induced granular sludge bed system, while the performance of other hydrogen production systems using biofilm sludge or granular sludge appeared to be less efficient with hydrogen production rate in the range of 0.15-2.36 L/L·h (Chang et al. 2002; Kumar and Das 2001; Oh et al. 2004; Rachman et al. 1998; Wu et al. 2003). The maximum hydrogen production rate achieved in the present system was 7.5 L/L·h, which is comparable to that obtained by Lee et al. (2004) and is much higher compared with those obtained in the other systems aforementioned. It is noted that the present hydrogen production rate is also higher comparing with 3.2 L/L·h obtained in the CSTR system containing granular sludge in a previous chapter (Chapter 4). This seems to suggest that column-shaped reactor favors hydrogen production in contrast to the CSTR system, due to elevated biomass retention. However, the highest hydrogen production rate achieved so far at 15.3 L/L·h was reported by Wu and his team (2006) in a granule-based CSTR system, indicating that besides reactor configuration, operating parameters and other factors also influenced biohydrogen production. For a given reactor volume of 1.4 L, only 0.6 L of the reactor volume was considered as effective immobilized sludge container. This led to a conclusion that it is possible to produce biohydrogen at a much higher rate with appropriate reactor design.

5.3.5 Implications for Research and Engineering

A rapid approach of acid incubation initially developed in a CSTR (Chapter 4) to initiating the granulation of hydrogen-producing bacteria is validated in terms of compatibility to the column-shaped upflow reactors, a most popular configuration used in the immobilized-cell processes. The successful application of acidification is based on the facts that the hydrogen-producing bacteria mainly consisting of *Clostridium* species form endospores which can be considered “survival structures” developed by these bacteria under a stressful environment. The microbial culture used in the present study was collected from an anaerobic digester. Applicability of the acid incubation approach developed in this work to other inocula from different natural sources such as compost and soil remains unclear and further testing on

these inocula is hence recommended from an engineering and application perspective. The process has a rapid startup period of granulation rendering a cost competitive operation.

Lowering culture pH to 2.0 immediately altered bacterial surface charges and created a benign electrostatic environment for microbial aggregation. Although a similar trend was observed on a same inoculum in the CSTR and AFBRs that charge numbers reduced with the acid incubation, zeta potentials increased from -11.6 mV to -3.5 mV in the CSTR, while from -25.4 to 11.9 in the AFBRs. As stated earlier, the most plausible factor resulting in an inconsistent measurement would appear to be the difference in floc sizes. This also indicates that culture properties have an important influence on the bacterial electrostatic environment. A pretest determining isoelectric pH of different hydrogen-producing cultures is recommended to minimize the electrostatic repulsive forces. Since the improvement of bacterial surface physicochemical characteristics was able to achieve immediately with the acid incubation, a 24-h incubation time used in the present work may be shortened, which would help activate hydrogen-producing bacteria from the acid incubation.

The mechanisms of pH change to accelerate the immobilization process of *Clostridia*-like bacteria remain unclear. Presumably, either pH change did stimulate the growth of immobilized hydrogen-producing bacteria, or improved adhesive properties of cells, due to the acidification, indirectly resulted in less cell detachment occurring on immobilized particles, or both mechanisms functioned. The denaturing gradient gel electrophoresis profile showed that only one strain of bacterium was predominant over the microbial population of the acclimated seed sludge (Figure 3-3). Due to its rapid self-flocculated properties, identification and purification of this strain of bacterium would help to provide microbial information to understand microbial immobilization. Purified bacterium is likely to serve as a biological flocculant in the processes of water treatment and inducing reagent for microbial immobilization. As the microbial nuclei play a critical role in the formation of granules, it is anticipated that methanogenic granulation is accelerated

via rapid formation of acidogens-rich starting nuclei for the further development of methanogens. This hypothesis is supported experimentally by the finding obtained by Vanderhaegen et al. (1992) who stated that sugar fermentative acidogens formed sufficient biomass and polymers to act as “nucleation” centers in which the rest of the methanogenic associations could develop.

5.4 Conclusions

An approach to initiating rapid immobilization of hydrogen-producing microbial culture in upflow column-type reactors was demonstrated with or without support carriers. A 24-h period of acidification on the microbial culture at pH 2.0 substantially accelerated the granulation, but not biofilm formation. In contrast to a much longer time (346 h) required for granulation of the culture without acidification, acid incubation was able to initiate a rapid formation of granules within 92 h. Use of support media as starting nuclei may result in rapid formation of biofilms without acidification within 36 h. Although microbial activity was inhibited by acid incubation for about 78 h, resulting in a retarded formation of biofilms of the acidified culture, these pre-acidified biofilms were able to develop much faster in the subsequent observation.

The experimental results indicated that acid-pretreated culture resulted in improvement in surface physicochemical properties of the culture favoring microbial adhesion and immobilization. Zeta potential increased from -25.3 mV to 11.9 mV, hydrophobicity in terms of contact angle improved from 31° to 38° and production of extracellular polymers increased from 66 mg/g-VSS to 136 mg/g-VSS. Formation of granules and biofilms allowed the hydrogen-producing biosystems to be operated at a HRT as short as 0.25 h, corresponding to an OLR of 40 g-glucose/L·h. The highest hydrogen production rates of 6.98 L/L·h and 7.49 L/L·h were achieved in granule-based and biofilm-based reactors, respectively at the shortest HRT condition, considerably higher than those reported by others.

CHAPTER 6 CHARACTERISTICS OF HYDROGEN-PRODUCING GRANULES AND BIOFILMS

6.1 Introduction

It is generally believed that spontaneous biofilm formation or granulation of hydrogen-producing bacteria can occur with reduced HRT in upflow column reactors or CSTRs (Fang et al. 2002a; Oh et al. 2004; Yu and Mu 2006). A long startup period of a few months, however, is needed for complete development of those bioparticles (Fang et al. 2002a; Yu and Mu 2006). For example, during the startup of an UASB hydrogen-producing reactor, Mu and Yu (Mu and Yu 2006) found that small granules was formed with diameters of 0.4-0.5 mm at the reactor bottom until 140 days of operation which developed rapidly to 2.0 mm after 60 more days. A long period of 86 days was also required to form hydrogen-producing biofilms on the support matrix in a thermal trickling biofilter (Oh et al. 2004). Understanding the cell immobilization process is highly relevant for improving reactor design and performance. However, the formation mechanisms of hydrogen-producing granules or biofilms have not been fully understood.

By examining methanogenic granules, it was found that microbial population and ECP are likely to play important roles in the granule formation (Hulshoff Pol et al. 2004; Schmidt and Ahring 1994; Schmidt and Ahring 1996). The physical, physicochemical and hydraulic characteristics of hydrogen-producing granules, such as bioparticle morphology, ECP contents, and settling velocity, have been investigated by several research groups (Fang et al. 2002a; Mu and Yu 2006; Zhang et al. 2004). Some researchers showed that polysaccharides might be the key ECP constituents for hydrogen-producing granules and hence might play a more important role in the formation of hydrogen-producing granules, compared to protein for methanogenic granules (Fang et al. 2002a; Mu and Yu 2006). However, as suggested in previous chapters, compared to ECP polysaccharides, proteins might be more crucial to the initiation of granulation. Knowledge with respect to

microbial species and spatial distribution of microbial components of the hydrogen-producing granules or biofilms remains limited, even though recently some advanced molecular techniques have been extensively applied to evaluate a microbial communities and their micro-environment of various granules and biofilms (Chiu et al. 2006; Fang et al. 2002a; Fang et al. 2002b; Tay et al. 2001b).

An approach to rapidly initiate granulation and biofilm formation of hydrogen-producing sludge was evaluated and factors affecting cell immobilization with respect to physicochemical characteristics of microbial culture were examined in Chapters 4 and 5. The role of biological and micrological factors would be very interesting to consider but was not conducted at that time. To well understand the mechanisms involved in the cell immobilization of hydrogen-producing cultures, the present work elucidated microbial community of the hydrogen-producing-granules and biofilms using the 16S rDNA-based techniques, and spatial distribution and internal structure of microbial components through examining the confocal laser scanning microscope (CLSM) images.

6.2 Materials and Methods

6.2.1 Experimental Approach

Only those rapidly immobilized bioparticles, i.e. acid-induced CSTR granules, acid-induced AFBR granules, and self-attached AFBR biofilms were examined in this Chapter. Cultivation conditions had been described in Chapter 4 and Chapter 5. All bioparticles were collected at a HRT of 0.5 h, corresponding to an OLR of 20 g-glucose/L·h. At that time, the CSTR granular sludge had been operated for about 8 months, whereas AFBR granules and AFBR biofilms were cultivated for more than 2 months.

6.2.2 Analytical Methods

The analytical protocols for gas composition, glucose concentration and VSS can be found in Chapter 3, and measurement of culture characteristics, including zeta

potential, contact angle and ECP composition, sludge flocs or bioparticles size and microbial species composition was performed according to the procedures described in [Chapter 4](#). The settling velocities of granules or biofilms in water were measured by recording the time taken for an individual granule or biofilm to fall from a certain height (1.0 m) in a measuring cylinder (10 cm in diameter; 1.5 m in height).

16S rDNA-based microbial analysis: The DNA from the granules and biofilm were extracted via enzymatic lysis using extraction buffer (100 mM Tris-HCl at pH 8.0, 100 mM EDTA at pH 8.0, and 1.5 M NaCl) containing Proteinase K (10 mg ml⁻¹). Samples were incubated at 37°C for 30 min and shaken at 150 rpm. The sodium dodecyl sulfate (SDS) was added (20%) to samples and incubated at 65°C for 90 min. Samples were subjected to 2 cycles of freeze-thawing. The supernatant was collected after centrifugation at 6000-g for 10 min, and the aqueous phase was extracted with phenol: chloroform: isoamyl alcohol (25:24:1, v:v:v). The DNA was precipitated utilizing isopropanol and pelleted by centrifugation (10,000g) for 10 min, then resuspended in water.

The DNA amplification reaction was performed with different sets of primers as tabulated in [Table 6-1](#). The PCR mixture consisted of 5 µl PCR buffer (10×), 2.5 µl MgCl₂ (25 mM), 2.5 µl dNTP (2.5 mM), 0.25 µl (100 mM) each of the primer solutions, 0.5 µl Taq. Polymerase (Promega, Madison, WI, USA), 100 ng of DNA template and ddH₂O to make 50µl. The DNA was amplified using an eppendorf mastercycler (Eppendorf AG, Germany).

DNA fragments were separated by DGGE according to the methods described by Muyzer and his co-workers (1993). Only a single band was identified in DGGE profile of the PCR-amplified DNA with 968F/1393R primer set and single band in gel electrophoresis for the other sets of primer, representing the DNA fragments from an individual bacterium.

Table 6-1. Primers and PCR program conditions used for 16S rDNA genes amplification.

Primer	Sequence (5'-3')	PCR Program	Cycles
968F ^a	AACGCGAAGAACCTTAC	94°C, 3',	1 cycle
1393R	ACGGAGGTAGCTGTCTGACATGA	94°C, 30"; 54°C, 55"; 72°C, 90"	30 cycles
		72°C, 7'	1 cycle
63F	CAGGCCTAACACATGCAAGTC	94°C, 3',	1 cycle
1512R	ACGGCTACCTTGTTACGACT	94°C, 45"; 55°C, 60"; 72°C, 90"	35 cycles
		72°C, 7'	1 cycle
27F	AGAGTTTGATCMTGGCTCAG ^b	94°C, 3',	1 cycle
1512R	ACGGCTACCTTGTTACGACT	94°C, 40"; 55°C, 60"; 72°C, 120"	35 cycles
		72°C, 7'	1 cycle

^aGC clamp of 40 base pairs (5'-CGCCCGGGGCCCCGGGCGGGGCGGGGGCACGGGGGG-3') was attached to 968F primer; ^bM = C: A in equal proportion.

Once a single band was confirmed, primers were changed for complete sequences. The extracted DNA was amplified by PCR using the primer set of 27F and 1512R to generate complete genome of 1485 base pairs. The PCR-amplified DNA was sequenced using the ABI Prism model 3730 (version 3.2) DNA sequencer.

Analysis of DNA sequences and homology searches were completed with standard DNA sequencing programs, and the BLAST server of National Centre for Biotechnology Information (NCBI) using the BLAST algorithm. The obtained DNA sequences and their closest 16S rDNA sequences of reference microorganism retrieved from the Ribosomal Database Project ([Maidak et al. 2000](#)) were aligned manually with the software Bioedit ([Hall 1999](#)) to construct phylogenetic tree using the neighbor-joining (NJ) method. Relative support for particular clades in the NJ tree topologies was estimated by using 500 replicates of the bootstrap procedure, performed with the Phylip software package ([Felsenstein 1989](#)).

The nucleotide sequences reported in the present work (please see [Appendix A](#)) have been submitted to the GenBank, EMBL, and DDBJ databases and assigned the following accession numbers: EF656615 AB308401 (AFBR-B), EF656616 AB308402 (AFBR-G), and EF656617 AB308403 (CSTR-G).

Staining and CLSM imaging: The collected bioparticles were maintained in a fully hydrated state during staining. Calcofluor white (Sigma Aldrich, St. Louis, MO) was used to stain the β -D-glucopyranose polysaccharides. The fluorescein isothiocyanate (FITC) (Molecular Probes, Carlsbad, CA) is an amine reactive dye that stains proteins and other amine-containing compounds such as amino-sugars in the bioparticles. Nile Red (Molecular Probes, Carlsbad, CA) is a phenoxazone dye that was utilized to stain lipids. Fluorescently labeled lectins Concanavalin A-tetramethylrhodamine conjugate (Molecular Probes, Carlsbad, CA) were used to bind to α -mannopyranosyl and α -glucopyranosyl sugar residues. As Con A can also bind with protein and glycoconjugate groups associated with cell walls, SYTO 63 (Molecular Probes, Carlsbad, CA), which is a cell wall-permeable nucleic acid stain, was used to differentiate the cells. The SYTOX blue (Molecular Probes, Carlsbad, CA), a cell wall-impermeable stain, was applied to stain the dead cells in the bioparticles.

The following quadruple staining scheme is proposed. The SYTO 63 (20 μ M) was first dripped on the sample and then placed on a shaker table for 30 min. Next, 0.1 M sodium bicarbonate buffer was added to the sample to keep amine group in non-protonated form; then, the FITC solution (10 g/L) was added to the sample for 1 h at room temperature. Finally, the Con A solution (0.2 g/L), Nile Red and calcofluor white were applied to the sample for 30 min in series followed by SYTOX Blue. After each of the six staining stages described above, the sample was washed twice to remove excess stain using phosphate-buffered saline (PBS). The stained bioparticles were embedded and frozen at -20 °C, from which 40- μ m sections were cut using a cryomicrotome and mounted on microscopic slides for observation.

The CLSM (Leica TCS SP2 Confocal Spectral Microscope Imaging System, Gmbh, Germany) was employed to probe the internal structure of granules and biofilms. The bioparticles were imaged using a x10 or x20 objective and analyzed using Leica confocal software. The fluorescence of calcofluor white was detected by excitation at 400 nm and from the emission width at 410-480 nm (blue). The FITC

probe was detected by excitation at 488 nm and emission at 500-550 nm (green). Excitation at 543 nm and emission at 550-600 nm (light blue) were used to detect Con A conjugates. The fluorescence of SYTO 63 (red) was determined from excitation at 633 nm and emission at 650-700 nm. The fluorescent intensity of SYTOX Blue (pink) was detected via excitation at 458 nm and emission at 460-500 nm. Excitation and emission wavelength were set at 514 nm and 625-700 nm for the detection of fluorescein Nile Red (yellow)

6.3 Results

6.3.1 Formation of Granules and Biofilms

The acclimated hydrogen-producing bacteria were immobilized by forming granules in a CSTR and AFBRs and forming biofilms in the AFBRs with GAC as support carriers ([Chapters 4 and 5](#)). Restated, formation of granules in the CSTR and AFBR was accomplished in a very short period of 3-5 days after the acclimated sludge was subject to an acid incubation for 24 h by shifting the culture pH from 5.5 to 2.0, which was much shorter than the formation times of several months required under normal conditions in other studies ([Fang et al. 2002a](#); [Yu and Mu 2006](#)). In the control tests without the acid incubation, hydrogen-producing granules were able to form in the AFBR after around 15 days of operation, but not in the CSTR, which suggested that reactor configuration had an important influence on microbial granulation. Use of support media as starting nuclei may result in rapid formation of biofilms without acidification within 36 h. Although microbial activity was inhibited by acid incubation for about 78 h, resulting in a retarded formation of biofilms of the acidified culture, these acidified biofilms were able to develop much faster in the subsequent observation. As a result, biofilm accumulation layers reached the maximum bed height in the AFBR cultures with or without acidification almost simultaneously after 104 h of operation.

Table 6-2. Hydrogen production performance of granular sludge and biofilm sludge.

Culture type	H ₂ yield	Specific H ₂ production rate
	(mol-H ₂ /mol-glucose)	(mmol/g-VSS·h)
CSTR-granule	1.81	8.31
AFBR-granule	1.66	8.77
AFBR-biofilm	1.71	8.96
Acclimated sludge	1.88	15.62

6.3.2 Hydrogen Production Performance

The ability of granules and biofilms to convert glucose into hydrogen in terms of hydrogen yield ranged from 1.66 to 1.81 mol-H₂/mol-glucose, while hydrogen production ability of the biomass (specific hydrogen production rate, mmol-H₂/g-VSS·h) was in a range of 8.31-8.96 mmol-H₂/g-VSS·h (Table 6-2). The reactor performance such as glucose conversion rate and volumetric hydrogen production rate of granules and biofilms had been presented previously (Chapters 4 and 5).

6.3.3 Physical and Physicochemical Characteristics

Physical and physicochemical characteristics of hydrogen-producing granules and biofilms are summarized in Table 6-3. The CSTR and AFBR granule sizes (in Sauter mean diameter) ranged from 0.2 to 4.2 mm (average 1.7 ± 0.9 mm) and from 0.2 to 3.5 mm (1.5 ± 0.3 mm), respectively. The sizes of biofilm particles ranged from 1.0 to 2.6 mm averaging at 1.8 ± 0.4 mm. The thickness of biofilms was estimated to be in a range of 0.3-1.5 mm. Bioparticle morphology was expressed in terms of roundness, and the roundness of CSTR granules, AFBR granules and AFBR biofilms were 2.6 ± 1.4 , 1.3 ± 0.5 and 1.6 ± 0.6 , respectively. Larger values of roundness indicated for the granules cultivated in the CSTR might be attributed to the fact that some scattered granules were found in the CSTR, which were not

granules in the real sense and thus called as “patch flocs” in the context. In contrast to the sphericity or sphericity-like of healthy granules, those patch flocs were in irregular shapes as shown in Table 6-3. The CSTR and AFBR hydrogen-producing granules had settling velocities of 13-69 m/h and 21-78 m/h in water, respectively, while the settling velocities of biofilms were 58-189 m/h, due to the presence of much heavier support cores.

Table 6-3. The physical characteristics of hydrogen-producing granules and biofilms.

	CSTR-granule	AFBR-granule	AFBR-biofilm

Diameter (mm)	0.2-4.2 (1.7±0.9) ^a	0.2-3.5 (1.5±0.3)	1.0-2.6 (1.8±0.4)
Roundness (-)	1.2-10.4 (2.6±1.4)	1.1-3.3 (1.3±0.5)	1.3-2.6 (1.6±0.6)
Settling velocity (m/h)	13-69 (47±18)	21-78 (64±9)	58-189 (94±15)
Ash (%)	2.1-2.8 (2.5±0.5)	8.3-9.4 (8.6±1.0)	8.7-9.8 (9.2±1.0)
ECP proteins (mg /g-VSS)	24-32 (28±3)	26-35 (29±4)	24-30 (27±4)
ECP polysaccharides (mg /g-VSS)	145-169 (152±11)	153-175 (158±9)	142-174 (154±14)

^ameasured range (average ± standard deviation).

The granules and biofilms consisted mainly of volatile organic materials as the ash contents were found to be $2.5 \pm 0.5\%$ for the CSTR granules, $8.6 \pm 1.0\%$ for the AFBR granules and $9.2 \pm 1.0\%$ for the AFBR biofilms, respectively. The low ash contents in the granules and biofilms were presumably attributed to the facts that synthetic glucose wastewater was fed wherein less inorganic materials were associated. Each gram of granules or biofilms (in VSS) contained (24-35) mg proteins and (142-175) mg polysaccharides, with a proteins/polysaccharides ratio of around 0.2. Other ECP contents in the hydrogen-producing granules and biofilms, such as lipids, uronic acid and nucleic acids were not quantified, due to their small

proportion in the hydrogen-producing granules as suggested by Fang et al. (2002a) in a recent study.

Figure 6-1. SEM photographs of (a) typical surface bacterial morphology and (b) porous structure of hydrogen-producing bioparticles.

SEM micrograph (Figure 6-1a) revealed that rod-shaped microorganisms were found in the CSTR granules, having a length of 2.5–7 μm and a width of 0.4–0.6 μm . Figure 6-1b illustrates the presence of cavities and holes among the immobilized bacterial clusters. The cavities may allow substrate, metabolic products as well as the release of biogas to be efficiently transferred. Similar bacterial morphologies and structural properties were exhibited by other granules and biofilms, which were not presented here for brevity.

6.3.4 Microbial Species Analysis

The diversity of microbial communities of granules and biofilms was analyzed and compared by PCR-DGGE techniques, with the DGGE profiles of 16S rDNA gene fragments, as shown in Figure 6-2. The DGGE profiles show a uniquely distinguishable band present in each microbial community as DNA fragments were amplified with primers 968F and 1393R. Such profiles were attained repeatedly when different sets of primers were employed. It was noted that the bands corresponding to different samples appeared at the same location of DGGE profiles, indicating a similar microbial composition of the different microbial communities. The PCR-amplified DNA genes were sequenced and the obtained sequences were

compared with those of the reference microorganisms available in the Genbank database by BLAST search. All bacterial sequences ([Appendix A](#)) were found to be most closely related to the bacterial species of *C. pasteurianum* with similarities of 98% ([Table 6-4](#)).

Figure 6-2. DGGE profiles of the PCR-amplified 16S rDNA fragments extracted from the CSTR-granular sludge (CSTR-G, left two lanes), AFBR-granular sludge (AFBR-G, middle two lanes) and AFBR-biofilm sludge (AFBR-B, right two lanes). The gradient concentration of the denaturant was 35-65%.

Phylogenetic analysis of the three sequenced bacterial strains designed as CSTR-G, AFBR-G and AFBR-B and their close relatives was performed by constructing 16S rDNA phylogenetic tree, using the neighbor joining algorithm with 500 bootstrappings (their similarities, please refer to [Appendix B](#)). [Figure 6-3](#) illustrates

the phylogenetic tree for three bacterial strains closely related to the *Clostridium* species.

Table 6-4. Phylogenetic affiliation of bacterial sequences.

Culture type	Number of bases	Phylogenetic relationship	
	used in establishing identity	Closest species in GenBank	Similarity (%)
CSTR-granule	1350	<i>C. pasteurianum</i> (CH7)	98
AFBR-granule	1359	<i>C. pasteurianum</i> (CH4)	98
AFBR-biofilm	1401	<i>C. pasteurianum</i> (CH7)	98

6.3.5 CLSM Images

Figure 6-4 and Figure 6-5 show the confocal images of the cross-section of a hydrogen-producing granule or biofilm by detecting the distribution of proteins, α -polysaccharides, β -polysaccharides, lipids, dead cells and living cells. Proteins were distributed uniformly in the healthy granules (Figure 6-4A and Figure 6-5A) and biofilm (Figure 6-5B), while other ECP contents (polysaccharides and lipids) and cells were mostly accumulated on the outer layer of granules or biofilms. Deeper inside the granule was a core made mainly of proteins. High bioactivity in the outer layer was expected. Unlike the layer structure of healthy granules, “patch flocs” had a uniform structure as cells and ECP contents were distributed relatively evenly in the granule’s interior (Figure 6-4B). This uniform structure might be caused by the presence of small pores as detected in the granule interior (Figure 6-4B, g), which could be functioned as mass transfer channels for the contact between substrate and microbial cells.

Figure 6-6 presents the distribution of the intensities of the fluorescent lights corresponding to proteins, lipids, α -polysaccharides, dead cells, living cells and β -polysaccharides (distance=0 refers to granule surface) for the AFBR granules shown in Figure 6-5A. Proteins were distributed uniformly over the granule interior, while other ECP contents and cells varied with the distance, generally increasing

from the centre towards the outside of granules. Other granules and biofilms were also examined (results not presented), and their distributions indicated similarly.

Figure 6-3. 16S rDNA phylogenetic tree for stains CSTR-G, AFBR-G and AFBR-B, and their close relatives in the genus *Clostridium*. The tree is based on the Jukes-Cantor distance and was constructed using a neighbor-jointing algorithm with 500 bootstrappings. *Anaerobacter polyendosporus* was selected as the outgroup species. The scale bar represents 0.02 nucleotide divergence. Numbers at the nodes are the bootstrap values (%). Nucleotide sequence accession numbers or stain names are indicated in brackets.

(A)

(B)

Figure 6-4. Confocal images of hydrogen-producing granules cultivated in the CSTR: (A) healthy granule and (B) patch flocs. Green: proteins (a); yellow: lipids (b); light blue: α -polysaccharides (c); red: living cells (d); pink: dead cells (e); blue: β -polysaccharides (f); light background: microscopic image (g); Last figure: merging of all (6 or 7) images(g or h).

(A)

(B)

Figure 6-5. Confocal images of hydrogen-producing granules (A) and biofilms (B) cultivated in the AFBRs. Green: proteins (a); yellow: lipids (b); light blue: α -polysaccharides (c); red: living cells (d); pink: dead cells (e); blue: β -polysaccharides (f). Last figure: merging of all (6) images (g).

6.4 Discussion

Acid incubation stimulated the formation of hydrogen-producing granules in both the CSTR and the AFBR. Changing culture pH resulted in improvement in surface physicochemical properties favoring microbial agglutination and immobilization, including reduced electrostatic repulsion, improved hydrophobic surface, more ECP

secretion and a higher ratio of proteins/polysaccharides (Chapters 4 and 5). Forming microbial nuclei was the crucial step to the development of granules, which was substantially accelerated by the acidification in contrast to the control tests. The roles of initial nuclei in microbial immobilization had been discussed in Chapter 5. Biofilm formation seems to be a mere phenomenon of an increase of biofilm thickness as a result of microbial multiplication on the inert nuclei, once physical attachment of cells to those nuclei had been established. Rapid formation of hydrogen-producing biofilms indicated that the hydrogen-producing bacteria used in the present study possessed a very strong affinity to attach to the surface of support media and an ability of rapid microbial colonization and propagation.

Although the sizes of granule cultivated in the CSTR and AFBRs were comparable, the granule shapes were quite different, as evidenced by their roundness. Violent shear forces from the stirring impellers of the CSTR made the granules disintegrated and amorphous, while moderate liquid shear force kept granules more uniform in shape in the AFBRs. The biofilm particles also displayed a uniform shape despite the irregular shape of GAC carriers under moderate shear force. The settling velocities of biofilms were substantially higher than those of granules, and statistic data showed that granules cultivated in the AFBR had a better settling ability compared to the CSTR granules. These findings indicate that column reactors seem to be favourable for the solid-liquid separation and biomass retention of hydrogen-producing biofilms and granules. Granule physical properties, such as granule size and settling velocities presented in the present study are consistent with those found for normal hydrogen-producing granules or bioflocs (Fang et al. 2002a; Mu and Yu 2006; Zhang et al. 2004).

Bacterial morphology of the bioparticles cultivated in the present study appeared to be very uniform. The microbial community of the granules or biofilms was uniquely constituted by the rod-shaped hydrogen-producing bacteria, which were identified to be *C. pasteurianum* by analyzing their nucleotide sequences (Figure 6-1a). Besides rod-like, coccus and filamentous bacteria (Mu and Yu 2006) or fusiform bacteria (Fang et al. 2002a; Wu et al. 2006) were also found in the sucrose-

fed hydrogen-producing granules. This is most likely due to different feedstocks and seed sludge used compared to the present experimental conditions. It is interesting to note that *C. pasteurianum*-like bacterium (similarity, 98%) was also found to be distinct microbial species in the self-flocculated hydrogen-producing granules cultivated by Wu et al. (2006) in a CSTR operated at a HRT of 0.5 h and a sucrose concentration of 30 g COD/L, which had a fusiform shape (3-5 μm in length and 1-2 μm in width), rather than a rod shape as observed in the present study.

The physical and structural properties of particle-supported biofilms and granules were similar, as were their microbial composition and distribution. Living cells were mostly accumulated on the outer layer of granules, while deeper inside of the granules was a core made of less active cells and mainly of ECP. Therefore, particle-supported biofilms and granules will be considered as a single category: particle-based biofilms. In fact, a comparable hydrogen production performance in terms of hydrogen yield and specific hydrogen production rate was demonstrated by the granular sludge and biofilms. Their microbial conversion efficiency of glucose to hydrogen, evaluated on the basis of a maximum of 4 mol- H_2 /mol-glucose, varied in a narrow range from 41.5% to 45.3%, which were compared well with the values previously reported (Fang et al. 2002a; Liu and Fang 2003; Wu et al. 2006). Nevertheless, specific hydrogen production rates of the immobilized sludge, i.e. granules and biofilms were nearly half of that of suspended sludge (15.62 mmol- H_2 /g-VSS $\cdot$ h). This indicates that, although numerous pores existed within the granules or biofilms, a large amount of ECP secreted might clog up these pores and hence reduced the mass transfer capability through the bioparticle interior, as evidenced by the presence of numerous dead cells throughout the granules or biofilms.

Bacterial community analysis using DGGE techniques is based on the separation of PCR-amplified same length fragments of genes coding for 16S rDNA. Each band on the DGGE profiles corresponded to a gene fragment of unique 16S rDNA sequences and accordingly represented a specific species in the microbial

community, whereas the intensity of a band represents the relative abundance of the corresponding microbial species (Fang and Liu 2002; Zhang and Fang 2001). It was somewhat surprising that only one distinct band was detected in each DGGE profile (Figure 6-2), which indicated the DNA fragments from an individual bacterium, and hence single microbial composition in the microbial communities. The simple microbial community of sludge samples tested was most likely attributed to the fact that only one predominant species was found in the seed sludge which had been acclimated in a CSTR by stepwise HRT reduction in more than 60 days (Chapter 3). In addition, synthetic wastewater was also in favor of maintaining such a pure microbial composition.

The phylogenetic tree revealed that strains CSTR-G and AFBR-G clustered closely with strain AFBR-B, as indicated by a high bootstrap value of 99% (Figure 6-3). These three strains further formed a very compact phylogenetic cluster with *C. pasteurianum*, and this relationship was robust, as supported by a bootstrap value of 100%. The sequence identities between the three strains were found to be larger than 99%, indicating that strains AFBR-G, CSTR-G and AFBR-B were very closely related and might be distinguishable at the strain level. *C. pasteurianum* is a classic volatile fatty acids (mainly, butyrate and acetate) and hydrogen producer, and its growth might be stimulated by the fermentable carbohydrate, such as sucrose and glucose (Dabrock et al. 1992).

Figure 6-6. A typical distribution of the intensities of the fluorescent lights corresponding to proteins, lipid, α -polysaccharides, dead cells, living cells and β -polysaccharides for granule shown in Figure 6-5A. Distance = 0 refers to granule surface.

It is interesting to note that bacterial species *C. pasteurianum*-like were found predominant in two recent reports studying on the microbial community of hydrogen-producing granules. Wu et al. (2006) found that bacterial compositions in a hydrogen-producing reactor became simple and a shift of microbial population occurred as the HRT decreased from 6 h to 0.5 h. Self-flocculated granules were

formed as the HRT was shortened to 0.5 h, at which a distinct bacterial species was detected by PCR-DGGE analysis whose sequence was found to be highly similar (98% similarity) to that of an uncultured *C. pasteurianum* strain. It should be realized that this bacterial strain was not found before the formation of self-flocculated granules. Fang et al. (2002a) also found that 46.2% of the clones derived from hydrogen-producing granules were most closely related to *C. pasteurianum* with a similarity of 94-96%. These findings seem to indicate that *C. pasteurianum* or the strains close to *C. pasteurianum* might be the crucial microbial species participating in the formation of hydrogen-producing granules.

It follows that microbial population and ECP are likely to play important roles in the formation of granules. A common viewpoint postulates that filamentous bacteria might be favorable for the microbial immobilization of anaerobes (Macleod et al. 1990; Morgan et al. 1991). For example, Wiegant (1998) observed that steady granules could be developed from the precursors mainly containing filamentous *Methanosaeta* in UASB reactors. Initially, *Methanosaeta* form very small aggregates via bridging between microbial cells or microflocs, or attaching to finely dispersed matter. Cellular multiplication and entrapment of non-attached bacteria lead to the growth of the precursors to form granules. However, there were no filamentous bacteria found in the hydrogen-producing granules and biofilms. Contrary to the complex microbial groups present in methanogenic granules, hydrogen-producing granules and biofilms consisted of single species of *Clostridia* because of the simplicity of the acidification process, as observed in the present study. *C. pasteurianum* is a nonhomoacetogenic hydrogen-producing species present either singly or in pairs and excretes intracellular polysaccharide reserve (Fang et al. 2002a). Because of its capability to secrete much more ECP, *C. pasteurianum*-like bacterium, that has a high affinity to attach to all kinds of surface, attach to other microbial cells or carrier materials thus forming granules or biofilm. In that case, ECP would serve as a bio-glue to facilitate *Clostridium* interaction and further strengthen hydrogen-producing aggregate structure through formation of a polymeric network. It can be concluded that, *C. pasteurianum*'s role

as the crucial microbial species to microbial granulation was likely attributed to its strong ability of ECP secretion.

The role of ECP in the microbial granulation had been noted previously. *Methanosarcina*, typical coccoid methanogens, are responsible for the nucleus formation as the anaerobic cultures are fed with acetate, especially at the high OLRs (Bochem et al. 1982; Wu et al. 1996). The formation of *Methanosarcina* granules are believed to be due to their capacity to secrete much more ECP compared to *Methanosaeta* and form clumps of bacteria (Chen and Lun 1993). The granules are developed as the clumps reach macroscopic dimensions and other species, such as *Methanosaeta* tend to inhabit in the *Methanosarcina* clumps.

The ECP composition affects the surface properties of the bacterial flocs, such as surface charges and hydrophobicity (Liao et al. 2001; Liu et al. 2003b). It should be stressed that the key ECP contents of *Clostridia*-predominated granules and biofilms are distinctly different with those of methanogenic granules, and the roles of ECP contents might also be different in the process of granulation. The ECP content of methanogenic granules has been reported to range between 0.6 and 20% of the VSS content, depending on the granular sludge examined, the extraction method, and the analytical methods for ECP (Schmidt and Ahring 1996). The ECP in methanogenic granules consists mainly of proteins and polysaccharides, typically in a ratio of 2:1-6:1. The same ECP composition was found for hydrogen-producing granules and biofilms compare with methanogens, but rather low ratios of proteins to polysaccharides ranging from 0.2 to 0.6 were observed in the present and other studies (Fang et al. 2002a; Mu and Yu 2006). Because of such a significant difference, some researchers pointed out that polysaccharides might be the key ECP constituent for hydrogen-producing granules and hence might play a more important role in the formation of hydrogen-producing granules, but proteins for methanogenic granules (Fang et al. 2002a; Mu and Yu 2006). In contrast to other ECP contents, however, the relative abundance of proteins in the granule centres suggested a possible mechanism involved in the evolution of hydrogen-producing granules (Figure 6-4a, Figure 6-5 and Figure 6-6). In such a hypothesis, microbial

aggregation starts as a protein-rich matrix or nucleus is formed. The protein matrix might contain microbial cells and proteins that could be enriched by secretion of the attached cells or by entrapping extraneous proteins in a form of macromolecules, colloids and slimes. Based on this hypothesis, many granulation phenomena due to acid incubation could be explained. The acid incubation stimulated the production of ECP proteins, and the concentration of ECP proteins could reach a relatively higher level within a short period, which promoted the process of granulation. On the other hand, a lower accumulation rate of ECP proteins under normal conditions without the acidification might limit the formation of hydrogen-producing granules in the control CSTR and AFBR.

6.5 Conclusions and Recommendations

The present study demonstrated that it is possible to achieve rapid formation of hydrogen-producing granules in the CSTR and AFBR by acidifying microbial culture for 24 h, whereas hydrogen-producing biofilms formed rapidly in the AFBRs with or without the acidification. Comparison of physical properties, and microbial composition and distribution suggested that both hydrogen-producing granules and biofilms could be considered as a single entity, viz. particle-based biofilms. As a consequence, comparable hydrogen production performance in terms of hydrogen yield and specific hydrogen production rate was demonstrated by both the granular sludge and biofilms.

Phylogenetic analysis of the microbial population in the granules and biofilms showed that only one distinct bacterial strain existed in respective microbial community, which exhibited a high similarity to the species of *C. pasteurianum*. Because *C. pasteurianum* was also found abundant in hydrogen-producing granules in the previous studies, it is postulated that *C. pasteurianum* might exist as the crucial bacterial species in the hydrogen-producing community in the formation of granules and biofilms. The strong agglutination property of *C. pasteurianum* might be attributed to its high productivity of ECP which would serve as a bio-glue to facilitate cell-to-cell or cell-to-substratum interaction. As abundant proteins were

noted in the granule cores, it can be derived that rapid formation of the hydrogen-producing granules could be due to the establishment of precursor protein-rich microbial nuclei.

The phylogenetic analysis showed that microbial diversity between CSTR-granules, AFBR-granules, and AFBR-biofilms was quite similar and only distinguishable in a strain level. Such a bacterial discrepancy in those microbial communities was not investigated in detail. Microbial mutation may occur. Gene cloning and structural analysis plus physiological characterization may be conducive to identifying and distinguishing those bacterial strains.

The physiology of bacteria in the granulation is of great importance for the control of granulation and interactions between the granules and the environment ([Schmidt and Ahring 1996](#)). Investigation on physiological changes of microbial culture during the acid incubation would be in favor of understanding the mechanisms involved in the granulation induced by the acidification, which is highly recommended.

Although the sizes of granules and biofilms were measured in the present study, the size effect on hydrogen production rate was not investigated. Because of most living cells locating outside of particles, smaller granules or thinner biofilms might enhance the hydrogen production efficiency. One challenge remains here is the control of biofilm thickness and granule size.

CHAPTER 7 A COMPARISON STUDY ON HYDROGEN-PRODUCING BIOSYSTEMS

7.1 Introduction

A CSTR (Figure 7-1a) is frequently used for continuous hydrogen production (Chen and Lin 2003; Majizat et al. 1997; Yu et al. 2003a). In such a conventional system, hydrogen-producing bacteria are well suspended in the mixed liquor and less suffered from the mass transfer resistance, but biomass retention is substantially influenced by the culture HRT and washout of biomass may occur at a shorter HRT. Hydrogen production rates are thus restricted considerably by a low CSTR biomass retention and a low hydraulic loading (Lay et al. 1999; Yu et al. 2002). Immobilized-cell systems have become popular alternatives to suspended-cell systems in continuous operations since they are more capable of maintaining higher biomass concentration and could operate at high dilution rates without biomass washout.

Biomass immobilization can be achieved through forming granules (Fang et al. 2002a), biofilms (Palazzi et al. 2000) or gel-entrapped bioparticles (Wu et al. 2003). Previous studies suggested that biofilms and granules showed advantages compared to the cell-entrapped bioparticles in the hydrogen production (Table 2-2). In continuous cultures without pH control, Yokoi et al. (1997a) found that hydrogen production rates and glucose consumption rates with *C. butyricum* immobilized on porous glass beads are higher than the corresponding values with cells immobilized in agar gel at both HRTs of 3 h and 5 h. In another study with a culture of *E. aerogenes* strain HO-39, Yokoi et al. (1997b) compared the hydrogen-producing properties of free cells, and cells immobilized in agar gel, or on porous glass beads in continuous cultures. The hydrogen production rate for agar gel and porous glass beads increased with decreasing HRT, but not so for free cells. The hydrogen production rate for glass beads was superior to that of agar gels at HRTs less than 3 h, and reached the maximum of about 0.85 L/L·h at a HRT of 1 h. The results

indicate that biofilm attachment on solid or porous supports has an advantage in improvement of hydrogen production rate compared to gel-entrapped bioparticles. The inferior performance of gel-immobilized sludge processes is mainly attributed to the low mass transfer efficiency. Furthermore, stability and durability of gel-entrapped bioparticles are generally questionable. Hence, gel immobilization techniques may not be the technology of choice for fermentative hydrogen production. Those immobilized hydrogen-producing cultures have been employed in different reactor systems as shown in [Figure 7-1b-f](#), i.e. CSTR, fixed- or packed-bed reactor, fluidized bed reactor, UASB reactor, and trickling biofilter. From the viewpoint of reactor configuration, those reactor systems can be divided into two types: tank-shaped reactor and column-shaped reactor.

Hydrogen production performances of immobilized-cell systems, however, are conflicting. For example, hydrogen production rate varying from 0.20 L/L·h with granules in an UASB reactor ([Yu and Mu 2006](#)) to 15.09 L/L·h with silicone-immobilized and self-flocculated granular sludge in a CSTR ([Wu et al. 2003](#)) as shown in [Table 2-2](#). It seems to indicate that microbial cell immobilization methods and reactor configuration play a crucial role in hydrogen production rate. Nevertheless, it is stressed that those systems contained different microbial cultures and were operated under different conditions. This should be taken into consideration when making comparison in the system performance. To realize a valid comparison, it is of importance to maintain a homogenous microbial culture in the reactors while operating under the same conditions. As tabulated in [Table 2-2](#), both biofilm ([Lee et al. 2003](#)) and granular sludge ([Lee et al. 2004](#); [Wu et al. 2006](#)) exhibited superior hydrogen production rates compared to gel-entrapped bioparticles ([Wu et al. 2003](#); [Yokoi et al. 1997a](#)). However, comparative studies between biofilm and granular sludge or between tank reactor and column reactor for hydrogen production remain limited so far. The present study was conducted to fill up the knowledge gap by summarizing the experimental results obtained.

Figure 7-1. Schematic diagram of hydrogen-producing reactors (a) conventional CSTR containing suspended sludge, (b) immobilized-cell CSTR, (c) fixed/packed-bed reactor, (d) fluidized bed reactor, (e) UASB reactor, and (f) trickling biofilter.

The objective of the present study was to evaluate the system performance with respect to hydrogen production and system stability. These systems included suspended sludge CSTR system, granular sludge CSTR system, granular sludge AFBR system and biofilm AFBR system.

7.2 Materials and Methods

7.2.1 Experimental Approach

Four biosystems were used in the present study, this is, suspended sludge CSTR system, granular sludge CSTR system, granular sludge AFBR system and biofilm AFBR system. The experimental materials including inoculum, substrate and setup, and operating conditions for the CSTR system and AFBR systems had been described in [Chapters 3, 4 and 5](#). The experimental data obtained were summarized in this chapter to evaluate the hydrogen production performance of those biosystems. In addition, a further evaluation on hydrogen production in the AFBRs with granular sludge and biofilm sludge was conducted at a pH of 5.5 and 37°C. The experimental protocols are as follows: Two AFBRs designated as Granule reactor and Biofilm reactor, respectively were run with HRTs of 0.25 h, 0.5 h, 0.75 h, 1 h, 1.5 h, 2 h, 3 h and 0.125 h in series, corresponding to influent glucose concentrations of 10, 20, 30, 40, 60, 80, 120 and 5 g/L to maintain a constant OLR of 40 g-glucose/L·h. The HRT was calculated on the basis of the total reactor volume, while the OLR was computed from the concentration of glucose loaded to the reactor per unit HRT. Pseudo steady-state conditions at each HRT level were considered attained when the biogas production and glucose conversion rate were relatively consistent within 5% for three consecutive days. Evaluation of the system performance was carried out during the pseudo steady-state conditions.

7.2.2 Analytical Methods

The analytical protocols can be found in [Chapter 3](#).

7.3 Results and Discussion

7.3.1 Suspended Sludge vs. Granular Sludge

As stated earlier ([Chapter 3](#)), biomass retention of suspended bacteria was related to the operating HRT in a completely mixed hydrogen-producing reactor. A relatively consistent biomass concentration of 0.84-1.01 g-VSS/L was observed as the HRT was operated in a range of 6-12 h ([Table 3-1](#)). However, further reducing HRT caused a substantial decrease in biomass retention to 0.33 g-VSS/L, and complete washout of hydrogen-producing bacteria took place at a HRT of 2 h, corresponding to a D of 0.5 h^{-1} . This suggests that the specific growth rate ($\mu_{\max}$) of hydrogen-producing culture used in the present study is close to 0.5 h^{-1} . The $\mu_{\max}$ value is the same to that obtained by Ghosh which was referred in a previous study ([Van Ginkel and Logan 2005b](#)) and is higher compared with those ($0.14\text{-}0.33 \text{ h}^{-1}$) reported by other researchers ([Chen et al. 2001](#); [Horiuchi et al. 2002](#)). It is noted that mixed cultures of hydrogen-producing bacteria were used and operated at different conditions in these aforementioned studies, and it is likely that they exhibit inconsistent growth rates due to the diversity of microbial population.

In contrast to the system upset occurring at a HRT of 2 h, steady hydrogen production was observed in the suspended-cell CSTR as the HRT ranged from 3 h to 12 h. [Figure 7-2a](#) illustrates the effect of HRT on hydrogen yield, hydrogen production rate and specific hydrogen production rate in the suspended-cell CSTR. Hydrogen yield stabilized at $1.92 \text{ mol-H}_2/\text{mol-glucose}$ at HRTs of 6-12 h, but it decreased substantially to $0.97 \text{ mol-H}_2/\text{mol-glucose}$ as the HRT was shortened to 3 h. Specific hydrogen production rate increased from 8.33 to $16.09 \text{ mmol-H}_2/\text{g-VSS}\cdot\text{h}$ with the decreased HRT from 12 h to 3 h, whereas hydrogen production rate increased from $0.21 \text{ L/L}\cdot\text{h}$ at a HRT of 12 h to $0.32 \text{ L/L}\cdot\text{h}$ at 6 h HRT, but decreased to $0.13 \text{ L/L}\cdot\text{h}$ when HRT was shortened to 3 h.

Figure 7-2. Hydrogen production by suspended sludge (a) and granular sludge (b) in a CSTR.

In contrast to the suspended sludge, granular sludge concentration increased with the reduction in HRT (Figure 7-3). The maximum concentration of granular sludge was estimated to be 16.0 g-VSS/L in the granule-based CSTR at a HRT of 0.5 h, which was far higher than that obtained in suspended-cell system. It was found that granules disaggregated and eventually evolved into dispersed cells at a HRT of 3 h, whose performance in hydrogen production was found to be close to the suspended sludge system at the same HRT, suggesting that HRT might play a crucial role in the stability of microbial granules. A HRT equal to or shorter than the critical washout point of suspended sludge is required in a granular sludge CSTR. This is consistent with the hypothesis proposed by Tijhuis et al. (1994) who stated that, in ideally mixed reactors, formation of biofilm covered carriers only takes place as the HRT is shorter than the inverse of the maximum growth rate (the dilution rate is larger than the maximum growth rate, $D > \mu_{\max}$).

Hydrogen yield, hydrogen production rate and specific hydrogen production rate increased significantly with the reduction in HRT from 2 h to 0.5 h as shown in Figure 7-2b. Their respective maximum values of 1.81 mol-H₂/mol-glucose, 3.20 L/L·h and 8.31 mmol-H₂/g-VSS·h were recorded at a HRT of 0.5 h. A HRT of 0.5 h was thus suggested as the optimum operating condition for the granular sludge, which was much shorter as compared with a HRT of 6 h required for the suspended sludge in the same CSTR. The ability to retain a large amount of biomass of granular sludge at a shorter HRT condition allowed high-rate hydrogen production to be accomplished in the granule-based CSTR system. As a sequence of the

enriched granular sludge, granular sludge exhibited a ten-fold increase in hydrogen production rate while keeping a comparable hydrogen yield to the suspended sludge.

Figure 7-3. Biomass concentrations of suspended sludge and granular sludge.

7.3.2 Granular Sludge vs. Biofilm Sludge

Granular sludge and biofilm sludge hydrogen production had been evaluated in [Chapter 5](#), and the characteristics of granules and biofilms were evaluated in [Chapter 6](#). The results indicated a comparable hydrogen production performance of granules and biofilms in terms of hydrogen yield and specific hydrogen production rate due to the presence of physical, physicochemical and biological similarities between granular sludge and biofilm sludge. However, a major drawback mostly cited in other biofilm processes is the requirement of a large amount of carrier materials. On the one hand, supporting materials occupy a considerable space in the reactor, which reduce the effective volume and hence reactor performance and efficiency. On the other hand, supporting materials need to be replaced periodically due to wear and tear. The cost of material replace could be a major economic consideration of maintenance.

It should be realized that the evaluation of biofilm process for biohydrogen production in Chapter 5 was only based on the data derived from a short period operation of about 20 days, and a much longer operation seems to be necessary to recognize its applicability. Therefore, an extended operation was conducted to evaluate hydrogen production by granular sludge and GAC-supported biofilm sludge in two AFBRs, designated as Granule reactor and Biofilm reactor, respectively. The HRT varied from 0.125 h to 3 h, corresponding to the change of glucose concentration from 5 g/L to 120 g/L to keep the OLR at a constant level of 40 g-glucose/L·h.

A close performance in hydrogen production was observed while two types of immobilized cultures were tested at different HRTs and influent substrate concentrations as shown in Figure 7-4. Hydrogen content increased slightly from 38% to 48% of the biogas produced as the HRT increased from 0.125 h to 3 h in the Granule reactor and Biofilm reactor with a similar variation trend Figure 7-4a. Hydrogen yield and hydrogen production rate increased as the HRT was shortened from 3 h to 0.25 h and decreased substantially with further reduction in HRT to 0.125 h (Figure 7-4b and c). While hydrogen yield at a given HRT was comparable for both reactors, a slightly higher hydrogen production rate was found in the Biofilm reactor. The hydrogen yield obtained in both reactors was within a range of 0.4-1.7 mol-H₂/mol-glucose with the maximum yield occurring at a HRT of 0.25 h and a glucose concentration of 10 g/L, in which hydrogen production rate also reached the respective maximum values of 7.6 and 6.6 L-H₂/L·h in Biofilm reactor and Granule reactor. It was noted that a 1.1 L-H₂/L·h increase in hydrogen production rate was achieved in Biofilm reactor under the optimum operating conditions as compared with Granule reactor. This was due to the relatively high glucose conversion rates obtained in Biofilm reactor as shown in Figure 7-4d. Glucose conversion rate in Biofilm reactor and Granule reactor were determined to be around 80% at HRT ranging between 0.25 h and 1.0 h, corresponding to glucose concentration of 10-40 g/L. Significant decrease in glucose conversion rate was noted when the HRT and glucose concentration reduced to 0.125 h and 5 g/L, at which 44% and 54% of the influent glucose were utilized. Such a decrease in

glucose conversion rate was also found when the HRT increased to 3 h, corresponding to an increase in glucose concentration to 120 g/L.

Based on the above observation and on hydrogen yield and hydrogen production rate, the optimal operating HRT and glucose concentration were determined to range between 0.25-0.75 h and 10-30 g/L for Biofilm reactor and Granule reactor, respectively. Shortening the HRT to 0.125 h caused inferior hydrogen fermentation, and a HRT of 0.25 h was considered to be critical for the AFBRs, regardless of biofilm or granule system. This value was at least one-fold lower than the HRT required by other hydrogen production bioreactors at optimum hydrogen production. For examples, Lee et al. (Lee et al. 2004) found a substantial decrease in hydrogen yield and hydrogen production rate when a granule-based column reactor was operated at a HRT of 0.25 h as compared with the peaks achieved at 0.5 h HRT. Furthermore, it was recognized that the HRT ranged between 1 h and 2 h for other immobilized-cell biosystems, i.e. UASB reactor (Yu et al. 2002) and packed-bed reactor (Chang et al. 2002) for optimum hydrogen production. It is speculated that a lower critical HRT obtained is attributed to the good mixing characteristics of bioparticles under fluidization state. On the other hand, while both reactors were operated at a glucose concentration of up to 30 g/L, satisfactory hydrogen production could still be achieved. This implied that critical glucose concentration was around 30 g/L for the present system as significant reduction in hydrogen yield and hydrogen production rate occurred when glucose concentration was further increased to 40 g/L for both reactors. This critical concentration of substrate was in agreement with the reported values for the granule- or biofilm-based processes (Wu et al. 2006). It was noted that inhibitory concentration of substrate on acidogenic bacteria was estimated to be about 5-6 g-glucose/L in the suspended cultures (Majizat et al. 1997; Van Den Heuvel and Beefink 1988). These results imply that microorganisms in the biofilm and granule biomass matrix might react more actively under a stressful environment in comparison to the biomass in the form of suspension.

Obviously, there was no apparent difference in hydrogen production between Granule reactor and Biofilm reactor as observed in the present study. This is largely owing to the relatively consistent and comparable biomass concentration maintained in both reactors as shown in [Figure 7-5](#). Biomass concentration in the reaction zone was maintained consistently within the ranges of 61-65 g-VSS/L and 61-66 g-VSS/L at HRTs between 0.125 h to 2 h for the Granule reactor and Biofilm reactor, respectively. Considering the total volume of reactor (1.4 L), overall biomass concentration corresponded to 34.7-36.9 g-VSS/L and 34.3-37.6 g-VSS/L, respectively. This indicated that adding support media in Biofilm reactor did not reduce the biomass retention as compared with the granule-based reactor. [Figure 7-6](#) reveals that proportion of biofilm decreased significantly with an increase of granular biomass in the Biofilm reactor. It was observed that with the evolution of biofilm, the thickness of biofilm increased significantly and attachment of microorganisms to the support media might become weaker. As a result, biofilm was separated from support media due to particle-particle collision, leaving fragmented biofilm in the reactor. These fragmented biofilm eventually developed into granules which had predominated over biofilm in Biofilm reactor after being operated for 50 days. As washout of support carriers is not a concern of granular sludge systems, this poses to be an advantage of the systems.

Figure 7-4. Effects of HRT and glucose concentration on (a) hydrogen content, (b) hydrogen yield, (c) hydrogen production rate, and (d) glucose conversion rate in Granule reactor and Biofilm reactor.

Figure 7-5. Effects of HRT and glucose concentration on biomass concentration in Granule reactor and Biofilm reactor.

These findings indicate that biofilm process might be not suitable for anaerobic hydrogen fermentation due to the rapid growth characteristics of hydrogen-producing bacteria. As stated earlier, $\mu_{\max}$ and yield coefficient of hydrogen-producing bacteria (0.12 g-VSS/g-glucose, [Appendix C](#)) would result in the production of a large amount of biomass (ca. 72-127 g-VSS per day) under an OLR (40 g-glucose/L·h). The maximum specific growth rate and biomass growth yield were also found in other studies, ranging from 0.17 to 0.5 h⁻¹ and from 0.08 to 0.33 g-VSS/g-COD, respectively. Furthermore, higher organic loading rates, such as 40 g-glucose/L·h in the present study and up to 80 g-COD/L·h in a study conducted by Wu et al. (2006), were generally employed in the anaerobic hydrogen fermentation. In a recent review, Nicolella (2000a) had mentioned that biofilm reactors are not particularly useful when fast-growing organisms (i.e. with a maximum specific growth rate > 0.1 h⁻¹). Rapid buildup of hydrogen-producing biofilms could result in a system upset due to the mass transfer limitation. Working on a trickling biofilter, for example, Oh et al. (2004) noted that microbial growth of hydrogen-producing

bacteria was too excessive under a mesophilic condition, which caused system upset just after one-week of operation.

Figure 7-6. Distribution profile of biofilm sludge and granule sludge in Biofilm reactor.

7.3.3 CSTR vs. AFBR

As stated earlier, there are two types of reactors used in hydrogen fermentation. The influence of reactor configuration on hydrogen production was evaluated by examining granular sludge hydrogen production in the CSTR and AFBR. Maximum hydrogen production rates of the suspended sludge, CSTR granular sludge, AFBR granular sludge and biofilm sludge were essentially correlated to the biomass concentration as shown in [Figure 7-7](#), indicating that the system performance is largely influenced by the reactor biomass retention. Compared to the CSTR, much more biomass retention was retained in the column reactors (35 vs. 16 g-VSS/L), which might be attributed to their intrinsic structure. A larger height/section diameter ratio of column reactors might be the advantages favoring solid/liquid separation and biomass retention, and hence the hydrogen production.

Figure 7-7. Hydrogen production rate ($R^2 = 1.00$, $p = 0.0002$) as a function of biomass concentration.

Since the recirculation flow rate was kept at consistently at 200 mL/min, its effect on mass transfer and reactor performance was not examined in the present study. Nevertheless, it is possible that increasing liquid recirculation ratio might improve mass transfer of immobilized culture and substrate in a column reactor. Fixed- or packed-bed reactor is operated under the conditions with a lesser extent of hydraulic turbulence, thus its immobilized cultures usually encounter mass transfer resistance which would result in inferior rates of substrate conversion and hydrogen production. Rachman et al. (1998) found that high hydrogen molar yield could not be maintained consistently in a packed-bed reactor, although the pH in the effluent was controlled at more than 6.0. This was owing to pH gradient distribution along the reactor column resulting in a heterogeneous distribution of microbial activity. In order to overcome the mass transfer resistance and pH heterogeneous distribution, they recommended that fluidized bed or expended bed reactor system with recirculation flow are more appropriate for further enhancing the hydrogen production rate and yield. In a later study, Kumar and Das (2001) observed that hydrogen production and substrate conversion rates of a packed-bed reactor both increased with the recycling ratio, and obtained the maximum hydrogen production

rate of 1.69 L/L·h at a recirculation ratio of 6.4. This means that the mass transfer resistance in a packed-bed reactor can be reduced by increasing slurry recycle ratio.

Appropriate reactor configuration would help to increase hydraulic turbulence of the column reactors, and result in an increase in hydrogen production. Kumar and Das (2001) investigated hydrogen production by *E. cloacae* attaching on coir in packed bed reactors with different configurations, i.e. tubular, tapered or rhomboid shape at a HRT of 1.08 h. The comparative study indicated that the rhomboid bioreactor with convergent-divergent configuration has a maximum hydrogen production rate of 1.60 L/L·h as compared with 1.46 L/L·h in tapered reactor and 1.40 L/L·h in tubular reactor. The enhancement in hydrogen production rate could be attributed to higher turbulence created by reactor geometry favorable mass transfer and reduced gas hold-up.

7.4 Conclusions

Formation of granules or biofilms substantially enhanced biomass retention. Reactor volumetric hydrogen production rate was highly related to the biomass retention. Rapid growth properties of hydrogen-producing cultures and higher OLR conditions might limit the application of biofilm processes to anaerobic hydrogen production since excessive production of fermentative biomass resulted in the washout of support carriers in a fluidized bed reactor or system upset in a fixed-bed reactor. As no support carrier is needed by the granule-based systems, this poses to be an advantage of the systems. It is concluded that the column-shaped reactor with granular sludge is a preferred choice of continuous biohydrogen production systems.

CHAPTER 8 CONCLUSIONS AND RECOMMENDATIONS

8.1 Conclusions

After an extensive investigation, two major tasks were completed in this dissertation: (1) an approach of acid incubation to rapidly initiate granulation of hydrogen-producing sludge was developed, which substantially promoted the granulation of hydrogen-producing culture in both CSTR and AFBR systems; (2) a column-shaped reactor with granular sludge was found most favorable for continuous hydrogen production. Fundamental studies were also carried out to provide insight into the formation mechanisms of hydrogen-producing granules and biofilms. The experimental results obtained enabled the following conclusions to be made:

During the acclimation of anaerobically digested sludge, the hydraulic retention time (HRT) appears to be a significant hydrodynamic selection on the mixed-microbial populations. A stable microbial population was established by washing out propionate producing bacteria as the HRT is shortened to 6 h. An increase in the hydrogen yield from 1.6 mol-H₂/mol-glucose to 1.9 mol-H₂/mol-glucose was measured when the HRT was shortened from 8 h to 6 h. Arising from the stabilized microbial population, the hydrogen composition and yield were maintained at about 64% and 1.9 mol-H₂/mol-glucose, respectively, in the subsequent operation at 8-12 h HRT.

An approach to initiating rapid formation of hydrogen-producing granules was originally demonstrated with an anaerobic CSTR. Compared with the absence of granules in the control run, applying acid incubation on the culture demonstrated an ability to initiate microbial granulation of sludge within 114 h of operation. The granulation was much shorter than that normally required of 2-6 months reported in other studies. Subsequently, effectiveness of the acid incubation on rapid microbial granulation was validated with an AFBR. However, applying acid incubation to the

hydrogen-producing culture did not shorten the time required by biofilm attachment on GAC support carrier. GAC-supported biofilm was able to spontaneously form within 36 h without the acid incubation.

Experimental results indicated that lowering the culture pH to 2.0 could result in improvement in bacterial surface physicochemical properties favoring microbial agglutination and immobilization, including reduced electrostatic repulsion, improved hydrophobic surface, more ECP secretion and a higher ratio of extracellular proteins/polysaccharides. The improved zeta potential, hydrophobicity and extracellular protein better facilitated the interactions between microbial cells which accelerated granule development. By comparing the formation of biofilms and granules, it is concluded that initial nuclei were important for microbial immobilization of hydrogen-producing bacteria and acid incubation substantially promoted the development of microbial nuclei and granules.

Phylogenetic analysis of the microbial population in the granules and biofilms showed that only distinct bacterial strain existed in the respective microbial community, which exhibited a high similarity (98%) to the species of *C. pasteurianum*. Because *C. pasteurianum* was also found abundant in hydrogen-producing granules in previous studies, it was believed that *C. pasteurianum* might be the crucial species in the hydrogen-producing community to the formation of granules and biofilms. The strong agglutination property of *C. pasteurianum* might be attributed to its high productivity of ECP which would serve as a bio-glue to facilitate cell-to-cell or cell-to-substratum interaction. As abundant proteins were noted in the granule cores indicated by the confocal laser scanning microscope images, it can be derived that rapid formation of the hydrogen-producing granules could be due to the establishment of precursor protein-rich microbial nuclei.

Formation of granular sludge and biofilm sludge substantially enhanced biomass retention, and hydrogen production rate was highly related to the reactor biomass retention. In contrast to a hydrogen production rate of 0.32 L/L·h obtained in the CSTR with suspended sludge, a 10-fold and a more than 20-fold increases in

hydrogen production rate while keeping comparable hydrogen yields ranged from 1.66-1.81 mol-H₂/mol-glucose were achieved in the granule-based CSTR and granule-based or biofilm-based AFBRs, respectively. Although a close performance in hydrogen production was demonstrated by granular sludge and biofilm sludge in the AFBRs, substantial washout of biofilms occurred and reactor biomass was replaced completely after the operation of 50 days. It is concluded that a column-typed reactor with rapidly formed granular sludge may work as a high-rate system for continuous hydrogen fermentation.

8.2 Limitations and Recommendations

It should be pointed out that all immobilized-cell systems for continuous hydrogen production were investigated only in laboratory scale, and scale-up study has not yet been examined. In addition, these studies were all performed on soluble glucose-rich wastewater, rather than on particulate wastewaters. Commercial methane-producing granule-based UASB reactors are undesirable when operating on substrates with high suspended solids contents ([Hawkes et al. 2007](#)). The same rationale might be applicable to hydrogen-producing granule reactors. The challenge now is to integrate what has been reported in this thesis into practical operation on industrial particulate-free or particulate feedstocks in the full scale.

From this thesis, it has been shown that it should be possible to promote the granulation by acidifying anaerobically digested sludge enriched with *C. pasteurianum*-like microorganisms in a tank-type reactor or a column-type reactor. The question arises as to whether or not acid incubation happens to be effective on microbial granulation due to the presence of *C. pasteurianum* in microbial community. Applicability of the acid incubation approach developed in this work to other bacterial species remains unclear and further testing on the inocula from different natural sources such as compost and soil is hence recommended from an engineering and application perspective.

Lowering culture pH to 2.0 immediately altered bacterial surface charges and created a benign electrostatic environment for microbial aggregation. Although a similar trend was observed in the CSTR and AFBR cultures that charge numbers reduced with the acid incubation, zeta potentials decreased from -11.6 mV to -3.5 mV in the CSTR, while from -25.4 to 11.9 in the AFBRs. The most plausible factor resulting in an inconsistent measurement would appear to be the difference in floc sizes ([Chapter 5](#)). This also indicates that culture conditions have an important influence on the bacterial electrostatic properties. A pretest determining isoelectric pH of different hydrogen-producing cultures is recommended to minimize the electrostatic repulsive forces. Since the improvement of bacterial surface physicochemical characteristics was able to achieve immediately with the acid incubation, a 24-h incubation time used in the present work may be shortened, which would help activate hydrogen-producing bacteria from the acid incubation.

Although phylogenetic analysis in [Chapter 6](#) showed that microbial diversity between CSTR-granules, AFBR-granules and AFBR-biofilms was quite similar and only different in a strain level, bacterial discrepancy in those microbial communities was not investigated in detail. Microbial mutation may occur. Gene cloning and structural analysis plus physiological characterization may be conducive to identifying and distinguishing those microorganisms.

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APPENDIX A BACTERIAL SEQUENCES

1. Bacterial strain CSTR-G (Accession number: EF656617 AB308403)

GCGTAACAAGGTAGGCTCTGTAAGGCGGCGGACGGGTGAGTAACACGT
GGGTAACCTGCCTCAAAGAGGGGAATAGCCTCCCGAAAGGGAGATTAA
TACCGCATAATATTACAGCTTCGCATGAAGCAGTAATTAAAGGAGTAAT
CCGCTTTGAGATGGACCCGCGGCGCATTAGCTAGTTGGAGAGGTAACGG
CTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCAC
ATTGGAAGTGAACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGG
AATATTGCACAATGGGCGAAAGCCTGATGCAGCAACGCCGCGTGAGTG
ATGACGGTCTTCGGATTGTAAAGCTCTGTCTTTTGGGACGATAATGACG
GTACCAAAGGAGGAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTA
ATACGTAGGTGGCAAGCGTTGTCCGGATTTACTGGGCGTAAAGGATGTG
TAGGCGGATACTTAAGTGAGATGTGAAAGCCCCGGGCTTAACTTGGGGA
CTGCATTTCAAACCTGGGTGTCTAGAGTGCAGGAGAGGAAAGCGGAATTC
CTAGTGTAGCGGTGAAATGCGTAGAGATTAGGAAGAACATCAGTGGCG
AAGGCGGCTTTCTGGACTGTAAGTACGCTGAGGCATGAAAGCGTGGGG
AGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTA
CTAGGTGTAGGAGGTATCGACTCCTTCTGTGCCGCAGTAAACACAATAA
GTACTCCGCCTGGGAAGTACGGTCGCAAGATTAAAACTCAAAGGAATTG
ACGGGGGCCCCGCACAAGCAGCGGAGCATGTGGTTTAATTCGAAGCAAC
GCGAAGACCCTTACCTAGACTTGACATCTCCTGAATAGCGTAGAGATAC
GTGAAGCCCTTCGGGCCAGGAAAACAGGTGGTGCATGGTTGTCGTCAGC
TCGTGTCGTGAGATGTTGGGTAAAGTCCCGCAACGAGCGCAACCCTTAT
CATTAGTTGCTACCATTAAGTTGAGCACTCTAGTGAGACTGCCCGGGTT
AACCGGGAGGAAGGCGGGGATGACGTCAAATCATCATGCCCCTTATGTC
TAGGGCTACACACGTGCTACAATGGTGAGAACAAACGAGATGCAATACC
GCGAGGTGGAGCCAACTTGAAAACATCCCAGTTCGGATTGTAGGCT
GAAATTCGCCTACATGAAGTTGGAGTTGCTAGTAATCGCGAATCAGAAT
GTCGCGGTGAATACGTTCCCGGGCCTTGTAACACACCGCCCGTCACACCA
TGAGAGCTGGTAACACCCGAAAGAGTTGAGCTATCC

2. Bacterial strain: AFBR-G (Accession number: EF656616 AB308402)

GGTTTAAAGCGGCGGACGGGGGAGTAACACGTGGGTAACCTGCCTCAA
 AGAGGGGAATAGCCTCCCGAAAGGGAGATTAATACCGCATAATATTAC
 AGCTTCGCATGAAGCATGTAATTAAAGGAGTAATCCGCTTTGAAATGGA
 CCCGCGCCGCATTAGCTAATTGGAGAGGTAACGGCTCACCAAGGCAACG
 ATGCGTAGCCGACCTGAAAGGGTGATCGGCCACATTGGAAGTGAAGACA
 CGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGG
 GCGAAAGCCTGATGCAGCAACGCCGCGTGAGTGATGACGGTCTTCGGAT
 TGTAAGCTCTGTCTTTTGGGACGATAATGACGGTACCAAAGGAGGAAG
 CCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGC
 GTTGTCCGGATTTACTGGGCGTAAAGGATGTGTAGGCGGATACTTAAGT
 GAGATGTGAAAGCCCCGGGCTTAACCTGGGGACTGCATTTCAAAGTGGG
 TGTCTAGAGTGCAGGAGAGGAAAGCGGAATTCCTAGTGTAGCGGTGAA
 ATGCGTAGAGATTAGGAAGAACATCAGTGGCGAAGGCGGCTTTCTGGA
 CTGTAAGTACGCTGAGGCATGAAAGCGTGGGGAGCAAACAGGATTAG
 ATACCCTGGTAGTCCACGCCGTAAACGATGAGTACTAGGTGTAGGAGGT
 ATCGACTCCTTCTGTGCCGCAGTAAACACAATAAGTACTCCGCCTGGGA
 AGTACGGTCGCAAGATTAAAGTCAAAGGAATTGACGGGGGGCCCGCAC
 AAGCAGCGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACC
 TAGACTTGACATCTCCTGAATAGCGTAGAGATACGTGAAGCCCTTCGGG
 GCAGGAAGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCTGTGAGATG
 TTGGGTAAAGTCCCGCAACGAGCGCAACCCTTATCATTAGTTGCTACCAT
 TAAGTTGAGCACTCTAGTGAGACTGCCCGGGTTAACCGGGAGGAAGGC
 GGGGATGACGTCAAATCATCATGCCCTTATGTCTAGGGCTACACACGT
 GCTACAATGGTGAGAACAACGAGATGCAATACCGCGAGGTGGAGCCAA
 ACTTGAAAACATCCCAGTTCGGATTGTAGGCTGAAATTCGCCTACAT
 GAAGTTGGAGTTGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACG
 TTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGCTGGTAACA
 CCCGATGTCCGTGAGGTAACCTCATAGACCAGCTGCCGCAACAGT

3. Bacterial strain: AFBR-B (Accession number: EF656615 AB308401)

GCGTGGCGCGTGCTTACCATGCAGTCGAGCGAGAAACCTTCGGGTTTCT
AGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTCAAAGAGGGG
AATAGCCTCCCGAAAGGGAGATTAATACCGCATAATATTACAGCTTCGC
ATGAAGCAGTAATTAAAGGAGTAATCCGCTTTGAGATGGACCCGCGGCG
CATTAGCTAGTTGGAGAGGTAAACGGCTCACCAAGGCGACGATGCGTAGC
CGACCTGAGAGGGTGATCGGCCACATTGGAACCTGAGACACGGTCCAAA
CTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCC
TGATGCAGCAACGCCGCGTGAGTGATGACGGTCTTCGGATTGTAAAGCT
CTGTCTTTTGGGACGATAATGACGGTACCAAAGGAGGAAGCCACGGCTA
ACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGG
ATTTACTGGGCGTAAAGGATGTGTAGGCGGATACTTAAGTGAGATGTGA
AAGCCCCGGGCTCAACTTGGGGACTGCATTTCAAACCTGGGTGTCTAGAG
TGCAGGAGAGGAAAGCGGAATTCCTAGTGTAGCGGTGAAATGCGTAGA
GATTAGGAAGAACATCAGTGGCGAAGGCGGCTTTCTGGACTGTAAGTGA
CGCTGAGGCATGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGT
AGTCCACGCCGTAAACGATGAGTACTAGGTGTAGGAGGTATCGACTCCT
TCTGTGCCGCAGTAAACACAATAAGTACTCCGCCTGGGAAGTACGGTCC
CAAGATTAAAACTCAAAGGAATTGACGGGGGGCCCGCACAAGCAGCGGA
GCATGTGGTTTAAATTCGAAGCAACGCGAAGAACCTTACCTAGACTTGAC
ATCTCCTGAATAGCGTAGAGATACGTGAAGCCCTTCGGGGCAAGAAGAC
AGGTGGTGCATGGTTGTCGTCAGCTCGTGTCTGAGATGTTGGGTAAAG
TCCCGCAACGAGCGCAACCCTTATCATTAGTTGCTACCATTAAGTTGAG
CACTCTAGTGAGACTGCCCCGGGTAAACCGGGAGGAAGGCGGGGATGAC
GTCAAATCATCATGCCCCCTTATGTCTAGGGCTACACACGTGCTACAATG
GTGAGAACAACGAGATGCAATACCGCGAGGTGGAGCCAAACTTGAAAA
CTCATCCCAGTTCGGATTGTAGGCTGAAATTCGCCTACATGAAGTTGGA
GTTGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACGTTCCCGGGC
CTTGACACACCGCCCGTCACACCATGAGAGTTGGTAACACCCGAAGTC
CGTGAGGTAACCTTTATGGGCCAGCGGCCGAAGGGGG

APPENDIX B SEQUENCE IDENTITY MATRIX

Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	1	0.992	0.986	0.981	0.923	0.965	0.972	0.842	0.921	0.922	0.84	0.831	0.904	0.831	0.908	0.827	0.917	0.927	0.922	0.922
2	-	1	0.99	0.987	0.926	0.968	0.976	0.845	0.924	0.925	0.845	0.833	0.907	0.837	0.909	0.829	0.921	0.93	0.925	0.927
3	-	-	1	0.979	0.92	0.961	0.968	0.84	0.918	0.919	0.842	0.83	0.9	0.83	0.905	0.825	0.914	0.923	0.919	0.92
4	-	-	-	1	0.924	0.965	0.971	0.847	0.924	0.926	0.842	0.833	0.906	0.833	0.911	0.829	0.919	0.932	0.926	0.923
5	-	-	-	-	1	0.929	0.926	0.868	0.985	0.978	0.846	0.834	0.9	0.848	0.906	0.821	0.941	0.912	0.978	0.942
6	-	-	-	-	-	1	0.973	0.853	0.934	0.933	0.842	0.833	0.902	0.837	0.907	0.831	0.923	0.932	0.933	0.93
7	-	-	-	-	-	-	1	0.848	0.925	0.927	0.851	0.837	0.905	0.836	0.91	0.829	0.918	0.936	0.927	0.927
8	-	-	-	-	-	-	-	1	0.869	0.857	0.827	0.806	0.849	0.852	0.845	0.822	0.855	0.851	0.857	0.855
9	-	-	-	-	-	-	-	-	1	0.983	0.847	0.832	0.897	0.849	0.907	0.825	0.932	0.914	0.983	0.941
10	-	-	-	-	-	-	-	-	-	1	0.837	0.831	0.897	0.85	0.907	0.82	0.935	0.911	1	0.937
11	-	-	-	-	-	-	-	-	-	-	1	0.858	0.839	0.827	0.843	0.881	0.847	0.835	0.837	0.857
12	-	-	-	-	-	-	-	-	-	-	-	1	0.832	0.819	0.828	0.857	0.843	0.84	0.831	0.84
13	-	-	-	-	-	-	-	-	-	-	-	-	1	0.831	0.891	0.834	0.903	0.93	0.897	0.9
14	-	-	-	-	-	-	-	-	-	-	-	-	-	1	0.826	0.833	0.841	0.828	0.85	0.844
15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	0.836	0.903	0.908	0.907	0.914
16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	0.833	0.834	0.82	0.838
17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	0.903	0.935	0.921
18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	0.911	0.912
19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	0.937
20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1

Strains: 1. AFB-R-G; 2. AFB-R-B; 3. CSTR-G; 4. Clostridium pasteurianum (ATCC 6013); 5. Clostridium acetobutylicum (NCP 262); 6. Clostridium akagii (CK58); 7. Clostridium acidisoli (CK74); 8. Clostridium bifermentans (NCIMB 10716); 9. Clostridium bifermentans (NCIMB 10716); 10. Clostridium butyricum (NCIMB 8082); 11. Clostridium cellobioparum (DSM 1351); 12. Clostridium cellobioparum (DSM 1351); 13. Clostridium kluyveri; 14. Clostridium lentocellum (DSM 5427); 15. Clostridium tetani (NCTC 279); 16. Clostridium thermolacticum (DSM 2911); 17. Clostridium thermobutyricum (Wiegel); 18. Clostridium tyrobutyricum (ATCC 25755); 19. Clostridium kainantoi (DSM 523); 20. Anaerobacter polyendosporus

APPENDIX C MICROBIAL GROWTH KINETIC CONSTANTS

Microbial kinetic parameters of suspended culture could be determined by employing the following equation (Chang and Lin 2004; Majizat et al. 1997):

$$D = Y \frac{D(S_0 - S)}{X} - k_d = Y \cdot V - k_d \quad (\text{C-1})$$

Where, V is specific substrate utilization rate (g-glucose/g-VSS·h); D is dilatation rate ($=1/\text{HRT}$, 1/h); Y is true yield coefficient expressing the cell mass produced per unit substrate (g-VSS/g-glucose); S_0 and S are influent and effluent concentrations of glucose (g/L), respectively; X is the biomass concentration (g-VSS/L); k_d is the endogenous decay coefficient (1/h). By dint of plotting D vs. V as indicated in Eq.(C-1), the kinetic constants of Y , k_d can be determined by using the experimental data presented in Table C-1.

Table C-1. Effluent concentrations of suspended culture operated under different HRT conditions.

HRT (h)	D (1/h)	Biomass concentration (mg-VSS/L)	Residual glucose concentration (mg/L)	$Y_{x/s}$ (-)
12	0.08	1013.33	56.4	0.10
10	0.10	843.33	375.6	0.09
8	0.13	897.64	836.8	0.10
6	0.17	847.50	2218.5	0.11
3	0.33	328.22	7047.5	0.11
2	0.50	washout		

The kinetic parameters were estimated to be k_d 0.016 1/h and Y 0.12 g-VSS/g-glucose (Figure C-1). The k_d value was extremely low, and Y value was quite close to specific growth yield of biomass, $Y_{x/s}$ indicating that operation conditions are suitable for the growth of hydrogen-producing bacteria. At pH 5.5, almost all the energy obtained by catabolism was mainly utilized to maintain cell growth, rather cell activity. The Y value is in the range of 0.08-0.16 g-VSS/g-COD reported in

other mixed hydrogen-producing cultures (Chen et al. 2001; Fang et al. 2002a; Kim et al. 2006b) , but much lower compared to 0.33 g-VSS/g-COD reported by Yu and Mu (2006) in a UASB reactor fed with sucrose.

Figure C-1. Determination of Y and k_d .