Identification and Description of *Clostridium* sp. and Metabolic Activities in Fermentative Hydrogen Production

A THESIS SUBMITTED

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Declaration

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ABBREVIATIONS

The following abbreviations have been used throughout this thesis.

A (a)	adenine
ABNC	active but non-culturable
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
Blastn	Basic Local Alignment Search Tool network service
bp	base pair(s)
C (c)	cytosine
C.	*Clostridium*
cDNA	complementary DNA
cfu	colony forming unit(s)
cm	centimetre
Ct	threshold cycle at which exponential PCR amplification begins
DCW	dry cell weight
DGGE	denaturing gradient gel electrophoresis
DMSO	dimethyl sulfoxide
dATP	deoxyadenosine triphosphate
dNTP	deoxynucleoside triphosphate
EDTA	ethylenediaminetetraacetic acid
FISH	fluorescence *in situ* hybridisation
[FeFe] H_2ase	[FeFe] hydrogenase
G	guanine
g	gram(s)
g	gravity
h	hour(s)
HPC	heterotrophic plate count
H_2	hydrogen
HydA	hydrogenase structural protein
hydA	gene of hydrogenase structural protein
IPCR	Inverse PCR
kbp	kilo base-pair(s)
km	kilometre(s)
L	litre(s)
LB	Luria-Bertani
m	metre(s)
M	moles L^{-1}
mg	milligram(s)
min	minute(s)
ml	millilitre(s)
mm	millimetre(s)
mM	millimoles L^{-1}
NA	nutrient agar
NCBI	National Center for Biotechnology Information
ng	[NiFe] hydrogenase
[NiFe] H_2ase	nanogram(s)
nm	nanometre(s)

O/N	overnight
ORF	open reading frame
PCR	polymerase chain reaction
R2A	Reasoner's 2A medium
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
s	second(s)
Shine-Dalgarno	SD
sp.	specie(s)
Tris	trishydroxymethylaminomethane
TAE	Tris/acetate/EDTA (buffer)
Taq	*Thermus aquaticus* DNA polymerase
TBE	Tris/borate/EDTA (buffer)
TGGE	temperature gradient gel electrophoresis
*T*m	melting temperature of PCR amplified DNA
T-RFLP	terminal restriction fragment length polymorphism
TSA	tryptone soy agar
µg	microgram(s)
µl	microliter(s)
µM	micromoles L^{-1}
U	unit(s)
UV	ultra-violet
VBNC	viable but non-culturable
v/v	volume per volume
w/v	weight per volume
WPW	Waste potato water
WPS	Waste potato starch
WTP	water treatment plant
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactoside

ABSTRACT

Hydrogen is an environmentally friendly and highly efficient energy source. Fermentative hydrogen production is an exciting R&D area that offers a means to produce hydrogen from a variety of renewable resources or even wastewaters. However, the development of fermentative hydrogen production processes has been hampered due to their low yield and relatively high costs. The aim of this thesis was to improve fundamental knowledge of hydrogen-producing bacteria, provide genetic information associated with the hydrogen evolution, and to optimise operating conditions to enhance hydrogen yield.

Isolation and identification of hydrogen producing bacteria from activated sludge were conducted using 16S rRNA gene-directed PCR-denaturing gradient gel electrophoresis (DGGE), clone library and heterotrophic plate isolation. The results showed that *Clostridium* sp. were dominant and active hydrogen producers. For the first time, three hydrogen producers, which harboured the [FeFe] hydrogenase gene, were characterised by 16S rDNA sequencing, and further physiologically identified as *Clostridium* sp. (W1), *Clostridium butyricum* (W4) and *Clostridium butyricum* (W5). The structure of the putative [FeFe] hydrogenase gene cluster of *C. butyricum* W5 was also described. The changes in [FeFe] hydrogenase mRNA expression of *C. butyricum* W5 during fermentation were monitored. Statistical analysis showed that both the [FeFe] hydrogenase mRNA expression level and cell growth have positive relationships with hydrogen production.

The newly isolated *C. butyricum* W5 demonstrated highly promising hydrogen fermentation performance and was therefore used as the working strain. Optimization of operating conditions in terms of carbon and nitrogen sources, pH, temperature and inoculum size was carried out in a laboratory scale batch system. Use of molasses and NH_4NO_3 resulted in a high hydrogen production yield. Under the optimized fermentation conditions, 100g/L molasses, 1.2g/L NH_4NO_3, and 9×10^4 cell/ml initial cell number at 39°C and pH 6.5, a maximum hydrogen yield of 1.85 mol H_2/ mol hexose was achieved. This corresponded to a hydrogen production rate of 17.38 mmol/h/L. Acetic, lactic and butyric acids were found to be the main by-products of the fermentation. The interrelations between the hydrogen yield and other yields of metabolites were statistically analysed corresponding to the variation in

operating conditions. The dry cell weight was found to have a power relationship with hydrogen production.

The results from this study have provided a better understanding of metabolic processes and gene expression involved in fermentative hydrogen production, and an improved bioengineering process for hydrogen production.

Publications in support of this thesis

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X. Wang, B. Jin, D. Mulcahy. Impact of Carbon and Nitrogen Sources on Hydrogen Fermentation by a Newly Isolated *Clostridium butyricum* W5. *International Journal of Hydrogen Energy,* accepted, 2008.

X. Wang, B. Jin, D. Mulcahy. Process optimization of biological hydrogen production from molasses by a newly isolated *Clostridium butyricum* W5. *Submitted to Journal of Bioscience and Bioengineering.*

X. Wang, P.T. Monis, B. Jin, C.P. Saint: Biochemical Kinetics of Hydrogen Fermentation with a Newly Isolated *Clostridium butyricum* W5. *Submitted to Journal of Applied Microbiology.*

Conference proceedings

Presentation: X. Wang, B. Jin and C. Saint. Impact of carbon and nitrogen sources on hydrogen fermentation by a newly isolated *Clostridium* sp. W5. 2nd International Hydrogen Energy Congress (IHEC 2007), Istanbul, Turkey. Presented by B. Jin.

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Presentation: X. Wang. Production of Fungal Biomass Protein and Biocontrol Agent Using Winery Wastes. Inaugural Australia China Workshop on Water Quality and Technology, Oct. 2006, Adelaide, South Australia.

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INTRODUCTION

Hydrogen (H_2) is an environmental friendly and highly efficient energy source. A hypothetical hydrogen economy, in which the energy is derived from reacting hydrogen with oxygen, was proposed in recent years on the purpose of replacing depleting fossil fuels to meet sustainable development goals. The energy yield of H_2 combustion is compatible with high-efficiency fuel cells and 2.75 times greater than that of hydrocarbon fuels. It produces water as the sole by-product with zero emissions of air pollutants such as NOx and SOx. Use of hydrogen as a fuel is recognised as a sustainable means of energy generation and a pathway to reduce greenhouse gas emissions. It can serve all sectors of the economy including transportation, electricity generation, heating, etc, or as an energy carrier in the form of fuel cells.

The conventional processes for hydrogen production from fossil fuel or water result in high yields and efficiency, but involve enormous energy consumption and greenhouse gas emissions. Considering global environmental benefits and the facilitation of resource recovery, biological hydrogen production from renewable biomass shows significant advantages. There are two biological hydrogen production processes which are most attractive to researchers at present, photo-fermentation by green algae and fermentation by anaerobic bacteria. Fermentative hydrogen production is an exciting R&D area that offers a potential means to produce H_2 from a variety of renewable resources or even wastewater *via* a low energy demand continuous process.

Although the fermentation process for hydrogen production appears to be attractive, commercialization of fermentative hydrogen production has been hampered in the past due to low hydrogen yields and relatively high costs. The majority of the previous research focused chiefly on improving the process performance of continuous fermentation to enhance hydrogen yield. The high production costs were due to the use of expensive substrates including sugar and organic nitrogen sources. Moreover, there are limitations due to the complexity and long duration of continuous fermentation, the interrelations of the operating parameters and the biochemical kinetics of the hydrogen fermentation process not being thoroughly understood and investigated. Improper operating conditions have led to low yield and consequently increased cost. Another noticeable value lost relates to the by-products, volatile fatty acids. The reported volatile fatty acids are mainly acetic, lactic and butyric, which are the most widely utilized multifunctional organic acids in food, pharmaceutical and

cosmetics industries. Unfortunately, the accumulation of these acids has not been reported in any detail and the value of these by-products has not been estimated.

The main reason for the observed low yield is the lack of efficient hydrogen producing bacteria or mixed cultures. Even though there have been a few studies focused on microbial community analysis of hydrogen-producing sludge, the relationship between hydrogen-producing bacteria and the whole microbial community has not been thoroughly studied. The gene regulation of the key hydrogen producing enzyme, hydrogenase, in these bacteria has been little investigated and its maturation is ill-defined. The impact of valuable by-products such as volatile fatty acids and solvents on hydrogen production has not been paid sufficient attention so far.

Therefore, to isolate and identify the most viable working bacteria or microbial flora, to advance knowledge of bacterial hydrogen evolution from the molecular level, to seek alternative carbon and nitrogen sources and to optimize the fermentation performance of hydrogen production are the driving forces of this thesis.

The overall aim was to develop a cost-effective fermentative process for the production of hydrogen from renewable resources. The specific objectives of this study included:

- Profiling microbial community structure in hydrogen-producing activated sludge and isolation and identification of hydrogen producers.
- Identification and description of the hydrogen evolution-related genes and gene clusters in the most efficient hydrogen producer.
- Selection of the most efficient hydrogen producer and suitable fermentation substrates and optimization of operating conditions of a batch fermentation process for hydrogen production based on these.
- Investigation of the impacts of intermediates and end-products on hydrogen production.
- Investigation of the relationship between hydrogenase gene expression and hydrogen production.

Besides Literature Review (Chapter 1), Materials and Methods (Chapter 2) and Conclusions and Recommendations (Chapter 8), a further five chapters (from Chapter 3 to Chapter 7) narrating the experimental results, are presented in this thesis.

Chapter 3, "Isolation and Microbial Community Analysis of Hydrogen Producing Bacteria from Activated Sludge", profiles microbial community structure in hydrogen-producing activated sludge by 16S rRNA gene-directed PCR-DGGE and 16S rRNA gene clone library. Bacterial strains conducive to hydrogen production were isolated onto solid media and their potential to produce hydrogen was investigated by PCR amplification of the hydrogenase gene. The production of hydrogen by those isolated bacteria containing the hydrogenase gene was confirmed by fermentation and production of hydrogen by the individual isolates.

Chapter 4, 'Profiling of Hydrogenase Gene Cluster in *Clostridium butyricum* W5', identifies the three hydrogen-producing isolates and describes the characteristics of a putative [FeFe] hydrogenase gene cluster from the newly isolated *Clostridium butyricum* W5. The sequence of the *Clostridium butyricum* W5 hydrogenase amplified by primer set E1f/E1r was used as "known sequence" for Inverse PCR to obtain further gene sequence information.

Chapter 5, 'Impact of Carbon and Nitrogen Sources on Hydrogen Fermentation by a Newly Isolated *Clostridium butyricum* W5', investigates the effects of different carbon and nitrogen sources on the production of hydrogen. Bottle tests were used for comparison of three isolates and preliminary screening of carbon and nitrogen sources was carried out. Six carbon and six nitrogen sources were selected. A bioreactor was used for further comparison of the selected carbon and nitrogen sources and different pHs.

Chapter 6, 'Process Optimization of Biological Hydrogen Production from Molasses by a Newly Isolated *Clostridium butyricum* W5', employs a batch process using *C. butyricum* W5, the most efficient hydrogen-producing isolate, as the working strain and molasses and NH_4NO_3 as raw materials. Carbon and nitrogen source concentrations, pH, temperature and inoculum size were investigated and optimised. Hydrogen yield, production rate, bacterial biomass, and VFAs were the key parameters for evaluation of the fermentation performance. The relationship between operational conditions and hydrogen yield were analysed

experimentally and statistically. The results are not only instructive to batch hydrogen fermentation, but could also be informative for continuous hydrogen production processes.

Chapter 7, 'Biochemical Kinetics of Hydrogen Fermentation with a Newly Isolated *Clostridium butyricum* W5', describes the biochemical kinetics of a hydrogen production process, including yield and rates, cell growth, volatile fatty acids accumulation and [FeFe] hydrogenase mRNA in the cell. The real-time RT-PCR procedure was employed and optimized for quantification of [FeFe] hydrogenase gene mRNA. The expression level of the [FeFe] hydrogenase was statistically analysed and its relationship with hydrogen production evaluated. Other possible indices involved in the hydrogen fermentation were also examined.

This research provided detailed information about fermentative hydrogen production. The studies on isolation and identification of hydrogen producing bacteria, genetic profiling, optimization of the fermentation process and the internal relationships between the metabolites and operating parameters all provided valuable information yielding a better understand of biological hydrogen production in both the science and engineering aspects. Production performance was significantly enhanced, making fermentative hydrogen production as valid technology for commercial biological hydrogen production in the near future.

CHAPTER 1

Literature Review

Fermentative hydrogen (H_2) production was firstly reported in the late 1970s as H_2 produced in solvent-producing process by some anaerobic bacteria (Karube et al., 1976). Our knowledge of how these and other microorganisms evolve H_2 has grown tremendously over the ensuing nearly 30 years (Armstrong, 2004; Nath and Das, 2004; Winter, 2005; Hawkes et al., 2007). In this literature review, an attempt was made to summarize recent advanced knowledge, science and engineering aspects associated with H_2 energy, fermentative H_2 production, and hydrogenase (H_2ase) gene and regulation. Additional features of advanced molecular techniques used in this thesis are also discussed in detail in this chapter.

1.1 H_2, a clean, renewable and sustainable energy source

As concerns increase over the supply of fossil fuels and changes in global climate, there is an urgent need to develop a clean and renewable energy source. The global climate change, environmental degradation, human health problems caused by the emission of pollutants like CO_x, NO_x, SO_x, C_xH_y, soot, ash, and tar aerosols (Elliott, 1997) have become world wide issues. World marketed energy consumption is projected to increase by 57 % from 2004 to 2030, according to the International Energy Outlook 2007. Production of unconventional energy resources is projected to increase to 9% of total world supply in 2030 (United States Energy Information Administration, 2007).

H_2 has emerged to be the current priority as an ideal alternative for fossil fuels because of its high energy yield and minimum impact on the environment. H_2 is the simplest and most abundant element in the universe and one of the most abundant on earth, which can be found in many different materials including water, natural gas and biomass. As Peter Hoffman writes in his book, *Tomorrow's Energy: Hydrogen, Fuel Cells and the Prospects for a Cleaner Planet* (Hoffman, MIT Press, 2001), H_2 is regarded as a "forever fuel" and can "propel airplanes, cars, trains and ships, run plants, and heat homes, offices, hospitals and schools….As a gas, H_2 can transport energy over long distances, in pipelines, as cheaply as electricity (under some circumstances, perhaps even more efficiently), driving fuel cells or other power-generating machinery at the consumer end to make electricity and water. As a chemical fuel, H_2 can be used in a much wider range of energy applications than electricity."

As a fuel, H_2 has the highest gravimetric energy density of any known fuel and is compatible with electrochemical and combustion processes for energy conversion. The energy yield of

combustion is 143kJ.g^{-1}, which is about 2.75 times greater than hydrocarbon fuels (Boyles, 1984). The only by-product produced during H_2 combustion or electricity generation is water, which is totally environmentally friendly. In addition, H_2 has great potential for chemical synthesis or for electrical storage and generation with fuel cells, which combine H_2 and O_2 to generate electricity. H_2 fuel cells and related technologies provide the essential link between renewable energy sources and sustainable energy services. Furthermore, H_2 can provide storage options for geothermal, seasonal and intermittent renewable resources when combined with engineering decarbonisation technologies (Winter, 2005).

1.2 Hydrogen production

H_2 production is commonly completed from fossil fuels via a chemical method, including steam reforming or thermal cracking of natural gas, partial oxidation of heavier than naphtha hydrocarbons, and coal gasification. H_2 may also be produced from water by electrolysis, photolysis, thermochemical process, direct thermal decomposition or thermolysis and biological production. Moreover, H_2 can be emitted from microorganisms as a by-product of metabolism. Table 1 summarises the relative advantages and disadvantages of the processes for H_2 production. H_2 production from fossil fuel and water results in a high yield. However, the usage of exhaustible energy and production of greenhouse gases are the major disadvantages for the process which uses fossil fuel and water. Considering global environmental benefits and the facilitation of resource recovery, biological H_2 production from renewable sources, H_2 production from biomass has overall advantages compared to other processes, despite the relatively low yield and immature technology. The discovery and development of H_2 production from renewable, especially waste, materials of bulk production will accelerate the establishment of a H_2 economy. (Das and Veziroğlu, 2001; van Ginkel et al., 2001; Hawakes et al. 2002).

Table 1.1 Advantages and disadvantages of H_2 production processes.

Processes	Advantages	Disadvantages
Fossil fuel	• High yield • Mature technology	• Use of exhaustible energy fossil fuel • Production of air pollution gas, CO_2 • Intensive energy consumption • High cost
Water	• High yield • Mature technology	• Need electricity • Energy intensive • High cost • Limited by water resources
Biomass	• Environmental friendly • No secondary pollution • Not energy intensive • Operates at ambient temperatures and pressures • Low cost • Use in exhaustible energy • Use of waste biomass	• Low yield • Immature technology

The early basic and applied research on the biological H_2 production was initiated after the energy crisis of the early 1970s (Gibbs et al., 1973). Biological H_2 production can be classified as direct biological photolysis, indirect biological photolysis, photo-fermentation and dark-fermentation. Direct biological photolysis is a process by which algae and cyanobacteria split water and direct transfer of electrons from water to protons, coupling the ferredoxin reducing reactions of photosynthesis to a [NiFe] H_2ase (Benemann, 2000; Das and Veziroğlu, 2001). Indirect biological photolysis is a two-step process which fixes CO_2 into carbohydrate and then uses it to produce H_2 to avoid O_2 inhibition in direct biological photolysis (Weare and Benemann, 1974). Similar to indirect biological photolysis, which is a two-step process, photo-fermentation uses microorganisms to produce fermentative volatile fatty acids (VFAs) that are then converted to H_2 by photosynthetic bacteria (Benemann, 1998). Table 1.2 summarizes the advantages and disadvantages of these biological H_2 production processes.

Table 1.2 Comparison of important biological H_2 production processes

Process	Microorganisms	Advantages	Disadvantages
Direct biological photolysis	Green algae	• Can produce H_2 directly from water and sunlight • Solar conversion energy increased by tenfold as compared to trees, crops	• Requires high intensity of light • O_2 can be danderous for the system
Indirect biological photolysis	Cyanobacteria	• Can produce H_2 from water • Has the ability to fix N_2 from atmosphere	• Uptake H_2ase enzymes are to be removed to stop degradation of H_2 • About 30% O_2 present in gas mixture • O_2 has an inhibitory effect on nitrogenase
Photo-fermentation	Photosynthetic bacteria	• A wide spectral range of light can be employed • Can use different waste materials like distillery effluents, waste etc	• Low conversion efficiency • O_2 is a strong inhibitor of H_2ase
Dark fermentation	Fermentative bacteria	• Can produce H_2 without light • A variety of carbon sources can be used as substrates • Produces valuable metabolites such as butyric, lactic and acetic acid as by-products • An anaerobic process, so there is no O_2 limitation problem	• Relatively lower achievable yields of H_2 • As yields increase, fermentative H_2 production becomes thermodynamically unfavourable • Product gas mixture contains CO_2, which has to be separated

Fermentative H_2 production referred in later parts of this thesis is dark fermentation process for H_2 production because of the infrequent usage of this term in scientific reports.

1.3 Fermentative H_2 production

1.3.1 Principle of fermentative H_2 production

Fermentative H_2 production occurs under anoxic or anaerobic conditions. Fig 1.1 illustrates the biochemical pathways of H_2 production from conversion of carbohydrate materials into H_2. When bacteria grow on organic substrates, substrates are degraded by oxidation to provide building blocks and metabolic energy for growth under aerobic environments. This oxidation generates electrons, which need to be disposed of in order to maintain electrical neutrality. In anaerobic or anoxic environments, other compounds such as protons, which are reduced to molecular H_2, need to act as electron acceptors (Nath and Das, 2004).

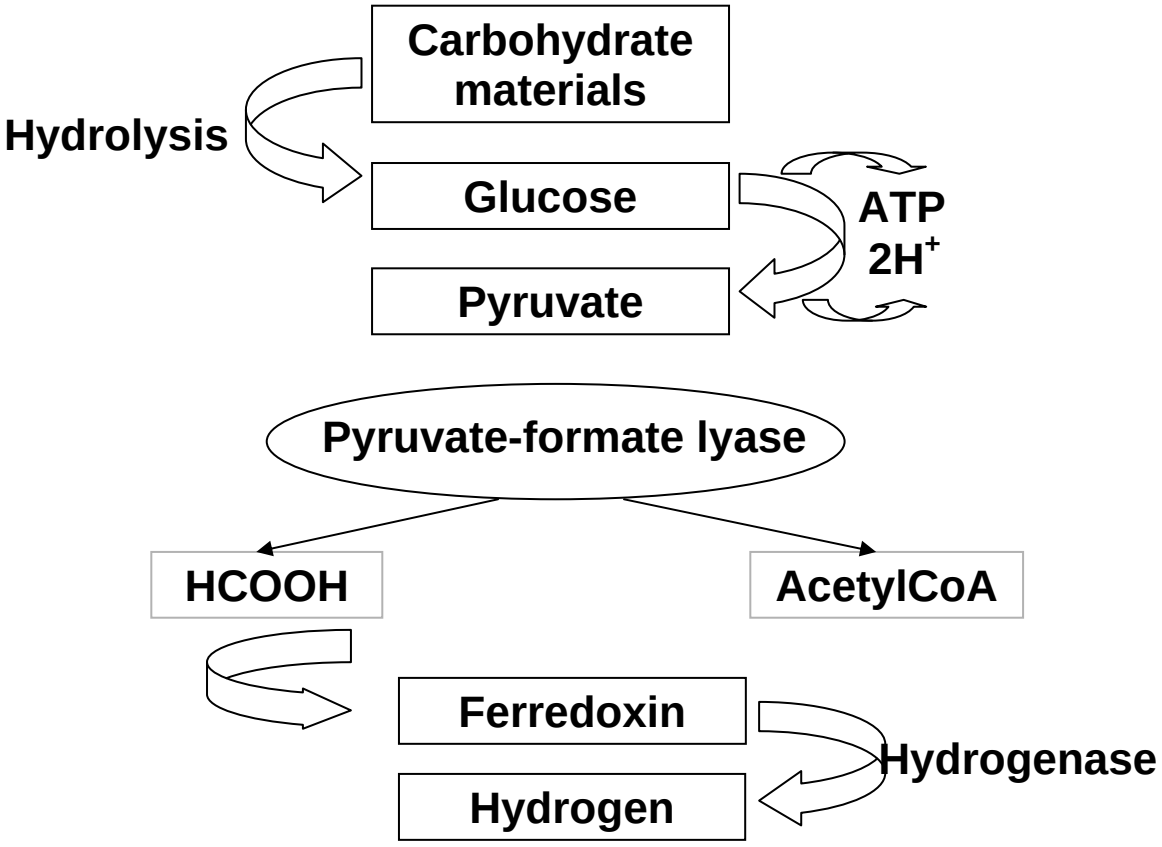


Fig 1.1 Biochemical pathway for conversion of renewable biomass to H_2 via fermentative H_2 production (Nath and Das, 2004). (ATP: adenosine triphosphate)

1.3.2 Metabolic pathway of fermentative hydrogen production

In fermentative H_2 production, theoretical yield of H_2 is 4 or 2 mol per mole hexose if acetic acid or butyric acid is the sole end-product, as shown in Fig 1.2. H_2 yield is even lower when more reduced organic compounds, such as lactic acid, propionic acid, and ethanol, are produced as fermentation products. It is believed that these products are produced via non-H_2 generating metabolic pathways (Angenent et al., 2004). Sugar-based substrates can be metabolized to hexose by enzyme digestion and further into pyruvate through several pathways. Both of these pathways produce two moles of pyruvate and two moles of NADH. Molecular H_2 in all known H_2 production systems is attributed by electrons derived from the reaction: oxidative decarboxylation of pyruvate by pyruvate:ferredoxin oxidoreductase (Fig 1.2) (Gottschalk, 1986). The H_2 partial pressure is believed to be the switch of these two metabolic pathways. When the H_2 partial pressure is lower than 60 Pa, the NADH produced in step 1 of pathway (a) as shown in Fig 1.2 will also be used to generate an additional 2 moles of H_2. In many cases, however, the NADH will probably be oxidized through other fermentation pathways, such as butyrate fermentation (Fig 1.2). Some fermentation products such as ethanol or lactic acid are products of alternative pathways for pyruvate metabolism which compete with step 2 in pathway (a) and (b) in Fig 1.2. Consequently, in practical systems, the yields are normally less than two moles of H_2 per mole of hexose (Angenent et al., 2004).

Anaerobic digestion of organic waste to produce H_2 is a technically feasible approach to biological H_2 production (Miyake et al., 1999). H_2 has been biologically produced from wastewater (Liu et al., 1995; Wang et al., 2003a; Mohan et al., 2007; Yang et al., 2007), solid wastes (Mizuno et al., 2000; Liu et al., 2006a; Alzate-Gaviria et al., 2007), or certain substances in water, like molasses (Tanisho and Ishiwata, 1994; Ren et al., 2006; Li et al., 2007b), glucose (Kataoka et al., 1997; Chittibabu et al., 2006), crystalline cellulose (Lay, 2001), peptone (Bai et al., 2001), and starch (Lay, 2000). Technologies to enhance H_2 production have been reported in the literature (Tanisho and Ishiwata, 1995; Mizuno et al., 2000; Sano et al., 2006; Kalogo and Bagley, 2008). During anaerobic digestion, methanogenic or sulfate-reducing bacteria could consume H_2 produced by acidogenic bacteria, which is a negative impact of biological H_2 production (Adams, 1998). Various pre-treatment methods, including heat, acid, alkali treatment, have been used in order to reduce

the methanogenic and sulphate reducing bacteria content and thus obtain satisfactory results (Chen et al., 2002; Wang et al., 2004; Wang et al., 2007b).

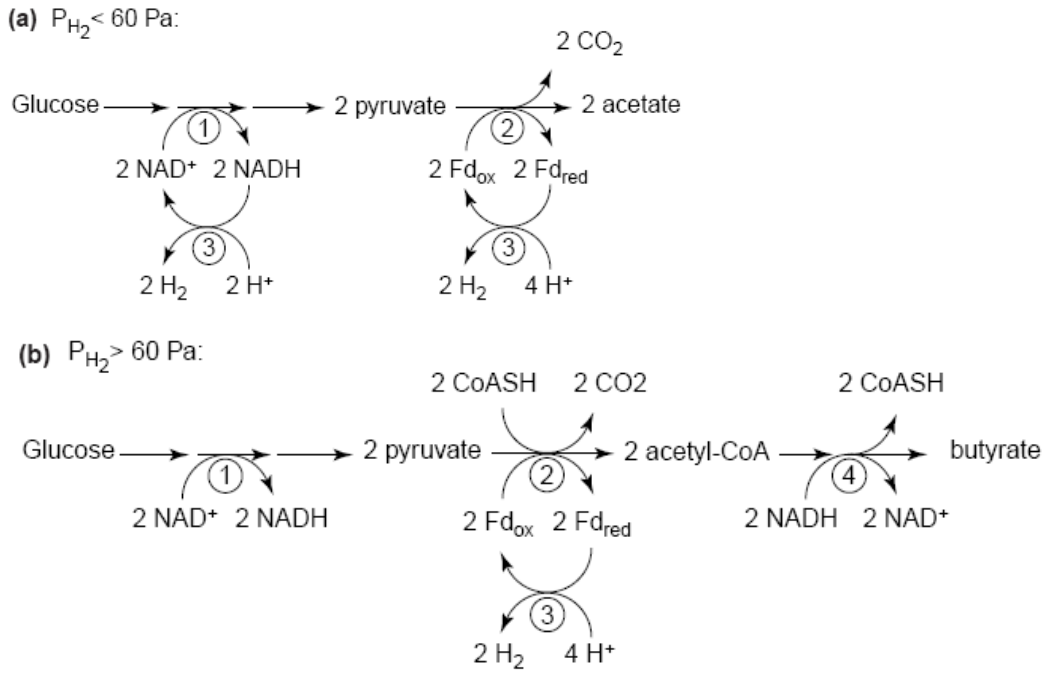


Fig 1.2 H_2-producing metabolic pathways in fermentative H_2 production (Angenent et al., 2004).

1.3.3 Microorganisms

H_2 production by microorganisms can be divided into two main categories: "photosynthesis" by microalgae, cyanobacteria, or photosynthetic heterotrophic bacteria, cultured under anaerobic light conditions and "fermentation" by chemotropic bacteria without photo-energy (Levin et al., 2004). Photosynthetic microorganisms can decompose water or use organic substrates like organic acids for H_2 production in the presence of light energy. Fermentative

Table 1.3 Imperative H_2-producing microbial strains reported and their H_2 yield (Kotay and Das, 2008)

Organism	**Substrate Maximum**	**Process**	**Reported yield of H_2 ($molH_2$/mol substrate)**	**References**
Enterobacter aerogenes HU-101 (mutant AY-2)	Glucose	Batch, (blocking metabolites formation)	1.17	Mahyudin et al., 1997
Enterobacter aerogens	Molasses	Ar sparging, batch	1.58	Tanisho et al., 1994
Enterobacter aerogens	Molasses	Batch	0.52	Tanisho et al., 1994
Clostridium butyricum	Glucose	N_2 sparging, continuous	1.4–2.3	Kataoka et al., 1997
Enterobacter cloacae IIT BT 08	Glucose	Continuous (immobilized bioreactor)	2.3	Kumar and Das, 2000
Enterobacter cloacae DM11	Glucose	Continuous (immobilized bioreactor)	3.8	Kumar et al., 2001
Citrobacter sp. Y19	Glucose	Ar sparging, batch	2.49	Oh et al., 2003
Rhodopseudomonas palustris P4	Glucose	Batch, with intermittent purging of Ar	2.76	Oh et al., 2002
Mixed culture (predominantly *Clostridium sp*.)	Glucose	N_2 sparging, continuous HRT: 8.5 h	1.43	Mizuno et al., 2000
Mixed microflora	Wheat starch co-product	N_2 sparging continuous	1.9	Hussy et al., 2003
Mixed microflora	0.75% soluble starch	Chemostat HRT: 17 h	2.14	Lay, 2000
Mixed microflora (Mesophilic)	Sewage-sludge	Anaerobic and acidogenic digestion	1.7	Lin and Chang, 1999

H_2 production, compared to biological photosynthetic processes, has additional advantages as (i) it can continually produce H_2 without the need of light; (ii) it can use a variety of cheap carbon sources, such as starch, contained in wastewater; (iii) by-products of the fermentation include butyric, lactic and acetic acid, which have alternative commercial value; and (iv) bacteria used for fermentative H_2 production are anaerobic and therefore do not require O_2 (Nath and Das, 2004). Fermentative H_2-producing bacteria can use organic substances as their sole carbon sources, converting the organic materials into H_2 (Hallenbeck, 2005), and thus can be used to both treat large quantities of organic wastes and generate clean energy.

Clostridia

Most bacteria belonging to the genus *Clostridium* can produce H_2 from carbohydrate in fermentative H_2 production. Clostridia are Gram-positive, spore-forming, rod-shaped obligate anaerobic bacteria (Fig 1.3). Endospores are very resistant to heat or harmful chemicals including acid and alkali, and cannot be destroyed easily. They grow very fast and have great variety on substrate utilization, which favours their industrial use (Brock et al., 1994). Recently, microbial community analysis of various H_2-producing activated sludge systems showed that *Clostridium* sp. are dominant and active H_2 producers. The presence of *Clostridium* sp. is normally more than 60% of total bacterial population after pre-treatment (Fang et al., 2002; Chang et al., 2006; 2007; Wang et al. 2007b). The dominance of *Clostridium* sp. is possibly enhanced by the resistance of endospores. Many *Clostridium* sp. have been used for H_2 production, Those include *C butyricum* (Yokoi et al. 2001), *C. thermolacticum* (Collet et al. 2004), *C. pasteurianum* (Liu and Shen, 2004; Lin and Lay, 2004), *C. paraputrificum* M-21 (Ewyernie et al., 2001), *C. bifermentants* (Wang et al. 2003a), *C. butyricum* CGS5 (Chen et al., 2005), *C. acetobutylicum* (Zhang et al. 2006a), *C. tyrobutyricum* ATCC 25755 (Liu et al., 2006b), and *C. butyricum* ZJUCB (Kong et al., 2006).

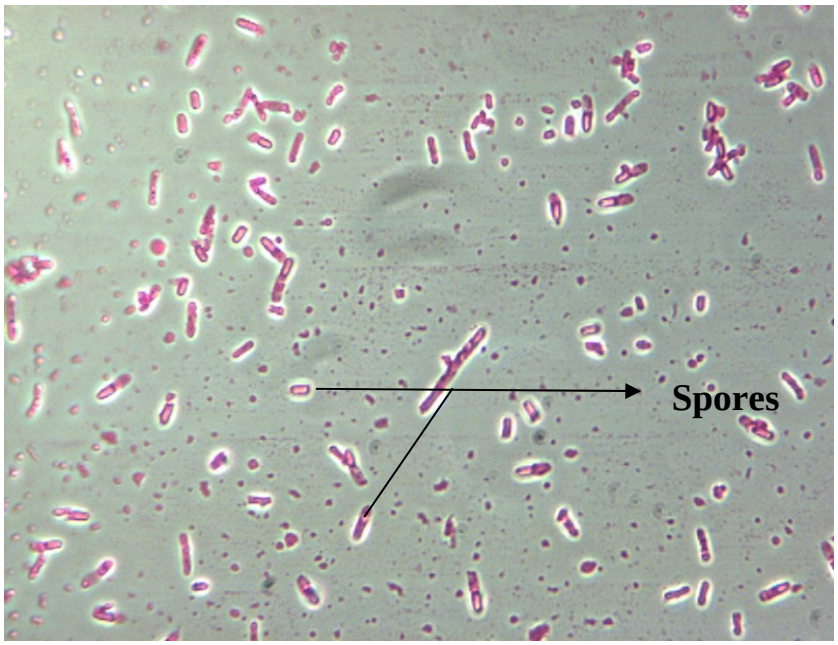


Fig 1.3 Microscopic images of *Clostridium butyricum* W5 (~1000X)

Many researchers, who already have experience with anaerobic digestion to produce methane, treat dark H_2 fermentation as a "black box". This means that H_2 was produced by an uncharacterised bacterial community in sludge. They found pre-treatment of sludge and keeping pH in a certain range to be crucial to gain high yield and production rate (Angenent et al., 2004; Lin et al., 2004). However, as discussed above, more and more H_2-producing activated sludge communities are being analysed and individual H_2-producing bacteria isolated and tested (Chang et al., 2006; Wang et al., 2007b). This work suggested that H_2-producing bacteria work individually in the community and that maintaining a high proportion of efficient H_2 producers is essential for high production rate and yield . Evidence supporting this includes:

- The dominant bacteria were shown to be able to produce H_2 independently.
    a) Normally, only spore-forming bacteria could survive after pre-treatment by high temperature, acid or alkali. Spore forming *Clostridium* sp have been observed to be dominant in most community analyses (Chang et al., 2006; Wang et al., 2007b).
    b) *Clostridium.* sp have been proved to be able to produce H_2 at around pH 6.5 (Liu et al., 2006b; Baek et al., 2006), which was the same as the suitable pH for H_2

production by sludge (Zhang et al., 2003; Mizuno et al., 2000). This suggests that *Clostridium* sp were active H_2 producers in H_2-producing sludge.

c) Unlike methanogens, clostridia showed a wide range of substrate utilization such as polysaccharides, polypeptides, fats and nucleic acids, and can produce H_2 independently (Liu et al., 2006a).

d) To my knowledge, until now, no other wild bacterial stain was found to have great advantages on both substrate utilization and H_2 evolution compared to *Clostridium* sp under similar operating conditions.

- Mixed cultures give lower yield and production rate when compared to pure cultures. Although we still do not have any comprehensive comparisons between pure cultures and mixed cultures under the same operating conditions, a few papers indicated that mixed microbial cultures did not produce H_2 with any greater efficiency than pure cultures (Liu et al., 2006b; 2006b;Chittibabu et al., 2006). The main reason for the low yield and efficiency could be explained by the fact that other bacteria, which do not contain H_2ase (key enzyme for H_2 evolution), use substrate without releasing H_2 or even consume H_2 (Wang et al., 2007b). Also, differences in H_2 evolution abilities of individual *Clostridium* sp isolated from one H_2 production system (Wang et al., 2007b) could lead to low yield and production rate compared with pure culture.

In conclusion: to control continuous process operation parameters including substrate concentration, pH and temperatures suitable for the most efficient H_2 production, *Clostridium.* sp, appeared to be important for a high yield and production rate.

Bacteria

Recently, H_2-producing aerobic bacteria were also investigated and identified, and the fermentation process was described. *Rhodopseudomonas palustris* P4 (2002) and *Citrobacter* sp. Y19 (2003) for CO-dependent H_2 production was studied by Oh et al. *R. palustris* P4 showed a high H_2 yield (2.76 mmol H_2/mmol glucose) and production rate (29.9 mmol H_2/g cell h) when using glucose as substrate, but an inefficient usage of disaccharides and starch. *Citrobacter.* sp.

Y19 showed a similar H_2 fermentation ability and substrate utilization. Moreover, *Actinomyces* sp. and *Porphyromonas* sp. were detected in an anaerobic granular sludge with lower H_2 yield (1 to1.2 mmol/mol glucose) compared to *Clostridium* sp. (Oh et al., 2003). *Thermotogales* sp. and *Bacillus* sp. have also been shown to be H_2 producers in acidogenic cultures (Shin et al., 2004). In order to overcome the theoretical limitation on dark fermentation yield, mutant and recombinant bacteria were constructed and their H_2 production was investigated. H_2 yield by a *Clostridium tyrobutyricum* PAK-Em mutant increased from 1.35 H_2 mol/mol glucose to 2.61 H_2 mol/mol glucose, and H_2/CO_2 ratio was also increased (Liu et al, 2006b). A recombinant *Escherichia coli* BL-21 which contains *E. cloacae* IIT-BT-08 Fe- H_2ases also yields 3.12 mol H_2/ mol glucose (Chittibabu et al., 2006). However, both mutant and recombinant bacteria showed slower growth and H_2 production rate compared to the original strains, which limits further industrial application. Some thermophilic anaerobic bacteria also attracted attention because temperature could be easily employed as a selective condition to maintain the dominance of H_2 producers in mixed culture. As methanogens are sensitive to temperature, no methane gas could be detected in thermophilic H_2 production (Shin et al., 2004; Zhang et al., 2003; Liu et al., 2003). H_2-producing microorganisms, *Thermoanaerobacterium thermosaccharolytium* and *Desulfotomaculum geothermicum*, were detected in a thermophilic H_2 production system. The yield reached 1.8 mol H_2/ mol hexose (Shin et al., 2004). A H_2-producing *Thermococcus kodakaraensis* KOD1 strain was reported to have a maximum growth rate at 85 °C (Kanai et al., 2005). A thermophilic *Clostridium thermolacticum* was also reported that can use lactose to produce H_2 at 58°C (Collet et al., 2004). Thermophilic H_2 production is suitable for places where cheap heating resources are available.

1.3.4 Fermentation substrates

In accordance with the sustainable development, use of renewable resources even waste materials substrates for H_2 production has attracted considerable attention in recent years. The most commonly used were single sugars including oligosaccharides or monosaccharide (Kataoka et al., 1997; Mahyudin et al., 1997; Oh et al., 2002; 2003). Glucose and sucrose are easily degradable carbon sources and used mostly for research purpose. Glucose/hexose or sucrose have been used for H_2 production, leading to a relatively high yield (Chittibabu et al., 2006; Ren

et al., 2006; Li et al., 2007). However, high costs related to use of these sugars limit their industry applications. The maximum H_2 yield on hexose was reported to be 1.7 mol/mol (Lin et al., 2004). A high yield, 2 mol/mol hexose was obtained in a sucrose fermentation process which is still far lower than the theoretical yield of 4 mol/mol hexose (Chen et al., 2000; 2001). The utilization of substrates as an energy and building block for cell growth is the main reason for obtaining the yields lower than theoretical yield (Kapdan and Kargi, 2006).

The products of other metabolic pathway such as acetate (Barbosa et al., 2001) and glycerol (Saint-Amans et al., 2001) were also considered in order to reduce the production cost, but the yield are not satisfactory. Other complex materials or wastes including sugar containing materials or wastes, cellulose-containing agricultural and food industry by-products and wastes, carbohydrate-rich industrial wastewaters and waste sludge from wastewater treatment plants (Wang et al., 2003; Zhang et al., 2003; Adamson, 2004; Hussy et al., 2005; Li et al., 2007b) have also been tested for H_2 production. Starch containing wastes were considered to be more suitable substrates than cellulose containing agricultural wastes for H_2 production because starch can be biological and chemically hydrolysed easily. The H_2 yield was reported to be around 0.8 mol/mol hexose by batch process, which was much lower than continuous fermentation, more than 2.5 mol/mol hexose (Yokoi et al., 1998; Zhang et al., 2003; Liu and Shen, 2004). Even the yield on dissolved cellulose and hemicellulose containing materials were similar with starch, the overall H_2 yields on cellulose were low because of the partial hydrolysed of substrates (Kapdan and Kargi, 2006). It was reported that the usage of food industry wastes resulted in a low yield, and a pre-treatment to remove undesirable components and for nutritional balancing is required (Kapdan and Kargi, 2006).

Because 30-40% of total production costs are associated with the use of substrates (Åkerberg et al., 2000), the price of carbon and nitrogen sources for bulk H_2 production became an important factor for hydrogen economy. The utilization efficiency of the substrates also affects the yields and consequently the cost. Therefore, seeking suitable and economical carbon and nitrogen sources is supposed to be the first step to industrialization. Unfortunately, detailed comparison of the effect of carbon and nitrogen sources and evaluation of carbon and nitrogen concentrations in

fermentation broth for certain bacterial fermentative H_2 production have not been found in the literature, to our knowledge.

1.3.5 Hydrogen production processes

Both batch and continuous fermentation processes were employed in biological H_2 production. Batch or semi-continuous fermentation was normally used for pure culture (Oh et al., 2002; 2003; Liu et al., 2006b). The key operating parameters in the batch or semi-continuous fermentation process include pH, temperature, composition and concentration of the culture medium, and general microbial growth conditions (King and Hossain, 1982). A few parameters, mostly pH and temperature were employed in most previous investigations (Baek et al., 2006; Chittibabu et al., 2006). There is no report which studied interrelationships and impact of these operating parameters in the H_2 production system.

Compared to batch process, the most significant benefit for operating a continuous process for H_2 production is higher yields based on the same substrates, microorganism, and operating parameters. The increase in yield is contributed by saving the substrates for providing the energy and building block of cell growth (Kapdan and Kargi, 2006). Continuous fermentation on solid wastes or waste water seeded with pre-treated activated sludge can possibly save the cost for sterilization of bioreactor system (Das and Veziroğlu, 2001; Adamson, 2004; Kapdan and Kargi, 2006). However, there are some key technique challenges which make the continuous H_2 production process invalid. This is due to the complexity of the fermentation system and long term of the operating period (Hawkes et al., 2002; Chittibabu et al., 2006).

Batch and continuous fermentation are the most commonly used bioengineering processes. To establish a highly efficient and high yield fermentation system, these two processes are be usually employed and studied. Because of the easy operation and short term, batch fermentation is generally used to seek the optimal pH, temperature or other operating parameters, and investigate the effects of substrates concentration. Optimized variables are instructive for the continuous processes (El-Mansi and Bryce, 1999). Unfortunately, there is a shortage of comprehensive comparison studies on the effects of operating parameters and substrate

concentrations in batch fermentation process for the hydrogen production. The operating parameters were arbitrally chosen and really less effort has been paid on the substrates. A few work investigated pH (Hwang et al., 2004; Mohan, 2007), temperature (Majizat et al., 1997; Nielsen et al., 2001) or substrates concentration (Wu and Lin, 2004), but only focused in one or two parameters and with a rough description.

1.4 Hydrogenases

1.4.1 Classification of hydrogenases

Hydrogenases (H_2ases) catalyse the simple redox reaction $H_2 \Leftrightarrow 2H^+ + 2e^-$ and play a key role in microbial energy metabolism. They exist widely in many microorganisms, using H_2 as a source of reducing power or protons as oxidants for the disposal of excess reducing equivalents (Vignais et al., 2001; Cammack et al., 2001). Most known H_2ases can catalyse the reaction in either direction for H_2 uptake or evolution *in vivo*, depending on the demands of the host organism. A H_2ase usually contains a metal ion at its active site. According to the metal content, therefore, the H_2ases can be grouped as [NiFe] H_2ases and [FeFe] H_2ases. Some H_2ases contain only iron, [FeFe]; but the majority of known H_2-activating enzymes contain nickel and iron at the active centre, [NiFe] (some [NiFe] H_2ases also contain selenium). Only one enzyme has been reported that displays H_2ase activity in methanogens that has been shown to contain no redox-activity metal at all (Robson, 2001; Cammack et al, 2001; Vignais et al., 2001; Wu and Mandrand, 1993). H_2-forming methylenetetrahydromethanopterin dehydrogenases (Hmd) contain only a monouclear Fe active site, but catalyze a reaction differing from the one indicated above (Pilak et al., 2006). Fig 1.4 shows the active sites of [FeFe] H_2ases and [NiFe] H_2ases (Armstrong, 2004).

In summary, the available data suggest that [FeFe] H_2ases are restricted to bacteria and Eucarya, while [NiFe] H_2ases, with only one possible exception, seem to be present only in Archaea and bacteria. Now, over 100 of these enzymes have been characterized genetically and/or biochemically. Compelling evidence from sequences and structures indicates that the [NiFe] H_2ases and [FeFe] H_2ases are phylogenetically distinct classes of proteins.

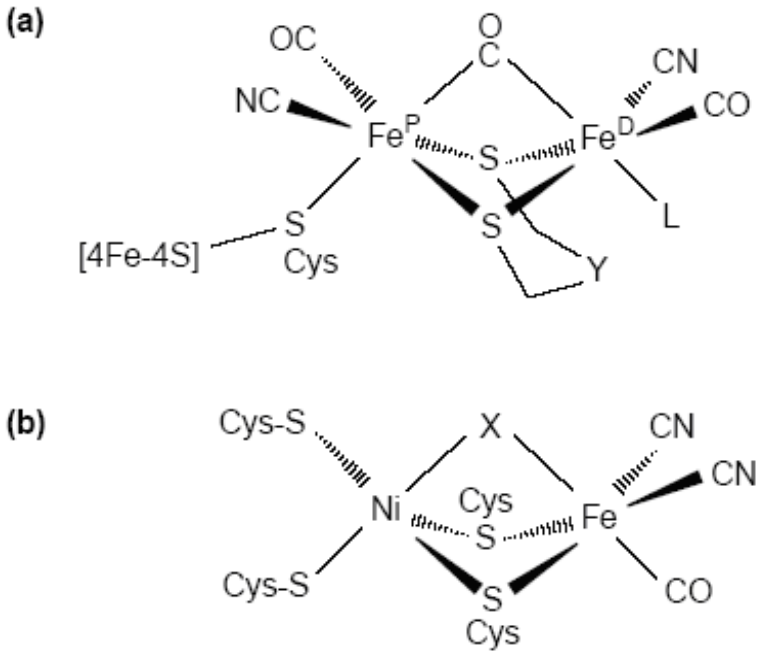


Fig 1.4 Active sites of [FeFe] H_2ases and [NiFe] H_2ases (Armstrong, 2004).

(a) The FeFe active site is called the H-cluster; FeP and FeD are proximal and distal, respectively, in relation to the [4Fe-4S] cluster; L is an exchangeable ligand (H_2O) and Y may be an amino-N atom, as recently proposed.

(b) In the NiFe active site, X is an additional bridging ligand, believed to be an oxo or hydroxo group in the inactive forms Ni-A and Ni-B, and a hydride in the active form Ni-C. One of the terminal cysteines might be replaced by selenocysteine, as is found in an enzyme from *Desulfomicrobium baculatum*.

1.4.2 Active site of hydrogenases

The active sites of [FeFe] and [NiFe] H_2ases have a dimetallic framework as shown in Fig 1.4, which is supposed to be required for their catalytic activity (Evans and Pickett, 2003; Tye et al. 2006). The CO and CN ligands are unique for iron (Nicolet et al. 2000; Fray et al., 2001). The catalytic core of the [NiFe] H_2ases is a heterodimeric protein, although additional subunits are present in a number of these enzymes. Functional classes of [NiFe] H_2ases have been defined, and they are consistent with categories defined by sequence similarity of the catalytic sub-units.

The catalytic core of the [FeFe] H_2ases is a ca. 350-residue domain that accommodates the active site (H-cluster) (Vignais et al., 2001; Armstrong, 2004).

1.4.3 Maturation of hydrogenases

H_2ases processing and maturation involve the products of several genes which have been identified and are currently being characterized in the case of the [NiFe] H_2ases. By analysing the [NiFe] H_2ases gene of *Helicobacter pylori*, *hypA, hypB, hypC, hypD*, *hypF*, *HP0635* (which the function was unknown, referred to as *hydE* here), and *HP06* (referred as *hydD* in the *H. pylori* 26695 genome database), genes are found to be required for [NiFe] H_2ases activity, since all the mutants have negligible [NiFe]H_2ases activity. HypA and HypB were shown to be required not only for [NiFe] H_2ase maturation, but also for urease maturation, suggesting that some of these accessory proteins were common to both maturation processes (Olson et al., 2001; Benoit et al., 2004). H_2ases activity in a *hypE* mutant was partly restored by adding 5 mM nickel to the growth medium (Olson et al., 2001; Benoit et al., 2004). In contrast, almost nothing is known regarding the maturation of the [FeFe] H_2ases. Inspection of the currently available genome sequences suggests that the [NiFe] H_2ases maturation proteins have no similar counterparts in the genomes of organisms possessing [FeFe] H_2ases only (Vignais et al., 2001; Armstrong, 2004; Meyer, 2007).

1.4.4 [FeFe] hydrogenases

The screening of hundreds of microbial genome sequences showed that [FeFe] H_2ases are present in only a small number of bacteria (Akhmanova et al., 1998), mainly Clostridia (Friedrich et al., 2005) or β-proteobacteria (Stephenson and Stickland, 1931). A few monomeric [FeFe] H_2ases are barely larger than the H-cluster domain. Many others are monomeric as well, but possess additional domains that contain redox centres, mostly iron-sulphur. Some [FeFe] H_2ases are oligomeric. Bacteria which accommodate the [FeFe] H_2ase gene normally have several copies of the structural gene, but most of them only have a single set of maturation genes (Meyer, 2007). The absence of *hydE, hydF* and *hydG* genes in several bacterial genomes result in no active [FeFe] H_2ase even if they contain the gene *hydA* (Böck et al., 2006; Posewitz et al., 2004; King et al., 2006). As the maturation of [FeFe] H_2ases is still regarded as a puzzle, genetic

manipulation of the expression of [FeFe] H_2ase was mainly performed within H_2-producing species (Liu et al., 2006b). In addition, although the identification of genes encoding [FeFe] H_2ases can be straightforwardly done based on the considerable amount of data available in the well known sequence databases such as GenBank, many [FeFe] H_2ases gene sequences are erroneously annotated and gene sequence structure is poorly described (Meyer, 2007).

1.5 Detection of H_2-producing bacteria from activated sludge

1.5.1 Factors impeding accurate detection by heterotrophic plate count VBNC bacteria in activated sludge

In order to resist adverse environments and survive, microorganisms adopt distinct ways to keep themselves alive and to propagate. Some bacteria form spores, which are highly resistant to temperature, desiccation, starvation, radiation, biological enzyme digestion and chemical disinfectants (Piggot and Hilbert, 2004), while others are capable of utilising active mechanisms for stress resistance through a series of genetic programs, during which a bacterium develops recalcitrance to culture whilst retaining viability, referred to as the Viable But Non-Culturable (VBNC) state. The viability is characterized in terms of their ability to reduce dyes (indicating metabolic activity) or prevent uptake of dyes (indicating an intact membrane). However, the cells are not able to form colonies when cultured under traditional standard methods (Oliver, 2005; Mukamolova et al. 2003; Kell et al., 1998). The starvation response of bacteria can not been included as a VBNC state as they are still readily culturable in suitable media (Keep et al., 2006). It is difficult to differentiate the VBNC state from the "culturable but needs special protocol" of some bacteria present in the environment (Mukamolova et al. 2003). Proteome analysis (Heim et al., 2002) and cell wall changes (Signoretto et al., 2000) were introduced to differentiate the physiological differences and recently have been regarded as more reliable diagnostics of the VBNC state.

In activated sludge, bacteria submitted to various environmental stresses (oxygen, light, temperature and so on) may form VBNC cells (Oliver et al. 1995; Rowan 2004). Traditional standard methods can only identify a small number of microorganisms from within a complex

microbial community. Microbial communities have mostly been analysed with culture-dependent methods, such as heterotrophic plate count (HPC) and the most probable number (MPN) technique, but those methods can only provide no more than 10% of total microorganisms present in the environment (Amann et al., 1995; Garcia-Armisen and Servais 2004; Garrec et al. 2005).

1.5.2 Molecular techniques for community analysis

Rapid DNA sequencing techniques have enabled microbial diversity studies and community analyses, resulting in significant improvement in the understanding of microbial ecology and phylogeny (Woese, 1987). Furthermore, developments in molecular techniques and the rapidly growing DNA sequence databases make microbial diversity determination faster and more reliable (Dahllöf, 2002). Total genomic DNA extracts from environmental samples can be analysed directly using microarrays or dot-blot hybridisation, or amplified using polymerase chain reaction (PCR) and characterised using a range of techniques including pattern analysis, cloning and sequencing, probe hybridisation and microarrays (Ranjard et al., 2000; Dahllöf, 2002). Advanced techniques employed in this thesis will be discussed in more detail below.

PCR based 16S rRNA gene-directed techniques

The polymerase chain reaction (PCR) is a widely used technique in molecular biology and microbiology research. With PCR it is possible to amplify a single or very few copies of a piece of DNA across several orders of magnitude, generating millions of DNA pieces which are sufficient for further detection/manipulation.

An important first step towards understanding the roles of the various bacteria in the H_2-producing process is not only to determine the numbers and the relevant abundances of different bacterial groups, but also to identify the bacteria responsible for the bulk of the activity. Methods employed for microbial community investigation are generally divided into culture-dependent or culture-independent methods. Traditional culture-dependent methods result in the characterization and isolation of bacteria which can be cultured on selective media. However,

about 90% of environmental bacteria remain “un-culturable” and are still undescribed (Amann et al., 1995). Culture-independent approaches such as PCR-DGGE, 16S ribosomal DNA (rDNA) clone libraries, fluorescent in situ hybridization (FISH), Phospholipid Fatty Acid (PLFA) Analysis etc compensate for the shortcomings of traditional culture methods by providing the structure of microbial communities irrespective of how conducive the organisms are to culture (You et al., 2000; van der Gucht et al., 2005).

A new trend in microbial community analysis is to employ two or more methods which can compensate for the shortcomings of each method, *eg.* PLFA is focused more on group changing, PCR-DGGE gives better results on dominant species and 16S rDNA clone library provides less quantitative data (Rattray and Craig, 2007; Droppo et al., 2007; Jin and Kelley, 2007; Toyota and Kuninaga, 2006; Moser et al., 2003). In this thesis, the total bacterial community profile of a H_2-producing process was assessed by 16S rRNA gene-directed PCR-DGGE and 16S rDNA clone library. These two techniques will be introduced in detail.

PCR-DGGE

Denaturing gradient gel electrophoresis (DGGE) was invented by Fischer and Lerman in 1979 (Fischer and Lerman, 1979). DGGE works by applying a small sample of double-stranded DNA (or RNA) to an electrophoresis gel that contains a denaturing agent, a mixture of urea and formamide, which is capable of inducing DNA to melt at various stages (Muyzer and Smalla, 1998). DNA fragments which having different sequences partially melt at different positions in the gradient and therefore "stop" at different positions in the gel (Helms, 1990). 16S rRNA genes amplified from environmental samples, with universal primers can then be differentiated by DGGE.

Community structure and diversity surveys of microorganisms in activated sludge based on PCR-DGGE have mainly focused on the polymorphism of 16SrDNA and 16SrRNA. For example, it has been used to characterise nitrifying bacterial communities (Schramm et al., 1996; You et al., 2000). The dominant bands observed from DGGE can be identified by cloning and sequencing them (Kowalchuk et al., 1997). In recent years, modified PCR-DGGE methods have improved the reliability and accuracy of PCR-DGGE. The nested

PCR-DGGE method can extend the possibilities of DGGE in microbial community analysis because it can better reveal subtle changes within or differences between microbial communities (Boon et al., 2002).

Clone library

A clone library is a gene bank containing DNA sequences (clones) with accompanying information. A 16S rDNA clone library can be constructed by amplifying part or the full-length of the 16S rRNA gene of total bacteria in environmental samples using universal primers. PCR products are cloned into plasmids. Library construction requires sufficient amounts of high-quality DNA which is representative of the microbial community present (Marsischky and LaBaer, 2004). Unlike PCR-DGGE, this approach has no method to screen clones and so a lot of sequencing is required to characterise the clones in the library.

16S rDNA clone libraries constructed from environmental samples have proven to be a powerful tool in detecting previously undescribed and potentially unculturable bacterial strains (Delong, 1992; Barns et al., 1994; Fuhrman 1994; Zhang et al., 2007a; Rattray and Craig, 2007). This technique has been used in microbial community analysis in activated sludge (Curtis et al., 2002; Curtis and Sloan, 2004; Kurata et al., 2004; Acinas et al., 2005). The microbial community structure of several activated sludge processes were profiled using 16S rDNA clone libraries (Dionisi et al., 2006; Blackall et al., 1998; Schuppler et al., 1995). However, compared with other microbial ecology techniques, this method attracted surprisingly little attention according to some commonly used scientific databases, partly because of the tedious and laborious library screening. Most of the reports combine 16S rDNA clone library with other techniques such as PCR-DGGE (Zhang et al., 2005a; Ogino et al., 2001; You et al., 2000), FISH (Figuerola and Erijman, 2007; Wong et al., 2004; Okabe et al., 2003; Lee et al., 2001), Restriction Fragment Length Polymorphism (RFLP) (Yi et al., 2003) or quantitative PCR (Mori et al., 2004).

1.6 Dynamics of functional gene expression

The structure and diversity of the microbial community based on 16S rDNA analysis gives us information on the total bacteria which are present. In this case too much information may be given, especially if we are primarily focused on specific functions in the activated sludge, such as nitrification or H_2 production. Functional genes can be good biomarkers. Such genes are often more variable than 16SrDNA genes but they also contain conserved regions which can be used for developing PCR assays (Palys et al., 1997). Some important functions are not associated with a specific taxonomic group. For example, the denitrification-related gene is a functional trait found within more than 50 genera (Zumft, 1997). Ingela et al. assessed the PCR primers targeting *nirS, nirK* and *nosZ* genes for a community survey of denitrifying bacteria and gained satisfactory results regarding the microbial community structure in soil (Ingela et al., 2004).

Nowadays, there is a new and prospective approach for evaluating H_2 production performance by measuring the functional enzyme activity (Chang et al., 2006; Fang et al., 2006; Jo et al., 2007). In activated sludge H_2-producing systems, *Clostridium.* sp. were found and proved to be the dominant and active H_2 producer (Chang et al., 2006; Wang et al., 2007b). The H_2ases in Clostridia are [FeFe] H_2ases and these appear to be highly conserved (Meyer, 2007). Because of the difficulty in culturing certain bacteria in activated sludge and the presence of VBNC bacteria, measurement of [FeFe] H_2ases gene expression level has been regarded as having great potential to be the H_2 production index of the activated sludge system (Chang et al., 2006; Chang et al.,2007).

In all living organisms, cells regulate their cellular activities by activating or deactivating the expression of certain genes. Gene expression corresponds to the number of copies of the particular gene's messenger RNA (mRNA). As mRNA is translated at the ribosome to synthesize functional proteins, mRNA copies tend to roughly correlate with protein expression level. The expression of the [FeFe] H_2ase gene was quantified by measuring mRNA copy number in environmental samples with real-time reverse transcriptase PCR (real-time RT-PCR) (Chang et al., 2006). As a highly conserved enzyme (Meyer, 2007), specific PCR assays targeting [FeFe] H_2ases gene can be easily designed.

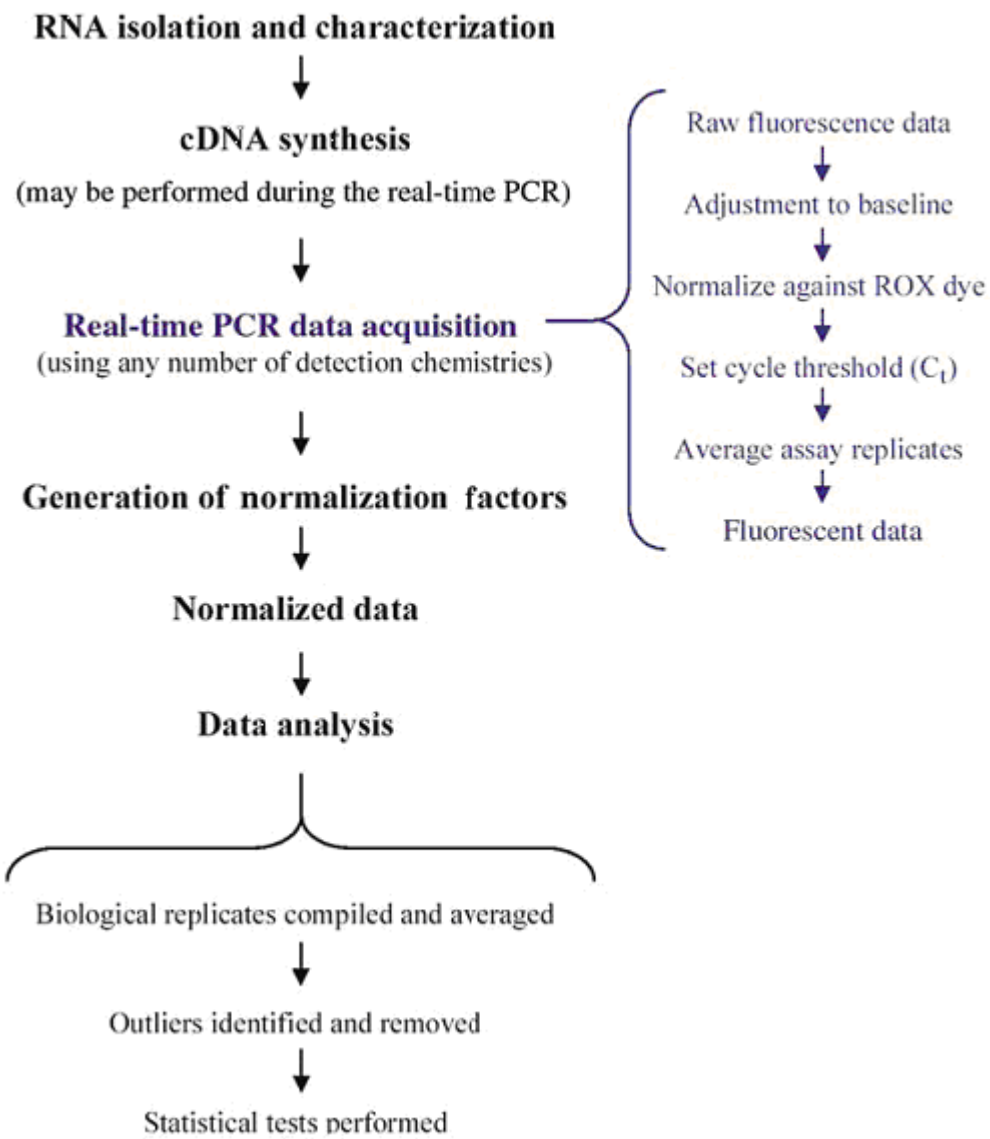


Fig 1.5 Steps performed when measuring gene expression using real-time PCR. RNA is first isolated and characterized for quantity and integrity. If performing a one-step reaction, RNA is used as a template for the real-time PCR assay, and reverse transcription occurs during the assay. During a two-step reaction, cDNA is first synthesized and then used as a PCR template in a separate reaction. The steps performed on the real-time PCR machine are shown in blue, the time during which raw fluorescence data are collected, adjusted, and manipulated to generate the output data used for analysis. For normalizing results with multiple housekeeping genes, a normalization factor must be calculated for each individual sample. Dividing the fluorescent data by its normalization factor produces the normalized data, which is followed by statistical analysis (Wong and Medrano, 2005).

Real-time RT-PCR is powerful and the most widely used technique for detecting and quantifying specific gene expression *in vitro* because of its accuracy and ease of operation (Wong and Medrano, 2005). Real-time RT-PCR has been successfully used to detect and monitor specific functional genes in various applications (Hristova et al., 2001; Hosoda et al., 2005). This technique has been used for the quantification of [FeFe] H_2ase, [NiFe] H_2ase and [NiFeSe] H_2ase in a number of applications and has proven to be an accurate assay for quantification of the H_2ase expression level in single or mixed bacterial cultures (Chang et al., 2006; Fang et al., 2006; Caffrey et al., 2007). However, being a new approach in H_2 production investigation, this technique has not yet been fully evaluated. The specific primers and real-time RT-PCR procedure used in Chang's research (2006) are not specific and have not been optimized. The general steps of a real-time RT-PCR are outlined in Fig 1.5 (Wong and Medrano, 2005)

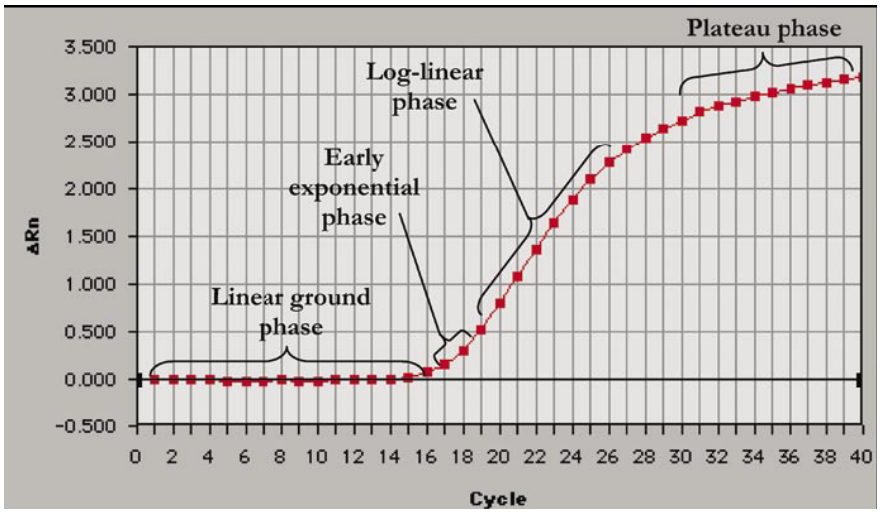
1.6.1 Reverse transcriptase PCR (RT-PCR)

The reverse transcription polymerase chain reaction (RT-PCR) is a technique for amplifying a defined piece of RNA. The RNA is firstly reverse transcribed into its DNA complement *in vitro*, followed by the amplification of the resulting DNA using polymerase chain reaction. RT-PCR can be performed as either a one-step or two-step reaction, depending on whether the entire reaction from cDNA synthesis to PCR amplification is performed in one or separate tubes. One-step real-time PCR is thought to minimize experimental variation and reduce the risk of RNase, RNA or DNA contaminations, because both enzymatic reactions occur in a single tube. However, as reverse transcriptase was reported to be able to inhibit PCR and simulate primer dimer formation (Chumakov, 1994), two-step RT-PCR was regarded to be more sensitive and accurate, especially in situations where using DNA or plasmid as a standard, or the same sample is to be assayed on several occasions over a period of time (Battaglia et al., 1998; Wong and Medrano, 2005). Normally, two-step real-time PCR is performed on dilutions of a single cDNA, which can reduce or eliminate the inhibition of PCR introduced by reverse transcriptase (Mannhalter et al., 2000). Data from two-step RT-PCR was considered reliable when Pearson correlation coefficients (R^2) ranged from 0.974 to 0.988 (Vandesompele et al., 2002).

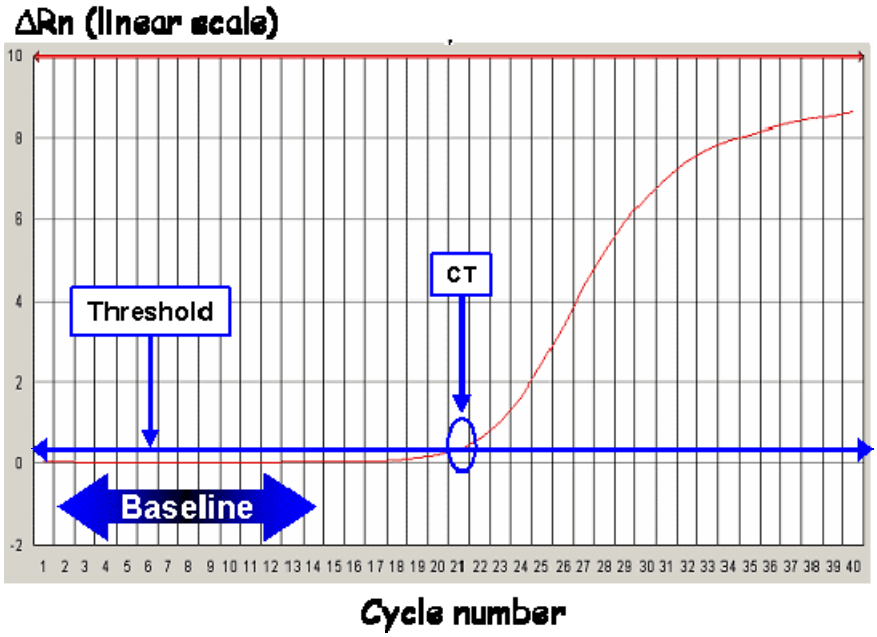
1.6.2 Real-time PCR

Real-time PCR, is an advanced molecular technique based on PCR, which is used to amplify and quantify a targeted DNA sequence simultaneously. Unlike traditional PCR, which only allows analysis of the product after the reaction completed, real-time PCR enables both detection and quantification of a specific sequence in a DNA sample during the exponential phase of amplification, which is more useful and reproducible than end-point analysis for quantification (Cockerill and Smith, 2002; Klein, 2002). A double-stranded DNA-specific binding dye produces fluorescence only when incorporated into double-stranded DNA (ie the amplified DNA). The fluorescence intensity increases with the increase in amplified DNA during PCR, and then the quantification is made according to the proportional relationship between the amount of starting template and the cycle at which amplification enters the exponential phase, therefore allowing DNA concentrations to be quantified (Chiang et al. 2007). The dynamic range of real-time PCR ranges from 7 to 8 log orders of magnitude (Morrison et al., 1998), which is 10,000- to 100,000-fold more sensitive than other detection methods such as RNase protection assays (Wang and Brown, 1999) and dot blot hybridization (Malinen et al., 2003). It can even detect a single copy of a specific RNA molecule (Palmer et al., 2003). Because of the melting curve analysis or the specificity of probes, real-time PCR is sensitive enough to discriminate between mRNA with almost identical sequences (Wong and Medrano, 2005).

The Real-time PCR principle is based on the properties of the PCR reaction kinetics. Like normal PCR, there are four major phases in a real-time PCR reaction, as shown in Fig 1.6 A, the linear ground phase, early exponential phase, log-linear (also known as exponential) phase, and plateau phase (Tichopad et al., 2003). During the first 10–15 cycles (linear ground phase), because the product concentration is still low, the fluorescence signal emitted at each cycle have not yet risen above baseline fluorescence. At the early exponential phase, the amount of fluorescence has reached a threshold where it is significantly higher than background levels and the signal is strong enough for detection, which is usually 10 times the standard deviation of the baseline (Fig 1.6 B). This cycle is known as Ct in ABI Prism® literature (Applied Biosystems, Foster City, CA, USA) or crossing point (CP) in LightCycler® literature (Roche Applied



(A)



(B)

Fig 1.6 Phases of the PCR amplification curve (A) and illustration of Threshold and Ct (B). Rn is the intensity of fluorescent emission of the reporter dye divided by the intensity of fluorescent emission of the passive dye (a reference dye incorporated into the PCR master mix to control for differences in master mix volume). ΔRn is calculated as the difference in Rn values of a sample and either no template control or background, and thus represents the magnitude of signal generated during PCR (Wong and Medrano, 2005).

Science, Indianapolis, IN, USA) (2,10). The Ct value is decided by the initial copy number in samples and is used to represent experimental results (Heid et al., 1996).

There are two common methods of quantification. One uses fluorescent dyes that intercalate with double-strand DNA, such as SYBR Green I (Haugland, 1999) and SYTO 9 (Monis et al., 2005), which bind to the minor groove of double stranded DNA. Such dyes bind to any double-stranded DNA, even non-specific products or primer dimers from PCR reactions. Therefore, DNA melting curve analysis, which determines the *T*m of the DNA after amplification, is necessary to characterise the amplification products at the end of the reaction. Because of the difference in lengths and G+C content of amplification products, they can be identified by their different *Tm* (Higgins et al., 2001).

The other method uses modified DNA oligonucleotide probes that fluoresce when hybridized with a complementary DNA (Wang et al., 2003c). Two separate sequence-specific oligonucleotides are used as hybridisation probes. Both of them are fluorescently labelled. The upstream probe has a donor fluorophore on the 3'-end and the downstream probe has an acceptor fluorophore on the 5'-end. Fluorescence will only occur when the probes have both hybridised next to each other on the target strand. As amplification occurs, either the decrease in the fluorescence of the donor or the increase in the fluorescence of the acceptor can be detected. By this method, as the probes are sequence-specific, there is no need for further product differentiation. Hydrolysis (Taqman) probes and Molecular beacons (MB) are the other two commonly used probe formats, which both employing dual labelling with a fluorophore at the 5'-end and a quencher for that fluorophore at the 3'-end, but have different mechanisms (Mackay et al., 2002).

1.7 Challenges and bottleneck associated with hydrogen fermentation

The ideal situation in an industrial bioprocess setting is to have a microorganism able to consume cheap substrates even waste materials during growth for production of the target product(s) and/or valuable by-product(s) with high production rate and yield. While it is possible to produce

clean H_2 energy from biological H_2 production methods, no commercial systems are yet available because of the low yield and high costs. Although much work has been done to find the most suitable operation conditions for H_2 production, current H_2 economy for the development and commercialization of fermentative H_2 production is still facing a challenge due to the low yield, and the high cost from substrate use (Das and Veziroğlu, 2001; Adamson, 2004; Nath and Das, 2004; Mertens and Liese, 2004; Schütz et al., 2004; Hallenbeck, 2005; Meyer, 2007; Winter, 2005; Kapdan and Kargi, 2006; Hawkes et al., 2007; Lattin and Utgikar, 2007; Marbán and Valdés-Solís, 2007; Rosyid etal., 2007; Kotay and Das, 2008).

Until now, little has been established regarding the factors which control the intermediary metabolism leading to the production of H_2. The major effort for understanding the H_2 production system lies in an appreciation of the environmental conditions and how these fermentation performances respond to the environmental changes that influence H_2 metabolism (Lee *et al*., 1999). Many research projects have been conducted focusing on the reactors and fermenting conditions (Kotay and Das, 2008) in early stage of biological H_2 research. Progress on increasing yield and lowering costs is still unsatisfactory. The substrates which normally lead to high yield were expensive sugar and organic nitrogen sources (Section 1.3.4). The operating parameters used for most processes were arbitrally chosen based on personal experiences without thorough and detailed optimization (Section 1.3.5).

More recently, some researchers have focused on the microbial content and structure of the H_2-producing activated sludge, and concentrated on problems about what the microbial impact on H_2 production is and what the hydrogenating activity is. One of the key topics which concerns researchers is strain development. Selection of efficient H_2 producers has never stopped (Oh et al., 2002; 2003; Kapdan and Kargi, 2006). Genetic improvements in the microorganisms were expected to be able to give better performance. However, the lack of the basic information about the gene cluster structure, gene expression regulation, enzyme maturation, substrate metabolism and by-products formation are all hampering the improvement in yields.

CHAPTER 2

Materials and Methods

The materials and methods described in this chapter are generally used in this thesis. Specific materials and methods will be described in detail in the relevant chapters.

2.1 Substrates

Molasses used in this research was obtained from Magill Grain Store Pty Ltd (Australia). The hexose concentration in the molasses was 44.4% (w/w) and the (Total Kjeldahl nitrogen) TKN was 0.7086%. Waste Potato Starch (WPS) was provided by Smiths Crisps Ltd (Australia). The WPS contained 90% (w/w) starch and 0.04055 % TKN.

2.2 Microorganisms and maintenance

The bacterial strains used in this thesis were three H_2 producers isolated in Chapter 3, *Clostridium* sp. W1 (GenBank Acc. No. DQ831125), *C. butyricum* W4 (GenBank Acc. No. DQ831126) and *C. butyricum* W5 (GenBank Acc. No. DQ831124). The identification of these three isolates is described in Chapters 3 and 4. The original strains were stored in 10% glycerol broths supplemented with 27.5 g/L Tryptone Soya Broth (TSB) (BO0077M, Columbia, HBA, Oxoid) and Microbank® Bacterial and Fungal Preservation System (Pro-Labs Diagnostics, Richmond Hill, Ontario, Canada) at -80°C. The working strains were grown on blood agar (PP2001, Columbia, HBA, Oxoid) and cultured anaerobically in ANAEROGEN system (AN0025, Columbia, HBA, Oxoid) at 37°C for 24 h and stored at 4°C.

2.3 Media

All media described in this section was prepared in Milli-Q water (Millipore Ultra-Pure Water System) with steam sterilization carried out at 121°C for 15 min.

2.3.1 Luria-Bertani (LB) broth for bacterial culture

LB broth used in this thesis consisted of 1% (w/v) tryptone (Oxoid), 0.5% (w/v) yeast extract (Oxoid), 0.5% (w/v) NaCl, pH 7.0.

2.3.2 LB agar for growth of transformed *E. coli*

Transformed *E.* coli were grown on LB agar consisting of 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, 1.5% (w/v) neutralised bacteriological agar (Oxoid), 100 μg/ ml ampicillin, pH 7.0.

2.3.3 Seed culture for fermentation

Tryptone Soya Broth (CM0129, Columbia, HBA, Oxoid) was used as the seed culture medium, following the manufacturer's instructions.

2.3.4 Medium for bottle test

Six carbon sources (glucose, fructose, lactose, sucrose, WPS and molasses) and 6 nitrogen sources, yeast extract (LP0021, Columbia, HBA, Oxoid), neutralised soya peptone (NSP) (LP0044, Columbia, HBA, Oxoid), urea, $(NH_4)_2SO_4$, KNO_3 and NH_4NO_3 were employed in the screening investigations for H_2 production. Yeast extract was used as the nitrogen source for the carbon source screening experiments. Glucose was used as the carbon source for the trials of nitrogen sources. The fermentation media contained carbon sources equal to 10g/L hexose and nitrogen sources equal to 3g/L yeast extract.

2.4.5 Medium for selection of carbon and nitrogen sources in the bioreactor

Carbon and nitrogen source concentrations in fermentation media were the same as those used in the bottle tests.

2.4.6 Medium for optimizing molasses and NH_4NO_3 concentration

A series of molasses concentrations from 20 to 120g/L were tested to find out the optimal carbon source concentration. This was then used afterwards to find the optimal NH_4NO_3 concentration (0-1.5g/L).

2.4.7 Medium for optimizing operating parameters

The fermentation media contained molasses 100g/L and NH_4NO_3 1.2g/L.

2.4 Fermentation process set-up

2.4.1 Bottle tests

Preliminary screening for strain selection and suitable carbon and nitrogen sources were carried out at 35°C with initial pH 7.0 in refitted 1 L bottles, which have two tubes fitted into the rubber stopper to allow inert gas purging and biogas emission. The working volume was 800 ml and 10% (v/v) seed culture was used as the inoculum. Biogas was collected by the water release method (Fig 2.1).

Fig 2.1 Bottle test device

2.4.2 Bioreactor system

The fermentation was performed in a laboratory-scale batch bioreactor BioFlo 110 (New Brunswick Scientific, USA) with a working volume of 1.5-liter (Fig 2.2). All the connections were sealed with silicon gel to maintain an anaerobic environment. The inoculum was transferred with a sterile hypodermic disposable syringe. Nitrogen purging of the medium was performed before the fermentation to maintain an anaerobic environment. Biogas produced was collected by

the water release method (Fig 2.1). The fermentation conditions were varied according to experimental requirements.

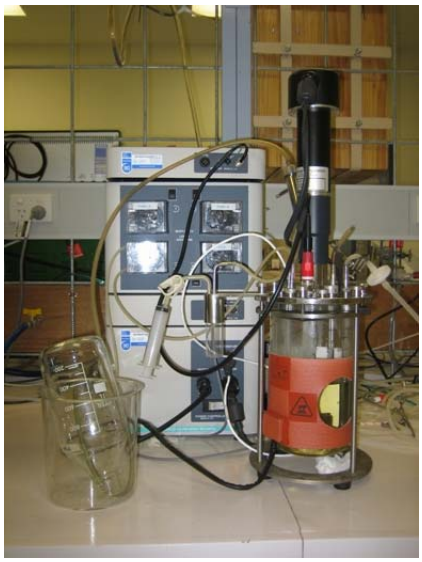


Fig 2.2 Bioreactor system used in this thesis

2.5 Sample collection and preparation

In the bottle test experiment, total biogas volume and H_2 concentration were measured. Twenty (20) ml of fermentation broth in the bottle was collected after 16 hours for analysis of H_2 production and biomass. For batch fermentation, except for those described in Chapter 7, biogas volume and H_2 concentration were measured and 20 ml of fermentation broth in the bioreactor was collected at certain intervals for investigation of the kinetics of H_2 accumulation, cell growth and formation of VFAs.

2.6 Chemical analysis

Fermentation broth samples were centrifuged at 4 ^{o}C, 3000 rpm for 20 min. Bacteria biomass was washed twice with Mill-Q water and weighed after drying at 60 ^{o}C for 4h for biomass measurement.

One hundred (100) µl of the supernatant was diluted 10 times and further filtered through a 0.22µm membrane for analysis of VFAs by HPLC. The HPLC analysis used a ROA Organic Acid Column (Phenomenex, 300×7.8) and a refractive index detector (Varian, Model 350). The mobile phase was 4 mM H_2SO_4 at a flow rate of 0.5 mL min^{-1} and the column temperature was 50^{o}C.

The biogas was sampled using a glass syringe and analyzed on a CP-3800 gas chromatograph (Varian Inc. CA, USA) equipped with a thermal conductivity detector (Wang et al., 2007b). The working temperatures of injector, detector, and column were maintained at 50°C, 140°C and 40°C, respectively. All results were obtained from the means of triplicate determinations.

2.7 Molecular techniques

2.7.1 Nucleic acids extraction

2.7.1.1 DNA extraction

Genomic DNA from pure cultures was extracted by rapid freeze-thawing (Kawai et al. 2002) in Chapter 3. Cell suspensions underwent three cycles of boiling at 100°C for 5 min followed by freezing in liquid nitrogen for 2 min, and then a cycle as follows: 56°C for 10 min, 99°C for 10 min and then 56°C for 4 min in a thermal cycler (Perkin Elmer GeneAmp 2400, Applied Biosystems Norwalk, CT) with 100 µl InstaGene™ Matrix (BIO-RAD Laboratories Inc., Hercules, CA) added. In other chapters, genomic DNA from pure cultures was extracted by QIAamp DNA Mini Kit (Qiagen GmbH, Germany).

2.7.1.2 RNA extraction

For total cellular RNA isolation, 200µl of fermentation broth was mixed thoroughly with 2 volumes of RNAprotect Bacteria Reagent (Qiagen GmbH, Germany) immediately after sampling, incubated for 5 min at room temperature and then centrifuged at 5000 rcf (4°C for 10 minutes). Pellets were stored at -20°C until further extraction with RNeasy® Protect Bacteria Mini Kit (Qiagen GmbH, Germany), which was carried out according to the Enzymatic lysis and Proteinase K digestion protocol described in the RNAprotect® Bacteria Reagent Handbook. Pellets can be frozen and stored at –20°C for up to 2 weeks, or at –70°C for up to 4 weeks.

2.7.2 PCR reactions

The PCR machine used in this research was a Perkin Elmer GeneAmp 2400 PCR system (Applied Biosystems Norwalk, CT) (Fig 2.3 A) for routine amplification, and a Mastercycler® ep Gradient S thermocycler (Eppendorf, Westbury, NY) for gradient amplification (Fig 2.3 B).The specific PCR protocols and primer sets used in this thesis are described in Chapters 3, 4 and 7.

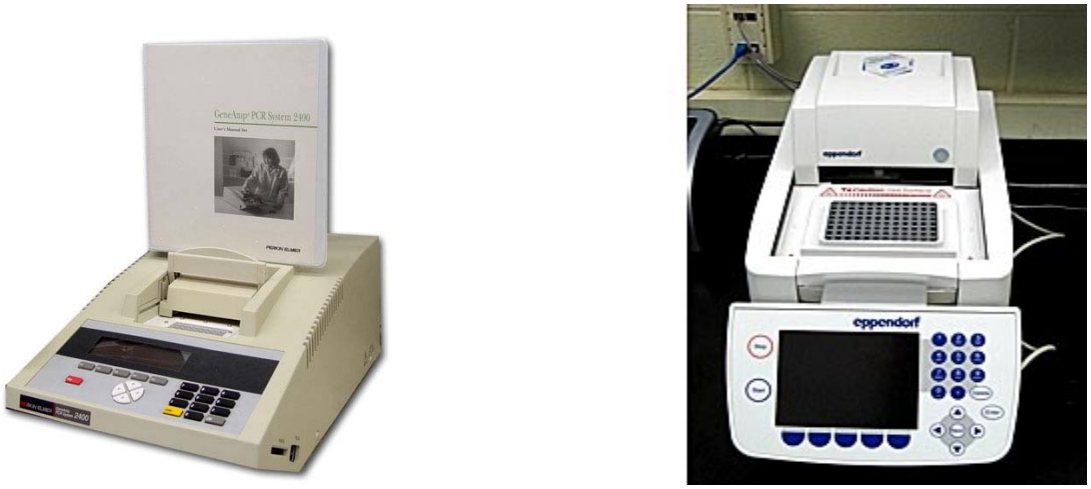
2.7.2.1 Universal eubacterial nested 16S rRNA gene-directed PCR

Nested PCR using primer set 27F / 1492R for the first round of amplification and primer set 357F-GC /518R for the second round (Suzuki and Giovannoni, 1996) was used in this thesis when template DNA concentration in environmental samples was too low to be detected in one PCR reaction. The sequences are listed in Table 2.1.

Table 2.1 Primers used for nested PCR amplification

Primer	**Sequence (5' – 3')**
27F	AGAGTTTGATCCTGGCTCAG
357F-GC	GC clamp-CCTACGGGAGGCAGCAG
518R	ATTACCGCGGCTGCTGG
1492R	TACGGTTACCTTGTTACGACT
GC clamp	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG

For the two rounds of PCR, each 50 µl reaction mixture contained 200 µM of each deoxynucleoside triphosphate, 1.0 µM of each primer, 2.5 mM $MgCl_2$, 1xPCR buffer II (Applied Biosystems, Foster City CA), 5% v/v DMSO, 2.5 U of Ampli *Taq* Gold DNA polymerase (Applied Biosystems) and 5 µl of genomic DNA template for the first round or 2 µl PCR product from first round for the second round. Before the addition of the DNA polymerase and template, the PCR mix was exposed to UV for 10 min to reduce the level of any contaminating bacterial DNA that might be present in any of the reagents (Goldenberger and Altwegg, 1995). Negative



(A) (B)

Fig 2.3 PCR machines used in this thesis

controls consisted of an equal volume of nuclease free water in place of the DNA template. Thermal cycling consisted of an initial denaturation at 95°C for 10 min to allow activation of the *Taq* polymerase, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 1 min and extension at 72°C for 2 min. A final extension at 72°C for 10 min was then performed. Reaction products were verified by 1.0 % (w/v) agarose gel electrophoresis (Section 2.7.3).

2.7.3 Agarose gel electrophoresis

DNA fragments generated from PCR and real-time PCR reactions, or restriction enzyme digestion (Chapter 4) were verified for correct size by electrophoresis at room temperature using

1.0% (w/v) agarose gels stained with 1x SYBR Safe™ nucleic acid stain (Invitrogen, Carlsbad, CA, USA). Five µl PCR products were mixed with 5 µl 2xloading buffer (Geneworks, SA, Australia) and electrophoresed in 1xTBE buffer (0.1 % [w/v] Tris-base, 0.93 % [w/v] EDTA, 0.055% [w/v] boric acid) at 100 V with an Owl electrophoresis chamber connected to an EC250-90 electrophoresis power supply. For each gel, a control marker consisting of a 100 or 500 bp ladder (Geneworks, SA, Australia) was used as marker (Chapter 4). After electrophoresis, DNA was visualised on a Dark Reader transilluminator (Clare Chemical Reseach, Dolores, CO, USA) and the image was captured using an electrophoresis documentation and analysis system (EDAS) 290 using Kodak version 3.5.3 1D Image Analysis Software (Eastman Kodak Company, NY, USA).

2.7.4 Cloning of PCR products

PCR products were cloned into *E. coli* directly using the TOPO TA Cloning® Kit (Invitrogen, CA, USA) following the manufacturer's instructions. The volumes of reagents are listed in Table 2.2. For direct cloning of DNA fragments, bands were excised from agarose or DGGE gels and 4 µl of DNA fragment (cleaned by an UltraClean™ GelSpin DNA Purification Kit (MoBio Laboratories Inc., Solana Beach, CA)) was used. For cloning from PCRs, 2 µl of PCR product was used.

After the addition of pCR®2.1-TOPO® vector (Fig 2.4), the reaction mix was incubated at room temperature for 5 min for PCR products and 30 min for inverse PCR products which were longer than 1kb. According to the manufacturer's instructions, increasing the reaction time will yield more colonies. 2 µl of the cloning reaction was added to a vial of One Shot® Chemically Competent *E. coli* and incubated on ice for 5 min. Cells were then heat shocked at 42°C for 30 s and then immediately added to 250 µl of pre-warmed SOC medium. For initial cell recovery the *E. coli* were incubated at 35°C for 1 h with horizontal shaking at 200 rpm. Transformants were then cultured overnight on LB agar containing 100 µg/ml X-gal and 100 µg/ml ampicillin. White colonies (which should to contain inserts) were randomly picked and tested by PCR and electrophoresis for the correct sized fragment.

Table 2.2 TOPO® Cloning reaction set up

Reagent	**Chemically Competent *E. coli* (μl)**
DNA fragments	N
Salt Solution	1
Dilute Salt Solution	--
Sterile Water	4 - N
TOPO® vector	1
Final Volume	6

2.7.5 Plasmid extraction

Colonies with correct inserts were inoculated into 5 ml LB broth containing 100 μg/ ml ampicillin and incubated overnight at 35°C. Plasmid DNA was isolated with the Spin Miniprep Kit (Qiagen GmbH, Germany), following the manufacturer's instructions. Final elution volume was 50 μl in the provided sterile Milli-Q water.

2.7.6 DNA sequencing of plasmid inserts

Inserts were sequenced by the Australian Genome Research Facility Ltd (Brisbane, QLD, Australia) using the BigDye Terminator Sequencing Reaction Kit (Applied Biosystems Norwalk, CT). Sequence chromatograms were analysed using SeqMan II version 4.05 (Dnastar, Madison, WI, USA). Sequence similarity searches were performed using the Basic Local Alignment Search Tool (BLAST) program to search the National Center for Biotechnology Information (NCBI) sequence database (http://www.ncbi.nlm.nih.gov /BLAST/).

2.8 Statistical analysis

The experimental data were analysed in a regression mode (Microsoft Excel 2003) to establish the relationships between H_2 yield and by-products of bacterial biomass and VFAs produced in the fermentation under different operational conditions. The model equation was determined by choosing the highest R^2 of each trendline. Data used for profiling the kinetic curves and further analysis were mean of the triplicates of laboratory test results. The standard deviations (SD) of

three samples were less than 3%. Appropriate tests of significance analysis of variances (ANOVA) and confidence limits at the 5% level were used in the data evaluations.

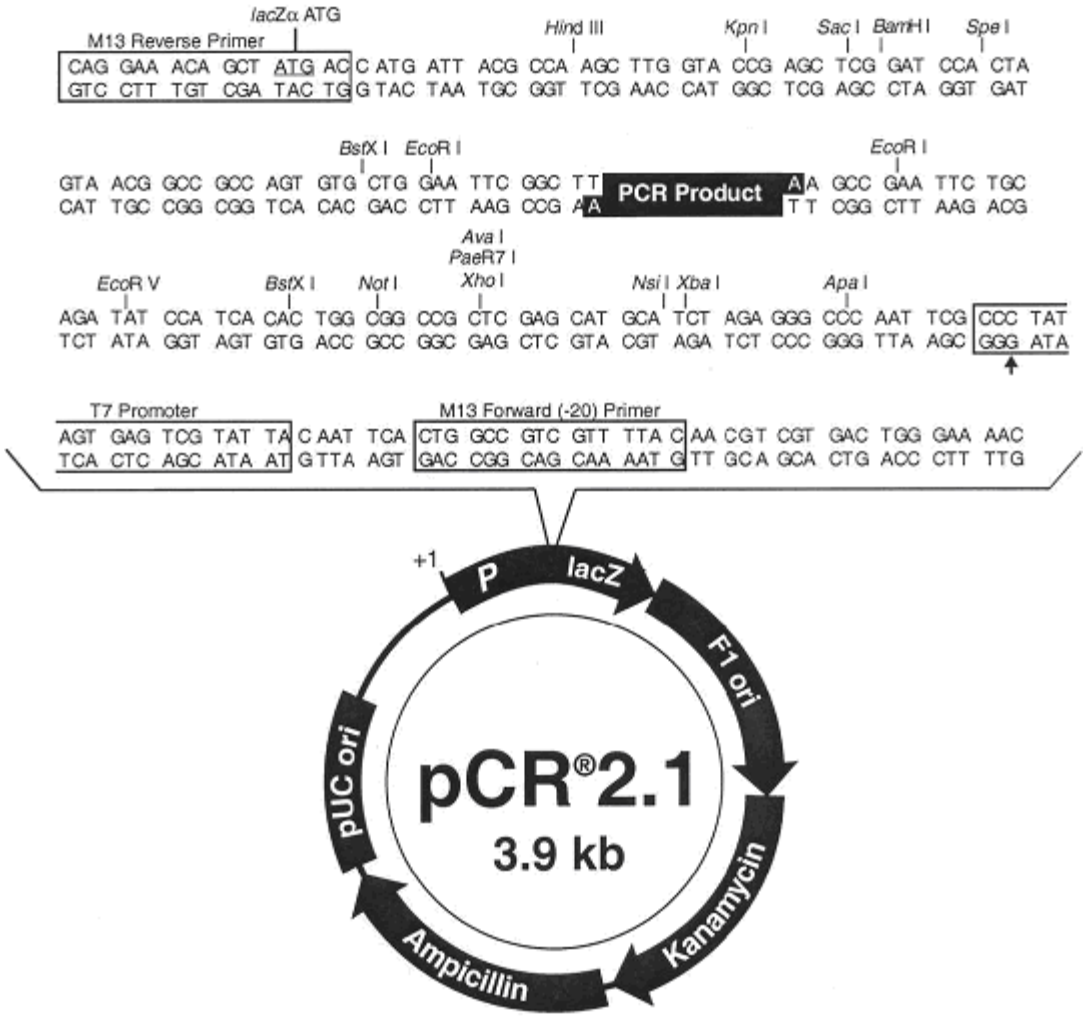


Fig. 2.4 Map of pCR®2.1-TOPO® (Invitrogen Corporation)

CHAPTER 3

The Isolation and Microbial Community Analysis of Hydrogen-producing Bacteria from Activated Sludge

3.1 Introduction

While it is possible to produce environmentally friendly hydrogen (H_2) energy from biological production methods, no commercial systems are yet available. As discussed in Chapter 1, the commercialization of H_2 production has been hampered in the past due to low yields of H_2 and relatively high production costs. Much work has attempted to seek the most suitable conditions for H_2 production. However, yields are still low (Lee et al., 1999; Kapdan and Kargi, 2006). To date, much of the research has concentrated on optimization of reactor and fermentation conditions (Nath and Das, 2004). Little work appeared to have focused on the microbial community structure in the fermentation system and how this impacts on H_2 production. Recently, using Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE), Chang et al. (2005) reported that clostridia represented the largest component of the microbial community in heat-treated activated sludge. However, the identity of the main H_2 producers is yet to be determined and it is not known if the production yield can be increased by manipulation of the community composition.

An important first step towards understanding the roles of the various bacteria in the H_2-producing process is not only to determine the numbers and the relevant abundances of different bacterial groups, but also to identify the bacteria responsible for the bulk of the activity. As discussed in Section 1.5, methods employed for microbial community investigation are generally divided into culture-dependent or culture-independent methods. Conventional culture-dependent methods result in the characterization and isolation of bacteria which can be cultured on selective media. However, about 99% of wild bacteria remain “un-culturable” and are still undescribed (Amann et al., 1995). Culture-independent approaches such as PCR-DGGE (Section 1.5.2) and 16S rDNA clone libraries (Section 1.5.2) compensate for the shortcomings of traditional culture methods by providing the structure of microbial communities irrespective of how conducive the organisms are to culture (You et al., 2000; Van der Gucht et al., 2005).

In this chapter, the total bacterial community profile of a H_2-producing process was assessed by 16S rRNA gene-directed PCR-DGGE, and subsequent identification of the major bands performed after cloning and DNA sequence analysis. A 16S rRNA gene clone library also confirmed the dominance of *Clostridium* sp. in this process. Organisms conducive to culture

were isolated onto solid media and their potential to produce H_2 was investigated by PCR amplification of the H_2ase gene. The production of H_2 by those isolated bacteria containing the H_2ase gene was confirmed by fermentation and production of H_2 by the individual isolates.

A paper based on the research of this chapter "Isolation and Microbial Community Analysis of Hydrogen-Producing Bacteria from Activated Sludge" has been published in the scientific journal, *Journal of Applied Microbiology,* 2007, 103(5):1415-1423 (Appendix).

3.2 Aims

In this chapter, an attempt was made to investigate the microbiological structure in heat-treated activated sludge and isolate efficient H_2 producers. The detailed aims were:

- Profile the bacterial community of heat-treated activated sludge with 16S rRNA gene-directed PCR-DGGE and 16S rDNA clone library.
- Isolate potential H_2 producers in the system.
- Identify the H_2 production ability of individual isolates.

3.3 Specific materials and methods

3.3.1 Seed activated sludge

Seed activated sludge was collected from anaerobically digested sludge at the Bolivar Wastewater Treatment Plant, South Australia. To eliminate methane-producing bacteria the sludge was heat-treated by three cycles of boiling at 100°C for 15 min and cultured at 35°C overnight (O/N), as described by Wang et al. (2004). The pretreated sludge was seeded into sterilized waste potato water (WPW) (provided by Smiths Crisps Ltd, Australia) in an 800ml working volume anaerobic reactor. TKN of WPW was 3.2g kg^{-1}. The total solid content was 0.01% and the starch content of the WPW was adjusted to 1%. The initial pH of fermentation was 6.2.

3.3.2 DNA extraction from activated sludge

Activated sludge from the exponential phase of the fermentative H_2 production was taken in sterile 1.5 ml microfuge tubes, immediately closed and stored at –20°C until further processing. For DNA extraction, the sludge was centrifuged at 18,000 g (4°C for 10 min) and the resulting pellet was resuspended with 100 µl 10mM Tris.HCl, pH 8.5. DNA was subsequently extracted by using the Ultra Clean™ Soil DNA Kit (MoBio Laboratories Inc., Solana Beach, CA), which has been described previously as a successful kit for extracting DNA from activated sludge (Kreuzinger et al., 2003). The extraction was confirmed by gel electrophoresis. DNA was stored at –20°C until further processing.

3.3.3 PCR-DGGE profiling microflora

Nested PCR amplification was performed as described by Hoefel (Hoefel et al., 2005). Primer set 27F / 1492R was used for the first round of amplification and primer set 357F-GC /518R for the second round (Table 3.1). Amplified DNA was examined by horizontal electrophoresis in 1% agarose with 5 µl aliquots of PCR product using standard methods (Section 2.7.3). Before the addition of the DNA polymerase and template, the PCR mix was exposed to UV for 10 min to reduce the level of any contaminating bacterial DNA that might be present in any of the reagents (Goldenberger and Altwegg, 1995).

DGGE was performed using the Bio-Rad D-GENE^{TM} Denaturing Gel Electrophoresis System (Bio-Rad, Hercules, CA, USA) as previously described (Hoefel et al. 2005). Nested-PCR products were applied directly to 8% (w/v) polyacrylamide gels in 1 x TAE (40 mmol/l, Tris-base, 0.12% (v/v) acetic acid (glacial), 1.0 mmol/l EDTA (pH 8.0)) with a denaturing gradient ranging from 40 to 70% (where 100% denaturant contained 7 mol/l urea and 40% formamide). Amplified DNA from *E. coli, Aeromonas hydrophila* and *Staphylococcus epidermidis* was used as a DNA reference marker. After 16h electrophoresis at a constant voltage of 60V at 56 ^{o}C, gels were stained with a 1 x SYBR Green I solution (FMC BioProducts; Rockland, ME, USA) in 1 x TAE for 2 h. A gel documentation and analysis system (EDAS 290 with Kodak version 3.5.3 1D Image Analysis Software, Eastman Kodak Company, Rochester, NY, USA) was used to analyze stained gels.

Bands excised from the DGGE gel were cleaned using an UltraClean™ GelSpin DNA Purification Kit (MoBio Laboratories Inc., Solana Beach, CA). Cloning, sequencing and sequence similarity searches were performed according to the description in Section 2.7.

3.3.4 Phylogenetic analysis

Full length sequences of *C. roseum* (Acc. No. Y18171), *C. diolis* (Acc. No. AJ458417), *C. acetobutylicum* (Acc. No. U16165), *C. beijerinckii* (Acc. No. X68179), *C. saccharoperbutylacetonium* (Acc. No. U16122), *C. butyricum* (Acc. No. X68178), *C. punicem* (Acc. No. X71857) and *C. tyrobutyricum* (Acc. No. M59133) were obtained from GenBank. Additional sequence data for isolates W1, W4 and W5 were generated by sequencing the 27F, 357F, 786F and 1492R amplicon. Sequences were aligned using ClustalX version 1.8 (Thompson et al., 1997). Phylogenetic analyses were conducted on an approximate 1401 nucleotide fragment of the 16S rRNA gene using MEGA 3.1 (Kumar et al., 2004).

In the case of W5 the 5' end was truncated by 116nt and *C. roseum* was truncated at the 3' end by 34nt. Distance-based analyses were conducted using Tamura-Nei distance estimates and the Neighbor Joining algorithm with the pair-wise deletion option selected. Maximum parsimony analysis was conducted using all sites and close neighbor interchange for tree construction, with a search level of 1 and random addition of trees with 10 replications. All analyses were conducted using 1000 bootstrap replicates to determine the robustness of the resulting tree topologies.

3.3.5 Cloning profile and sequencing

Nested PCR products were cloned into *E. coli* directly using the TOPO TA Cloning® Kit (Invitrogen, CA, USA) following the manufacturer's instructions. Plasmid DNA was extracted from positive colonies by QIAprep® Spin Miniprep Kit (Qiagen GmbH, Germany) and amplified by 16S rRNA gene specific primer set 357F and 518R. After analysis by DGGE, amplicons with distinct migration positions were sequenced as described above.

Table 3.1 Primers used for PCR amplification in this chapter

Primer	Sequence (5' – 3')	Target	Reference
E1f	GCTGATATGACAATAATGGAAGAA	H_2ase gene	Jui-Jen Chang et al., 2005
E1r	GCAGCTTCCATAACTCCACCGGTTGCACC	H_2ase gene	Jui-Jen Chang et al., 2005
L1f	AAATCACCACAACAAATATTTGGTGC	H_2ase gene	Jui-Jen Chang et al., 2005
L1r	ACATCCACCAGGGCAAGCCATTACTTC	H_2ase gene	Jui-Jen Chang et al., 2005
27F	AGAGTTTGATYMTGGCTCAG	Bacteria 16S rDNA	Suzuki and Giovannoni, 1996
357F	CCTACGGGAGGCAGCAG	Bacteria 16S rDNA	Muyzer et al., 1993
357F-GC	GC clamp-CCTACGGGAGGCAGCAG	Bacteria 16S rDNA	Muyzer et al., 1993
786F	GATTAAATACCCTGGTAG	Bacteria 16S rDNA	Neimark and Kocan, 1997
518R	ATTACCGCGGCTGCTGG	Bacteria 16S rDNA	Muyzer et al., 1993
1492R	TACGGYTACCTTGTTACGACT	Bacteria 16S rDNA	Suzuki and Giovannoni, 1996
GC clamp	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACG GGGGG		Muyzer et al., 1993

3.3.6 Heterotrophic plate isolation

Two media were used to culture and isolate total bacteria in H_2-producing activated sludge: nutrient agar and blood agar (PP2001, Columbia, HBA, Oxoid). The plates were cultured in a MK3 Anaerobic Work Station (Don Whitley Scientific Limited, West Yorkshire, England) in a 10% H_2 and 90% nitrogen gas atmosphere at 35°C for 48 h. DNA was extracted from colonies picked randomly, and amplified using primer set 357F-GC and 518R, and then analyzed by DGGE. As described above, amplicons with distinct migrations were sequenced.

3.3.7 H_2ase gene amplification

The primers of Jui-Jen Chang et al. (2005) were used to amplify a segment of the H_2ase gene (Table 3.1). Two sets of the primers were designed to target the same H_2ase gene and to be partially overlapping to verify if any resulting amplicons came from the same domain. The reaction conditions were modified to reduce non-specific amplification as follows: 95^{o}C for 10 min to activate the *Taq* polymerase (Amplitaq Gold, Applied Biosystems, Foster, California), followed by 40 cycles of denaturation (30s at 94 ^{o}C), annealing (1 min at 53^{o}C), and extension (1 min at 72 ^{o}C), with a final extension at 72 ^{o}C for 10 min. Amplicons were examined by horizontal electrophoresis in a 1% agarose gel. Amplicons of the expected sizes were cleaned using an UltraClean^{TM} PCR Clean-up Purification Kit (MoBio, Laboratories Inc., Solana Beach, CA) and sequenced as described above.

3.4 Results and discussion

3.4.1 PCR-DGGE profiling of H_2-producing bacteria in activated sludge

Total genomic DNA was extracted from H_2-producing activated sludge at a time when maximum H_2 was produced. This DNA was used as a template for profiling the bacterial community composition by 16S rRNA gene-directed PCR-DGGE. As shown in Fig 3.1, the community profile consisted of 4 major bands indicating a relatively low diversity of bacteria within the H_2-producing community. This result is consistent with other investigations on H_2 production by activated sludge which showed that only a few bacterial species survive after heat or acid

pretreatment (Wang et al., 2004; Chang et al., 2005). These major DGGE bands were excised, and cloned into *E. coli.* Cloned inserts were verified against the original DGGE profile

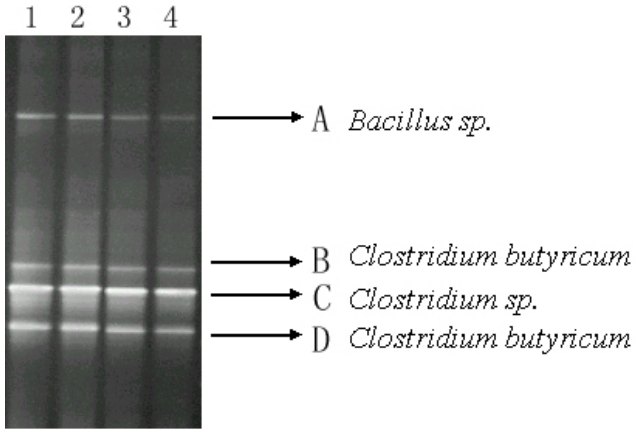


Fig 3.1 PCR-DGGE profile of bacteria in H_2-producing activated sludge. DNA fragments amplified by nested PCR were performed in quadruplicate. The major bands are shown by arrows.

following re-amplification using the original PCR-DGGE protocol. DNA sequence analysis revealed band A to have 96% sequence similarity to a species of *Bacillus,* band B to have 99% similarity to *C. butyricum* (Acc. No. X68178), band C to have 99% sequence similarity to *C. sp.* (Acc. No. AY082483) and band D to have 100% sequence similarity to *C. butyricum* (Acc. No. X68178).

3.4.2 16S rRNA gene clone library

DGGE successfully profiled the most dominant bacteria in the H_2-producing activated sludge. Further attempts to identify the presence of other less abundant bacteria were performed by construction of a 16S rRNA gene clone library using the products of the same PCR separated by DGGE. Twenty seven clones were analyzed by PCR-DGGE. DNA sequence analysis was performed and five inserts were subsequently further analyzed. This revealed these as representing *C. butyricum* (Acc. No. AY442812, 100% similarity), *C. sp.* (Acc. No. AY082483, 100% similarity), *Aneurinibacillus aneurinilyticus* (Acc. No. AB211018, 100% similarity), *C. sp.* (Acc. No. DQ168199, 100% similarity) and *C. sp.* (Acc. No. AY082483, 96% similarity). The bulk of clones (80%) in the library belonged to the genus *Clostridium*. The bacterial

community structure determined by the 16S rDNA clone library method was slightly different to the PCR-DGGE result, possibly because the library has the potential to detect minor species within the population that fail to amplify sufficiently to be detectable by PCR-DGGE (Rainey et al., 1996; Vallaeys et al., 1997; von Wintzingerode et al., 1997).

3.4.3 Heterotrophic plate isolation

Attempts were made to isolate as many types of bacteria as possible from the H_2-producing activated sludge. Following culture onto solid medium, colonies were randomly selected and characterized using the primer set 357F-GC/518R and PCR-DGGE. The results showed that five different bacterial species were isolated and designated as W1, W2, W3, W4 and W5. They differed in colony shape and haemolytic characteristics on blood agar (Table 3.2). The isolates were identified using 16S rRNA gene sequence analysis. One isolate, that was designated as W1, was similar (99%) to *C. diolis* (Acc. No. AJ458417) and only grew on nutrient agar. The other four isolates, designated W2 to W5, were similar to *Bacillus sp.* (Acc. No. AF169535, 96%), Swine manure bacterium (Acc. No. AY167932, 100%), *C. butyricum* (Acc. No. X68178, 99%) and *C. butyricum* (Acc. No. X68178, 99%) respectively and grew only on blood agar (Table 3.2). The 16S rRNA gene sequences of isolates W1, W4 and W5 have been deposited onto GenBank with accession numbers DQ831125, DQ831126 and DQ831124 respectively.

3.4.4 H_2ase gene-targeted PCR analyses

In order to test their potential to produce H_2, isolates were screened by PCR for the presence of H_2ase gene using primer sets E1f/E1r and L1f/L1r. All of the isolates were PCR positive using primer set E1f/E1r, while only W3, W4 and W5 were PCR positive using primer set L1f/L1r. The amplicons were sequenced and identified by BLAST searching the NCBI sequence database (GenBank accession numbers AAT76847, CAB13459, ZP_00800425, AAT76847 and AAT76847 for W1 – W5 respectively). The results (Table 3.2) suggest that primer sets E1f/E1r and L1f/L1r are not specific for H_2ase genes because one amplicon was similar to a dehydratase gene and another had a relatively low similarity (67%) to other H_2ase genes. This lack of

Table 3.2 Summary of the isolates

Isolate	16S rDNA sequence results		Colony characters*	Haemolytic type	H_2ase gene targeted primers PCR results		H_2 production yield (ml H_2/g glucose) **
	Closest similar sequence	Similarity			Closest similar sequence	Similarity	
W1	*C. diolis,* Acc. No. AJ458417	99% (1375/1383)	Round and clear edge, 1mm, cream	-	H_2ase, *C. diolis* isolate Z2, Acc. No. AY652732	96% (110/115)	63/150
W2	*Bacillus sp.,* Acc. No. AF169535	96% (1329/1379)	Round and clear edge, 2mm, gray	-	L-serine dehydratase (alpha chain), *Bacillus subtilis* subsp. *subtilis* str. 168, Acc. No. CAB13459	85% (101/118)	0
W3	*Swine manure bacterium,* Acc. No. AY167932	100% (1296/1296)	Irregular shape and edge, 4mm, white	+	Ferredoxin: 4Fe-4S ferredoxin H_2ase, *Alkaliphilus metalliredigenes* QYMF, Acc. No. ZP_00800425	67% (95/141)	0
W4	*C. butyricum,* Acc. No. X68178	99% (1434/1435)	Irregular shape and edge, 8mm, gray	-	H_2ase, uncultured *C. sp.,* Acc. No. AAT76847	99% (129/130)	70/167
W5	*C. butyricum,* Acc. No. X68178	99% (1078/1079)	Irregular shape and edge, 2mm, gray	-	H_2ase, uncultured *C.* sp., Acc. No. AAT76847	99% (146/147)	86/220

*As W1 can't grow on blood agar, the colony characteristics were determined by nutrient agar culture. Others were all determined by blood agar culture.

** Data are shown for H_2 production yield with both mineral salt medium and improved mineral salt medium.

specificity might lead to over estimation of H_2ase gene expression if these primers are used to measure transcription copies, if it served as an activity index for the H_2 production system. The amplicons from isolates W1, W4 and W5 had high similarity (96%, 99%, 99% respectively) with H_2ase genes from *Clostridium* sp.

3.4.5 Hydrogen production

The five isolates were seeded into mineral salt medium to test their ability to produce H_2. Only W1, W4 and W5 could produce H_2 after adjustment of the initial pH and fermentation temperature (data not shown). H_2 yields for isolates W1, W4 and W5 were 63ml H_2/g glucose, 70ml H_2/g glucose and 86 ml H_2/g glucose respectively (Table 3.2). One hundred and fifty (150) ml H_2/g glucose, 167 ml H_2/g glucose and 220 ml H_2/g glucose were achieved after further testing with an improved mineral salt medium. The fermentation time was shortened from 48 hours with mineral salt medium to 24 hours with improved mineral salt medium. As assessed by HPLC, the main organic acid by-products produced by W1 were acetic and butyric acids, while W4 and W5 produced acetic, butyric, malic and fumaric acids. Production of malic and fumaric acids may correlate with the higher H_2 production yields because more protons would be released (Hallenbeck and Benemann, 2002; Vijayaraghavan and Amin Mohd Soom, 2006). For W4 and W5, strict anaerobic conditions were required, which included nitrogen purging of the medium and fermentation device before fermentation. However, unlike W4 and W5, W1 grew and produced H_2 well under non-strict anaerobic conditions.

The H_2 production yields for isolates W1, W4 and W5 were much higher than those for *Clostridium* sp. reported elsewhere under the same reaction conditions, batch fermentation, without pH adjustment and no nitrogen sparging during fermentation (Kumar and Das, 2000; Oh et al., 2002; 2003; Shin et al., 2004). The great decrease of fermentation period was also very attractive for this fermentation to be industrialized. These three isolates will also allow the more detailed investigation of the relationship between H_2 yield and by-products composition. One hypothesis is that the higher H_2 yield produced by these isolates is associated with production of both malic and fumaric acids, which were not reported previously as by-products accumulated during fermentation. This may result in the release of more protons (Hallenbeck and Benemann, 2002; Vijayaraghavan and Amin Mohd Soom,

2006). Further investigation could be done to compare the metabolic pathways for H_2 production in these three isolates and to determine if the pathways can be manipulated to produce useful by-products and at the same time improve the H_2 yield, which will make biological H_2 production more profitable and practicable.

The ability of isolates to produce H_2 in culture correlated with the detection of a H_2ase gene with high DNA sequence similarity to a H_2ase gene described from *Clostridium* sp. Phylogenetic analysis using 16S rRNA gene sequences from the H_2-producing isolates established that these two bacteria were *C. butyricum*, while the other could not be definitively identified but was similar to *C. roseum*, *C. diolis*, *C. acetobutylicum* and *C. beijerinckii.*

Another interesting outcome was the isolation of W1, which appeared to grow and produce H_2 in non-strict anaerobic conditions. Isolates W4 and W5, as well as the other species of *Clostridium* reported in the literature to produce H_2, require strict anaerobic conditions. The requirement for strict anaerobic conditions greatly increases production costs because of the relatively high price of gas or reducing agents, such as argon, nitrogen, H_2 gas and L-cystine·HCL, required to remove trace amounts of oxygen from the medium, and the additional constraints placed on reactor design and operation (Fabiano and Perego, 2002; Kapdan and Kargi, 2006). A mixed fermentation using *Enterobacter aerogenes* and *C. butyricum* was used to solve this problem, as *E. aerogenes* would remove oxygen from the reactor. However, *E. aerogenes* became dominant, which led to lower H_2 yields (Yokoi et al., 2001). A *Clostridium* species with the ability to tolerate dissolved oxygen will be highly beneficial and make dark fermentation more economical and practical. To my knowledge, there are two possible reasons to explainan this oxygen tolerance in W1. First, the H_2ase generated by W1 may be more tolerant to oxygen compared with other H_2ases, Secondly; there may be a protective system against oxygen presence during H_2 fermentation. Although the H_2 production yield by W1 is lower compared with W4 and W5, more work focusing on the differences between their respective H_2ase genes and gene regulation will be helpful to determine if there is any relationship between oxygen tolerance and H_2 yield or whether the H_2 metabolic pathway can be modified to improve yields in oxygen-tolerant H_2 producers.

3.4.6 Phylogenetic analysis of isolated strains

The three isolates capable of H_2 production were more rigorously identified by phylogenetic analysis using 16S rRNA gene sequences from a variety of *Clostridium* species associated with H_2 production (Ogino et al., 2005; Zhang et al., 2006; Liu et al., 2006b). A larger fragment of the 16S rRNA gene was amplified and sequenced from isolates W1, W4 and W5 using primers 27F, 357F, 786F and 1492R. Phylogenetic analysis (Fig 3.2) suggested that isolates W4 and W5 are closely related to (or the same species as) *C. butyricum*. Whilst most similar to *C. diolis*, isolate W1 was placed in an unresolved cluster of species including *C. roseum*, *C. diolis*, *C. acetobutylicum* and *C. beijerinckii* and possibly represents a distinct species.

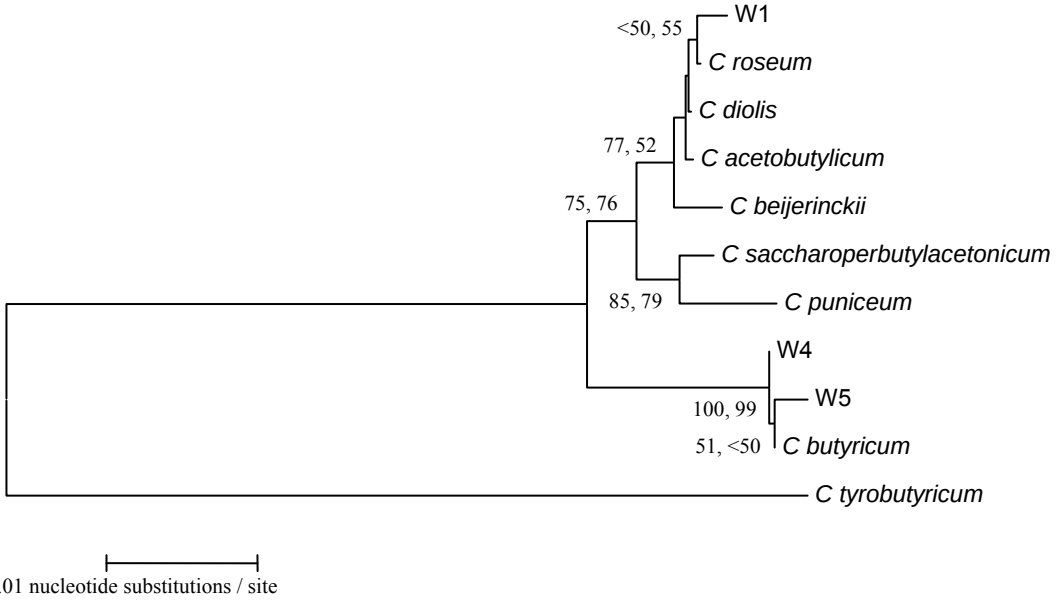


Fig 3.2 Phylogenetic tree resulting from Neighbor Joining analysis of 16S rDNA sequences of the 3 isolates and published sequences of *Clostridium* species known to produce H_2. Percent bootstrap support (≥50%) from 1000 replicates is indicated at supported nodes for distance-based and parsimony analyses, respectively.

3.5 Summary

In this chapter, culture independent methods, including PCR-DGGE and 16S rDNA clone library construction, in association with culture dependent isolation techniques, were used to profile the microbial community structure of H_2-producing bacteria in activated sludge. Profiling the community composition of the micro flora suggested that the majority of bacteria were various *Clostridium* species. This was confirmed by clone library analysis,

where 80% of the cloned inserts were *C.* sp. A total of 5 isolates were established from solid media. Three of them, designated as W1, W4 and W5, harbored the H_2ase gene as determined by PCR and DNA sequence analysis (99% similarity). These isolates were similar to *C. butyricum* and *C. diolis* as determined by 16S rRNA gene sequence. A maximum H_2 production yield of 220ml H_2 g^{-1} glucose was achieved by W5, which was grown on improved mineral salt medium by batch fermentation without pH adjustment and nitrogen sparging during fermentation. W1 is the first reported *Clostridium* sp. that can tolerate microaerobic conditions for producing H_2.

Specific genetic markers for *Clostridium* sp. W1, W4 and W5 would be of great utility for investigating H_2 production at the molecular level. Two H_2ase primer sets reported by previous researchers were found not to be specific for H_2ase, amplifying other genes from non- H_2 producers. The H_2-producing bacteria isolated in this chapter have great value for investigating H_2 production, gene regulation and enzyme structure.

CHAPTER 4

Profiling of Hydrogenase Gene Cluster

in *Clostridium butyricum* W5

4.1 Introduction

[FeFe] hydrogenases ([FeFe] H_2ases) play a vital role in hydrogen (H_2) evolution in clostridia. A few attempts were made by genetic manipulation to improve the hydrogen evolution rates and yields. Chittibabu et al. (2006) ferment glucose by a recombinant *E. coli* containing a clostridia [FeFe] H_2ase and obtained 3.12 mol H_2/mol glucose, which was close to the theoretical yield, 4 mol/mol, but led to a low productivity due to slow bacterial growth rate. Liu et al. (2006) mutated the genes of acetate kinase and phosphotransacetylase in order to increase butyric acid yield. They were surprised by the improved H_2 yield. Until now, the gene regulation and expression of [FeFe] H_2ases are still regarded as a puzzle. An attempt to clear the basic information towards advanced manipulation, the sequence of [FeFe] H_2ases gene and gene cluster structure, was carried out in this work.

As described in Chapter 3, isolates from heat-treated activated sludge were screened by PCR for the presence of the [FeFe] H_2ase gene using primer sets E1f/E1r and L1f/L1r. The results showed that the amplicons from isolates W1, W4 and W5 had high similarity (96%, 99%, and 99% respectively) with H_2ases genes from *Clostridium* sp.

The functions of [NiFe] H_2ases and the characterization of the genes encoding them have been thoroughly described (Blokesch et al., 2002). However, although HydA and its gene *hydA* were studied in the 1990's (Vasconcelos et al., 1994; Gorwa et al., 1996). Knowledge of the gene regulation and maturation of [FeFe] H_2ases is still at an early stage (Blokesch et al., 2002). The [FeFe] H_2ase structural genes are not located in the same operon with putative maturase-encoding genes. Moreover, the homologs of [NiFe] H_2ase maturation genes have not been detected in bacteria with [FeFe] H_2ases, which has led to the prediction that [NiFe] H_2ases and [FeFe] H_2ases have different regulatory systems (Vignais et al., 2001).

Manipulating *Clostridium* sp. to selectively produce H_2 will require an understanding of how [FeFe] H_2ase gene expression is regulated. Although the regulation of H_2ase activity at the enzymatic level has been established by Vasconcelos et al. (1994), the investigation of regulation at the genetic level has been very limited. Furthermore, H_2 was assumed to cause feedback inhibition on H_2 production, as the removal of H_2 by nitrogen sparging was observed to improve H_2 production by 50% (Mizuno et al. 2000). Gene cluster structure

information will be helpful for finding out whether H_2 will induce feedback inhibition of H_2ase gene expression.

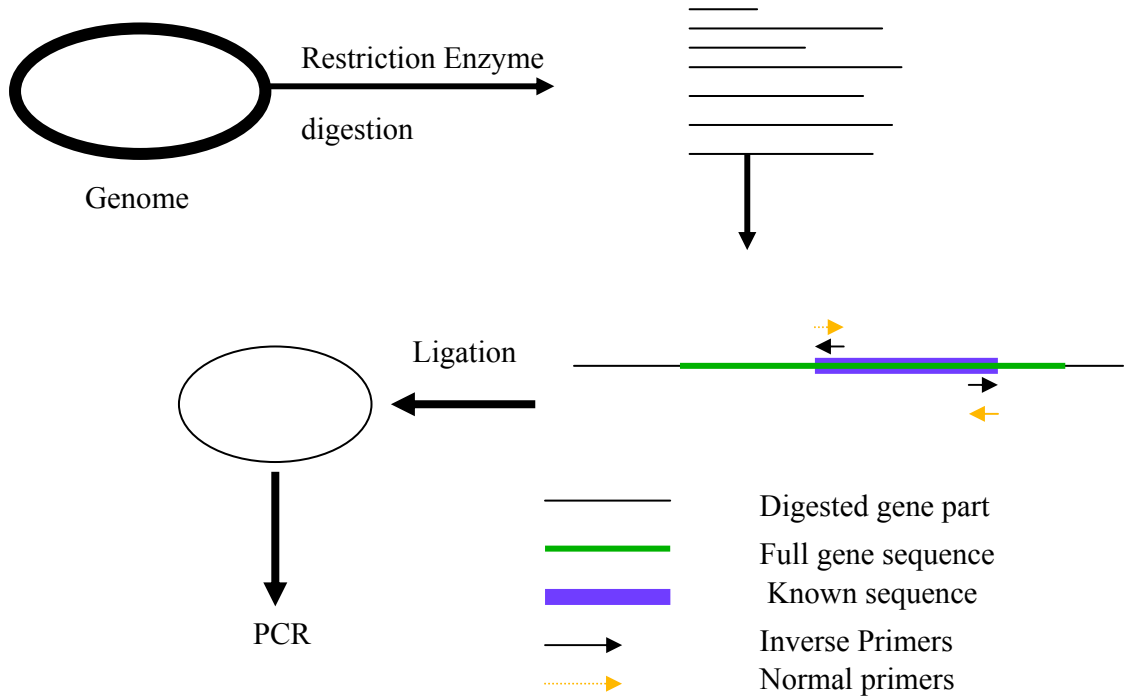


Fig 4.1 Illustration of IPCR reaction

Inverse PCR (IPCR) is a modification of PCR first described by Ochman et al. in 1988. It allows rapid *in vitro* amplification of DNA sequences that flank a region of "known sequence". The word "inverse" means that the primers are oriented in the reverse of the usual orientation. As shown in Fig 4.1, genomic DNA is digested with a restriction enzyme which is selected to have one or a few recognition sites within the known gene sequence of interest (but no restriction site between the two primers). The fragments of genomic DNA are circularized by self-ligation and amplified by PCR. The circularization allows the primers to amplify across the unknown region of genomic DNA, which can then be sequenced. The process can be used iteratively as new sequence data are generated from each round of IPCR. The self-ligation step is considered as the most difficult step to optimize because ligation conditions need to favor intramolecular ligation (to allow circularization) rather than intermolecular ligation (e.g. joining of different DNA fragments). Low concentrations of DNA in a small volume favor intramolecular ligation. The circular fragments generated after

ligation can potentially be larger than can be readily amplified by conventional PCR. For this reason, systems such as the Elongase® Enzyme Mix (Invitrogen, CA, USA), which is designed to amplify DNA fragments larger than 5 kb, should be used for IPCR. Unlike *Taq* polymerase, which generally adds a single adenine (A), to the 3′-ends of amplified DNA fragments (Clark, 1988), the Elongase® Enzyme Mix contains a proofreading enzyme, which is claimed by the manufacturer to produce greater than 95% blunt-end fragments. To facilitate cloning into TA vector systems, which contains 3′T overhangs at each end to complement the A overhang added by the *Taq* polymerase (*eg* the TOPO® TA Cloning Kit), the Elongase PCR products need to be tailed with dATP using *Taq* DNA polymerase (Kobs, 1997).

This chapter identifies the three H_2-producing isolates described in the previous chapter and reports the characterisation of a putative 3.3kb [FeFe] H_2ase gene cluster from *C. butyricum* W5. The sequence of the *C. butyricum* W5 H_2ase amplified by primer set E1f/E1r was used as "known sequence" for IPCR to obtain further gene sequence information.

4.2 Aims

To provide [FeFe] H_2ase gene sequence and gene cluster structure for further understanding of [FeFe] H_2ase gene and subsequently genetic modification to improve H_2 production work, there aims for this chapter were made as follows:

- Identify three H_2-producing isolates;
- Sequence and analyze the *C. butyricum* [FeFe] H_2ase gene cluster;
- Predict the [FeFe] H_2ase gene cluster structure in *C. butyricum* W5.

4.3 Specific materials and methods

4.3.1 RapID™ ANA II System identification

The physiological identification of three H_2-producing isolates was performed using the RapID™ ANA II System (Remel, Inc., Lenexa, Kansas, USA), following the manufacturer's instructions. The resulting pattern of positive and negative test scores is used as the basis for

identification of the test isolate by comparison of test results to reactivity patterns stored in a database or through the use of a computer-generated Code Compendium.

4.3.2 IPCR

Primer design

Primer sets used for IPCR are designed by "Fast PCR" software, which is available online at http://www.biocenter.helsinki.fi/bi/programs/fastpcr.htm. The selection criteria for Left (Right) primer are listed in Table 4.1. For other parameters default values were used. Additional criteria considered were the requirement that primers should have a C or G at the 3'-end, primers should not start or finish with CCC or GGG and the annealing temperature of the primer set should be ≥ 58°C. The primer sets used in this chapter are listed in Table 4.2

Table 4.1 Selection criteria for IPCR primer sets.

Parameter	**value**
Tm Range (°C)	60-70
GC % Range	50-65
Tm at 3'-end (°C)	35-48
Length Range	21-30
Quality threshold	80

Table 4.2 Primers set used in this chapter.

Primer	**Sequence (5'-3')**	**Tm (°C)**	**MW[2]**
R 75-96	GCT TGC CTA ACC CAT GCA GGA C	58	6680
F 376-398	TGA GAA GGC AGA TCCAGC AAT GG	57	7147
R 258-278	GCG GCT ACA CAT TGA CCG CAT	56	6391
F 1357-1380	CCA GGA CAA CAT AAA GCC CAT GAA	56	7333

Restriction enzyme digestion

For restriction enzyme digestion, two enzymes that generate sticky ends (*Hin*dIII and *Eco*RI) and two that produce blunt ends (*Eco*RV and *Dra*I) were chosen. The recognition sites were required to have 6bp or more. All the enzymes were purchased from New England Biolab Inc. (Ipswich, Maryland, USA).

The restriction enzymes were kept on ice when used to ensure integrity and stored at -20 °C. The volume of enzyme stock used for digestion was required to be lower than 10% of the final digestion volume as glycerol contained in the enzyme storage buffer has the potential to inhibit digestion. Too much or too little enzyme may result in non canonical cleavage of DNA or partial digestion. Allowing 2.5 U of enzyme per µg DNA will generally ensure complete digestion.

Digestions were performed in a heating block at 37°C overnight and inactivated as specified in Table 4.3. Each 20 µl reaction mixture contained 2.5 U restriction enzyme, 2 µg acetylated BSA (Ambion, Applied Biosystems, Foster City CA), 1× reaction buffer and 20 µg of genomic DNA template. Reaction products were verified by 1.0 % (w/v) agarose gel electrophoresis (Section 2.7.3).

Table 4.3 Inactivation conditions of restriction enzymes

Enzyme	Inactivation temperature (°C)	Inactivation time (min)
*Hin*dIII	65	20
*Eco*RI	65	20
*Eco*RV	65	20
*Dra*I	80	20

Ligation

T4 DNA ligase (New England Biolab Inc, Ipswich, MA) was used to ligate digested genomic DNA fragments. Ligation was performed at 4°C O/N for sticky ended fragments digested by *Hin*dIII and *Eco*RI, and at room temperature O/N for blunt ended fragments digested by *Eco*RV and DraI. Each 40 µl reaction mixture contained 12U T4 DNA ligase, 1× reaction

buffer and 8 µl of digested genomic DNA. Heat treatment at 70°C for 10 min was used to inactive T4 DNA ligase.

PCR amplification with Elongase® Enzyme Mix

Ligated DNA fragments were amplified with Elongase® Enzyme Mix. Each 50 µl reaction mixture contained 200 µM of each dNTP, 400 nM of forward and reverse primers, 2 µl of 5 × buffer A, 8 µl of 5 × buffer B, 10 µl of ligated DNA mix, and 1 U of Elongase® Enzyme Mix. Buffer A and B were provided with the Elongase® Enzyme Mix Kit. Thermal cycling consisted of an initial denaturation at 94°C for 10 min to allow activation of the *Taq* polymerase, followed by 35 cycles of denaturation at 94°C for 30 s and finally annealing at 58°C for 30 s. As the ligated DNA fragment sizes were unknown, a long extension time, 15 min at 68°C, was used to ensure adequate time for extension. A final extension at 72°C for 10 s was then performed. Reaction products were verified by 1.0 % (w/v) agarose gel electrophoresis (Section 2.7.3).

"Known sequence" for IPCR

The sequence of a 442 bp length fragment of the *C. butyricum* W5 [FeFe] H_2ase gene was used as a "known sequence" for IPCR and is presented below:

AATAATGGAAGAAGCAACAGAGTTTATTGAAAGAATAAATAATAATGGACCATT
TCCTATGTTTACATCATGTTGTCCTGCATGGGTTAGGCAAGCAGAAAATTACTAC
CCAGAACTTTTAGGTAATTTATCTTCAGCTAAATCACCACAACAAATATTTGGAG
CAGCAAGTAAAACATATTACCCTACAGTGGAAGGATTAGATCCTAAGAGTGTTT
ATACAGTTACAATAATGCCTTGCACTGCTAAAAAATATGAAGCTGATAGAACAG
AAATGGAAAATGAAGGTTTAAGAAATATAGATGCTGTTTTAACTACAAGAGAAT
TAGCTAAAATGATTAAGGATGCAAAAATTAATTTTGCTACATTAGAAGATGAGA
AGGCAGATCCAGCAATGGGTGAATATACTGGAGCTGGAGTTATATTTGGTGCAA
CCGGTGGA

4.3.3 A-tailing protocol

A-tailing step was performed according to a protocol described by Kobs (1997) with minor modification. Each 10 µl reaction mixture contained 1 µl Ampli *Taq* Gold DNA polymerase (Applied Biosystems), 2.5 mM $MgCl_2$, 10 mM dATP, 1×reaction buffer and 2 µl PCR

products. The reaction mixture was kept at 70°C for 15 min, and cleaned up before cloning into pCR®2.1-TOPO® vector.

4.3.4 ORF analysis

ORF analysis was performed at the National Centre for Biotechnology Information (NCBI) using ORF Finder. (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) (Rombel et al, 2002)

4.4 Results and discussion

4.4.1 Identification of the H_2-producing isolates

Three H_2-producing isolates were identified using the RapID™ ANA II System. This system qualitatively identifies medically important anaerobic bacteria by their utilization of conventional and chromogenic substrates. Table 4.4 summaries the utilization pattern of the 3 isolates. Isolates W4 and W5 were confirmed to be *C. butyricum*, while W1 showed a distinct substrate utilization pattern compared to all the listed anaerobic bacteria provided with the system.

Isolate W1 may be a new species of *Clostridium*, as we compared substrate utilization patterns with other known *Clostridium* sp. and could not find a match species (Durre, 2004). As discussed in Chapter 3: i) W1 showed oxygen tolerant H_2 production, which has not previously been reported in the literature; ii) Whilst most similar to *C. diolis* (99% similarity), the 16S rDNA sequence of W1 is different from any known *Clostridium* sequence and been placed in an unresolved cluster of species including *C. roseum, C. diolis, C. acetobutylicum* and *C. beijerinckii* in a phylogenetic tree (Fig 3.2). The ability to tolerate oxygen, distinction in phylogenetic analysis and the unique metabolic profile displayed with the RapID ANA II system all suggest that *Clostridium* sp. W1 is very likely a new species of *Clostridium*.

Table 4.4 Substrates utilization pattern of three isolates

		Value	W1	W4	W5
Before RapID™ ANA II reagent addition	URE	1	-	-	-
	BLTS	2	+	-	-
	αARA	4	-	+	+
	ONPG	1	+	+	+
	αGLU	2	+	+	+
	βGLU	4	+	-	-
	αGAL	1	+	+	+
	αFUC	2	-	-	-
	NAG	4	±	-	-
	PO_4	1	-	-	-
After RapID™ ANA II reagent addition	LGY	2	-	-	-
	GLY	4	-	-	-
	PRO	1	-	-	-
	PAL	2	+	-	-
	ARG	4	+	-	-
	SER	1	-	-	-
	PYR	2	-	-	-
Spot Indole	IND	4	-	-	-
Value total (Code)			275(1)060	431000	431000

4.4.2 IPCR

IPCR products from 4 different restriction enzyme digestions were verified by agarose gel electrophoresis. As shown in Fig 4.2, a bright band was obtained from *Eco*RI digests and faint bands were obtained for the *Eco*RV and *Hin*dIII digests. No product could be observed for the DraI digest. The IPCR product from *Hin*dIII digests was only about 1.5 kb and so was not further characterised. The products form *Eco*RI and *Eco*RV IPCR were about 2.5kb, so these were A-tailed, purified, cloned into the pCR®2.1-TOPO® vector and sequenced.

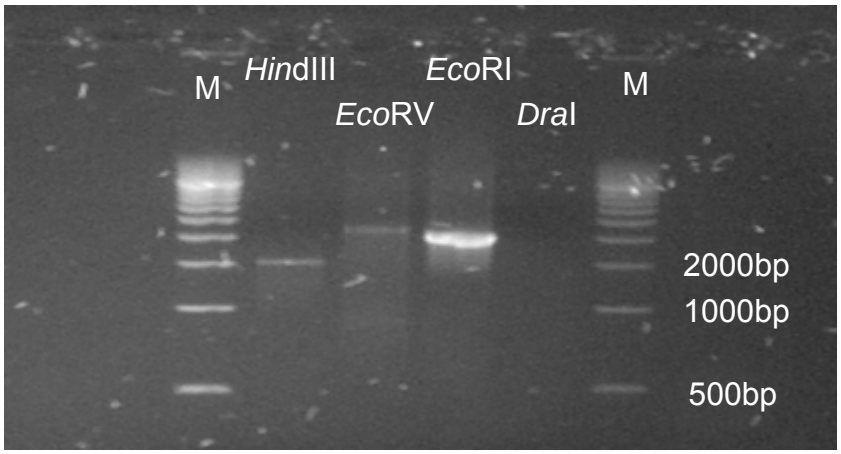
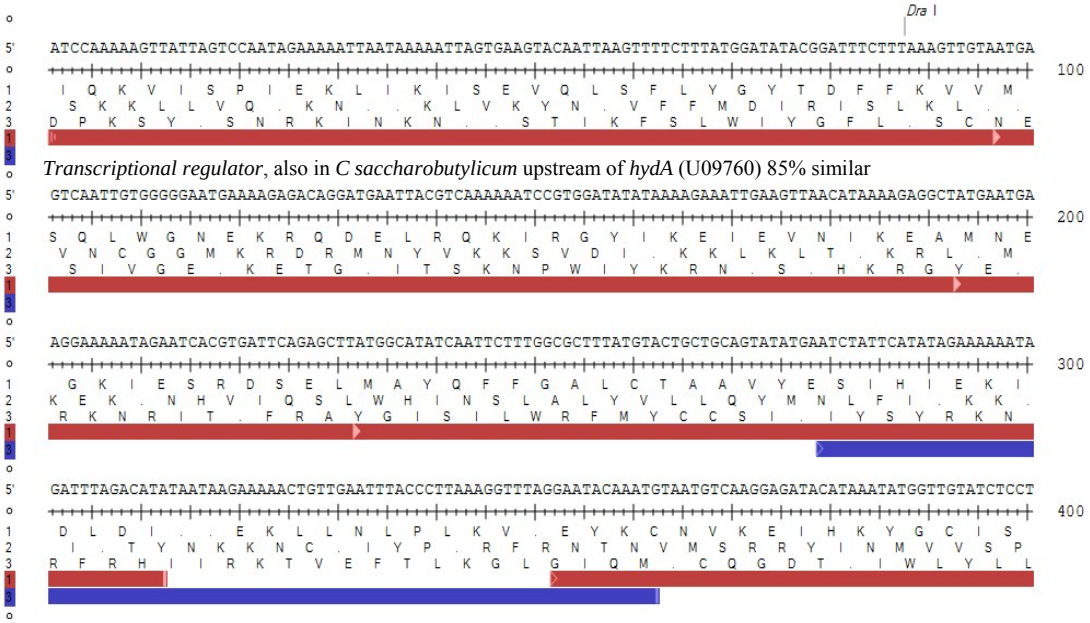


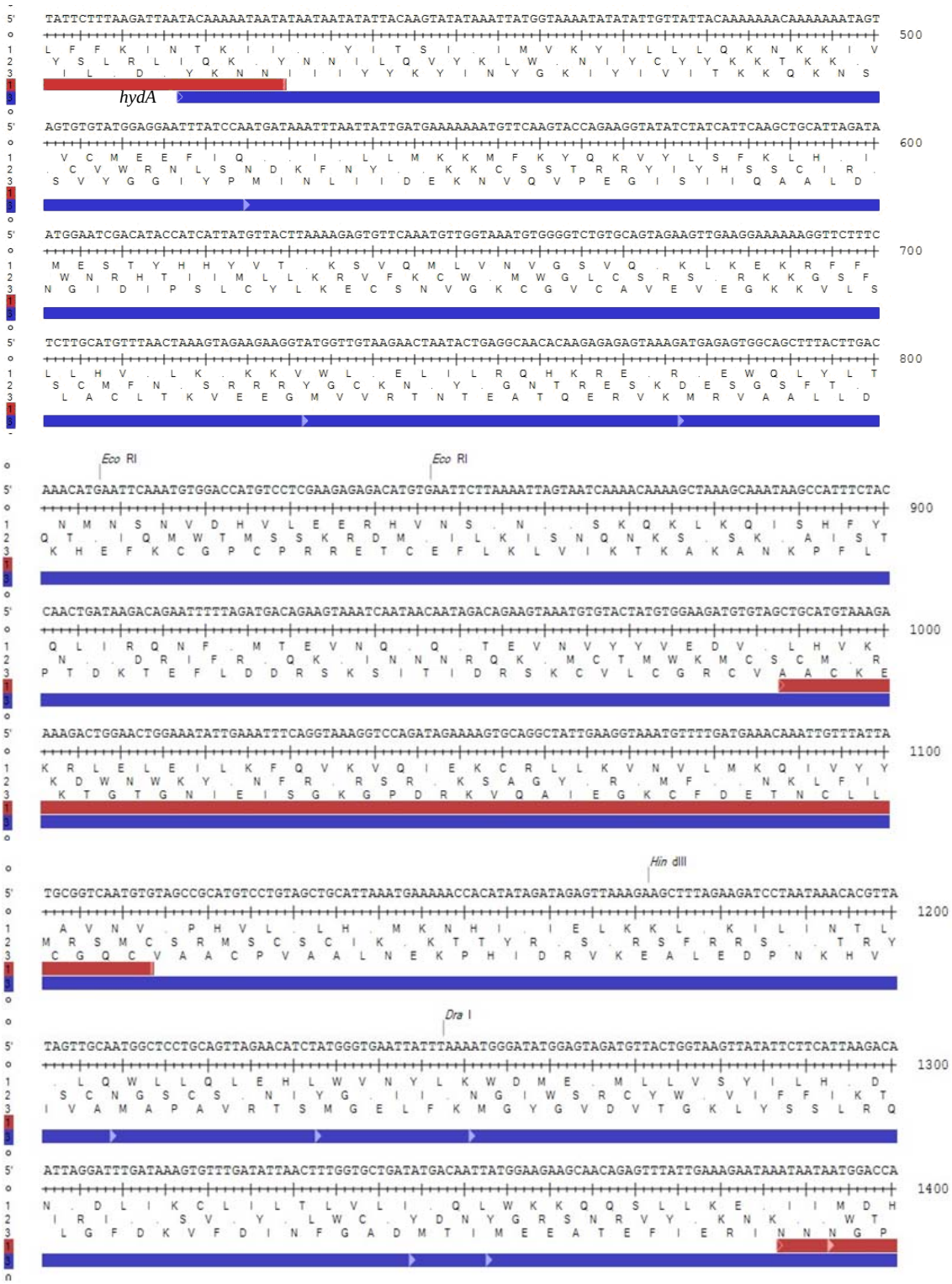
Fig 4.2 Electrophoresis picture of IPCR products.

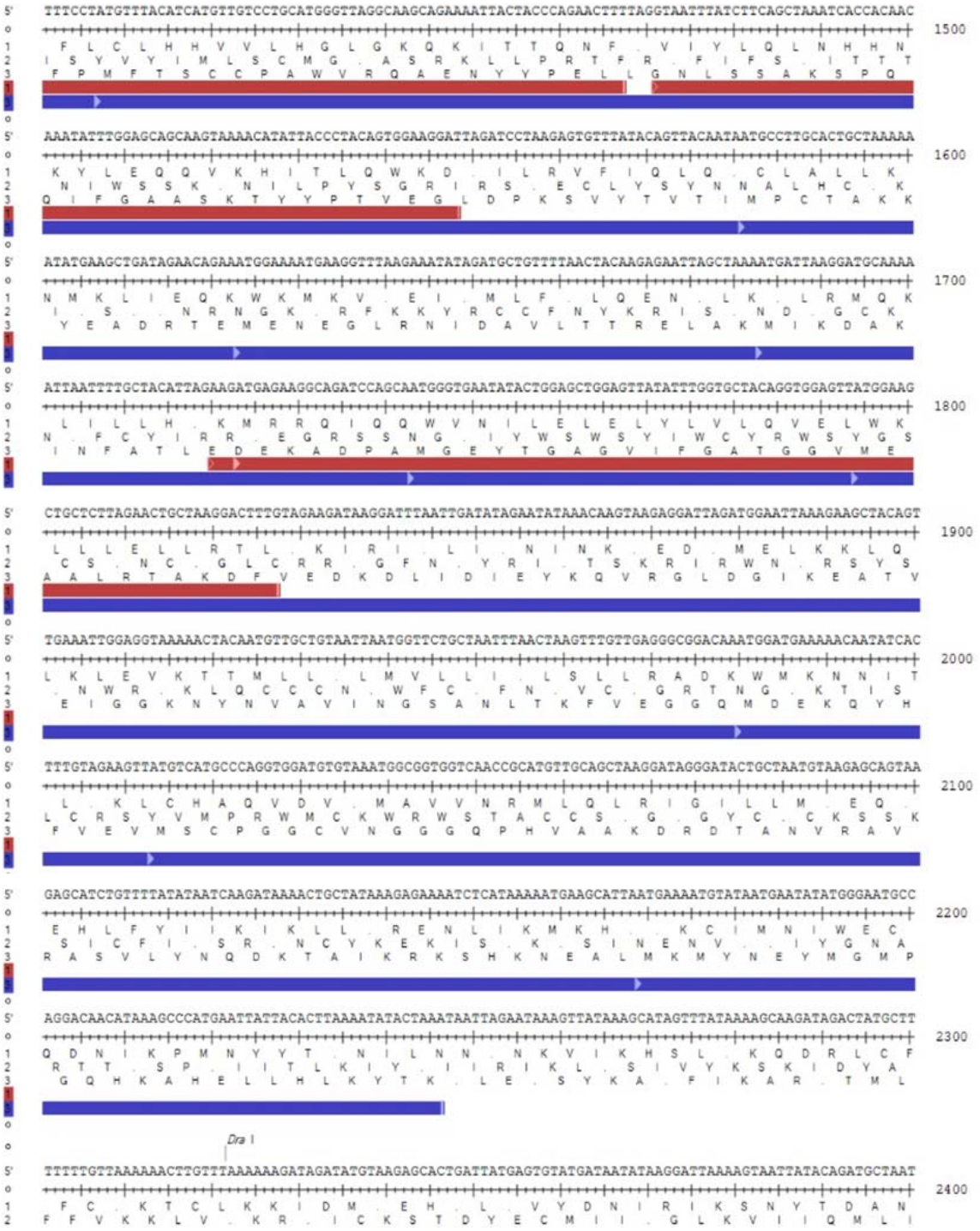
4.4.3 Sequence results

4.4.3.1 Sequence assembly and annotation

A 3331bp sequence was obtained from alignment of the *Eco*RI and *Eco*RV IPCR amplicon sequences. This sequence has been deposited onto GenBank with accession EU366290. The sequence, also showing all 3 reading frames and ORFs for the 1st and 3rd reading frame, is below:




hydA
Eco RI
Eco RI
Hin dIII
Dra I


Dra I

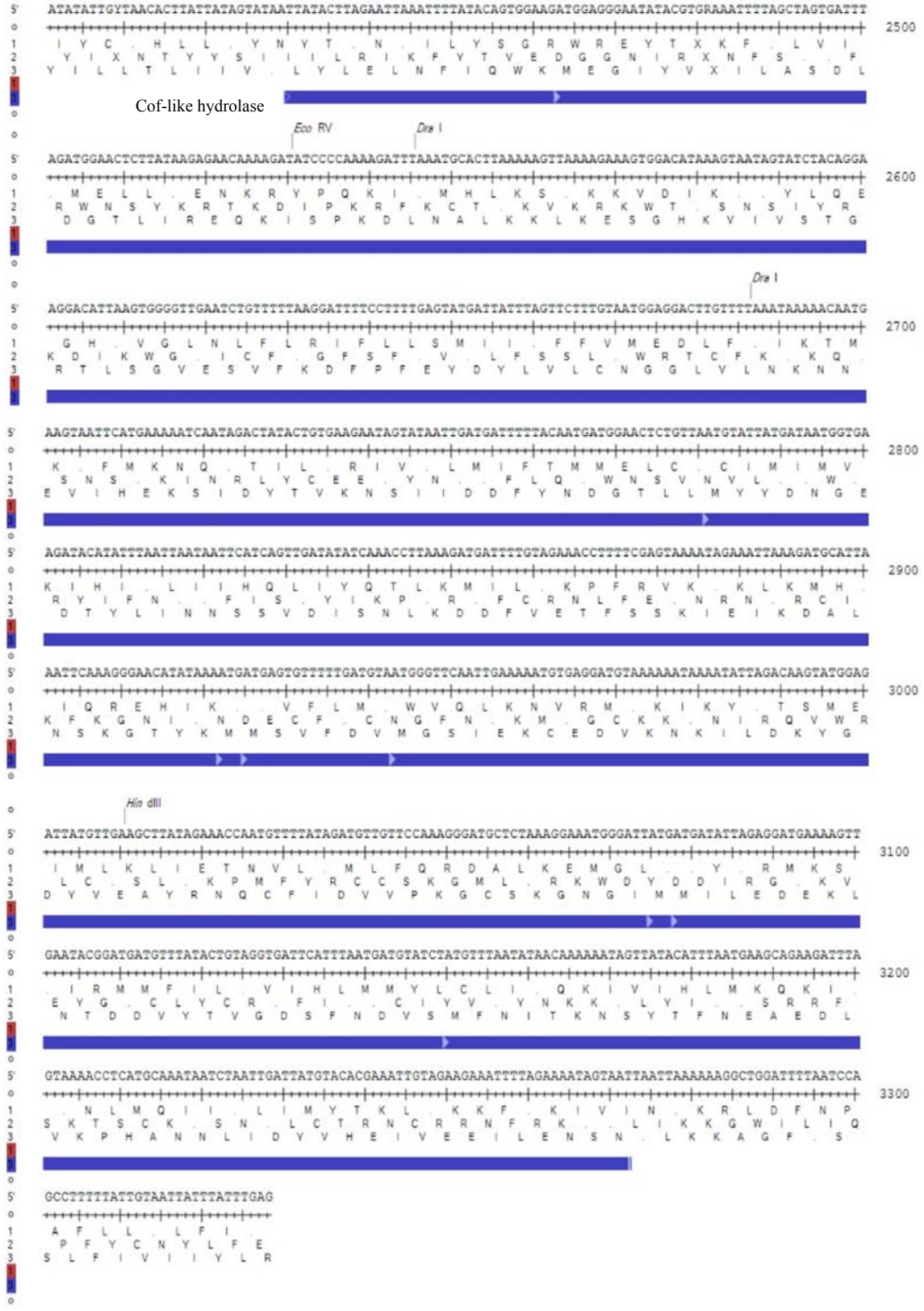


Fig 4.3 Sequence from alignment of the *Eco*RI and *Eco*RV IPCR amplicon sequences

4.4.3.2 ORF identification and mapping of *hydA* and flanking regions

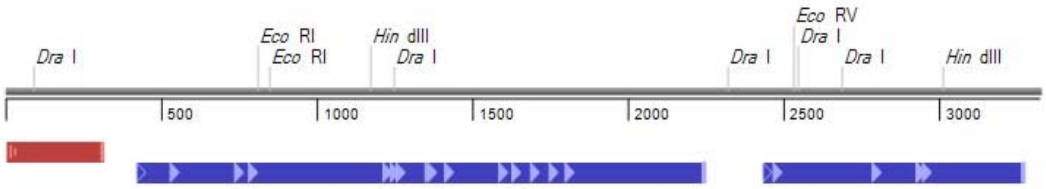


Fig 4.4 Map of the [FeFe] H_2ase gene cluster in *C. butyricum* W5

Following sequencing and alignment, a map was constructed to illustrate the relationship between the original known gene fragment, the complete *hydA* sequence, the IPC fragments and the restriction sites used in the initial IPCR experiments (Figure 4.3). Three recognition sites were detected for *Dra*I (TTT'AAA), 88, 1248 and 2322, were screened in the aligned sequence, while no IPCR product was detected.

Three open reading frames (ORFs) were identified in the sequence (1 in frame 1, 2 in frame 3, Fig 4.4). The largest ORF was 1725bp. It starts with the start codon "ATG" at the 525 bp position, which encodes a 574 amino acid (aa) protein from base position 528-2252. The aa sequence had 99% similarity with a recently sequenced *C. butyricum* H_2ase (Acc. No. ABR25256) by Klein and Hartmeier (2007). The second ORF of 219bp was found upstream of the putative H_2ase, with a 66% similarity with a 72aa transcriptional regulator in *C. beijerincki* NCIMB 8052. A similar sequence is also present upstream of the H_2ase of *C. saccharobutylicum* (85% similarity). Similar predicted transcriptional regulators in other *Clostridium* sp. which have relatively low similarity with our sequence were also listed by tBlastn. Although the function of this upstream regulator protein has not been investigated or described previously, a possibly close relationship between transcription regulators with [FeFe] H_2ase expression deserves more attention. The third ORF was downstream of the putative H_2ase and was highly similar to a putative Cof-like hydrolase in *C. butyricum* (97%) (Acc. No. ABO42544) (Wang et al., 2007a).

A Shine-Dalgarno (SD)-like sequence, TGGAGG (the consensus sequence is AGGAGG) was found before the -10 upstream area of the H_2ase-like ORF. Shine-Dalgarno sequences

are the ribosome binding sites used to initiate protein synthesis and are usually 5-6 nucleotides upstream of the start codon (Shine and Dalgarno, 1975). No strong SD sequences were found near the other two ORFs, although the Cof-like hydrolase ORF has the sequence AGTGGA near the start codon, which might be sufficient to promote ribosome binding. Pribnow boxes, which are centered at -10 from the start codon, are part of the promoter in prokaryotes and are required for DNA transcription. The consensus sequence is TATAAT, but the actual sequence can vary, with the main criterion being that the sequence is AT rich. All three ORFs have AT-rich regions of 6 nucleotides or greaterin the -10 upstream region.

4.5 Summary

Three H_2-producing isolates obtained in Chapter 3 were identified by biochemical phenotyping using the RapID^{TM} ANA II System in this chapter. For strain W4 and W5, the results are consistent with 16S rDNA sequence identification, which both (W4, W5) identified as *C. butyricum.* W1 showed a distinct substrate utilization pattern compared to other reported *Clostridium* sp. As indicated in Chapter 3, W1 can produce H_2 in a microaerobic environment, which is unique among clostridia. W1 is possibly a new species of *Clostridium* and has great potential to be used as a working strain to investigate the relationship between H_2 production and metabolism. More knowledge about the differences of W1's H_2ase from those of other clostridia and its metabolism will be useful information to understand H_2 evolution in *Clostridia* and to make the fermentative H_2 production more practicable.

A 3.3kb length DNA sequence from *C. butyricum* W5, which contains the [FeFe] H_2ase gene, was obtained by IPCR in this chapter. The [FeFe] H_2ase gene cluster structure was predicted by ORF analysis from this sequence. The predicted transcriptional regulator upstream of the [FeFe] H_2ase gene occurs in many *Clostridium* sp. and was also found in our sequence, which might have important influence on the regulation of the [FeFe] H_2ase gene expression. The downstream putative Cof-like hydrolase indicates the end of the gene cluster, which is similar to other *Clostridium* sp.

CHAPTER 5

Impact of Carbon and Nitrogen Sources on Hydrogen Production by *Clostridium butyricum* W5

5.1 Introduction

One of the major factors limit the application of hydrogen production for an industrial process is the high production cost (Chapter 1, section 1.7). As the 30-40% of total fermentation costs are associated with the substrates (Åkerberg et al., 2000), the price of carbon and nitrogen sources for bulk H_2 production become an important factor for controlling cost. However, because of the easy degradability of sugar based carbon sources and the abundant nutrient contents in organic nitrogen sources, expensive glucose or sucrose and organic nitrogen sources are widely used in many applications (Kapdan and Kargi, 2006) (Section 1.3.4). Efforts were also made to find cheap or even waste materials to lower the costs (Kapdan and Kargi, 2006). However, the low utilization rate of substrates, resulting in a low yield and consequently increasing the production cost remains to be a crucial issue for biological hydrogen production .

A number of renewable materials can be used as carbon sources for H_2 production. These include sugar or cellulose-containing agricultural and food industry by-products and wastes, carbohydrate-rich industrial wastewaters and waste sludge from wastewater treatment plants (Adamson, 2004; Hussy et al., 2005). The biological H_2 production using waste materials can also be environmentally friendly waste treatment processes (van Ginkel et al., 2005; Li et al., 2007a; Mohan et al., 2007). Many previous investigations have focused on special waste materials in continuous H_2 production processes, such as starch waste water (Zhang et al., 2003) and waste biosolids (Wang et al., 2003b). However, there is a little information about how carbon and nitrogen sources affect the production of H_2 and by-products in the fermentation process available in the literature. Considering that the total production costs are greatly affected by the substrates price (Åkerberg et al., 2000), detailed investigations on finding suitable and cheap carbon and nitrogen sources, and evaluation of carbon and nitrogen concentrations in fermentation broth for certain bacterial H_2 production are clearly necessary.

Three H_2-producing *Clostridium* species W1, W4 and W5 isolated in Chapter 3 were further identified physiologically, which is described in Chapter 4. Because H_2-producing bacteria work individually in the community, maintaining high proportion of efficient H_2 producers is essential to improve fermentation performance for achieving high production yield and rate

(Chapter 1, Section 1.3.3). The behaviour of the dominant H_2 producer becomes extremely important in the whole system. On this basis, the selection of an efficient H_2 producer and the identification of suitable fermentation substrates appeared to be the first two steps towards the development of an efficient H_2 production system.

We compared the H_2 production performance of three isolates and preliminarily investigated their ability to use carbon and nitrogen sources for H_2 production. A pH test was also carried out to probe the effect of pH on H_2 fermentation using glucose. Bottle tests were used for comparison of the three isolates and preliminary screening of carbon and nitrogen sources. Six carbon (glucose, fructose, lactose, sucrose, waste potato starch (WPS) and molasses) and six nitrogen (yeast extract, neutralised soya peptone, urea, $(NH_4)_2SO_4$, KNO_3 and NH_4NO_3) sources were employed. Bach fermentation process using the laboratory scale bioreactor system was carried out for further comparison of carbon (glucose, molasses and WPS) and nitrogen (yeast extract and NH_4NO_3) sources and different pHs (6.0, 6.5 and 7.0).

A paper based on the research of this chapter "Impact of Carbon and Nitrogen sources on Hydrogen Fermentation by a newly isolated *Clostridium butyricum* W5" has been accepted by *International Journal of Hydrogen Energy.*

5.2 Aims

This chapter was trying to select the most efficient H_2 producer from three *Clostridium* sp. isolated previously, then seek cheap and appropriate fermentation substrates. The detailed aims are:

- Compare the H_2 production ability of the three isolates.
- Select suitable carbon and nitrogen sources for fermentative H_2 production by bottle test.
- Establish how the carbon and nitrogen sources and pH affect fermentation performances in the bioreactor in terms of H_2 production and yield, bacterial growth and production of by-products.

5.3 Specific materials and methods

5.3.1 Strain selection

Three H_2-producing isolates (*Clostridium* sp W1, *C. butyricum* W4 and *C. butyricum* W5) were employed in the strain selection. Four carbon sources (glucose, lactose, sucrose and fructose) were used to compare their H_2 production. The fermentation media contained carbon sources which carbon concentration equal to 10g/L hexose and 3g/L yeast extract.

5.3.2 Preliminary screening of carbon and nitrogen sources

Six carbon sources (glucose, fructose, lactose, sucrose, WPS and molasses) and 6 nitrogen sources, yeast extract (LP0021, Columbia, HBA, Oxoid), neutralised soya peptone (NSP) (LP0044, Columbia, HBA, Oxoid), urea, $(NH_4)_2SO_4$, KNO_3 and NH_4NO_3 were employed in the screening investigations for H_2 production. WPS was provided by Smiths Crisps Ltd (Australia). The WPS contained 90% (w/w) starch and 0.04055 % TKN, as determined by previous research (Zhang et al., 2007c). Molasses was obtained from Magill Grain Store Pty Ltd (South Australia). The hexose concentration in the molasses was 44.4% (w/w) and the TKN was 0.7086%. Yeast extract was used as the nitrogen source for the carbon source screening experiments. Glucose was used as the carbon source for the trials of nitrogen sources. The fermentation media contained carbon sources which carbon concentration equal to 10g/L hexose and nitrogen sources equal to 3g/L yeast extract.

5.3.3 Comparison of different carbon and nitrogen sources

The concentrations of carbon and nitrogen sources in the fermentation media were set up the same as those as for the bottle tests. Fermentations were performed at 35 °C, pH 6.5, 300 rpm with an inoculum size of 100ml seed culture in 20 hours.

5.4 Results and Discussion

5.4.1 Strain selection

As discussed in Chapter 3, the three isolates showed different H_2 production capabilities on glucose. In order to find out a suitable H_2 producer with respect to its ability to use carbon

and nitrogen sources, we compared the H_2 production of three isolates by using four basic sugar carbon sources in this chapter. The experimental data revealed that *C. butyricum* W5 showed a greater advantage against *Clostridium* sp. W1 and *C. butyricum* W4. Sucrose and glucose appeared to be favored substrates for the three isolates, while they grew poorly in the lactose and fructose media (Table 5.1 and Fig 5.1). The results indicated that glucose and sucrose based waste materials could be good candidates for further investigation.

Table 5.1 Production of H_2 by three isolates using different carbon sources

Carbon source	Bacteria strain	H_2 /(ml/L)
Glucose	W1	378
Lactose	W1	90
Sucrose	W1	349
Fructose	W1	210
Glucose	W 4	439
Lactose	W 4	143
Sucrose	W 4	520
Fructose	W 4	378
Glucose	W 5	550
Lactose	W 5	138
Sucrose	W 5	558
Fructose	W 5	518

* Cumulative H_2 production calculated as ml H_2 per litre fermentation broth.

5.4.2 Screening of carbon and nitrogen sources

Glucose was the most commonly used carbon source for determining H_2 production ability in laboratories (Oh et al., 2003; Zhang et al., 2006). WPS and molasses were also considered as potential raw materials for H_2 production because of their wide availability, low cost, high carbohydrate concentration and high biodegradability (Yokoi et al., 2001; Jin et al., 2003). In consideration of lowering the production costs for industrial applications, several inorganic nitrogen sources were also selected. Among six organic or inorganic nitrogen sources, urea ($CO(NH)_2$) has the highest nitrogen content (46.7%), followed by NH_4NO_3 (35%), $(NH_4)_2SO_4$ (21%), KNO_3 (13.9%), Yeast extract (9.8%) and NSP (8.7%). Experimental data on the screening of carbon and nitrogen sources for the H_2 production are given in Table 5.2.

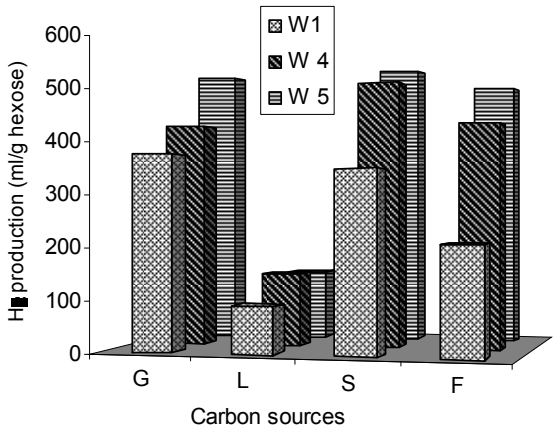


Fig 5.1 Production of H_2 by three isolates using glucose (G), lactose (L), sucrose (S) and fructose (F).

The results from carbon source screening experiments showed that the highest concentrations of H_2 (950ml/L) and biomass (1.45g/L) were measured in the fermentation system using molasses, followed by WPS, with 648ml/L and 1.35g/L, respectively. The high biomass and H_2 production compared with monosaccharide and disaccharide are possibly due to the biological accessible nitrogen sources existing in WPS and molasses (Zhang et al., 2007c; Ren et al., 2006; Li et al., 2007b). A similar H_2 production performance was given in the fermentation system using glucose, fructose and sucrose. These showed that the newly isolated *C. butyricum* W5 has a capability to use those monosaccharides and disaccharides, which also are the main sugar content of hydrolysed WPS and molasses (Oh et al., 2003). Lactose fermentation resulted in a poor performance in terms of H_2 production and bacterial growth, indicating that lactose containing substrates may not be a suitable carbon source for the H_2 production by *C. butyricum* W5. The results of nitrogen source screening revealed that the highest H_2 production could be achieved by using yeast extract (950ml/L). Fermentation of the inorganic nitrogen sources $(NH_4)_2SO_4$ and NH_4NO_3 also led to a relatively high yield (460 and 483ml/L, respectively), which showed great industrial potential because of their low cost. Urea and KNO_3 appeared not to be a favoured nitrogen source for *C. butyricum* W5.

Table 5.2 Production of H_2 and biomass using different carbon and nitrogen sources

Carbon source	Carbon source concentration (g/L)	Nitrogen source	Nitrogen source concentration (g/L)	Biomass (g/L)	Cumulative H_2 production (ml/L*)
Glucose	10.0	Yeast extract	3.00	1.28	550
Fructose	10.0	Yeast extract	3.00	1.22	518
Lactose	9.5	Yeast extract	3.00	0.45	138
Sucrose	9.5	Yeast extract	3.00	1.30	558
WPS	10.0	Yeast extract	3.00	1.35	648
Molasses	22.5	Yeast extract	3.00	1.45	950
Glucose	10.0	NSP	3.38	1.02	482
Glucose	10.0	Urea	0.63	0.56	256
Glucose	10.0	$(NH_4)_2SO_4$	1.40	0.97	460
Glucose	10.0	KNO_3	2.12	0.80	380
Glucose	10.0	NH_4NO_3	0.84	1.09	483

* Cumulative H_2 production calculated as ml H_2 per litre fermentation broth.

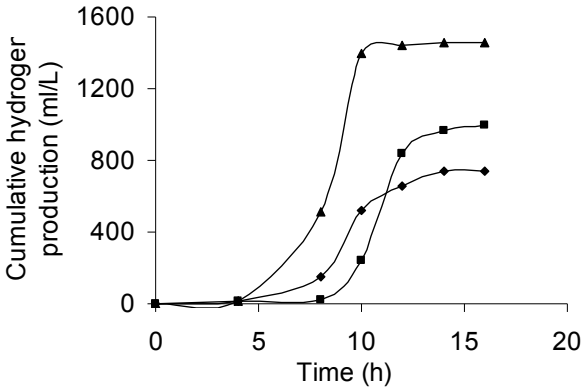
Table 5.3 Effect of carbon and nitrogen source on production of VFA and H_2 yield

Experiment Number	Carbon source	Nitrogen source	Lactic acid (g/L)	Acetate acid (g/L)	Butyric acid (g/L)	Sugar usage (%)	H_2 yield (mol/mol hexose)
E1	Glucose	Yeast extract	5.30	1.35	1.79	97%	0.61
E2	WPS	Yeast extract	1.60	1.47	2.87	75%	1.11
E3	Molasses	Yeast extract	2.92	1.62	3.65	96%	1.22
E4	Glucose	NH_4NO_3	4.09	0.81	1.72	97%	0.51

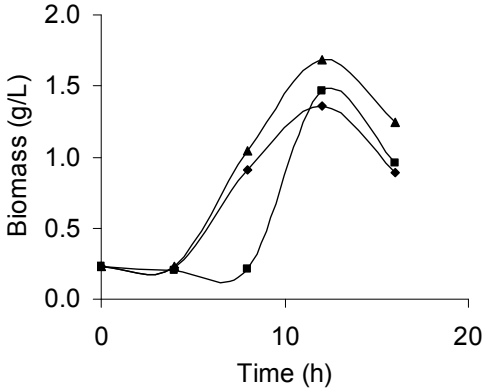
5.4.3 Effects of glucose, waste potato starch and molasses

According to the results obtained from carbon source screening, two promising carbon sources WPS and molasses were further investigated by a batch bioreactor process. As a comparison trial, a batch fermentation using glucose as carbon source was carried out under the same operating conditions. Analytical data revealed that lactic acid, acetic acid and butyric acid were the end by-products, and other VFAs and alcohols either were measured at a very low level or were undetectable. Fig 5.2 presents kinetic profiles of biochemical parameters during 20 h fermentation, showing the effect of the three carbon sources on production of H_2 (A), bacterial biomass (B), lactic acid (C), acetic acid (D) and butyric acid (E). A shorter lag phase leading to a higher rate of H_2 production and biomass growth was given in the fermentation using molasses as carbon source (Fig 5.2A and B). The use of WPS corresponded to a relatively longer lag phase but higher rates of H_2 production and biomass growth than for glucose. The shortest lag phase (6 h) was obtained in the fermentation of molasses, followed by glucose (8 h) and WPS (12 h). VFAs produced along with H_2 synthesis are valuable by-products. In theory, 4 or 2 mol of H_2 per mole hexose can be produced if acetic acid or butyric acid is the sole other end-product (Angnent et al., 2004). It is worthwhile to note that the fermentation using a pure culture of *C. butyricum* W5 as H_2 producer produced mainly lactic acid, acetic acid and butyric acid as by-products, and lactic acid was found to be a dominant VFA in the fermentation using molasses and glucose.

Compared with glucose, the molasses fermentations resulted in a high production rate of H_2 and biomass, while accumulating more acetic acid and butyric acid (Fig 5.2D and E). Angnent and co-workers reported that the formation of other reduced organic compounds like lactic acid, propionic acid and ethanol will lead to a much lower H_2 yield (Angnent et al., 2004). They stated that these metabolic pathways bypass the major H_2-producing reaction in carbohydrate fermentations (Angnent et al., 2004). A small amount of propionic acid (less than 0.1g/L) was observed at 8 h when fermented molasses was consumed by bacteria (data not shown). No ethanol production was observed during the fermentations using *C. butyricum* W5. Fig 5.2C shows that a higher level of lactic acid was produced by glucose compared to molasses. The WPS fermentation demonstrated an unfavourable performance in terms of H_2 yield, production of VFAs and sugar consumption rate (Table 5.3).



(A)



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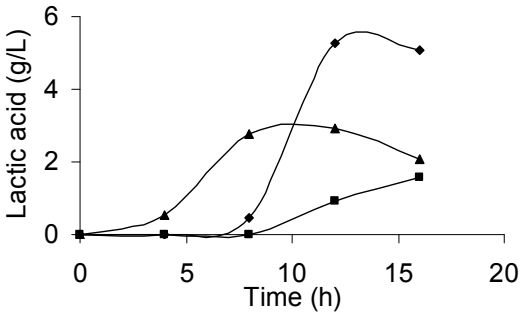
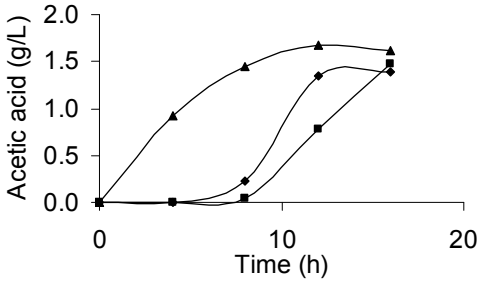
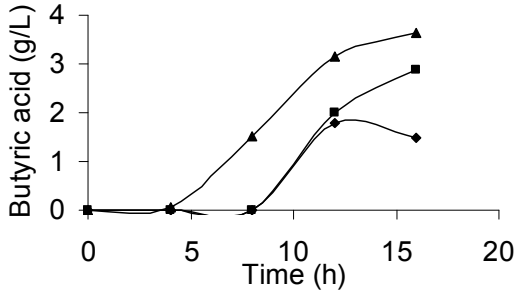


(C)



(D)



(E)

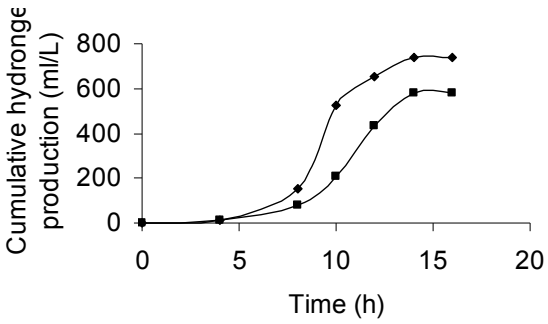
Fig 5.2 Kinetic profiles of H_2 (A), biomass (B) and lactic acid (C), acetate acid (D) and butyric acid (E) produced using glucose (♦), WPS (■) and molasses (▲) as carbon sources.

5.4.4 Effects of NH_4NO_3 and yeast extract

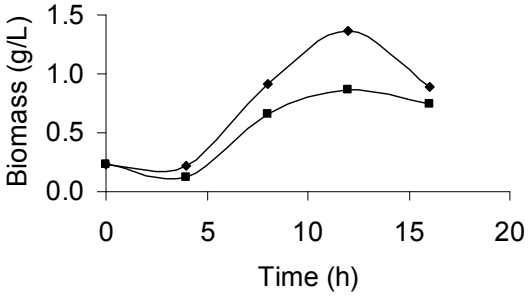
Use of a cheap inorganic nitrogen source instead of expensive organic nitrogen sources would be favoured in an industrial process. NH_4NO_3, a widely used inorganic nitrogen source, was tested in comparison with yeast extract. The results from experiments on nitrogen source impact are shown in Fig 5.3. Similar lag phases (8 h) were obtained from the fermentation of both nitrogen sources. It is reasonable to note that NH_4NO_3 led to slow bacterial growth. A slightly higher butyric acid concentration was obtained in the fermentation using NH_4NO_3 than using yeast extract. Comparing the experimental data for the first 12 h fermentation, the use of NH_4NO_3 demonstrated a weaker performance in terms of production of H_2 (Fig 5.3A), biomass (Fig 5.3B), lactic acid (Fig 5.3C) and acetic acid (Fig 5.3D) than those fermentations using yeast extract. Obviously, yeast extract was the favoured nitrogen source for the growth of *C. butyricum* W5, leading to a high yield of H_2 and by-products. Data on experiments E2 and E3 presented in Table 5.3 show that the extra nitrogen content in WPS and molasses may be responsible for the high H_2 yield and VFA production.

5.4.5 Effects of operation pH

Broth pH considerably affects the H_2 productivity and the behaviour of the bacterial community in continuous fermentation processes for the H_2 production. The optimal starting pH for H_2 production (Minnan et al., 2005; Li and Chen, 2007) and the optimal operational pH for continuous fermentation (Hussy et al., 2005) have been investigated in previous studies. The effect of operation pH on batch fermentation is of primary importance to both process performance and operation costs in an industrial process. Preliminary studies work on W5 showed that a pH lower than 6 and/or higher than 7 was not suitable for bacterial growth (data not shown). The effect of operation pH in batch fermentation was investigated by varying it between 6 and 7. As shown in Fig 5.4 (A) and (B), both H_2 production and biomass reached the highest levels at pH 6.5. A shorter lag phase and earlier butyric acid production were observed at pH 6.0, but a shorter fermentation duration and lower cumulative H_2 production and biomass were obtained at pH 6.5. A shorter fermentation duration was also observed at pH 7.0, resulting in a high yield of H_2 and biomass. Poor H_2 production was interpreted as being related to the change of the metabolic pathway



(A)



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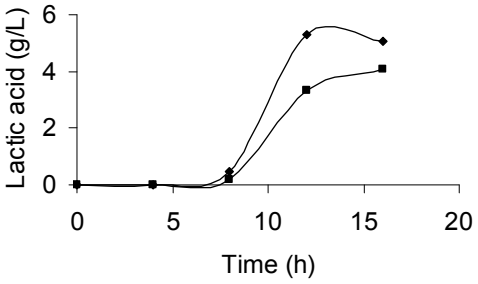

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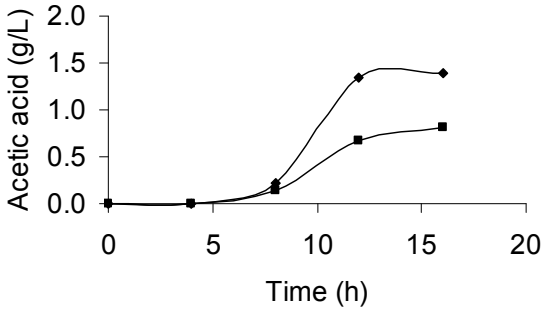

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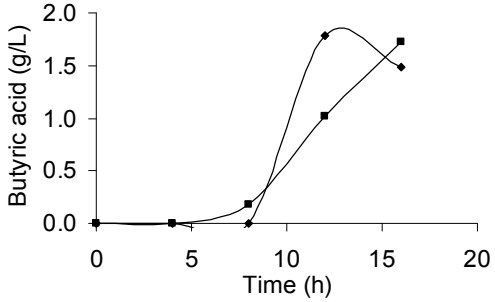
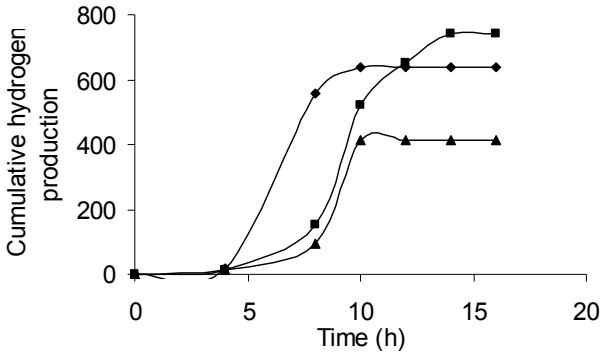


(E)

Fig 5.3 Kinetic profiles of H_2 (A), biomass (B) and Lactic acid (C), Acetate acid (D) and Butyric acid (E) produced using yeast extract (♦) and NH_4NO_3 (■) as nitrogen sources.



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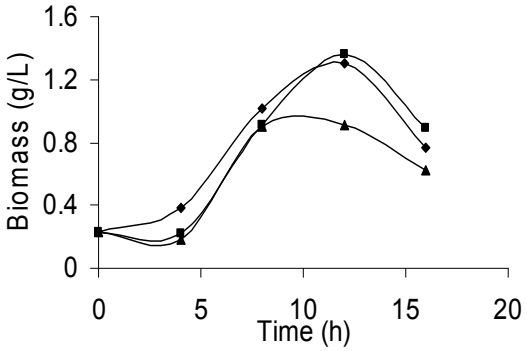

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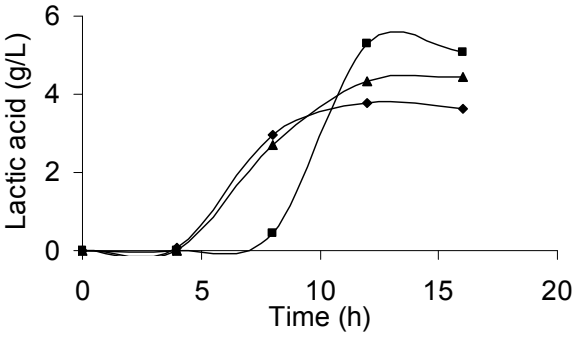

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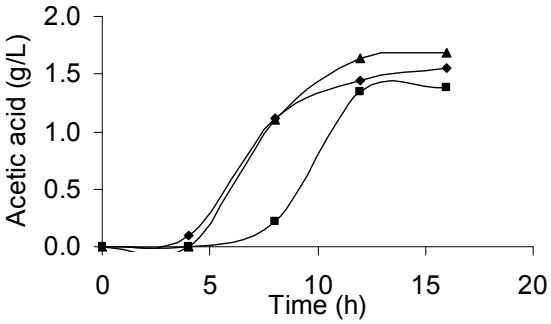

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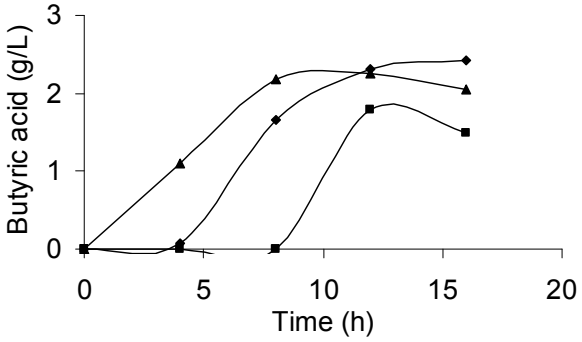


(E)

Fig 5.4 Kinetic profiles of H_2 (A), biomass (B) and lactic acid (C), acetate acid (D) and butyric acid (E) produced at operational pHs 6.0 (♦), 6.5(■) and 7.0 (▲).

with formation of acidic or alcoholic metabolites. Those could inhibit cell growth by changing the internal pH (Bowles et al., 1985). The optimum pH 6.5 for maximum H_2 yield and biomass growth is consistent with previous reports on H_2 production by batch or continuous fermentation processes, in which an optimal pH between 6.0 and 7.0 was reported (Chang et al., 2006).

5.5 Summary

The experimental data revealed that the newly isolated *C. butyricum* W5 is a promising H_2 producer that can use a wide range of carbon and nitrogen sources either as raw material or in the form of wastes. Molasses and waste potato starch can be used as carbon sources for large scale H_2 production. While yeast extract is the most favoured nitrogen source for bacterial growth and H_2 production, NH_4NO_3 was proven to be suitable for fermentative H_2 production. The use of the cheaper carbon and nitrogen sources of molasses, WPS and NH_4NO_3 shows great potential for a commercial H_2 production process. The operation pH 6.5 is beneficial mostly for the bacterial growth and hydrogen production. In association with H_2 production, the fermentation using *C. butyricum* W5 produced lactic acid, acetic acid and butyric acid as by-products. Differently, lactic acid was found to be the main VFA produced when using glucose as carbon source, while butyric acid was the main VFA produced using molasses and WPS.

CHAPTER 6

Process Optimization of Biological H_2 Production from Molasses by *Clostridium butyricum* W5

6.1 Introduction

Optimization of the operation parameters is a central step for development of a fermentation process. The inappropriate operating parameters such as pH and temperature result in a poor fermentation performance and undesirable low yield and process efficiency. The pH, temperature, composition and concentration of the culture medium, and microbial population were the most important parameters employed in a number of previous investigations. Because of the easy operation and short term, batch fermentation is generally used to seek the optimal pH, temperature or other operating parameters, and investigate the effects of substrates concentration. Optimized variables by batch fermentation are instructive for the continuous processes (El-Mansi and Bryce, 1999). However, only a few parameters, such as pH and temperature, were investigated in most previous studies on fermentative hydrogen production (Baek et al., 2006; Chittibabu et al., 2006). Most reported work to determine the values of operating parameters was based on subjective personal experience. There is lack of studies on the effects of operating parameters on the hydrogen yield and intercalations of these parameters with H_2 synthesis, bacterial growth and production of by-products. A few work investigated pH (Hwang et al., 2004; Mohan, 2007), temperature (Majizat et al., 1997; Nielsen et al., 2001) or substrates concentration (Wu and Lin, 2004), but only focused in one or two parameters and in a limited range. Some groups optimized initial pH, temperature and other parameters for certain H_2-producing bacteria such as *Rhodopseudomonas palustris* P4 (Oh et al., 2002) and *Escherichia coli* BL-21 (Chittibabu et al., 2006), but in bottle tests.

Due to the complexity of batch or continuous processes, a fixed parameter set was also used to study fermentation characteristics (Fang et al., 2002). However, some researchers noticed that operational parameters such as pH and temperature could significantly affect H_2 yield and volatile fatty acids (VFAs) formation. Hwang et al. (2004) worked on anaerobic fermentation at different pH ranges and concluded that pH plays an important role in determining the type of anaerobic fermentation pathway in biological H_2 processes. Mu et al. (2006) used mixed cultures and found that glucose consumption rate, H_2 yield, and bacterial cell growth rate increased as the temperature increased from 33 to 41 ^oC, while the specific H_2 production rate increased with increasing temperature from 33 to 39 ^oC, and then decreased as the temperature was further increased to 41 ^oC. Although many works indicated that operating parameters affected H_2 production and VFA formation greatly, there was still no research focusing on the optimization of the production conditions in a fermentation in

which the key operating parameters of pH, temperature, concentration of carbon, nitrogen and initial bacterial cell concentration were valued and investigated.

Therefore, in Chapter 5, we observed that all three isolates can produce H_2 individually but showed different H_2 production abilities. Adjusting the fermentation environment to suit the dominant and most efficient H_2 producer appeared to be the key for both batch and continuous processes with mixed bacteria. Results from Chapter 5 indicated that *C. butyricum* W5 appeared to be the best candidate for development of an efficient H_2 production system. Molasses and NH_4NO_3 showed great potential for highly efficient H_2 production and yield.

In this chapter, *C. butyricum* W5, the most efficient H_2-producing isolate obtained in Chapter 5, was used as the working strain to optimize fermentation parameters in a batch H_2 production process. Molasses and NH_4NO_3 were found to be the suitable raw materials for hydrogen production by *C. butyricum* W5. Carbon and nitrogen source concentrations, pH, temperature and inoculumn size were investigated and optimised. H_2 yield, production rate, bacterial biomass, and VFAs were measured. The relationship between operational conditions and H_2 yield was analysed. The results are not only instructive to batch H_2 fermentation, but also could be informative for continuous H_2 production processes.

On the basis of substrate cost and production efficiency, molasses, which contains mainly sucrose, glucose and fructose, was employed as the carbon source to optimize H_2 fermentation parameters in this chapter. There are several significant advantages of using molasses as a raw material for H_2 production. Molasses is much cheaper than glucose. A large amount of molasses is produced as a by-product of the sugar cane and sugar beet refining industry, which would provide sufficient raw material for this application. The molasses contains high amounts of organic nitrogen sources which remain after sugar cane or sugar beet processing (Beshay and Moreira, 2005). These are biochemically accessible to the fermentation bacteria. Therefore, addition of expensive organic nitrogen sources, such as yeast extract and peptone, is unlikely to be necessary. The molasses should also be able to accelerate bacterial growth as the non-sugar content includes high amounts of essential vitamins and salts (He et al., 2007). It has been reported that the use of molasses as the substrate in continuous fermentation processes leads to much higher H_2 yield and production

rate compared to glucose fermentation (Chittibabu et al., 2006; Ren et al., 2006; Li et al., 2007b).

A paper based on the research of this chapter "Process optimization of biological hydrogen production from molasses by a newly isolated *Clostridium butyricum* W5" has been submitted to *International Journal of Hydrogen Energy*.

6.2 Aims

The study in this chapter was to optimize operation conditions and find out the relationships between the metabolites and H_2 evolution. The aims were to:

- Identify the most suitable concentrations of molasses and NH_4NO_3.
- Optimize pH, temperature and initial cell concentration.
- Statistically analyse the possible kinetic relationships between fermentation parameters and metabolites.

6.3 Specific materials and methods

6.3.1 Molasses

Molasses was obtained from Magill Grain Store Pty Ltd (South Australia). Its original total sugar concentration was 44.0% in terms of hexose, which was similar to previous analysis of Australian-produced molasses (Papageorgiou et al, 1997). Its Total Kjeldahl nitrogen (TKN) was 0.7086%.

6.3.2 Experimental setup

In general, the optimization experiments were carried out in a batch fermentation bioreactor system under operation conditions with 100g/L molasses, 1.5g/L NH_4NO_3 and 1.2×10^5 cell/ml and operation pH 6.5, and the fermentation was performed at a agitation rate of 300 rpm and 35°C,. However, a range of each of these parameters were initially selected and tested in the relevant optimization experiments. A series of molasses concentrations from 20 to 120g/L were tested to find out the optimal carbon source concentration. This was then used

afterwards to find the optimal NH_4NO_3 concentration (0-1.5g/L). A series of pHs (5.5-7.5), temperatures (30-45°C) and initial cell concentrations (3×10^4-1.2×10^5 cell/ml) were also tested to find the optimal operating conditions based on the optimized medium composition.

6.3.3 Data analysis

The experimental data were analysed in a regression mode (Microsoft Excel 2003) to establish the relationships between H_2 yield and the by-products of bacterial biomass and VFAs produced in the fermentation under different operational conditions. Only results from the carbon and nitrogen source optimization and some results from temperature optimization (35°C, 38°C and 40°C) were used to fit the data because bacterial growth was not affected greatly by pH, or temperature or low initial cell number. The model equation was determined on the basis of the highest R^2 values of each trendline. The results reported in Fig 7 and Table 1 were the arithmetic mean values of the tests in triplicate. Appropriate tests of significance analysis derivations (ANOVA) and confidence deference at 5% level were used in the data evaluations.

6.4 Results and discussion

6.4.1 Effect of molasses concentration

H_2 production and bacterial growth

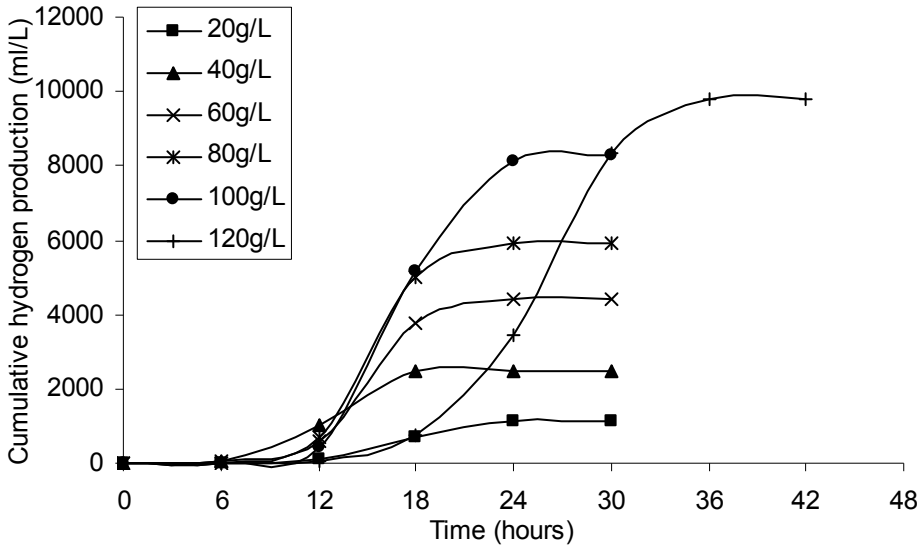
In dark fermentation, H_2 comes from metabolism of the carbon source (Das and Veziroğlu, 2001). The molasses concentration is crucial to the final H_2 yield. However, as high hexose and oligosaccharide concentrations may induce substrate inhibition by modifying metabolic pathways, the usage of high sugar-based carbon source concentrations is limited (Chittibabu et al., 2006; Oh et al., 2003). Therefore, in order to optimize molasses concentration in the batch H_2 fermentation, a series of molasses concentrations (20, 40, 60, 80, 100 and 120g/L) were tested in the reactor process. Kinetic profiles of the H_2 production and biomass growth associated with various molasses concentrations are depicted in Fig 6.1 A and Fig 6.2 A. H_2 production and yield, total and residual sugar in terms of hexose and sugar usage rate are presented in Fig 6.1 B and Table 6.1.

Table 6.1 Effect of molasses concentration on H_2 production and sugar usage

Parameters	**Initial molasses concentration (g/l)**					
	20	**40**	**60**	**80**	**100**	**120**
H_2 production (ml/L[a])	782	2485	4403	5914	8311	9811
Total sugar (g/l[b])	8.89	17.78	26.67	35.56	44.44	53.33
Residual sugar (g/l[b])	0.35	1.47	2.01	3.21	3.59	4.61
H_2 yield (mol/mol[c])	0.74	1.22	1.44	1.47	1.63	1.62
Sugar usage rate (%)	96.06	91.74	92.45	90.97	91.92	91.36

[a] Data are shown as ml H_2 per litre fermentation medium.
[b] Data are shown as gram hexose per litre fermentation medium
[c] Data are shown as mol H_2 per mol hexose.

As molasses concentration increased, both biomass and H_2 production increased, while the yield of H_2 increased and reached the highest level of 1.63mol H_2/mol hexose at 100g/L molasses. Similar variation of H_2 volume and biomass was found in the fermentation using 120g/L molasses, which produced 1.62mol H_2/mol hexose. A long lag phase of 18 h was observed at 120g/L molasses concentration. This may be due to the high osmotic pressure of the medium (Esener et al., 1980; Mille et al., 2005). A similar trend was reported by Wu and Lin (2004), in that the highest H_2 yield was reached at 40g Chemical Oxygen Demand (COD)/L, and a long accumulation time of more than 20 hours was required for fermentation of molasses over 60g COD/L. From Table 6.1, it can be seen that our isolated H_2 producer is capable of using over 91% of the sugar in molasses in the batch fermentation, while 96% of total sugars were consumed when the molasses concentration was 20g/L. Molasses concentration significantly affected the H_2 production (Fig 6.1 B) and bacterial growth (Fig 6.2 B). Statistical data analysis showed that the H_2 production and biomass growth have a linear relationship with the carbon source concentration. Linear equations were $y = 1780.6x - 887.42$ with $R^2 = 0.9948$ and $y = 0.9739x - 0.2443$ with $R^2 = 0.9872$.



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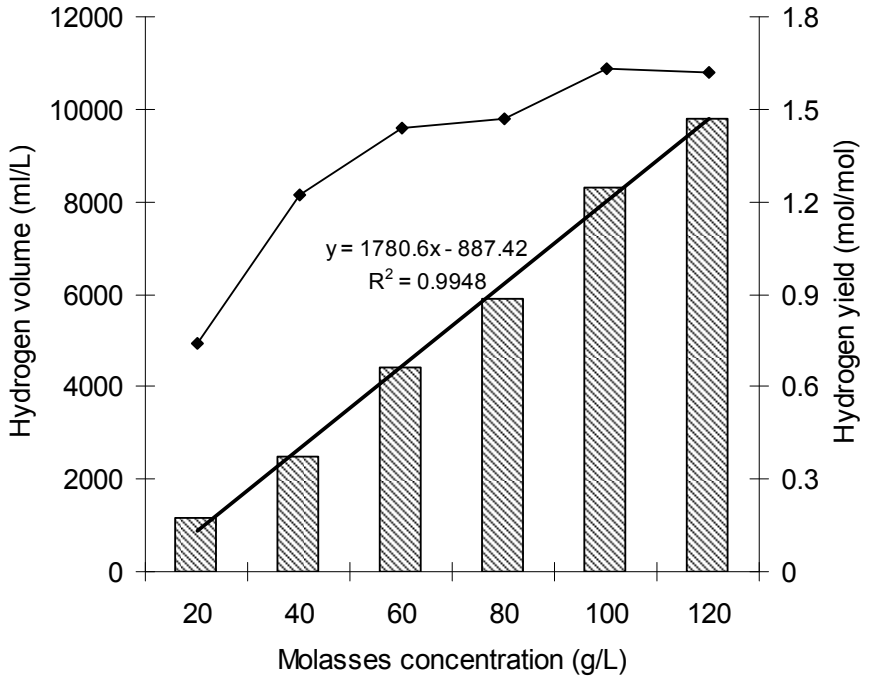
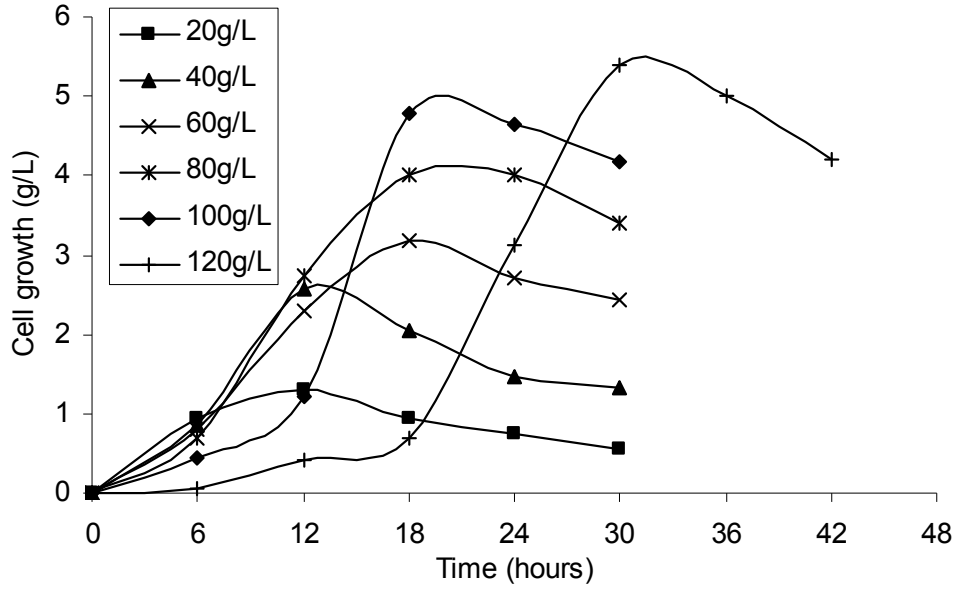


(B)

Fig 6.1 Time profile of cumulative H_2 production (A) and H_2 production (▨) and yields (♦) at different molasses concentrations (B).



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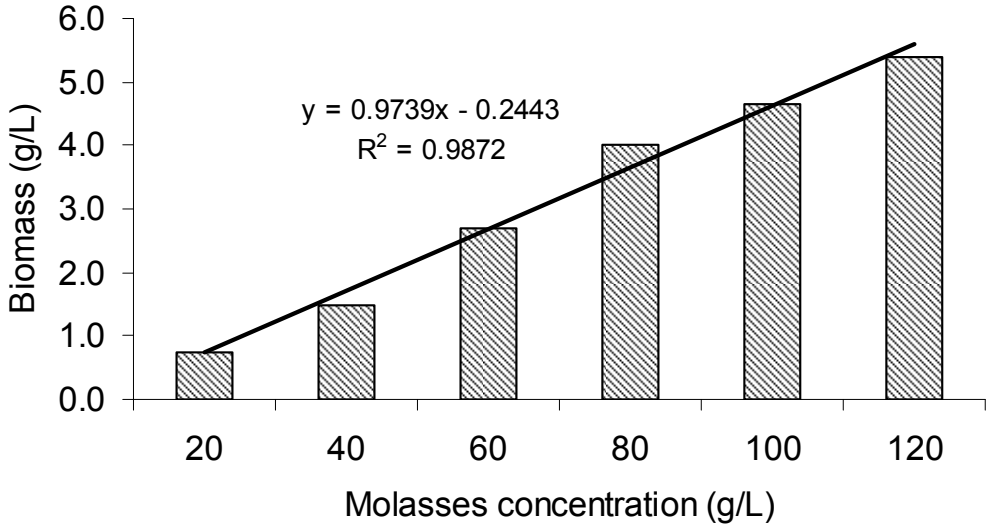


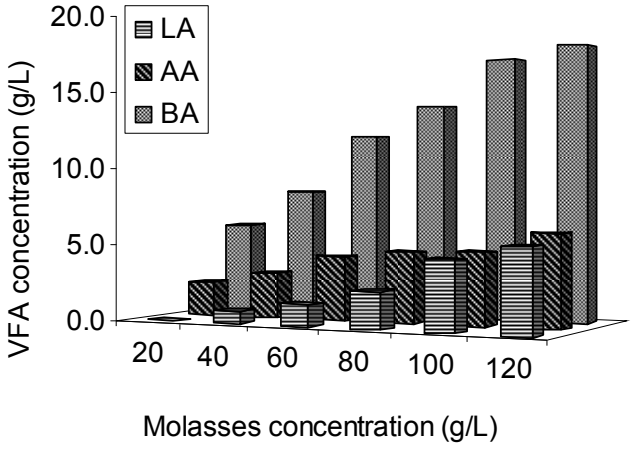
(B)

Fig 6.2 Time profile of cell growth (in terms of DCW) (A) and Biomass (B) at different molasses concentrations.

VFA production

Acetic, butyric and lactic acids have been reported as the main by-products produced in glucose-grown culture with *C. butyricum* VPI3266 (Saint-Amans et al., 2001). These were confirmed by our research. According to the literature, a high acetic/butyric acid ratio in the final fermentation broth is associated with a high H_2 yield. Lactic acid is a product from a non-H_2 generating metabolic pathway and it leads to low H_2 yield (Angenent et al., 2004). However, a PAK-Em mutant of *C. tyrobutyricum* tested by Liu et al. (Liu et al., 2006b) achieved a higher yield of H_2 and butyric acid compared to the original strain, while acetic acid yield varied a little. As shown in Fig 6.3, the acetic acid and butyric acid production increased (Fig 6.3 A), while both the lactic acid production (Fig 6.3 A) and yield (Fig 6.3 B) increased. It was also found that the final concentrations of the three VFAs increased with the increase in molasses concentration, and the mass ratio of acetic and butyric acids maintained constant values around 0.30 (Fig 6.3 C). At the same time, H_2 yield increased from 0.74 (20g/L molasses) to 1.63 (100g/L molasses) and 1.62 (120g/L molasses). Therefore, the relationship between acetic/butyric acid ratio and H_2 production should be reconsidered. The increase in lactic acid fraction with the increase in hexose concentration and H_2 yield is noteworthy and warrants further investigation.

Production of propionic acid was confirmed to be associated with low H_2 yield (Angenent et al., 2004). It has been detected as the main by-product of H_2 production by other researchers (Khanal et al., 2004; Ren et al., 2006). The results from present study revealed that little propionic acid was produced in the *C. butyricum* W5 fermentation. When molasses concentration was 20g/L, a noticeable butyric acid yield 0.34g/g hexose was obtained in this study, which was unexpectedly twice higher than those study on *C. butyricum* (Kong et al., 2006). The H_2 yield in this study was comparably higher than those fermentations using two *C. tyrobutyricum* mutants (*pta*-deleted mutant (PPTA-Em) and *ack*-deleted mutant (PAK-Em), which were constructed to improve their potential to produce butyric acid (Liu et al., 2006b). Butyric acid yield decreased to 0.27g/g (Table 6.2) with an increase in molasses concentration. This decrease was possibly induced by the increase in lactic acid.



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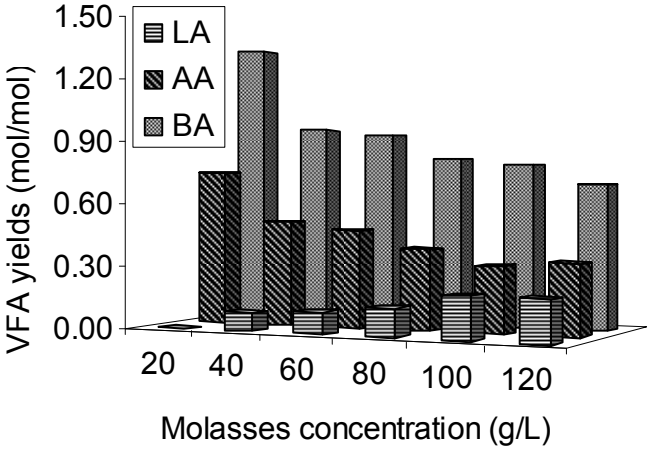

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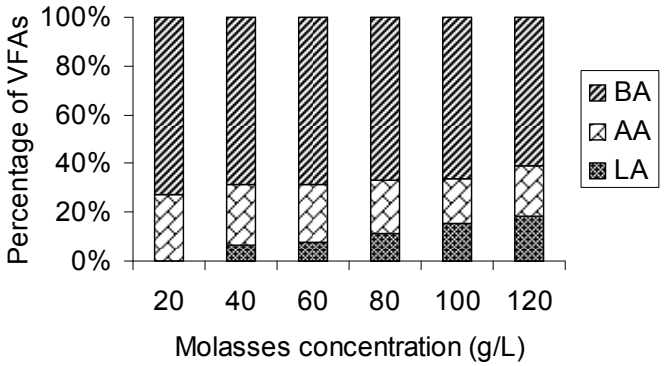


(C)

Fig 6.3 VFA concentration (A), yields (B) and percentage (C) at different molasses concentrations.

Table 6.2 Effect of initial cell concentration on H_2 production

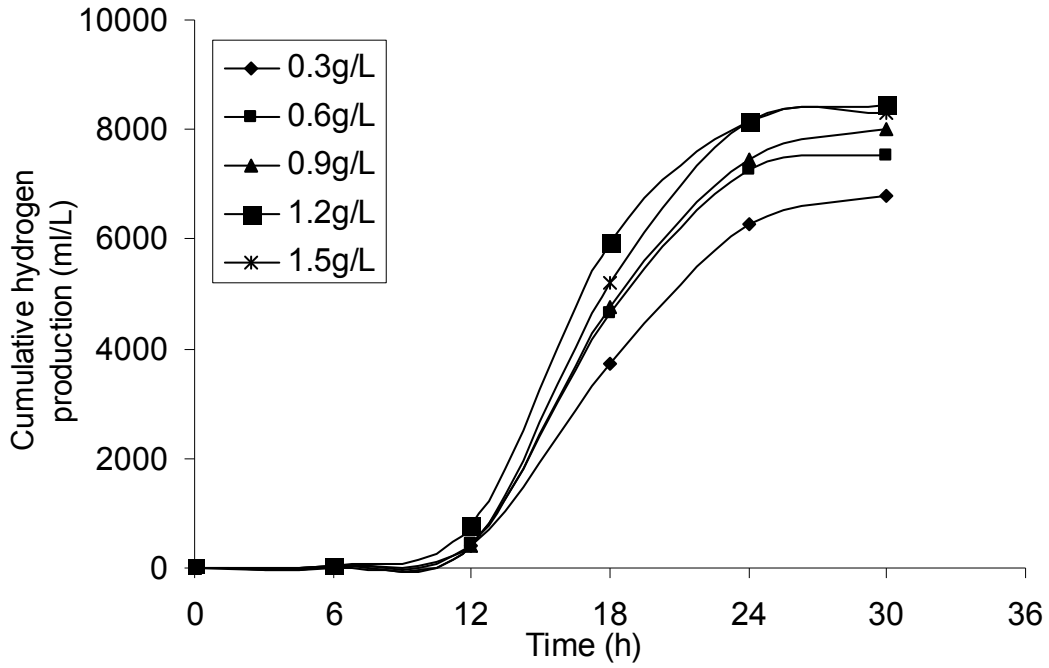
Parameters	**Initial cell numbers (cell/ml)**			
	3×10^4	**6×10^4**	**9×10^4**	**1.2×10^5**
H_2 yield (mol/mol[a])	1.47	1.56	1.85	1.85
H_2 production rate (mmol/h/l[b])	8.68	12.83	17.38	17.38
Lag time (h)	18	12	10	10
Fermentation duration (h)	42	30	24	24
Biomass (g/l[c])	4.03	4.36	4.43	4.48
Butyric acid (g/g hexose)	0.18	0.22	0.27	0.27

[a] Data are shown as mol H_2 per mol hexose.
[b] Data are shown as mmol H_2 per litre fermentation medium per hour.
[c] Data are shown as gram biomass per litre fermentation medium

A solventogenic phase (ethanol, butanol and acetone produced) in the H_2 fermentation has been reported in the late exponential phase after the acidogenic phase (Baek et al., 2006). This was explained as an effect of the acetic and butyric acids produced, which could consume extra NAD(P)H (2 mol than in the acidogenic pathways (Lin et al., 2006)). However, these by-products were found to be at a low or undetectable level in our H_2 fermentation system. H_2 removal and pH control could be employed as explanations because they keep protons going to molecular H_2, therefore reducing the concentration of NAD(P)H. The detailed metabolic pathway of H_2 fermentation with *C. butyricum* W5 is worthy of investigation in future research to better understand the H_2 evolution and related by-product production.

6.4.2 Effect of NH_4NO_3 concentration

NH_4NO_3 has been widely used as a nitrogen source for many fermentation processes because of its high nitrogen content (35%) and low cost. Molasses employed in this research contains 0.7086% nitrogen, mainly organic nitrogen, depending on its production process (Beshay et al., 2005). NH_4NO_3 was used in this research as a supplementary nitrogen source. The experiments were performed in additional 0 - 1.5g/L, at 0.3g/L interval, of NH_4NO_3 to investigate the effect of nitrogen source concentration on the fermentation performance, and concequently to find an optimal value of the nitrogen supplementation.



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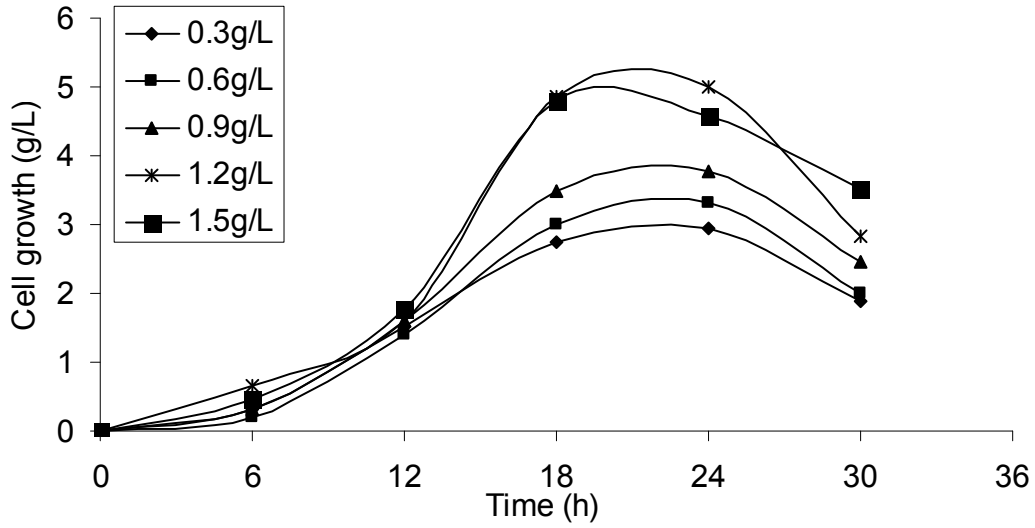


(B)

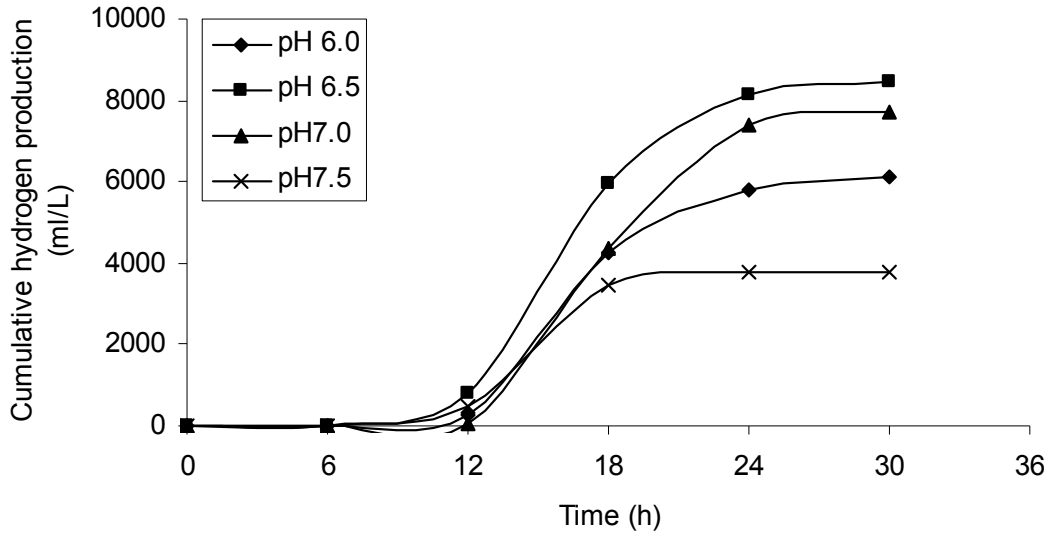
Fig 6.4 Time profiles of cumulative H_2 production (A) and cell growth (B) (in terms of DCW) at different NH_4NO_3 concentrations.

The results are shown in Fig 6.4. Both H_2 final yield (Fig 6.4 A) and dry cell weight (DCW) (Fig 6.4 B) reached the highest points at NH_4NO_3 concentration 1.2g/L. Only a short fermentation time was need to achieve a peak value of H_2 yield when the NH_4NO_3 concentration was higher than 1.2g/L. Further increase in the NH_4NO_3 concentration resulted in little improvement of H_2 yield and bacterial growth. The overall results indicate that the organic nitrogen compounds existing in the molasses would provide suitable nitrogen sources for the H_2-producing bacterium. A low level of inorganic nitrogen supplementation should be sufficient.

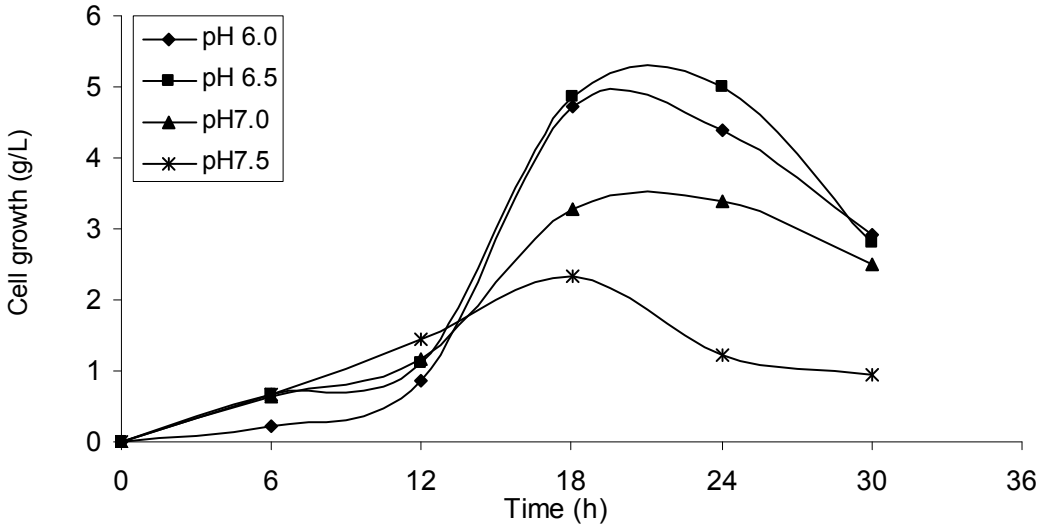
6.4.3 Effect of growth pH

The effect of operation pH on H_2 production was also examined in this chapter. Preliminary bottle experiments showed that there was no cell growth and H_2 evolution at pH 5.0 and 8.0 and a slow growth took place at pH 5.5 (data not shown). Kinetic studies on pH ranging from 6.0-7.5 were conducted in the reactor system. As shown in Fig 6.5, the optimum pH value for achieving a high H_2 production rate and yield was 6.7, followed by 7.0. It is interesting to note that our H_2-producing strain showed a sensitive performance at an operational pH around 6.5. Significant reductions in H_2 production and DCW were observed at pH 6.0 and 7.5. The optimum pH for cell growth was also 6.5. But unlike H_2 yield and production rate, a similar DCW curve could be observed at pH 6.0. The lowest DCW was given at pH 7.0. In previous research, the optimum pH for H_2 evolution of *C. butyricum* NCIB 9576 H_2ase was found to be 7.0 (Baek et al., 2006).

Much optimization work has been done especially to focus on the impact of initial pH on individual types of bacteria or mixed microorganisms (Khanal et al., 2004; Chittibabu et al., 2006). Little attention has been given to the investigation of the impact of the operation pH on *C. butyricum* growth in the batch process for H_2 production. The optimum pH for a continuous fermentation process by mixed microorganisms was investigated by Hwang et al. (2004) and Mohan (2007). Our results indicate that H_2 production using *C. butyricum* W5 is sensitive to pH change.



(A)



(B)

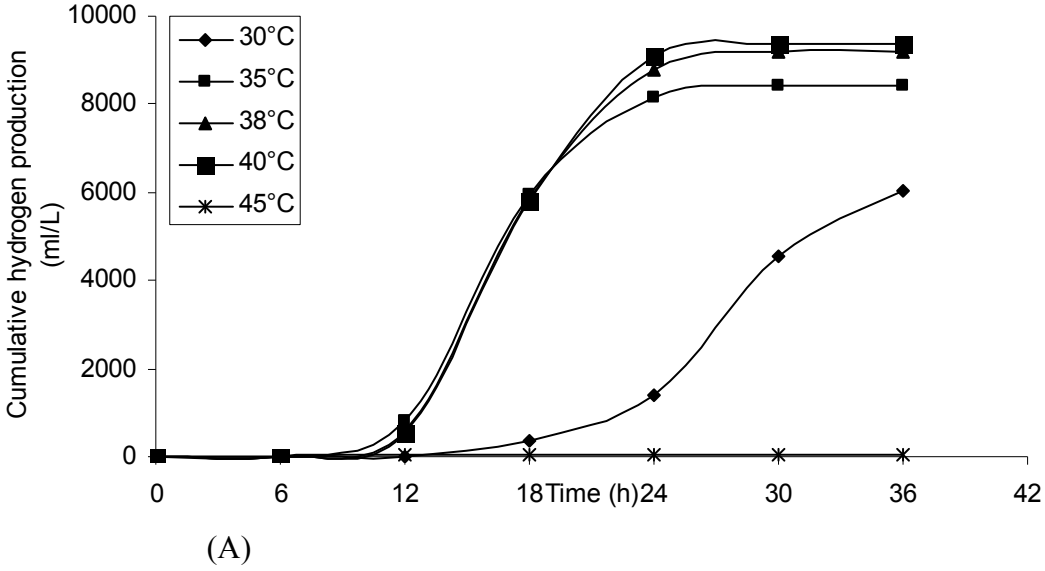
Fig 6.5 Time profiles of cumulative H_2 production (A) and cell growth (B) (in terms of DCW) as a function of pH.

6.4.4 Effect of temperature

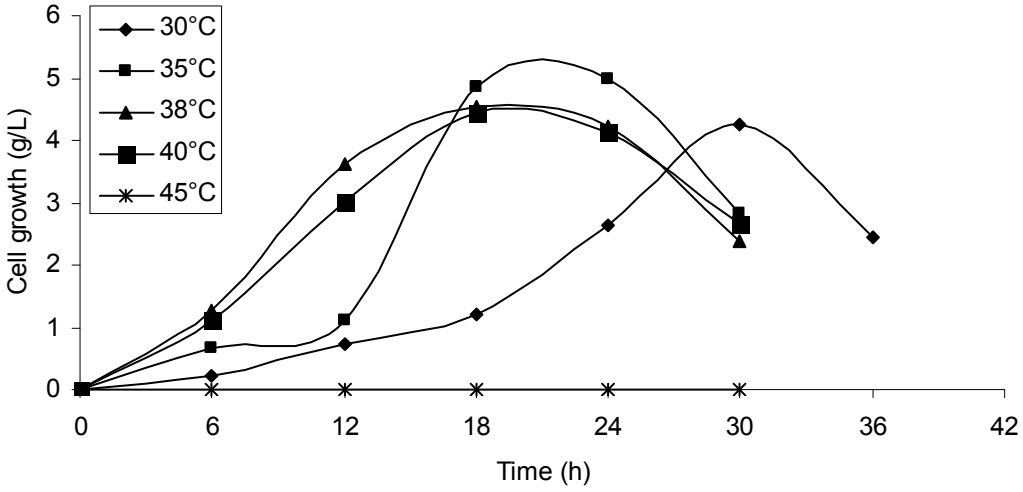
In general, the optimum temperatures for the key enzyme involved in yielding the target product and for cell growth are different. The mesophilic range (30-37°C) was considered as the favourable temperature range for H_2 production (Majizat et al., 1997; Nielsen et al., 2001). Previous research showed that optimum temperatures for *C. butyricum* NCIB 9576 cell growth and H_2ase activity were 37°C and 45°C, respectively (Baek et al., 2006). 35°C was used widely by previous researchers when H_2 was produced from other *Clostridium sp.* (Wang et al., 2004) or by anaerobic digestion (Zhang et al., 2005b; Wang et al., 2003b). In our research, as shown in Fig 6.6, 35°C was the most favourable temperature for *C. butyricum* W5 cell growth but not for H_2 production. The optimum temperature for H_2 production was from 38°C to 40°C. When the temperature was higher than 42°C, *C. butyricum* W5 could not grow at all (data not shown). This may be due to denaturation of other key enzymes (Fabiano et al., 2002). Mu *et al*. (2006) studied various temperatures in the mesophilic range of H_2 production by anaerobic sludge, and found that H_2 yield and production rate increased with an increase in temperature from 33-39°C and decreased as the temperature further increased to 41 ^{o}C.

6.4.5 Effect of initial inoculum size

An optimum initial bacterial population may affect significantly the lag phase of cell growth, metabolic reaction, product synthesis and productivity. A series of inoculum sizes ranging from 3×10^4 to 1.2×10^5 cell/ml were tested in this chapter. As shown in Table 6.2 and Fig 6.7, with the increase of initial cell number up to 9×10^4 cell/ml, a significant decrease in lag phase from 18 h to 10h was found, resulting in a decrease in the fermentation period required to achieve the stationary growth phase from 42h to 24h. No significant improvement in H_2 production rate and yield, or biomass growth was found when the inoculum size increased from 9×10^4 to 1.2×10^5 cell/ml. Therefore, 9×10^4 cell/per can be regarded as the optimum inoculum size for the H_2 production.



(A)



(B)

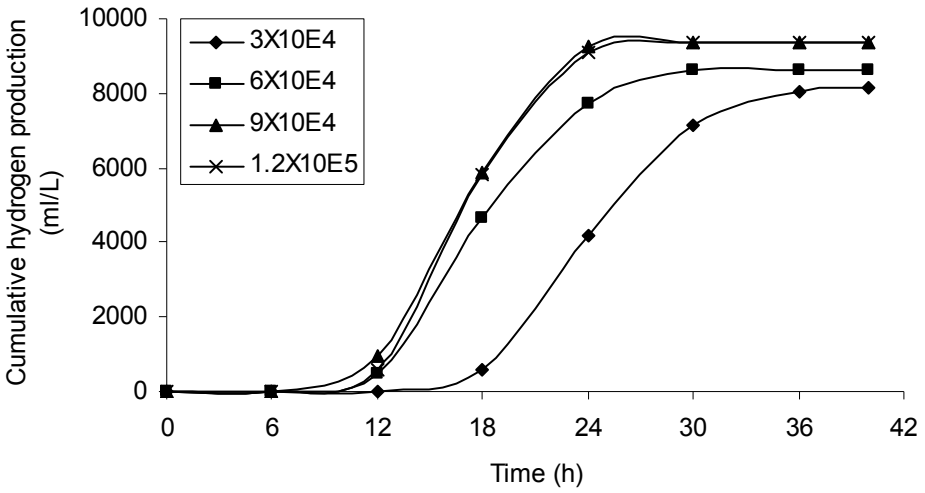
Fig 6.6 Time profiles of cumulative H_2 production (A) and cell growth (in terms of DCW) (B) at different temperature.

As shown in Figures 6.1 to 6.7, a lag phase of H_2 synthesis took place after the lag phase of bacterial cell growth. The profile curves of cell growth and H_2 accumulation with respect to variation in the optimal initial cell number displayed in Fig 6.7 showed that biomass reached a peak point in 6 h, while the highest H_2 volume was found in 24 h in the fermentation process. The highest production rate was also observed between the late potential phase and early stationary phase of cell growth.

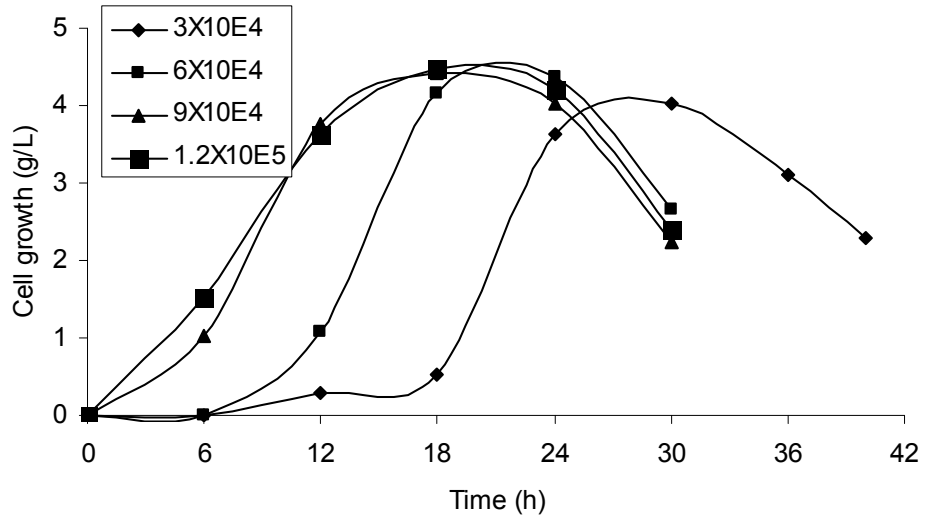
H_2 yield shown in Table 6.2 reached 1.85 mol H_2/ mol hexose under the optimized fermentation condition. Theoretically, 4 moles H_2/mol hexose could be gained if hexose was metabolized to acetic acid and 2 moles H_2/mol hexose is associated with a fermentation in which butyric acid is the final VFA (Angenent et al., 2004). It was reported in most previous studies that the maximum yield that could be gained from glucose is around 1.0-1.4 mol H_2/ mol glucose in a glucose fermentation using a wild strain, while mutant and recombinant strains could yield around 3 mol H_2/ mol glucose (Liu et al., 2006b; Chittibabu et al., 2006; Minnan et al., 2005). However this comes along with low growth rate and expensive substrates. Production rate in our process reached 17.38 mmol/h/L, which was much higher than previously reported for *C. theromolacticum* (5mmol H_2 /g cell/h (Collet et al., 2004) and 5.57 m^3 H_2/ m^3 reactor/day (He et al., 2007)). The results confirmed that pure culture fermentation for H_2 production has advantages over use of mixed microorganisms. High yield, a fast production rate of H_2 and a valuable by-product, butyric acid, could be achieved using a cheap substrate combination.

6.4.6 Data analysis

In order to seek correlations between H_2 yield and by-product formation, regression analysis was applied to analyse data of H_2 yield, biomass, ratio of acetic acid/butyric acid and lactic acid yield. The relationship between fermentation parameters and by-product formation with H_2 production has attracted the interest of many researchers (Kong et al., 2006; Chang et al., 2006). Angenent et al.(2004) attemped to find statistic correlation in the fermentation process and no mathematical relationship between ratio of acetic acid/ butyric acid and H_2 yield has been reported. The expression level of the clostridia H_2ase gene was proved to have a positive relationship on H_2 production, further application was labour intensive and limited by

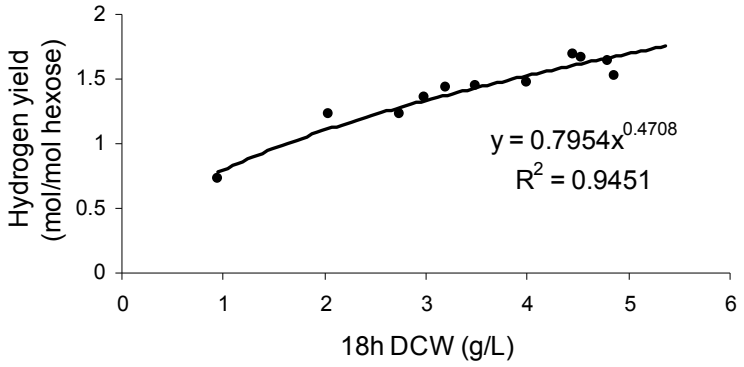


(A)

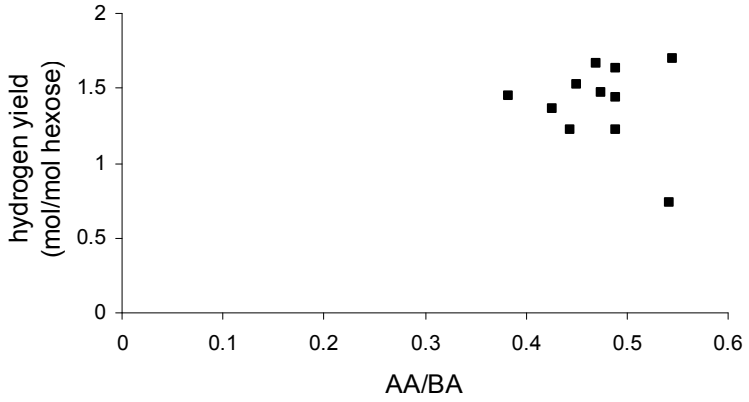


(B)

Fig 6.7 Time profiles of cumulative H_2 production (A) and cell growth (B) (in terms of DCW) at different initial cell concentrations.



(A)



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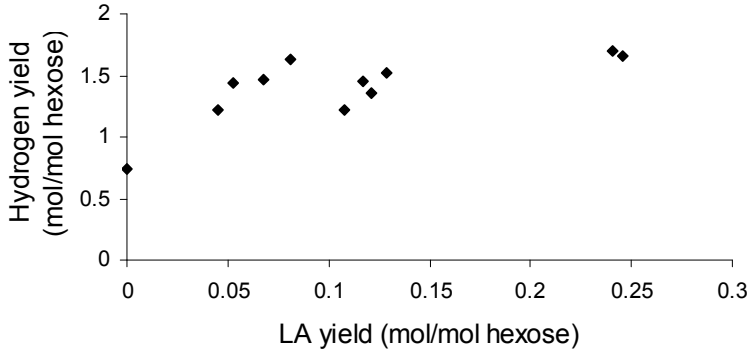


(C)

Fig 6.8 Statistical analysis data of H_2 yield against 18h DCW with H_2 yield (A), H_2 yield against acetic/butyric ratio (B) and H_2 yield against lactic acid yield (C).

the analysis costs (Chang et al, 2007). The relationship between lactic acid yield and H_2 yield was also probed in our research. Lactic acid is one of three main acids produced by *C. butyricum* W5 and was reported as a product of the competitive non-H_2-producing metabolic pathway which may lead to low H_2 yield. Fig 6.8 (A) shows that a power regression equation $Y = 0.7954X^{0.4708}$ with $R^2 = 0.9451$ best fitted the relationship between H_2 yield and biomass, indicating that H_2 yield increased with the increase in cell growth. Fig 6.8 (B) and (C) show that there is no clear mathematical relationship between the H_2 yield and either ratio of acetic acid /butyric acid or lactic acid yield. Further investigation is needed to clarify the metabolic pathways of H_2 and VFA production.

6.5 Summary

This chapter firstly optimized a batch fermentative H_2 production process by *C. butyricum* W5 using molasses involving a comprehensive study of operational parameters. Under the optimized fermentation condition, 100g/L molasses, 1.2g/L NH_4NO_3, and 9×10^4 cell/ml initial cell number at 39°C and pH 6.5, a maximum H_2 yield of 1.85 mol H_2/ mol hexose was achieved, corresponding to a H_2 production rate of 17.38 mmol/h/L. The VFAs were found to be mainly butyric acid, followed by acetic acid and lactic acid. The butyric acid yield was 0.27 g/g hexose. Lactic acid is an unfavourable by-product accumulated at high molasses concentration. Solvents and propionic acid were found at low or undetectable levels in the fermentation process.

The statistical analysis revealed a significant correlation between bacterial growth and H_2 yield. DCW could be employed as a H_2 yield indicator because H_2 yield increased in a power relationship with the increase in cell growth. However, the acetic/butyric acid ratio and lactic acid yield showed no specific relationship with either H_2 yield or biomass accumulation. Optimized parameters could provide basic information for the scale up of a continuous fermentative H_2 production process.

CHAPTER 7

Biochemical Kinetics of Fermentative Hydrogen Production by Clostridium butyricum W5

7.1 Introduction

In Chapter 6, optimal operational parameters of a batch H_2 fermentation process by *C. butyricum* W5 were obtained and DCW was proposed as a potential H_2 yield index because H_2 yield increased with the increase in cell growth. However, as discussed in the Chapter 1, Section 1.2.2, although anaerobic digestion of organic waste to produce H_2 has become technically feasible, and probably the most economical means of to bio-H_2 production, using cell growth of a certain bacterium or genus as an index of performance in sludge systems is technically difficult to perform.

Recently, the expression level of [FeFe] H_2ases, key enzymes which are responsible for H_2 production in most *Clostridium* sp., were reported to have a positive relationship to H_2 production in continuous processes and were been considered to be a potential biological activity index for H_2 production systems (Chang et al., 2005; Fang et al., 2006). As discussed in Chapter 1: i) functional gene expression levels can serve as a biomarker of certain functions of mixed microbial communities because it is more specific to target function and can separate out genes with the same function but belong to different species or even different genera (Chapter 1, Section 1.5); ii) *Clostridium* sp have been proved to be the major H_2 producers (Chapter 1, Section 1.2.4); iii) [FeFe] H_2ases exist in almost all H_2-producing clostridia and are conserved (Chapter 1, Section 1.3.4), the [FeFe] H_2ases gene expression level, in terms of mRNA numbers, has great potential to be a biological activity index for H_2 production systems.

However, the recent research described by Chang et al. (2006) and Fang et al. (2006) either used primers lacking in absolute specificity for H_2ase (Chang et al., 2006) as demonstrated by our research (Wang et al., 2007b) or targeted the DNA sequence of [FeFe] H_2ase gene (Fang et al., 2006) which could over estimate the number of active bacteria since this would detect the DNA from non-viable and inactive cells, as well as from active H_2 producers. The specificity of the real-time PCR assays previously described was not guaranteed because of the low annealing temperatures, 48°C and 52°C respectively (Section 1.4.2.1.2), and poor accuracy of quantification (Chang et al, 2006).

In order to clarify the relationship between gene expression and H_2 production, and to test the reliability of using it as a H_2 production index, the biochemical kinetics of H_2 production, cell

growth, measurement of VFAs accumulation and [FeFe] H_2ase gene expression level was reported in this chapter. The qRT-PCR procedure was optimized for quantification of [FeFe] H_2ase gene mRNA. The expression level of [FeFe] H_2ase was statistically analysed and its relationship with H_2 production was evaluated. Other possible indices involved in the H_2 fermentation were also examined.

This thesis has highlighted the importance of biological H_2 production and developed a H_2-producing system with high efficiency. This chapter will further evaluate the H_2 production index and provide information for understanding the metabolism of H_2 evolution at the cell level.

A paper based on the research of this chapter, "Biochemical Kinetics of Fermentative Hydrogen Production by *Clostridium butyricum* W5", has been submitted to *Journal of Applied Microbiology*.

7.2 Aims

The aims of this chapter were to

- Optimize the qRT-PCR procedure for [FeFe] H_2ase gene mRNA quantification.
- Profile biochemical kinetics of H_2 production, cell growth, VFAs accumulation and [FeFe] H_2ase gene mRNA in a batch fermentative H_2 production.
- Seek possible H_2 production indices and evaluate their reliability by statistical analysis.

7.3 Specific materials and methods

7.3.1 Measurement of cell growth

Heterotrophic plate count (HPC) using TSB agar were used to describe cell growth in this chapter. One hundred (100) µl of fermentation broth was diluted serially in tenfold steps in saline (0.9% NaCl), spread on TSB agar and cultured in an anaerobic jar at 39 ^{o}C immediately to determine Colony Forming Units (CFU)/ml. DCW, used in the previous chapter represents total bacteria including active, alive but not functional and inactivated cells.

As mRNA is mainly synthesized by active cell, CFU used in this chapter represents active cells and is therefore more precise to calculate mRNA number per cell.

7.3.2 Batch fermentation

The fermentation was performed at 39 °C, pH 6.5, and 300 rpm. The fermentation medium contained 10g/L 'LAB-LEMCO' Powder (LP0029, Columbia, HBA, Oxoid), 3g/L yeast extract (LP0021, Columbia, HBA, Oxoid) and 10g/L glucose.

7.3.3 Analytical methods

Five ml of fermentation broth in the bioreactor was collected in 0, 2, 4, 5, 6, 7, 8, 9, 10, 11 h for investigating the kinetics of H_2 accumulation, cell growth, glucose consumption and VFA production.

7.3.4 Reverse transcriptase-PCR (RT-PCR)

After heating at 95°C for 10 min to denature RNA and inactivate RNases, each RNA sample was reverse transcribed using 50U MuLV Reverse Transcriptase (Applied Biosystems, Foster City, California, USA), 20U RNase inhibitor (Applied Biosystems, Foster City, California, USA), 1mM deoxynucleoside triphosphate (dNTP), 5mM $MgCl_2$, 0.75μM reverse primer E1r and 1× PCR buffer in a total volume of 20μl at 42°C for 30min. The reaction was terminated by heating at 95°C for 10 min.

7.3.5 PCR and Real-Time PCR

For conventional PCR, a 465bp fragment encoding part of the clostridia H_2ase gene was amplified using primers E1f (5'-GCTGATATGACAATAATGGAAGAA-3')/E1r (5'-GCAGCTTCC ATAACTCCACCGGTTGCACC-3') (Chang, 2005; Wang et al., 2007b), with a Mastercycler® ep Gradient S thermocycler (Eppendorf, Westbury, New Yark, USA). PCR mixing and reaction conditions were performed as described previously (Wang et al., 2007b). The annealing temperature was optimised using a temperature gradient from 48°C to 60°C (48°C, 48.2°C, 48.9°C, 50°C, 51.4°C, 52.9°C, 54.5°C, 56.1°C, 57.5°C, 58.7°C, 59.6°C and 60°C).

Real-time PCR was performed in 100 µl thin-walled tubes in a Rotor-Gene 6000 HRM (Corbett Research, Sydney, Australia). Each 25µl reaction mixture contained 0.5µM of forward primer E1f, 3mM $MgCl_2$, 1× PCR Rnx Buffer II (Invitrogen, Carlsbad, California, USA), 1U of Platinum® *Taq* DNA Polymerase (Invitrogen, Carlsbad, California, USA), 2µM SYTO 9 (Invitrogen, Carlsbad, California, USA) (Monis et al., 2005), and 5µl from the reverse transcription reaction. The reactions were conducted at 2 different annealing temperatures, 48 ^{o}C (published annealing temperature) and 57 ^{o}C (optimum annealing temperature for conventional PCR identified in this study), for comparison. 57 ^{o}C (15s) was selected for use in PCR reactions with initial denaturation at 95^{o}C for 10 min to activate the *Taq* polymerase followed by 35 cycles of denaturation (30s at 94 ^{o}C), annealing (1 min), and extension (1 min at 72 ^{o}C), with a final extension at 67 ^{o}C (published extension temperature) for 7 min. Data collection was performed at 78°C for 10 s. Following amplification, melting curves were acquired on the FAM high channel using 0.2°C steps with a hold of 30s at each step from 70 to 85 °C. The differentiated data were analysed using the Rotor-Gene 6000 Series Software 1.7 (Build 34). The copy number of cDNA was quantified by reference to a standard curve using amplified plasmid DNA.

7.3.6 Construction of plasmids and standard curves

For the estimation of [FeFe] H_2ase mRNA in a sample, a plasmid standard was generated by cloning the PCR product from primer set E1f/E1r. Briefly, PCR product was cleaned using an UltraClean™ GelSpin DNA Purification Kit (MoBio Laboratories Inc., Solana Beach, California, USA) and cloned into *E. coli* using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, California, USA), following the manufacturer's instructions. Plasmid DNA was extracted from positive colonies by QIAprep® Spin Miniprep Kit (Qiagen GmbH, Germany) and quantified on a GeneQuant Pro RNA/DNA calculator (Biochrom, Cambridge, UK). Plasmids were diluted in tenfold steps representing 10^8-10^3 copies/µl in BPC grade water (W3513, Sigma-Aldrich, Ayrshire, UK) and amplified by real-time PCR. Ct values were calculated by the Rotor-Gene 6000 Series Software 1.7 (Build 34). The standard curve was obtained by plotting the Ct value versus the logarithm of the number of plasmid copies per reaction.

7.4 Results and discussion

7.4.1 Effect of annealing temperature on PCR amplification

Annealing temperature is an important factor affecting PCR specificity (Diaco, 1995; Fang, 2005). Low annealing temperature allows primer dimers and non-specific amplification while high annealing temperature reduces binding efficiency of primers to the target sequence. When using double stranded DNA-specific intercalating dyes, the fluorescent signal detected by real-time PCR caused by primer dimers and non-specific amplification can affect the precision of quantification of target DNA (Fang, 2005). The optimal annealing temperature for PCR was determined using a temperature gradient from 48°C to 60°C. Electrophoresis showed that brightest bands were obtained when PCR annealing temperatures were T7 (56.1^{o}C), T8 (56.5^{o}C) and T9 (57.5°C) (Fig. 7.1).

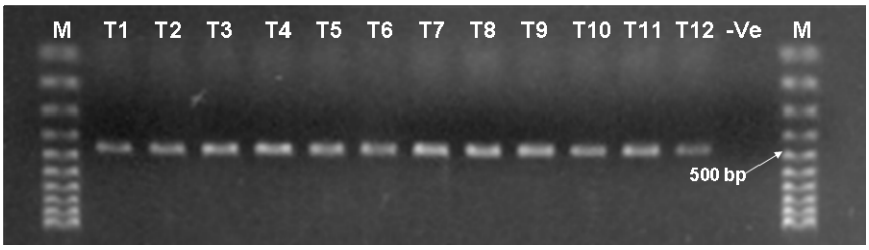


Fig 7.1 Electrophoresis of temperature gradient PCR products, M=100bp marker, T1=48°C annealing temperature, T2=48.2°C, T3=48.9°C, T4=50°C T5=51.4°C, T6=52.9°C, T7=54.5°C, T8=56.1°C, T9=57.5°C, T10=58.7°C, T11=59.6°C, T12=60°C.

Real-time PCR was conducted using annealing temperatures of 48°C and 57°C and the amplified material compared using DNA melting curve analysis. This is a precise method which can be used to verify the identity of an amplicon following PCR (Robinson, et al., 2006). Non-specific amplicons, generally having low *Tm* and can be easily detected by melting curve analysis. As shown in Fig 1, non-specific amplification was eliminated when the PCR annealing temperature rose from 48 ^{o}C (Fig. 7.2 (A)) to 57 ^{o}C (Fig. 7.2 (B)).

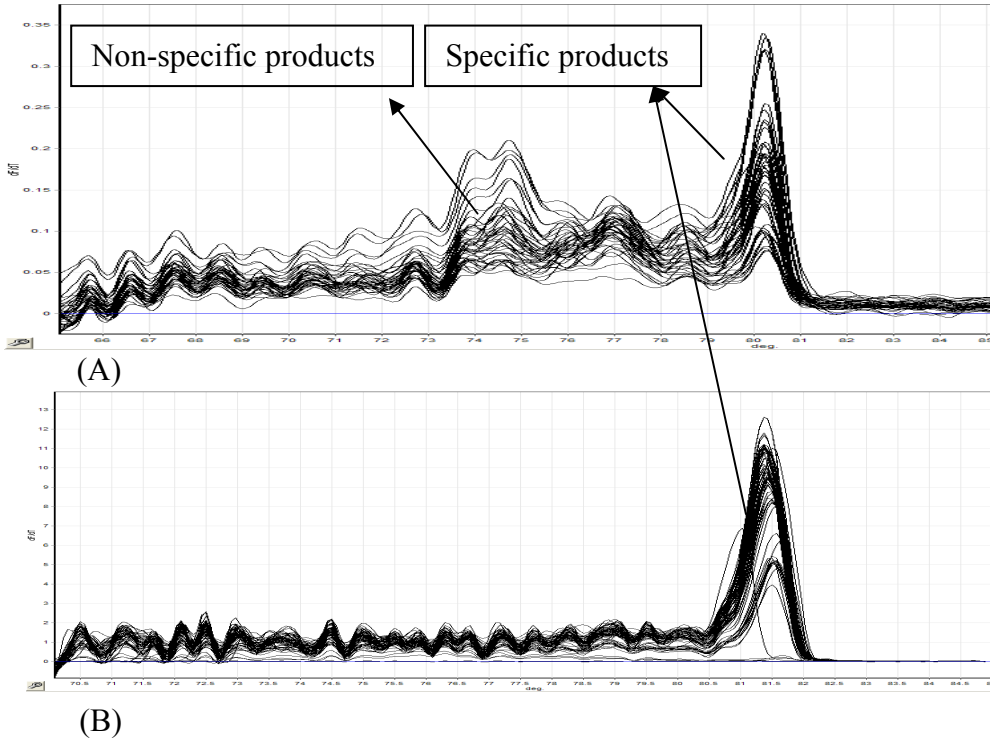


Fig 7.2 (A) Melting curve analysis of PCR products amplified with an annealing temperature of 48 ^{o}C. (B) Melting curve analysis of PCR products amplified with an annealing temperature of 57 ^{o}C.

7.4.2 Kinetics of H_2 production, cell growth and glucose consumption

Following inoculation, the bacterium started to grow almost immediately. Bacterial biomass was quadrupled at the 2 h sample point (Fig 7.3 and Table 7.1). CFU/ml reached the highest point at 8 h and dropped dramatically afterwards. Exponential H_2 production started after 4 h and H_2 reached a stationary level at 9 h, which was 1 h later than the peak for cell growth. Glucose concentration dropped considerably while cell number increased at 2 h and kept stable after 9 h, at which time no more H_2 was produced. The H_2 yield on glucose was 0.81 mol/mol.

H_2 has previously been reported as a bacterial cell growth-associated product (Chittibabu et al., 2006). Therefore, the growth rate of the working strain is crucial for gaining a high H_2 yield and production rate, which allows a shorter fermentation period and saves operating cost. *C. butyricum* W5 tested in our system showed a comparable shorter lag phase (2h) and fermentation period (9h) than the previously reported *Clostridium* sp. (Liu et al.., 2006;

Zhang et al., 2006) and other H_2-producing bacteria (Oh et al.. 2002; 2003). A similar short lag phase and fast growth were also observed previously with *C. butyricum* ZJUCB (Kong et al., 2006), which indicated that *C. butyricum* has a large potential for H_2 production via fermentation and great potential value as a target for genetic modification to maximize H_2 evolution.

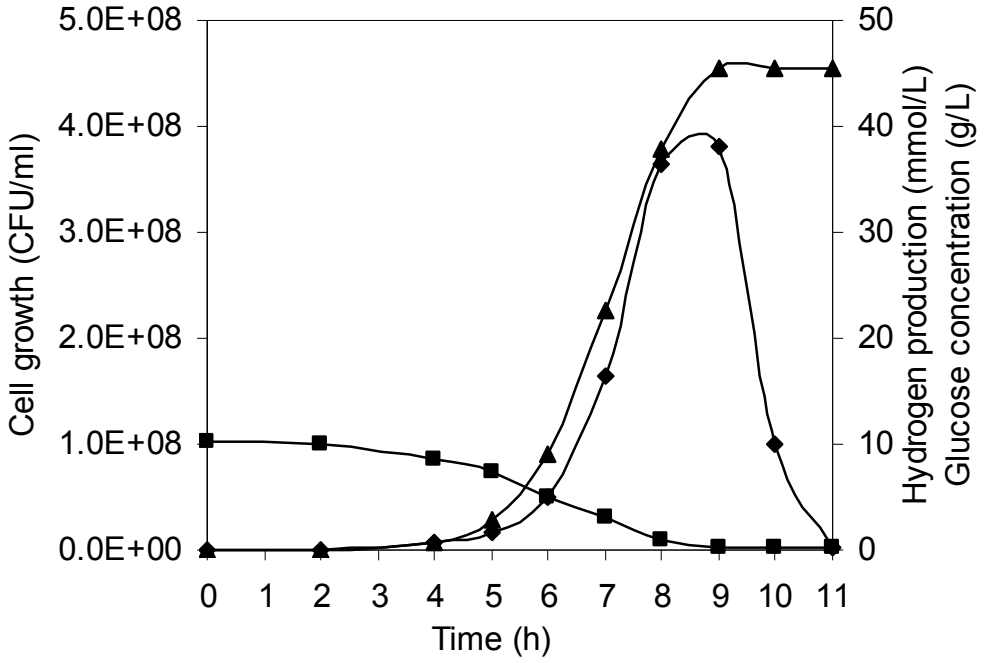


Fig 7.3 Time profiles of H_2 production (▲, mmol/L), cell growth (♦, CFU/ml) and glucose concentration (■, g/L) during fermentation.

Table 7.1 Kinetic parameters during fermentative H_2 production by *C. butyricum* W5.

Sample time (h)	H_2 Production (mmol/l)	H_2 Production rate (mmol/l/h)	Cell concentration (CFU/ml)	Cell growth rate (CFU/ml/h)	Glucose concentration (g/l)	*hydA* mRNA concentration (copies/ml)	*hydA* mRNA concentration (copies/CFU)
0	0.00	0.00	7.10E+04	0	10.14	1.01E+09	1.43E+04
2	0.00	0.00	3.02E+05	1.16E+05	9.89	1.44E+09	4.78E+03
4	0.76	0.38	6.87E+06	3.28E+06	8.54	1.94E+10	2.83E+03
5	2.90	1.07	1.61E+07	9.23E+06	7.42	1.93E+11	1.20E+04
6	8.97	3.04	4.95E+07	3.34E+07	4.93	6.08E+11	1.23E+04
7	22.59	6.81	1.65E+08	1.16E+08	3.10	3.99E+11	2.42E+03
8	37.81	7.61	3.65E+08	2.00E+08	0.88	1.46E+11	4.00E+02
9	45.45	3.82	3.82E+08	1.70E+07	0.22	8.87E+09	2.32E+01
10	45.45	0.00	1.01E+08	-2.81E+08	0.15	9.73E+09	9.63E+01
11	45.45	0.00	2.46E+06	-9.85E+07	0.14	4.86E+09	1.98E+03

7.4.3 VFA Production

Acetic acid, butyric acid and lactic acid were reported as the main metabolic products of *C. butyricum* (Yokoi et al., 1998; Saint-Amans et al., 2001). Among these metabolites, acetic acid and butyric acid have been recognized as the key VFAs in most fermentative H_2 production processes. The ratio of acetate acid/butyric acid was reported to be associated with H_2 yield by previous research (Angenent et al., 2004). Lactic acid was not a favoured product as it comes from a non H_2-producing metabolic pathway (Angenent et al., 2004).

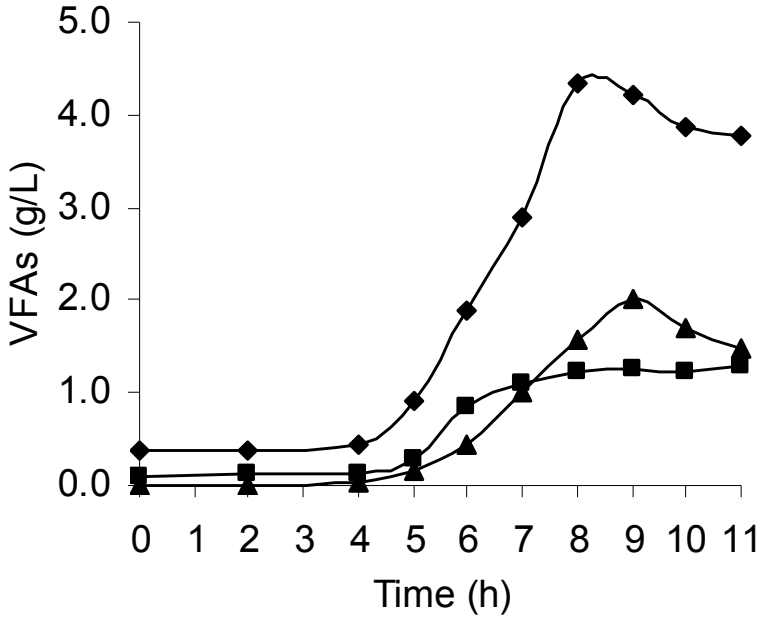


Fig 7.4 Time profiles of production of lactic acid (♦, LA), acetic acid (■, AA) and butyric acid (▲, BA).

As shown in Fig 7.4, it is interesting to note that lactic acid was the main VFA product form *C. butyricum* W5, while acetic and butyric acids accumulated less. To our knowledge, lactic acid formation during fermentative H_2 production with *Clostridium* sp. has not been reported in the literature. Our previous research showed that lactic acid formation was affected greatly by substrate (data not shown). More work focusing on the accumulation of the unfavoured by-product, lactic acid, will be helpful to manipulate the H_2 production metabolic pathway to gain higher yields. Lactic acid and butyric acid were consumed after 8 or 9 h. Acetic acid

remained at a stable level after 8 h. As glucose was nearly depleted after 8 h, bacteria started to consume lactic acid and butyric acid, which is likely to be responsible for the decrease in the observed levels of these organic acids (Matsumoto, 2007). Butyric acid is considered as an important industrial feedstock and has a high value for pharmaceutical usages (Beyer-Sehlmeyer et al., 2003). Butyric acid yield from glucose by *C. butyricum* W5 (0.20g/g) was comparable to butyric acid production from *C. butyricum* ZJUCB (~0.125g/g) (Kong et al., 2006) and *C. butyricum* CGS5 (~0.18g/g) (Chen, 2005).

7.4.4 Quantification of [FeFe] H_2ase gene expression

Real-time RT-PCR can be carried out by either a one-step reaction (reverse transcription and PCR amplification is performed in a single tube) or two-step reaction (reverse transcription and PCR amplification are performed in separate tubes). It was reported that two-step protocols are more acuate than one-step protocols, and the two-step protocols are more suitable in a situation where the same sample is assayed on several occasions over a period of time (Battaglia et al., 1998).

A two-step real-time RT-PCR was used in this work. Fig 7.5 shows the standard curve made from 10-fold serial dilution of plasmid containing the amplified region of the [FeFe]H_2ase gene. The threshold, which was automatically calculated by the RotorGene software, was 0.1692. The standard curve showed a linear correlation ($R^2 = 0.99889$) between input plasmid DNA and Ct. [FeFe]H_2ase mRNA concentrations determined according to the standard curve in terms of copies per millilitre fermentation broth and per CFU are shown in Table 1. Time profiles of [FeFe]H_2ase mRNA copies per cell with H_2 production rate, and cell growth rate from inoculation (0 h) to late exponential phase (9 h) are displayed in Fig 7.6. [FeFe]H_2ase mRNA levels in cells reached the highest point in 2 h before the H_2 production rate and cell growth approached maximum levels. The high initial [FeFe]H_2ase mRNA concentration per cell was possibly due to inoculation with bacteria already in the exponential growth phase.

Our results confirmed Chang et al.'s (2005) conclusion that a high expression of [FeFe]H_2ase gene was observed before optimal H_2 production and was positively related with H_2 production rate. The time interval between peaks of [FeFe]H_2ase mRNA levels in cells with H_2 production rate is possibly due to the translation of new enzyme from mRNA.

[FeFe]H_2ase mRNA levels in cells could be considered as an early indicator for H_2 production.

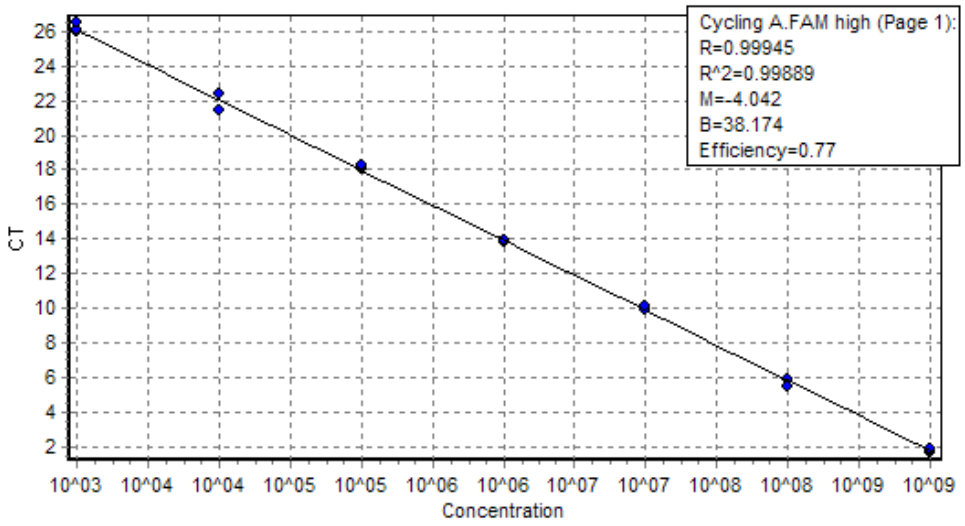
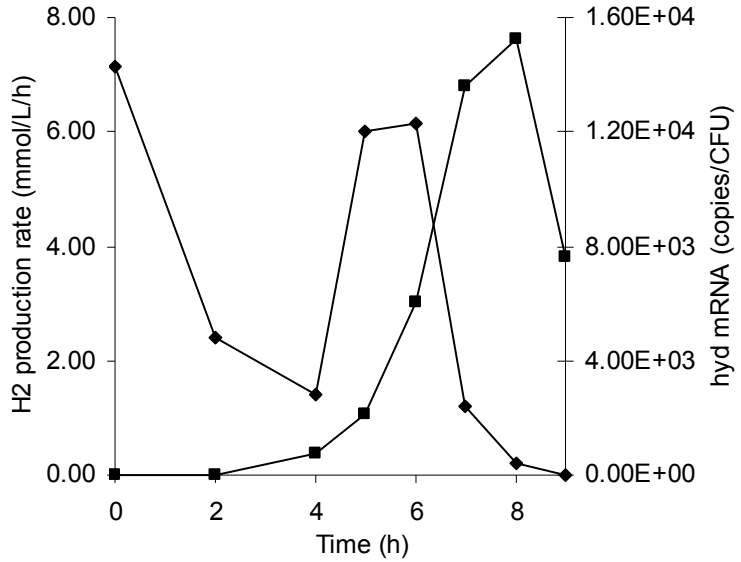


Fig 7.5 Standard curve with the correlation coefficient (R^2) by plotting the Ct against the input DNA plasmid quantity in logarithmic scale (copies/µl).

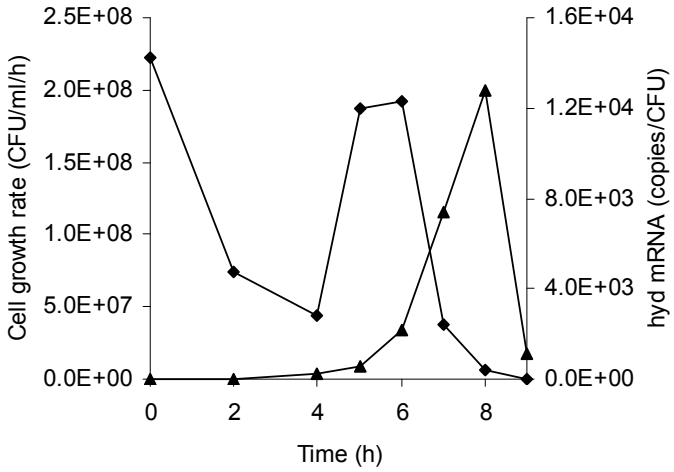
7.4.5 Data analysis

In order to seek correlations between H_2 production and cell growth, regression analysis was employed to analyse data from H_2 production and cell growth (0-9h). Fig 7.7 shows a linear regression with equation y = 1E-07x + 1.1824 with R^2 = 0.9839 best fitting the relationship between H_2 production and cell growth in terms of CFU/ml. This indicates that before the cell death phase, H_2 production increased with the increase in cell growth. Live cell number is positively related with H_2 production synchronously.

Currently, fermentative H_2 production processes mainly use mixed bacteria such as activated sludge (Mohan et al., 2007; Zhang et al., 2007b). A small number of studies have employed pure culture (Chittibabu et al., 2006). As bacterial numbers are difficult to quantify in some mixed culture processes, [FeFe] H_2ase mRNA level in the cell could be considered to be an advanced indicator for H_2 production. Otherwise, cell growth rate can be used as an indicator because of the low cost and ease of operation.



(A)



(B)

Fig 7.6 Time profiles of H_2 production rate (■, mmol/L/h) (A), cell growth rate (▲, CFU/ml/h) (B) and *hyd* mRNA (♦, copies/CFU)

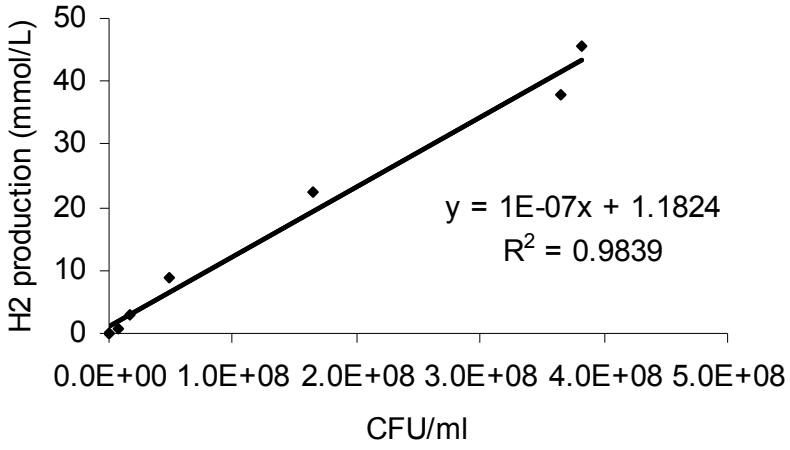


Fig 7.7 Statistical analysis data of H_2 production against CFU/ml (0-9 h).

7.5 Summary

This is the first comprehensive study on the biochemical kinetics of a H_2 fermentation process by *C. butyricum*. H_2 production, cell growth, VFA formation and [FeFe]H_2ase expression levels were profiled in detail and statistical analysis was used to determine relationships between different parameters. 57 ^oC was the optimal annealing temperature for qRT PCR after optimization. Peak expression of the [FeFe]H_2ase gene was observed before peak H_2 production and was positively related to the H_2 production rate. Cell growth proved to have a linear relationship with H_2 production. [FeFe]H_2ase expression level and cell growth rate have great potential to be used as H_2 production indices in different fermentation systems. The results are informative for further understanding of the metabolism of H_2 production from both engineering and molecular microbiology aspects.

CHAPTER 8

Conclusions and Recommendations

8.1 Conclusions

Fermentative hydrogen production has been an attractive research area in recent decades, as H_2 has great potential to be a highly efficient and environmentally friendly energy carrier in the future. However, this process suffers inherent limitations for commercialization in that its low yield and relative high production costs. Studies on engineering and/or technical aspects have been the main research topics in most previous investigations of hydrogen production processes. Although the majority of previous research focused on process optimization and seeking cheap substrates (even waste materials), the problems were still not solved. Moreover, fundamental knowledge related to biochemical and microbial mechanisms, metabolic and enzymatic activities have not been paid sufficient attention so far.

This research started from the investigation of the microbial community structure in a heat-treated activated sludge, isolating and identifying the potential hydrogen producers, and then used the most efficient hydrogen producer, *C. butyricum* W5, as a working strain. The [FeFe] hydrogenase gene cluster structure of *C. butyricum* W5 was profiled using advanced molecular techniques. The optimization of the batch fermentation process was carried out in a laboratory scale bioreactor system. This is the first report involved in all key operation parameters associated with fermentative H_2 production process.

In the study of the microbial community of the hydrogen-producing activated sludge system, culture independent methods, including PCR-DGGE and 16S rDNA clone library construction, combined with culture dependent isolation techniques, were used to profile the microbial community structure of hydrogen-producing bacteria in activated sludge. Three potential hydrogen producers were isolated and identified. Their ability for hydrogen production was subsequently confirmed. Results of the 16S rDNA-targeted profiling and plate isolation showed that clostridia species were dominant in the hydrogen fermentation. The ability of these isolates to produce hydrogen in culture correlated with the detection of a hydrogenase gene with high DNA sequence similarity to one described from *Clostridium* sp. Phylogenetic analysis, using 16S rRNA gene sequences from the hydrogen-production isolates and physiological identification, determined that W4 and W5 are *C. butyricum*, while the other, W1 could not be definitively identified. It was placed in an unresolved cluster of species including *C. roseum*, *C. diolis*, *C. acetobutylicum* and *C. beijerinckii* and showed a distinct substrate utilization pattern compared to other reported *Clostridium* sp. The hydrogen

production yields by isolates *Clostridium* sp. W1, *C. butyricum* W4 and *C. butyricum* W5 were much higher than those for *Clostridium* species reported elsewhere under the same reaction conditions.

A 3.3kb length DNA sequence from *C. butyricum* W5, which contains the [FeFe] hydrogenase gene, was obtained by IPCR. The [FeFe] hydrogenase gene cluster structure was predicted by ORF analysis from this sequence. The predicted transcriptional regulator upstream of the [FeFe] hydrogenase gene occurs in many *Clostridium* sp. and was also found in this sequence, which might have important influence on the regulation of the [FeFe] hydrogenase gene expression. The downstream putative Cof-like hydrolase indicates the end of the gene cluster, which is similar with other *Clostridium* sp. The absence of "TATA" boxes in the -10 areas of 3 genes may indicate that they are co-expressed. This genetic information on the [FeFe] hydrogenase gene cluster provides an important base for further investigation on the manipulation of [FeFe] hydrogenase gene expression to achieve high hydrogen production.

The screening of fermentation substrates revealed that the newly isolated *C. butyricum* W5 is a promising hydrogen producer which can use a wide range of carbon and nitrogen sources in either raw material or wastes. Molasses and waste potato starch can be used as carbon sources for a large scale hydrogen production. While yeast extract is the most favoured nitrogen source for bacterial growth and hydrogen production, NH_4NO_3 was proven to be a suitable nitrogen source for fermentative hydrogen production. The statistical analysis revealed that hydrogen production and yield have lineal relationships with molasses concentration. The use of the cheaper carbon and nitrogen sources of molasses, waste starch and NH_4NO_3 shows great potential for a commercial process for hydrogen production.

Optimization of the batch fermentation process was carried out with *C. butyricum* W5 using molasses. Under the optimized fermentation conditions: 100g/L molasses, 1.2g/L NH_4NO_3, and 9×10^4 cell/ml initial cell number at 39°C and pH 6.5, a maximum hydrogen yield of 1.85 mol H_2/ mol hexose was achieved, corresponding to a hydrogen production rate of 17.38 mmol/h/L. The statistical analysis revealed a significant correlation between bacterial growth and hydrogen yield. DCW could be employed as a hydrogen production yield indicator because hydrogen yield increased with the increase in cell growth. This is the first thorough investigation of batch hydrogen fermentation using molasses as substrate. The high yield and

production rate obtained from this process using *C. butyricum* W5 indicate that biological hydrogen production feasible for an industrial process.

In association with hydrogen production, the fermentation using *C. butyricum* W5 produced lactic acid, acetic acid and butyric acid as by-products. Lactic acid was found to be the main VFA produced when using glucose as carbon source. In contrast, butyric acid was the main VFA produced using molasses and waste potato starch. A high level of acetic acid and butyric acid was found to be associated with a high level of hydrogen and biomass, but the yields of these two VFAs decreased as the hydrogen production yield increased. Under optimized conditions, the butyric acid yield was 0.27 g/g hexose. Lactic acid accumulated at high molasses concentrations. Solvents and propionic acid were found at low or undetectable levels in the fermentation process. The ratio of acetic acid/butyric acid and lactic acid yield showed no statistical relationship with either hydrogen yield or biomass accumulation. Although no further enzymatic analysis of by-product metabolism was performed, statistical analysis and hypothesises should have great value for the understanding of by-product formation and its influence on hydrogen production, and provide basic support data for genetic manipulation towards high hydrogen production and yield.

The biochemical kinetics of a hydrogen production process by *C. butyricum* W5 were studied a during batch fermentation. Hydrogen production, cell growth, VFA formation and [FeFe] hydrogenase expression levels were examined. The optimal annealing temperature of real-time RT PCR was clarified and the [FeFe] hydrogenase gene mRNA detection procedure was optimized. Peak expression of the [FeFe] hydrogenase gene was observed before peak hydrogen production and was positively correlated with hydrogen production rate. Cell growth was shown to have a linear relationship with hydrogen production. [FeFe] hydrogenase expression level and cell growth rate were found to have great potential to be used as hydrogen production indices in different fermentation systems. These results are useful for understanding the metabolism of hydrogen formation *in vivo* and monitoring hydrogen production in engineering processes.

This work has demonstrated a new approach for the study of biological hydrogen production. Besides the promising results for the development of hydrogen production process, new molecular microbiological techniques have been applied to study the microbial community

structure, profile the [FeFe] hydrogenase gene cluster structure and describe the change of [FeFe] hydrogenase gene expression *in vivo* in a batch hydrogen production system. The results presented in this thesis have advanced knowledge of the metabolic flux network of the biological hydrogen production system.

8.2 Recommendations

The results of this research provide important information for further investigation of biological hydrogen production. The following recommendations can be made.

The three isolates, *Clostridium* sp W1, W4 and W5, will allow detailed investigation of the relationship between hydrogen yield and by-product composition. Further investigation could be done to compare the metabolic pathways for hydrogen production in these three isolates and to determine if the pathways can be manipulated to produce useful by-products and at the same time to improve the hydrogen yield. More work focusing on the differences between the three isolates in terms of hydrogenase genes and gene regulation will be helpful to determine if there is any relationship between oxygen tolerance and hydrogen yield or whether the hydrogen metabolic pathway can be modified to improve yields in oxygen-tolerant hydrogen producers.

C. butyricum W5 can be employed as a working strain to produce hydrogen from molasses efficiently on a large scale. Based on the information of the [FeFe] hydrogenase gene cluster obtained in this study, manipulation of the [FeFe] hydrogenase gene expression in *C. butyricum* W5 may be a promising approach to increase the yield and efficiency of the hydrogen production. Moreover, fermentative hydrogen production represents a metabolic flux network. During the fermentation, various extracellular metabolites including ethanol, acetate, butyrate and lactate can be produced. The conversion of pyruvate to these reduced end products involves oxidation of NADH. The concentration of NADH will increase if the formation of these metabolites can be blocked. H_2 evolution through the NADH pathway is driven by the necessity of reducing the residual NADH of metabolic reactions such as NADH + $H^+ \rightarrow$ NAD + H_2. Thus, if metabolic reactions can be controlled to increase the amount of residual NADH, the H_2 yield can be improved further. My hypothesis is that H_2 yield can be improved by eliminating the formation of some of these reduced products through redirection of the metabolic pathways towards H_2 synthesis**.** Except for the recognition of its complexity,

the metabolic flux network associated with H_2 production is not well understood. There is a need for extensive analysis and detailed understanding of metabolic fluxes and their regulatory circuits leading to H_2 formation. Such investigation might elucidate potential means for redesigning or redirecting the cellular metabolic pathways via metabolic engineering.

Bibliography

Acinas SG, Sarma-Rupavtarm R, Klepac-Ceraj V, Polz MF. PCR-induced sequence artifacts and bias: insights from comparison of two 16S rRNA clone libraries constructed from the same sample. *Appl Environ Microbiol* 2005, 71(12):8966-8969.

Adams MW, Stiefel EI. Biological hydrogen production: not so elementary. *Science* 1998, 282(5395):1842-1843.

Adamson KA. Hydrogen from renewable resources-the hundred year commitment. *Energy Policy* 2004, 32:1231-1242.

Åkerberg C, Zacchi G. An economic evaluation of the fermentative production of lactic acid from wheat flour. *Bioresource Technol* 2000, 75(2):119-126.

Akhmanova A, Voncken F, van Alen T, van Hoek A, Boxma B, Vogels G, Veenhuis M, Hackstein JHP. A hydrogenosome with a genome. *Nature* 1998, 396:527-528.

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

Amann RI, Ludwig W and Schleifer KH. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol Rev* 1995, 59(1):143-169.

Angenent LT, Karim K, Al-Dahhan MH, Wrenn BA, Domíguez-Espinosa R. Production of bioenergy and biochemicals from industrial and agricultural wastewater. *TRENDS Biotechnol* 2004, 22(9):477-485.

Armstrong F A. Hydrogenases: active site puzzles and progress. *Curr Opin Chem Biol* 2004, 8: 133-140.

Baek J, Choi E, Yun Y, Kim S, Kim M. Comparison of hydrogenases from *Clostridium butyricum* and *Thiocapsa roseopersicina*: Hydrogenases of *C. butyricum* and *T. roseopersicina. J Microbiol Biotechnol* 2006, 16:1210-1215.

Bai MD, Cheng SS, Tseng IC. Biohydrogen produced due to peptone degradation by pretreated seed sludge. *The IWA Asia-Pacific Regional Conference, Asian WATERQUAL,* 2001, Fukuoka, Japan.

Barbosa MJ, Rocha JMS, Tramper J, Wijffels. Acetate as a carbon source for hydrogen production by photosynthetic bacteria. *J Biotechnol* 2001, 85: 25-33.

Barns SM, Fundyga RE, Jefferies MW, Pace NR. Remarkable archaeal diversity detected in Yellowstone National Park hot spring environment. *Proc Natl Acad Sci* 1994, 91(5):1609-1613.

Battaglia M, Pedrazzoli P, Palermo B, Lanza A, Bertolini F, Gibelli N, Da Prada GA, Zambelli A, Perotti C, Robustelli della Cuna G. Epithelial tumour cell detection and

the unsolved problems of nested RT-PCR: a new sensitive one step method without false positive results. *Bone Marrow Transpltation* 1998, 22(7):693-698.

Benemann JR. Processes Analysis and Economics of Biophotolysis of Water. A Preliminary Assessment. Report to the International Energy Agency Hydrogen Program, Subtask B, Annex 10, Photoproduction of Hydrogen, IEA/H2/10/TR2-98, 1998.

Benemann JR. Hydrogen production by microalgae. *J Appl Phycol* 2000, 12:291-300.

Benoit S, Methta N, Ge W, Gatlin M, Maier RJ. Requirement of hydD, hydE, hypC and hypE genes for hydrogenase activity in *Helicobacter pylori*. *Microb Pathog* 2004, 36: 153-157.

Beshay U, Moreira M. Production of alkaline protease with *Teredinobacter turnirae* in controlled fed-batch fermentation. *Biotechnol Lett* 2005, 27:1457-1460.

Beyer-Sehlmeyer G, Glei M, Hartmann E, Hughes R, Persin C, Böhm V, Rowland I, Schubert R. Butyrate is only one of several growth inhibitors produced during gut flora-mediated fermentation of dietary fibre sources. *Brit J Nutr* 2003, 90:1057-1070.

Blackall LL, Burrell PC, Gwilliam H, Bradford D, Bond PL, Hugenholtz P. The use of 16S rDNA clone libraries to describe the microbial diversity of activated sludge communities. *Water Sci Techol* 1998, (4-5): 451-454.

Blokesch M, Paschos A, Theodoratou E, Bauer A, Hube M, Huth S, Böck A. Metal insertion into NiFe-hydrogenases. *Biochem Soc Trans* 2002, 30:674-680.

Böck A, King PW, Blokesch M, Posewitz M C. Maturation of Hydrogenases. *Adv Microb Physiol* 2006, 51:1-71.

Boon N, De Windt W, Verstraete W, Top EM. Evaluation of nested PCR-DGGE (denaturing gradient gel electrophoresis) with group-specific 16S rRNA primers for the analysis of bacterial communities from different wastewater treatment plants. *FEMS Microbiol Ecol* 2002, 39:101-112.

Boyles D. Bioenergy technology-Thermodynamics and costs. 1984, Wiley, New York.

Brock TD, Madigan MT, Martinko JM. Biology of microorganisms. 1994, Prentice-Hall, New York

Caffrey SM, Park HP, Voordouw JK, He Z, Zhou J, Voordouw G. Function of periplasmic hydrogenases in the sulfate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough. *J Bacteriol* 2007, 189(7):6159-6167.

Cammack R, Frey M, Robson R. Hydrogen as a fuel: Learning from nature. 2001, Taylor & Francis, London.

Clark JM. Novel non-templated nucleotide addition reactions catalyzed by procaryotic and eucaryotic DNA polymerases. *Nucl Acids Res* 1988, 16:9677-9686.

Clifton CE. The unitization of amino acids and related compounds by *Clostridium tetani. J Bacteriol* 1942, 44:179-183.

Chang JJ, Chen W, Shih SY, Yu SJ, Lay JJ, Wen FS, Huang CC. Molecular detection of the clostridia in an anaerobic biohydrogen fermentation system by hydrogenase mRNA-targeted reverse transcription-PCR. *Appl Microbiol Biotechnol* 2006, 70:598-604.

Chang JJ, Chou CH, Hsu PC, Yu SJ, Chen WE, Lay JJ, Huang CC, Wen FS. Flow-FISH analysis and isolation of clostridial strains in an anaerobic semi-solid bio-hydrogen producing system by hydrogenase gene target. *Appl Microbiol Biotechnol* 2007, 74:1126-1134

Chen CC, Lin CY. Using sewage sludge as seed in an anaerobic hydrogen producing reactor. In: Proceedings 25th Wastewater Treatment Technology Conference, December 1–2. 2000:368-372.

Chen CC, Lin CY, Chang JS. Kinetics of hydrogen production with continuous anaerobic cultures utilizing sucrose as limiting substrate. *Appl Microbiol Biotechnol* 2001, 57:56-64.

Chen CC, Lin CY, Lin MC. Acid-base enrichment enhances anaerobic hydrogen production process. *Appl Microbiol Biotechnol* 2002, 58:224-228.

Chen W, Tseng Z, Lee K, Chang J. Fermentative hydrogen production with *Clostridium butyricum* CGS5 isolated from anaerobic sewage sludge. *Int J Hydrogen Energy* 2005, 30:1063-1070.

Chiang YC, Fan CM, Liao WW, Lin CK, Tsen HY. Real-time PCR detection of *Staphylococcus aureus* in milk and meat using new primers designed from the heat shock protein gene *htrA* sequence. *J Food Protect* 2007, 70 (12): 2855-2859.

Chittibabu G, Nath K, Das D. Feasibility studies on the fermentative hydrogen production by recombinant *Escherichia coli* BL-21. *Process Biochem* 2006, 41:682-688.

Chumakov KM. Reverse transcriptase can inhibit PCR and simulate primer dimmer formation. *PCR Meth Appl* 1994, 4(1): 62-64.

Cockerill RRI, Smith TF. Rapid-cycle real-time PCR: a revolution for clinical microbiology automated extractions, self-contained PCR instruments, and other improvements yield easy-to-run diagnostic tests. *ASM News* 2002, 68:77-83.

Collet C, Adler N, Schwitzguébel JP, Péringer P. Hydrogen production by *Clostridium thermolacticum* during continuous fermentation of lactose. *Int J Hydrogen Energy* 2004, 29:1479-85.

Curtis TP, Sloan WT, Scannell JW. Estimating prokaryotic diversity and its limits. *Proc Natl Acad Sci USA* 2002, 99:10494-10499.

Curtis TP, Sloan WT. Prokaryotic diversity and its limits: microbial community structure in nature and implications for microbial ecology. *Curr Opin Microbiol* 2004, 7:221-226.

Dahllöf I, Baillie H, Kjelleberg S. *rpoB*-based microbial community analysis avoids limitations inherent in 16S rRNA gene intraspecies heterogeneity. *Appl Environmen Microb* 2000, 66:3376-3380.

Das D, Veziroğlu TN. Hydrogen production by biological processes: a survey of literature. *Int J Hydrogen Energy* 2001, 26:13-28.

Delong EF. Archaea in coastal marine environments. *Proc Natl Acad Sci* 1992, 89:5685-5689.

Diaco R. Practical considerations for the design of quantitative PCR assays. 1995, In: Innis MA, Gelfand DH, Sinsky JJ, editors. PCR Strategies. NY, USA, Academic Press, Inc: 85-108.

Dionisi D, Majone M, Vallini G, Di Gregorio S, Beccari M. Effect of the applied organic load rate on biodegradable polymer production by mixed microbial cultures in a sequencing batch reactor. *Biotechnol Bioeng* 2006, 93(1):76-88.

Droppo Ian G, Ross N, Skafel M, Liss SN. Biostabilization of cohesive sediment beds in a freshwater wave-dominated environment. *Limnol Oceanogr* 2007, 52(2):577-589.

Durre P. Handbook on Clostridia. 2004 Routledge, USA.

El-Mansi M, Bryce CFA. Fermentation Microbiology and Biotechnology. 1999 CRC Press, FL, USA.

Elliott D. Energy, Society and the Environment: Technology for a Sustainable Future. 1997, Routledge, London.

Esener AA, Bol G, Kossen NW. Roels JA. Effect of water activity on microbial growth. *Adv Biotechnol* 1980, 3:339-344.

Evans DJ, Pickett CJ. Chemistry and the hydrogenases. *Chem Soc Rev* 2003, 32:268-275.

Ewyernie D, Morimoto K, Karita S, Kimura T, Sakka K, Ohmiya K. Conversion of chitinous waste to hydrogen gas by *Clostridium paraputrificum* M-21. *J Biosci Bioeng* 2001, 91:339-43.

Fabiano B, Perego P. Thermodynamic study and optimization of hydrogen production by *Enterobacter aerogenes*. *Int J Hydrogen Energy* 2002, 27:149-56.

Fang HHP, Zhang T, Liu H. Microbial diversity of mesophilic hydrogen producing sludge. *Appl Microbiol Biotechnol* 2002, 58:112-118.

Fang HHP, Zhang T, Li C. Characterization of Fe-hydrogenase genes diversity and hydrogen-producing population in an acidophilic sludge. *J Biotechnol* 2006, 126:357-364.

Ferchichi M, Crabbe E, Gil GH, Hintz W, Almadidy A. Influence of initial pH on hydrogen production from cheese whey. *J Biothchnol* 2005 120: 402-409.

Figuerola ELM, Erijman L. Bacterial taxa abundance pattern in an industrial wastewater treatment system determined by the full rRNA cycle approach. *Environ Microbiol* 2007, 9(7):1780-1789.

Fischer SG, Lerman LS. Length-independent separation of DNA restriction fragments in two-dimensional gel electrophoresis. *Cell* 1979,16(1):191-200.

Friedrich B, Buhrke T, Burgdorf T, Lenz O. A hydrogen-sensing multiprotein complex controls aerobic hydrogen metabolism in *Ralstonia eutropha*. *Biochem Soc Trans* 2005, 33:97-101.

Fuhrman JA, Lee SH, Masuchi Y, Davis AA, Wilcox RM. Characterization of marine prokaryotic communities *via* DNA and RNA. *Microb Ecol* 1994, 28:133-145.

Garcia-Armisen T, Servais P. Enumeration of viable *E. coli* in rivers and wastewaters by fluorescent in situ hybridization. *J Microbiol Methods* 2004, 58:269–279.

Garrec N, Besnard V, Magras C, Pourcher AM, Federighi M. Behaviour of *Listeria monocytogenes* Scott A and a wild strain in different sewage sludge matrices: evaluation of culturability and viability and determination of potential virulence in mice bioassay. *Revue Méd Vét* 2005, 156:148-154.

Gibbs M, Hollaender A, Kok B, Krampitz LO, San Pietro A. Proceedings of the Workshop on Bio Solar Hydrogen Conversion. 1973, 5–6. Bethesda Maryland.

Goldenberger D, Altwegg M. Eubacterial PCR: contaminating DNA in primer preparations and its elimination by UV light. *J Microbiol Meth* 1995, 21:27-32.

Gorwa M, Croux C, Soucaille P. Molecular Characterization and Transcriptional Analysis of the Putative Hydrogenase Gene of *Clostridium acetobutylicum* ATCC 824. *J Bacteriol* 1996, 178(9):2668–2675.

Gottschalk G. Bacterial Metabolism, 1986, Springer-Verlag

Haugland RP. Molecular probes: handbook of fluorescent probes and research chemicals. 1999, 7th edn., Molecular Probes Inc., Eugene, Oreg.

Hallenbeck PC. Fundamentals of the fermentative production of hydrogen. *Water Sci Technol* 2005, 52:21-29.

Hawkes FR, Dinsdale R, Hawkes DL, Hussy I. Sustainable fermentative hydrogen production: challenges for process optimisation. *Int J Hydrogen Energy* 2002, 27: 1339-1347.

Hawkes FR, Hussy I, Kyazze G, Dinsdale R, Hawkes DL. Continuous dark fermentative hydrogen production by mesophilic microflora: Principles and progress. *Int J Hydrogen Energy* 2007, 32:172-184.

He X, Guo X, Liu N, Zhang B. Ergosterol production from molasses by genetically modified *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol* 2007, 75:55-60.

Heid CA, J Stevens, KJ Livak, PM Williams. Real time quantitative PCR. *Genome Res* 1996, (6):986-994.

Heim S, Lleo MDM, Bonato B, Guzman CA, Canepari P. The viable but nonculturable state and starvation are different stress responses of *Enterococcus faecalis* as determined by proteome analysis. *J Bacteriol* 2002,184:6379–6745.

Helms C. Method: Denaturing gradient gel electrophoresis (DGGE). 1990.

Higgins JA, Fayer R, Trout JM, Xiao L, Lal AA, Kerby S, Jenkins MC. Real-time PCR for the detection of *Cryptosporidium parvum*. *J Microbiol Meth* 2001, 47:323-337.

Hoefel D, Monis PT, Grooby WL, Andrews S, Saint CP. Profiling bacterial survival through a water treatment process and subsequent distribution system. *J Appl Microbiol* 2005, 99:175-86.

Hoffman P. Tomorrow's Energy: Hydrogen, Fuel Cells and the Prospects for a Cleaner Planet. 2001, MIT Press, Massachusetts, USA.

Hosoda A, Kasai Y, Hamamura N, Takahata Y, Watanabe K. Development of a PCR method for the detection and quantification of benzoyl-CoA reductase genes and its application to monitored natural attenuation. *Biodegradation* 2005, 16:591-601.

Hristova KR, Lutenegger CM, Scow KM. Detection and quantification of methyl tert-butyl ether-degrading strain PM1 by realtime TaqMan PCR. *Appl Environ Microbiol* 2001, 67:5154-5160.

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

Hussy I, Hawkes FR, Dinsdale R, Hawkes DL. Continuous fermentative hydrogen production from sucrose and sugarbeet. *Int J Hydrogen Energy* 2005, 30:471-483.

Hwang MH, Jang NJ, Hyun SH, Kin IS. Anaerobic bio-hydrogen production from ethanol fermentation: the role of pH. *J Biothech* 2004, 111:297-309.

Ingela MT, Karin E and Asa J. Reassessing PCR primers targeting *nirS, nirK* and *nosZ* genes for community surveys of denitrifying bacteria with DGGE. *FEMS Microbiol Ecol* 2004, 49: 401-417.

Jin B, Huang LP, Lant P. *Rhizopus arrhizus*-a producer for simultaneous saccharification and fermentation of atarch waste materials to L(+)-Lactic acid. *Biotechnol Lett* 2003, 25: 1983-1987.

Jin G, Kelley TR. Characterization of microbial communities in a pilot-scale constructed wetland using PLFA and PCR-DGGE analyses. *J Environ Sci Health A Tox Hazard Subset Environ Eng* 2007, 42(11):1639-1647.

Jo JH, Jeon CK, Lee DS, Park JM. Process stability and microbial community structure in anaerobic hydrogen-producing microflora from food waste containing kimchi. *J Biotechnol* 2007, 131:300-308.

Kalogo Y, Bagley D. Fermentative hydrogen gas production using biosolids pellets as the inoculum source. *Bioresource Technol* 2008, 99(3):540-546

Kanai T, Imanaka H, Nakajima A, Uwamori K, Omori Y, Fukui T. Continuous hydrogen production by the hyperthermophilic archaeon, *Thermococcus kodakaraensis* KOD1. *J Biotechnol* 2005, 116:271-82.

Kapdan IK, Kargi F. Bio-hydrogen production from waste materials. *Enzym Microb Tech* 2006, 38: 569-582.

Karube I, Matsunaga T, Tsuru S, Suzuki S. Continuous hydrogen production by immobilized whole cells of *Clostridium butyricum*. *Biochim Biophys Acta* 1976, 444:338-343.

Kataoka N, Miya A, Kiriyama K. Studies on hydrogen production by continuous culture system of hydrogen producing anaerobic bacteria. *Water Sci Technol* 1997, 36:41-47.

Kawai M, Matsutera E, Kanda H, Yamaguchi N, Tani K, Nasu M. 16S ribosomal DNA based analysis of bacterial diversity in purified water used in pharmaceutical manufacturing processes by PCR and denaturing gradient gel electrophoresis. *Appl Environ Microbiol* 2002, 68:699-704.

Keep NH, Ward JM, Robertson G, Cohen-Gonsaud M, Henderson B. Bacteria resuscitation factors: revival of viable but non-culturable bacteria. *Cell Mol Life Sci* 2006, 63: 2555-2559.

Kell DB, Kaprelyants AS, Weichart DH, Harwood CR, Barer MR. Viability and activity in readily culturable bacteria: a review and discussion of the practical issues. *Antonie van Leeuwenhoek* 1998, 73:169-187.

Khanal SK, Chen WH, Li L, Sung S. Biological hydrogen production: effects of pH and intermediate products. *Int J Hydrogen Energy* 2004, 29(11):1123-1131.

Klein D. Quantification using real-time PCR technology: applications and limitations. *TRENDS Mol Med* 2002, 8:257-260.

King FG, Hossain MA, King PW, Posewitz MC, Ghirardi ML, Seibert M. Functional studies of [FeFe] hydrogenase maturation in an *Escherichia coli* biosynthetic system. *J Bacteriol* 2006, 188:2163-2172.

Kobs G. Cloning blunt-end DNA fragments into the pGEM®-T vector systems. *Promega Notes Magazine* 1997, 62:15-19.

Kong Q, He GQ, Chen F, Ruan H. Studies on a kinetic model for butyric acid bioproduction by Clostridium butyricum. *Lett Appl Microbiol* 2006, 43:71-77.

Kotay SM, Das D. Biohydrogen as a renewable energy resource—Prospects and potentials. *Int J Hydrogen Energy* 2008, 33(1):258-263.

Kowalchuk GA, Stephen JR, de Boer W, Prosser JI, Embley TM and Woldendrop JW. Analysis of ammonia oxidizing bacteria of the b-subdivision of the class Proteobacteria in coastal sand dunes by denaturing gradient gel electrophoresis and sequencing of PCR amplified 16S ribosomal DNA fragments. *Appl Environ Microbiol* 1997, 63:1489-1497.

Kumar N, Das D. Enhancement of hydrogen production by *Enterobacter cloacae* IIT- BT 08. *Process Biochem* 2000, 35:589-93.

Kumar N, Ghosh A, Das D. Redirection of biochemical pathways for the enhancement of H_2 production by *Enterobacter cloacae*. *Biotechnol Lett* 2001, 23:537-541.

Kumar S, Tamura K, Nei M. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Briefings in Bioinformatics* 2004, 5:150-163.

Kurata S, Kanagawa T, Magariyama Y, Takatsu K, Yamada K, Yokomaku T, Kamagata Y. Reevaluation and reduction of a PCR bias caused by reannealing of templates. *Appl Environ Microbiol* 2004, 70:7545-7549.

Lattin WC, Utgikar VP. Transition to hydrogen economy in the United States: A 2006 status report. *Int J Hydrogen Energy* 2007, 32:3230-3237.

Lay JJ. Modeling and optimization of anaerobic digested sludge converting starch to hydrogen. *Biotechnol Bioeng* 2000, 68: 269-278.

Lay JJ. Biohydrogen generation by mesophilic anaerobic fermentation of microcrystalline cellulose. *Biotechnol Bioeng* 2001, 74: 280-287.

Lee JW, Choi ES, Gil KK, Lee HW, Lee SH, Lee SY, Park YK. Removal behavior of biological nitrogen and phosphorus, and prediction of microbial community composition with its function, in an anaerobic-anoxic system from weak sewage. *J Microbiol Biotechnol* 2001, 11(6):994-1001.

Lee YJ, Miyahara T, Nolke T. Effect of pH on the microbial hydrogen fermentation. 1999 Asian Waterqual 99, 7th IAWQ Asia-Pacific Regional Conference 1, 215–220.

Levin DB, Pitt L, Love M, Biohydrogen production: prospects and limitations to practical application. *Int J Hydrogen Energy* 2004, 29:173-185.

Li D, Chen H. Biological hydrogen production from steam-exploded straw by simultaneous saccharification and fermentation. *Int J Hydrogen Energy* 2007a, 32:1742-1748.

Li J, Li B, Zhu G, Ren N, Bo L, He J. Hydrogen production from diluted molasses by anaerobic hydrogen producing bacteria in an anaerobic baffled reactor (ABR). *Int J Hydrogen Energy* 2007b, 32:3274-3283.

Lin CY, Chang RC. Hydrogen production during anaerobic acidogenic conversion of glucose. *J Chem Technol Biotechnol* 1999, 74(6):496-500.

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

Liu SJ, Yang WF, Zhou PQ. The research on hydrogen production from the treatment of bean products wastewater by immobilized photosynthetic bacteria. *J Environ Sci* 1995, 16: 42–44.

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

Liu H, Zhang T, Fang HPP. Thermophilic H_2 production from cellulose containing wastewater. *Biotechnol Lett* 2003, 25(4):365–369.

Liu G, Shen J. Effects of culture medium and medium conditions on hydrogen production from starch using anaerobic bacteria. *J Biosci Bioeng* 2004, 98:251–256.

Liu X, Zhu Y, Yang S. Butyric acid and hydrogen production by *Clostridium tyrobutyricum* ATCC 25755 and mutants. *Enzyme Microb Tech* 2006b, 38:521-528.

Mackay IM, Arden KE, Nitsche A. Real-time PCR in virology. *Nucleic Acids Res* 2002, 30(6): 1292-1305.

Mahyudin AR, Furutani Y, Nakashimada Y, Kakizono T, Nishio N. Enhanced hydrogen production in altered mixed acid fermentation of glucose by *Enterobacter aerogenes*. *J Ferment Bioeng* 1997, 83(4):358-363.

Majizat A, Mitsunori Y, Mitsunori W, Michimasa N, Junichiro M. Hydrogen gas production from glucose and its microbial kinetics in anaerobic system. *Water Sci Tech* 1997, 36:279-286.

Malinen E, Kassinen A, Rinttila T, Palva A. Comparison of real-time PCR with SYBR Green I or 5'-nuclease assays and dot-blot hybridization with rDNA-targeted oligonucleotide probes in quantification of selected faecal bacteria. *Microbiology* 2003, 149:269-277.

Mannhalter C, Koizar D, Mitterbauer G. Evaluation of RNA isolation methods and reference genes for RT-PCR analyses of rare target RNA. *Clin Chem Lab Med* 2000, 38:171-177.

Marbán G, Valdés-Solís T. Towards the hydrogen economy? *Int J Hydrogen Energy* 2007, 32:1625-1637.

Marsischky G, LaBaer J. Many paths to many clones: a comparative look at high-throughput cloning methods. *Genome Res* 2004 14:2020-2028.

Matsumoto M, Nishimura Y. Hydrogen production by fermentation using acetic acid and lactic acid. *J Biosci Bioeng* 2007, 103(3):236-241.

Mertens R, Liese A. Biotechnological applications of hydrogenases. *Current Opin Biotechn* 2004, 15:343–348.

Meyer J. [FeFe] hydrogenases and their evolution: a genomic perspective. *Cell Mol Life Sci* 2007, 64:1063-1084.

Mille Y, Beney L, Gervais P. Compared tolerance to osmotic stress in various microorganisms: Towards a survival prediction test. *Biotechnol Bioeng* 2005, 92(4):479-484.

Minnan L, Jinli H, Xiaobin W, Huijuan X, Jinzao C, Chuannan L, Fengzhang Z, Liangshu X. Isolation and characterization of a high H_2-producing strain *Klebsiella oxytoca* HP1 from a hot spring. *Res Microbiol* 2005, 156:76-81.

Miyake J, Miyake M, Asada Y. Biotechnological hydrogen production: research for efficient light energy conversion. *J Biotechnol* 1999, 70:89-101.

Mizuno O, Ohara T, Shinya M, Noike T. Characteristics of hydrogen production from bean curd manufacturing waste by anaerobic microflora. *Water Sci Technol* 2000, 42:345-350.

Mohan SVY, Bhaskar YV, Krishna PM, Rao NC, Babu VL, Sarma PN Biohydrogen production from chemical wastewater as substrate by selectively enriched anaerobic mixed consortia: Influence of fermentation pH and substrate composition. *Int J Hydrogen Energy* 2007, 32:2286-2295.

Monis PT, Giglio S, Saint CP. Comparison of SYTO9 and SYBR Green I for real-time polymerase chain reaction and investigation of the effect of dye concentration on amplification and DNA melting curve analysis. *Anal Biochem* 2005, 340:24-34.

Mori K, Iriye R, Hirata M, Takamizawa K. Quantification of Bacillus species in a wastewater treatment system by the molecular analyses. *Biotechnol Bioproc Eng* 2004, 9(6):482-489.

Morrison TB, Weis JJ, Wittwer CT. Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. *BioTechniques* 1998, 24:954-962.

Moser DP, Onstott TC, Fredrickson JK, Brockman FJ, Balkwill DL, Drake GR, Pfiffner SM, White DC, Takai K, Pratt LM, Fong J, Lollar BS, Slater G, Phelps TJ, Spoelstra N, Deflaun M, Southam G, Welty AT, Baker BJ, Hoek J. Temporal shifts in the geochemistry and microbial community structure of an ultradeep mine borehole following isolation. Geomicrobial J 2003, 20 (6):517-548.

Mu Y, Zheng XJ, Yu HQ, Zhu RF. Biological hydrogen production by anaerobic sludge at various temperatures. *Int J Hydrogen Energy* 2006, 31: 780-785.

Mukamolova GV, Kaprelyants AS, Kell DB, Young M. Adoption of the transiently non-culturable state – a bacterial survival strategy? *Adv Microb Physiol* 2003, 47:65-129.

Muyzer G, Smalla K. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie van Leeuwenhoek* 1998, 73:127-141.

Nath K, Das D. Improvement of fermentative hydrogen production: various approaches. *Appl Microbiol Biotechnol* 2004, 65:520-529.

Neimark H, Kocan KM. The cell wall-less rickettsia *Eperythrozoon wenyonii* is a Mycoplasma. *FEMS Microbiol Lett* 1997, 156:287-291.

Nicolet Y, Lemon BJ, Fontecilla-Camps JC, Peters JW. A novel FeS cluster in Fe-only hydrogenases. *Trends Biochem Sci* 2000, 25(3):138-143.

Nielsen AT, Amandusson H, Bjorklund R, Dannetun H, Ejlertsson J, Ekedahl LG, Lundstrom I, Sevensson BH. Hydrogen production from organic waste. *Int J Hydrogen Energy* 2001, 26(6):547-550.

Ochman H, Gerber AS, Hartl DL. Genetic applications of an inverse polymerase chain-reaction. *Genetics* 1988, 120(3):621-623.

Ogino A, Koshikawa H, Nakahara T, Uchiyama H. Succession of microbial communities during a biostimulation process as evaluated by DGGE and clone library analyses. *J Appl Micobiol* 2001, 91(4):625-635.

Oh YK, Seol E, Lee EY, Park S. Fermentative hydrogen production by a new chemoheterotrophic bacterium *Rhodopseudomonas Palustris* P4. *Int J Hydrogen Energy* 2002, 27:1373-1379.

Oh YK, Park MS, Seol EH, Lee SJ, Park S. Isolation of hydrogen producing bacteria from granular sludge of an upflow anaerobic sludge blanket reactor. *Biotechnol Bioprocess Eng* 2003, 8:54-57.

Okabe S, Ito T, Satoh H. Sulfate-reducing bacterial community structure and their contribution to carbon mineralization in a wastewater biofilm growing under microaerophilic conditions. *Appl Microbiol Biot*2003, 63(3):322-334.

Oliver JD, Hite F, Mc Dougald D, Andon NL, Simpson LM. Entry into, and resuscitation from, the viable but nonculturable state by *Vibrio vulnificus* in an estuarine environment. *Appl Environ Microbiol* 1995, 61:2624-2630.

Oliver JD. The viable but nonculturable state in bacteria. *J Microbiol* 2005, 43:93-100.

Olson JW, Mehta NS, Maier RJ. Requirement of nickel metabolism proteins HypA and HypB for full activity of both hydrogenase and urease in *Helicobacter pylori*. *Mol Microbiol* 2001, 39(1):176–182.

Palmer S, Wiegand AP, Maldarelli F, Bazmi H, Mican JM, Polis M, Dewar RL, Planta A. New real-time reverse transcriptase-initiated PCR assay with single-copy sensitivity for human immunodeficiency virus type 1 RNA in plasma. *J Clin Microbiol* 2003, 41:4531-4536.

Palys T, Nakamura LK, Cohan FM. Discovery and classification of ecological diversity in the bacterial world: the role of DNA sequence data. *Int J Syst Bacteriol* 1997, 47: 1145-1156.

Papageorgiou J, Bartholomew HC, Doherty WWS. HPAE-PAD: A repaid and precise method for sugar analysis. *Proceedings of Australian Society of Sugar Cane Technologists* 1997:379-385.

Piggot PJ, Hilbert DW. Sporulation of *Bacillus subtilis*. *Curr Opin Microbiol* 2004, 7(6):579–586.

Pilak O, Mamat B, Vogt S, Hagemeier CH, Thauer RK, Shima S, Vonrhein C, Warkentin E, Ermler U. The crystal structure of the apoenzyme of the iron-sulphur cluster-free hydrogenase. *J Mol Biol* 2006, 358(3):798-809.

Podestá JJ, Navarro AMG, Estrella CN, Estesom MA. Electrochemical measurements of trace concentrations of biological hydrogen produced by *Enterobacteriaceae*. *Inst Pasteur* 1997 148:87-93.

Posewitz MC, King PW, Smolinski SL, Zhang L, Seibert M, Ghirardi ML. Discovery of two novel radical S-adenosylmethionine proteins required for the assembly of an active [Fe] hydrogenase. *J Biol Chem* 2004, 279(24):25711-25720.

Ranjard L, Poly F, Nazaret S. Monitoring complex bacterial communities using culture-independent molecular techniques: application to soil environment. *Res Microbiol* 2000, 151(3):167-177.

Rattray R, Craig A. Molecular characterization of sheep ruminal enrichments that detoxify pyrrolizidine alkaloids by denaturing gradient gel electrophoresis and cloning. *Microb Ecol* 2007, 54(2):264-275.

Ren N, Li J, Li B, Wang Y, Liu S. Biohydrogen production from molasses by anaerobic fermentation with a pilot-scale bioreactor system. *Int J Hydrogen Energy* 2006, 31:2147-2157.

Robinson BS, Monis PT, Dobson PJ. Rapid, Sensitive, and Discriminating Identification of *Naegleria* spp. by Real-Time PCR and Melting-Curve Analysis. *Appl Environ Microbiol* 2006, 72(9):5857–5863.

Robson R. Biodiversity of hydrogenases. In: Hydrogen as a Fuel. Learning from Nature (Cammack R, Frey M, Robson R, eds) 2001:9-32.

Rombel IT, Sykes KF, Rayner S, Johnston SA. ORF-FINDER: a vector for high-throughput gene identification. *Gene* 2002, 282(1-2): 33-41.

Rowan NJ. Viable but non-culturable forms of food and waterborne bacteria: Quo Vadis? *Trends Food Sci Technol* 2004, 15(9):462-467.

Rosyid OA. Jablonski D, Hauptmanns U. Risk analysis for the infrastructure of a hydrogen economy. *Int J Hydrogen Engergy* 2007, 32:3194-3200.

Rychlik W, Spencer WJ, Rhoads RE. Optimization of the annealing temperature for DNA amplification *in vitro*. *Nucleic Acids Res* 1990, 18(21):6409-6412.

Saint-Amans S, Girbal L, Andrade J, Ahrens K, Soucaille P. Regulation of carbon and electron flow in *Clostridium butyricum* VPI 3266 grown on glucose-glycerol mixtures. *J Bacteriol* 2001,138(5):1748-1754.

Sano A, Yasuda K, Kato Y, Bando Y, Nakamura M. Hydrogen fermentation by using heat-shocked granular sludge. *J Chem Eng Jpn* 2006, 39(5): 580-582.

Schramm A, Larsen LH, Revsbech NP, Ramsing NB, Amann R and Schleifer KH. Structure and function of a nitrifying biofilm as determined by in situ hybridization and the use of microelectrodes. *Appl Environ Microbiol* 1996, 62(12): 4641-4647.

Schuppler M, Mertens F, Schon G, Gobel UB. Molecular characterization of nocardioform actinomycetes in activated sludge by 16S rRNA analysis. *Microbiology* 1995, 141: 513-521.

Schütz K, Happe T, Troshina O, Lindblad P, Leitão E, Oliveira P, Tamagnini P. Cyanobacterial H_2 production -a comparative analysis. *Planta* 2004, 218: 350-359.

Shen L, Guo Y, Chen X, Ahmed S, Issa JPJ. Optimizing annealing temperature overcomes bias in bisulfite PCR methylation analysis. *Biotechniques* 2007, 42(1): 48-58.

Shin HS, Youn JH, Kim SH. Hydrogen production from food waste in anaerobic mesophilic and thermophilic acidogenesis. *Int J Hydrogen Energy* 2004, 29(13):1355-1363.

Shine J, Dalgarno L. Determinant of cistron specificity in bacterial ribosomes. *Nature* 1975, 254 (5495): 34-38.

Signoretto C, Lleò MM, Tafi MC, Canepari P. Cell wall chemical composition of *Enterococcus faecalis* in the viable but nonculturable state. *Appl Environ Microbiol* 2000, 66:1953-1959.

Stephenson M, Stickland LH. Hydrogenase: a bacterial enzyme activating molecular hydrogen: The properties of the enzyme. *Biochem J* 1931, 25(1):205-214.

Suzuki MT, Giovannoni SJ. Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. *Appl Environ Microb* 1996, 62:625-630.

Tanisho S, Ishiwata Y. Continuous hydrogen production from molasses by the bacterium *Enterobacter aerogenes*. *Int J Hydrogen Energy* 1994, 19:807-812.

Tanisho S, Kuromoto M, Kadokura N. Effect of CO_2 removal on hydrogen production by fermentation. I*nt J Hydrogen Energy* 1998, 23:559-63.

Tichopad A, Dilger M, Schwarz G, Pfaffl MW. Standardized determination of real-time PCR efficiency from a single reaction set-up. *Nucleic Acids Res* 2003, 31:e122.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 1997, 25: 4876-4882.

Toyota K, Kuninaga S. Comparison of soil microbial community between soils amended with or without farmyard manure. *Appl Soil Ecol* 2006, 33(1):39-48.

Tye JW, Darensbourg MY, Hall MB. *De novo* design of synthetic di-iron(I) complexes as structural models of the reduced form of iron-iron hydrogenase. *Inorg Chem* 2006, 45(4):1552-1559.

United States Energy Information Administration. International energy outlook 2007. U.S. Department of Energy, 2007.

van der Gucht K, Vandekerckhove T, Vloemans N, Cousin S, Muylaert K, Sabbe K, Gillis M, Declerk S, De Meester L, Vyverman W. Characterization of bacterial communities in four freshwater lakes differing in nutrient load and food web structure. *Fems Microbiol Ecol* 2005, 53(2):205-220.

van Ginkel S, Sung S, Lay JJ, Biohydrogen production as a function of pH and substrate concentration. *Environ Sci Technol* 2001, 35(24): 4726-4730.

van Ginkel SW, Oh SE, Logan BE. Biohydrogen gas production from food processing and domestic wastewaters. *Int J Hydrogen Energy* 2005, 30(15):1535-1542.

Vandesompele J, De Paepe A, Speleman F. Elimination of primer-dimer artifacts and genomic coamplification using a two-step SYBR green I real-time RT-PCR. *Anal Biochem* 2002, 303(1):95-98.

Vallaeys T, Topp E, Muyzer G, Macheret V, Laguerre G, Rigaud A, Soulas G. Evaluation of denaturing gradient gel electrophoresis in the detection of 16S rDNA sequences variation in rhizobia and methanotrophs. *FEMS Microbiol Ecol* 1997, 24(3):279-285.

Vasconcelos I, Girbal L, Soucaille P. Regulation of carbon and electron flow in *Clostridium acetobutylicum* grown in chemostat culture at neutral pH on mixtures of glucose and glycerol. *J Bacteriol* 1994, 176(5):1443-1450.

Vignais PM, Billoud B, Meyer J. Classification and phylogeny of hydrogenases. *FEMS Microbiol Rev* 2001, 54: 455-501.

von Wintzingerode F, Göbel UB, Stackebrandt E. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. *FEMS Microbiol Rev* 1997, 21(3):213-229.

Wang CC, Chang CW, Chu CP, Lee DJ, Chang BV, Liao CS. Producing hydrogen from wastewater sludge by *Clostridum bifermentans. J Biotechnol* 2003a, 102(1):83-92.

Wang CC, Chang CW, Chu CP, Lee DJ, Chang BV, Liao CS, Tay JH. Using filtrate of waste biosolids to effectively produce bio-hydrogen by anaerobic fermentation. *Water Res* 2003b, 37(11):2789-2793.

Wang CC, Cheng CW, Chu CP, Lee DJ, Chang BV, Liao CS. Efficient production of hydrogen from wastewater sludge. *J Chem Technol Biotechnol* 2004, 79:426-427.

Wang L, Gaigalas AK, Blasic J, Holden MJ, Gallagher DT, Pires R. Fluorescence resonance energy transfer between donor-acceptor pair on two oligonucleotides hybridized adjacently to DNA template. *Biopolymers* 2003, 72(6):401-412.

Wang MY, Olson BH, Chang JS. Improving PCR and qPCR detection of hydrogenase A (*hydA*) associated with Clostridia in pure cultures and environmental sludges using bovine serum albumin. *Appl Microbiol Biotechnol* 2007a, 77(3):645-656.

Wang T, Brown MJ. mRNA quantification by real time TaqMan polymerase chain reaction: validation and comparison with RNase protection. *Anal Biochem* 1999, 269:198-201.

Wang X, Hoefel D, Saint CP, Monis PT, Jin B. The isolation and microbial community analysis of hydrogen producing bacteria from activated sludge. *J Appl Microbiol* 2007b, 133:1415-1423.

Weare NM, Benemann JR. Nitrogenase activity and photosynthesis by *Plectonema boryanum* 594. *J Bacteriol* 1974, 119:258-268.

Winter CJ. Into the hydrogen energy economy—milestones. *Int J Hydrogen Energy* 2005, 30(7):681-685.

Woese CR. Bacterial evolution. *Microbiol Rev* 1987, 51(2):221-271.

Wong ML, Medrano JF. Real-time PCR for mRNA quantitation. *Biotechniques* 2005, 39(1): 75-85.

Wong MT, Tan FM, Ng WJ, Liu WT. Identification and occurrence of tetrad-forming *Alphaproteobacteria* in anaerobic-aerobic activated sludge processes. *Microbiology* 2004, 150:3741-3748.

Wu JH, Lin CY. Biohydrogen Production using food wastewater mesophilic fermentation. *Water Sci Technol* 2004, 49:223-238.

Wu LF, Mandrand MA. Microbial hydrogenases: primary structure, classification, signatures and phylogeny. *FEMS Microbiol Rev* 1993, 10(3-4): 243-270.

Yang P, Zhang R, McGarveyc JA, Benemann J. Biohydrogen production from cheese processing wastewater by anaerobic fermentation using mixed microbial communities. *Int J Hydrogen Energy* 2007, 32(18):4761-4771.

Yi S, Tay JH, Maszenan AM, Tay STL, Sun DD, Englande AJ, Tay JH. A culture-independent approach for studying microbial diversity in aerobic granules. *Water Sci Technol* 2003, 47(1):283-290.

Yokoi H, Tokushige T, Hirose J, Hayashi S, Takasaki Y. H_2 production from starch by mixed culture of *Clostridium butyricum* and *Enterobacter aerogenes*. *Biotechnol Lett* 1998, 20(2):143-147.

Yokoi H, Saitsu AS, Uchida H, Hirose J, Hayashi S, Takasaki Y. Microbial hydrogen production from sweet potato starch residue. *J Biosci Bioeng* 2001, 91(1):58-63.

You SJ, Ouyang CF, Lin SF, Liu WT. Characterization of the microbial diversity in a biotreatment process using non-culture based methods. *Water Sci Technol* 2000, 42 (3-4):143-148.

Zhang H, Bruns MA, Logana BE. Biological hydrogen production by *Clostridium acetobutylicum* in an unsaturated flow reactor. *Water Res* 2006a, 40:728-734.

Zhang T, Liu H, Fang HHP. Biohydrogen production from starch in wastewater under thermophilic conditions. *J Environ Manag* 2003, 69:149-156.

Zhang HS, Bruns MA, Logan BE. Biological hydrogen production by *Clostridium acetobutylicum* in an unsaturated flow reactor. *Water Res* 2006b, 40:728-734.

Zhang X, Yan X, Gao P, Wang L, Zhou Z, Zhao L. Optimized sequence retrieval from single bands of temperature gradient gel electrophoresis profiles of the amplified 16S rDNA fragments from an activated sludge system. *J Microbiol Meth* 2005a, 60:1-11.

Zhang Y, Liu G, Shen J. Hydrogen production in batch culture of mixed bacteria with sucrose under different iron concentrations. *Int J Hydrogen Energy* 2005b, 30: 855-860.

Zhang ZP, Tay JH, Show KY, Yan R, Liang DT, Lee DJ, Jiang WJ. Biohydrogen production in a granular activated carbon anaerobic fluidized bed reactor. *Int J Hydrogen Energy* 2007c, 32:185-191.

Zhang ZY, Jin B, Kelly M. Production of lactic acid and byproducts from waste potato starch by *Rhizopus arrhizus*: role of nitrogen sources. *World J Microb Biot* 2007d, 23:229-236.

Zumft WG. Cell biology and molecular basis of denitri-fication. *Microbiol Mol Biol Rev* 1997, 61(4): 533-616.

Appendix

X Wang, D Hoefel, CP Saint, PT Monis, B Jin. Isolation and Microbial Community Analysis of Hydrogen Producing Bacteria from Activated Sludge. *Journal of Applied Microbiology* 2007, 103(5)7: 1415-1423.