MONASH University

Highly efficient biohydrogen production by diverse microbial community in landfill leachate sludge using dark fermentative batch reactor

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This thesis includes five original papers published in peer reviewed journals and three unpublished publications. The core theme of the thesis is Highly Efficient Bio-hydrogen Production Revealed in a Diverse Microbial Community from Landfill Leachate Sludge using Dark Fermentative Batch Reactor. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the School of Science under the supervision of Assoc. Pro. Dr. Juan Joon Ching.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of Chapter 2, 3, 4, 5, 6 and 6a my contribution to the work involved the following:

Thesis chapter	Publication title	Publication status	Nature and extent of candidate's contribution
2	A review of sustainable hydrogen production using seed sludge via dark fermentation doi.org/10.1016/j.rser.2014.03.008	Published	I was responsible for 95% of drafting and writing.
3	High efficiency bio-hydrogen production from glucose revealed in an inoculum of heat-pretreated landfill leachate sludge doi: 10.1016/j.energy.2014.05.088	Published	I was responsible for 100% of analysis and interpretation of data and 95% of drafting and writing.
4	Comparison of microbial communities at different fermentation phase of hydrogen production using Illumina MiSeq	Submitted	I was responsible for 100% of analysis and interpretation of data and 90% of drafting and writing.
5	Production of bio-hydrogen from dairy wastewater by using pretreated landfill leachate sludge as an inoculum	Submitted	I was responsible for 100% of analysis and interpretation of data and 95% of drafting and writing.
6	Discovery of three new Clostridium strains provided a new insight for biohydrogen production	Submitted	I was responsible for 100% of analysis and interpretation of data and 95% of drafting and writing.
6 a	Draft Genome Sequence of Clostridium perfringens Strain JJC, a Highly Efficient Hydrogen Producer Isolated from Landfill Leachate Sludge doi:10.1128/genomeA.00064-14	Published	I was responsible for 100% of analysis and interpretation of data and 90% of drafting and writing.
	Draft Genome Sequence of Clostridium bifermentans Strain WYM, a Promising Biohydrogen Producer Isolated from Landfill Leachate Sludge doi:10.1128/genomeA.00077-14	Published	I was responsible for 100% of analysis and interpretation of data and 90% of drafting and writing.
	Draft Genome Sequence of Clostridium sp. Strain Ade.TY, a New Biohydrogen- and Biochemical-Producing Bacterium Isolated from Landfill Leachate Sludge doi:10.1128/genomeA.00078-14	Published	I was responsible for 100% of analysis and interpretation of data and 90% of drafting and writing.

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis

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Abstract

This study investigated the microbiology in landfill leachate sludge for biological H_2 production. This sludge originated from sanitary landfill, hence the microbial community which survived in the sludge may have unique H_2-producing features. The landfill leachate sludge was pretreated at different temperatures and landfill leachate sludge pretreated at 65 ºC revealed the maximum H_2 yield of 6.43 ± 0.16 mol H_2/mol glucose under the optimum conditions of 37ºC and pH 6. This new record of high H_2 has exceeded the conventional theoretical yield of 4 mol H_2/ mol glucose. Under the same optimum condition (37ºC and pH 6), the H_2 produced from dairy wastewater was 113.2 ± 2.9 mmol H_2/g COD (12.8 ± 0.3 mmol H_2/g carb.). The H_2 productions from glucose and wastewater were thermodynamically favourable with the Gibb's free energy of –34 and –40 kJ/mol, respectively. The microbial community was successfully revealed by 16S-rRNA Metagenomics using Illumin Miseq. Sludge pretreated at 65 ºC was revealed to contain 98% of H_2-producing bacteria which mainly belong to the genera *Clostridium*, *Bacillus*, *Eubacterium* and *Sporacetigenium*. In comparison, untreated sludge which contained mainly H_2-consuming bacteria including genera *Pseudomonas*, *Sulfurimonas* and *Treponema*. The sludge pretreatment has successfully eliminated H_2-consuming bacteria and enriched H_2-producing bacteria which in turn improved H_2 yield up to 53%. Three H_2-producing bacteria were successfully isolated and identified as *Clostridium perfringens* strain JJC (AWRZ00000000), *C. bifermentans* strain WYM (AVSU00000000) and *Clostridium* sp. strain Ade.TY (AVSV00000000). Based on multiple genome alignment, *Clostridium* sp. strain Ade.TY is likely to be a new H_2-producing species as it does not aligned to the genome of its closest neighbour. The batch mode optimization showed that the maximum H_2 production attained from these isolates are in the descending order of *C. perfringens* strain JJC > *C. bifermentans* strain WYM > *Clostridium* sp. strain Ade.TY with yield of 4.68 ± 0.12, 3.29 ± 0.11, 2.87 ± 0.10 mol H_2/ mol glucose, respectively. This shows that *C. perfringens* strain JJC could convert substrates to H_2 more efficiently as compared to the others. Nonetheless, it is important to note that the H_2 yield from single H_2-producing bacterium was less efficient as compared to the performance of H_2 production using landfill leachate sludge as inoculum. In summary, landfill leachate sludge contains functional microbial community for efficient H_2 production with good potential in industrial application.

Abbreviations

Bio-H_2	Biological hydrogen
HPB	Hydrogen producing bacteria
HCB	Hydrogen consuming bacteria
POME	Palm oil mill effluent
UASB	Up flow anaerobic sludge blanket
TVS	Total volatile solid
VS	Volatile solid
BES	2-bromoethanesulfonate
MSW	Municipal solid waste
VFA	Volatile fatty acid
Glu.	Glucose
Carb.	Carbohydrate
WGS	Whole genome sequencing
NGS	Next generation sequencing

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Chapter 1
Introduction

1.0. Impending of fossil fuel depletion drives the development of alternative fuels

Fossil fuels is the leading energy source (1). Our current life style relies exclusively on fossil fuels to generate energy for electricity, transportation and industry. Considering the rapid growth of world population, energy experts predicted the end of fossil fuel reserves arrives in the year of 2042 based on modified Donald Klass model (2). Furthermore, extensive use of fossil fuels caused irreversible global environmental damages including global warming and pollution. Therefore, an environmentally friendly and sustainable replacement is required. As refer to Figure 1.1, it is predicted that the world's energy usage will progressively move towards gas fuels such as hydrogen gas (3). Hydrogen is the most promising replacement for fossil fuel as it is a clean and sustainable fuel.

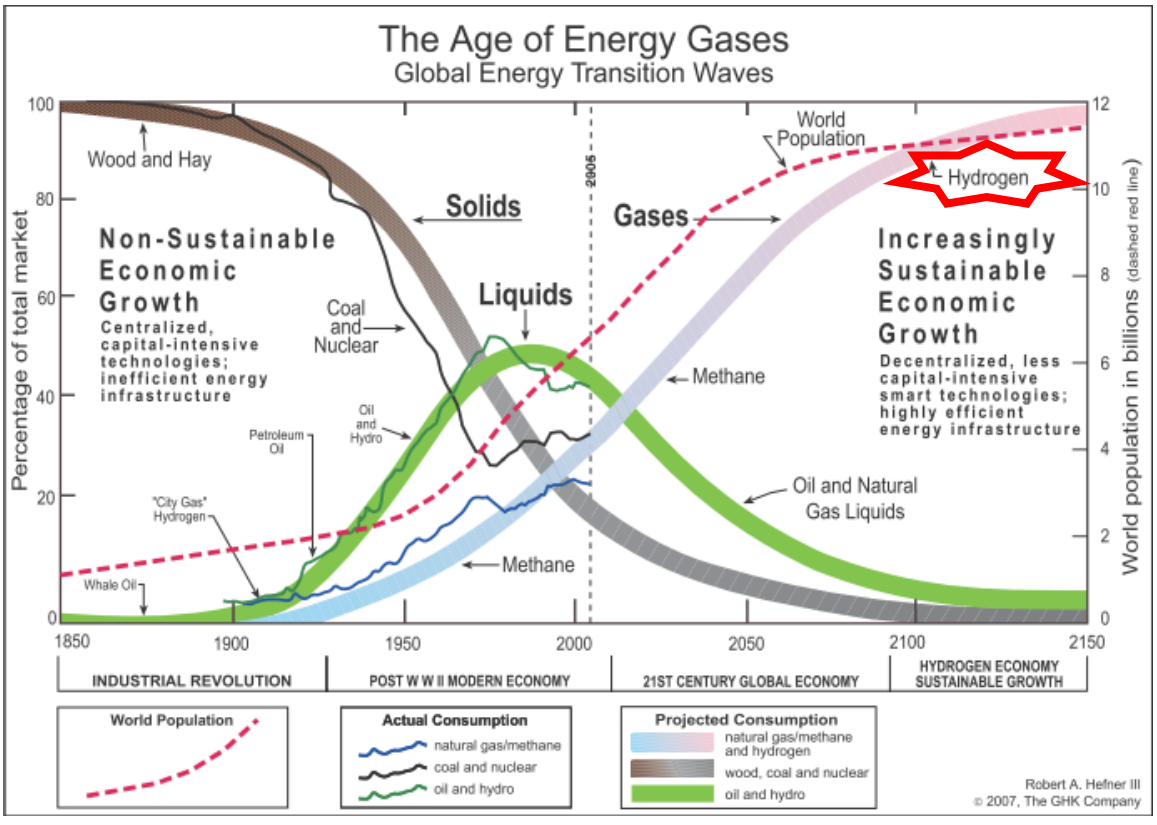

Figure 1.1: Concept of The Age of Energy Gases suggests energy transition towards H_2 as an ultimate energy carrier (Hefner, 2007)

1.1. Hydrogen as alternative fuel

Hydrogen (H_2) is appeared as an odourless, colourless, tasteless and non-toxic gas (4). The value of H_2 is enhances with its non-polluting nature, as the complete combustion of H_2 produces water as the only end product. Hydrogen also has the highest energy yield (141.9 J/kg) as compared to other fuel types (Table 1.1). However, H_2 does not usually exist as gas but primarily found in combination with other elements such as carbon and oxygen, forming a variety of compounds including water and hydrocarbons (5). Therefore, H_2 is an energy carrier that has to be extracted from other compounds. Currently, more than 96% of the global H_2 production requires fossil fuels as raw material or as source of energy (6, 7). Considering environmental problems arise from fossil fuels, H_2 derived from renewable sources is more environmentally friendly.

Table 1.1: Assessment of energy value and specific carbon emission different fuel types (8)

Fuel type	Energy per unit mass (J/kg)	Specific carbon emission (kg C/kg fuel)
Liquid hydrogen	141.90	0.00
Gaseous hydrogen	141.90	0.00
Natural gas	50.00	0.46
Liquefied natural gas (LNG)	50.00	-
Liquefied petroleum gas (LPG)	48.80	-
Gasoline	47.40	0.86
Fuel oil	45.50	0.84
Biodiesel	37.00	0.50
Charcoal	30.00	0.50
Ethanol	29.90	0.50
Methanol	22.30	0.50

1.2. Methods of producing bio-hydrogen

There are two main approaches for H_2 production which are categorized under chemical and biological methods. Chemical process is heavily relying on natural gas, heavy oils, coal and naphtha as primary feedstock (9). In addition, processes like steam reforming and partial oxidation require high temperature (up to 1500 ˚C) and pressure (30 atm). Consequently, chemical processes are usually energy intensive and expensive (10, 11). In contrast, biological hydrogen (bio- H_2) production offers an environmentally friendly alternative for H_2 production. In nature, a variety of H_2-producing bacteria including photosynthetic, photoheterotrophic and fermentative bacteria were found to be capable of producing H_2 (12). Among the biological processes, dark fermentation has attracted much

attention. Dark fermentation produces H_2 in the absence of light (13). Hence, it does not require solar input and the configuration of the bioreactor is simpler and cheaper (14). Dark fermentation can use a versatile range of substrate, particularly renewable resources that are organically rich such as stillage, sludge, leachate, pomace, stalks and bagasse (15-20). Therefore, H_2 production via dark fermentation is potentially to be integrated into waste management and to achieve the ultimate goal of converting waste into energy.

Currently, bio-H_2 production via fermentation methods are still at the research and development (R&D) stage. As reported by the U.S. Department of Energy (25), bio-H_2 production approach is clean, self-sustaining and tolerant of diverse water conditions. However, several critical challenges are precluding the application of this method in industries. The R&D needs are (1) to develop efficient microorganisms for sustainable bio-H_2 production; (2) to identify and characterise new microorganisms; (3) to develop inexpensive methods to grow and maintain microorganisms; (4) to develop cheap and durable bioreactors' materials; and (5) to design cheap and high-value manufacturing processes.

Table 1.2: Comparison between advantages and disadvantages of different methods producing hydrogen

Methods	**Advantages**	**Disadvantages**	**Ref.**
Steam reforming	✓ Cheaper than biological methods ✓ Most established commercial process	χ Heavily dependent on non-renewable feedstock χ Requires high temperature χ Expensive & Energy intensive	(10, 11)
Water electrolysis	✓ Capable of producing highly pure H_2	χ Expensive χ High electricity consumption	(10, 11)
Biophotolysis	✓ Capable of producing H_2 from water	χ Low conversion efficiency χ Requires solar energy χ Large operation space χ Expensive and complicated photo-bioreactor	(12-14)
Photofermentation	✓ High conversion efficiencies ✓ Ability to utilize broad spectrum of light ✓ Utilize organic substrate from waste	χ Expensive photo-bioreactor χ Presence of H_2 consumption pathway χ Lack of transparency in fermented broth which limits absorption of light energy	(12-14)
Dark fermentation	✓ Less expensive & energy intensive ✓ Does not require solar energy ✓ Technically simple ✓ Utilize organic renewable feedstock ✓ Production organic acids and solvents with commercial interest	χ Relatively lower achievable H_2 yield as compared to photofermentation χ Metabolic shift to solventogenesis pathway when pH is less than 5.0	(12-14)

1.3. Applications and economical value of hydrogen

Hydrogen can replace fossil fuel and reduce the over reliance as the primary energy. Generally, H_2 fuel can be used in conventional gasoline engines with modifications in order to generate energy via combustion in the air (21, 22). Alternatively, H_2 can be used in fuel cells to generate electricity. Hydrogen fuel cell is applicable in powering vehicles, electricity infrastructures and electrical utilities (3, 23). According to the U.S. energy Information Administration (24), The United State of America produces 10 – 11 million metric tons of H_2 every year which is sufficient to power 20 – 30 million cars or 5 – 8 million households. On another hand, automobile running with the H_2 fuel cell has better fuel efficiency as compared to diesel engines. Figure 1.2 shows the second generation of Mercedes-Benz B-class running with a H_2 fuel cell. Comparing between the fuel efficiency of Mercedes-Benz B-class running with a H_2 fuel cell and a diesel engine, the model with a H_2 fuel cell has the better fuel efficiency with the fuel consumption of 0.97 kg H_2 / 100 km (3.3 L diesel / 100 km equivalent) as compared to diesel engine with the fuel consumption of 5.4 – 5.6 L diesel / 100 km (25, 26). Moreover, the model with a H_2 fuel cell can achieve zero emission with 0.0 g / km carbon dioxide as compare to the model with a diesel engine which has the emission of 114 – 121 g / km carbon dioxide, according to the model specifications (26). The features of high energy yield and non-polluting nature enhance the value of H_2 as the replacement of fossil fuels.

Figure 1.2: The Mercedes-Benz B-class runs with the H_2 fuel cell developed by Daimler AG. (Image taken from the exhibition in the World Hydrogen Technology Conference (WHTC) 2013, Shanghai)

Apart from alternative fuel, H_2 is also raw materials in many industries such as the manufacture of ammonia and methanol. In addition, H_2 is also important for other industrial applications such as fertilizers, vitamins, semi-conductor circuits, toothpaste, glass, food processing, refined metals lubricants and detergents. Other processes used H_2 in desulfurization and denitrogenation of coal; hydrocracking of crude oil into gasoline or liquefied petroleum gas; as a fuel in rocket engines and coolant in electrical generator (13, 27, 28). In general, H_2 is a versatile element that is applicable is a wide range of industries.

1.4. Problem statement

As discussed, dark fermentation offers an opportunity of producing H_2 from organic rich waste. However, dark fermentation is less efficient in terms of converting substrates to H_2 because most thermal enthalpies are lost in the formation of volatile fatty acids (VFA). In theory, 1 mole of glucose ($C_6H_{12}O_6$) should produce 12 moles of H_2 (eq. 1), while 1 mole of lactose ($C_{12}H_{22}O_{11}$) will produce 23 moles of H_2 (eq. 2).

$$C_6H_{12}O_6 + 6\ H_2O \rightarrow \mathbf{12\ H_2} + 6\ CO_2 \qquad \text{(eq. 1)}$$

$$C_{12}H_{22}O_{11} + 12\ H_2O \rightarrow \mathbf{23\ H_2} + 12\ CO_2 \qquad \text{(eq. 2)}$$

Typically, the maximum energy conversion from glucose to H_2 is only 33% via the acetate pathway (eq. 3) and 17% via the butyrate pathway (eq. 4). Meanwhile the efficiency of lactose conversion to H_2 is only 31% via the acetate and methane pathways (eq. 5 and 6) (18, 29, 30).

Glucose fermentation

Acetate pathway : $C_6H_{12}O_6 + 2H_2O \rightarrow \mathbf{4H_2} + 2CH_3COOH + 2CO_2$ (eq. 3)

Butyrate Pathway : $C_6H_{12}O_6 \rightarrow \mathbf{2H_2} + CH_3CH_2CH_2COOH + 2CO_2$ (eq. 4)

Lactose fermentation

Acetate pathway : $C_{12}H_{22}O_{11} + 5H_2O \rightarrow \mathbf{8H_2} + 4CO_2 + 4CH_3COOH$ (eq. 5)

Methane pathway : $C_{12}H_{22}O_{11} + 5H_2O \rightarrow \mathbf{8H_2} + 8CO_2 + 4CH_4$ (eq. 6)

Currently, the highest reported H_2 yield is only 2.3 mol H_2/mol glucose which is only about 50% of the theoretical maximum H_2 yield (31). This phenomenon is caused by (1) the rapid conversion of substrate into cell biomass instead of H_2 (32-35) and (2) an inappropriate combination of fermentation conditions. It is postulated that mixed microflora in sludge could

overcome this problem via the synergetic interaction among the different bacteria because they can adapt to a wider range of conditions.

Accessibility of H_2-producing bacteria to substrates directly influences the performance of H_2 production. Simple substrates such as glucose and lactose are easily accessible for H_2 production. In contrast, complex substrates such as lignocellulose and cellulose have to be broken down into simpler substrate like glucose to improve accessibility. Therefore it is logic that combinations of microorganisms which could utilize different substrates for hydrogen production are essential for a better H_2 production performance. The community of H_2-producing bacteria is naturally found in soil, sediment and sludge. There were many reports on H_2 production using various sludge as inoculum such as sludge from brewery industry, oil palm mill, sewage, paper mill and cattle farm (29, 36-43). However, most of the reports encountered inefficiency in H_2 yield from different types of sludge. As mentioned, the highest reported H_2 yield is less satisfactory because it only represents 23% of H_2 production from a complete conversion of glucose. Therefore, there is a need in developing a better sludge inoculum to produce H_2 more efficiently.

1.5. Objectives

Landfill leachate sludge is expected to contain diverse microflora which might possess good H_2-producing property that has not been discovered. Hence, it is hypothesized that landfill leachate sludge is a new inoculum that contains H_2-producing microflora that produce H_2 efficiently. To answer the research question, the research approach are:

(1) To investigate H_2 production performance of landfill leachate sludge in batch fermentation using glucose and dairy wastewater;
(2) To analyze microbial community in the landfill leachate sludge with 16S rDNA-metagenomic using Illumina MiSeq;
(3) To isolate and identify H_2-producing bacteria from landfill leachate sludge; and
(4) To investigate H_2 production performance of the isolated bacteria in batch fermentation using glucose and wastewater.

Chapter 2
Literature Review

The review presented in this chapter has been partly submitted for peer review:

Wong YM, Wu TY, Juan JC. A review of sustainable hydrogen production using seed sludge via dark fermentation. Renewable and Sustainable Energy Reviews. 2014; 34(0):471-82.

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Declaration for Thesis Chapter 2

Declaration by candidate

In the case of Chapter 2, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Drafting and writing the publication	95

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
A/P Dr. Juan Joon Ching	Reviewed the publication	N.A.
Dr. Wu Ta Yeong	Reviewed the publication	N.A.

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	Date 9 Sept 2014
Main Supervisor's Signature	[redacted]	Date 9 Sept 2014

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

2.0. Introduction

Hydrogen gas (H_2) is an alternative fuel to reduce the over reliance on fossil fuels as the primary energy used in vehicles and machines. Generally, H_2 fuel can be used in conventional gasoline engines with modifications in order to generate energy via combustion in the air (21, 22). The combustion of H_2 is sustainable and environmentally friendly because it does not generate greenhouse gases such as carbon dioxide and methane (1, 44). Hydrogen also possesses high energy yield (141.9 J/kg) among the known fuel types such as methane (55.7 J/kg), natural gas (50 J/kg), biodiesel (37 J/kg) and ethanol (29.9 J/kg) (8). However, more than 96% of global H_2 is generated from fossil fuels (6, 7). Therefore, there is an urgency to develop a more cost-effective and environmentally friendly technology to for H_2 production.

Dark fermentation is a biological approach commonly used to produce H_2 in the absence of light (13). This process does not require solar input and hence the configuration of the bioreactor is simpler and cheaper (14). Most importantly, this technology has attracted attention because it can use a versatile range of substrate, particularly renewable resources that are organically rich such as stillage, sludge, leachate, pomace, stalks and bagasse (45-47). Due to cost and environmental concerns, organic waste material is a better choice of substrates than pure compounds such as sugar or starch. This technology allows dark fermentation to be integrated into wastewater treatment systems to produce H_2 and to treat wastewater.

Seed sludge contains diverse microflora that can produce H_2 via dark fermentation (48-51). Microorganisms found in the seed sludge are more beneficial than pure cultures because they are more adaptive to environmental stresses including limited substrates, and changes in pH and temperature. Moreover, the diverse microflora present in the seed sludge might provide synergistic interactions that improve substrate degradation and thus enhance H_2 production. Unfortunately, microflora in the seed sludge usually consists of both H_2-consuming and H_2-producing bacteria (Table 2.1). Therefore, it is essential to eliminate the activity of H_2-consuming bacteria (HCB) in order to increase H_2 production from H_2-producing bacteria (HPB). To achieve this, seed sludge can be pretreated using various physical and chemical pretreatment methods to enrich HPB. However, the search for the most effective pretreatment method for this purpose is still under intensive research.

Table 2. 1: Example of H_2 producing and consuming bacteria with their characteristics

Organisms	Functions	Characteristics	Ref.
Clostridium spp.	H_2 production	Obligate and mesophilic anaerobes The most popular H_2 producer Ferment a wide range of carbohydrates and produce H_2. E.g. *Clostridium butyricum, C. acetobutylicum, C. tyrobutyricum, C. saccharolyticum*	(31, 52-54)
Thermoanaerobacterium spp.	H_2 production	Obligate and thermophilic anaerobes E.g. *Thermoanaerobacterium thermosaccharolyticum*	(55)
Ethanoligenens spp.	H_2 production	Obligate anaerobes Produce solvent during H_2 production E.g. *Ethanoligenens harbinens*	(56)
Bacillus spp.	H_2 production	Facultative anaerobes May possess important features such as salt tolerance E.g. *Bacillus megaterium*	(57)
Enterobacter spp.	H_2 production	Facultative anaerobes Have better tolerance against oxidative stress E.g. *Enterobacter aerogenes*	(56)
Klebsiella spp.	H_2 production	Facultative anaerobes Have better tolerance against oxidative stress E.g. *Klebsiella pneumonia*	(58)
Methanogens	H_2 consumption	Obligate anaerobes Utilize H_2 for methane production E.g. *Methanobacterium* spp., *Methanococcus* spp. etc.	(59)
Other H_2 consuming bacteria	H_2 consumption	Obligate/ facultative anaerobes Utilize H_2 as electron donor and precursors for metabolic compounds E.g. *Lactobacillus* spp. and *Bifidobacterium* spp.	(60, 61)

Apart from the variety of HPB involved in dark fermentation, high H_2 yield is also associated with fermentation conditions including pH, temperature and types of substrate. These factors influence H_2 production by altering the physiological properties such as the enzymatic activities of HPB. In addition, H_2 production can be further enhanced by supplements or constrained by inhibitors. Theoretically, a maximum of 12 moles of H_2 is produced from 1 mole of glucose.

$$C_6H_{12}O_6 + 6\ H_2O \rightarrow 12\ H_2 + 6\ CO_2 \qquad \text{(eq. 1.1)}$$

However, currently the highest reported H_2 yield is only about 20% of this maximum yield. Therefore, in order to improve H_2 yield, it is important to recognize the major contributing factors in H_2 production.

This paper critically reviews the challenges of H_2 production using seed sludge as inoculum, focusing mainly on (1) the strengths and weaknesses of different pretreatment methods on the seed sludge; and (2) the effects of different factors including types of potential substrate, operation conditions, nutrients and inhibitors, and the diverse microflora in seed sludge.

2.1. Factors affecting hydrogen production by seed sludge

2.1.1. Effects of sludge pretreatment

In order to enhance H_2 production, pretreatment is commonly used to enrich HPB. Pretreatment must be able to selectively preserve HPB while eliminating HCB. Untreated seed sludge generally produces low H_2 yield (< 1.0 mol H_2/mol glucose) and pretreated seed sludge successfully improves H_2 yield (Appendix 1-1). This is verified by the hydrogenase (primary H_2-producing enzyme) activity in the pretreated seed sludge that has been reported to be three fold higher when compared to the untreated seed sludge (62). These indicate that the pretreatment successfully enriches HPB and increased H_2 yield.

Pretreatment methods are divided into physical and chemical pretreatments. Physical pretreatments are further separated into heat, ultrasonication, ultraviolet irradiation, aeration, and freeze and thaw methods, while chemical pretreatments include pH pretreatment, and chemical activation and inhibition. The selection of pretreatment methods is important because bacteria react differently to the stress applied. For example, it has been shown that acid pretreated seed sludge that was dominated by HCB, such as *Propionibacterium granulosum*, produced 10.4 fold less H_2 compared to heat treated seed sludge (56). This suggests that heat pretreatment is the more effective method to eliminate *P. granulosum*. It demonstrates that the type of pretreatment serves an important role in controlling H_2 yield as it directly affects the variety of bacteria that is present in the seed sludge.

Table 2. 2: Comparison of H_2 yield between heat pretreated and untreated sludge

	H_2 yield		Pretreatment		
	Heat pretreatment	**Untreated**	**condition**	**Sludge source**	**Ref.**
1.	2.38 mol H_2/mol glu.	N.A.	100˚C, 15 min	Sewage treatment plant	(63)
2.	2.30 mol H_2/mol glu.	0.43 mol H_2/mol glu.	65˚C, 30 min	Sewage treatment plant	(31)
3.	1.95 mol H_2/mol glu.	0.43 mol H_2/mol glu.	95˚C, 30 min	Sewage treatment plant	(31)
4.	1.04 mol H_2/mol glu.	0.70 mol H_2/mol glu.	70˚C, 30 min	Sewage treatment plant	(37)
5.	0.90 mol H_2/mol glu.	0.38 mol H_2/mol glu.	95˚C, 30 min	Sewage treatment plant	(64)
6.	[b] 0.40 mol H_2/mol glu.	[b] 0.20 mol H_2/mol glu.	100˚C, 30 min	Intertidal zone	(61)
7.	1.61 mol H_2/ mol hex.	0.3 mol H_2 / mol hex.	100˚C, 60 min	POME treatment plant	(55)
8.	[a] 0.0106 mol H_2/ g carb.	[a] 0.0191 mol H_2/ g carb.	70˚C, 30 min	Sewage treatment plant	(49)
9.	0.0000122 mol H_2/g COD	0.0018 mmol H_2/g COD	100˚C, 60 min	H_2 producing reactor	(65)
10.	0.00041 mol H_2/g COD	0.00012 mol H_2/g COD	100 ˚C, 60 min	POME treatment plant	(66)
11.	[a] 0.00233 mol H_2/g VS	[a] 0.0265 mol H_2/g VS	90˚C, 60 min	Anaerobic treatment plant	(67)
12.	[a] 0.0498 mol H_2	[a] 0.0341 mol H_2	100˚C, 90 min	Intertidal zone	(68)
13.	[a] 0.0488 mol H_2	[a] 0.0366 mmol H_2	80˚C, 20 min	Intertidal zone	(57)
14.	[a] 0.0325 mol H_2	[a] 0.0366 mmol H_2	100˚C, 20 min	Intertidal zone	(57)

[a] H_2 yield was estimated using PV = nRT at standard condition where P = 1 atm; R = 8.21 × 10^{-5} m^3.atm/mol.K and T = 300 K
[b] Estimated value

2.1.2. Physical pretreatments

A. Heat pretreatment

In physical pretreatment, heat pretreatment is the most commonly used method (Appendix 1-2). This is a simple method that eliminates HCB effectively and has a high potential for commercialization. A review of studies shows that the highest H_2 yield was produced by seed sludge pretreated at 65˚C which yielded 2.30 mol H_2/mol, that is 8.85 fold higher than the untreated sludge (Table 2.2, no. 2). Seed sludge pretreated at this temperature preserved the most types of HPB, and no HCB were detected (31). This indicates that heat pretreatment successfully eliminated HCB and improved H_2 production. On the other hand, heat pretreated seed sludge also significantly enhances the reduction of chemical oxygen demand (COD) in wastewater. It has been shown that COD of POME was reduced up to 89% and H_2 yield was increased 3.4 fold compared to that of untreated seed sludge (66). The reduction of COD in wastewater during H_2 production signifies the potential of applying heat pretreated sludge for wastewater treatment via dark fermentation.

It is challenging to identify the best combination of pretreatment temperature and heating duration in order to further improve H_2 yields (Figure 1). Among the reported combinations, seed sludge pretreated at 65˚C for 30 minutes and 100 ˚C for 15 minutes are the most promising combinations (Table 2.2, no. 1 & 2). This suggests that a lower pretreatment temperature may require a longer pretreatment time and vice versa in order to achieve similar H_2 yields. In contrast, preheating seed sludge at higher temperatures for longer durations has shown lethal effects on HPB which reduces H_2 yield (31, 57, 69). For example, in one study seed sludge pretreated at 70˚C for 30 min produced even lower H_2 than the untreated seed sludge (Table 2.2, no. 8). Similarly, H_2 produced by seed sludge pretreated at 95˚C was 4.53 fold lower than seed sludge pretreated at 65˚C (Table 2.2, no. 2 & 5). This variation between H_2 productions and pretreatment combinations could be due to the density of cells and the type of microorganisms present in the seed sludge (70, 71). It is reasonable to assume that seed sludge that contains higher cell density requires longer pretreatment durations to ensure all HCB are eliminated. However, the appropriate pretreatment combination is complicated due to the variety of bacteria present in the seed sludge. Depending on the source of the seed sludge, HCB such as Homoacetogens can survive under intensive heat, while HPB such as *Enterobacter* spp. is easily destroyed during heat pretreatment (64, 72). Therefore, the optimum heat pretreatment temperature and time are dependent on the types of HPB and HCB present in the seed sludge.

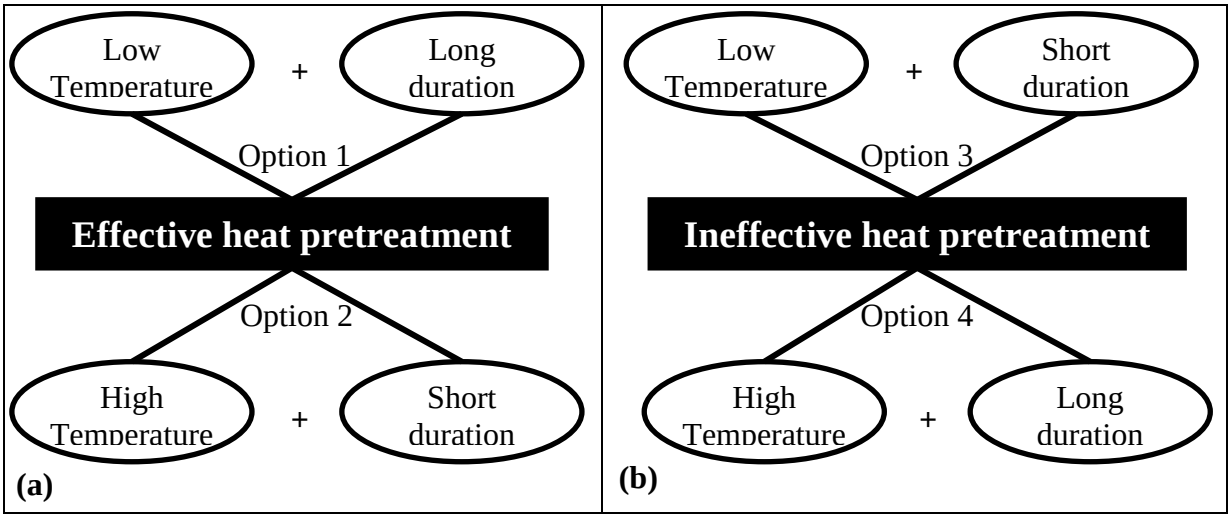

Figure 2.1: Relationship between pretreatment temperature and duration. (a) Effective heat pretreatment is resulted from the combination of low temperature with long duration (option 1) or high temperature with short duration (option 2); (b) ineffective heat pretreatment is resulted from the combination of low temperature with short duration (option 3) or high temperature with long duration (option 4).

B. Ultrasonication

Ultrasonication uses sound waves to eliminate HCB. A summary of the H_2 yield using this pretreatment is listed in Table 2.3. This method eliminates HCB by destroying their cell walls. Although the same damage may also occur to HPB, this can be prevented by controlling the pretreatment duration and intensity (73, 74). Ultrasonic pretreatment improves H_2 production because ultrasonic waves break the sludge particles into smaller sizes, disintegrate coenobium and increase the interaction between the HPB and the substrate (75, 76). Studies have shown the maximum yield obtained from this method was only 1.03 mol H_2/mol glucose. This yield was only about 8% of the maximum H_2 yield that can be produced from 1 mole of glucose (eq. 2.1). This shows that this method is less satisfactory than heat pretreatments. Therefore, optimization studies are required, including studies on the power of the ultrasonic waves, time of exposure, and heat control, before this pretreatment method can produce satisfactory results.

C. Ultraviolet irradiation

Ultraviolet irradiation has bactericidal action that can eliminate HCB and enrich HPB. The recommended pretreatment condition is 15 minutes of UV irradiation. This condition has successfully eliminated methanogens and increased H_2 yield 0.39 fold compared to untreated sludge (Table 2.3, no. 7). However, the radiation can only be transmitted through the smaller sludge particles that are lighter in color. Hence, HCB inside the larger and darker colored sludge particles are protected from UV irradiation and only HCB present on the surface are eliminated (77). This method is not as efficient as other physical treatments.

D. Aeration

Aeration pretreatment applies oxidative stress to deactivate anaerobic HCB. This pretreatment method is aimed at eliminating methanogens that are sensitive to oxidative stress. However, oxidative stress also damages obligate anaerobic HPB and is less effective against facultative HCB. The inefficiency of aeration pretreatment has been shown to result in low H_2 yield (0.7 mol H_2/mol glucose) that is far below the maximum H_2 yield (Table 2.3, no. 8). Hence, this may not be an effective method to enrich HPB in most of the seed sludge.

E. Freeze and thaw

This pretreatment method involves freezing and thawing seed sludge simultaneously at an extreme temperature. The freeze and thaw method appears to be the least effective physical pretreatment because seed sludge pretreated using this method has been shown to produce very low H_2 yield (0.15 mol H_2/mol glucose) compared to other pretreatment methods (Table 2.3, no. 11 & 12). Freezing and thawing seed sludge instantaneously lysed the bacteria, including both HCB and HPB, which in turn reduced H_2 yield. Therefore, the freeze and thaw method is not a favored method to enrich HPB due to its detrimental effects on HPB cells.

Table 2.3: H_2 production from sludge pretreated with ultrasonication, ultraviolet irradiation, aeration and freeze and thaw method

Pretreatment condition	H_2 Yield	Sludge source	Ref.
Ultrasonication			
1. Ultrasonic, 20 min	1.03 mol H_2/mol glucose	Sewage treatment plant	(37)
2. Ultrasonic, 30 min	[a] 0.00016 mol H_2/g VS	Sewage treatment plant	(78)
3. Ultrasonic, 30 min	[a] 0.00423 mol H_2/g TS	Sewage treatment plant	(74)
4. Ultrasonic, 30 min	[a] 0.00126 mol H_2/g COD	Sewage treatment plant	(79)
5. Ultrasonic, 30 min	[a] 0.0058 mol H_2/g cornstalk	Sewage treatment plant	(47)
Ultraviolet irradiation			
6. UV, 15 min	[a] 0.00565 mol H_2/g TS	Sewage treatment plant	(74)
7. UV, 15 min	[a] 0.00434 mol H_2/g TS	Sewage treatment plant	(74)
Aeration			
8. Aeration, 24 hr	0.70 mol H_2/mol glucose	Sewage treatment plant	(64)
9. Aeration, 24 hr	[a] 0.00406 mol H_2/ g glucose	Sewage treatment plant	(80)
10. Aeration, 12 hr	[a] 0.00912 mol H_2	Sewage treatment plant	(56)
Freeze and thaw			
11. Freeze (-25 ˚C, 24 hr) and thaw (R.T., 5 hr)	[b] ~0.15 mol H_2/mol glucose	Intertidal zone	(61)
12. Freeze (-10 ˚C, 24 hr) and thaw (30˚C)	0.00019 mol H_2/g COD	POME treatment plant	(66)

[a] H_2 yield was estimated using PV = nRT at standard condition where P = 1 atm; R = 8.21 × 10^{-5} m^3.atm/mol.K and T = 300 K
[b] Estimated value

2.1.3. Chemical pretreatments

A. pH pretreatment

The most popular chemical pretreatment method used to enrich HPB is pH pretreatment (Appendix 1-3). This method involves adjusting the pH of seed sludge to an extreme pH such as pH 3 or 12 (Table 4) and attempting to lyse HCB. In principle, extreme pH induces HPB to form spores. Bacterial spores have rigid cell walls that are difficult to break and hence HPB should survive this pretreatment. However, in reality, most HCB do not form spores and their cell walls are easily disrupted at extreme pH levels (37, 61, 64, 81). Evidently, an acidic pretreatment is more effective compared to an alkali pretreatment. This is evidenced by the H_2 yield produced by acidic pretreated seed sludge which was found to be 1.67 fold higher than alkaline pretreated seed sludge (Table 2.4 no. 2 and 19). This suggests that HPB is more susceptible to alkaline pretreatment but the reason is unknown as there are no reports on the effects of alkalinity on HPB survival. Nonetheless, pH pretreatment is still not as effective as heat pretreatment because the H_2 yield is not as high.

B. Chemical activation and inhibition

Chemical activation pretreatment enriches HPB by spiking or shocking the seed sludge with a selected substrate or medium such as sucrose or rice (Table 2.5, no. 1–6). This method is useful in terms of enriching selective HPB. For example, seed sludge activated with clostridium enforcement medium can enrich HPB like *Clostridium* spp (52). It has also been claimed that spiking the seed sludge with sucrose is highly effective in enriching thermophilic HPB such as *Thermoanaerobacterium* sp. (55). However, this method is practical only if the specific medium or substrate for the targeted HPB is identified which is often challenging.

The chemical inhibition pretreatment can employ toxic chemicals such as chloroform and 2-bromoethanesulfonate (BES) into the seed sludge to inhibit HCB (Table 2.5, no. 7–13). However, these inhibitors are often lethal to the HPB (55, 64, 66, 67) and highly toxic and harmful to humans and the environment. Consequently, it is extremely challenging to search for a suitable and yet environmentally benign inhibitor. Therefore, chemical inhibition pretreatment is the least favorable method for enriching HPB.

Table 2.4: Comparison of H_2 yield between pH pretreatment and untreated sludge

H_2 yield		Pretreatment		
pH pretreatment	**Untreated**	**condition**	**Sludge source**	**Ref.**
Acid pretreatment				
1. 2.25 mol H_2/mol glu.	N.A.	Acid (pH 3), 24 hr	Sewage treatment plant	(81)
2. 1.51 mol H_2/mol glu.	0.38 mol H_2/mol glu.	Acid (pH 3), 24 hr	Sewage treatment plant	(64)
3. 1.11 mol H_2/mol glu.	0.70 mol H_2/mol glu.	Acid (pH 3), 24 hr	Sewage treatment plant	(37)
4. [b] 0.85 mol H_2/mol glu.	[b] 0.20 mol H_2/mol glu.	Acid (pH 3–4), 24 hr	Intertidal zone	(61)
5. 0.65 mol H_2 / mol hex.	0.3 mol H_2 / mol hex.	Acid (pH 3–4), 24 hr	POME treatment plant	(55)
6. 0.0018 mol H_2/gCOD	0.0018 mmol H_2/g COD	Acid (pH 5), 24 hr	H_2 producing reactor	(65)
7. 0.00032 mol H_2/g COD	0.00012 mol H_2/g COD	Acid (pH 3), 24 hr	POME treatment plant	(66)
8. [a] 0.00189 mol H_2/g VS	[a] 0.0265 mol H_2/g VS	Acid (pH 3), 24 hr	Anaerobic treatment plant	(67)
9. [a] 0.026.8 mol H_2	[a] 0.0341 mol H_2	Acid (pH 3), 30 min	Intertidal zone	(68)
10. [a] 0.00074 mol H_2	[a] 0.0071 mol H_2	Acid (pH 3), 24 hr	Sewage treatment plant	(56)
Base pretreatment				
11. 1.34 mol H_2/mol glu.	0.38 mol H_2/mol glu.	Base (pH 10), 24 hr	Sewage treatment plant	(64)
12. 0.68 mol H_2/mol glu.	0.70 mol H_2/mol glu.	Base (pH 10), 24 hr	Sewage treatment plant	(37)
13. [b] 0.10 mol H_2/mol glu.	[b] 0.20 mol H_2/mol glu.	Base (pH 12), 30 min	Intertidal zone	(61)
14. [a] 0.00569 mol H_2/ g glu.	[a] 0.72 mmol H_2/ g glu.	Base (pH 10), 24 hr	Sewage treatment plant	(80)
15. 0.51 mol H_2 / mol hex.	0.3 mol H_2 / mol hex.	Base (pH 12), 24 hr	POME treatment plant	(55)
16. 0.00037 mol H_2/g COD	0.00012 mol H_2/g COD	Base (pH 12), 24 hr	POME treatment plant	(66)
17. [a] 0.00240 mol H_2/g VS	[a] 0.0265 mol H_2/g VS	Base (pH 12), 24 hr	Anaerobic treatment plant	(67)
18. [a] 0.00006 mol H_2/g VS	[a] 0.00005 mol H_2/g VS	Base (pH 12), 5 min	Sewage treatment plant	(78)
19. [a] 0.0154 mol H_2	[a] 0.0341 mol H_2	Base (pH 10), 30 min	Intertidal zone	(68)
20. [a] 0.00211 mol H_2	[a] 0.0071 mol H_2	Base (pH 11), 24 hr	Sewage treatment plant	(56)

[a] H_2 yield was estimated using PV = nRT at standard condition where P = 1 atm; R = 8.21 × 10^{-5} m^3.atm/mol.K and T = 300 K

[b] Estimated value

Table 2.5: H_2 production from sludge pretreated with chemical activation and inhibition methods

Pretreatment condition	**H2 Yield**	**Sludge source**	**Ref.**
Chemical activation			
1. Reactivated in clostridium enforcement medium, 15 days	2.19 mol H_2/mol hexose	Cattle farm	(52)
2. Loading shock (50 g sucrose/L), 2 days	1.96 mol H_2/ mol hexose	POME treatment plant	(55)
3. Loading shock (50 g sucrose/L), 2 days	0.199 mol H_2/ L POME	POME treatment plant	(55)
4. Reactivated in rice medium, 1 month	[a] 0.00212 mol H_2/g TS	Composting plant	(35)
5. Reactivated in rice medium, 1 month	[a] 0.00517 mol H_2/g TS	Composting plant	(35)
6. KNO_3 (10 mmol/L)	[a] 0.0345 mol H_2	Intertidal zone	(68)
Chemical inhibition			
7. BES (10 mmol/L), 24 hr	0.33 mol H_2/mol glucose	Sewage treatment plant	(64)
8. BES (10 mmol), 30 min	1.01 mol H_2 / mol hexose	POME treatment plant	(55)
9. BES (0.2 g/L), 24 hr	0.0000317 mol H_2/g COD	H_2 producing reactor	(65)
10. Chloroform (1%), 24 hr	0.61 mol H_2/mol glucose	Sewage treatment plant	(64)
11. Chloroform (2%), 24 hr	[a] 0.00353 mol H_2/ g glucose	Sewage treatment plant	(80)
12. Chloroform (0.1%), 24 hr	0.00023 mol H_2/g COD	POME treatment plant	(66)
13. Chloroform (0.2%)	[a] 0.00134 mol H_2/g VS	Anaerobic treatment plant	(67)

[a] H_2 yield was estimated using PV = nRT at standard condition where P = 1 atm; R = 8.21 × 10^{-5} m^3.atm/mol.K and T = 300 K
[b] Estimated value

2.1.4. Combination pretreatments

Combination pretreatment methods combine the strengths of physical and chemical pretreatment methods to improve the selection of HPB. Combination methods employ dual pretreatments such as repeated heating (Table 2.6, no. 2 & 6) or a combination of several pretreatments (Table 2.6). Studies have shown that heat coupled with acid, acid coupled with BES, and heat coupled with ultrasonic treated seed sludge produced at least two fold more H_2 compared to that of individual pretreatments. In addition, the sequence of combination pretreatments plays an important role (82). For example, it is crucial that heat pretreatment be applied before pretreatment with chloroform (82). Research has shown that the yield obtained by using heat pretreatment followed by chloroform generated 22% more H_2 compared to using chloroform followed by heat (Table 2.6, no. 4 & 5). This is because HPB sporulates from heat pretreatment. Since spores are more stress-resistant, the subsequent chemical pretreatment further eliminates HCB and enriches HPB. Currently, the best combination pretreatment, which is heat pretreatment followed by aeration pretreatment, has only

produced 1.83 mol H_2/mol glucose (Table 2.6, no. 1). It is interesting to note that the result of this combination pretreatment is still lower than that of heat pretreatment. This method is established as an alternative to physical or chemical methods when individual pretreatments cannot effectively enrich HPB.

Table 2.6: Hydrogen production from sludge pretreated with combination pretreatment

Pretreatment	H_2 Yield	Sludge source	Ref.
1. Heat (boiling) + aeration (4 min)	1.83 mol H_2/mol glucose	River sludge	(83)
2. Repeated boiling (2× for 5 hr)	1.00 mol H_2/mol glucose	Beer Industry	(82)
3. Heat (77 ˚C) + Ultrasonic (20 min)	1.55 mol H_2/mol glucose	Sewage treatment plant	(37)
4. Heat (repeated boiling) + chloroform (0.05%)	0.51 mol H_2/mol glucose	Beer Industry	(82)
5. Chloroform (0.05%) + heat (repeated boiling)	0.44 mol H_2/mol glucose	Beer Industry	(82)
6. Repeated boiling (2× for 5 hr)	0.33 mol H_2/mol glucose	Bakers yeast industry	(82)
7. Heat 90˚C + Ultrasonic	[a] 1.32–1.50 mol H_2/g COD	Sewage treatment plant	(73)
8. Acid (pH 5) + BES (0.2 g/L)	2.90×10^{-5} mol H_2/g COD	H_2 producing reactor	(65)
9. Heat (100 ˚C) + acid (pH 5)	2.07×10^{-5} mol H_2/g COD	H_2 producing reactor	(65)
10. Acid (pH 5) + heat (100 ˚C) + BES (0.2 g/L)	1.08×10^{-5} mol H_2/g COD	H_2 producing reactor	(65)
11. Heat (100 ˚C) + BES (0.2 g/L)	8.40×10^{-6} mol H_2/gCOD	H_2 producing reactor	(65)
12. Heat (boiling) + freeze -20˚C + thaw (4˚C)	0.41 mol H_2/mol glycerol	Sewage treatment plant	(84)
13. Heat (95 ˚C) + acid (pH 3–5), 48 hr	[a] 0.0545 mol H_2	Cattle farm	(70)
14. Water soak (3 hr) + Reactivated in glucose (3 days)	[a] 0.011 mol H_2/g substrate	Cattle farm	(85)
15. Aeration (4 days) + Reactivated in glucose (3 days)	[a] 0.010 mol H_2/g substrate	Cattle farm	(85)
16. UV (3 hr) + Reactivated in glucose (3 days)	[a] 0.010 mol H_2/g substrate	Cattle farm	(85)

[a] H_2 yield was estimated using PV = nRT at standard condition where P = 1 atm; R = 8.21×10^{-5} m^3.atm/mol.K and T = 300 K
[b] Estimated value

2.2. Microbial diversity

Sludge containing diverse microorganisms and functional seed sludge that produces H_2 is usually enriched by pretreatment methods (Appendix 1-5). Different pretreatment methods have shown different preservation effects on a variety of bacteria (55, 63) and this directly influences the H_2 yield. For example, HPB such as *Clostridium acetobutylicum* is predominant in heat pretreated sludge (Appendix 1-5: Table A5, no. 1, 2, 25); *Clostridium* spp. is also found in pH pretreated sludge (Appendix 1-5: Table A5, no. 12, 13 & 23);

Thermoanaerobacterium sp. is found in load shock pretreated sludge (Appendix 1-5: Table A5, no. 9); and *Bacillus* sp. is found in BES pretreated sludge (Appendix 1-5: Table A5, no. 11). There are also some HCB such as *Lactobacillus* spp. and *Bifidobacterium* spp. which persist even after pretreatment (Appendix 1-5: Table A5, no. 2, 5, 6, 12). Typically, pretreated seed sludge that contains more varieties of HPB and less of HCB produces a higher amount of H_2 (Table 2.7, no. 1–9). For example, seed sludge containing only HPB (Table 2.7, no. 1–5) was found to produce a higher amount of H_2 than seed sludge containing both HPB and HCB (Table 2.7, no. 6–9). On top of that, seed sludge containing several strains of *Clostridium* spp. also produced a higher amount of H_2 compared to a single strain or pure culture (49, 53, 60, 86). This is because different bacteria may utilize different substrates or cooperate in breaking down complex substrates in order to produce H_2. This synergistic interaction among a variety of bacteria in seed sludge is more beneficial than a pure culture in terms of H_2 production from complex substrates such as wastewater.

The variety of HPB which belongs to the family of strict anaerobes Clostridiaceae has the greatest potential in H_2 production via dark fermentation (31, 52, 53). Besides high H_2 production, *Clostridium spp.* can also produce H_2 from a wide range of substrates such as maltose, cellobiose, starch, glucose, sucrose, xylose, dextrin, paper cellulose, powder cellulose, casein and ground nut oil (54). This allows *Clostridium spp.* to produce H_2 from waste streams that contain diverse substrates. *Ethanoligenens harbinens* is a newly identified HPB enriched from aerated seed sludge (56). It is a strict anaerobe that produces ethanol and H_2 simultaneously. This HPB is highly resistant against the bactericidal effect of ethanol during H_2 production. This suggests that *Ethanoligenens harbinens* can be used in ethanol-rich waste for H_2 production. *Bacillus megaterium* is another newly identified HPB (57) isolated from intertidal sludge and it tolerates high salinity levels of up to 15% (87). This is useful in H_2 production from high salinity wastewater or even polluted sea water.

In contrast to strict anaerobe, some researchers have suggested that facultative-HPB could be the better H_2 producers. Most of the identified facultative-HPB such as *Enterobacter* spp. and *Klebsiella* spp. (56, 58) belong to the family of Enterobacteriaceae. Their higher tolerance to oxygen stress, allows facultative-HPB to act as a shelter for hydrogenase. Hydrogenase can be irreversibly inhibited by oxygen regardless of whether it is present in a strict or facultative-HPB (88, 89). Facultative-HPB is able to recover the activity of hydrogenase by rapidly depleting oxygen which accidentally enters the fermentation medium (89-91). However, the trade-off to this is that facultative-HPB generates lower amounts of H_2 compared to strict anaerobes such as *Clostridium spp.* Therefore, facultative-HPB in sludge

can function as a defense against oxidative stress while maintaining an oxygen free condition for strict anaerobes to produce H_2. This shows that the symbiotic interaction between strict and facultative-HPB in seed sludge is important to sustain H_2 production.

Table 2.7: Type of pretreated sludge that contains only H_2 producing bacteria and both H_2 producing and consuming bacteria

	Source of Sludge	Microbial community	Pretreatment	H_2 yield	Ref.
Sludge contains only H_2 producing bacteria					
1.	Sewage treatment plant B	*Clostridium acetobutyricum* (AE0011437.1) *Clostridium butyricum* (DQ831124.1) *Clostridium* sp. HPB-21 (AY862509.1) Uncultured *Clostridium* sp. (EF700377.1)	Heat	2.30 mol H_2/mol glucose	(31)
2.	POME treatment plant	*Thermoanaerobacterium sp.* (AY999015) *Thermoanaerobacterium thermosaccharolyticum* (AY999014) *Clostridium thermopalmarium* (AF286862)	Loading shock	1.96 mol H_2/ mol hexose	(55)
3.	Sucrose-based synthetic wastewater sludge	*Clostridium butyricum* CGS5	Heat	2.78 mol H_2/ mol sucrose	(53)
4.	Sewage treatment plant	*Klebsiella* sp. HE1 (AY540111)	N.A.	0.92 mol H_2/ mol sucrose	(58)
5.	Sewage treatment plant	*Clostridium acetobutyricum* (FM994940.1) *Klebsiella pneumonia* (GQ214541.1) *Clostridium butyricum* (DQ831124.1) Uncultured bacterium (DQ464539.1 and DQ414811.1)	Heat	0.0106 mol H_2/ g carbohydrate	(49)
Sludge contains both H_2 producing and consuming bacteria					
6.	POME treatment plant	*Lactobacillus* sp. (AY363384) *Bacillus* sp. (AB193859) *Clostridium* sp. (AB234007)	Acid	0.65 mol H_2 / mol hexose	(55)
7.	Sewage treatment plant C	*Bacillus* sp. (DQ168845.1) *Clostridium butyricum* (DQ831124.1) *Clostridium acetobutyricum* (DQ235219.1 and FM994940.1) *Clostridium* sp. (DQ168846.1) *Lactobacillus delbrueekii* (FJ915706.1) Uncultured bacterium (DQ235219.1) Uncultured *Bacillus* sp. (DQ168845.1) Uncultured *Clostridium* (DQ168846.1)	Heat	2.18 mol H_2/mol glucose	(60)
8.	Sewage treatment plant D	*Bifidobacterium boum* (AY166529.1) *Clostridium* sp. (FJ876436.1)	Heat	1.32 mol H_2/mol glucose	(60)

		Clostridium butyricum (DQ831124.1) *Clostridium acetobutyricum* (FM994940.1) *Lactobacillus fermentum* (GQ131282.1) *Lactobacillus delbrueekii* (FJ915705.1 and FJ915706.1) Uncultured bacterium (AB441617.1)			
9.	Intertidal sludge	*Bacillus* sp. (GQ180912) *Lactobacillus plantarum* (GQ180905 and GQ180906) *Clostridium* sp. (GQ180907, GQ180908, GQ180910 and GQ180911) *Enterococcus faecium* (GQ180909)	Freeze and thaw	0.15 mol H_2/mol glucose	(61)

2.3. Effects of operation conditions on hydrogen production by sludge inocula

2.3.1. Effects of substrate

H_2 research aims to integrate dark fermentation with waste management. Therefore, many researchers are focusing on H_2 production from organic waste in various streams of waste (Appendix 1-1 to 1-4). Organic substrates found in wastewaters are cheap and easily available. Hence, they can be used in dark fermentation for H_2 production. However, wastewaters are not usually sufficiently nutritious to support H_2 production and it is not practical to continuously supply the fermentation process with costly nutrients such as glucose, peptone and yeast extract. One of the solutions is to improve the nutrient content using a combination of different types of wastewaters (92). The production of H_2 significantly increases by combining two different types of waste. For example, food wastewater or cassava stillage is rich in carbohydrate and sewage sludge is rich in nitrogen and other micro nutrients. When these combined substrates were applied in dark fermentation, H_2 yield increased by 0.63 fold (92). This shows that mixing carbohydrate and nitrogen-rich substrates improves the nutrient content in fermentations and increases H_2 yield. In addition, waste from different resources contains varieties of bacteria. The synergetic interaction between microflora from different waste resources also contributes to improved H_2 yield from the combined wastes (48). A combination of wastes from different sources provides an opportunity to enhance H_2 production by improving the nutrient content and microbiological profile in the fermentation system.

A balanced concentration of substrate also plays an important role in H_2 production. It is logical to assume that H_2 production increases with substrate concentration. For example, it was found that when the cellobiose concentration increased 2 fold, the H_2 yield increased

from 1.57 to 2.19 mol H_2/mol hexose (52). A relatively low substrate concentration is only sufficient to support biomass growth and hence H_2 production is restricted (32-35). However, an excessive amount of substrate does not always ensure high H_2 production. This is because an excessive amount of substrate increases osmotic pressure and hence inhibits HPB growth. Furthermore, excess substrate inhibits H_2 production by shifting fermentation pathways to produce alcohol and/or lactic acid. This will be further discussed in the next section (46). On the other hand, in the case of ineffective sludge pretreatment, a high substrate concentration provokes methane production from methanogens. When the substrate is in excess, it is rapidly converted into H_2 and this leads to the accumulation of H_2. The increase in H_2 partial pressure triggers methane production from methanogens that are still in the sludge because H_2 is the intermediate precursor for methane production (93). This can be prevented by reducing the substrate input for H_2 production as suggested by Chen *et al*. (93). Thus, a reasonable amount of substrate in the fermentation is important because limited or excessive substrates inhibit H_2 production.

Accessibility of HPB to substrates directly influences the sustainability of H_2 production. Simple substrates such as glucose and lactose are easily accessible for H_2 production. Theoretically, 1 mole of glucose ($C_6H_{12}O_6$) should produce 12 moles of H_2 (eq. 2.1), while 1 mole of lactose ($C_{12}H_{22}O_{11}$) will produce 23 moles of H_2 (eq. 2.2).

$$C_{12}H_{22}O_{11} + 12\ H_2O \rightarrow \mathbf{23\ H_2} + 12\ CO_2 \qquad \text{(eq. 2.2)}$$

Nevertheless, dark fermentation is less efficient in terms of converting substrates to H_2 because most thermal enthalpies are lost in the formation of volatile fatty acids (VFA). Typically, the maximum energy conversion from glucose to H_2 is only 33% via the acetate pathway (eq. 2.3) and 17% via the butyrate pathway (eq. 2.4). Meanwhile the efficiency of lactose conversion to H_2 is only 31% via the acetate and methane pathways (eq. 2.5 and 2.6) (18, 29, 30).

Glucose fermentation

Acetate pathway : $C_6H_{12}O_6 + 2H_2O \rightarrow \mathbf{4H_2} + 2CH_3COOH + 2CO_2$ (eq. 2.3)

Butyrate Pathway : $C_6H_{12}O_6 \rightarrow \mathbf{2H_2} + CH_3CH_2CH_2COOH + 2CO_2$ (eq. 2.4)

Lactose fermentation

Acetate pathway : $C_{12}H_{22}O_{11} + 5H_2O \rightarrow \mathbf{8H_2} + 4CO_2 + 4CH_3COOH$ (eq. 2.5)

Methane pathway : $C_{12}H_{22}O_{11} + 5H_2O \rightarrow \mathbf{8H_2} + 8CO_2 + 4CH_4$ (eq. 2.6)

However, achieving the theoretical maximum H_2 yield is not feasible. Currently, the highest reported H_2 yield is only 2.3 mol H_2/mol glucose which is only about 50% of the theoretical maximum H_2 yield (31). This phenomenon is caused by (1) the rapid conversion of substrate into cell biomass instead of H_2 (32-35) and (2) an inappropriate combination of fermentation conditions as discussed in this paper. It is postulated that mixed microflora in the seed sludge could overcome this problem via the synergetic interaction among the different bacteria because they can adapt to a wider range of conditions.

2.3.2. Effects of pH

The reported optimum pH for H_2 production is in the range of pH 6 – 8 (Appendix 1-1 to 1-4). This represents the pH range that supports the growth of many HPB including *C. butyricum*, *C. beijerinckii*, *C. tyrobutyricum* and *C. saccharoperbutylacetonicum* (35, 42, 46, 53, 61, 94-96). An optimum pH helps to maintain the surface charge on the cell membrane which facilitates nutrient uptake and hence sustains growth of HPB (46, 94). In addition, HPB contains the essential enzyme, hydrogenase, which plays the most important role in H_2 production. Hydrogenase is reported to function optimally at a pH range of 6 – 6.5 (97, 98). Evidence of this was seen in a study when H_2 production at a pH level lower than 6 was reduced by half (50) or completely ceased (42). This shows that pH plays a critical role in sustaining the growth of HPB and the activity of hydrogenase in H_2 production.

It is also noteworthy that the buffer capacity of the fermentation medium plays an important role in regulating the pH in order to achieve optimum H_2 production. Unlike synthetic mediums, natural buffering capacity does not occur in most of the waste resources, hence utilizing waste resources to produce H_2 is hampered (67). Some researchers have suggested that batch fermentation should be initiated at a higher pH level (pH 8–10) (78, 99, 100) because high initial pH will buffer acid production accordingly and prevent a sharp pH reduction (94). Zhao *et al*. (100) and Lee *et al.* (101) stressed that the medium will become more acidic over time due to the production and accumulation of organic acids during the fermentation process. Hence, a stable pH in the medium is essential to sustain optimum H_2 production.

2.3.3. Effect of temperature

Temperature determines the physiological activities of HPB. The fermentation temperature for most of the H_2 productions was reported in the mesophilic range (20–45˚C) (Appendix 1-1 to 1-4). This is because most of the HPB present in the seed sludge are mesophiles such as *Clostridium* spp., *Enterobacter* spp., and *Bacillus* spp. that grow in this temperature range (53, 102, 103). However, H_2 production is only vigorous in a narrow range of temperatures even though HPB may grow in a wide temperature range. For example, Mu *et al.* (104) detected HPB growth at 33–41˚C but the highest H_2 yield was obtained at 39˚C. From the literature, the most promising temperature range for H_2 production is 35–37˚C (31, 63, 81). This suggests that HPB are only physiologically active in a narrow temperature range for H_2 production despite their ability to grow in a wide temperature range.

Furthermore, it is argued that H_2 production at higher temperatures (>45 ˚C) is favorable. This is because H_2 gas is less soluble at high temperatures (46, 105, 106). It is also interesting to note that hydrogenase is reported to function optimally in the range of 50–70˚C despite many HPB being identified as mesophiles (46, 105, 106). This leads to the identification of several thermophiles that belong to the *Thermoanaerobacterium* genus which produce H_2 at thermophilic temperatures (>45 ˚C) (55, 70, 107). These bacteria can produce up to 1.96 mol H_2/mol hexose at 60˚C after 48 hours (55). Thus, thermophiles are suitable to be used in warm or even hot wastewater, such as beverage producing, food processing or pulp and paper industries, because they are able to tolerate a high operation temperature. Temperature is a crucial parameter in dark fermentation because temperatures outside the suitable range will restrain H_2 production.

2.3.4. Effects of nutrients and inhibitors

A. Effects of organic acids

Fermentative H_2 production is accompanied by the formation of volatile fatty acids (VFA) such as acetate, butyrate, propionate, lactate, formate and ethanol. Productions of VFA via different fermentation pathways are influenced by the variety of HPB present in the seed sludge which in turn is determined by the pretreatment method. These pathways are indicated by the ratio of acetate to butyrate which is clearly listed in (Appendix 1-1 to 1-4). When the ratio of acetate to butyrate is larger than one, it represents an acetate pathway (eq. 2.3). Meanwhile, a ratio that is smaller than one indicates the butyrate pathway (eq. 2.4). This further emphasizes that H_2 yield is strongly related to the selection of the pretreatment method because this determines the variety of HPB that produces H_2.

In strict anaerobes, the fermentative pathways are divided into two main routes: acidogenesis (acid production) and solventogenesis (solvent production) (Figure 2.2). These pathways are usually efficiency indicators of H_2 production (35, 108). Generally, glucose undergoes glycolysis to produce pyruvate with NADH as the electron donor. The electrons generated from the oxidative decarboxylation of pyruvate are transferred to protons and then hydrogenase reduces the protons to molecular H_2 gas. In acidogenesis, the production of acetate is normally the preferred pathway in H_2 production (35, 37, 60, 109). The ideal H_2 yield is 4 mol/mol of hexose via the acetate pathway but it is halved via the butyrate pathway (35, 40, 110, 111). It has been reported that a protein-rich substrate favors the acetate pathway but a carbohydrate-rich substrate favors the butyrate pathway (92). On the other hand, other acidogenesis pathways which produce VFA such as lactate or propionate have been reported to inhibit H_2 production (35, 60, 105). In contrast to acidogenesis, H_2 production in solventogenesis is accompanied by the production of solvents such as ethanol and buthanol. However, solventogenesis usually does not encourage high H_2 yield because solvents like ethanol has bactericidal effects (35). During batch fermentation, the switch from the acidogenesis to the solventogenesis pathway triggers the buildup of biogas partial pressure, the accumulation of VFA, and the reduction of fermentative pH (13, 35, 84, 112-114). The direction of the fermentative pathway directly influences the quality of H_2 yield in which the acetate and butyrate pathways are the more favorable directions.

Organic acids can act as a supplementary and/or inhibitory factor to H_2 production. Productions of acetate and butyrate are usually associated with high H_2 production but an accumulation of these acids will negatively impact H_2 production (Figure 2.3a). For instance, it has been found that fermentation supplemented with excess butyrate inhibited H_2 production from kitchen waste (109) and acetate inhibited H_2 production from glycerol (84). Studies have also shown that the H_2 yield from apple pomace was reduced by at least 5% with the addition of acetate and butyrate (74). In contrast, other organic acids that have been reported as indicators of low H_2 production such as lactate and propionate can be potential supplements when present at a threshold concentration (74). When added to fermentations, lactate and propionate can trigger a positive reaction to induce higher H_2 production via the pyruvate pathway (Figure 2.3b). For example, it was reported that the addition of lactate at a concentration of 650 mg/L enhanced H_2 production by up to 37%; and propionate increased H_2 yield by 28% (74). The concentration of organic acids in the fermentation medium regulates H_2 production with the control of different feedback mechanisms.

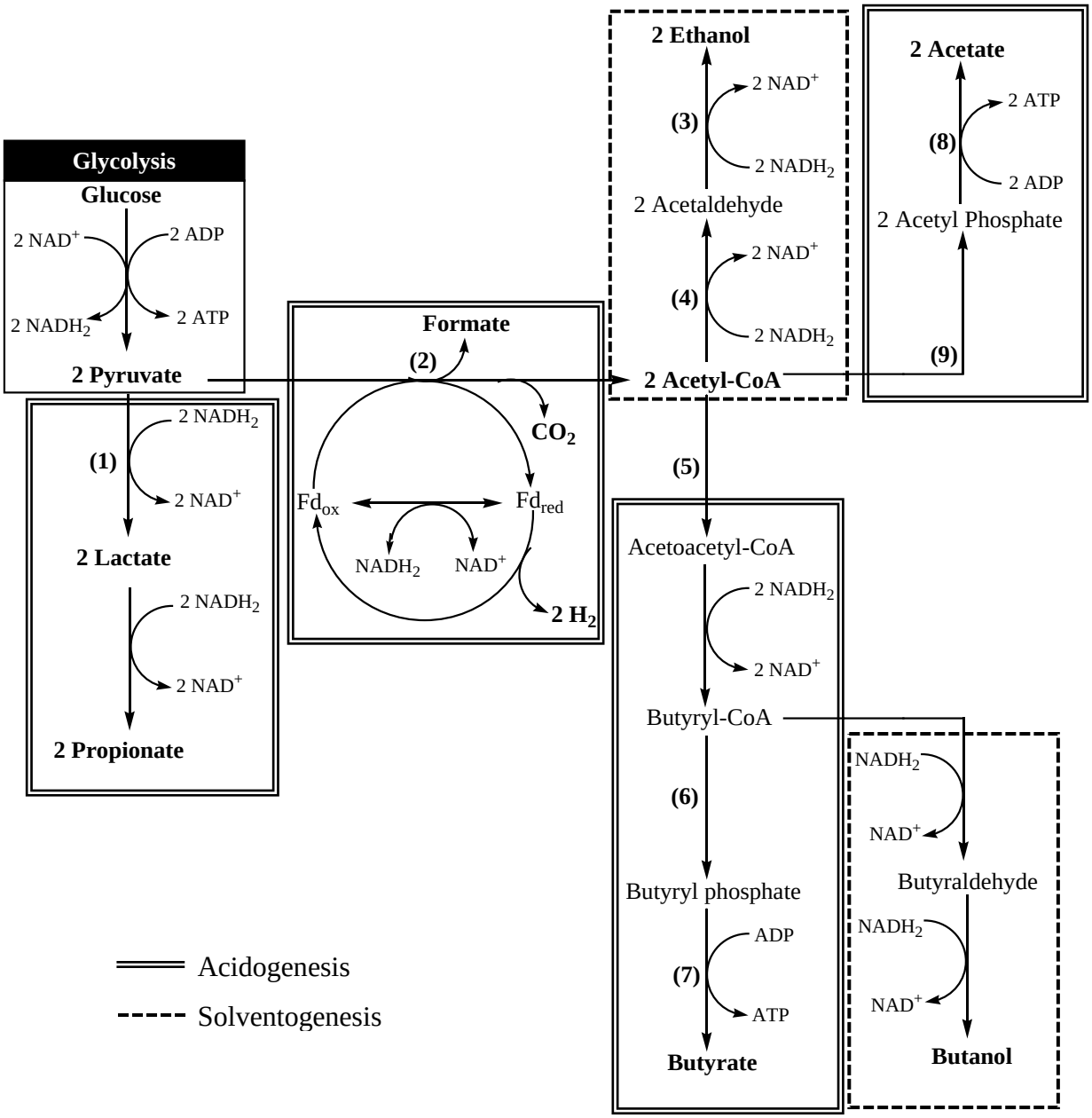

Figure 2.2: The connection of glycolytic pathway for glucose fermentation with organic acid and solvent production from pyruvate by strict anaerobes. Numbers in brackets represents key enzymes: (1) lactate dehydrogenase; (2) pyruvate formate lyase; (3) ADH: alcohol dehydrogenase; (4) acetaldehyde dehydrogenase; (5) thiolase; (6) phosphotransbutylase; (7) butyrate kinase; (9) phosphotransacetylase; (8) acetate kinase.

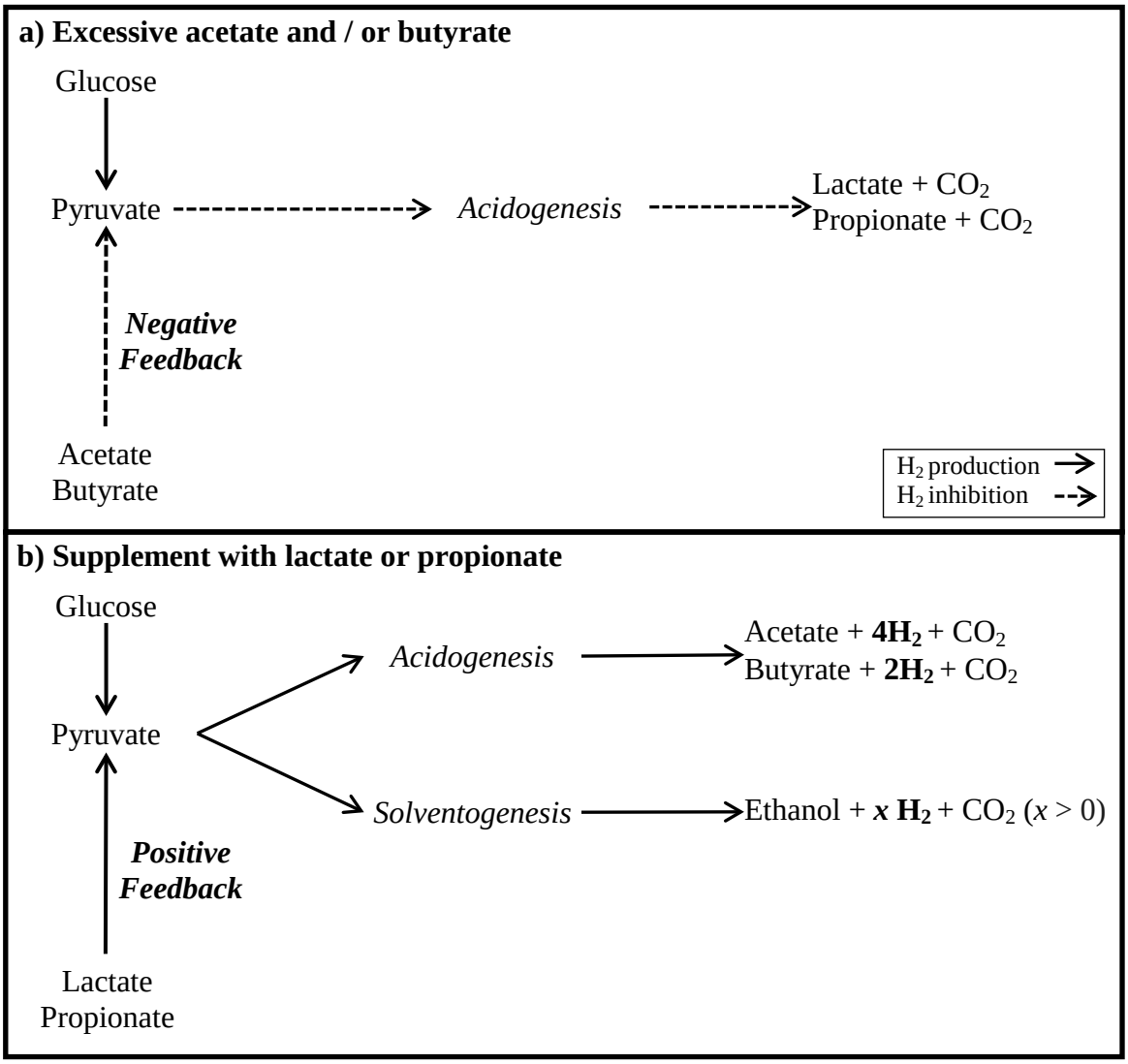

Figure 2.3: Relationship between fermentation products and H_2 production. (a) event of H_2 inhibition due to accumulation of acetate and/or butyrate; (b) event of H_2 production supplemented by lactate and propionate.

B. Effects of macro- and micronutrients

The macronutrients in dark fermentation are carbon (C), nitrogen (N) and phosphorous (P) and these are usually the essential nutrients (45, 51, 106, 115). Carbon content is solely contributed by the substrate from which H_2 is produced as discussed in section 2.3. Nitrogen can be in various forms such as protein, nitrate, nitrite and ammonium. The presence of ammonium in fermentation is essential because it does not only serve as a nutrient for bacterial growth but also provides a slight buffering capacity in the medium against the production of organic acids (116, 117). However, it is argued that nitrogen content

is not essential because it does not influence the production of total biogas but might affect the lag time of gas production (84). Phosphorous is usually present in the form of phosphate. Argun *et al*. (118) showed that the maximum H_2 was produced at C/P ratio of 1000 (equivalent to C/N/P of 100/0.5/0.1). Consequently, a balanced nutrient level is essential for optimum H_2 production.

Metal ions are micronutrients for fermentation. Nickel (Ni) and iron (Fe) serve as the co-factors for hydrogenase (119). Hydrogenase is the main enzyme responsible for H_2 production. It is classified according to the metal component in the active site commonly Ni-Fe and Fe-Fe (120, 121). Therefore, fermentation medium supplemented with Ni and Fe enhances H_2 production (48, 63, 122). It was reported that a fermentation medium supplemented with 0.1 mg/L Ni resulted in a 2.4 fold increase in H_2 yield compared to non-supplemented fermentations (63), whereas a fermentation medium with increased Fe concentration of 18 to 55 mg/L improved H_2 yield by 1.5 fold and shortened the lag phase by 0.33 fold (48). Other metal ions including zinc (Zn), copper (Cu) and chromium (Cr) are also found to be beneficial to other key enzymes including dehydrogenase, dismutase, hydrogenase and methyltransferase (123). The threshold concentrations of Zn, Cu and Cr are reported as 0.24 mg/L, 3.0 mg/L and 15 mg/L respectively. Once the concentration exceeds the threshold limit, these elements become toxic to HPB. For instance, the yield of H_2 was reduced by half when the concentration of Zn, Cu and Cr exceeded the threshold concentration (124). Additional metal ions at appropriate concentrations can enhance H_2 production accordingly by regulating the activity of the enzymes involved in the process.

Metal ions can also stabilize H_2 production and improve the H_2 production processes (50, 119). Calcium (Ca) concentration in the range of 50–150 mg/L stabilizes and improves H_2 production (48, 125, 126). Adding molybdate (Mo) favors the H_2 production process because it inhibits sulphate reduction and methane production (122, 127). Niu *et al*. (50) reported that a low concentration of Mo (0.0042 mg/L) could increase H_2 yield by 29%. Overall, metal ions assist in obtaining high H_2 yield by alleviating fermentation conditions.

2.4. Conclusion

The benchmark of H_2 production from sludge via dark fermentation is summarized in Table 2.8. The selection of pretreatment methods determines the variety of HPB preserved in the seed sludge. The activity of HPB is influenced by various fermentation conditions,

including type of substrate, fermentation pH and temperature, and types of nutrients, supplements and inhibitors. With the identification of the strengths and weaknesses of these conditions, we can further enhance H_2 production via dark fermentation.

Table 2.8: Summary of factors responsible and recommended conditions for high H_2 production from seed sludge via dark fermentation

Parameters	Reported range	Recommended conditions
Pretreatment temperature	65 – 100˚C for 15 – 90 min	65˚C for 30min or 100˚C for 15 min
Fermentation pH	pH 6 – 8	pH 6.0 – 6.5
Fermentation temperature	20 – 45˚C	35 – 37˚C
Micronutrients concentration	N.A.	Ni 0.1 mg/L; Fe 55 mg/L; Zn ≤ 0.24 mg/L; Cu ≤ 3.0 mg/L; Cr ≤ 15 mg/L; Ca 50–150 mg/L; Mo 0.0042 mg/L

Hydrogen production from seed sludge via dark fermentation can be a sustainable approach for long term fuel supply. We have presented the importance of sludge enrichment using different pretreatment methods and have revealed that heat pretreatment is the most frequently applied and the most effective method to eliminate HCB while preserving HPB. In addition, the enriched sludge requires optimum fermentation conditions in order to produce H_2 optimally through the correct fermentation pathway. However, the current fermentation conditions only enable the enriched sludge to produce up to 2.3 mol H_2/mol glucose via dark fermentation. This is still far from the theoretical value of 4 mol H_2/mol glucose. To further enhance the H_2 yield from seed sludge as inoculum, the challenges ahead are to investigate:

(1) The type of pretreatment methods with appropriate condition and duration that can effectively enrich HPB in seed sludge in order to achieve maximum H_2 production;

(2) The combination of fermentation conditions that can direct HPB into the correct fermentation pathway for optimum H_2 production.

Chapter 3

High efficiency bio-hydrogen production from glucose by an inoculum of heat-pretreated landfill leachate sludge

The work presented in this chapter has been partly submitted for peer review:

Wong YM, Juan JC, Ting A, Wu TY. High efficiency bio-hydrogen production from glucose revealed in an inoculum of heat-pretreated landfill leachate sludge. Energy. 2014;72(0): 628 – 635

Monash University

Declaration for Thesis Chapter 3

Declaration by candidate

In the case of Chapter 3, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Analysis and interpretation of data	100
Drafting and writing the publication	95

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
A/P Dr. Juan Joon Ching	Reviewed the publication	N.A.
Dr. Adeline Ting	Reviewed the publication	N.A.
Dr. Wu Ta Yeong	Reviewed the publication	N.A.

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	Date 9 Sept 2014
Main Supervisor's Signature	[redacted]	Date 9 Sept 2014

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

3.0. Introduction

Hydrogen (H_2) is a promising alternative to fossil fuels. Unlike fossil fuels, combustion of H_2, combustion of H_2 does not produce greenhouse gases and hence will not induce global warming (128-130). It possesses high energy yield per unit weight (141.9 J/kg) (8). Moreover, H_2 can be produced biologically from various types of organic-rich waste such as stillage, sludge, leachate, pomace, stalks and bagasse (66, 110, 131-134). Hence, bio-H_2 production assists in waste reduction. In conjunction with rapid depletion of fossil fuels reserves, H_2 has growing importance as the clean and sustainable energy.

It has recently been noted that mixed microbial communities in wastewater sludge is a convenient source of inoculum for fermentative H_2 production (135). Mixed microbial community helps to enhance H_2 production due to the symbiotic interactions between H_2-producing bacteria (HPB) and other bacteria (136, 137). In order to enrich HPB in seed sludge, many researchers have reported on the application of heat pretreatment on various sources of sludge including sludge of sewage (31, 63), compost (130), river sediments (138) and cow dung (139) as inoculum for H_2 production. It was reported that sewage sludge pretreated at 65 ˚C successfully improved H_2 yield by 8.85 fold to 2.30 mol H_2/mol glucose as compared to that of untreated sludge (31). Heat pretreatment is the simplest and relatively most effective technique used to remove H_2-consuming bacteria (HCB) and preserve HPB (31, 36, 41, 78, 140, 141). The difference between HPB and HCB is that majority of HPB belongs to the family Clostridiaceae which forms spores that are heat resistant (31, 140). This allows HPB to survive from the heat pretreatment while removing HCB in the sludge inoculum.

Landfill is a facility built to dispose daily municipal waste. It is rich in organic waste and hence it is usually active in biodegradation. Hence, both landfill leachate and its sludge, which are originated from the landfill, contain diverse microorganism. Currently, there is only one report on using leachate as inoculum to produce H_2. Watanabe and Yoshino (134) reported that the H_2-producing microbial community in the landfill leachate was capable of producing 2.67 mol of H_2 per mol of carbohydrate. However, no heat pretreatment was employed to increase H_2 from the landfill leachate sludge. In this study, we study employed landfill leachate sludge as inoculum to produce H_2. We comprehensively investigated the optimization of H_2 production from the inoculum of heat-pretreated landfill leachate sludge. We also examined the effect of initial pH, fermentation temperature and substrate concentration which have been reported to play a significant role in H_2 production (32-35, 42,

50, 53, 102, 103). To verify the high efficiency of H_2 production from the inoculum of landfill leachate sludge, the kinetics and thermodynamics were analyzed with modified Gompertz model and Gibbs free energy (31, 142-144).

3.1. Methods and Materials

3.1.1. Sampling Sites and samples collection

Jeram Sanitary Landfill (3.189424, 101.366703) is located in Jeram, Selangor, Malaysia. It is designed to receive up to 2000 metric tonnes of municipal solid waste daily with the leachate generation of about 1000 m^3/day. The sludge was collected from landfill leachate collection pond (Figure 3.1). Simple on-site measurements suggest that the landfill leachate has a slight alkaline pH and low dissolved oxygen (Table 3.1).

Figure 3.1: Landfill leachate collection ponds in Jeram, Selangor

Table 3.1: Landfill leachate characteristics measured in sampling site

Characteristics	Value
pH	8.28 ± 0.08
Dissolved oxygen	0.15 ± 0.03 ppm

Haphazard sampling method was adopted in sample collection. Haphazard sampling is a non-systematic sampling method without classical sampling design (145, 146). Sludge was collected from the deepest point of the collection pond using sediment dredge (77933, Forestry-Suppliers, USA) and fresh landfill leachate was collected using water sampler (77222, Forestry-Suppliers, USA). This is method was selected because the leachate

collection ponds is designed in such a way that the sludge is channel to a specific spot, hence a more complex randomized method was not suitable for sludge collection. The collected landfill leachate and sludge was stored in autoclaved bottles. The sludge was sieved through a 400 μm sieve and stored in at 4˚C.

3.1.2. Pretreatment of the landfill sludge

The landfill leachate sludge was heat-pretreated at temperatures of 40, 55, 65, 80 and 95˚C for 30 min. Subsequently, the pretreated sludge was enriched in Reinforcement Clostridial Medium at 37˚C for 24 h. The enrichment is necessary to ensure the consistency of cell count inoculated into the bottles. The untreated inoculum was used as a control.

3.1.3. Hydrogen production from pretreated landfill sludge

Batch mode H_2 production was carrying out in a 200 mL serum bottle containing 150 mL of fermentation media. For each bottle, 2% v/v of seed sludge was added to 150 mL of reaction medium. Biogas produced was collected and the volume was measured using water displacement method as shown in Figure 3.2. All the fermentation processes were conducted in triplicate.

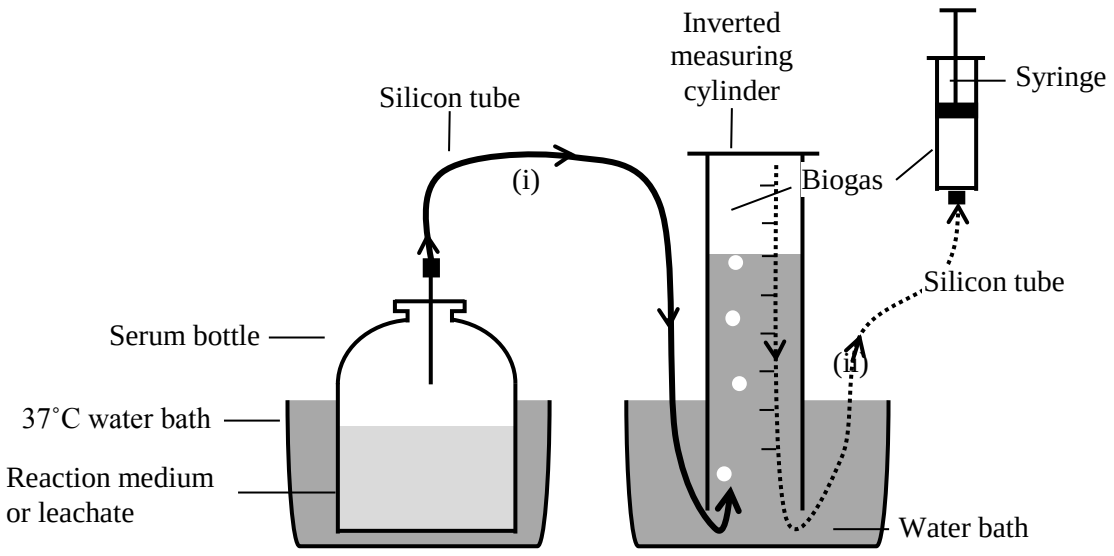

Figure 3.2: Experimental setup of batch mode dark fermentation. Direction (i): the collection of biogas flow from serum bottle to measuring cylinder; direction (ii): the collection of biogas from measuring cylinder to syringe. Silicon tube and syringe were flushed with argon gas.

The effect of initial pH was examined by adjusting the fermentation medium to pH 4, 5, 6, 7, 8 using 5 M NaOH or HCl respectively. The final pH was determined by filtering 5 mL of reaction medium through 0.20 μm millipore filter following by pH measurement using a pH meter (Hanna instruments, HI991001).

In order to investigate the effect of control pH, neutralization with NaOH was adopted from Yang et al. (147) with modifications. The pH control in batch fermentation was conducted in media containing 10 and 20 g/L glucose. The media were spiked with 0.5 mL of 5 M NaOH at specific time intervals as follows and then followed by 48 hours of incubation.

i. Single neutralization at 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 and 24 h
ii. Continuous neutralization at 12 h intervals at 12, 24 and 36h

Batch fermentation with pH control was also conduction in the absence of acetate.

The temperature effects on the hydrogen production were studied by operating batch fermentation at 25, 30, 37, 45, and 50ºC. The effects of glucose concentration against the H_2 yield were investigated by using different substrate concentration of 3, 5, 10, 15, 20 and 25 g/L.

3.1.4. Preparation of fermentation medium

Six media (A, B, C, D, E, & to F) with different composition were used as fermentation medium to test the best medium suitable for hydrogen production. The suitable media were selected based on hydrogen production. The composition of the media was summarized as follows and the detail of the composition was listed in Table 3.2:

1. Medium A contained a double-phosphate buffer system;
2. Medium B contained a single-phosphate buffer system;
3. Medium C and D contained a phosphate buffer system with different varieties of metals;
4. Medium E contained acetate buffer system and it is a commercially produced semisolid medium specialized for Clostridial as well as other anaerobes;
5. Medium F was modified from Medium E with similar composition excluding ‘Lab-Lemco’ Powder (beef extract), soluble starch and agar.

Since media E is a commercial product and its content was unable to modify, the pH of the other media was adjusted to pH 6 ± 0.05, in accordance to the measured pH of Media E, using 5M NaOH and 5M HCl. A volume of 150 mL of media was added to 200 mL serum

bottle and purged with argon gas for 2 minute. Fermentative media were subsequently autoclaved at 115 ºC for 15 min.

Table 3.2: Compositions of different fermentation media

Media	Composition (g/L)	Ref.
Medium A (Double-phosphate buffer system)	Glucose, (5.0)*; peptone (5.0); K_2HPO_4 (14.0); KH_2PO_4 (6.0); $(NH_4)_2SO_4$ (2.0); trisodium citrate dehydrate (1.0); $MgSO_4$ (0.2.0)	(148)
Medium B (Single-phosphate buffer system)	Glucose (5.0)*, peptone (1.0), yeast extract (5.0), $NaHPO_4$ (0.1), NaCl (0.5)	(134)
Medium C (Double-phosphate buffer system with metals B)	Glucose (5.0)*; NH_4Cl (0.5); KH_2PO_4 (0.5); K_2HPO_4 (0.5); $NaHCO_3$ (4.0); $FeCl_2.4H_2O$ (0.15); $MgCl_2.6H_2O$ (0.085); $ZnSO_4.7H_2O$ (0.01); $MnCl_2.4H_2O$ (0.03); H_3BO_3 (0.03); $CoCl_2.6H_2O$ (0.02); $CaCl_2.2H_2O$ (0.01); $NiCl_2.6H_2O$ (0.02); $Na_2MoO_4.2H_2O$ (0.03)	(64)
Medium D (single-phosphate buffer system with metals A)	Glucose (5)*, peptone (2); KH_2PO_4 (0.1190); $CaCl_2.6H_2O$ (0.3232); $MgCl_2.6H_2O$ (0.2323); KCl (0.1688); $MnCl_2.4H_2O$ (0.0639); $CoCl_2.6H_2O$ (3.87×10^{-3}); H_3BO_3 (0.74×10^{-3}); $CuCl_2.2H_2O$ (0.35×10^{-3}); $Na_2MoO_4.2H_2O$ (0.33×10^{-3}); $ZnCl_2$ (0.27×10^{-3}); $FeCl_2.4H_2O$ (0.0106); sodium thioglycolate (0.02174)	(149)
Medium E (Reinforced Clostridial Medium)	Glucose (5.0)*; yeast extract (3.0); `Lab-Lemco' powder (10.0); peptone (10.0); soluble starch (1.0); sodium acetate (3.0); NaCl (5.0); cysteine hydrochloride (0.5); ager (0.5)	CM0149, Oxoid
Medium F (Modified Reinforced Clostridial Medium)	Glucose (5.0)*; yeast extract (3.0); peptone (10.0); sodium acetate (3.0); NaCl (5.0); cysteine hydrochloride (0.5)	N.A.

*Glucose concentration was standardized at 5 g/L

3.1.5. Analysis

i. Cell dry mass

Cell dry mass was determined by centrifuging the reaction medium at 5000 ×*g* for 15 minutes, washing twice with distilled water and drying at 105 ºC until three constant weights were gained.

ii. Substrate and product analysis

Glucose and metabolites concentration including acetic acid, butyric acid, lactic acid, formic acid, propionic acid and ethanol were analysed by using a high performance liquid chromatography system (1200 series, Agilent Technologies) equipped with a refraction index detection (RID) and Animex Hi-Plex H column (300 × 7.7 mm, Agilent). The column temperature and detector temperature were adjusted to 65 and 55ºC respectively.

Biogas produced was collected and measured using water displacement method. Subsequently, the yield and composition of biogas was analyzed via a gas chromatograph (GC) equipped with thermal conductivity detector (TCD) with 2.7 m Hayesep Q column packed with molecular sieve. Helium was used as carrier gas at a flow rate of 2 mL/min. Injector, detector and oven temperatures were kept at 100, 150 and 60ºC, respectively.

3.1.6. Kinetics study

a) Kinetic of H_2 production by modified Gompertz model

The modified Gompertz equation was used to fit the cumulative volume of H_2 produced over the time course (31, 143, 144):

$$\boldsymbol{H = H_{max}\left\{-e\left[\frac{R_{max}\cdot e}{H_{max}}(\lambda - t) + 1\right]\right\}} \qquad \text{(eq. 3.1)}$$

where H is the cumulative H_2 production (mol H_2/mol glucose), H_{max} is the maximum H_2 production (mol H_2/mol glucose), R_{max} is the maximum H_2 production rate (mol H_2/mol glucose/h), λ is the lag phase time (h) and t is the incubation time (h), and $\frac{R_{max}\cdot e}{H_{max}}$ represents the rate constant (k). The modified Gompertz equation was used to fit the cumulative H_2 data, using the OriginPro 8.5.

b) Kinetic of microbial growth by Logistic model

The Logistic model was used to predict microbial growth (144)

$$\boldsymbol{X = \frac{X_o e^{(K_c t)}}{1 - \left(\frac{X_o}{X_{max}}\right)\left(1 - e^{(K_c t)}\right)}} \qquad \text{(eq. 3.2)}$$

where X is the cell mass concentration (g/L); X_o is the initial cell mass concentration; X_{max} is the maximum cell mass concentration; K_c is the specific growth rate; and t is time (h). The data was plotted using the OriginPro 8.5.

3.1.7. Thermodynamic analysis

a) Activation enthalpy of fermentation and thermal deactivation

Enthalpy can be determined using Arrhenius approach based on the relationship between the reaction rate constant (*k)* and temperature (T):

$$k = Ae^{\left(\frac{-\Delta H}{R.T}\right)} \quad \text{(eq. 3.3)}$$

$$\ln k = \ln A - \frac{\Delta H}{RT} \quad \text{(eq. 3.4)}$$

Where ΔH is the activation enthalpy, A is the Arrhenius pre-exponential factor and R is the ideal gas constant (8.3144621 J/K/mol). However, Arrhenius equation is strictly limited to an elementary process and only correlates well to the rate measurements for a single reaction which is free of diffusion and thermal resistance.

Biological H_2 production involves complex enzymatic reactions. Hence, the reaction rate of biological H_2 production increases with temperature up to the threshold temperature (T_{opt}). At temperature higher than T_{opt} biological H_2 production is subjected to thermal deactivation due to denaturation of key enzymes and cell death. According to Fabiano and Perego (142), this situation can be represented by the modified Arrhenius equation as follow:

$$\ln H_{max} = \ln(A.X.Y) - \frac{\Delta H}{RT} \quad , T < T_{opt} \quad \text{(eq. 3.5)}$$

$$\ln H_{max} = \ln(B.X.Y) - \frac{\Delta H*}{RT} \quad , T > T_{opt} \quad \text{(eq. 3.6)}$$

Where H_{max} is the maximum H_2 productivity obtained from modified Gompertz equation, A and B are the Arrhenius pre-exponential factors, X is the cell mass concentration (g/L), Y is the H_2 yield per unit cell mass (mol H_2/ g cell mass), R is the ideal gas constant (8.3144621 J/K/mol) and T is temperature in Kelvin (K). In term of enthalpy, ΔH is the fermentation activation enthalpy whereas the thermal deactivation enthalpy (ΔH_d) is determined by

$$\Delta H_d = \Delta H + |\Delta H^*| \quad \text{(eq. 3.7)}$$

Thermal deactivation enthalpy represents the threshold energy for enzymatic denaturation and microbial death.

b) Activation entropy of fermentation and thermal deactivation

Activation entropy of fermentation and thermal deactivation can be determined using the following equation which derived from Eyring and Arrhenius equations (142)

$$\Delta S = R\left(ln\frac{Ah}{k_bT}\right) \qquad \text{(eq. 3.8)}$$

$$\Delta S_d = R\left(ln\frac{Bh}{k_bT}\right) \qquad \text{(eq. 3.9)}$$

Where A and B are the Arrhenius pre-exponential factors, h is the Planck's constant (6.63×10^{-34} J.s) and k_b is the Boltzmann's constant (1.38×10^{-23} J/K).

c) Gibbs free energy

Gibbs free energy is determined using the following equation

$$\Delta G = \Delta H - T\Delta S \qquad \text{(eq. 3.10)}$$

Where ΔH is the activation enthalpy obtained from equation (eq. 3.5) and ΔS is the activation entropy obtained from equation (eq. 3.8).

3.2. Results and discussion

3.2.1. Screening of fermentation media for hydrogen production

Five media composed of different buffering system were compared with respect to H_2 yield. The recorded H_2 yield and glucose consumption in each medium was presented in Figure 3.3. It was clearly showed that Media E and F were similar with the yield of 3.37 and 3.30 mol H_2/mol glucose, respectively. H_2 production from Media A and B were found to be less satisfaction with lower yield of 1.31 ± 0.14 and 1.12 ± 0.05 mol H_2/mol glucose respectively as compared to Media E and F. In contrast, H_2 was not detected from the established Media C and D employed in Chang et al. (64) and Lin et al. (149) studies. Furthermore, the recorded glucose consumption in Media C and D was 0%, indicates that substrate was not consumed for growth and H_2 production. Likewise, glucose consumption in Media A, B, E and F was recorded at 97, 94, 98 and 99% respectively which indicate substrates were utilized for H_2 production. In comparison with Media A, B, E and F, Media C and D consisted of additional metal salts such as iron (Fe), nickel (Ni), calcium (Ca) and molybdate (Mo) which were micronutrients and enzyme co-factors (48, 50, 63, 119, 122). Nonetheless, the addition of trace metals did not improve bacteria performance in producing

H_2. Hence, it is postulated that different buffering agents could cause the variation in H_2 production.

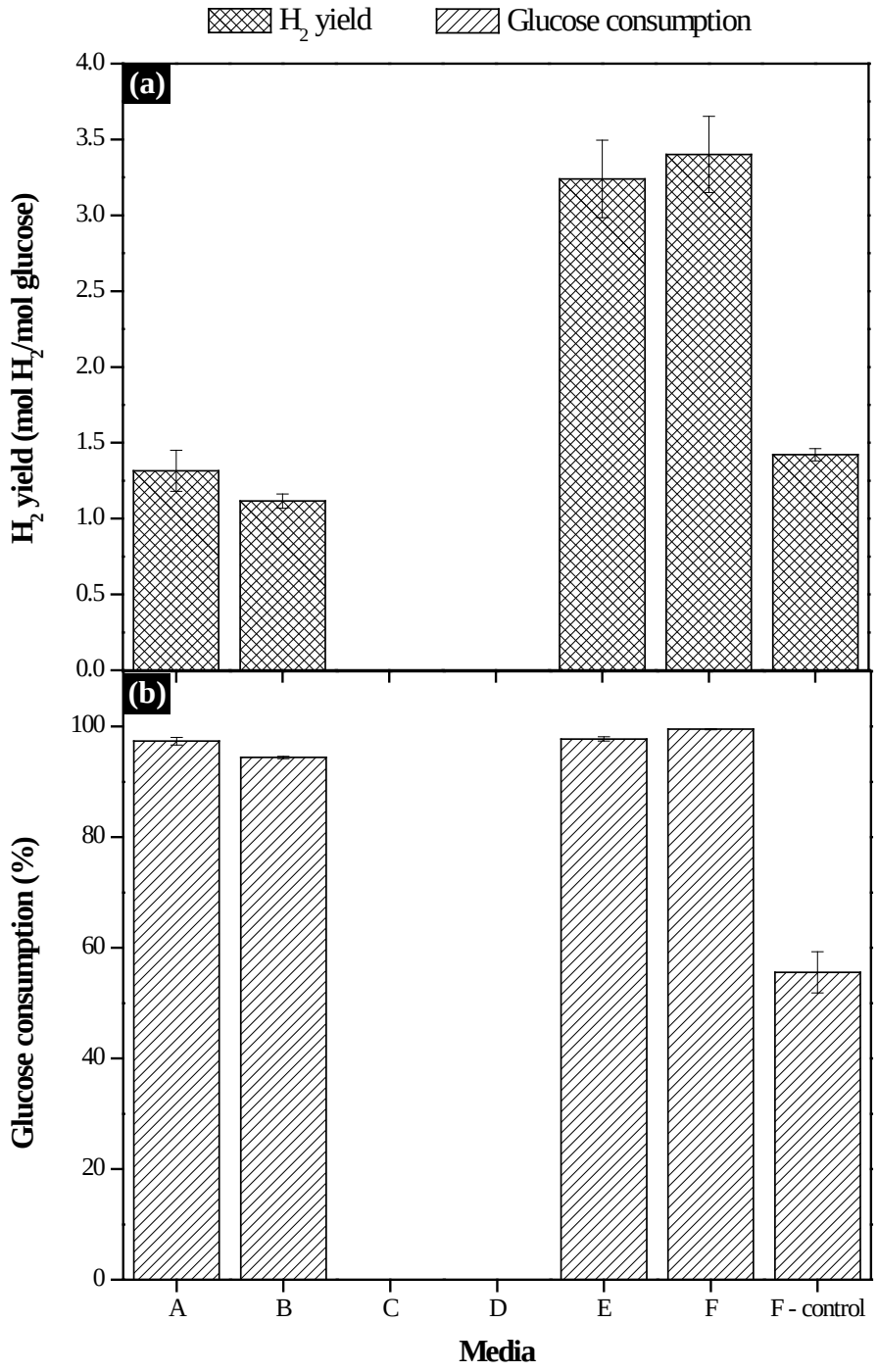

Figure 3.3: (a) Hydrogen production and (b) glucose consumption in media with different buffering system. Medium A and B composed of phosphate buffering system; Medium C and D composed of phosphate buffering system with various trace metal content; Medium E and F composed of acetate buffering system; Medium F-control was a positive control of Medium F but excluding sodium acetate as the buffer. (Fermentation condition: 5 g/L glucose, 37 ˚C, 48 h and pH 6)

To understand the effect of buffering agents, the characteristics of buffering agent used in the media is summarized in Table 3.3. The calculated buffering capacities in the media fall in the typical buffer range of 0.01 – 0.0001 (150) except for Media D which has the value of 8.78×10^{-5} that was slightly outside this range. This indicates that the buffering system in Media D may not be functioning well. Phosphate buffer system used in Media A – D has a pK_a value of 7.2 (150). According to Henderson-Hasselbach equation, the acceptable buffer range lies around $pK_a \pm 1$. Hence, the phosphate buffering system in Media A – D were estimated to function appropriately in the pH between 6.2 and 8.2. In contrast, acetate buffer adopted in Media E and F has a pK_a value of 4.7, with an effective buffering range of 3.7 to 5.7. As the initial fermentation pH was adjusted to 6.0 ± 0.05 which is closer to the working pH range of sodium acetate buffer and hence it is more effective for pH maintenance. Likewise, phosphate buffer covers buffer region at a higher pH might be the plausible reason of low H_2 yield in Media A – D because it could not withstand the pH drop during fermentation. To further confirm the role of sodium acetate, Medium F without sodium acetate was included as a positive control and the recorded H_2 yield was reduced by 58% as compared to Medium F. This proves that acetate buffer plays a significant role in buffering the pH change during fermentation.

Table 3.3: Characteristics of buffering agents in different fermentation media

Media	Buffer type	pK_a	Buffering pH range [a]	Buffering capacity [b]
A	Phosphate	7.2	6.2 – 8.2	1.60×10^{-2}
B	Phosphate	7.2	6.2 – 8.2	1.07×10^{-4}
C	Phosphate	7.2	6.2 – 8.2	8.41×10^{-3}
D	Phosphate	7.2	6.2 – 8.2	8.78×10^{-5}
E	Acetate	4.7	3.7 – 5.7	3.83×10^{-3}
F	Acetate	4.7	3.7 – 5.7	3.83×10^{-3}

[a] Buffering range was determined by the formula: $pH = pK_a \pm 1$
[b] Buffering capacity was calculated using the Van Slyke equation (151)

Other than using sodium acetate as effective buffering agent, Media E and F were composed of of yeast extract, peptone, glucose, sodium chloride and L-cysteine hydrochloride hydrate. This nutrient formulation is rather simple as compared to Media A – D which included various trace metals. Organic nitrogen was supplied by yeast extract and peptone which contain various free amino acids could be used as building blocks for cell

metabolism and other biochemical reactions (152). Glucose was the sole carbon source and it is well known as the preferred carbon sources by majority of bacteria. Cysteine hydrochloride acted as a reducing agent which reduced oxidation-reduction potential of the fermentation medium and fostered growth of anaerobic bacteria (153). Sodium chloride was the only trace metal in the medium. It is an enzyme cofactor (154) and act as the conjugate base to the acetate buffering system. In comparison with Medium E, Medium F lacked of 'Lab-Lemco' powder, soluble starch and agar. As observed, the amount of H_2 produced from these media was comparable hence excluding these three components did not affect the overall H_2 performance. Thus, Medium F was desired to be used as the fermentation medium for subsequent optimization.

3.2.2. Effects of sludge pretreatment

The effect of sludge pretreatment on H_2 production performance is presented in Figure 1. The results are well fitted using the Modified Gompertz Equation with R^2 value of more than 0.98 (Table 3.4). According to Gompertz model, the maximum H_2 yield of untreated sludge was only 3.02 mol H_2/mol glucose but drastically increased two fold to 6.43 mol H_2/mol glucose after it was pre-treated at 65˚C. It was clearly shown that there was an increased in H_2 yield with increasing pretreatment temperature up to 65˚C but reduced at higher temperature up to 95˚C. The results suggest that sludge pretreated at 65˚C is the best temperature to enrich H_2-producing bacteria, producing twice as much H_2 yield as untreated sludge. This indicates that heat pretreatment has successfully eliminated H_2-producing bacteria and enriched H_2-producing bacteria. Landfill leachate sludge is originated from landfill that is very active in biological decomposition. Thus, it contains diverse microbial community which can be categorized as H_2-consuming and H_2-producing bacteria. The difference between H_2-consuming and H_2-producing bacteria is that majority of H_2-producing bacteria sporulate at high temperature. Sporulation protects H_2-producing bacteria, such as genus *Clostridium*, from heat damage. Likewise, H_2-consuming bacteria which do not form spores are prone to cell lysis at high temperature. This observation is with agreement the study conducted by Baghchehsaraee et al. (31), which they reported that H_2 produced from sludge pretreated at 65˚C was increased by 81% as compared to untreated sludge. It is also noteworthy that, the biogas produced from all heat-pretreated sludge only contained H_2 and carbon dioxide. There was no sign of methane in all samples throughout the batch fermentations which indicates that the system is free from methanogenic activities. Since

sludge pretreated at 65˚C produced the highest amount of H_2, the same condition is applied in the following optimization studies.

It is surprising that a heat-pretreated sludge was found to give high H_2 yield. One-might argue that the outstandingly high activity of the heat-pretreated sludge could be due to co-digestion effect between the sludge and the fermentation medium. Therefore, the co-digestion effect was investigated by measuring the weight difference of the sludge before and after fermentation. To measure the dried weight of sludge, sludge and cell biomass must be separated by centrifugation at 800 rpm for 5 min. It was reported that bacteria cell biomass pelleting at the speed of at least 4000 rpm for 15 min (Peterson et al., 2012, Tazehkand et al., 2008). Since sludge was heavier, centrifugation of the sludge-cell mixture at the minimal speed is sufficient to pelletize sludge while bacteria cell remained in the supernatant. This cycle was repeated for three times by rinsing the pelletized sludge with distilled water to maximize the cell removal. The measured sludge dried weight from 2 % v/v sludge inoculum for before and after fermentation was 0.5771 ± 0.0107 and 0.5751 ± 0.0130 g, respectively. This result demonstrated that there is no significant difference between the weight of sludge before and after fermentation, and thus co-digestion did not occur during fermentation. Furthermore, the productivity of H_2 at different inoculum volume of 1, 2, 5 and 10 % v/v was 3.25 ± 0.18, 3.40 ± 0.25, 3.30 ± 0.26 and 3.46 ± 0.23 mol H_2/mol glucose, respectively. This result shows that the inoculum volume does not affect the H_2 productivity under the same fermentation condition (pH 6, 48 h, 37 ˚C and 5 g/L glucose), which indicate that the compounds naturally present in the sludge inoculum did not co-digested for H_2 production. This is because if co-digestion took place during the fermentation, the H_2 yield will increase with inoculum volume. Therefore, it is concluded that the high H_2 productivity is contributed from the fermentation medium.

Table 3.4: Kinetic parameters of production H_2 in the effect of sludge pre-treatment from Modified Gompertz Equation

Initial pH	H_{max} (mol H_2/ mol glu.)	R_{max} (mol H_2/ mol glu./h)	k	λ (h)	$\mathbf{R^2}$
untreated	3.02 ± 0.05	0.22 ± 0.00	0.20 ± 0.02	9.82 ± 0.49	0.9960
40	3.27 ± 0.04	0.24 ± 0.00	0.20 ± 0.01	8.47 ± 0.41	0.9971
50	4.45 ± 0.09	0.29 ± 0.00	0.17 ± 0.02	8.44 ± 0.60	0.9943
65	6.43 ± 0.16	0.37 ± 0.00	0.16 ± 0.02	6.85 ± 0.73	0.9920

80	6.35 ± 0.22	0.34 ± 0.00	0.15 ± 0.02	6.97 ± 0.99	0.9862
95	4.28 ± 0.09	0.27 ± 0.00	0.17 ± 0.02	6.03 ± 0.68	0.9926

H_{max}, maximum H_2 production; R_{max}, maximum H_2 production rate; k, rate constant; λ, lag time

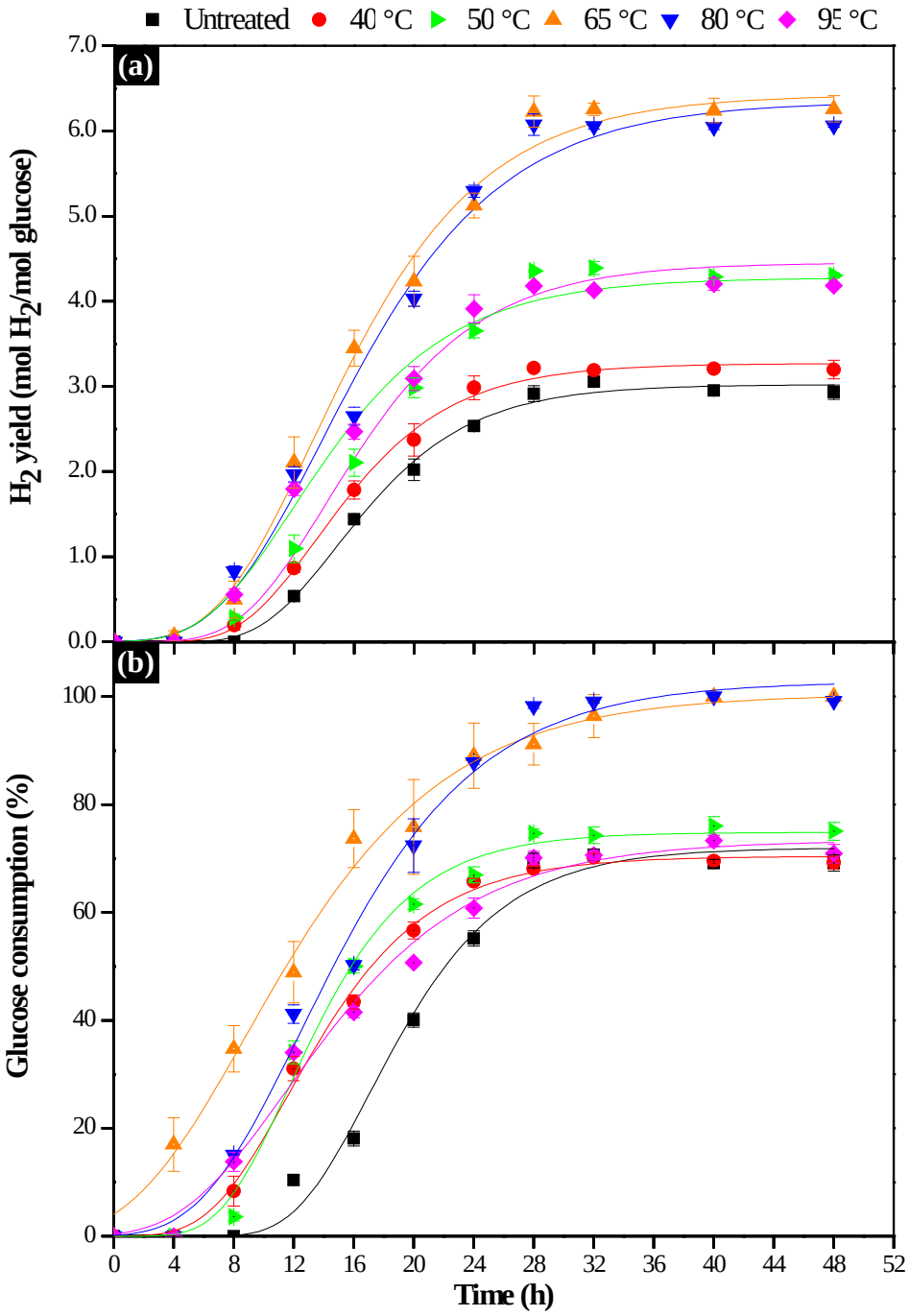

Figure 3.4: (a) Hydrogen production performance and (b) glucose consumption in the effects of pretreatment. Final pH for all conditions were measured at around 4.2 to 4.6. (Fermentation condition: 10 g/L glucose, 37 ˚C, 48 h and pH 6).

3.2.3. Effects of initial pH

The effect of initial pH on H_2 production performance is displayed in Figure 3.5. The results are well fitted using the Modified Gompertz Equation with R^2 value of more than 0.99 (Table 3.5). The maximum yield of 6.43 mol H_2/mol glucose was recorded at initial pH 6 with the maximum production rate of 0.37 ± 0.00 mol H_2/mol glucose/h at a lag time of 6.85 ± 0.73 h. In comparison, varied H_2 yield but comparable glucose consumption (100%) was observed at initial pH 8. At pH 8, H_2 production was reduced by 18% to 5.31 mol H_2/mol glucose but the measure cell dry mass was higher (2.512 ± 0.308 g/L) with the shorter lag time of 2.50 ± 0.60 h. This suggests that substrate was rapidly converted into cell mass at higher initial pH. This could be related to original growth pH for the bacteria as the measured pH of landfill leachate was around pH 8 (Section 3.1.1). Therefore, the recorded optimum initial pH for H_2 production and bacterial growth was varied. In contrast, at initial pH 4 H_2 production was completely inhibited with no measured cell mass whereas at initial pH 5 H_2 production was delayed for 16.78 ± 0.35 h with a reduced H_2 yield of 5.48 ± 0.15 mol H_2/mol glucose. This shows that H_2 production and bacterial growth were severely affected by acidic pH and the favourable pH for H_2 production occurred at the slightly acidic condition, pH 6. It has been reported that the functional pH for hydrogenase falls in the range of pH 6 – 6.5 (97, 98). Besides, this pH could suppress the growth of hydrogen consuming bacteria such as methanogens and acetobacteria (42, 61, 130, 155). In addition, pH of medium is the determinant factor of bacterial growth because it affects cell membrane surface charge which eventually influences nutrients absorptions and activities of enzymes (46, 94). Therefore, the optimum initial pH for H_2 production using landfill leachate sludge as inoculum is pH 6.

Table 3.5: Kinetic parameters of production H_2 in the effect of initial pH from Modified Gompertz Equation

Initial pH	H_{max} (mol H_2/ mol glu.)	R_{max} (mol H_2/ mol glu./h)	k	λ (h)	$\mathbf{R^2}$
4	0.00	0.0000	0.00	0.00	0.0000
5	5.48 ± 0.15	0.20 ± 0.00	0.10 ± 0.01	16.78 ± 0.35	0.9986
6	6.43 ± 0.16	0.37 ± 0.00	0.16 ± 0.02	6.85 ± 0.73	0.9920

7	6.22 ± 0.15	0.33 ± 0.00	0.14 ± 0.01	4.58 ± 0.74	0.9920
8	5.31 ± 0.09	0.34 ± 0.00	0.17 ± 0.01	2.50 ± 0.60	0.9928

H_{max}, maximum H_2 production; R_{max}, maximum H_2 production rate; k, rate constant; λ, lag time

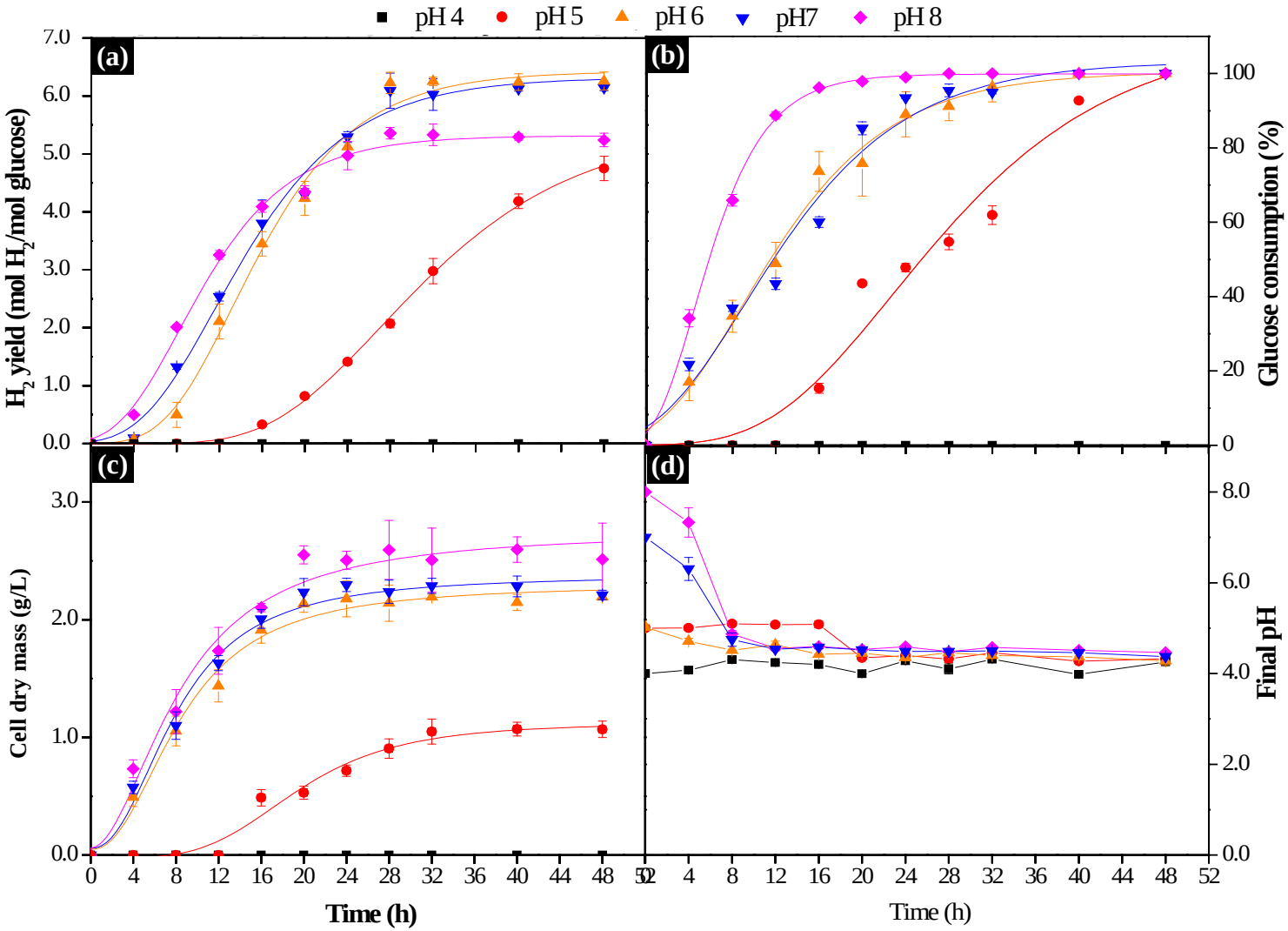

Figure 3.5: Effect of initial pH on (a) H_2 yield; (b) glucose consumption; (c) cell dry mass; and (d) final pH. (Fermentation conditions: 10g/L glucose, 48h and 37˚C)

3.2.4. Effects of fermentation temperature

The effect of fermentation temperature on H_2 production performance is displayed in Figure 3.6. The results are well fitted using the Modified Gompertz Equation with R^2 value of more than 0.97 (Table 3.6). The optimum temperature is recorded at 37˚C. At lower temperature, H_2 yield was reduced up to 66% with prolonged lag time up to 27.35 ± 2.82 h. In contrast at high temperature, H_2 yield was reduced by 31% at 45˚C and completely inhibited at 50˚C. It is observed that H_2 production is associated with microbial growth. The highest cell mass concentration was measured at 37˚C when H_2 production was at the maximum. Similarly, when cell growth was not detected at 50˚C, H_2 production was inhibited. This is a logic observation as biological H_2 is produce from enzymatic reaction within bacterial cells. The optimum temperature, 37˚C, falls in the mesophilic range (15 – 45˚C). This is because most of the H_2-producing bacteria are mesophiles such as *Clostridium* spp., *Enterobacter* spp., and *Bacillus* spp. which grow in this temperature range (53, 102, 103). This is in agreement with the literature which reported that 35 – 39˚C is the ideal range of temperature for H_2 production (31, 63, 81, 104). This suggests that HPB are only physiologically active in a narrow temperature range for H_2 production despite their ability to grow in a wide temperature range.

Table 3.6: Kinetic parameters of production H_2 in the effect of fermentation temperature from Modified Gompertz Equation

Temp.	H_{max} (mol H_2/ mol glu.)	R_{max} (mol H_2/ mol glu./h)	k	λ (h)	$\mathbf{R^2}$
25˚C	2.19 ± 0.14	0.05 ± 0.01	0.07 ± 0.03	27.35 ± 2.82	0.9717
30˚C	3.40 ± 0.14	0.15 ± 0.00	0.12 ± 0.01	11.92 ± 0.95	0.9909
37˚C	6.43 ± 0.16	0.37 ± 0.00	0.16 ± 0.02	6.85 ± 0.73	0.9920
45˚C	4.46 ± 0.08	0.33 ± 0.00	0.20 ± 0.02	8.21 ± 0.55	0.9953
50˚C	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.0000

H_{max}, maximum H_2 production; R_{max}, maximum H_2 production rate; k, rate constant; λ, lag time

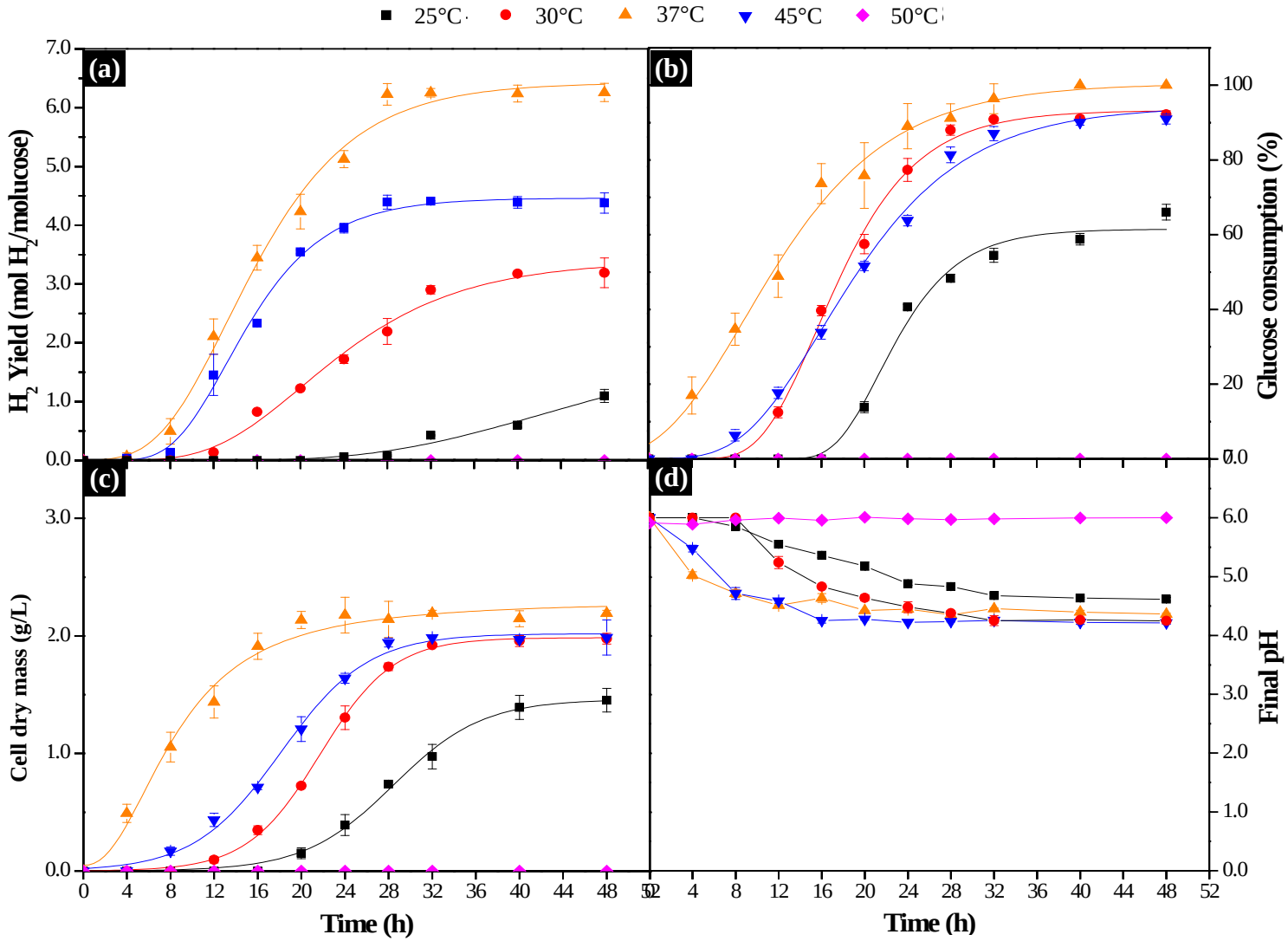

Figure 3.6: Effect of fermentation temperature on (a) H_2 yield; (b) glucose consumption; (c) cell dry mass; and (d) final pH. (Fermentation conditions: 10g/L glucose, 48h and 37˚C)

3.2.5. Thermodynamic study

Figure 4 represents the modified Arrhenius plot described by eq. 3.7 and 3.8 with a good regression of 0.9993 and 0.9099, respectively. Table 4 summarizes the thermodynamic parameters calculated from the modified Arrhenius model. The intersection point of the linear lines represents the optimum fermentation temperature which is 37.6˚C. The calculated Gibbs free energy is –34 kJ/mol. The negative sign represents that H_2 production is an exergonic reaction. This suggests that the high efficiency of H_2 production using landfill leachate sludge as inoculum is thermodynamically favorable.

The calculated activation enthalpy of fermentation (ΔH) is 68 kJ/mol suggests that H_2 production is an endothermic reaction. This was expected because many enzymatic reactions involve in microbial growth required energy from the hydrolysis of ATP (60, 156). Nonetheless, this value is still within the range for microbial growth (54 – 71 kJ/mol) (142) and for enzymatic reaction (18 – 83 kJ/mol) (72, 142). In contrast, activation enthalpy of thermal deactivation (ΔH_d) represents the threshold energy for enzymatic denaturation and microbial death. The calculated ΔH_d is 113 kJ/mol which is lower than the reported value 290–380 kJ/mol for microbial death (157). This indicates that H_2 production is sensitive to thermal deactivation in term of enzymatic reaction. Nonetheless, this value is similar to the ΔH_d for H_2 production reported by Fabiano and Perego (142) that is 118.1 kJ/mol.

In this study, the activation entropy of fermentation (ΔS) is 0.331 kJ/mol/K. This indicates that the H_2 production is a random reaction. In contrast, activation entropy of thermal deactivation (ΔS_d) is –0.586 kJ/mol/K. The negative sign represents a reduced randomness. This result is similar to those reported by Fabiano and Perego (142) and other enzymes such as cytochrome (158). Interestingly, this is contradicted with the other literatures which reported that deactivation of enzymes increases the randomness of a system ($+\Delta S_d$). However, the significance of negativity was not explained in the literatures (158, 159).

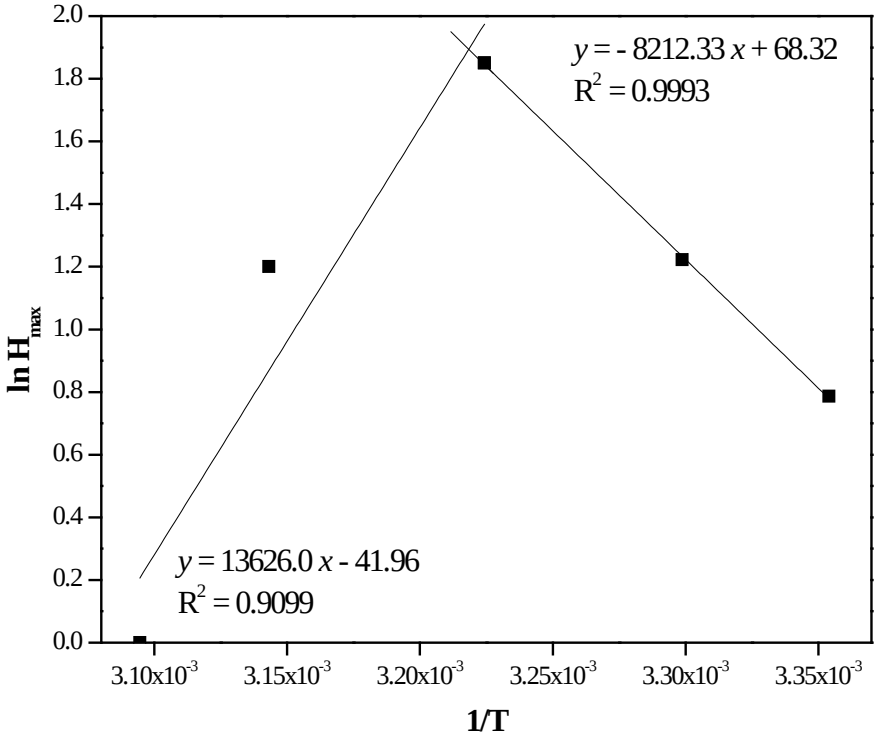

Figure 3.7: Modified Arrhenius plot for the evaluation of enthalpies and entropies. The intersection point of the linear lines represents the optimum fermentation temperature

Table 3.7: Thermodynamics quantities of H_2 production using landfill leachate sludge inoculum at optimum condition

Thermodynamic parameter	**Values**
Gibbs free energy (kJ/mol)	– 34
Activation enthalpy of fermentation (kJ/mol)	68
Activation entropy of fermentation (kJ/mol/K)	0.331
Activation enthalpy of thermal deactivation (kJ/mol)	113
Activation entropy of thermal deactivation (kJ/mol/K)	– 0.586

Reference temperature: 310.15 K. (37ºC)

3.2.6. Effects of substrate concentration

The effect of varying substrate concentration on H_2 fermentation performance is shown in Table 3.8. The maximum H_2 yield was exhibited by glucose concentration of 10 g/L. Apparently, same amount of H_2 (0.19 ± 0.03 mmol) was still being detected even no glucose was supplied to the fermentation (0 g/L glucose). This is because the bacteria might be utilizing nitrogen source to generate trace amount of H_2. However, this amount is negligible because it is only represents 0.35% yield. In contrast, there was an increased in H_2 yield with substrate concentrations from 0 to 10 g/L glucose. Increasing glucose concentration improves substrate availability. Substrate, in this case glucose, is the precursor for subsequent metabolites formation and H_2 production via decarboxylation and proton reduction (35, 108). Therefore, increasing glucose concentration improves H_2 production significantly. However, at subsequent high glucose concentration up to 25 g/L, a reduction in H_2 production with a higher measured cell dry mass was observed. This suggests that substrate was rapidly converted into cell mass instead of H_2. Evidently, glucose was also less effectively utilized with the glucose consumption of 52–67%. Therefore, at higher substrate concentration, H_2 production is subjected to substrate inhibition.

Table 3.8: Effects of substrate concentration on H_2 yield, final pH, glucose concentration and cell dry mass

	Glucose conc. (g)	C:N	Hydrogen yield		Final pH	Glucose Consumption (%)	Cell dry mass (g/L)
			mol H_2 / mol glu.	mmol H_2			
1.	0	0.0	N.A.	0.19 ± 0.03	5.49 ± 0.01	0.00 ± 0.00	N.D. [a]
2.	5	1.0	3.40 ± 0.25	14.66 ± 1.08	4.44 ± 0.06	99.53 ± 0.09	1.22 ± 0.12
3.	10	2.5	6.43 ± 0.16	53.98 ± 1.51	4.20 ± 0.01	100.00 ± 0.00	2.19 ± 0.02
4.	10 [b]	0.0	N.D.	N.D.	N.D.	N.D.	N.D.
5.	15	4.0	3.60 ± 0.12	46.22 ± 1.58	4.39 ± 0.10	67.49 ± 2.02	2.36 ± 0.05
6.	20	5.0	3.08 ± 0.13	46.23 ± 1.61	4.17 ± 0.05	51.73 ± 3.76	2.54 ± 0.10
7.	25	6.0	2.06 ± 0.10	42.94± 1.99	4.28 ± 0.02	52.61 ± 2.53	2.57 ± 0.04

N.A., Not available; N.D., Not detected

[a] Upon the end of fermentation, the media turned slightly cloudy but cell dry mass was too small to be measured.

[b] No nitrogen source was added to the optimum condition.

According to law of mass action, rate of fermentation increases with the substrates concentration (160). This phenomenon occurred when glucose concentration was increased from 5 to 10 g/L, the H_2 yield was doubled. At higher glucose concentration, the availability of glucose prompted the reaction towards product formation (in this case H_2 and organic acids production) in order to reach equilibrium. However, like other enzymatic reactions, maximum H_2 production rate increases only up to the optimum substrate concentration (160). It is observed that H_2 production was reduced by 68% when glucose concentration was elevated to 25 g/L. On the basis of law of mass action, it is reasonably assumed more organic acids will be produced along with H_2 production due to higher rate of glucose uptake. Nevertheless, higher substrate concentrations could induce a "shock load" during fermentation and eventually lead to product or feedback inhibition (161). As a consequence, feedback inhibition might be triggered in which glucose consumption is reduced. This causes chain effects that reduce the availability of subsequent metabolites, such as reduced-ferredoxin which is responsible for proton reduction, and hence inhibit H_2 production.

It is also important to note that nitrogen source plays an important role in H_2 production. When no nitrogen source was supplied to the fermentation, no sign of growth and production of H_2 were detected (Table 5, no. 4). The proportion of substrate and nitrogen in fermentation medium is measured by the ratio of carbon to nitrogen (C:N). The highest amount of H_2 was produced from the C:N of 2.5 (Table 5, no. 3). Ratio that deviates from 2.5 represents a disproportion of substrate and nitrogen which leads to reduction of H_2 yield. Despite H_2 yield was reduced, the measured cell dry mass was higher at ratio 4.0 – 6.0. This suggests that substrate was rapidly converted into cell mass instead of H_2 production and this was also observed by Bao et al. (136). The reduced H_2 yield could be related to the electron flow interference caused by the carbon-concentrated but nitrogen-limited growth. When electron flow was interrupted, metabolic pathways shifted to form more reduced compounds like butyrate and lactate. This observation is with agreement the studies conducted by Lin and Lay (115). Therefore, appropriate ratio, in this case C:N 2.5, diverts metabolic pathways to one which promotes H_2 production.

3.2.7. Effects of neutralization with NaOH on H_2 production and glucose consumption

H_2 production from media containing higher substrate concentration can be improved by NaOH neutralization at specific time intervals. As shown in Figure 3.8(b), H_2 produced from 20 g/L glucose improved when the media was neutralized with NaOH. The highest yield was exhibited by neutralization at exponential phase (10 and 12 h) with the yield of 4.55 ± 0.12 and 4.56 ± 0.15 mol H_2/mol glucose respectively. It is interesting to note that the H_2 yield did not improve even after neutralization from 14 h onwards. Without NaOH neutralization in 20 g/L glucose, substrate consumption was recorded at 46% whereas with neutralization, the percentage of substrate consumption was increased accordingly. In contrast, H_2 produced from 10 g/L glucose was not influenced by NaOH neutralization and substrate consumption was recorded at 53% by 12h and completely consumed by 48h (Figure 3.9). The final pH in all fermentations were recorded in the range of 4.2 – 4.4. The variation in H_2 production can be explained by acid crash. Acid crash occurs when the medium pH is too low which in turn leads to deactivation of key enzymes, inhibition of H_2 production and cell growth. In this study, acid crush was not observed at 10 g/L glucose but at 20 g/L glucose. It is observed that acid crush can be prevented by NaOH neutralization between 10 – 12 h but not after 12 h. This result is agreed well with the previous report (147) that acid crash can be prevented by neutralization at exponential phase. Failing to control pH at the specific time frame induces permanent inhibition effect on cell growth. In addition, adequate substrate concentration prevents acid crash (162). This is also consistent with the results in this study in which H_2 yield produced from optimum substrate concentration (10 g/L glucose) was not influenced by neutralization. Therefore, NaOH neutralization in media containing higher substrate concentration is essential to prevent acid crash that inhibits H_2 production.

To further improve substrate consumption and H_2 production, continuous neutralization was conducted in a 12 h interval. As shown in Figure 3.10, continuous neutralization improved glucose consumption 100% which in turn enhanced H_2 production. It is observed that final pH from continuous neutralization was in the range of 5.11 to 5.41 which is higher than controls. This observation is logical because neutralization at 12 h resets the starting point of fermentation which means 24 h marks the second exponential phase. However, the higher final pH suggested that the third exponential phase (36 h) was not essential because the remaining substrate could be little and hence organic acids and H_2 production were slowing down or stopped. This shows that continuous neutralization is important to improve substrate consumption and H_2 production but the frequency can be varied depending on the remaining substrate in the medium.

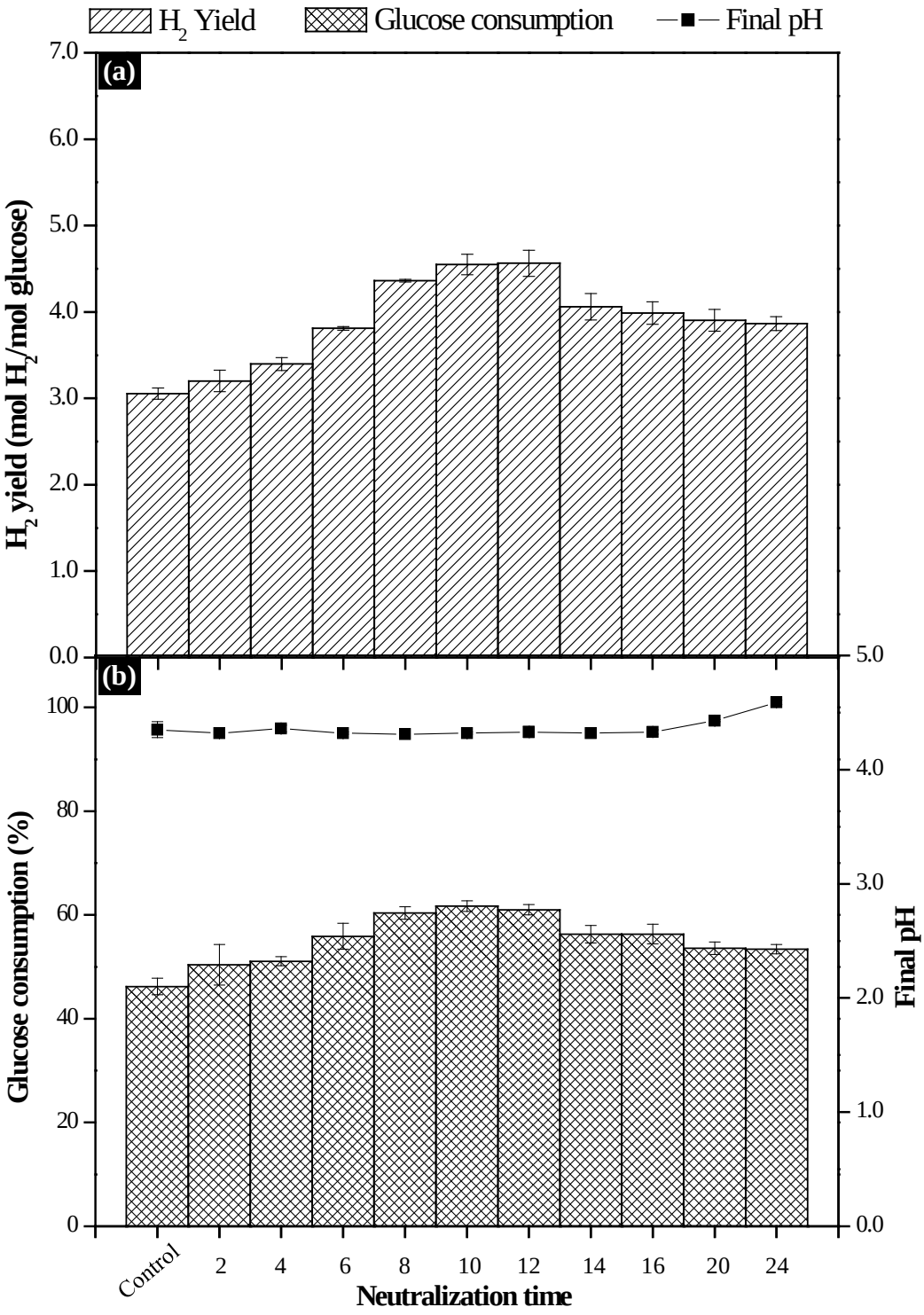

Figure 3.8: Effect of NaOH neutralization on substrate concentration 20 g/L glucose at specific time intervals (a) H_2 production performance and (b) glucose consumption and final pH. Control represents no neutralization took place. (Fermentation condition: 20 g/L glucose, 48h and NaOH neutralization at specific time intervals).

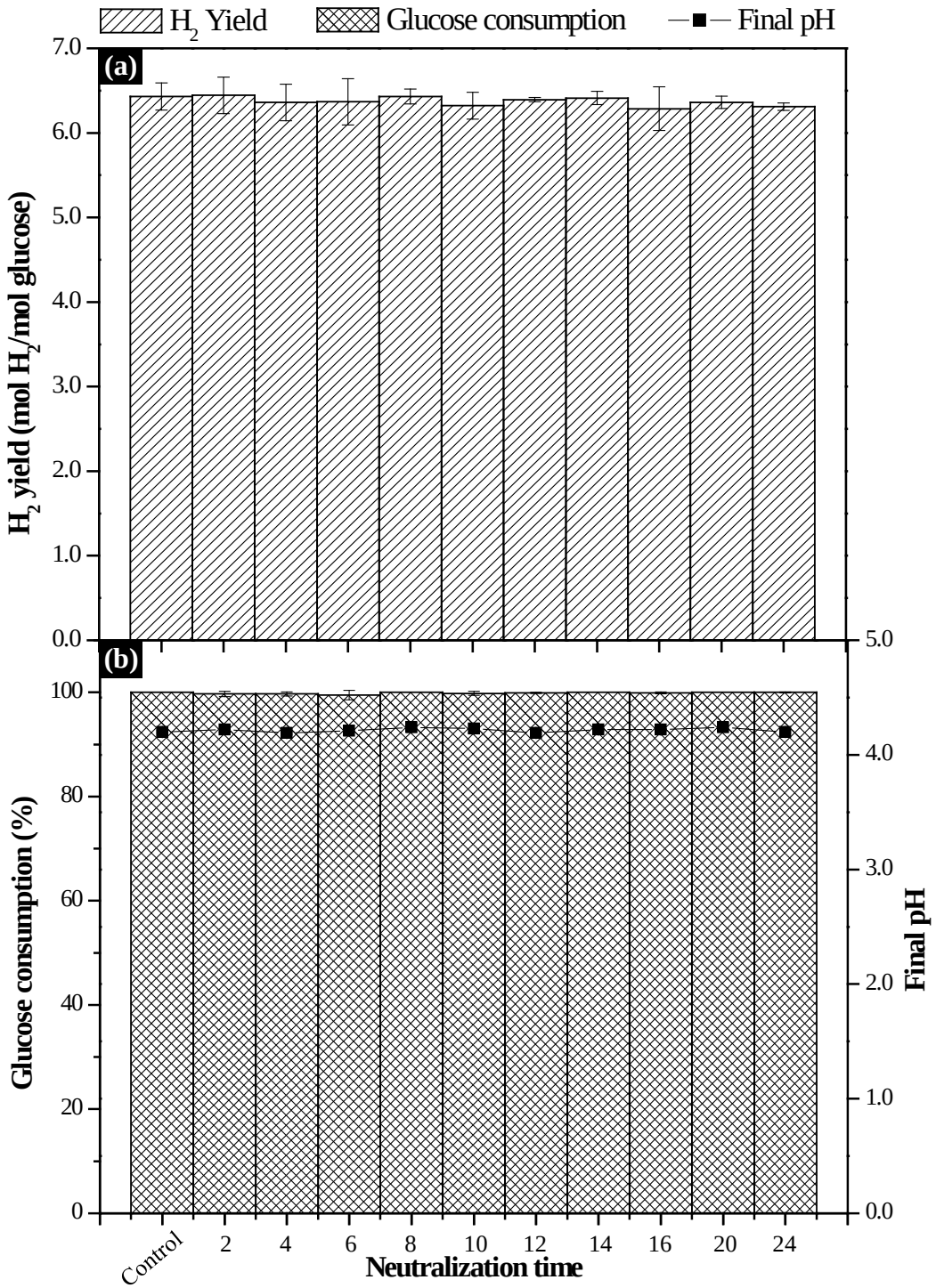

Figure 3.9: Effect of NaOH neutralization on substrate concentration 10 g/L glucose at specific time intervals (a) H_2 production performance and (b) glucose consumption and final pH. Control represents no neutralization took place. (Fermentation condition: 10 g/L glucose, 48h and NaOH neutralization at specific time intervals).

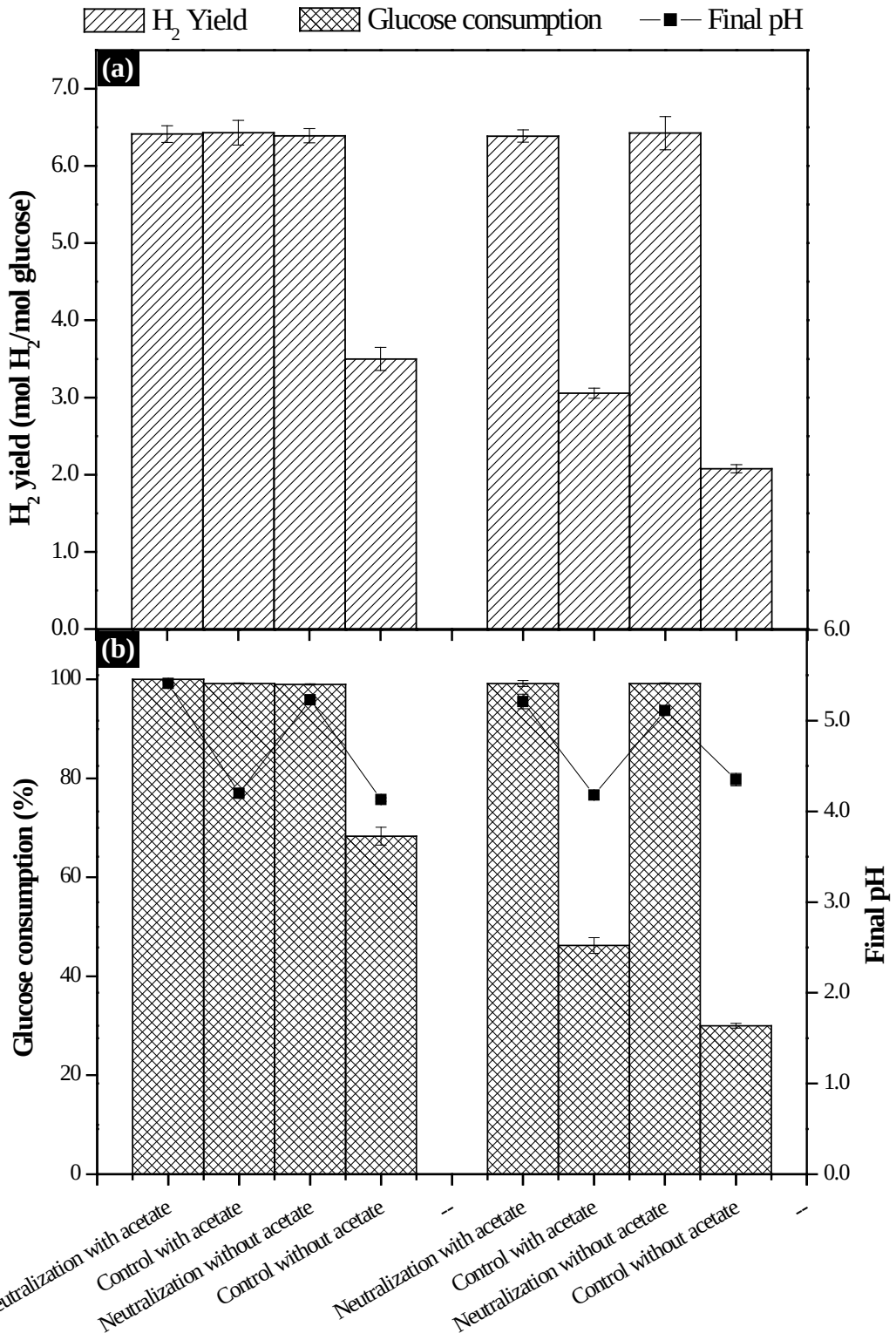

Figure 3.10: H_2 production performance with and without acetate in the effect of continuous NaOH neutralization at 12 h intervals. Control represents no neutralization took place. (Fermentation condition: 10 and 20 g/L glucose, 48 h and NaOH neutralization at specific time intervals).

3.2.8. Formation of volatile fatty acids and solvents

Profile of volatile fatty acids (VFAs) and solvents at different fermentation conditions are shown in Table 3.9. The predominant VFAs produced from all fermentation conditions were acetate and butyrate, accompanied with a small amount of lactate (less than 20 mmol/L). Other VFAs and solvent including formate, propionate and ethanol were not detected. The measured ratio of acetate to butyrate (Ace:But) for all fermentation conditions are summarized in Table 3.9. At optimum condition, the highest Ace:But ratio was 1.53. The efficiency of H_2 production was commonly monitored by the Ace:But ratio (31, 40, 56, 60, 78, 81). Hydrogen production is conveniently represents by the acetate and butyrate pathways (eq. 3.10 and eq. 3.11)

Acetate pathway: $C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2$ (eq. 3.10)

Butyrate pathway: $C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2$ (eq. 3.11)

Since acetate pathway produces higher amount of H_2 than butyrate pathway, a higher ratio from a mixed fermentation is normally representing the more efficient H_2 production.

It is remarkable that the maximum H_2 yield produced from this process is 6.43 mol H_2/mol glucose. Thus, the proposed theoretical stoichiometric equation of H_2 production from glucose based on the experimental Ace:But ratio is as follows:

$3.5\ C_6H_{12}O_6 + 12\ H_2O \rightarrow 14\ CO_2 + 26\ H_2 + 1.5\ CH_3COOH + CH_3CH_2CH_2COOH$ (eq. 3.12)

According to the proposed equation (eq. 3.12), the stoichiometry theoretical yield of H_2 is 7.43 mol H_2/mol glucose. Therefore, the maximum yield from this study represents about 87% of H_2 conversion efficiency. The remaining 13% of glucose might be converted into microbial biomass. Nonetheless, this is only a preliminary equation based on the ratio of butyrate to acetate ratio, glucose consumption and H_2 yield. We are undergoing further research into this newly discovered phenomenon.

Table 3.9: Profile of VFA and solvent with respective ratio of acetate to butyrate at different fermentation conditions

Conditions	Concentration (mmol/L)			Ace:But
	Lac	Ace	But	
Optimum	11.6 ± 2.1	117.1 ± 5.2	78.5 ± 4.3	1.53
pH 4	N.A.	N.A.	N.A.	N.A.
pH 5	21.4 ± 2.8	86.9 ± 11.2	80.5 ± 6.2	1.08
pH 7	14.6 ± 0.3	74.8 ± 8.3	85.5 ± 2.8	0.88
pH 8	16.2 ± 1.5	69.3 ± 4.9	71.8 ± 4.8	0.96
25ºC	4.3 ± 0.2	33.0 ± 2.1	25.7 ± 0.8	1.28
30ºC	4.1 ± 0.7	43.2 ± 1.7	37.0 ± 1.3	1.17
45 ºC	8.8 ± 0.2	87.9 ± 2.7	67.2 ± 1.6	1.31
50 ºC	N.A.	N.A.	N.A.	N.A.
0 g/L (C:N 0.0)	N.A.	N.A.	N.A.	N.A.
5 g/L (C:N 1.0)	4.0 ± 0.4	25.9 ± 4.8	29.2 ± 4.2	0.89
15 g/L (C:N 4.0)	11.6 ± 2.1	117.1 ± 5.2	78.5 ± 4.3	1.49
20 g/L (C:N 5.0)	8.2 ± 0.6	53.3 ± 1.0	35.4 ± 1.5	1.50
25 g/L (C:N 5.0)	7.3 ± 2.3	65.9 ± 6.0	44.0 ± 1.3	1.50

Abbreviation: N.A., Not available; Lac, lactate; Ace, acetate; But, butyrate; Ace:But, ratio of acetate to butyrate

No formate, propionate and ethanol were detected in all batch fermentations.

3.3. Conclusion

This batch mode optimization showed that H_2 production using landfill leachate sludge as inoculum achieved the maximum H_2 yield of 6.43 mol H_2/mol glucose with 100% of substrate consumption, under the conditions of 37ºC, pH 6.0 and 10 g/L glucose. This high H_2 yield is thermodynamically favourable with the Gibbs free energy, fermentation activation enthalpy and entropy of –34 kJ/mol, 68 kJ/mol and 0.3311 kJ/mol/K, respectively.

Investigation on the effect of pretreatment temperature revealed that 65˚C was the most effective temperature to enrich H_2 producing bacteria. H_2 production using pretreated landfill leachate sludge was found to occur in a wide range of pH which covers from slightly acidic to alkaline condition (pH 5 to 8) with the optimum pH of 6.0. In addition, H_2 production was observed in the mesophilic range, from 25 to 45ºC suggests that H_2 producing bacteria presented in the sludge are mesophiles. Increasing temperature was found to increase the overall performance with maximum H_2 yield obtained at 37ºC but further temperature increment up to 50ºC inhibited H_2 production. Study on the effect of glucose concentration showed that H_2 yield was significantly improved with increasing substrate concentration with optimum level reached at 10 g/L glucose. The results present in this paper require further studies to reveal the mechanism in which high efficient H_2 production was achieved. Nonetheless, these results provide a new insight in biological H_2 production.

Chapter 4

Comparison of microbial communities at different fermentation phases of hydrogen production using new generation sequencer

The work presented in this chapter has been partly submitted for peer review:

WONG, Y. M., GAN H.M., ADELINE TING, C.M. AUSTIN, JUAN, J. C. Comparison of microbial communities at different fermentation phase of hydrogen production using Illumina MiSeq. Applied and Environmental Microbiology. (Submitted).

Monash University

Declaration for Thesis Chapter 4

Declaration by candidate

In the case of Chapter 4, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Analysis and interpretation of data	100
Drafting and writing the publication	90

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
A/P Dr. Juan Joon Ching	Reviewed the publication	N.A.
Dr. Adeline Ting	Reviewed the publication	N.A.
Dr. Gan Han Ming	Reviewed the publication	N.A.
Prof. Chris M Austin	Reviewed the publication	N.A.

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	Date 9 Sept 2014
Main Supervisor's Signature	[redacted]	Date 9 Sept 2014

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

4.0. Introduction

Biological H_2 is a renewable energy which can be produced from dark fermentation. Dark fermentation produces H_2 in the absence of light (13). Hence, it does not require solar input and the configuration of the bioreactor is simpler and cheaper (14). Dark fermentation can use a versatile range of substrate, particularly renewable resources that are organically rich such as stillage, sludge, leachate, pomace, stalks and bagasse (15-20, 163). Therefore, H_2 production via dark fermentation can potentially to be integrated into waste management and to achieve the ultimate goal of waste into energy.

The key player in dark fermentation is H_2-producing bacteria. Particularly, H_2-producing microbial community which naturally subsists in wastewater sludge is a convenient source of inoculum for fermentative H_2 production. This leads to the emphasis of our study on landfill leachate sludge. Landfill is commonly understood as a facility built to dispose municipal waste. Bacteria which survived in such environment usually have a better adaption to harsh living conditions such as poor nutrients, extreme temperature and pH. To survive in such conditions, the microbial community usually has special synergistic interactions to improve food and nutrients availability through decomposting organic matters. With these features, the microbial community could possess unique H_2 production performance which is not yet to be discovered. Therefore, it is crucial to analyse the relationship between microbial community and H_2 production.

Many studies analysed the microbial community with the conventional method coupling PCR of 16S rRNA, denaturing gradient gel electrophoresis (DGGE) and Sanger sequencing (31, 36, 49, 53, 57, 60, 61, 131, 139, 164-168). However, this approach has a low coverage in microbial community, time consuming process and non-reproducible method (169). These drawbacks can be overcomed with the advancement in the next generation sequencing (NGS). Furthermore, NGS provides new opportunities (1) to investigate the diversity and composition of microbial communities in depth with a high sample throughput (170) and (2) to elucidate low abundance microbial community in rare biosphere with a better sensitivity (171-173). Currently, the most common NGS methods used to study the microbial community in biogas productions are 454 pyrosequencing and SOLiD™ (sequencing by oligo ligation and detection) (174-178). Biological H_2-producing microbial communities have not been analysed by Illumina Mi-Seq yet. It was reported that the sequencing cost of Illumina for every megabase is 50- and 12,000-fold cheaper than 454 pyrosequencing and

Sanger sequencing, respectively (169). Moreover, this approach adopted paired-end sequencing which is rapid, comprehensive, and reproducible.

In this study, we have analysed the role of microbial diversity in biological H_2 production from glucose using landfill leachate sludge as inoculum with Illumina Mi-Seq. This study provides a better understanding on the relationship between the effect of microbial diversity and bio-H_2 production. This will enable better understanding on the effect of pre-treatment and selection of inoculum in industrial application.

4.1. Materials and methods

4.1.1. Inoculum and treatment conditions

The H_2-producing sludge inoculum was collected from a leachate collection pond located at the Jeram Sanitary Landfill in Selangor, Malaysia. Prior to its use, the sludge inoculum was sieved through a 400 μm screen and stored at 4˚C. In order to enrich the H_2 producing bacteria, the landfill leachate sludge was heat-pretreated at temperatures of 65˚C for 30 min. Subsequently, the pretreated sludge was enriched in Reinforced Clostridial Medium at 37˚C for 24 h. The enrichment is necessary to ensure the consistency of cell count inoculated into the bottles. The untreated inoculum was used as a control.

4.1.2. Experimental setup

Batch fermentations were conducted in triplicate, in 200 mL serum bottles containing 150 mL of fermentative media as report in our previous publication (179). The seed sludge was added to the serum bottles as inoculum with the concentration of 2% v/v with medium solution. The fermentation was conducted at the initial pH 6, 37°C and 10 g/L of glucose.

Repeated batch fermentation was conducted to examine the sustainability of H_2 yield. Inoculated sludge from the first batch fermentation was reused as the inoculum for the next fermentation and hence forth. To reuse the sludge, the media were centrifuged at 5000 rpm for 10 min upon the end of fermentation. The pellet was rinsed three times with saline to ensure no residue was carried forward to the subsequent fermentation. The pellet was re-suspended in saline in order to readjust the sludge concentration to 2% v/v. This recycled sludge was re-inoculated into the fresh medium to resume fermentation.

4.1.3. Analysis

Biogas produced was collected and measured using water displacement method. Subsequently, the yield and composition of biogas was analyzed via a gas chromatograph (GC) equipped with thermal conductivity detector (TCD) with 2.7 m Hayesep Q column packed with molecular sieve. Helium was used as carrier gas at a flow rate of 2 mL/min. Injector, detector and oven temperatures were kept at 100, 150 and 60ºC, respectively.

4.1.4. Genomic DNA extraction

Microbial communities in three different types of landfill leachate sludge sample were studied.

1. Untreated sludge – raw sludge collected from landfill leachate pond was used without treatment.
2. Pretreated sludge – landfill leachate sludge pretreated at 65ºC
3. Recycled sludge – reused sludge from the third cycle of fermentation

Bacterial genomic DNA in sludge was extracted using UltraClean Soil DNA Isolation Kit (MoBio Inc.) with an extra centrifugation step following each centrifugation step as per protocol.

4.1.5. 16S rRNA-metagenomics sequencing with next generation sequencing

The concentration of gDNA was quantified using Qubit® 2.0 Fluorometer. The V3 region on 16S rRNA was amplified using KAPA HiFi PCR kit with barcoded primer as follow:

Forward primer: 5'-AATGATACGGCGACCGACATCTACA-3'

Reverse primer: 5'-CAAGCAGAAGACGGCATACGAGATGA-5'

The amplification cycle was 95˚C for 3 min; 25 cycles of 98˚C for 20 s, 63˚C for 15 s, 72˚C for 5 s and 72˚C for 1 min. Upon the completion of PCR amplification, the amplified sequence with the size of approximately 300 bp was selected using 2% gel electrophoresis. The sequence was then recovered using the QIAquick Gel Extraction Kit (Qiagen). The extracted DNA sequence was dilute 10,000× with 0.05% Tween 20. Quantitative PCR (qPCR) was performed to quantify the concentration of amplified sequence at 95˚C for 5 min; 35 cycles of 95˚C for 30 s, 60˚C for 30 s, 72˚C for 10 s. The sample preparation and sequencing

kit used for sequencing were Nextera XT DNA Sample Preparation Kit and MiSeq Reagent Kits v2, respectively. Whole genome sequencing was performed using the Illumina MiSeq Benchtop Sequencer (2 × 150 bp paired-end sequencing).

4.1.6. Bioinformatics analysis

Sequence analysis and similarity search was conducted using USEARCH (180). Metagenome analysis was conducted using MEGAN5 (181). The taxonomy distributions were represented using Krona interactive visualization program (182).

4.2. Results and discussion

4.2.1. H_2 production from repeated batch fermentation

The H_2 produced using the pretreated sludge as inoculum in the first cycle was measured at 6.43 ± 0.16 mol H_2 / mol glucose, as compared to that of untreated sludge which was reduced to 3.02 ± 0.05 mol H_2 / mol glucose (179). Furthermore, the H_2 production using the pretreated sludge as inoculum in the second and third cycle was reduced to 2.99 ± 0.10 and 2.9 ± 0.33 mol H_2 / mol glucose, respectively (Figure 4.1). This shows that the H_2 production performance was reduced up to 55% and remained consistent in the subsequent cycle. According to Baghchehsaraee and colleagues (31, 60), the efficiency of H_2 production can be strongly related to the microbial diversity of the inoculum. It was claimed that the H2 yield increased with diversity of microbial community.

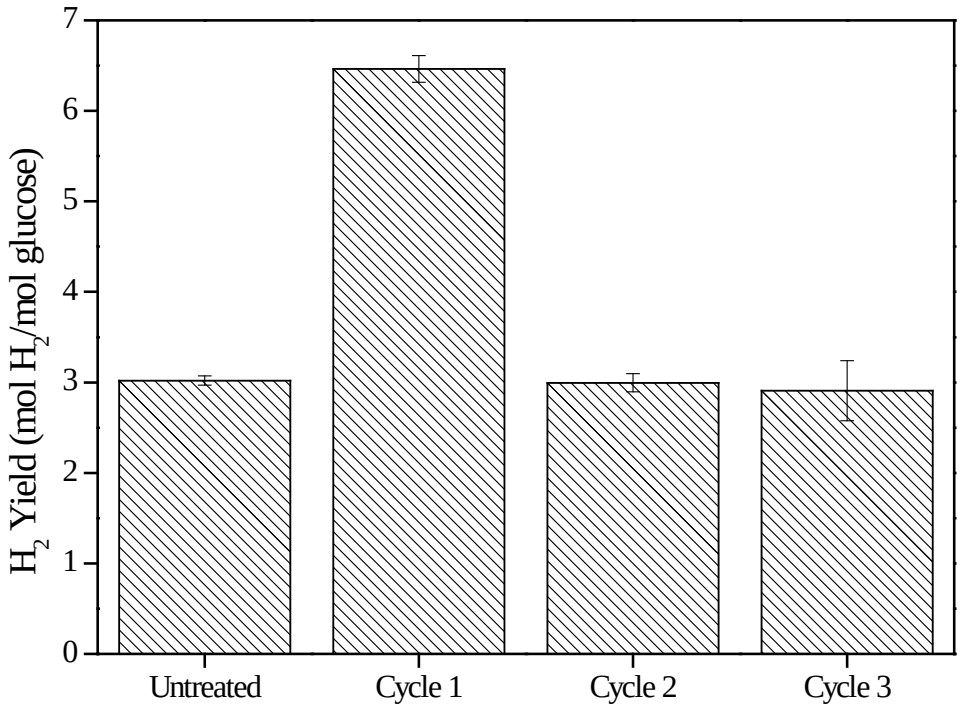

Figure 4.1: Hydrogen production performance on repeated batch fermentation using untreated sludge and pretreated sludge at 65˚C.

4.2.2. Relationship between microbial community and H_2 production from different sludge samples

Taxonomic distributions of the three sludge samples are display in Figure 4.2 and the distribution at genus level are listed in Table 4.1, 4.2 and 4.3. It is obvious that there is a vast difference in the three types of sludge samples.

In the untreated sludge (Figure 4.2 (a), Table 4.1), microbial community was very diverse. It harbored over 100 different families of bacteria but the most abundant were belonged to the family of H_2-consuming bacteria including Flavobacteriaceae (22%), Pseudomonadaceae (17%) and Helicobacteraceae (14%). The families of H_2-producing bacteria such as Clostridiaceae and Peptostreptococcaceae were only accounted for 6%. This suggests that huge amount of H_2-consuming bacteria have restricted the performance of H_2 production from the untreated sludge. Consequently, this revealed that H_2 yield is significantly clearly affected by the presence of H_2-consuming bacteria.

The H_2 production was significantly increased after heat pretreatment, which also accordance with previous reports (31, 60, 179). In this study, we found out that the microbial community in pretreated sludge was greatly reduced to less than 60 families but was

dominated by family Clostridiaceae (66%) and Peptostreptococcaceae (32%) (Figure 4.2 (b), Table 4.2). These families represent the source of H_2-producing bacteria (54, 183-185). For this result, we can conclude that the pretreatment method has successfully eliminated H_2-consuming bacteria and also enriched H_2-producing bacteria. The Illumina Mi-Seq revealed heat pretreatment is as an effective and simple method to enrich H_2-producing bacteria. The principle of this method is that H_2-producing bacteria such as the genus *Clostridia* survive from the heat pretreatment due to the sporulation characteristics (186). Likewise, H_2-consuming bacteria such as the genus *Pseudomonas* did not sporulate and easily deactivated under heat treatment. Therefore, H_2 yield improved by 53% due to enrichment of H_2-producing microbial community in the pretreated sludge.

In the recycled sludge, it is surprising that the H_2 production reduced from 6.43 ± 0.16 to 2.90 ± 0.33 mol H_2 / mol glucose. As shown in Figure 4.2 (c) and Table 4.3, the recycled sludge only contain limited diversity of H_2-producing bacteria community which mainly consist of 99% family Clostridiaceae The other families detected in the first cycle were no longer surviving in subsequent cycles. It is obvious that the diversity of microbial community has drastically reduced after several cycles of repeated fermentation. Microbial diversity can be reduced by the variation of doubling time of different H_2-producing bacteria. The fast growing bacteria has short doubling time. Hence, they will displace the slow growing bacteria and eventually dominate the microbial community in the recycled sludge. This evidenced that the diversity of H_2-producing microbial community might be the contributing factor for H_2 production. It has been reported that the diversity of H_2-producing microbial community assists high H_2 production performance due to the synergistic interaction between H_2-producing bacteria (31, 136, 164, 187-189). Furthermore, efficient H_2-producing bacteria in the recycled sludge might be outcompeted by the less efficient H_2-producing bacteria. This could also be another contributing factor for the reduced H_2 yield.

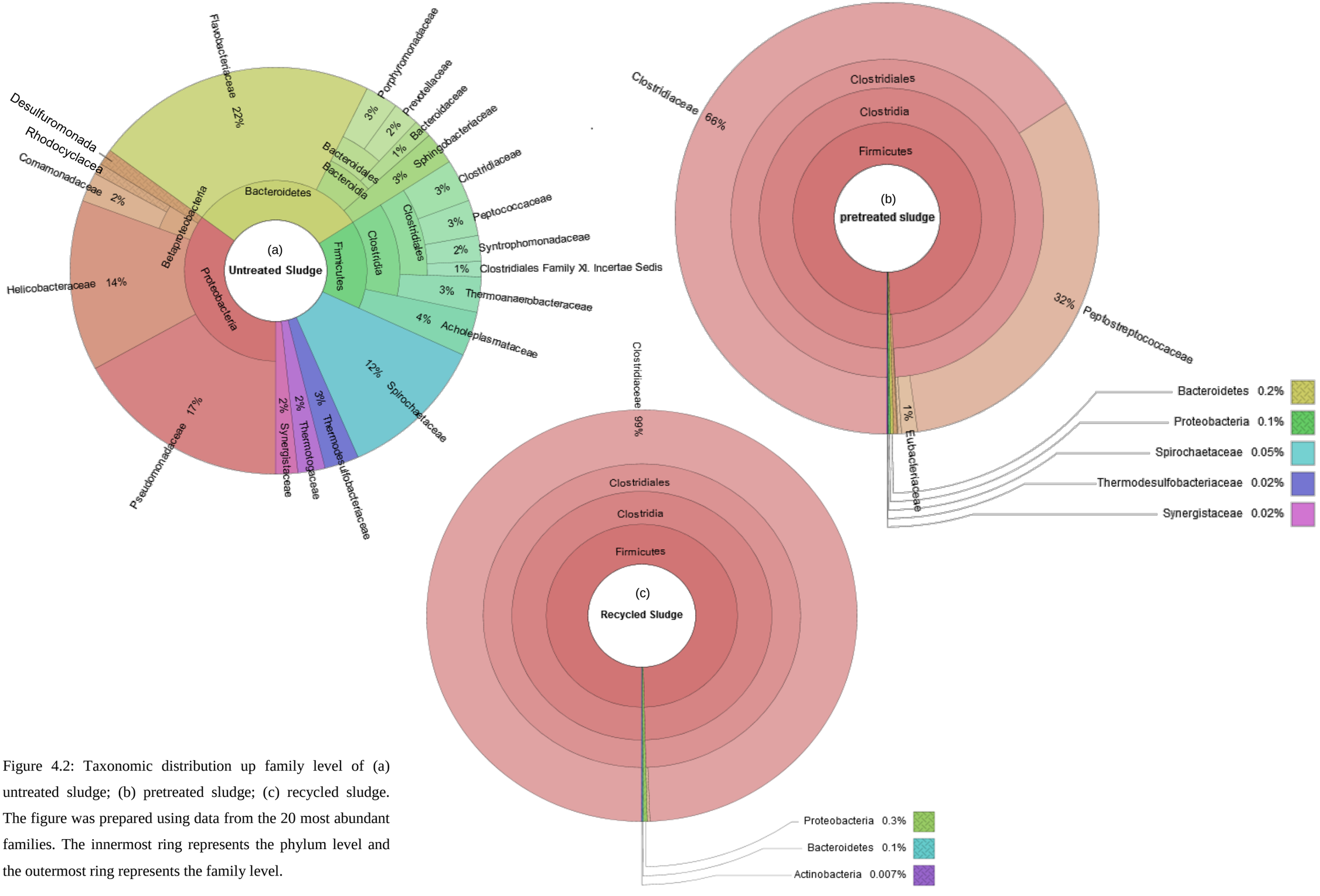

Figure 4.2: Taxonomic distribution up family level of (a) untreated sludge; (b) pretreated sludge; (c) recycled sludge. The figure was prepared using data from the 20 most abundant families. The innermost ring represents the phylum level and the outermost ring represents the family level.

Table 4.1: The 20 most abundant genera in untreated landfill leachate sludge

Family	Genus	Number of reads
Pseudomonadaceae	*Pseudomonas*	12848
Helicobacteraceae	*Sulfurimonas*	11325
Spirochaetaceae	*Treponema*	4101
Spirochaetaceae	*Sphaerochaeta*	3393
Flavobacteriaceae	*Capnocytophaga*	2656
Acholeplasmataceae	*Acholeplasma*	2611
Sphingobacteriaceae	*Parapedobacter*	2085
Thermoanaerobacteraceae	*Thermanaeromonas*	2060
Thermotogaceae	*Petrotoga*	1747
Syntrophomonadaceae	*Syntrophomonas*	1321
Porphyromonadaceae	*Proteiniphilum*	1259
Bacteroidaceae	*Bacteroides*	1215
Clostridiaceae	*Clostridium*	1201
Peptococcaceae	*Desulfotomaculum*	1157
Thermodesulfobacteriaceae	*Thermodesulfobacterium*	1110
Desulfobulbaceae	*Desulfuromonas*	1070
Thermodesulfobacteriaceae	*Caldimicrobium*	1061
Clostridiaceae	*Thermohalobacter*	880
Oceanospirillaceae	*Marinospirillum*	686
Cloacimonetes	*Candidatus Cloacimonas*	676

Table 4.2: The 20 most abundant genera in pretreated landfill leachate sludge

Family	Genus	Number of reads
Clostridiaceae	*Clostridium*	76400
Peptostreptococcaceae	Unclassified Peptostreptococcaceae	11400
Eubacteriaceae	*Eubacterium*	1603
Bacillaceae	*Bacillus*	364
Flavobacteriaceae	*Capnocytophaga*	124
Pseudomonadaceae	*Pseudomonas*	102
Syntrophomonadaceae	*Syntrophomonas*	61
Peptococcaceae	*Desulfotomaculum*	53
Acholeplasmataceae	*Acholeplasma*	49
Clostridiales Family XI. Incertae Sedis	*Soehngenia*	49
Thermoanaerobacteraceae	*Thermanaeromonas*	47
Spirochaetaceae	*Treponema*	46
Porphyromonadaceae	*Proteiniphilum*	43
Clostridiaceae	*Natronincola*	41
Prevotellaceae	*Prevotella*	40
Peptostreptococcaceae	*Sporacetigenium*	35
Clostridiaceae	*Caloramator*	32
Clostridiaceae	*Thermohalobacter*	29
Brevibacteriaceae	*Brevibacterium*	24
Helicobacteraceae	*Sulfurimonas*	21

Table 4.3: The 20 most abundant genera in recycled landfill leachate sludge

Family	Genus	Number of reads
Clostridiaceae	*Clostridium*	137368
Rhodobacteraceae	*Roseivivax*	112
Rhodobacteraceae	*Yangia*	82
Eubacteriaceae	*Eubacterium*	59
Pseudomonadaceae	*Pseudomonas*	52
Hyphomicrobiaceae	*Rhodoplanes*	51
Prevotellaceae	*Prevotella*	41
Clostridiales Family XI. Incertae Sedis	*Peptoniphilus*	36
Flavobacteriaceae	*Flavobacterium*	28
Bacteroidaceae	*Bacteroides*	20
Porphyromonadaceae	*Proteiniphilum*	18
Xanthobacteraceae	*Ancylobacter*	17
Acetobacteraceae	*Acidocella*	15
Sphingobacteriaceae	*Nubsella*	13
Xanthobacteraceae	*Xanthobacter*	11
Brevibacteriaceae	*Brevibacterium*	10
Ruminococcaceae	*Ruminococcaceae*	9
Clostridiaceae	*Caloramator*	8
Helicobacteraceae	*Sulfurimonas*	0
Spirochaetaceae	*Treponema*	0

4.2.3. Microbial community in landfill leachate sludge

A. Hydrogen producing microbial community

H_2-producing bacteria in landfill leachate sludge belong to four main genera, namely which are *Clostridium*, *Bacillus*, *Eubacterium* and *Sporacetigenium*, respectively (Table 4.2). Pretreated sludge was found to contain abundant genus *Clostridium.* This genus is reported as the most popular H_2 producers which is spore-forming obligate anaerobe (113). Generally, they are found in the environment rich in decaying plant materials. Therefore, they are capable of hydrolyzing a wide range of carbohydrates including monosaccharide, disaccharides, xylan, cellulose, starch, chitin, pectin and others (190). The main enzyme that is responsible for H_2 production is hydrogenase, which trigger H_2 production by proton reduction. Commonly, *Clostridia* spp. contain multi-subunits hydrogenase including the [FeFe] hydrogenases and [NiFe] hydrogenases. Three species of H_2-producing bacteria have been successfully isolated from the pretreated sludge, namely *C. perfringens* strain JJC, *C. bifermentans* strain WYM and *Clostridium* strain Ade.TY, which possess unique genomic characteristics and good H_2 production performance (191-193). Therefore, the abundance of *Clostridia* in landfill leachate sludge could be the key factor for the high H_2 production.

H_2 producers from other genus especially facultative species are less popular e.g genus *Bacillus* is a facultative H_2 producer. It is agreed that the presence of facultative bacteria in H_2-producing sludge act as the defence mechanism for strict anaerobic H_2-producer. Facultative H_2-producer is able to consume oxygen rapidly which accidentally enters the fermentation medium and recover the activity of anaerobic H_2-producer before the inhibition effect become permanent (89, 91, 92). In contrast, genus *Eubacterium* was found in H_2-producing sewage sludge (163) but the capacity of H_2 production from individual isolates was not reported. The plausible reason is that the identity of genus *Eubacterium* is often confused with other genus, typically saccharolytic species of *Eubacterium* share similar phenotypic features as *Clostridium*. Moreover, the genera *Eubacterium* and *Clostridium* are phylogenetically related (194). Another less common H_2 producer found in landfill leachate sludge belong to the genus *Sporacetigenium,* family Peptostreptococcaceae. They produce volatile fatty acids such as acetic and propionic acids (195, 196). Based on literatures, *S. mesophilum* strain ZLJ115T is the only studied H_2-producing strain (197). Although these two genera are less studied, their role in H_2 production using landfill leachate sludge appears to be important. As observed from Figure 2 and 3, the absence of these genera from families

Eubacteriaceae and Peptostreptococcaceae in the recycled sludge may be the contributing factor to the reduced H_2 yield.

B. Hydrogen consuming microbial community

In untreated sludge, the top three most abundant genera were H_2-consuming bacteria, belonging to genera *Pseudomonas*, *Sulfurimonas* and *Treponema*. Most of the bacteria from these genera utilize H_2 as the electron donor as their energy metabolism. Genus *Psuedomonas* and *Sulfurimonas* are facultative anaerobes that oxidize H_2 and donate electrons to either oxygen to form water or to produce ATP (114, 198, 199). In contrast, genus *Treponema* is obligate anaerobe which oxidizes H_2 with oxygen and nitrate forming hydrogen peroxide and nitrogen gas. They can also utilized H_2 and carbon dioxide as their sole substrate (200, 201). The H_2 consuming characteristics of these genera is undesirable in fermentative H_2 productions. Hence, the reduced H_2 yield from untreated sludge could be due to the high abundance of H_2-consuming bacteria.

4.3. Conclusion

Metagenomics by 16S rRNA using Illumina Mi-Seq has favourably shown the relationship of the microbial community in landfill leachate and the performance of H_2 production. In summary, sludge inoculum containing H_2-consuming bacteria such as genera *Pseudomonas*, *Sulfurimonas* and *Treponema* did not fovour H_2 production. Efficient H_2 production was only observed in sludge inoculum contained diverse H_2-producing bacteria including genera *Clostridium*, *Bacillus*, *Eubacterium* and *Sporacetigenium*. However, the high efficient H_2 production was unsustainable due to reduced diversity in H_2-producing bacteria and/or the high efficient H_2 producers were eliminated. Nonetheless, this study revealed the importance of microbial diversity that may improve H_2 production and hence contribute to industrial application.

Chapter 5

Production of bio-hydrogen from dairy wastewater using pretreated landfill leachate sludge as an inoculum

The work presented in this chapter has been partly submitted for peer review:

Wong YM, Wu TY, Juan JC. Production of bio-hydrogen from dairy wastewater using pretreated landfill leachate sludge as an inoculum. *International Journal of Hydrogen Energy*. (Submitted)

Monash University

Declaration for Thesis Chapter 5

Declaration by candidate

In the case of Chapter 5, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Analysis and interpretation of data	100
Drafting and writing the publication	95

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
A/P Dr. Juan Joon Ching	Reviewed the publication	N.A.
Dr. Wu Ta Yeong	Reviewed the publication	N.A.

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	Date 9 Sept 2014
Main Supervisor's Signature	[redacted]	Date 9 Sept 2014

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

5.0. Introduction

Hydrogen (H_2) is a potential alternative energy. The value of H_2 is increases with its non-polluting nature, as the complete combustion of H_2 produces water as the only end product. Hydrogen does not naturally exist as gas but more commonly found in combination with other elements and form a variety of compounds such as water and hydrocarbons (5). Hence, H_2 is an energy carrier that has to be extracted from other compounds. Currently, more than 96% of the global H_2 production requires fossil fuels as raw material or as source of energy (6, 7). Considering environmental problems arise from fossil fuels, H_2 derived from renewable sources is more environmentally friendly.

Bio-H_2 converts from wastewater using mixed microbial inoculum found in sludge materials is a sustainable approach for energy production. The current wastewater treatment incorporates biological process to breakdown organic compounds in wastewater (202). This approach is less feasible because energy is lost in the conversion of organic compounds to greenhouse gases such as carbon dioxide (203). To improve the sustainability of biological wastewater treatment, bio-H_2 production, typically using mixed microbial community found in various sludge can be integrated into wastewater management. Sludge is a byproduct from wastewater treatment (204), which contain abundant of bacteria that survive in the sludge. These bacteria may have unique features in production of bio-H_2, decomposition of organic materials and adaptation to harsh living conditions (135-137). Furthermore, these features represent the natural symbiotic interaction within the mixed microbial community in sludge, which might potentially enhance H_2 production (31, 63, 130, 138, 139). Therefore, bio-H_2 production is an alternative approach of converting waste into energy.

In this study, bio-H_2 was produced from dairy wastewater as organic feedstock using landfill leachate sludge as an inoculum. Dairy products are the important source of proteins, vitamins and minerals but they have a short shelf life. The expired products are often returned and then followed by biological treatment. Since the expired products are unsafe for human consumption, they provide good resources for bio-H_2 production. According to Venkata Mohan (65), sludge of a H_2 producing bioreactor produces 1.8 mmol H_2/g COD from dairy wastewater at optimum condition. Hence, dairy wastewater treatment has the potential to be integrated with bio-H_2 production. On the contrary, landfill leachate sludge is originated from a sanitary landfill and deposited in leachate treatment plant. From our previous literature (179), landfill leachate sludge has a great potential as the inoculum for bio-H_2 production with a high efficient H_2 yield of 6.43 mol H_2/mol glucose (179). In this study, we have

attempted to use the same sludge inoculum to investigate the effect of initial pH and temperature on bio-H_2 production from dairy wastewater on the basis of batch-dark fermentation. Thermodynamic analysis performed using the rate constant calculated from the modified Gompertz model and the activation and thermal deactivation of enthalpy and entropy as well as the Gibbs free energy were determined from the modified Arrhenius plot. The importance of this study represents the practical application of H_2 production from dairy wastewater using landfill leachate sludge as inoculum, as well as the theoretical investigation of process thermodynamics.

5.1. Materials and methods

5.1.1. Inoculum and treatment conditions

The H_2-producing sludge inoculum was collected from a leachate collection pond located at the Jeram Sanitary Landfill in Selangor, Malaysia. Prior to its use, the sludge inoculum was sieved through a 400 μm screen and stored at 4˚C. In order to enrich the H_2 producing bacteria, the landfill leachate sludge was heat-pretreated at temperatures of 65˚C for 30 min (179). Subsequently, the pretreated sludge was enriched in Reinforcement Clostridial Medium at 37˚C for 24 h. The enrichment is necessary to ensure the consistency of cell count inoculated into the bottles. The untreated inoculum was used as a control.

5.1.2. Wastewater collection

Fresh landfill leachate was collected from the same sanitary landfill as described in Section 3.1.1. Dairy wastewater was collected from a manufacturer of dairy products in Selangor which the company chooses to remain as anonymous. It was collected freshly from wastewater discharge point where hot wastewater is channelled to the treatment plant. The wastewater was not collected from wastewater treatment plant because it was constantly mixed treated wastewater and hence, it had diluted soluble carbohydrates and may contaminate with microfloral existing in the treatment plant. The characteristics of landfill leachate and dairy wastewater are summarized in Table 5.1. The mains substrate from dairy wastewater were composed of a mixture of glucose, sucrose, lactose and fructose which the total concentration was represented in total carbohydrate.

Table 5.1: Characteristics of landfill leachate and dairy wastewater

Parameters	Landfill leachate	Dairy wastewater
pH	8.27 ± 0.25	5.90 ± 0.26
Total COD (mg/L)	17617 ± 530	14150 ± 600
Soluble COD (mg/L)	10650 ± 450	9567 ± 293
Kjeldahl N (mg/L)	427.3 ± 7.4	305.7 ± 13.1
Soluble carbohydrate (g/L)	N.D.	84.63 ± 4.75
Soluble protein (g/L)	3.94 ± 0.12	3.16 ± 77

5.1.3. Experimental setup

Batch fermentations were conducted in triplicate, in 200 mL serum bottles containing 150 mL of dairy wastewater. The seed sludge was added to the serum bottles as inoculum with the concentration of 2% v/v with medium solution. H_2 production from dairy wastewater was tested in the effect of organic load (Dilution factor: 0, 20, 40, 60 and 80%), initial pH (4, 5, 6, 7, 8) and temperature (25, 30, 37, 45, 50˚C).

5.1.4. Analysis

Chemical oxygen demand (COD) in wastewaters was measured using Hach method 8000 with COD digestion reagent vials, high range (20 – 1500 mg/L) (Hach Co., 2014). Soluble carbohydrates in wastewater were measured using phenol/sulphuric acid method (205). Kjeldahl nitrogen was tested using Hach Total Kjeldahl Nitrogen Method 8075.

Soluble metabolites concentration including acetic acid, butyric acid, lactic acid, formic acid, propionic acid and ethanol were analysed by using a high performance liquid chromatography system (1200 series, Agilent Technologies) equipped with a refraction index detection (RID) and Animex Hi-Pex H column (300 × 7.7 mm, Agilent). The column temperature and detector temperature were adjusted to 65 and 55ºC respectively.

Biogas produced was collected and measured using water displacement method. Subsequently, the yield and composition of biogas was analyzed via a gas chromatograph (GC) equipped with thermal conductivity detector (TCD) with 2.7 m Hayesep Q column

packed with molecular sieve. Helium was used as carrier gas at a flow rate of 2 mL/min. Injector, detector and oven temperatures were kept at 100, 150 and 60ºC, respectively.

5.1.5. Kinetics and thermodynamic analysis

A. Kinetic of H_2 production by modified Gompertz model

The modified Gompertz equation was used to fit the cumulative volume of H_2 produced over the time course (31, 143, 144):

$$\boldsymbol{H = H_{max}\left\{-e\left[\frac{R_{max}\cdot e}{H_{max}}(\lambda - t) + 1\right]\right\}} \qquad \text{(eq. 1)}$$

where H is the cumulative H_2 production, H_{max} is the maximum H_2 production, R_{max} is the maximum H_2 production rate, λ is the lag phase time (h) and t is the incubation time (h), and $\frac{R_{max}\cdot e}{H_{max}}$ represents the rate constant (k). The modified Gompertz equation was used to fit the cumulative H_2 data, using the OriginPro 8.5.

B. Activation enthalpy of fermentation and thermal deactivation

Enthalpy can be determined using Arrhenius approach based on the relationship between the reaction rate constant (*k)* and temperature (T) (142):

$$k = Ae^{\left(\frac{-\Delta H}{R.T}\right)} \qquad \text{(eq. 2)}$$

$$\ln k = \ln A - \frac{\Delta H}{RT} \qquad \text{(eq. 3)}$$

Where ΔH is the activation enthalpy, A is the Arrhenius pre-exponential factor and R is the ideal gas constant (8.3144621 J/K/mol). However, Arrhenius equation is strictly limited to an elementary process and only correlates well to the rate measurements for a single reaction which is free of diffusion and thermal resistance.

Bio-H_2 production involves complex enzymatic reactions. Hence, the reaction rate of bio-H_2 production increases with temperature up to the threshold temperature (T_{opt}). At temperature higher than T_{opt} bio-H_2 production is subjected to thermal deactivation due to denaturation of key enzymes and cell death. According to Fabiano and Perego (142), this situation can be represented by the modified Arrhenius equation as follows:

$$\ln H_{max} = \ln(A.X.Y) - \frac{\Delta H}{RT} \quad , T < T_{opt} \qquad \text{(eq. 4)}$$

$$\ln H_{max} = \ln(B.X.Y) - \frac{\Delta H*}{RT} \quad , T > T_{opt} \qquad \text{(eq. 5)}$$

Where H_{max} is the maximum H_2 productivity obtained from modified Gompertz equation, A and B are the Arrhenius pre-exponential factors, X is the cell mass concentration, Y is the H_2 yield per unit cell mass, R is the ideal gas constant (8.3144621 J/K/mol) and T is temperature in Kelvin. In term of enthalpy, ΔH is the fermentation activation enthalpy whereas the thermal deactivation enthalpy (ΔH_d) is determined by

$$\Delta H_d = \Delta H + |\Delta H^*| \qquad \text{(eq. 6)}$$

Thermal deactivation enthalpy represents the threshold energy for enzymatic denaturation and microbial death.

C. Activation entropy of fermentation and thermal deactivation

Activation entropy of fermentation and thermal deactivation can be determined using the following equation which derived from Eyring and Arrhenius equations (142)

$$\Delta S = R\left(ln\frac{Ah}{k_bT}\right) \qquad \text{(eq. 7)}$$

$$\Delta S_d = R\left(ln\frac{Bh}{k_bT}\right) \qquad \text{(eq. 8)}$$

Where A and B are the Arrhenius pre-exponential factors, *h* is the Planck's constant (6.63×10^{-34} J.s) and k_b is the Boltzmann's constant (1.38×10^{-23} J/K).

D. Gibbs free energy

Gibbs free energy is determined using the following equation

$$\Delta G = \Delta H - T\Delta S \qquad \text{(eq. 9)}$$

Where ΔH is the activation enthalpy obtained from eq. 4 and ΔS is the activation entropy obtained from eq.8.

5.2. Results and discussion

5.2.1. H_2 production from landfill leachate and dairy wastewater

In the comparison of H_2 production capacity between landfill leachate and dairy wastewater, no gas production was detected from landfill leachate where as 15.78 ± 0.22 mmol H_2/ g COD (1.78 ± 0.02 mmol H_2/ g carbohydrate) was measured from dairy

wastewater Table 5.1 shows landfill leachate has high COD but does not contain soluble carbohydrate whereas dairy wastewater has a lower COD with 84.63 ± 4.75 g/L soluble carbohydrate. High COD is commonly encountered from landfill leachate because it contains complex organic materials such as humic and fulvic acids that are difficult to degrade (206). This suggests that H_2 cannot be produced from complex organic materials in landfill leachate. In contrast, dairy wastewater contains soluble carbohydrates in which HPLC analysis revealed that they are mixture of glucose, fructose, sucrose and lactose. These compounds are easily access by H_2-producing bacteria to produce H_2.

5.2.2. Effects of initial pH and fermentation temperature on H_2 production from dairy wastewater

Initial pH and temperature are the most important factors in bioprocesses as they influence the bacterial metabolism. The best H_2 production performance was observed at the optimum conditions of pH 6 and 37˚C. This falls within the reported range of pH (6 – 8) and temperature (35 – 39˚C) for H_2 production (31, 35, 42, 46, 53, 61, 63, 81, 94-96, 104). The results were well fitted using the Modified Gompertz Equation with R^2 value of more than 0.99 (Table 5.2). The recorded maximum yield at the optimum condition was 113.2 ± 2.9 mmol H_2/g COD (12.8 ± 0.3 mmol H_2/g carb.) at the maximum production rate of 3.73 ± 0.01 mmol H_2/g COD/h (0.42 ± 0.00 mmol H_2/g carb./h) with a lag time of 25.9 ± 0.8 h. The H_2 production from dairy wastewater using landfill leachate sludge as inoculum was more efficient as compared to other inoculums (Table 5.4). The efficient H_2 production is commonly related to the type and diversity of H_2-producing bacteria present in the inoculum. The performance could be efficient if the inoculum contains little or no H_2-consuming bacteria. In addition, H_2-producing bacteria belonging to the family of strict anaerobes Clostridiaceae has the greatest potential in H_2 production via dark fermentation (31, 52, 53), as compared to facultative H_2-producing bacteria such as the family of Enterobacteriaceae. Hence, the performance of H_2 production could be improved with a high diversity of strict anaerobic H_2-producing bacteria. As reported in our previous publication (179), the sludge originated from landfill shows great potential in H_2 production from glucose could be due to the diverse microflora in the inoculum.

Overall, the optimum initial pH for H_2 production is pH 6. At initial pH 8, H_2 production was reduced by approximately 22 % but the measured cell mass was 6.6 % higher with the shorter lag time of 24.2 ± 1.2 h. This shows that at higher initial pH, carbohydrate

was rapidly converted into cell mass. This observation is also well agreed with our previous study [16]. In comparison, no cell mass was measured at initial pH 4 and hence H_2 productions were completely inhibited. This shows that the favourable initial pH is 6 because H_2 production favourable under slightly acidic condition. This represents the functional pH for hydrogenase which is reported in the range of pH 6 – 6.5 (97, 98). It is also reported that this pH range suppresses the growth of hydrogen consuming bacteria such as methanogens and acetobacteria (42, 61, 130, 155). This suggests that H_2 production could enhance due to suppression of H_2 consumption process. On the contrary, medium pH could alter the membrane potential and intracellular pH of bacteria cell (46, 94). This directly influences the activity of proteins for nutrients uptake and synthesis of ATP which in turn affect bacteria growth. For example, porin is an integral protein found in membrane, responsible for the transporting nutrients such as glucose and amino acids into the cells. It was reported that the porin's channel is closed by half at pH 5.4, thus the reduced pore size limits uptake of nutrients (207). Therefore, different initial pH alters bacteria activities which in turn influence H_2 production.

The performance of H_2 production and measured cell mass were the highest at temperature 37˚C regardless of the different initial pH (Table 5.2 and 5.3). At temperature beyond 37˚C, the measured cell mass was reduced along with H_2 production. It is obvious at 50˚C in which H_2 production was completely inhibited when no cell mass was detected. Temperature could manipulate growth of H_2-producing bacteria by changing the permeability of cell membrane. The membrane solidify at low temperature whereas liquefy at high temperature and this may inactivate integral proteins for transport of nutrients and ions (208). Moreover, H_2 is the product of deprotonation from a series of enzymatic reactions. Since enzymatic activities are susceptible for thermal deactivation, hence it is logic that H_2 production temperature sensitive. Furthermore, most of the H_2-producing bacteria are reported to be mesophiles such as *Clostridium* spp., *Enterobacter* spp., and *Bacillus* spp. (53, 102, 103). They survive in a wide range of temperature from 15 – 45˚C but most commonly fermentative H_2 production was reported at a narrow range of 35 – 39˚C (186).

Table 5.2: Kinetic parameters of production H_2 from dairy wastewater using Modified Gompertz Equation

Initial pH	Temp (˚C)	H_{max}		R_{max}		k	λ (h)	R^2
		mmol H_2/ g COD	mmol H_2/ g carb	mmol H_2/g COD/h	mmol H_2/g carb/h			
5	25	10.9 ± 0.3	1.2 ± 0.0	0.48 ± 0.00	0.05 ± 0.00	0.120 ± 0.007	49.4 ± 0.3	0.999
	30	39.5 ± 1.1	4.5 ± 0.1	1.10 ± 0.00	0.12 ± 0.00	0.076 ± 0.004	35.6 ± 0.4	0.999
	37	54.1 ± 4.1	6.1 ± 0.5	1.40 ± 0.01	0.16 ± 0.00	0.071 ± 0.008	38.4 ± 0.7	0.996
	45	30.3 ± 2.1	3.4 ± 0.2	0.73 ± 0.00	0.08 ± 0.00	0.066 ± 0.006	38.3 ± 0.6	0.997
6	25	39.8 ± 0.7	4.5 ± 0.1	1.40 ± 0.00	0.16 ± 0.00	0.096 ± 0.004	40.1 ± 0.3	0.999
	30	78.9 ± 2.1	8.9 ± 0.2	2.99 ± 0.01	0.34 ± 0.00	0.103 ± 0.007	37.9 ± 0.5	0.998
	37	113.2 ± 2.9	12.8 ± 0.3	3.73 ± 0.01	0.42 ± 0.00	0.090 ± 0.007	25.9 ± 0.8	0.997
	45	43.0 ± 2.6	4.9 ± 0.3	1.01 ± 0.01	0.11 ± 0.00	0.064 ± 0.008	24.3 ± 1.3	0.992
7	25	20.2 ± 0.6	2.3 ± 0.1	0.74 ± 0.00	0.08 ± 0.00	0.100 ± 0.005	45.4 ± 0.3	0.999
	30	42.4 ± 1.1	4.8 ± 0.1	1.73 ± 0.00	0.20 ± 0.00	0.111 ± 0.008	37.3 ± 0.6	0.998
	37	88.5 ± 3.2	10.0 ± 0.4	2.66 ± 0.01	0.30 ± 0.00	0.082 ± 0.009	21.1 ± 1.2	0.993
	45	32.8 ± 0.5	3.7 ± 0.1	1.41 ± 0.00	0.16 ± 0.00	0.117 ± 0.007	29.4 ± 0.5	0.998
8	25	37.7 ± 3.5	4.2 ± 0.4	0.90 ± 0.01	0.10 ± 0.00	0.065 ± 0.009	36.9 ± 0.9	0.994
	30	38.2 ± 1.6	4.3 ± 0.2	3.00 ± 0.01	0.16 ± 0.00	0.098 ± 0.012	31.1 ± 1.1	0.993
	37	89.2 ± 3.7	10.1 ± 0.4	2.57 ± 0.01	0.29 ± 0.00	0.078 ± 0.008	24.2 ± 1.2	0.993
	45	39.0 ± 1.4	4.4 ± 0.2	1.43 ± 0.01	0.16 ± 0.00	0.100 ± 0.011	30.3 ± 1.0	0.994

No data from initial pH 4 and temperature 50 ˚C were not displayed as there were no H_2 productions detected.

Table 5.3: Characteristics of wastewater after fermentation at various temperature and initial pH

Initial pH	Temp. (˚C)	Final pH	Cell mass (g/L)	COD reduction		Soluble carbohydrate consumption (%)
				tCOD (%)	sCOD (%)	
5	25	3.37 ± 0.07	0.86 ± 0.05	81.4 ± 0.9	80.8 ± 1.1	38.0 ± 2.4
	30	3.43 ± 0.01	1.11 ± 0.20	81.7 ± 1.0	82.2 ± 2.8	47.4 ± 1.5
	37	3.31 ± 0.03	1.56 ± 0.18	81.9 ± 0.5	80.0 ± 1.6	59.8 ± 1.6
	45	3.31 ± 0.08	0.97 ± 0.08	82.1 ± 0.7	80.8 ± 0.8	47.9 ± 1.3
6	25	3.42 ± 0.08	1.73 ± 0.20	80.7 ± 0.8	82.9 ± 4.4	57.0 ± 2.6
	30	3.50 ± 0.09	2.80 ± 0.08	82.0 ± 1.4	82.6 ± 0.8	64.7 ± 1.7
	37	3.54 ± 0.01	3.54 ± 0.16	81.7 ± 1.2	83.3 ± 1.6	85.6 ± 0.9
	45	3.45 ± 0.05	2.78 ± 0.14	81.4 ± 1.7	81.4 ± 2.0	66.2 ± 1.1
7	25	3.52 ± 0.09	1.31 ± 0.21	81.4 ± 0.7	79.8 ± 1.6	54.4 ± 2.4
	30	3.54 ± 0.02	2.36 ± 0.22	82.6 ± 2.5	80.8 ± 3.5	66.1 ± 0.9
	37	3.44 ± 0.01	3.63 ± 0.16	80.8 ± 0.9	82.4 ± 1.3	87.4 ± 0.6
	45	3.46 ± 0.05	2.85 ± 0.17	81.6 ± 0.4	82.1 ± 1.3	63.3 ± 2.5
8	25	3.48 ± 0.07	1.62 ± 0.11	82.0 ± 1.5	81.0 ± 2.4	47.7 ± 1.7
	30	3.52 ± 0.06	2.36 ± 0.14	81.7 ± 0.5	80.8 ± 1.3	66.5 ± 0.9
	37	3.47 ± 0.02	3.79 ± 0.09	80.0 ± 0.5	82.2 ± 1.4	85.6 ± 0.5
	45	3.85 ± 0.58	2.60 ± 0.15	81.3 ± 0.9	81.9 ± 1.8	51.1 ± 2.3

No data from initial pH 4 and temperature 50 ˚C were not displayed as there were no H_2 productions detected.

Table 5.4: Comparison of H_2 yield from this study with other studies

Sludge inoculum	H_2 Yield	Sludge pretreatment	Substrate source	Initial pH and temp. (˚C)	Substrate consumption	Ref.
Landfill leachate sludge	113.2 ± 2.9 mmol H_2/g COD 12.8 ± 0.3 mmol H_2/ g carb.	Heat	Dairy wastewater	pH 6; 37.0 ˚C	81.7 ± 1.2% COD 85.6 ± 0.9% carb.	This study
H_2 producing reactor	0.0018 mmol H_2/g COD 1.8 mmol H_2/gCOD 0.0122 mmol H_2/g COD 0.0317 mmol H_2/g COD	Untreated Acid Heat BES	Dairy wastewater	29.0 ˚C	79% COD 63% COD 69% COD 87% COD	(65)
Palm oil mill effluent (POME) treatment plant	0.41 mmol H_2/g COD 0.32 mmol H_2/g COD 0.23 mmol H_2/g COD 0.12 mmol H_2/g COD	Heat Acid Chloroform Untreated	POME	pH 5.5; 35.0 ˚C	86 % COD 51 % COD 51 % COD 66 % COD	(66)
Sewage treatment plant	[a] 0.42 mmol H_2/ g COD	Base	Glucose & peptone	pH 10.5; 37.0 ˚C	N.A.	(209)
Sewage treatment plant	[a] 10.6 mmol H_2/ g carb.	Heat	Stillage of ethanol plant	pH 5.05; 37.0 ˚C	90% carb.	(49)

[a] H_2 yield was estimated using PV = nRT at standard condition where P = 1 atm; R = 8.21 × 10^{-5} m^3.atm/mol.K and T = 300 K

5.2.3. Thermodynamics of H_2 production from dairy wastewater

Table 5.5 summarizes the thermodynamic parameters calculated from the modified Arrhenius model. The calculated Gibbs free energy at initial pH 5, 6, 7, and 8 was –17, –40, –20 and –16 kJ/mol, respectively. It is observed that highest Gibbs free energy was belonged to the highest H_2 yield under initial pH 6. This suggests that H_2 production at initial pH 6 is more favourable as compared to that of other pH value. Figure 1 represents the modified Arrhenius plot described by eq. 3 and 4 with a good regression. The intersection point of the linear lines represents the actual optimum fermentation temperature. It is interesting that the linear lines intersect at the same point at all initial pH and hence the optimum fermentation temperature was 38.4 °C. The thermodynamic analysis suggests that H_2 production from dairy wastewater using landfill leachate sludge as inoculum is thermodynamically favourable.

Activation enthalpy of fermentation represents the thermodynamic potential of a reaction, it measures the amount of heat released or absorbed in a reaction. The calculated activation enthalpy of fermentation (ΔH) was 99, 65, 62 and 57 kJ/mol at initial pH 5, 6, 7 and 8, respectively. As expected, these values represent that energy was absorbed during H_2 production because ATP is hydrolyzed to drive enzymatic conversions of substrate into molecular H_2 (60, 156, 179). The ΔH obtained in this study falls within the range for microbial growth (54 – 71 kJ/mol) (142) and for enzymatic reaction (18 – 83 kJ/mol) (179). However, it is surprising that the ΔH at initial pH 5 was out of the range for microbial growth and enzymatic reaction. This phenomenon occurred because more energy was required to drive H_2 production as compared to optimum pH 6. This would mean that the bacteria need more ATP to drive H_2 production at pH 5 and hence it is thermodynamically less favourable. In contrast, activation enthalpy of thermal deactivation (ΔH_d) represents the threshold energy for enzymatic denaturation and microbial death. The calculated ΔH_d was 236, 282, 268 and 267 kJ/mol at initial pH 5, 6, 7 and 8, respectively. These values fall within the reported value 290–380 kJ/mol for microbial death (157) and therefore it is less sensitive to thermal deactivation. In comparison with the ΔH_d reported by Fabiano and Perego (142) and Wong *et al*. (179) for H_2 production was 118 and 113 kJ/mol, respectively. This study suggested that H_2 production from dairy wastewater using landfill leachate sludge as inoculum was less sensitive to thermal deactivation.

Entrophy measures the randomness of a reaction and the activation entropy of fermentation (ΔS) in this study was 0.054, 0.128, 0.265 and 0.052 kJ/mol/K at initial pH 5, 6, 7 and 8, respectively. This indicates that H_2 production from dairy wastewater was a random

reaction. In contrast, the calculated activation entropy of thermal deactivation (ΔS_d) was 0.424, - 1.17, 0.521 and 0.516 kJ/mol/K at initial pH 5, 6, 7 and 8, respectively. It is interesting to note that the value of ΔS_d at initial pH 5, 7 and 8 was higher than the value of ΔS. This is reasonable because thermal deactivation of enzymes increases with randomness of a system (158, 159). However, the negative value of ΔS_d at initial pH 6 represents a reduced randomness. This result is similar to those reported earlier by others (142, 158, 179). However, the significance of negativity was not explained in the literatures (158, 159).

Table 5.5: Thermodynamics quantities of H_2 production from dairy wastewater using landfill leachate sludge inoculum

Thermodynamic parameter	pH 5	pH 6	pH 7	pH 8
Gibbs free energy (kJ/mol)	- 17	- 40	-20	-16
Activation enthalpy of fermentation (kJ/mol)	99	65	62	57
Activation entropy of fermentation (kJ/mol/K)	0.054	0.128	0.265	0.052
Activation enthalpy of thermal deactivation (kJ/mol)	236	282	268	267
Activation entropy of thermal deactivation (kJ/mol/K)	0.424	- 1.17	0.521	0.516

No data from initial pH 4 was not displayed, as there were no H_2 productions detected.

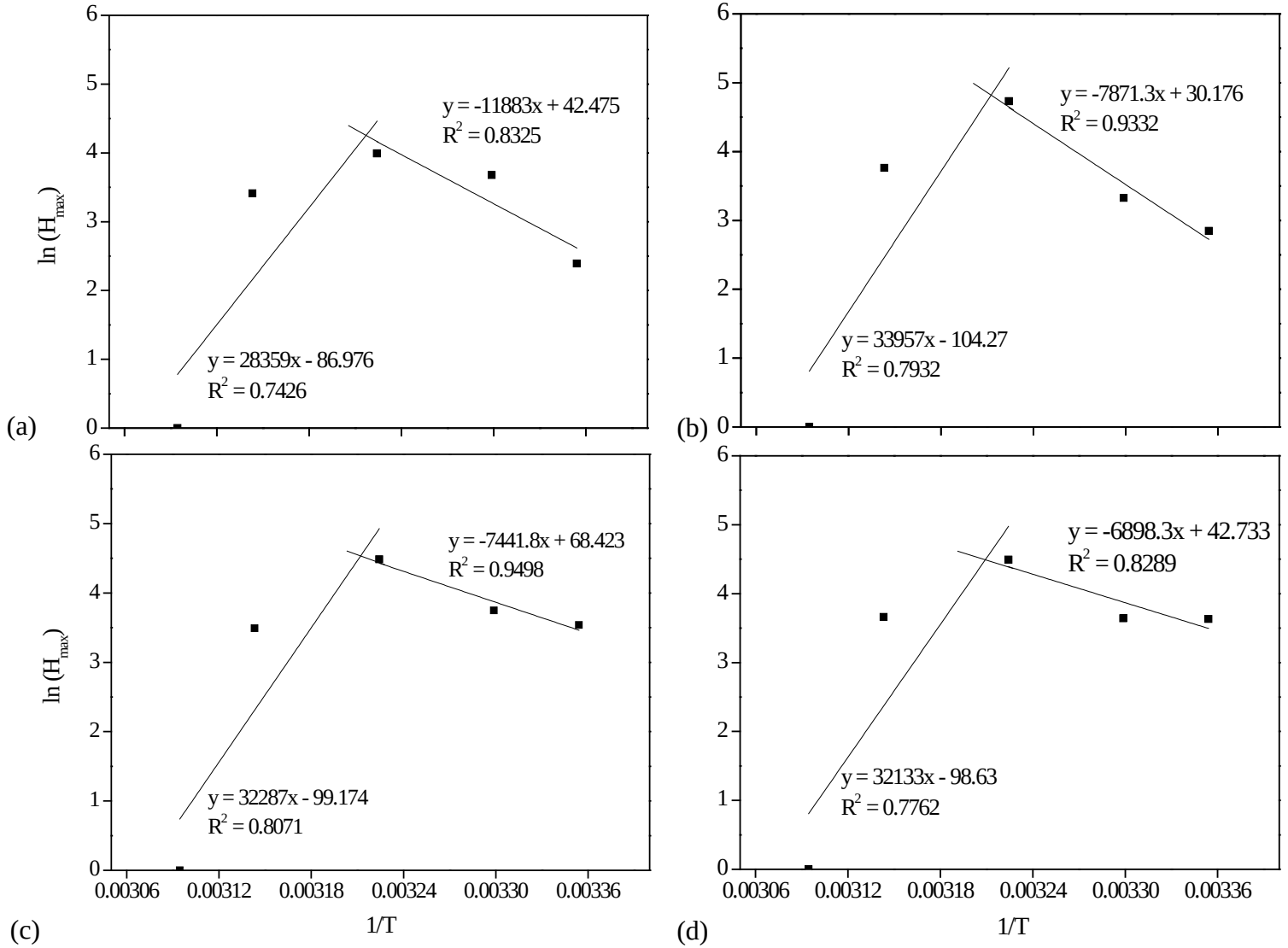

Figure 5.1: Modified Arrhenius plot for the evaluation of enthalpies and entropies at different initial pH (a) pH 5, (b) pH 6, (c) pH 7 and (d) pH 8. The intersection point of the linear lines represents the optimum fermentation temperature that is 38.4 °C at all initial pH

5.2.4. Effect of organic load on H_2 production from dairy wastewater

The starting organic load plays an essential role on the production of H_2. It is observed that H_2 performance was enhanced at an increasing dilution factor with the highest yield at 80% dilution (Table 5.6). The yield of H_2 from 80% dilution was 63% higher than that of undiluted wastewater. According to the law of mass action which stated that the rate of fermentation increases with substrates concentration (160). However it is interesting to observe that the fermentative H_2 production that driven by enzymatic reaction is prone to inhibition by high organic load. This results were accordance with an early study conducted by Roychowdhury et al. (1988) which found that high carbohydrate concentrations counteract fermentation performance (161). In this study, high amount of carbohydrates in undiluted wastewater leads to a product or feedback inhibition that triggered the reduction of carbohydrate consumption. This induces chain effects that reduce the availability of subsequent metabolites, such as reduced-ferredoxin which is responsible for proton reduction(179) and hence inhibits H_2 production.

Table 5.6: H_2 production at different organic load and characteristics of wastewater after fermentation

Dilution factor	0 %	20 %	40 %	60 %	80 %
H_2 yield					
mmol H_2/ g COD	41.7 ± 0.4	55.4 ± 1.6	60.6 ± 0.9	84.0 ± 2.3	113.2 ± 2.9
mmol H_2/ g carb.	4.71 ± 0.05	6.26 ± 0.18	6.85 ± 0.10	9.49 ± 0.26	12.8 ± 0.3
Final pH	3.85 ± 0.06	3.81 ± 0.02	3.71 ± 0.03	3.62 ± 0.04	3.54 ± 0.01
COD reduction					
tCOD (%)	83.7 ± 0.6	85.0 ± 0.3	86.1 ± 0.6	84.5 ± 0.6	81.7 ± 1.2
sCOD (%)	75.9 ± 0.9	77.8 ± 0.4	87.2 ± 0.8	88.4 ± 1.2	83.3 ± 1.6
Soluble carbohydrate consumption (%)	11.1 ± 1.7	19.3 ± 1.4	31.3 ± 1.9	43.3 ± 1.5	85.6 ± 0.9

Fermentation conditions: pH 6, 37°C and 3 days

5.2.5. Productions of volatile fatty acids and alcohol

Bio-H_2 production is accompanied by the production of volatile fatty acids (VFA) and alcohol. The concentration of VFA and alcohol different fermentation conditions is displayed in Table 5.7. It is notable that the profile of VFA and alcohols was different at various fermentation conditions, in which the predominated VFA were acetate and butyrate with trace amounts of lactate, formate and ethanol. At optimum condition (initial pH 6 at 37˚C), the ratio of acetate to butyrate was estimated to be 0.4 and this ratio increased depending on the fermentation conditions. The concentration of lactate increases with temperature and initial pH. Ethanol was only detected at fermentative temperature below 30°C. This indicates that there is a change in fermentation pathway at different fermentation conditions.

The ratio of acetate to butyrate (Ace:But) indicates the direction of fermentation pathway. If the Ace: But ratio is smaller than one, it represents the reaction was prone towards butyrate production whereas when the Ace: But ratio is larger than one, it favour towards acetate formation (149, 210, 211). It is commonly known that the production of acetate usually represents a higher H_2 yield. In theory, each mole of glucose will be converted to two mole of acetate with four mole of H_2 or one mole of butyrate with two mole of H_2, which can be represented by eq. 10 and 11.

Acetate pathway: $C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2$ (eq. 10)

Butyrate pathway: $C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2$ (eq. 11)

However, it is surprising that the highest H_2 yield was obtained at pH 6 and the Ace:But ratio is 0.4, which is less than 1. This suggested that the production of hydrogen under optimum condition follow butyrate pathway. This could be due to the key enzymes involved in the production of H_2 and butyrate are butyrate kinase and hydrogenase, respectively. It is reported that these genes are parallel regulated in some H_2-producing bacteria such as *C. perfringens* (212). Since H_2 production increased with higher expression of hydrogenase gene, the production of butyrate will also increase due to the parallel expression of both key enzymes. In contrast, when H_2 production was less efficient, the expression of both enzymes are suppressed, which has led to the lower yield of H_2 and butyrate.

Apart from Ace:But ratio, the efficiency of bio-H_2 production also monitored by the overall profile of VFA and alcohol. Typically, high concentrations of lactate and ethanol

represent a less efficient H_2 production. As shown in Table 5.7, at optimum condition, the amount of lactate only represents about 1.5 % of the overall VFA and alcohol production as compared to 7% from fermentation occurred at initial pH 5 and 25˚C. Furthermore, ethanol was only observed at 25 and 30˚C. It was reported that pH and temperature alters bioactivity of enzymes [52]. Therefore, formation of VFA and alcohol are highly related to fermentation conditions because the pH and temperature will alters bioactivity of the enzymes. For example, phosphotransbutyrylase (butyrate formation) (213) , NAD-independent lactate dehydrogenanse (pyruvate formation) (214), and phosphotransacetylase (acetate formation) (214) was only physiologically active at pH 6, 7.5, and 5, respectively. Besides that pH also influences the electron flow in the fermentation (115). When there are excess protons and substrates, fermentation pathways are shifted to produce more reduced metabolites including ethanol and lactate which eventually reduces H_2 yield. Therefore the fermentation condition is an important factor to control the pathway for VFA and alcohol formation in order to achieve efficient bio-H_2 production.

Table 5.7: Concentration of volatile fatty acids and alcohol different fermentation conditions

Initial pH	Temp. (˚C)	Concentration (mmol/L)					Ace:But
		Lac	For	Ace	EtOH	But	
5	25	13.1 ± 0.2	2.61 ± 0.41	111.8 ± 6.5	14.7 ± 0.9	44.2 ± 1.2	2.5
	30	6.2 ± 0.2	2.50 ± 0.24	154.2 ± 4.2	11.1 ± 1.3	71.3 ± 2.6	2.2
	37	5.2 ± 0.4	2.36 ± 0.28	88.1 ± 3.5	N.D.	61.6 ± 5.3	1.4
	45	3.1 ± 2.7	2.39 ± 0.32	137.9 ± 4.8	N.D.	58.1 ± 2.4	2.4
6	25	6.6 ± 0.5	2.72 ± 0.45	145.9 ±6.4	9.5 ± 0.9	71.6 ± 4.5	2.0
	30	4.1 ± 0.3	2.30 ± 0.20	87.0 ±5.3	6.5 ± 0.6	92.0 ± 3.4	0.9
	37	2.9 ± 0.1	2.22 ± 0.17	54.6 ± 0.8	N.D.	125.3 ± 1.0	0.4
	45	7.2 ± 0.2	2.42 ± 0.22	140.4 ± 6.0	N.D.	78.4 ± 3.0	1.8
7	25	9.8 ± 0.8	2.39 ± 0.21	122.0 ± 7.6	10.2 ± 1.3	50.6 ± 2.3	2.4
	30	7.0 ± 0.2	2.62 ± 0.20	138.6 ± 3.0	6.3 ± 0.7	76.9 ± 4.0	1.8
	37	3.3 ± 0.2	2.34 ± 0.13	99.7 ± 9.7	N.D.	98.0 ± 3.4	1.0
	45	7.6 ± 0.3	2.57 ± 0.10	145.4 ± 4.2	N.D.	62.8 ± 3.2	2.3
8	25	6.4 ± 0.3	2.46 ± 0.14	158.8 ± 2.1	10.6 ± 0.9	69.8 ± 0.7	2.3
	30	6.1 ± 0.4	2.54 ± 0.17	149.2 ± 6.8	7.9 ± 0.7	71.7 ± 2.5	2.1
	37	3.3 ± 0.2	2.53 ± 0.34	99.3 ± 7.8	N.D.	98.8 ± 7.2	1.0
	45	6.4 ± 0.4	2.52 ± 0.14	146.8 ± 4.4	N.D.	75.3 ± 6.1	1.9

Abbreviation: N.D. (Not detected); Lac (lactate); For (formate); Ace (acetate); EtOH (ethanol); But (butyrate); Ace:But (ratio of acetate to butyrate)

5.3. Conclusion

In summary, dairy wastewater is a potential feedstock for bio-H_2 production using landfill leachate sludge as inoculum. The optimum conditions were identified at initial pH 6 and 37°C with the maximum H_2 yield of 113.2 ± 2.9 mmol H_2/g COD (12.8 ± 0.3 mmol H_2/g carb.). The wastewater was diluted by 80% to prevent product inhibition. The fermentation is accompanied with the production of various volatile fatty acids and alcohol. The predominated acids were acetate and butyrate with trace amount of lactate, formate and ethanol. At optimum condition, the ratio of acetate:butyrate was 0.4. This ratio increased with a change in fermentation conditions beyond the optimum. The kinetic and thermodynamic analysis revealed that the experimental results are consistent with the complex activated theory and in good agreement with the deactivation mechanism of enzymes. This study provide a framework for further research on bio-H_2 production from dairy wastewater using landfill leachate sludge as inoculum and contribute to the knowledge of scale-up operations.

Chapter 6

Discovery of three new Clostridia strains provided new insight to biohydrogen production

The work presented in this chapter has been partly submitted for peer review:

Wong YM, Juan JC, Ting A, Wu TY. Discovery of three new Clostridium strains provided new insight to biohydrogen production. Nature Biotechnology (Submitted)

Monash University

Declaration for Thesis Chapter 6

Declaration by candidate

In the case of Chapter 6, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Analysis and interpretation of data	100
Drafting and writing the publication	95

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
A/P Dr. Juan Joon Ching	Reviewed the publication	N.A.
Dr. Adeline Ting	Reviewed the publication	N.A.
Dr. Wu Ta Yeong	Reviewed the publication	N.A.

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	Date 9 Sept 2014
Main Supervisor's Signature	[redacted]	Date 9 Sept 2014

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

6.0. Introduction

Hydrogen (H_2) is a clean and environmental friendly fuel because combustion of H_2 produces water as the only end product (1). Biological H_2 production is a sustainable process because it can be produced from inexpensive waste biomass such as agricultural and organic-rich industrial waste via dark fermentation (45-47). Dark fermentation is an anaerobic process that converts biomass into biological H_2 in the absence of light (13). The advantages of this method are (1) it is a light-independent process and hence the configuration of the bioreactor is simpler and cheaper (14); and (2) it can utilize a wide range of carbohydrates as substrates to produce bio-H_2 (45-47). Therefore, dark fermentation can be easily integrated into waste management to achieve waste reduction and bio-energy production concurrently.

In dark fermentation, several H_2 producing bacteria (HPB) such as *Clostridium sp.*, *Bacillus* sp., *Klebsiella* sp. and *Enterobacter* sp. and *Ethanoligenens* sp. have been isolated from natural environment (53, 57, 95, 96, 142, 215-219). Among the reported the HPB, *Clostridium spp*. are the most popular H_2 producers due to its high efficiency in H_2 production (53, 95, 96, 215-218). They are obligate anaerobes that produce H_2 as well as volatile fatty acids and alcohols such acetate, butyrate, lactate, formate, ethanol and butanol that have industrial applications. However, the current highest H_2 yield was reported at 3.35 mol H_2/mol glucose by *Clostridium* sp. DMHC-10 (54). In theory, a maximum of 12 moles of H_2 can be produced from each mole of glucose.

$$C_6H_{12}O_6 + 6\ H_2O \rightarrow 12\ H_2 + 6\ CO_2 \qquad \text{(eq. 1)}$$

However, the highest reported H_2 yield is only about 28% of this maximum yield. Therefore, the search for the more efficient H_2 producer is desirable.

A sanitary landfill is a facility built to dispose municipal waste. Landfill leachate, as the name implies, is the garbage juice produced during the decomposition of organic waste within the landfill. Since an active landfill contains diverse microflora, sludge that deposited in leachate collection pond carry similar microflora. However, H_2-producing bacteria isolated from this source has not yet been reported. We have isolated three new H_2-producing bacteria and identified them as *Clostridium perfringens* strain JJC, *Clostridium bifermentans* strain WYM and *Clostridium* sp. strain Ade.TY using whole genome sequencing (191-193). Based on the gene annotation, strain Ade.TY contains an unique hydrogenase, namely energy-converting hydrogenase, which is commonly found in archaea that is uncommon in *Clostridium* sp. (220). Other than that, this strain also contains additional dimeric-periplasmic

[Fe] hydrogenase and two [Ni-Fe] hydrogenases. For strain JJC, it contains two different types of hydrogenases which are [Fe] hydrogenase *HydA* and a dimeric cytoplasmic [Fe] hydrogenase, whereas strain WYM contains a dimeric [Ni-Fe] hydrogenase. These features have prompted us to investigate their performance in H_2 production via batch mode optimization using dark fermentation.

6.1. Materials and methods

6.1.1. Isolation of bacteria strains and growth conditions

Sludge of landfill leachate was collected from Jeram Sanitary Landfill in Selangor, Malaysia. The sludge was pretreated at 65˚C for duration of 30 min. Culturable H_2-producing bacteria was isolated using enrichment method as described by Tolvanen *et. al.* (221) with slight modifications. Eight 30 mL serum bottles were arranged in series. Each serum bottle was filled with 15 mL of the reaction medium and they are connected using tubing (Cole-Parmer tygon tubing) with needles (0.8×38 and 0.6×25 mm) fixed at either ends. To initiate the enrichment process, 0.15% w/v of sludge was inoculated to the first bottle. The enriched medium was serial diluted and inoculated onto Columbia horse blood agar (CM0331, Oxoid) and reinforced clostridia agar (CM0151, Oxoid). The agars were prepared as the modified Hungate roll tube in 30 mL serum bottles (222). The inoculated agar was incubated anaerobically for 48 hrs. The colonies formed were re-streaked at least three times to obtain pure cultures.

6.1.2. Morphological test

The cell morphology was performed using the standard gram staining and spore staining method (merck). The morphological morphology was observed with a light microscope (Olympus BX51).

6.1.3. Genome project accession numbers

The H_2-producing isolates were identified using whole genome sequencing with Illumina Mi-seq. The draft genome sequences are deposited in the NCBI genome project under the accession number as follows: *Clostridium perfringens* strain JJC

(AWRZ00000000.1), *Clostridium bifermentans* strain WYM (AVSU00000000.1) and *Clostridium* sp. strain Ade.TY (AVSV00000000.1) (191-193).

6.1.4. H_2 production in batch fermentation

Batch fermentations were conducted in triplicate, in 200 mL serum bottles containing 150 mL of media. To each serum bottle, 2% v/v inoculum corresponding to 10^9 c.f.u was added in to the fermentation medium. In all samples, the medium solution contained glucose (0, 5, 10, 15, 20, 25 g/L) as the model substrate with the following nutrients: peptone (10 g/L), yeast (3 g/L), NaCl, (5 g/L); CH_3OONa (3 g/L), and cysteine (0.5 g/L). Prior to operation, each vial was purged with argon gas for 2 min and sterilized at 115˚C for 15 min. In order to optimize the H_2 production, the effects of initial pH (4, 5, 6, 7 and 8) at various temperatures (25, 30, 37, 45 and 50 °C) were investigated. The volume and composition of the biogas produced were measured and the concentrations of volatile fatty acids (VFA) were also analysed.

6.1.5. Analysis

Glucose and metabolites concentration including acetic acid, butyric acid, lactic acid, formic acid, propionic acid and ethanol were analysed by using a high performance liquid chromatography system (1200 series, Agilent Technologies) equipped with a refraction index detection (RID) and Animex Hi-Pex H column (300 × 7.7 mm, Agilent). The column temperature and detector temperature were adjusted to 65 and 55ºC respectively.

Biogas produced was collected and measured using water displacement method. Subsequently, the yield and composition of biogas was analyzed via a gas chromatograph (GC) equipped with thermal conductivity detector (TCD) with 2.7 m Hayesep Q column packed with molecular sieve. Helium was used as carrier gas at a flow rate of 2 mL/min. Injector, detector and oven temperatures were kept at 100, 150 and 60ºC, respectively.

6.1.6. Kinetics and thermodynamic analysis

A. Kinetic of H_2 production by modified Gompertz model

The modified Gompertz equation was used to fit the cumulative volume of H_2 produced over the time course (31, 143, 144):

$$\boldsymbol{H = H_{max}\left\{-e\left[\frac{R_{max}\cdot e}{H_{max}}(\lambda - t) + 1\right]\right\}} \qquad \text{(eq. 1)}$$

where H is the cumulative H_2 production (mol H_2/mol glucose), H_{max} is the maximum H_2 production (mol H_2/mol glucose), R_{max} is the maximum H_2 production rate (mol H_2/mol glucose/h), λ is the lag phase time (h) and t is the incubation time (h), and $\frac{R_{max}\cdot e}{H_{max}}$ represents the rate constant (k). The modified Gompertz equation was used to fit the cumulative H_2 data, using the OriginPro 8.5.

B. Activation enthalpy of fermentation and thermal deactivation

Enthalpy can be determined using Arrhenius approach based on the relationship between the reaction rate constant (*k)* and temperature (T) (142):

$$k = Ae^{\left(\frac{-\Delta H}{R.T}\right)} \qquad \text{(eq. 2)}$$

$$\ln k = \ln A - \frac{\Delta H}{RT} \qquad \text{(eq. 3)}$$

Where ΔH is the activation enthalpy, A is the Arrhenius pre-exponential factor and R is the ideal gas constant (8.3144621 J/K/mol). However, Arrhenius equation is strictly limited to an elementary process and only correlates well to the rate measurements for a single reaction which is free of diffusion and thermal resistance.

Biological H_2 production involves complex enzymatic reactions. Hence, the reaction rate of biological H_2 production increases with temperature up to the threshold temperature (T_{opt}). At temperature higher than T_{opt} biological H_2 production is subjected to thermal deactivation due to denaturation of key enzymes and cell death. According to Fabiano and Perego (142), this situation can be represented by the modified Arrhenius equation as follow:

$$\ln H_{max} = \ln(A.X.Y) - \frac{\Delta H}{RT} \quad , T < T_{opt} \qquad \text{(eq. 4)}$$

$$\ln H_{max} = \ln(B.X.Y) - \frac{\Delta H*}{RT} \quad , T > T_{opt} \qquad \text{(eq. 5)}$$

Where H_{max} is the maximum H_2 productivity obtained from modified Gompertz equation, A and B are the Arrhenius pre-exponential factors, X is the cell mass concentration (g/L), Y is the H_2 yield per unit cell mass (mol H_2/ g cell mass), R is the ideal gas constant (8.3144621 J/K/mol) and T is temperature in Kelvin (K). In term of enthalpy, ΔH is the fermentation activation enthalpy whereas the thermal deactivation enthalpy (ΔH_d) is determined by

$$\Delta H_d = \Delta H + |\Delta H^*| \qquad \text{(eq. 6)}$$

Thermal deactivation enthalpy represents the threshold energy for enzymatic denaturation and microbial death.

C. Activation entropy of fermentation and thermal deactivation

Activation entropy of fermentation and thermal deactivation can be determined using the following equation which derived from Eyring and Arrhenius equations (142)

$$\Delta S = R\left(ln\frac{Ah}{k_bT}\right) \qquad \text{(eq. 7)}$$

$$\Delta S_d = R\left(ln\frac{Bh}{k_bT}\right) \qquad \text{(eq. 8)}$$

Where A and B are the Arrhenius pre-exponential factors, *h* is the Planck's constant (6.63×10^{-34} J.s) and k_b is the Boltzmann's constant (1.38×10^{-23} J/K).

D. Gibbs free energy

Gibbs free energy is determined using the following equation

$$\Delta G = \Delta H - T\Delta S \qquad \text{(eq. 9)}$$

Where ΔH is the activation enthalpy obtained from eq. 4 and ΔS is the activation entropy obtained from eq.8.

6.2. Results and discussion

6.2.1. Characterisation of H_2-producing isolates

The three isolates were identified as Gram positive and rod shape bacteria. Endospore staining revealed that strain JJC and WYM are endospore-forming bacteria but not strain Ade.TY (Figure 6.1). Based on whole genome sequencing, heat plot from multiple genome alignment (Appendix 5-2) revealed that strain JJC is a *Clostridium perfringens* and strain

WYM is a *Clostridium bifermentans*. However, multiple genome alignment shows that strain Ade.TY is a *Clostridium* species but it does not align with any existing genome sequences and hence suggests that strain Ade.TY could be a new species. The relationship of the isolates with their closely related species is represented in a phylogenetic tree based on 16S rRNA sequences (Figure 6.2). The phylogeny confirmed that strain JJC and WYM are *C. perfringens* and *C. bifermentans*, respectively. As for strain Ade.TY, it brunched away from the closely related species and hence further indicates that it may be a new H_2-producing species.

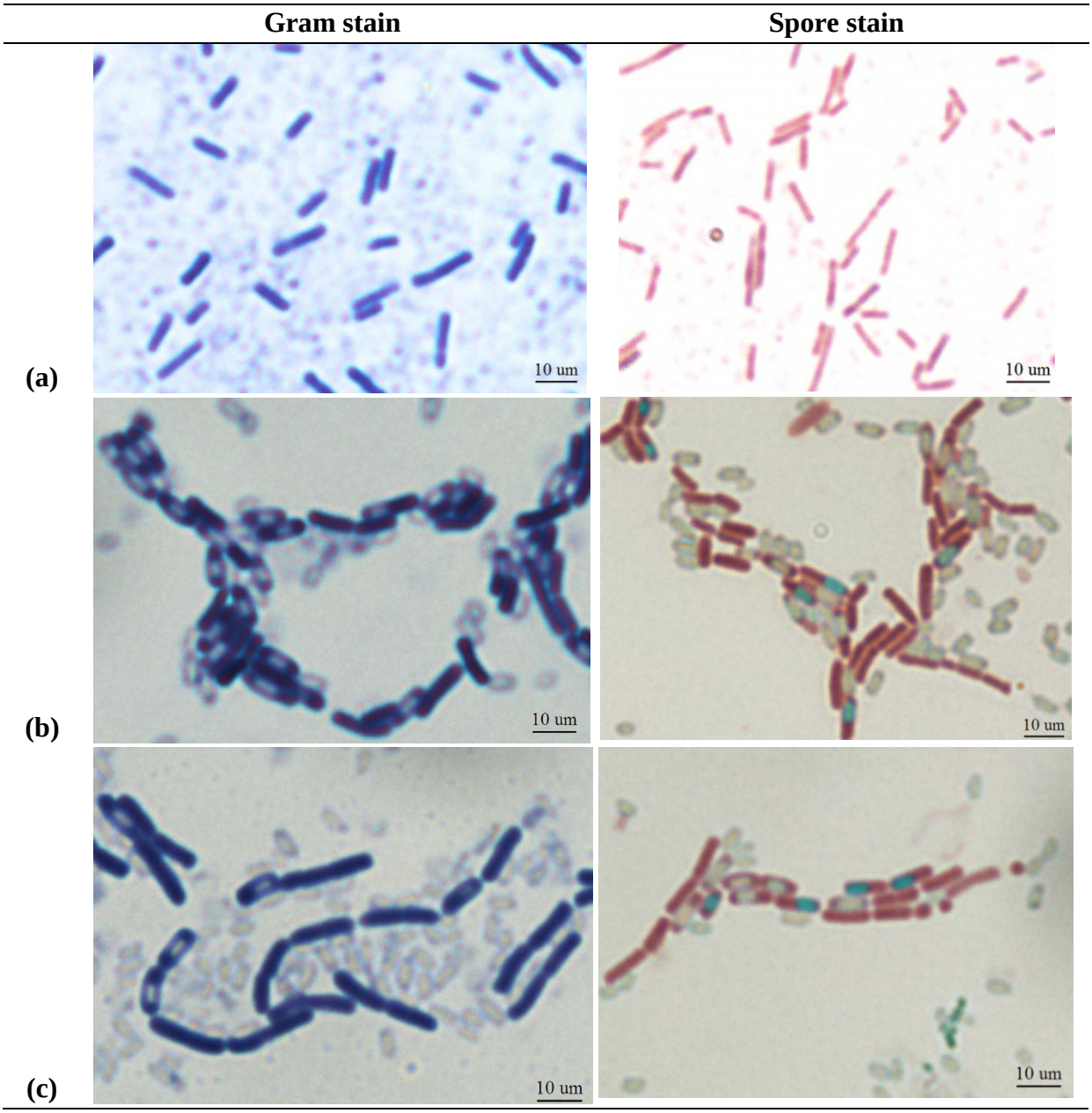

Figure 6.1: Microscopy examination: Gram and spore staining of (a) *Clostridium* sp. Strain Ade.TY, (b) *Clostridium perfringens* strain JJC and (c) *Clostridium bifermentans* strain WYM. Observations were made at (1000×).

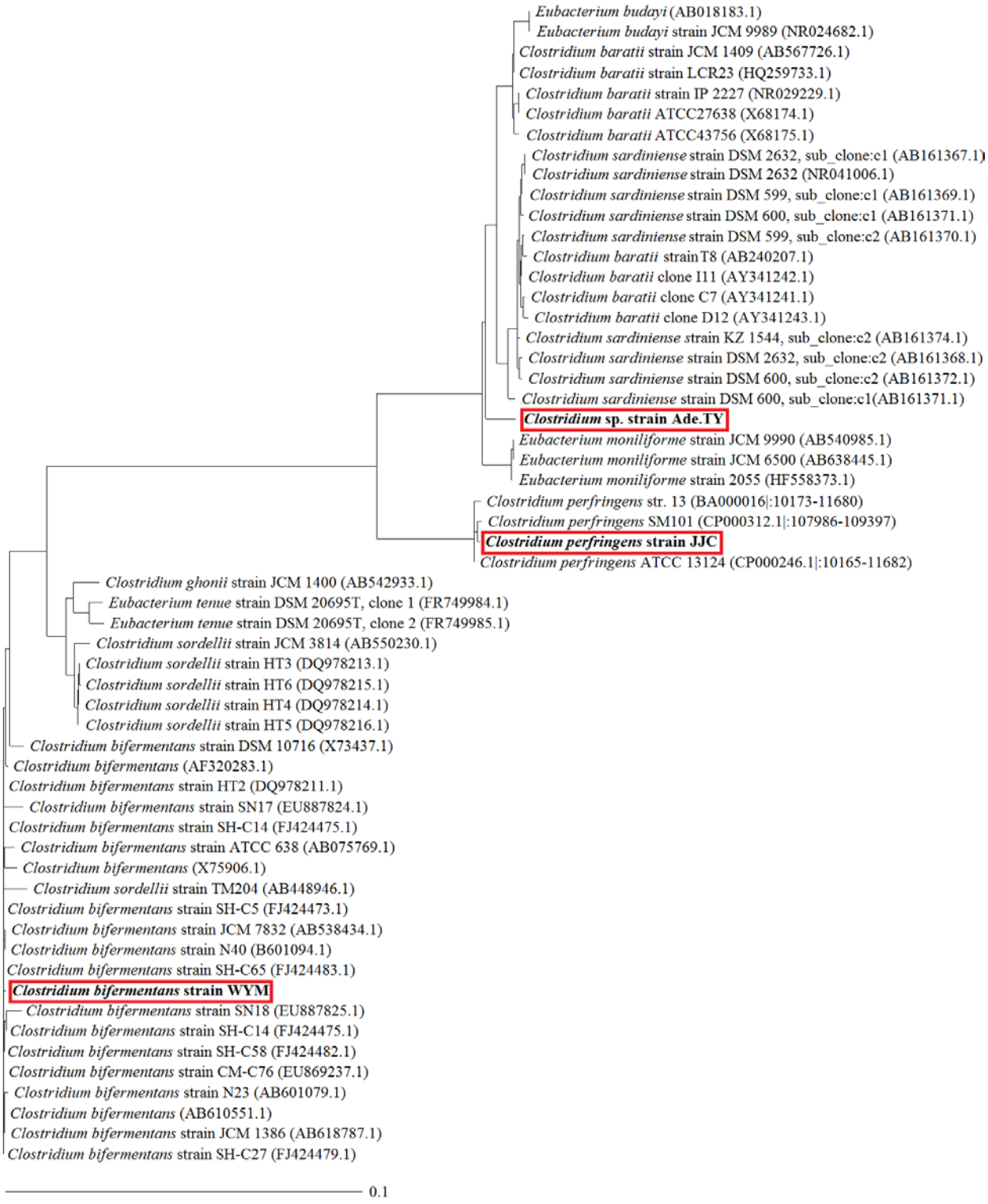

Figure 6.2: Phylogeny of *C. perfringens* strain JJC, *C. bifermentans* strain WYM and *Clostridium* sp. strain Ade.TY with their respective relatives based on 16S rRNA gene sequences

6.2.2. Effect of initial pH on H_2 production

Hydrogen production from the isolates varies with initial pH. The results are well fitted by the modified Gompertz model with the correlation of more than 0.98 (Table 6.1). For all isolates, the maximum H_2 production was attained at initial pH 6, in the descending order of strain *C. perfringens* strain JJC > *C. bifermentans* strain WYM > *Clostridium* sp. strain Ade.TY with yield of 4.68 ± 0.12, 3.29 ± 0.11, 2.87 ± 0.10 mol H_2/ mol glucose, respectively. This was expected because hydrogenase was reported to be active at pH 6 – 6.5 (97, 98). Furthermore, initial pH 6 also has been reported as the optimum initial pH for other *Clostridium* spp. including *C. butyricum* (53, 95), *C. beijerinckii*, *C. tyrobutyricum* (95), *C. saccharoperbutylacetonicum* (96), and *Clostridium* sp. R1 (215). Conversely, the range of initial pH for H_2 production differs between isolates (Table 1). There is no H_2 production and cell growth was detected from *C. perfringens* strain JJC at initial pH 4. Although H_2 production was attained from *C. bifermentans* strain WYM and *Clostridium* sp. strain Ade.TY at initial pH 4, the lag phase of strain Ade.TY was prolonged until 40 h as compared to that of strain WYM which had a shorter lag phase of 35 h. This result suggests that strain JJC does not grow at pH 4 whereas strain WYM can survive in an acidic environment better than strain Ade.TY. In contrast, it is also observed that H_2 production from strain JJC was drastically reduced at different initial pH (Table 1). This indicates that the efficient H_2 production from strain JJC is more affected by pH as compared to the other two isolates. Furthermore, the cell mass increased with initial pH which suggests that substrates were rapidly converted into cell mass instead of H_2. The results are consistent with the growth pH range of *C. perfringens* (pH 5 – 9) and *C. bifermentans* (pH 4 – 10) (211, 223-225). Cell growth and H_2 production are interrelated to the pH of medium as pH changes membrane potential and intracellular pH. These changes might directly influence the synthesis of ATP and activity of proteins for nutrients uptake. For example, it was reported that the size of protein channel for the transport of nutrients is closed by half at pH 5.4 and hence limits the uptake of nutrients such as glucose and amino acids (46, 94, 207). Bacteria physiology is closely related to the effect of pH which in turn affect H_2 production performance.

The high efficient H_2 production observed from the H_2-producing bacteria isolated in this study, especially *C. perfringens* strain JJC, was highly replicable. The maximum H_2 production from *C. perfringens* strain JJC is about 28% higher than *Clostridium* sp. DMHC-10 (Table 6.2). One-might argue that the outstandingly high activity of *C. perfringens* strain JJC is against the theoretical yield of dark fermentation. This could be related to the origin of H_2-

producing bacteria, the landfill leachate sludge. Landfill is an environment rich in microbial activity but has a harsh living condition with constantly low and imbalance nutrient level. Therefore, bacteria survived in this environment may possess unique substrate utilization mechanism which leads to efficient H_2 production. Our previous study (179) showed that landfill leachate sludge also exhibit extraordinary H_2 production of 6.43 mol H_2/glucose which exceed the theoretical yield. This indicates that the microbial community was indeed possess unique H_2 production property. Furthermore, whole genome sequencing revealed that this isolate contains two hydrogenases and three [FeFe]-hydrogenase maturation proteins (191). Further research is undergoing to analyse the pathway involve in H_2 production. Nonetheless, the high efficient H_2 production from *C. perfringens* strain JJC is a new discovery.

6.2.3. Effect of fermentation temperature on H_2 production

Fermentation temperature is another influencing factor in H_2 production. The results revealed that all three isolates produced H_2 over a wide range of temperature but the highest H_2 production was achieved at 37˚C. The modified Gompertz model gave a good correlation coefficient of more than 0.98 (Table 6.3). Interestingly, H_2 production and cell growth were not detected at temperature higher than 50˚C for all isolates. This suggests that these isolates are mesophiles whereby they neither produce H_2 nor grow at high temperature. This is consistent with the growth temperature of *C. perfringens* and *C. bifermentans* which are reported to be in the mesophilic range of 15 – 50˚C (211, 223-225). This study also revealed that the potentially new *Clostridium* sp. strain Ade.TY is active at the temperature range of 25 – 45 ˚C. Additionally, the results suggest that substrates were actively converted into H_2 at 37˚C but at 30˚C substrates were rapidly converted into cell mass. Both H_2 production and cell growth are the outcome of complex enzymatic reactions. As enzymes are thermal sensitive, therefore, it is logical that a change in temperature from 30 to 37˚C would shift the cell response from cell growth to H_2 production. Furthermore, most H_2 production was reported to occur optimally at a narrow temperature range of 35 – 39˚C (186). Besides, temperature manipulates the permeability of cell membrane and enzyme activity. Membranes liquefy at high whereas solidify at low temperature. The change in permeability may interfere the integration of membrane proteins which in turn deactivate nutrient transport (208). Hence, H_2 production was restricted at lower and higher temperatures (25, 45 and 50˚C).

Table 6.1: Kinetic parameters of production H_2 in the effect of fermentation pH from modified Gompertz equation

Strain	pH	H_{max} (mol H_2/ mol glu.)	R_{max} (mol H_2/ mol glu./h)	k	λ (h)	R^2	Final pH	Glucose Consumption (%)	Cell dry mass (g/L)
JJC	4	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
	5	3.21 ± 0.13	0.19 ± 0.00	0.16 ± 0.02	12.5 ± 0.9	0.9866	4.34 ± 0.01	84.7 ± 0.3	1.33 ± 0.11
	6	4.68 ± 0.12	0.24 ± 0.00	0.14 ± 0.01	6.8 ± 0.7	0.9933	4.41 ± 0.01	100.0 ± 0.0	1.39 ± 0.08
	7	3.22 ± 0.06	0.20 ± 0.00	0.17 ± 0.01	9.2 ± 0.6	0.9954	4.63 ± 0.01	100.0 ± 0.0	1.87 ± 0.17
	8	2.22 ± 0.06	0.12 ± 0.00	0.15 ± 0.01	8.4 ± 0.7	0.9935	4.84 ± 0.00	100.0 ± 0.0	2.11 ± 0.10
WYM	4	0.96 ± 0.04	0.04 ± 0.00	0.10 ± 0.00	35.3 ± 0.1	0.9999	4.02 ± 0.07	54.2 ± 3.3	0.82 ± 0.03
	5	2.76 ± 0.17	0.08 ± 0.00	0.08 ± 0.01	18.4 ± 0.5	0.9966	4.23 ± 0.01	65.0 ± 3.2	1.40 ± 0.11
	6	3.29 ± 0.11	0.17 ± 0.00	0.14 ± 0.02	7.1 ± 0.9	0.9871	4.31 ± 0.01	100.0 ± 0.0	1.81 ± 0.11
	7	2.97 ± 0.13	0.11 ± 0.00	0.10 ± 0.01	8.2 ± 0.9	0.9899	4.42 ± 0.03	100.0 ± 0.0	1.92 ± 0.06
	8	3.16 ± 0.07	0.14 ± 0.00	0.12 ± 0.01	8.1 ± 0.5	0.9965	4.36 ± 0.12	100.0 ± 0.0	1.97 ± 0.06
Ade.TY	4	0.27 ± 0.02	[a] N.A.	[a] N.A.	[a] N.A.	[a] N.A.	4.04 ± 0.02	18.2 ± 3.5	0.13 ± 0.00
	5	1.70 ± 0.03	0.13 ± 0.00	0.21 ± 0.01	22.4 ± 0.4	0.9978	4.34 ± 0.02	92.7 ± 0.4	1.54 ± 0.05
	6	2.87 ± 0.10	0.14 ± 0.00	0.14 ± 0.02	9.1 ± 0.9	0.9891	4.37 ± 0.04	100.0 ± 0.0	1.88 ± 0.10
	7	2.60 ± 0.15	0.13 ± 0.00	0.14 ± 0.02	12.8 ± 1.3	0.9778	4.52 ± 0.00	100.0 ± 0.0	2.12 ± 0.08
	8	2.71 ± 0.13	0.10 ± 0.00	0.10 ± 0.01	8.5 ± 1.1	0.9870	4.48 ± 0.06	100.0 ± 0.0	2.13 ± 0.13

Fermentation conditions: 37 °C, 48 h and 5 g/L glucose

N.A., Not available

[a] H_2 production was detected after 40 h, thus kinetic parameters were unable to generate using modified Gompertz model.

Table 6.2: Comparison of H_2 yield of the isolates from this study and other reported H_2 producing bacteria

Isolate	H_2 yield (mol H_2/mol glucose)	Source	Reference
Clostridium perfringens strain JJC	4.68	Landfill leachate sludge	This study
Clostridium sp. DMHC-10	3.35	Lab scale anaerobic reactor	(54)
Clostridium bifermentans strain WYM	3.31	Landfill leachate sludge	This study
Clostridium sp. strain Ade.TY	2.85	Landfill leachate sludge	This study
Thermoanaerobacter mathranii A3N (HM17901)	2.64	Oil producing well	(183)
Clostridium butyricum EB6	2.20	Palm oil mill effluent sludge	(184)
Clostridium perfringens strain W11	1.53	Cattle dung	(185)

Table 6.3: Kinetic parameters of production H_2 in the effect of fermentation temperature from Modified Gompertz Equation

Strain	Temp. (˚C)	H_{max} (mol H_2/ mol glu.)	R_{max} (mol H_2/ mol glu./h)	k	λ (h)	R^2	Final pH	Glucose Consumption (%)	Cell dry mass (g/L)
JJC	25	1.45 ± 0.04	0.08 ± 0.00	0.16 ± 0.01	26.8 ± 0.3	0.9983	4.66 ± 0.06	39.6 ± 0.7	0.89 ± 0.11
	30	2.56 ± 0.06	0.12 ± 0.00	0.13 ± 0.01	16.5 ± 0.4	0.9978	4.68 ± 0.02	92.7 ± 1.7	1.65 ± 0.06
	37	4.68 ± 0.12	0.24 ± 0.00	0.14 ± 0.01	6.8 ± 0.7	0.9933	4.41 ± 0.01	100.0 ± 0.0	1.39 ± 0.08
	45	3.12 ± 0.08	0.16 ± 0.00	0.14 ± 0.01	5.1 ± 0.8	0.9914	4.56 ± 0.04	98.4 ± 0.1	1.16 ± 0.08
	50	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
WYM	25	1.20 ± 0.10	0.05 ± 0.00	0.12 ± 0.02	24.0 ± 0.7	0.9916	4.64 ± 0.03	93.9 ± 2.6	1.36 ± 0.09
	30	2.49 ± 0.07	0.15 ± 0.00	0.16 ± 0.01	16.5 ± 0.6	0.9955	4.44 ± 0.02	100.0 ± 0.0	2.07 ± 0.11
	37	3.29 ± 0.11	0.17 ± 0.00	0.14 ± 0.02	7.1 ± 1.0	0.9871	4.31 ± 0.01	100.0 ± 0.0	1.81 ± 0.11
	45	2.60 ± 0.09	0.13 ± 0.00	0.14 ± 0.02	8.4 ± 0.9	0.9899	4.33 ± 0.02	100.0 ± 0.0	1.72 ± 0.02
	50	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
Ade.TY	25	1.12 ± 0.03	0.07 ± 0.00	0.17 ± 0.02	17.4 ± 0.6	0.9953	4.76 ± 0.04	28.5 ± 4.5	1.46 ± 0.04
	30	2.24 ± 0.10	0.08 ± 0.00	0.10 ± 0.01	12.4 ± 0.8	0.9938	4.43 ± 0.02	100.0 ± 0.0	2.14 ± 0.02
	37	2.87 ± 0.10	0.14 ± 0.00	0.14 ± 0.02	9.1 ± 0.9	0.9891	4.37 ± 0.04	100.0 ± 0.0	1.88 ± 0.10
	45	2.10 ± 0.08	0.09 ± 0.00	0.12 ± 0.01	7.3 ± 0.9	0.9893	4.37 ± 0.08	99.5 ± 0.9	1.66 ± 0.03
	50	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.

Fermentation conditions: pH 6, 48 h and 5 g/L glucose

N.A., Not available

6.2.4. Thermodynamics of H_2 production from the isolates

Table 6.4 summarises the thermodynamic parameters estimated from the modified Arrhenius model. The Gibbs free energy for H_2 production from the isolates were estimated at –35, –34 and –33 kJ/mol for *C. perfringens* strain JJC, *C. bifermentans* strain WYM and *Clostridium* sp. strain Ade.TY, respectively (Table 6.4). Figure 6.3 also shows a good regression of the modified Arrhenius plots. The intersection point of the linear lines represents the actual optimum fermentation temperature in which the temperature for H_2 production from strain JJC and WYM was estimated at 37.4 ˚C and strain Ade.TY at 36.4 ˚C. The thermodynamic analysis suggests that the high efficient H_2 production from these isolates are thermodynamically favourable.

Activation enthalpy of fermentation (ΔH) measured the thermodynamic potential which represents the amount of energy released or absorbed in a reaction. The estimated ΔH was 75, 63 and 58 kJ/mol for H_2 production from *C. perfringens* strain JJC, *C. bifermentans* strain WYM and *Clostridium* sp. strain Ade.TY, respectively (Table 6.4). This represents that energy was absorbed during H_2 production. This outcome is logical because biological H_2 is the product of a series of enzymatic reactions which requires energy from ATP hydrolysis (60, 156, 179). The ΔH obtained in this study is also in agreement with the energy for microbial growth (54 – 71 kJ/mol) (142) and for enzymatic reaction (18 – 83 kJ/mol) (179). Conversely, activation enthalpy of thermal deactivation (ΔH_d) represents the threshold energy for enzymatic denaturation and microbial death. The estimated ΔH_d was 93, 71, 64 kJ/mol H_2 production from strain JJC, strain WYM and strain Ade.TY, respectively. This indicates that the three isolates are sensitive to thermal deactivation because these values are lower than the reported value 290–380 kJ/mol for microbial death (157) and slightly lower than the ΔH_d reported for H_2 production (113 – 118 kJ/mol) (142, 179). Thermodynamic analysis in this study suggests that H_2-producing bacteria isolated from landfill leachate sludge are endothermic (require energy for enzymatic reactions) and sensitive to thermal deactivation.

Entropy represents the randomness of a reaction. The calculated activation entropy of fermentation (ΔS) was 0.354, 0.312 and 0.294 kJ/mol/K for strain JJC, strain WYM and strain Ade.TY, respectively. The positive values of ΔS suggest that H_2 productions from the three isolates were random reactions. Conversely, the calculated activation entropy of thermal deactivation (ΔS_d) was 0.053, -0.017 and -0.039 /mol/K for strain JJC, strain WYM and strain Ade.TY, respectively. It is reasonable to obtain a positive value of ΔS_d from strain JJC.

This is because when enzymes are deactivated, the randomness of a system is increased (158, 159). However, it is interesting to note that negative values of ΔS_d were obtained for strain WYM and strain Ade.TY, as these represent that deactivation of enzymes reduced randomness of a system. This result is similar to other those reported earlier by others (142, 158, 179) but the significance of negativity is remained unknown (158, 159).

Table 6.4: Thermodynamics quantities of H_2 production from the H_2-producing isolates

Thermodynamic parameter	Strain JJC	Strain WYM	Strain Ade.TY
Gibbs free energy (kJ/mol)	–35	–34	–33
Activation enthalpy of fermentation (kJ/mol)	75	63	58
Activation entropy of fermentation (kJ/mol/K)	0.354	0.312	0.294
Activation enthalpy of thermal deactivation (kJ/mol)	93	71	64
Activation entropy of thermal deactivation (kJ/mol/K)	0.053	-0.017	-0.039

No data from initial pH 4 was not displayed, as there were no H_2 productions detected.

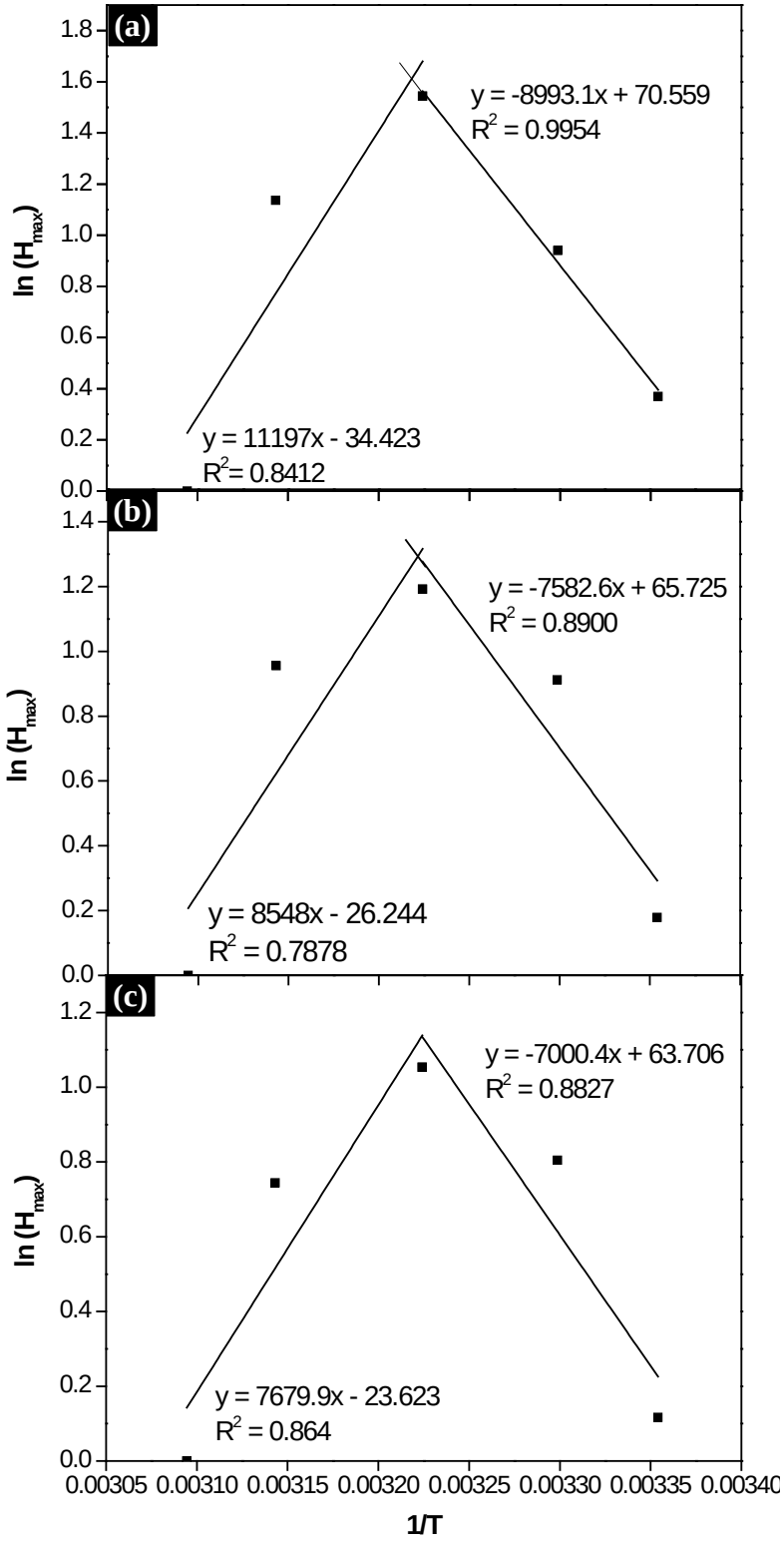

Figure 6.3: Modified Arrhenius plot for the evaluation of enthalpies and entropies: (a) *C. perfringens* strain JJC, (b) *C. bifermentans* strain WYM and (c) *Clostridium* sp. strain Ade.TY. The intersection point of the linear lines represents the optimum fermentation temperature.

6.2.5. Effect of organic load on H_2 production

The initial organic load also plays an important role on the H_2 production. The maximum H_2 yield from all three isolates was unveiled by glucose concentration of 5 g/L which is equivalent to C:N 1.0. Interestingly, small amount of H_2 in all the isolates ranging from 0.046 to 0.095 mmol H_2 was detected even without any input of glucose (Table 6.5). This indicates that the bacteria are using nitrogen and water to produce trace amount of H_2 that was only accounted for 0.23, 0.67 and 0.51% from strain JJC, WYM and Ade.TY, respectively. There was a remarkable increase in H_2 yield with glucose concentrations from 0 to 5 g/L glucose. This observation is reasonable because H_2 is produce from glucose decarboxylation and proton reduction. (35, 108). Hence, increasing glucose concentration enhances substrate accessibility which in turn improves H_2 production. Conversely, there was a reduction in H_2 yield with increased of cell dry mass at substrate concentration of 10 – 20 g/L glucose. This indicates that substrate was rapidly converted into cell mass instead of H_2. Seemingly, glucose was not utilized efficiently by the bacteria with the measured glucose consumption range of 20 to 70 % (Table 5). Hence, this suggests that H_2 production is subjected to substrate inhibition at higher substrate concentration.

The law of mass action states that the rate of formation increases with substrates concentration (160). This is observed when substrate concentration was increased from 2 to 5 g/L with an increase in H_2 yield of 59, 72 and 53% for strain JJC, WYM and Ade.TY, respectively. At 5 g/L glucose, the availability of substrate stimulates the fermentation towards H_2 and organic acids production to reach equilibrium. Nonetheless, enzymes involve in H_2 production are subject to substrate inhibition (160). Generally, it is assumed that more H_2 will be produced from higher availability of substrates. However, the H_2 yield from strain JJC, WYM and Ade.TY displayed a reduction up to79, 66 and 74%, respectively, when the glucose concentration increased up to 20 g/L. This phenomenon was observed because at higher substrate concentration, the accumulation of organic acids triggered a product or feedback inhibition which eventually hinders glucose uptake. This phenomenon was also encountered by other researchers (161). As a consequence, the availability of subsequent metabolites, such as reduced-ferredoxin responsible for proton reduction, is also reduced and causes a chain effect to inhibit H_2 production.

Nitrogen source also has an important role in H_2 production. The best H_2 production performance was observed at C:N 1.0 whereas no H_2 production and cell growth were detected from three isolates without the presence of yeast extract and peptone. This suggests

that nitrogen source is essential for bacteria cell growth and consequently inhibit H_2 production. Furthermore, reduced H_2 yield with higher cell dry mass was obtained at C:N 2.5 and 5.0. This evident that substrate was rapidly converted into cell mass instead of H_2 which was also observed by Bao et al. (136). The reduced H_2 yield could be associated with the electron flow within the fermentation. Fermentation condition with concentrated carbon but limited nitrogen source interrupts electron flow and shifts metabolic pathways to produce more reduced compounds like butyrate and lactate. Similar phenomena was also observed in the study conducted by Lin and Lay (115). Therefore, appropriate ratio, in this case C:N 1.0, directs metabolic pathways to one which enhances H_2 production.

Table 6.5: H_2 production H_2 in the effect of substrate concentration

Strain	Gluc. conc. (g/L)	C:N	Hydrogen yield		Final pH	Glucose Consumption (%)	Cell dry mass (g/L)
			mol H_2 / mol glu.	mmol H_2			
JJC	0	0.0	N.A.	0.046 ± 0.005	5.84 ± 0.04	N.A.	0.02 ± 0.01
	2	0.5	1.91 ± 0.04	3.39 ± 0.07	4.28 ± 0.05	100.0 ± 0.0	0.82 ± 0.04
	5	1.0	4.68 ± 0.12	19.9 ± 0.49	4.41 ± 0.01	100.0 ± 0.0	1.39 ± 0.08
	10	2.5	1.96 ± 0.07	16.9 ± 0.59	4.43 ± 0.03	65.8 ± 0.9	2.04 ± 0.06
	20	5.0	0.96 ± 0.03	16.8 ± 0.55	4.61 ± 0.05	20.3 ± 1.7	2.22 ± 0.07
WYM	0	0.0	N.A.	0.095 ± 0.050	5.65 ± 0.04	N.A.	0.22 ± 0.02
	2	0.5	0.90 ± 0.14	1.46 ± 0.22	4.44 ± 0.00	100.0 ± 0.0	1.43 ± 0.08
	5	1.0	3.29 ± 0.11	14.0 ± 0.48	4.31 ± 0.01	100.0 ± 0.0	1.81 ± 0.11
	10	2.5	2.52 ± 0.05	21.1 ± 0.42	4.41 ± 0.01	69.8 ± 0.7	2.06 ± 0.11
	20	5.0	1.12 ± 0.06	18.7 ± 0.98	4.46 ± 0.00	23.3 ± 3.8	2.14 ± 0.13
Ade.TY	0	0.0	N.A.	0.062 ± 0.009	5.72 ± 0.02	N.A.	0.14 ± 0.02
	2	0.5	1.33 ± 0.06	2.16 ± 0.10	4.53 ± 0.01	100.0 ± 0.0	1.12 ± 0.05
	5	1.0	2.87 ± 0.10	12.8 ± 0.43	4.37 ± 0.04	100.0 ± 0.0	1.88 ± 0.10
	10	2.5	1.78 ± 0.03	15.4 ± 0.27	4.48 ± 0.01	72.6 ± 1.9	2.20 ± 0.05
	20	5.0	0.75 ± 0.03	12.4 ± 0.43	4.39 ± 0.01	47.7 ± 1.5	2.16 ± 0.21

Fermentation conditions: pH 6, 37 °C and 48 h

No H_2 production was detected at 0 g/L nitrogen source and hence results are not shown.

N.A., Not available

6.2.6. Production of volatile fatty acids

The formation of H_2 is always associated with production of volatile fatty acids (VFA) and alcohol. The profile of volatile fatty acids and alcohol from the three isolates at different conditions are display in Figure 4. Acetate and butyrate were identified as the main fermentative products. Commonly, the pathway of fermentative H_2 production is monitored using the ratio of acetate to butyrate (Ace:But) (186). At optimum condition, Ace:But was 0.77, 0.65 and 0.80 for strain JJC, WYM and Ade.TY, respectively. These results are surprising because it disagree with the other literatures which reports that efficient H_2 production is associated with Ace:But ration greater than 1.0 (149, 210, 211). In theory, production of acetate is accompanied with 4 mole of H_2, while production of butyrate is accompanied with 2 mole of H_2. Therefore, it is expected that high Ace:But ratio in mix fermentation will favour H_2 production. In this study, the unexpected results from *C. perfringens* strain JJC could be due to the arrangement and activation of gene for the key enzymes involved. The key enzymes involved in acetate, butyrate and H_2 production are acetate kinase, butyrate kinase and hydrogenase. In *C. perfringens*, the butyrate kinase gene is located at the upstream of hydrogenase gene and they are parallel regulated in the response of glucose availability (Figure 4) (212). This suggests that the Ace:But may not be an accurate indicator to signify high H_2 production. In fact, the H_2 yield might be closely related to the gene activation of key enzymes. In contrast, there are limited or/and no relevant information for strain WYM and Ade.TY, respectively. Further studies are in progress to analyse the gene interactions using the genome sequences, cloning and hybridization tests.

It is interesting to note that the composition of VFA and alcohol varies at different fermentation conditions and different isolates. For *C. perfringens* strain JJC, the proportion of butyrate reduced with an increased composition of lactate, formate and ethanol at conditions beyond optimum. For *C. bifermentans* strain WYM, formate was detected at 0 g/L glucose and propionate was detected at 10 and 20 g/L glucose. For *Clostridium* sp. strain Ade.TY, the composition of acetate and lactate increased with a reduced composition of butyrate. Evidently, the fermentation pathways changes at different fermentation conditions. This reflects a shift in bioactivity of key enzymes that are extremely sensitive to pH, temperature and organic load. Activities of key enzymes such as phosphotransbutyrylase (butyrate formation) (213), NAD-independent lactate dehydrogenanse (pyruvate formation) (214), and phosphotransacetylase (acetate formation) (214) was only physiologically active at pH 6, 7.5, and 5, respectively. Furthermore, enzymatic activities might also influenced by the

imbalance of electron flow (115). Excess protons and substrates shift fermentation pathways to produce the more reduced metabolites including ethanol and lactate and hence reduced H_2 yield. Therefore, the appropriate combination of fermentation conditions is critical to ensure the key enzymes function properly and efficient H_2 production.

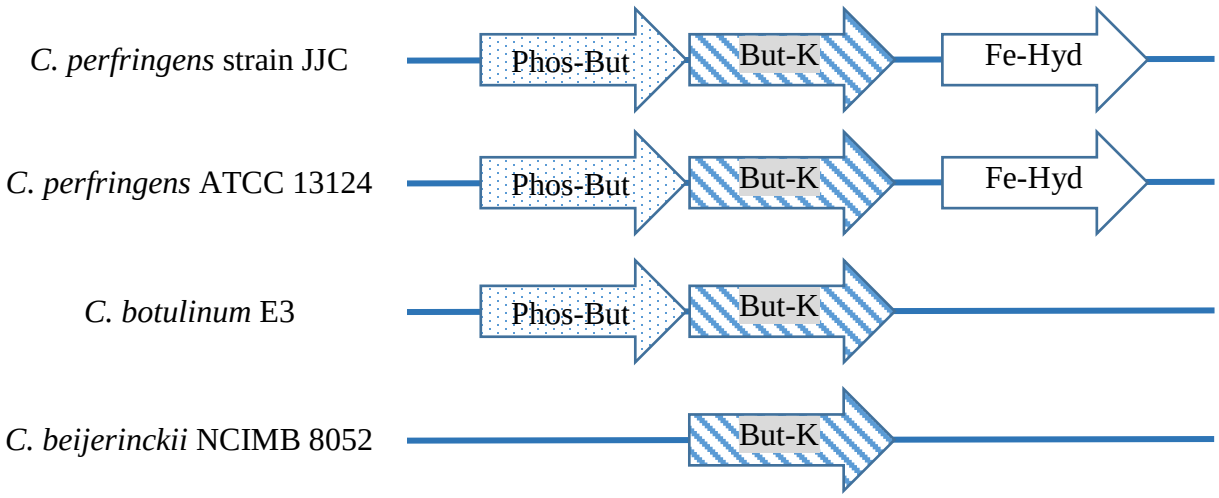

Figure 4: Gene arrangement of Phos-But (phosphate butyryltransferase, EC 2.3.1.19), But-K (Butyrate kinase, EC 2.7.2.7) and Fe-Hyd (Periplasmic [Fe] hydrogenase large EC 1.12.7.2) in different *Clostidium* spp. (RAST 2.0)

Table 7: Acetate : Butyrate at different fermentation conditions

Conditions	**Acetate : Butyrate**		
	Strain JJC	**Strain WYM**	**Strain Ade.TY**
pH 4	N.A.	1.14	0.96
pH 5	1.42	1.17	1.24
pH 6	0.77	0.65	0.80
pH 7	1.48	0.81	0.78
pH 8	1.78	1.22	0.79
25˚C	1.10	1.02	0.73
30˚C	0.46	0.95	1.19
37˚C	0.77	0.65	0.80
45˚C	1.32	0.87	1.32
50˚C	N.A.	N.A.	N.A.
0 g/L	12.1	5.37	4.01
2 g/L	1.61	2.77	1.07
5 g/L	0.77	0.65	0.80
10 g/L	1.44	2.43	0.55
20 g/L	1.16	1.84	0.27

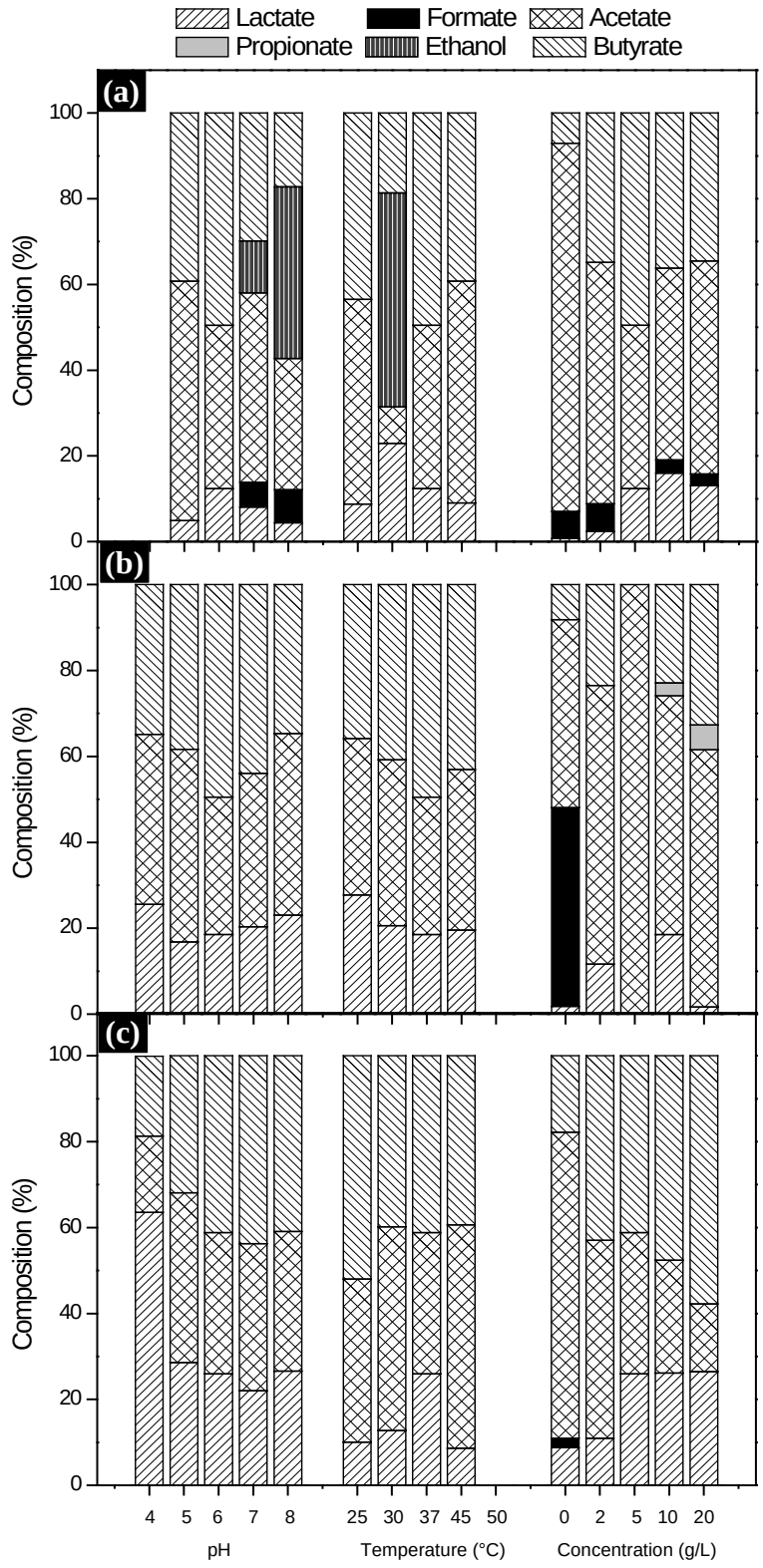

Figure 4: Profile of volatile fatty acids and alcohol from (a) *C. perfringens* strain JJC, (b) *C. bifermentans* strain WYM and (c) *Clostridium* sp. strain Ade.TY, at different conditions.

6.3. Conclusion

Three new H_2-producing bacteria, namely *Clostridium perfringens* strain JJC, *Clostridium bifermentans* strain WYM and *Clostridium* sp. strain Ade.TY, isolated from landfill leachate sludge demonstrate good performance in H_2 production. The maximum H_2 yield attained from these isolates are in the descending order of strain *C. perfringens* strain JJC > *C. bifermentans* strain WYM > *Clostridium* sp. strain Ade.TY with yield of 4.68 ± 0.12, 3.29 ± 0.11, 2.87 ± 0.10 mol H_2/ mol glucose, respectively. Interestingly, the high efficient and thermodynamically favourable H_2 production and, especially from *C. perfringens* strain JJC has been discovered. Based on this study, profile of fermentative products suggested that the low ratio of Ace: But is also capable of producing high H_2 yield. Instead, fermentative H_2 production involves complex gene interactions that are not yet known. This study provides a new insight on the potential of unique bacteria in H_2 production and opens a new opportunity for future investigation on H_2 production via the enzymatic and molecular basis.

Chapter 6a

Isolation and identification of H_2-producing bacteria

The work presented in this chapter has been published as Genome Announcement:

Wong YM, Juan JC, Gan HM, Austin CM. Draft Genome Sequence of Clostridium perfringens Strain JJC, a Highly Efficient Hydrogen Producer Isolated from Landfill Leachate Sludge. Genome Announcements. 2014;2(2).

Wong YM, Juan JC, Gan HM, Austin CM. Draft Genome Sequence of Clostridium bifermentans Strain WYM, a Promising Biohydrogen Producer Isolated from Landfill Leachate Sludge. Genome Announcements. 2014;2(2).

Wong YM, Juan JC, Ting A, Wu TY, Gan HM, Austin CM. Draft Genome Sequence of Clostridium sp. Strain Ade.TY, a New Biohydrogen- and Biochemical-Producing Bacterium Isolated from Landfill Leachate Sludge. Genome Announcements. 2014;2(2).

Monash University

Declaration for Thesis Chapter 6a

Declaration by candidate

In the case of Chapter 7, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Analysis and interpretation of data	100
Drafting and writing the publication	90

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
A/P Dr. Juan Joon Ching	Reviewed the publication	N.A.
Dr. Gan Han Ming	Reviewed the publication	N.A.
Prof. Chris M Austin	Reviewed the publication	N.A.
Dr. Adeline Ting	Reviewed the publication	N.A.
Dr. Wu Ta Yeong	Reviewed the publication	N.A.

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	Date 9 Sept 2014
Main Supervisor's Signature	[redacted]	Date 9 Sept 2014

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author to agree on the respective contributions of the authors.

6a.1. Introduction

Hydrogen-producing bacteria are naturally found in the environment including the landfill leachate sludge (48, 51). This sludge provides a harsh living environment that is hazardous and constantly nutrient-scarce. Hence, H_2-producing bacteria that live in this sludge could be less susceptible to external stress which in turn contribute to industrial application including bio-H_2 and biochemical production.

Among the existing H_2-producing bacteria, members of the genus *Clostridium* are obligate anaerobes that produce hydrogen more efficiently than do facultative anaerobes, such as *Bacillus* sp., *Klebsiella* sp., and *Enterobacter* sp. (53, 95, 96, 215-218). In addition, all production of biological hydrogen is accompanied by the production of useful organic acids and solvents, such as acetate, butyrate, lactate, formate, ethanol, and butanol, which have industrial applications. Hence, *Clostridium* spp. have promising potential applications in industrial biotechnology.

Three H_2-producing were isolated from landfill leachate sludge and were successfully identified with the whole genome sequencing (WGS) using Illumina MiSeq. This method identifies unknown species effectively, at the same time, unveils the characteristics of bacteria at the genome level (226-228). Hence, it is a rapid and accurate process that provides depth of information of a bacteria strain. This chapter summarises the genome characteristics of these isolated.

6a.2. Methods and Materials

6a.2.1. Isolation of H_2 producing bacteria

Landfill leachate sludge was pretreated at 65˚C for 30 min as described in Chapter 3. Culturable H_2-producing bacteria bacteria were isolated using enrichment method as described by Tolvanen *et. al.* (229) with slight modifications. Eight 30 mL serum bottles were arranged in series (Figure 6a.1). Each serum bottle was filled with 15 mL of the reaction medium as described in Chapter 3, Section 3.1.4 and they are connected using tygon tubing (Cole-Parmer) with needles (0.8×38 and 0.6×25 mm) fixed at either ends. To ensure unidirectional fluid flow, the tip of the longer needle was adjusted to just above the liquid surface of the first bottle and the shorter needle was adjusted to the head space of the neighboring bottle. To initiate the enrichment process, 0.15% w/v of sludge was inoculated to

the first bottle. As bacteria grew in the first bottle, accumulation of biogas increased air pressure in the head space and the medium carrying H_2-producing bacteria was transferred into the neighboring bottle through the needles. The principle of this method is that the fast-growing H_2-producing bacteria will be transferred to the next bottle before other bacteria such as the H_2-consuming bacteria have a chance to grow. The enrichment process was terminated when growth was visible in the last bottle. The enriched medium was serially diluted (10^{-1} to 10^{-8}) and 100 µL was directly-inoculated on Columbia horse blood agar (CM0331, Oxoid) and reinforced clostridial agar (CM0151, Oxoid). The media were prepared as the modified Hungate roll tube in 30 mL serum bottles (230). The inoculated agar was incubated anaerobically for 48 h. The colonies formed were streak-plated on Columbia horse blood agar at least three times to obtain pure cultures.

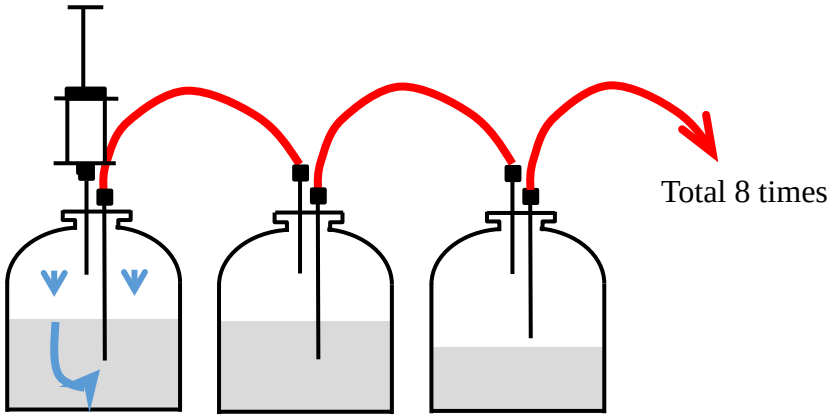

Figure 6a.1: Experimental setup for enrichment of H_2 producing bacteria.

6a.2.2. Sample preparation for whole genome sequencing using Illumina MiSeq

Genomes of the three isolates were sequenced using Illumina MiSeq for genomic analysis and species identification. The bacteria culture was prepared as described in Section 6A.1.1. DNA was extracted using the protocol design for Gram-positive bacteria in the QiagenDNeasy® Blood & Tissue Kit and then purified using the Agencourt AMPure XP purification kit. The Qubit® 2.0 Fluorometer was used to quantify the concentration and purity of gDNA. The quality control on size, quantity and purity of gDNA extractions was performed using the Bioanalyzer 2100 (Agilent). Sample preparation and sequencing kit used were Nextera XT DNA Sample Preparation Kit and MiSeq Reagent Kits v2 respectively. Whole genome sequencing was performed using the Illumina MiSeq Benchtop Sequencer (2 × 150 bp paired-end sequencing).

6a.2.3. Bioinformatics analysis

The reads generated from Illumina MiSeq were trimmed and assembled *de novo* using CLC Genomics Workbench 6.0 (CLC Bio, Denmark). Multiple genome alignment was conducted using Gegenees 2.0.3. The average similarities of the conserved core and size of core was set at 20% (231). The genome sequence was annotated with the Rapid Annotations using Subsystems Technology (RAST) server (232). Rnammer 1.2 and tRNA-scan-SE 1.21 were used to predict rRNA and tRNA respectively (233, 234).

6a.3. Results and discussion

6a.3.1. Identification of H_2-producing isolates

A. *Clostridum perfringens* strain JJC

Based on 16S rRNA analysis, strain JJC has a 100% identity score to *C. perfringens* ATCC 13124 and 99% to *C. perfringens* strains 13 and SM101 (Appendix 6-1). In addition, the heat plot from multiple-genome alignment revealed that strain JJC shares 95% similarities to strains 13 and ATCC 13124 and 88% to strain SM101 (Appendix 6-2). The results proved that strain JJC is a new strain of *C. perfringens*. The draft genome sequence of strain JJC comprises 3,259,329 bases in 69 contigs. It has a G+C content of 28.12% and contains 2,986 genes, 5 rRNAs, and 67 tRNAs. The characteristics of the genome of strain JJC are summarized in Table 6a.1. The draft genome sequence has been deposited in the GeneBank with the accession number AWRZ01000000 and published in Genome Announcement (191).

C. perfringens strain JJC contains two hydrogenases: [Fe] hydrogenase HydA and a dimeric cytoplasmic [Fe] hydrogenase. These proteins are activated and modified by three [FeFe]-hydrogenase maturation proteins, namely, HydE, HydF, and HydG (220, 235). In addition, it contains genes encoding products such as butyrate kinase (212) and acetate kinase (236) that are involved in the production of organic acids and solvents, including butyrate and acetate.

B. *Clostridum bifermentans* strain WYM

According to 16S rRNA analysis, strain WYM has 99 to 100% identity with many *C. bifermentans* strains, including strains E006 and E019 (Appendix 6-1). In addition, the heat plot from the multiple-genome alignment revealed that strain WYM shares up to 95% similarity with *C. bifermentans* ATCC 19299 AVNB01 and 88% with ATCC 638 AVNC01 (Appendix 6-2). These results suggest that strain WYM is a new strain of *C. bifermentans*. The draft genome sequence of strain WYM comprises 3,475,995 bases in 180 contigs. It has a G+C content of 28.02% and contains 3,380 genes, 5 rRNAs, and 51 tRNAs. The characteristics of genome are summarized in Table 6a.1. It is denoted as strain WYM and the draft genome sequence has been deposited in the GeneBank with the accession number of AVSU00000000. The draft genome is also published in Genome Announcement (192).

C. bifermentans WYM contains a dimeric [NiFe] hydrogenase that is regulated by the genes *hypA* and *hypB*. In addition, it contains genes encoding products such as acetate kinase, butyrate kinase, and ethanol dehydrogenase that are involved in the production of organic acids and solvents, including acetate, butyrate, and ethanol.

C. *Clostridum* sp. strain Ade.TY

The 16S rRNA analysis revealed that strain Ade.TY has a 99% identity score with several uncultured bacteria strains, and the 16S-rRNA phylogenetic tree also revealed that *Clostridium* sp. strain Ade.TY is a branch that is distant from other *Clostridium* species (Appendix 6-1). This finding suggests that *Clostridium* sp. strain Ade.TY may be a new hydrogen-producing species. This is further demonstrated by the heat plot from multiple-genome alignment, which revealed that strain Ade.TY has <50% similarity to the existing complete and draft genome databases of *Clostridium* species (Appendix 6-2). The draft genome sequence comprises 3,113,901 bases in 66 contigs. It has a GC content of 26.75% and contains 3,104 genes and 9 rRNAs and 68 tRNAs. The characteristics of genome of strain ADE.TY are summarized in Table 6a.1. It is denoted as *Clostridium* sp. strain Ade.TY and the draft genome sequence has been deposited in the GeneBank with the accession number of AVSV00000000. The draft genome is published in Genome Announcement (193).

Clostridium sp. strain Ade.TY contains a dimeric-periplasmic [Fe] hydrogenase and two [Ni–Fe] hydrogenases. It has an energy-converting hydrogenase that is regulated by six

gene clusters, *hypA, hypB, hypC, hypD, hypE,* and *hypF,* and a dimeric [Ni-Fe] hydrogenase (220).

Table 6a.1: Characteristics of genomes of the three selected isolates

Isolates	***C. perfringens* Strain JJC**	***C. bifermentans* Strain WYM**	***Clostridium* sp. Strain Ade.TY**
Genome size (bases)	3,259,329	3,113,901	3,475,995
No. of contigs	69	66	180
GC content	28.12%	26.75%	28.02%
No. of genes	2986	3104	3380
No. of rRNA	5	9	5
No. of tRNA	67	68	51

6a.4. Conclusion

Three culturable H_2-producing bacteria isolated from landfill leachate sludge were successfully identified as *Clostridium* sp. Strain Ade.TY, *C. perfringens* Strain JJC and *C. bifermentans* Strain WYM, respectively. These information aid in the future of cloning and metabolic engineering of the isolates.

Chapter 7

Conclusion and future work

7.0. Major findings and contributions of this study

The primary aims of this project were to investigate the H_2 production performance from landfill leachate sludge via dark fermentation and to analyse the microbial property responsible for H_2 production.

1. In this study, it can be concluded that landfill leachate sludge possesses great potential in H_2 production with the highest H_2 yield achieved was almost three-fold higher than the previously reported yield. The maximum H_2 yield of 6.43 mol H_2/mol glucose was achieved by the sludge pretreated at 65 ºC, with 100% of substrate consumption, under the conditions of 37ºC, pH 6.0 and 10 g/L glucose. This new record of high H_2 has break the conventional theoretical yield of 4 mol H_2/ mol glucose. The proposed theoretical stoichiometric equation of H_2 production from glucose based on the experimental Ace:But ratio is as follows:

   $$3.5\ C_6H_{12}O_6 + 12\ H_2O \rightarrow 14\ CO_2 + 26\ H_2 + 1.5\ CH_3COOH + CH_3CH_2CH_2COOH \quad \text{(eq. 3.12)}$$

   This process is thermodynamically favourable with the Gibbs free energy, fermentation activation enthalpy and entropy of – 34 kJ/mol, 68 kJ/mol and 0.331 kJ/mol/K, respectively (Chapter 3).
2. It is revealed by 16S-rRNA Metagenomics that the pretreated sludge at 65 °C contained 98% of H_2-producing bacteria from the genera *Clostridium*, *Bacillus*, *Eubacterium* and *Sporacetigenium*. In comparison, untreated sludge contained over 100 families of bacteria, mainly H_2-consuming bacteria including genera *Pseudomonas*, *Sulfurimonas* and *Treponema*. This difference explained the high efficient H_2 production from 65 ºC pretreated sludge could be due to the synergism between H_2-producing bacteria (Chapter 4).
3. The H_2-producing community in the 65 ºC pretreated landfill leachate sludge also showed good performance in H_2 production from dairy wastewater with the maximum H_2 yield of 113.2 ± 2.9 mmol H_2/g COD (12.8 ± 0.3 mmol H_2/g carb.) at the optimum condition of 37ºC and pH 6.0. The amount of H_2 produced from dairy wastewater using landfill leachate sludge was higher than the reported yield which suggests that H_2-producing community in this sludge has high competency in H_2 production (Chapter 5).
4. Three high efficient H_2-producing bacteria were successfully isolated from landfill leachate sludge, namely *C. perfringens* strain JJC, *C. bifermentans* strain WYM, *Clostridium* sp. strain Ade.TY (Chapter 7). The performance of H_2 production of the

isolates was in the descending order of *C. perfringens* strain JJC > *C. bifermentans* strain WYM > *Clostridium* sp. strain Ade.TY, with yield of 4.68 ± 0.12, 3.29 ± 0.11, 2.87 ± 0.10 mol H_2/ mol glucose, respectively. The high H_2 yields from the isolates are thermodynamically favourable with the Gibb's free energy of 35, –34 and –33 kJ/mol for *C. perfringens* strain JJC, *C. bifermentans* strain WYM and *Clostridium* sp. strain Ade.TY, respectively (Chapter 6). This shows that *C. perfringens* strain JJC could convert substrates to H_2 more efficiently as compared to the others. It is important to note that the H_2 yield from single H_2-producing bacteria was less efficient as compared to the performance of H_2 production using landfill leachate sludge as inoculum. In summary, landfill leachate sludge contains functional microbial community for efficient H_2 production with good potential in industrial application.

7.1. Future directions

This project provides new insight on the H_2 production via dark fermentation. Conventionally, it is assumed the highest H_2 yield could not exceed the theoretical yield of 4 mol H_2/mol glucose due to conversion of substrate into cell biomass, organic acid and alcohol, but this was subvert in this study. Therefore, future research could be focus on understanding the mechanism of the uniqueness in microbial community in landfill leachate sludge.

For H_2 production from glucose using 65 ºC pretreated sludge as inoculum:

(1) Functional metagenomics can be conducted to analyse the uniqueness of the genome and to analyse the potential gene interactions which in turn deduce the key enzymes that involved in H_2 production.

(2) Biochemical essays or transcriptomics analysis can be carried out to analyse the activities of key enzymes. With the profile of enzymatic analysis, a metabolic pathway can be generated to further validate eq. 3.12.

For H_2 production from dairy wastewater:

(3) H_2 production can be conducted in an up-scale continuous bioreactor to further investigate the potential of industrial application of using landfill leachate sludge to produce H_2 from wastewater

For the H_2-producing isolates:

(4) Gene knockout and cloning can be conducted in the isolates to investigate the role of important enzymes such as hydrogenase in H_2 production. These isolates were living in a landfill which is nutrient limited and has a complex combination of organic compound. In order to survive in the harsh environment, these bacteria may possess unique genetic features as compared to other strains which allow them to have good performance in H_2 production. Identifying the key genes could contribute to a better knowledge in metabolic engineering to improve H_2 production.

(5) Further studies can be conducted to improve H_2 production from the isolates such as genetically modify hydrogenase in the isolates to enhance performance of hydrogen production.

(6) *Clostridium* sp. strain Ade.TY. is suspected to be a new species of H_2-producing bacteria. Further analysis such as the average-nucleotide identity (ANI) analysis can be conducted to verify the bacteria species delineation.

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Appendix 1-1: Supplementary table for literature review on H_2 production from untreated sludge

Table A1: Summary of H_2 production from untreated sludge at their reported fermentation conditions

	Pretreatment	H_2 Yield	Initial pH and temp.	Substrate	Substrate consumption	Acetate : butyrate	Sludge source	Ref.
Untreated								
1.	N.A.	0.70 mol H_2/mol glucose	pH 6.5; 37.0˚C	Glucose	100% glucose	0.8	Sewage treatment plant	(37)
2.	N.A.	0.43 mol H_2/mol glucose	pH 6.7; 37.0 ˚C	Glucose	N.A.	1.5	Sewage treatment plant B	(31)
3.	N.A.	0.38 mol H_2/mol glucose	pH 7.0; 35.0 ˚C	Glucose	68.5% glucose	3.17	Sewage treatment plant	(141)
4.	N.A.	0.26 mol H_2/mol glucose	pH 6.7; 37.0 ˚C	Glucose	N.A.	1.5	Sewage treatment plant A	(31)
5.	N.A.	[b] ~0.20 mol H_2/mol glucose	pH 7.2; 37.0 ˚C	Glucose	N.A.	N.A.	Intertidal zone	(61)
6.	N.A.	[a] 0.72 mmol H_2/ g glucose	pH 7.0; 35.0 ˚C	Glucose	N.A.	N.A.	Sewage treatment plant	(80)
7.	N.A.	[a] 0.0366 mmol H_2	pH 7.2; 37.0 ˚C	Glucose	N.A.	N.A.	Intertidal zone	(57)
8.	N.A.	2.0 mol H_2/mol arabinose	pH 8.0; 37.0 ˚C	Arabinose	28.5% arabinose	[b] 0.29	H_2 producing reactor	(42)
		1.3 mol H_2/mol arabinose	pH 6.0; 37.0 ˚C		22.2% arabinose	[b] 0.27		
		0.1 mol H_2/mol arabinose	pH 4.5; 37.0 ˚C		8.8% arabinose	[b] 1.00		
9.	N.A.	0.3 mol H_2 / mol hexose	pH 5.5; 60.0 ˚C	Sucrose	92% sucrose	0.26	Palm oil mill effluent (POME) treatment plant	(36)
10.	N.A.	1.15 mol H_2/mol glucose	pH 6.5	Cassava stillage	N.A.	N.A.	Cassava stillage treatment plant	(38)
		1.22 mol H_2/mol glucose	pH 7.5					
		1.24 mol H_2/mol glucose	pH 5.5					
		0.87 mol H_2/mol glucose	pH 8.5					
		0.60 mol H_2/mol glucose	pH 4.5					
11.	N.A.	[a] 0.0341 mol H_2	pH 7.0; 35.0 ˚C	Sucrose	N.A.	N.A.	Intertidal zone	(138)

Table A1: Summary of H_2 production from untreated sludge at their reported fermentation conditions (CONTINUE)

Pretreatment	H_2 Yield	Initial pH and temp.	Substrate	Substrate consumption	Acetate : butyrate	Sludge source	Ref.
12. N.A.	[a] 0.0224 mol H_2	pH 6.5 – 7.5	Cassava stillage	N.A.	N.A.	Cassava stillage treatment plant	(38)
13. N.A.	[a] 0.0071 mol H_2	pH 6.8; 35.0 ˚C	Glucose	N.A.	[b] 2.5	Sewage treatment plant	(167)
14. N.A.	[a] 0.0265 mol H_2/g VS	pH 6.0; 60.0 ˚C	Cassava stillage	N.A.	N.A.	Anaerobic treatment plant	(67)
15. N.A.	[a] 0.0125 mol H_2/g total VS	pH 6.0; 60.0 ˚C	Cassava stillage	14.3% soluble carb	N.A.	Cassava stillage treatment plant	(92)
16. N.A.	[a] 1.29 mmol H_2/g total VS	pH 6.0; 60.0 ˚C	Cassava stillage and sewage sludge (ratio 3:1)	60.0% soluble carb	N.A.	Cassava stillage treatment plant	(92)
17. N.A.	0.0018 mmol H_2/g COD	29.0 ˚C	Dairy wastewater	79% COD	N.A.	H_2 producing reactor	(65)
18. N.A.	1.81 mol H_2/mol hexose	pH 6.0; 35.0 ˚C	Food waste	89% carbohydrate	N.A.	Food waste	(39)
19. N.A.	[a] 1.10 mol H_2/g VS	50.0 ˚C	Food waste	N.A.	N.A.	Anaerobic treatment plant	(86)
20. N.A.	2.10 mol H_2/mol hexose	pH 6.0; 35.0 ˚C	Food waste + sewage sludge	91% carbohydrate	0.21	Food waste	(39)
21. N.A.	0.00012 mol H_2/g COD	pH 5.5; 35.0 ˚C	POME	66 % COD	N.A.	Palm oil mill effluent (POME) treatment plant	(66)
22. N.A.	[a] 0.00073 mol H_2/g total VS	pH 6.0; 60.0 ˚C	Sewage sludge	15.2% soluble carb	N.A.	Cassava stillage treatment plant	(92)
23. N.A.	[a] 0.00031 mol H_2/g VS	pH 11.5; 37.0 ˚C	Sewage sludge	N.A.	2.1	Sewage treatment plant	(78)
24. N.A.	[a] 0.00005 mol H_2/g VS	pH 7.0; 37.0 ˚C	Sewage sludge	N.A.	2.1	Sewage treatment plant	(78)
25. N.A.	[a] 0.0191 mol H_2/ g carb	pH 5.05; 37.0 ˚C	Stillage of ethanol plant	90% carb	N.A.	Sewage treatment plant	(49)

[a] H_2 yield was estimated using PV = nRT at standard condition where P = 1 atm; R = 8.21×10^{-5} m^3.atm/mol.K and T = 300 K
[b] Estimated value

Appendix 1-2: Supplementary table for literature review on H_2 production from physically pretreated sludge

Table A2: Summary of H_2 production from physically pretreated sludge at their reported fermentation conditions

	Pretreatment	H_2 Yield	Initial pH and temp.	Substrate	Substrate consumption	Acetate : butyrate	Sludge source	Ref.
Heat treatment								
1.	60˚C, 40 min	0.00430 mol H_2/ g cellulose	pH 8.0; 55.0˚C	Cellulose	N.A.	N.A.	Sewage treatment plant	(131)
2.	65˚C, 30 min	2.30 mol H_2/mol glucose	pH 6.7; 37.0 ˚C	Glucose	N.A.	1.5	Sewage treatment plant B	(31)
3.	65˚C, 30 min	2.18 mol H_2/mol glucose	pH 7.0; 37.0˚C	Glucose	N.A.	0.69	Sewage treatment plant C	(60)
4.	65˚C, 30 min	1.64 mol H_2/mol glucose	pH 6.7; 37.0 ˚C	Glucose	N.A.	0.9	Sewage treatment plant A	(31)
5.	65˚C, 30 min	1.32 mol H_2/mol glucose	pH 7.0; 55.0˚C	Glucose	N.A.	0.69	Sewage treatment plant D	(60)
6.	65˚C, 30 min	1.25 mol H_2/mol glucose	pH 7.0; 55.0˚C	Glucose	N.A.	0.47	Sewage treatment plant C	(60)
7.	65˚C, 30 min	0.56 mol H_2/mol glucose	pH 7.0; 37.0˚C	Glucose	N.A.	0.74	Sewage treatment plant D	(60)
8.	65˚C, 30 min	[a] 0.00114 mol H_2/g dry algae	pH 7.5; 35.0 ˚C	Seaweed	N.A.	N.A.	Sewage treatment plant	(237)
9.	70˚C, 30 min	1.04 mol H_2/mol glucose	pH 6.5; 37.0˚C	Glucose	100% glucose	1.4	Sewage treatment plant	(37)
10.	70˚C, 30 min	0.00333 mol H_2/ g cellulose	pH 8.0; 55.0˚C	Cellulose	N.A.	N.A.	Sewage treatment plant	(131)
11.	70˚C, 50 min	0.00427 mol H_2/ g cellulose	pH 8.0; 55.0˚C	Cellulose	N.A.	N.A.	Sewage treatment plant	(131)
12.	70˚C, 30 min	[a] 0.0106 mol H_2/ g carbohydrate	pH 5.05; 37.0 ˚C	Stillage of ethanol plant	70% carb.	N.A.	Sewage treatment plant	(49)
13.	80˚C, 30 min	2.12 mol H_2/mol glucose	pH 6.7; 37.0 ˚C	Glucose	N.A.	1.8	Sewage treatment plant B	(31)
14.	80˚C, 30 min	1.32 mol H_2/mol glucose	pH 6.7; 37.0 ˚C	Glucose	N.A.	1.2	Sewage treatment plant A	(31)
15.	80˚C, 30 min	1.04 mol H_2/mol glucose	pH 8.5; 30.0 ˚C	Glucose	75% glucose	0.22	Agro-food organic wastes treatment plant	(40)
16.	80˚C, 10 min	1.02 mol H_2/mol glucose	pH 8.5; 30.0 ˚C	Glucose	84% glucose	0.03	Beetroot and bioethanol industry	(40)
17.	80˚C, 10 min	1.00 mol H_2/mol glucose	pH 8.5; 30.0 ˚C	Glucose	78% glucose	0.26	Agro-food organic wastes treatment plant	(40)
18.	80˚C, 30 min	0.96 mol H_2/mol glucose	pH 8.5; 30.0 ˚C	Glucose	78% glucose	0.2	Beetroot and bioethanol industry	(40)

Table A2: Summary of H_2 production from physically pretreated sludge at their reported fermentation conditions (CONTINUE)

Pretreatment	H_2 Yield	Initial pH and temp.	Substrate	Substrate consumption	Acetate : butyrate	Sludge source	Ref.
19. 80˚C, 20 min	0.00370 mol H_2/ g cellulose	pH 8.0; 55.0˚C	Cellulose	N.A.	N.A.	Sewage treatment plant	(131)
20. 80˚C, 40 min	0.00378 mol H_2/ g cellulose	pH 8.0; 55.0˚C	Cellulose	N.A.	N.A.	Sewage treatment plant	(131)
21. 80˚C, 60 min	0.00366 mol H_2/ g cellulose	pH 8.0; 55.0˚C	Cellulose	N.A.	N.A.	Sewage treatment plant	(131)
22. 80˚C, 20 min	[a] ~0.0488 mol H_2	pH 7.2; 37.0 ˚C	Glucose	N.A.	N.A.	Intertidal zone	(57)
23. 90˚C, 120 min	0.17 mol H_2/mol glucose	pH 5.5; 35.0 ˚C	Glucose	N.A.	~ 0.8	Sewage treatment plant	(238)
24. 90˚C, 60 min	0.07 mol H_2/mol glucose	pH 5.5; 35.0 ˚C	Glucose	N.A.	~ 0.3	Sewage treatment plant	(238)
25. 90˚C, 180 min	0.05 mol H_2/mol glucose	pH 5.5; 35.0 ˚C	Glucose	N.A.	~ 0.3	Sewage treatment plant	(238)
26. 90˚C, 30 min	0.05 mol H_2/mol glucose	pH 5.5; 35.0 ˚C	Glucose	N.A.	~ 0.2	Sewage treatment plant	(238)
27. 90˚C, 30 min	0.00395 mol H_2/ g cellulose	pH 8.0; 55.0˚C	Cellulose	N.A.	N.A.	Sewage treatment plant	(131)
28. 90˚C, 50 min	0.00402 mol H_2/ g cellulose	pH 8.0; 55.0˚C	Cellulose	N.A.	N.A.	Sewage treatment plant	(131)
29. 90˚C, 60 min	[a] 0.00233 mol H_2/g VS	pH 6.0; 60.0 ˚C	Cassava stillage	N.A.	N.A.	Anaerobic treatment plant	(67)
30. 90 – 95 ˚C, 30 min	[a] 0.047 mol H_2/LPOME	pH 6.0; 37 – 55 ˚C	POME	N.A.	N.A.	POME treatment plant	(168)
31. 90 – 100 ˚C, 60 min	1.37 mol H_2/mol reducing sugar	pH 7.0; 37.0 ˚C	Textile wastewater	N.A.	N.A.	Sewage treatment plant	(239)
32. 95˚C, 30 min	1.95 mol H_2/mol glucose	pH 6.7; 37.0 ˚C	Glucose	N.A.	2.4	Sewage treatment plant B	(31)
33. 95˚C, 30 min	0.90 mol H_2/mol glucose	pH 7.0; 35.0 ˚C	Glucose	92%	1.25	Sewage treatment plant	(141)
34. 95˚C, 30 min	0.19 mol H_2/mol glucose	pH 6.7; 37.0 ˚C	Glucose	N.A.	0.5	Sewage treatment plant A	(31)
35. 97˚C, 40 min	0.00032 mol H_2/ g cellulose	pH 8.0; 55.0˚C	Cellulose	N.A.	N.A.	Sewage treatment plant	(131)
36. 95 – 100 ˚C, 60 min	0.00948 mol H_2/ g starch	pH 6.0; 37.0 ˚C	Cassava starch	N.A.	0.17	Sewage treatment plant	(240)
37. 100˚C, 15 min	2.38 mol H_2/mol glucose	pH 7.0; 35.0 ˚C	Glucose	98% glucose	0.41	Sewage treatment plant	(63)
38. 100˚C, 30 min	0.63 mol H_2/mol glucose	pH 5.5; 35.0 ˚C	Glucose	N.A.	~ 1.8	Sewage treatment plant	(238)
39. 100˚C, 60 min	0.50 mol H_2/mol glucose	pH 5.5; 35.0 ˚C	Glucose	N.A.	~ 1.8	Sewage treatment plant	(238)
40. 100˚C, 120 min	0.43 mol H_2/mol glucose	pH 5.5; 35.0 ˚C	Glucose	N.A.	~ 1.3	Sewage treatment plant	(238)
41. 100˚C, 30 min	[b] ~0.40 mol H_2/mol glucose	pH 7.2; 37.0 ˚C	Glucose	N.A.	N.A.	Intertidal zone	(61)

Table A2: Summary of H_2 production from physically pretreated sludge at their reported fermentation conditions (CONTINUE)

Pretreatment	H_2 Yield	Initial pH and temp.	Substrate	Substrate consumption	Acetate : butyrate	Sludge source	Ref.
42. 100˚C, 15 min	0.07 mol H_2/mol glucose	pH 5.5; 35.0 ˚C	Glucose	N.A.	~ 0.3	Sewage treatment plant	(238)
43. 100˚C, 15 min	[a] 0.01214 mol H_2/g glucose	pH 7.0; 35.0 ˚C	Glucose	98% glucose	2.31	Sewage treatment plant	(116)
44. 100˚C, 15 min	[a] 0.00039 mol H_2/g glucose	pH 7.1; 37.8˚C	Glucose	N.A.	N.A.	Sewage treatment plant	(133)
45. 100˚C, 20 min	[a] ~0.0325 mol H_2	pH 7.2; 37.0 ˚C	Glucose	N.A.	N.A.	Intertidal zone	(57)
46. 100˚C, 15 min	[a] 0.00036 mol H_2/ g COD	pH 5.5; 37.0 ˚C	Glucose and peptone	N.A.	N.A.	Sewage treatment plant	(209)
47. 100˚C, 60 min	1.61 mol H_2/ mol hexose	pH 5.5; 60.0 ˚C	Sucrose	96% sucrose	0.88	POME treatment plant	(36)
48. 100˚C, 90 min	[a] 0.0498 mol H_2	pH 7.0; 35.0 ˚C	Sucrose	N.A.	N.A.	Intertidal zone	(138)
49. 100˚C, 15 min	[a] 0.34 mol H_2	pH 7.0; 55.0 ˚C	Xylose	95% xylose	0.15	Sewage treatment plant	(241)
50. 100˚C, 45 min	[a] 0.65 mol H_2	pH 7.0; 40.0 ˚C	Xylose	80% xylose	0.23	Sewage treatment plant	(241)
51. 100˚C, 15 min	[a] 1.10 mol H_2/g VS	pH 6.5; 35.0 ˚C	Wheat straw	N.A.	0.92	Anaerobic treatment plant	(110)
52. 100˚C, 60 min	0.01339 mol H_2/ g TVS	pH 5.0; 55.0 ˚C	Bagasse	N.A.	N.A.	Sewage treatment plant	(242)
53. 100˚C, 60 min	[a] 0.0000616 mol H_2/g TVS	pH 6.0; 37.0 ˚C	Pulping sludge and paper waste	N.A.	N.A.	Sewage treatment plant	(243)
54. 100˚C, 15 min	[a] 0.00668 mol H_2/g TS	pH 7.0; 50.0 ˚C	Cornstalk waste	N.A.	N.A.	River sludge	(94)
55. 100˚C, 15 min	a 0.00597 mol H_2/g TS	pH 7.0; 36.0 ˚C	Cornstalk waste	N.A.	N.A.	River sludge	(244)
56. 100˚C, 60 min	0.0000122 mol H_2/g COD	29.0 ˚C	Dairy wastewater	69% COD	N.A.	H2 producing reactor	(65)
57. 100 ˚C, 60 min	0.00041 mol H_2/g COD	pH 5.5; 35.0 ˚C	POME	86 % COD	N.A.	POME treatment plant	(66)
58. 100˚C, 60 min	0.130 mol H_2/ L POME	pH 5.5; 60.0 ˚C	POME	N.A.	0.62	Palm oil mill effluent (POME) treatment plant	(36)
59. 102˚C, 30 min	[a] 0.00109 mol H_2/ g VSS	pH 10.0; 37.0 ˚C	bovine serum albumin and dextran	55% and 87% respectively	N.A.	Sewage treatment plant	(155)
60. 102˚C, 90 min	2.00 mol H_2/mol sucrose	pH 5.5; 35.0 ˚C	Sucrose	N.A.	0.3	Soybean-processing wastewater treatment plant	(41)
61. 105˚C, 90 min	5.02 mol H_2/g COD	pH 7.0; 37.0 ˚C	Cassava wastewater	N.A.	N.A.	Sewage treatment plant	(245)

Table A2: Summary of H_2 production from physically pretreated sludge at their reported fermentation conditions (CONTINUE)

Pretreatment	H_2 Yield	Initial pH and temp.	Substrate	Substrate consumption	Acetate : butyrate	Sludge source	Ref.
62. 105˚C, 2 hr	3.00 mol H_2	pH 5.5; 35.0 ˚C	Corn hover	N.A.	N.A.	Sewage treatment plant	(246)
63. 121˚C, 20 min	[a] ~0.0244 mol H_2	pH 7.2; 37.0 ˚C	Glucose	N.A.	N.A.	Intertidal zone	(57)
64. 121˚C, 30–120 min	2.5 mol H_2/mol arabinose	pH 7.2; 37.0 ˚C	Arabinose	22% arabinose	[b] 0.55	Brewery industry	(42)
65. 121˚C, 30–120 min	1.7 mol H_2/mol arabinose	pH 8.0; 37.0 ˚C	Arabinose	40% arabinose	[b] 0.37	Brewery industry	(42)
66. 121˚C, 30–120 min	1.5 mol H_2/mol arabinose	pH 6.0; 37.0 ˚C	Arabinose	72% arabinose	[b] 0.32	Sewage treatment plant B	(42)
67. 121˚C, 30–120 min	1.5 mol H_2/mol arabinose	pH 8.0; 37.0 ˚C	Arabinose	98% arabinose	[b] 0.28	Sewage treatment plant B	(42)
68. 121˚C, 30–120 min	1.3 mol H_2/mol arabinose	pH 4.5; 37.0 ˚C	Arabinose	20% arabinose	[b] 0.29	Brewery industry	(42)
69. 121˚C, 30–120 min	1.2 mol H_2/mol arabinose	pH 6.0; 37.0 ˚C	Arabinose	51% arabinose	[b] 0.20	Sewage treatment plant A	(42)
70. 121˚C, 30–120 min	0.9 mol H_2/mol arabinose	pH 8.0; 37.0 ˚C	Arabinose	55% arabinose	[b] 0.11	Sewage treatment plant A	(42)
71. 121˚C, 30–120 min	0.8 mol H_2/mol arabinose	pH 4.5; 37.0 ˚C	Arabinose	42% arabinose	[b] 0.25	Sewage treatment plant A	(42)
72. 121˚C, 30–120 min	0.0 mol H_2/mol arabinose	pH 4.5; 37.0 ˚C	Arabinose	20% arabinose	[b] 0.00	Sewage treatment plant B	(42)
73. Autoclave, 20 min	[a] 0.0077 mol H_2	pH 6.8; 35.0 ˚C	Glucose	N.A.	2.41	Sewage treatment plant	(167)
74. Autoclave, N.A.	[a] 0.00244 mol H_2	pH 6.5; 37.0 ˚C	Arabinose	51% arabinose	4.6	Brewery industry	(247)
75. Autoclave, 30 min	[a] 0.00066 mol H_2/g VS	pH 6.8; 37.0 ˚C	Sewage sludge	N.A.	3.43	Sewage treatment plant	(117)
76. Autoclave, 15 min	0.00259 mol H_2/g VS	pH 7.5; 35.0 ˚C	Synthetic kitchen waste	N.A.	N.A.	Marsh (methane) gas plant	(248)
77. Autoclave, 30 min	[a] 0.00035 mol H_2/g VS	pH 6.8; 37.0 ˚C	Sewage sludge	N.A.	4.9	Sewage treatment plant	(78)
78. Boiling, 40 min	1.46 mol H_2/mol glucose	pH 7.0; 37.0 ˚C	Glucose	N.A.	N.A.	Candy Industry	(29)
79. Boiling, 15 min	[a] 0.00894 mol H_2/ g glucose	pH 7.0; 35.0 ˚C	Glucose	N.A.	N.A.	Sewage treatment plant	(80)
80. Boiling, 30 min	0.0058 mol H_2/ mg COD	pH 7 – 9; 35.0 ˚C	Glucose	15% COD	. N.A.	Sewage treatment plant	(249)
81. Boiling, 40 min	3.1 mol H_2/mol lactose	pH 7.0; 37.0 ˚C	Lactose	N.A.	N.A.	Candy Industry	(29)
82. Boiling, 40 min	3.6 mol H_2/mol lactose	pH 7.0; 37.0 ˚C	Cheese whey powder	N.A.	N.A.	Candy Industry	(29)
83. Boiling, 5 hr	[a] 11.4 mol H_2/g starch	pH 7.0; 37.0 ˚C	Wheat powder solution	N.A.	[b] 2.3	Bakers yeast industry	(118)

84.	Boiling, 30 min	[a] 0.47 mol H_2/ g VS	pH 5 – 6; 70.0 ˚C	Grass silage	N.A.	N.A.	Cattle farm	(106)

Table A2: Summary of H_2 production from physically pretreated sludge at their reported fermentation conditions (CONTINUE)

Pretreatment		H2 Yield	Initial pH and temp.	Substrate	Substrate consumption	Acetate : butyrate	Sludge source	Ref.
85.	Boiling, 30 min	[a] 0.00756 mol H_2/g TVS	pH 6.0; 37.0 ˚C	Defatted milk product food waste and lactose	N.A.	N.A.	Marsh (methane) gas plant	(30)
86.	Boiling, 15 min	[a] 0.00366 mol H_2/g TS	pH 7.0; 37.0 ˚C	Apple pomace	N.A.	N.A.	River sludge	(46)
87.	Boiling, 30 min and drying (110–115˚C)	0.0181 mol H_2/ mg COD	pH 7.9; 37.0 ˚C	Glucose	18% COD	0.27	Sewage treatment plant	(249)
88.	Repeated boiling (2× for 5 hr)	0.33 mol H_2/mol glucose	pH 7.0; 37.0 ˚C	Wheat powder solution	N.A.	N.A.	Beer Industry	(82)
89.	Repeated boiling (2× for 5 hr)	1.00 mol H_2/mol glucose	pH 7.0; 37.0 ˚C	Wheat powder solution	N.A.	N.A.	Bakers yeast industry	(82)
90.	Infrared oven (2 hr)	[a] 0.00521 mol H_2/ g TVS	pH 5.0; 36.0 ˚C	Wheat barn		0.57	Paper mill	(43)

[a] H_2 yield was estimated using PV = nRT at standard condition where P = 1 atm; R = 8.21×10^{-5} m^3.atm/mol.K and T = 300 K

[b] Estimated value

Appendix 1-3: Supplementary table for literature review on H_2 production from chemically pretreated sludge

Table A3: Summary of H_2 production from chemically pretreated sludge at their reported fermentation conditions

Pretreatment	H_2 Yield	Initial pH and temp.	Substrate	Substrate consumption	Acetate : butyrate	Sludge source	Ref.
pH pretreatment							
1. Acid (pH 2), 5 min	[a] 0.00013 mol H_2/g VS	pH 7.0; 37.0 ˚C	Sewage sludge	N.A.	5.1	Sewage treatment plant	(78)
2. Acid (pH 3), 24 hr	2.25 mol H_2/mol glucose	pH 6.0; 35.0 ˚C	Sucrose	N.A.	N.A.	Sewage treatment plant	(250)
3. Acid (pH 3), 24 hr	1.51 mol H_2/mol glucose	pH 7.0; 35.0 ˚C	Glucose	90.90%	0.77	Sewage treatment plant	(141)
4. Acid (pH 3), 24 hr	1.11 mol H_2/mol glucose	pH 6.5; 37.0˚C	Glucose	100% glucose	1.2	Sewage treatment plant	(37)
5. Acid (pH 3), 24 hr	[a] 0.00406 mol H_2/ g glucose	pH 7.0; 35.0 ˚C	Glucose	N.A.	N.A.	Sewage treatment plant	(80)
6. Acid (pH 3), 24 hr	0.00032 mol H_2/g COD	pH 5.5; 35.0 ˚C	POME	51 % COD	N.A.	Palm oil mill effluent (POME) treatment plant	(66)
7. Acid (pH 3), 24 hr	[a] 0.00189 mol H_2/g VS	pH 6.0; 60.0 ˚C	Cassava stillage	N.A.	N.A.	Anaerobic treatment plant	(67)
8. Acid (pH 3), 30 min	[a] 0.026.8 mol H_2	pH 7.0; 35.0 ˚C	Sucrose	N.A.	N.A.	Intertidal zone	(138)
9. Acid (pH 3), 24 hr	[a] 0.00074 mol H_2	H 6.8; 35.0 ˚C	Glucose	N.A.	2.95	Sewage treatment plant	(167)
10. Acid (pH 3–4), 24 hr	[b] ~0.85 mol H_2/mol glucose	pH 7.2; 37.0 ˚C	Glucose	N.A.	N.A.	Intertidal zone	(61)
11. Acid (pH 3–4), 24 hr	1.30 mol H_2/mol sucrose	pH 5.5; 35.0 ˚C	Sucrose	N.A.	1.07	Soybean-processing wastewater treatment plant	(41)
12. Acid (pH 3–4), 24 hr	0.65 mol H_2 / mol hexose	pH 5.5; 60.0 ˚C	Sucrose	95% sucrose	1.48	Palm oil mill effluent (POME) treatment plant	(36)
13. Acid (pH 3–4), 24 hr	[a] 0.00691 mol H_2/g of sugar	pH 6.6; 25.0 ˚C	mixture of fruit and vegetables waste compost of sucrose, fructose and glucose	[b] 96% total sugar	N.A.	Sewage treatment plant	(132)
14. Acid (pH 3–5), 48 hr	[a] 0.00254 mol H_2	pH 7.0; 55.0 ˚C	glucose	N.A.	N.A.	Cattle farm	(251)
15. Acid (pH 5), 24 hr	0.0018 mol H_2/gCOD	29.0 ˚C	Dairy wastewater	63% COD	N.A.	H_2 producing reactor	(65)
16. Base (pH 10), 24 hr	1.34 mol H_2/mol glucose	pH 7.0; 35.0 ˚C	Glucose	90.10%	0.84	Sewage treatment plant	(141)

Table A3: Summary of H_2 production from chemically pretreated sludge at their reported fermentation conditions (CONTINUE)

Pretreatment	H_2 Yield	Initial pH and temp.	Substrate	Substrate consumption	Acetate : butyrate	Sludge source	Ref.
17. Base (pH 10), 24 hr	0.68 mol H_2/mol glucose	pH 6.5; 37.0˚C	Glucose	100% glucose	0.9	Sewage treatment plant	(37)
18. Base (pH 10), 24 hr	[a] 0.00569 mol H_2/ g glucose	pH 7.0; 35.0 ˚C	Glucose	N.A.	N.A.	Sewage treatment plant	(80)
19. Base (pH 10), 30 min	[a] 0.0154 mol H_2	pH 7.0; 35.0 ˚C	Sucrose	N.A.	N.A.	Intertidal zone	(138)
20. Base (pH 11), 24 hr	[a] 0.00211 mol H_2	pH 6.8; 35.0 ˚C	Glucose	N.A.	2.95	Sewage treatment plant	(167)
21. Base (pH 12), 30 min	[b] ~0.10 mol H_2/mol glucose	pH 7.2; 37.0 ˚C	Glucose	N.A.	N.A.	Intertidal zone	(61)
22. Base (pH 12), 24 hr	0.51 mol H_2 / mol hexose	pH 5.5; 60.0 ˚C	Sucrose	92% sucrose	0.67	Palm oil mill effluent (POME) treatment plant	(36)
23. Base (pH 12), 24 hr	0.38 mol H_2/mol sucrose	pH 5.5; 35.0 ˚C	Sucrose	N.A.	0.95	Soybean-processing wastewater treatment plant	(41)
24. Base (pH 12), 24 hr	[a] 0.42 mol H_2/ g COD	pH 10.5; 37.0 ˚C	Peptone and Glucose	N.A.	N.A.	Sewage treatment plant	(209)
25. Base (pH 12), 24 hr	0.00037 mol H_2/g COD	pH 5.5; 35.0 ˚C	POME	59 % COD	N.A.	Palm oil mill effluent (POME) treatment plant	(66)
26. Base (pH 12), 24 hr	[a] 0.00240 mol H_2/g VS	pH 6.0; 60.0 ˚C	Cassava stillage	N.A.	N.A.	Anaerobic treatment plant	(67)
27. Base (pH 12), 5 min	[a] 0.00006 mol H_2/g VS	pH 7.0; 37.0 ˚C	Sewage sludge	N.A.	2.1	Sewage treatment plant	(78)
28. Base (pH 12), 5 min	[a] 0.00047 mol H_2/g VS	pH 11.5; 37.0 ˚C	Sewage sludge	N.A.	5.3	Sewage treatment plant	(78)
Chemical activation							
29. Reactivated in clostridium enforcement medium, 15 days	2.19 mol H_2/mol hexose	pH 6.0; 35.0 ˚C	Cellobiose	N.A.	N.A.	Cattle farm	(139)
30. Loading shock (50 g sucrose/L), 2 days	1.96 mol H_2/ mol hexose	pH 5.5; 60.0 ˚C	Sucrose	97% sucrose	10.59	Palm oil mill effluent (POME) treatment plant	(36)
31. Loading shock (50 g sucrose/L), 2 days	0.199 mol H_2/ L POME	pH 5.5; 60.0 ˚C	POME	N.A.	1.34	Palm oil mill effluent (POME) treatment plant	(36)

Table A3: Summary of H_2 production from chemically pretreated sludge at their reported fermentation conditions (CONTINUE)

Pretreatment	H_2 Yield	Initial pH and temp.	Substrate	Substrate consumption	Acetate : butyrate	Sludge source	Ref.
32. Reactivated in rice medium, 1 month	[a] 0.00212 mol H_2/g TS	36.0 ˚C	Untreated sweet sorghum stalk	89.12% sugar; 15% hemi-cellulose; 14% cellulose	N.A.	Composting plant	(130)
33. Reactivated in rice medium, 1 month	[a] 0.00517 mol H_2/g TS	36.0 ˚C	Alkali treated sweet sorghum stalk	99 % sugar; 54% hemi-cellulose; 42% cellulose	N.A.	Composting plant	(130)
34. KNO_3 (10 mmol/L)	[a] 0.0345 mol H_2	pH 7.0; 35.0 ˚C	Sucrose	N.A.	N.A.	Intertidal zone	(138)
Chemical inhibition							
35. BES (10 mmol/L), 24 hr	0.33 mol H_2/mol glucose	pH 7.0; 35.0 ˚C	Glucose	70.20%	4.18	Sewage treatment plant	(141)
36. BES (10 mmol), 30 min	1.01 mol H_2 / mol hexose	pH 5.5; 60.0 ˚C	Sucrose	95% sucrose	0.84	Palm oil mill effluent (POME) treatment plant	(36)
37. BES (0.2 g/L), 24 hr	0.0000317 mol H_2/g COD	29.0 ˚C	Dairy wastewater	87% COD	N.A.	H_2 producing reactor	(65)
38. Chloroform (1%), 24 hr	0.61 mol H_2/mol glucose	pH 7.0; 35.0 ˚C	Glucose	70.70%	1.88	Sewage treatment plant	(141)
39. Chloroform (2%), 24 hr	[a] 0.00353 mol H_2/ g glucose	pH 7.0; 35.0 ˚C	Glucose	N.A.	N.A.	Sewage treatment plant	(80)
40. Chloroform (0.1%), 24 hr	0.00023 mol H_2/g COD	pH 5.5; 35.0 ˚C	POME	51 % COD	N.A.	Palm oil mill effluent (POME) treatment plant	(66)
41. Chloroform (0.2%)	[a] 0.00134 mol H_2/g VS	pH 6.0; 60.0 ˚C	Cassava stillage	N.A.	N.A.	Anaerobic treatment plant	(67)

[a] H_2 yield was estimated using PV = nRT at standard condition where P = 1 atm; R = 8.21×10^{-5} m^3.atm/mol.K and T = 300 K

[b] Estimated value

Appendix 1-4: Supplementary table for literature review on H_2 production from sludge pretreated with physical coupled with chemical pretreatment

Table A4: Summary of H_2 production from sludge pretreated with physical coupled with chemical pretreatment at their reported fermentation conditions

	Pretreatment	H_2 Yield	Initial pH and temp.	Substrate	Substrate consumption	Acetate : butyrate	Sludge source	Ref.
1.	Heat (boiling) + aeration (4 min)	1.83 mol H_2/mol glucose	pH 6.0; 37.0 ˚C	Glucose	N.A.	N.A.	River sludge	(83)
2.	Heat (77 ˚C) + Ultrasonic (20 min)	1.55 mol H_2/mol glucose	pH 6.5; 37.0˚C	Glucose	100% glucose	1.9	Sewage treatment plant	(37)
3.	Heat (repeated boiling) + chloroform (0.05%)	0.51 mol H_2/mol glucose	pH 7.0; 37.0 ˚C	Wheat powder solution	N.A.	N.A.	Beer Industry	(82)
4.	Chloroform (0.05%) + heat (repeated boiling)	0.44 mol H_2/mol glucose	pH 7.0; 37.0 ˚C	Wheat powder solution	N.A.	N.A.	Beer Industry	(82)
5.	Heat 90˚C + Ultrasonic	[a] 1.32–1.50 mol H_2/g COD	pH 6.5; 36.0˚C	Sucrose	N.A.	N.A.	Sewage treatment plant	(73)
6.	Acid (pH 5) + BES (0.2 g/L)	2.90×10^{-5} mol H_2/g COD	29.0 ˚C	Dairy wastewater	83% COD	N.A.	H_2 producing reactor	(65)
7.	Heat (100 ˚C) + acid (pH 5)	2.07×10^{-5} mol H_2/g COD	29.0 ˚C	Dairy wastewater	86% COD	N.A.	H_2 producing reactor	(65)
8.	Acid (pH 5) + heat (100 ˚C) + BES (0.2 g/L)	1.08×10^{-5} mol H_2/g COD	29.0 ˚C	Dairy wastewater	83% COD	N.A.	H_2 producing reactor	(65)
9.	Heat (100 ˚C) + BES (0.2 g/L)	8.40×10^{-6} mol H_2/gCOD	29.0 ˚C	Dairy wastewater	81% COD	N.A.	H_2 producing reactor	(65)
10.	Heat (boiling) + freeze -20˚C + thaw (4˚C)	0.41 mol H_2/mol glycerol	pH 6.0; 37.0 ˚C	Glycerol	N.A.	N.A.	Sewage treatment plant	(84)
11.	Heat (95 ˚C) + acid (pH 3–5), 48 hr	[a] 0.0545 mol H_2	pH 5.0; 55.0 ˚C	Glucose	N.A.	N.A.	Cattle farm	(251)
12.	Water soak (3 hr) + Reactivated in glucose (3 days)	[a] 0.011 mol H_2/g substrate	pH 5– 5.5; 36.0 ˚C	Stale corn	N.A.	[b] 2.12	Cattle farm	(252)
13.	Aeration (4 days) + Reactivated in glucose (3 days)	[a] 0.010 mol H_2/g substrate	pH 5– 5.5; 36.0 ˚C	Stale corn	N.A.	N.A.	Cattle farm	(252)
14.	UV (3 hr) + Reactivated in glucose (3 days)	[a] 0.010 mol H_2/g substrate	pH 5– 5.5; 36.0 ˚C	Stale corn	N.A.	N.A.	Cattle farm	(252)

[a] H_2 yield was estimated using PV = nRT at standard condition where P = 1 atm; R = 8.21×10^{-5} m^3.atm/mol.K and T = 300 K

[b] Estimated value

Appendix 1-5: Supplementary table for literature review on Comparison of microbial diversity in various pretreated and untreated sludge

Table A5: Comparison of microbial diversity in various pretreated and untreated sludge inocula

Source of Sludge	Microbial community	Pretreatment	H_2 yield	Ref.
1. Sewage treatment plant B	*Clostridium acetobutyricum* (AE0011437.1) *Clostridium butyricum* (DQ831124.1) *Clostridium* sp. HPB-21 (AY862509.1) Uncultured *Clostridium* sp. (EF700377.1)	Heat	2.30 mol H_2/mol glucose	(31)
2. Sewage treatment plant C	*Bacillus* sp. (DQ168845.1) *Clostridium butyricum* (DQ831124.1) *Clostridium acetobutyricum* (DQ235219.1 and FM994940.1) *Clostridium* sp. (DQ168846.1) *Lactobacillus delbrueekii* (FJ915706.1) Uncultured bacterium (DQ235219.1) Uncultured *Bacillus* sp. (DQ168845.1) Uncultured *Clostridium* (DQ168846.1)	Heat	2.18 mol H_2/mol glucose	(60)
3. Intertidal sludge	*Bacillus megaterium* (HM104462)	Heat	1.65 mol H_2/mol glucose	(57)
4. Sewage treatment plant A	*Clostridium acetobutyricum* (AE001437.1) *Clostridium* sp. *JRI19* (EF067828.1) *Bacillus thuringiensis* (EF210289.1) *Clostridium butyricum* (DQ831124.1) Uncultured bacterium (DQ795258.1)	Heat	1.64 mol H_2/mol glucose	(31)
5. Sewage treatment plant D	*Bifidobacterium boum* (AY166529.1) *Clostridium* sp. (FJ876436.1) *Clostridium butyricum* (DQ831124.1) *Clostridium acetobutyricum* (FM994940.1) *Lactobacillus fermentum* (GQ131282.1) *Lactobacillus delbrueekii* (FJ915705.1 and FJ915706.1) Uncultured bacterium (AB441617.1)	Heat	1.32 mol H_2/mol glucose	(60)

Table A5: Comparison of microbial diversity in various pretreated and untreated sludge inocula (CONTINUE)

Source of Sludge	Microbial community	Pretreatment	H_2 yield	Ref.
6. Intertidal sludge	*Bacillus* sp. (GQ180912) *Lactobacillus plantarum* (GQ180905 and GQ180906) *Clostridium* sp. (GQ180907, GQ180908, GQ180910 and GQ180911) *Enterococcus faecium* (GQ180909)	Freeze and thaw	0.15 mol H_2/mol glucose	(61)
7. Sewage treatment plant	*Clostridium cellulosi* *Clostridium acetobutylicum* *Clostridium tyrobutyricum* *Streptococcus bovis* *Citrobacter* sp.	N.A.	0.261 mol H_2/g glucose	(164)
8. Cattle manure composting plant	*Clostridium saccharolyticum* strain HAWE3 (AY604565) *Clostridium butyricum* strain W4 (DQ831126) *Clostridium aerotolerans* (X76163) *Clostridium sphenoides* (X73449) *Enterococcus gallinarum* (EF025908) *Enterococcus saccharolyticus* (U30931)	Chemical activation	2.19 mol H_2/mol hexose	(139)
9. POME treatment plant	*Thermoanaerobacterium sp.* (AY999015) *Thermoanaerobacterium thermosaccharolyticum* (AY999014) *Clostridium thermopalmarium* (AF286862)	Loading shock	1.96 mol H_2/ mol hexose	(36)
10. POME treatment plant	*Thermoanaerobacterium thermosaccharolyticum* (AF247003 and AY999014)	Heat	1.61 mol H_2/ mol hexose	(36)
11. POME treatment plant	*Thermoanaerobacterium* sp. (AY350594) *Thermoanaerobacterium thermosaccharolyticum* (AY999014) *Clostridium* sp. (AF252325) *Bacillus* sp. (AB020196)	BES	1.01 mol H_2 / mol hexose	(36)
12. POME treatment plant	*Lactobacillus* sp. (AY363384) *Bacillus* sp. (AB193859) *Clostridium* sp. (AB234007)	Acid	0.65 mol H_2 / mol hexose	(36)

13. POME treatment plant	*Clostridium* sp. (AB234007 and AF252325) *Bacillus* sp. GB02-25 (DQ079010)	Base	0.51 mol H_2 / mol hexose	(36)

Table A5: Comparison of microbial diversity in various pretreated and untreated sludge inocula (CONTINUE)

Source of Sludge	Microbial community	Pretreatment	H_2 yield	Ref.
14. POME treatment plant	*Bacillaceae bacterium* NS1-3 (AY466703) *Clostridiales bacterium* NS5-4 (AY466717) *Clostridium* sp. L1/6 (AY188846) *Thermoanaerobacterium thermosaccharolyticum* (AY999014) *B. circulans* (AY294321) *Thermoanaerobacterium* sp. (AB034720)	Untreated	0.3 mol H_2 / mol hexose	(36)
15. Sucrose-based synthetic wastewater sludge	*Clostridium butyricum* CGS5	Heat	2.78 mol H_2/ mol sucrose	(53)
16. Sewage treatment plant	*Klebsiella* sp. HE1 (AY540111)	N.A.	0.92 mol H_2/ mol sucrose	(165)
17. Mangrove sludge	*Pantoea agglomerans*	Chemical activation	0.021 mol H_2/ L biogas	(166)
18. Intertidal sludge	*Bacillus* sp. B2 (JF449443)	Heat	0.0325 mol H_2	(57)
19. Sewage treatment plant	*Ethanoligenens harbinens* YUAN-3 (AY295777) *Enterobacter aerogenes* strain Aq16 (EU554442) *Ethanoligenens harbinens* YUAN-3 (AY295777) *Bacteroides vulgatus* ATCC8482 (CP000139)	Aeration	0.00912 mol H_2	(167)
20. Sewage treatment plant	*Clostridium tyrobutyricum* stain MPP-41 (DQ911273) Clostridium vincentii CGS6 (AY540110)	Heat	0.0077 mol H_2	(167)
21. Sewage treatment plant	*Acidovorax facilis strain* LMG 2193 (EU024133) *Clostridium tyrobutyricum* stain MPP-41 (DQ911273)	Untreated	0.0071 mol H_2	(167)
22. Sewage treatment plant	*Propionibacterium granulosum* cryptic plasmid PG01 (AY150274) *Clostridium tyrobutyricum* stain MPP-41 (DQ911273) *Clostridium longisporum* strain DSM8431 (X76164)	Acid	0.00211 mol H_2	(167)

Table A5: Comparison of microbial diversity in various pretreated and untreated sludge inocula (CONTINUE)

Source of Sludge	Microbial community	Pretreatment	H_2 yield	Ref.
23. Sewage treatment plant	*Clostridium tyrobutyricum* stain MPP-41 (DQ911273) *Clostridium vincentii* CGS6 (AY540110) *Bacteroides vulgatus* ATCC8482 (CP000139) *Clostridium longisporum* strain DSM8431 (X76164)	Base	0.00211 mol H_2	(167)
24.				
25. Sewage treatment plant	*Thermoanaerobacterium* genus	Heat	0.00430 mol H_2/ g cellulose	(131)
26. Sewage treatment plant	*Clostridium acetobutyricum* (FM994940.1) *Klebsiella pneumonia* (GQ214541.1) *Clostridium butyricum* (DQ831124.1) Uncultured bacterium (DQ464539.1 and DQ414811.1)	Heat	0.0106 mol H_2/ g carbohydrate	(49)
27. POME treatment plant	Clostridium paraputrificum JCM 5237 (AB627080.1) Clostridium bovipellis B30 (EF512134.1) Weissella soli strain NS26 (EU180607.1) Clostridium tyrobutyricum A1-3 (GU227148.1) Clostridium butyricum TM-9B (FR734080.1) Clostridium thermopalmarium KU-M1 (HM756303.1) Thermoanaerobacterium thermosaccharolyticum D120-70 (AF247003.1) Clostridium hydrogeniformans BL-20 (DQ196623.2) Clostridium sp. BS-1 (FJ805840.2) Clostridium beijerinckii HU-2 (AB626806.1) Clostridium baratii LCR23 (HQ259733.1) Clostridium sp. M-43 (AB504378.1)	Heat	0.047 mol H_2/LPOME	(168)

Appendix 2-1: Calculation for hydrogen yield

1. Concentration of hydrogen (ppm_v) in calibration gas

Formula:

In volume fraction (vol./vol.)

$$ppm_v = \frac{Vol_{H2}}{Vol_{total}} \times 10^6$$

In mole fraction (mol/mol)

$$ppm_v = \frac{mol_{H2}}{mol_{total}} \times 10^6$$

*Note: 10^6 is a conversion factor for ppm_v. 10^9 should be used for ppb_v

In the calibration gas for GC, 5% represents hydrogen gas

5% mol = 5% vol. = 0.05 v/v

Hence,

Concentration of hydrogen = 0.05×10^6

= $\underline{5 \times 10^4\ ppm_v}$

For example, if the calibration peak area for hydrogen was measured at ~61, then this area represents 5×10^4 ppm_v of hydrogen in the calibration gas.

2. Volume of bio-hydrogen in sample

Example:

Total biogas = 0.270 L (0.27×10^{-3} m^3); Peak area of hydrogen for sample: 1400

$$Concentration\ of\ hydrogen = \frac{1400}{61} \times (5 \times 10^4 ppm_v)$$

= $\underline{1.148 \times 10^6\ ppm_v}$

$$ppm_v = \frac{Vol_{H2}}{Vol_{total}} \times 10^6$$

$$Vol_{H2} = \frac{ppm_v}{1 \times 10^6} \times vol_{total}$$

$$= \frac{1.148 \times 10^6}{1 \times 10^6} \times (0.27 \times 10^{-3} m^3)$$

= $\underline{0.30996 \times 10^{-3}\ m^3}$

3. Mol of bio-hydrogen in sample

$$PV = nRT$$

where,

P = 1 atm,

R = 8.205 ×10^{-3} m^3.atm/mol.K,

T = 310.15 K (37°C)

When V = 0.30996 × 10^{-3} m^3,

$$mol\ of\ hydrogen = \frac{(1)(0.30996 \times 10^{-3})}{(8.205 \times 10^{-5})(310.15)}$$

$$= \underline{0.01218\ mol}$$

4. Hydrogen yield

Mol of glucose:

No. of moles = concentration (g/L) × volume (L)

When 10 g/L glucose was used in 150 mL of fermentation medium

No. of moles = 10 g/L × 0.15 L

$$= \underline{0.00833\ mol}$$

Therefore

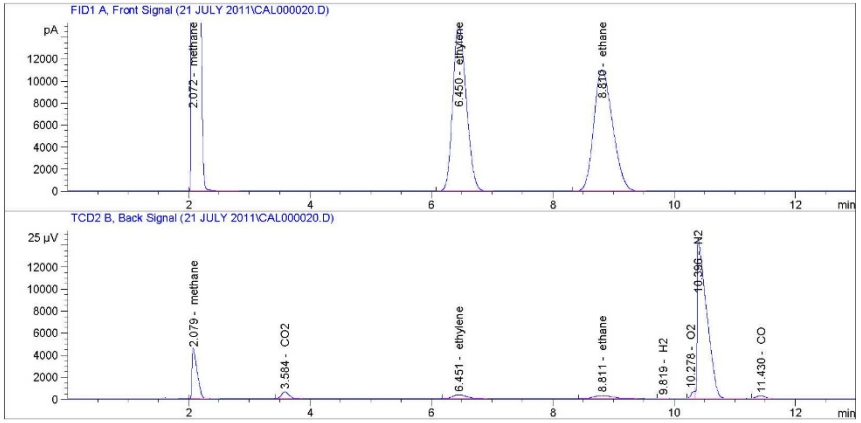
$$Hydrogen\ yield = \frac{0.01218}{0.00833}$$

$$= \underline{1.46\ mol\ H_2\ /\ mol\ glucose}$$

Appendix 2-2: Chromatogram of biogas profile for H_2 production from glucose using landfill leachate sludge at optimum conditions

Data File C:\CHEM32\1\DATA\21 JULY 2011\CAL000020.D
Sample Name: Cal

```
Acq. Operator   : yee meng
Acq. Instrument : Instrument 1                  Location : Vial 1
Injection Date  : 7/21/2011 10:58:04 AM
                                              Inj Volume : Manually
Acq. Method     : C:\CHEM32\1\METHODS\ANALYSIS.M
Last changed    : 7/21/2011 10:52:34 AM by yee meng
Analysis Method : C:\CHEM32\1\METHODS\SHUTDOWN.M
Last changed    : 7/21/2011 1:11:05 PM by yee meng
                  (modified after loading)
Method Info     : Training

Sample Info     : Cal
```




```
                    External Standard Report

Sorted By             :      Signal
Calib. Data Modified  :      7/21/2011 12:44:13 PM
Multiplier:                   :      1.0000
Dilution:                     :      1.0000
Sample Amount:                :      1.00000  [mole%]   (not used in calc.)
Use Multiplier & Dilution Factor with ISTDs
```

Data File C:\CHEM32\1\DATA\21 JULY 2011\CAL000020.D
Sample Name: Cal

Signal 1: FID1 A, Front Signal

RetTime [min]	Type	Area [pA*s]	Amt/Area	Amount [mole%]	Grp	Name
2.072	BB S	8.87269e5	1.69058e-5	15.00000		methane
6.450	BB	2.31431e5	8.64189e-6	2.00000		ethylene
8.810	BB	2.36452e5	8.45838e-6	2.00000		ethane
Totals :				19.00000		

Signal 2: TCD2 B, Back Signal

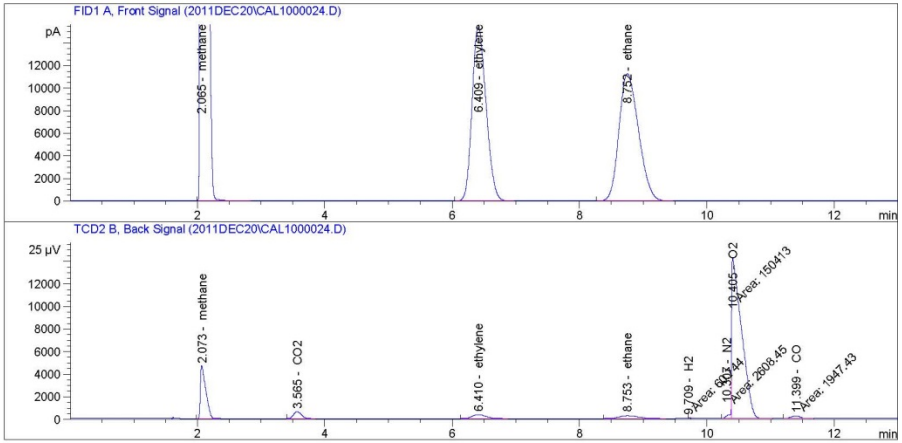
RetTime [min]	Type	Area [25 µV*s]	Amt/Area	Amount [mole%]	Grp	Name
2.079	BB	2.64708e4	5.66662e-4	15.00000		methane
3.584	BB	5098.37451	3.92282e-4	2.00000		CO2
6.451	BB	5164.02441	3.87295e-4	2.00000		ethylene
8.811	BB	5436.59961	3.67877e-4	2.00000		ethane
9.819	MM N	61.12198	8.18036e-2	5.00000		H2
10.278	MF	2460.35645	5.08056e-4	1.25000		O2
10.396	FM	1.55574e5	4.61195e-4	71.75000		N2
11.430	MM	2278.77881	4.38832e-4	1.00000		CO
Totals :				100.00000		

Instrument 1 7/21/2011 1:16:30 PM yee meng Page 1 of 5

Figure A1: Chromatogram for GC calibration of gas standards on July

Data File C:\CHEM32\1\DATA\2011DEC20\CAL1000024.D
Sample Name: cal1

```
==================================================================
Acq. Operator   : meng
Acq. Instrument : Instrument 1                    Location : Vial 1
Injection Date  : 12/21/2011 12:47:21 AM
                                                 Inj Volume : Manually
Acq. Method     : C:\CHEM32\1\METHODS\ANALYSIS.M
Last changed    : 12/21/2011 12:46:09 AM by meng
Analysis Method : C:\CHEM32\1\METHODS\SHUTDOWN.M
Last changed    : 12/21/2011 1:31:21 AM by meng
                  (modified after loading)
Method Info     : Training

Sample Info     : cal1
```




```
==================================================================
                    External Standard Report
==================================================================

Sorted By             :      Signal
Calib. Data Modified  :      Wednesday, December 21, 2011 1:31:20 AM
Multiplier:                   :      1.0000
Dilution:                     :      1.0000
Sample Amount:                :     1.00000  [mole%]   (not used in calc.)
Use Multiplier & Dilution Factor with ISTDs
```

Data File C:\CHEM32\1\DATA\2011DEC20\CAL1000024.D
Sample Name: cal1

Signal 1: FID1 A, Front Signal

RetTime [min]	Type	Area [pA*s]	Amt/Area	Amount [mole%]	Grp	Name
2.065	BB S	9.13246e5	1.64249e-5	15.00000		methane
6.409	BB	2.35503e5	8.49247e-6	2.00000		ethylene
8.752	BB	2.40390e5	8.31981e-6	2.00000		ethane
Totals :				19.00000		

Signal 2: TCD2 B, Back Signal

RetTime [min]	Type	Area [25 µV*s]	Amt/Area	Amount [mole%]	Grp	Name
2.073	BB	2.71963e4	5.51545e-4	15.00000		methane
3.565	BB	5192.37354	3.85180e-4	2.00000		CO2
6.410	BB	5294.27197	3.77767e-4	2.00000		ethylene
8.753	BB	5578.08057	3.58546e-4	2.00000		ethane
9.709	MM N	60.74398	8.23127e-2	5.00000		H2
10.307	MF	2608.44971	2.75068e-2	71.75000		N2
10.405	FM	1.50413e5	8.31043e-6	1.25000		O2
11.399	MM	1947.43274	5.13497e-4	1.00000		CO
Totals :				100.00000		

1 Warnings or Errors :

Warning : Calibration warnings (see calibration table listing)

```
==================================================================
                      *** End of Report ***
```


Figure A2: Chromatogram for GC calibration of gas standards on Dec.

Data File C:\CHEM32\1\DATA\YEE MENG\2012MAY02000213.D
Sample Name: cal

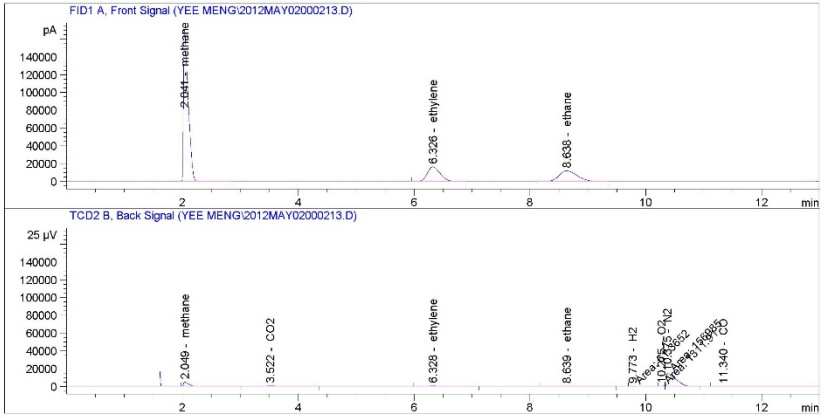
==========

Location : Vial 1

Inj Volume : Manually

Acq. Method : C:\CHEM32\1\METHODS\ANALYSIS.M
Last changed : 4/27/2011 12:30:32 PM by J
Analysis Method : C:\CHEM32\1\METHODS\ANALYSIS.M
Last changed : 4/24/2014 11:18:28 AM by LUTFI
(modified after loading)
Method Info : Training

Sample Info : cal



==========

Normalized Percent Report

==========

Sorted By : Signal
Calib. Data Modified : 4/24/2014 11:17:40 AM
Multiplier: : 1.0000
Dilution: : 1.0000
Sample Amount: : 1.00000 [mole%] (not used in calc.)
Use Multiplier & Dilution Factor with ISTDs

Signal 1: FID1 A, Front Signal

RetTime [min]	Type	Area [pA*s]	Amt/Area	Norm %	Grp	Name
2.041	BB S	9.58268e5	1.62902e-5	12.706510		methane
6.326	BB	2.47437e5	8.37714e-6	1.687225		ethylene
8.638	BB	2.53351e5	9.12351e-6	1.881472		ethane

Data File C:\CHEM32\1\DATA\YEE MENG\2012MAY02000213.D
Sample Name: cal

				Norm %	Grp	Name
				5.275207		

Signal 2: TCD2 B, Back Signal

RetTime [min]	Type	Area [25 µV*s]	Amt/Area	Norm %	Grp	Name
2.049	BB	2.78754e4	5.34790e-4	12.134399		methane
3.522	BB	5375.77295	3.86680e-4	1.692024		CO2
6.328	BB	5461.09570	3.92365e-4	1.744147		ethylene
8.639	BV	5769.66895	4.13381e-4	1.941398		ethane
9.773	MM N	61.36522	6.03272e-2	3.013346		H2
10.275	MF	1311.91199	4.43881e-4	0.474008		O2
10.375	FM	1.56985e5	4.85206e-4	62.000984		N2
11.340	BBA	1917.40344	4.64198e-4	0.724487		CO

Totals : 83.724793

1 Warnings or Errors :

Warning : Calibration warnings (see calibration table listing)

==========

*** End of Report ***

Figure A3: Chromatogram for GC calibration of gas standards on May

Data File C:\CHEM32\1\DATA\YEE MENG\2012MAY02000218.D
Sample Name: 48hr pH6 Rep2

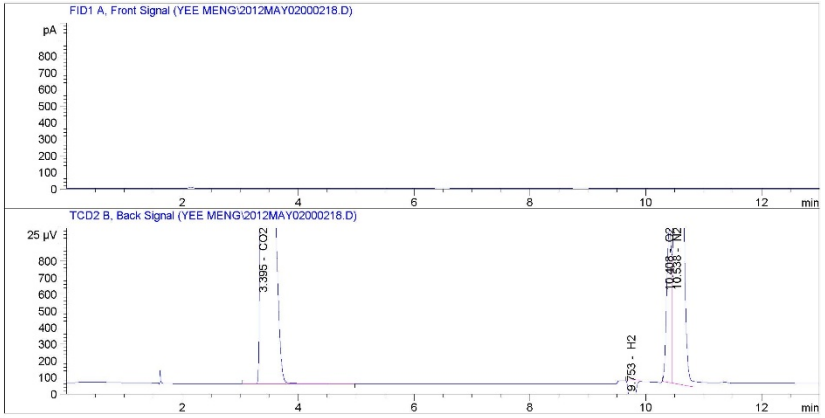
==========

Location : Vial 1

Inj Volume : Manually

Acq. Method : C:\CHEM32\1\METHODS\ANALYSIS.M
Last changed : 5/2/2012 10:26:01 PM by yee meng
Analysis Method : C:\CHEM32\1\METHODS\ANALYSIS.M
Last changed : 4/24/2014 11:20:39 AM by LUTFI
(modified after loading)
Method Info : Training

Sample Info : 48hr pH6 Rep2



==========

Normalized Percent Report

==========

Sorted By : Signal
Calib. Data Modified : 4/24/2014 11:20:43 AM
Multiplier: : 1.0000
Dilution: : 1.0000
Sample Amount: : 1.00000 [mole%] (not used in calc.)
Use Multiplier & Dilution Factor with ISTDs

Signal 1: FID1 A, Front Signal

RetTime [min]	Type	Area [pA*s]	Amt/Area	Norm %	Grp	Name
2.041		-	-	-		methane
6.326		-	-	-		ethylene
8.638		-	-	-		ethane

Data File C:\CHEM32\1\DATA\YEE MENG\2012MAY02000218.D
Sample Name: 48hr pH6 Rep2

Norm % Grp Name
0.000000

Signal 2: TCD2 B, Back Signal

RetTime [min]	Type	Area [25 µV*s]	Amt/Area	Norm %	Grp	Name
2.049		-	-	-		methane
3.395	BB	5.32964e4	3.86680e-4	11.492490		CO2
6.328		-	-	-		ethylene
8.639		-	-	-		ethane
9.753	MM N	2418.62061	6.03272e-2	78.002244		H2
10.408	MF	5451.44775	4.43881e-4	1.349408		O2
10.538	FM	3.38383e4	4.85206e-4	9.155858		N2
11.340		-	-	-		CO

Totals : 100.000000

2 Warnings or Errors :

Warning : Calibration warnings (see calibration table listing)
Warning : Calibrated compound(s) not found

==========

*** End of Report ***

Figure A4: Chromatogram for batch fermentation at optimum conditions pH 6, 37˚C and 10 g/L glucose for 48 h. 04 May 2012 Rep 1

Data File C:\CHEM32\1\DATA\YEE MENG\2012MAY02000218.D
Sample Name: 48hr pH6 Rep2

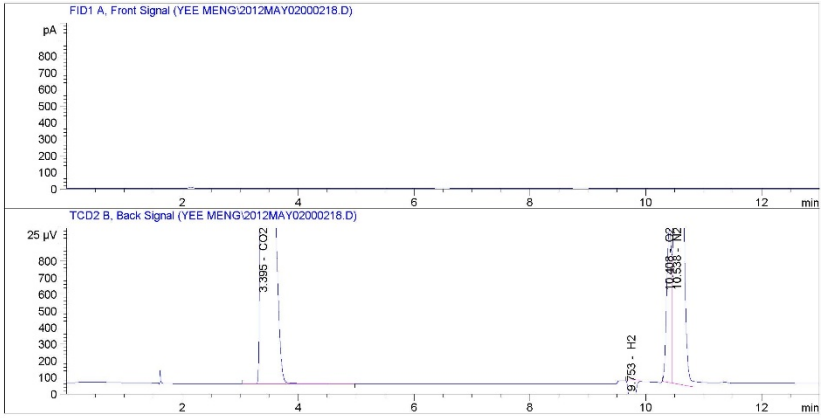
==========

Location : Vial 1

Inj Volume : Manually

Acq. Method : C:\CHEM32\1\METHODS\ANALYSIS.M
Last changed : 5/2/2012 10:26:01 PM by yee meng
Analysis Method : C:\CHEM32\1\METHODS\ANALYSIS.M
Last changed : 4/24/2014 11:20:39 AM by LUTFI
(modified after loading)
Method Info : Training

Sample Info : 48hr pH6 Rep2



==========

Normalized Percent Report

==========

Sorted By : Signal
Calib. Data Modified : 4/24/2014 11:20:43 AM
Multiplier: : 1.0000
Dilution: : 1.0000
Sample Amount: : 1.00000 [mole%] (not used in calc.)
Use Multiplier & Dilution Factor with ISTDs

Signal 1: FID1 A, Front Signal

RetTime [min]	Type	Area [pA*s]	Amt/Area	Norm %	Grp	Name
2.041		-	-	-		methane
6.326		-	-	-		ethylene
8.638		-	-	-		ethane

Data File C:\CHEM32\1\DATA\YEE MENG\2012MAY02000218.D
Sample Name: 48hr pH6 Rep2

Norm % Grp Name
0.000000

Signal 2: TCD2 B, Back Signal

RetTime [min]	Type	Area [25 µV*s]	Amt/Area	Norm %	Grp	Name
2.049		-	-	-		methane
3.395	BB	5.32964e4	3.86680e-4	11.492490		CO2
6.328		-	-	-		ethylene
8.639		-	-	-		ethane
9.753	MM N	2418.62061	6.03272e-2	78.002244		H2
10.408	MF	5451.44775	4.43881e-4	1.349408		O2
10.538	FM	3.38383e4	4.85206e-4	9.155858		N2
11.340		-	-	-		CO

Totals : 100.000000

2 Warnings or Errors :

Warning : Calibration warnings (see calibration table listing)
Warning : Calibrated compound(s) not found

==========

*** End of Report ***

Figure A5: Chromatogram for batch fermentation at optimum conditions pH 6, 37˚C and 10 g/L glucose for 48 h. 04 May 2012 Rep 2

Data File C:\CHEM32\1\DATA\YEE MENG\2012MAY02000220.D
Sample Name: 48hr pH6 Rep3

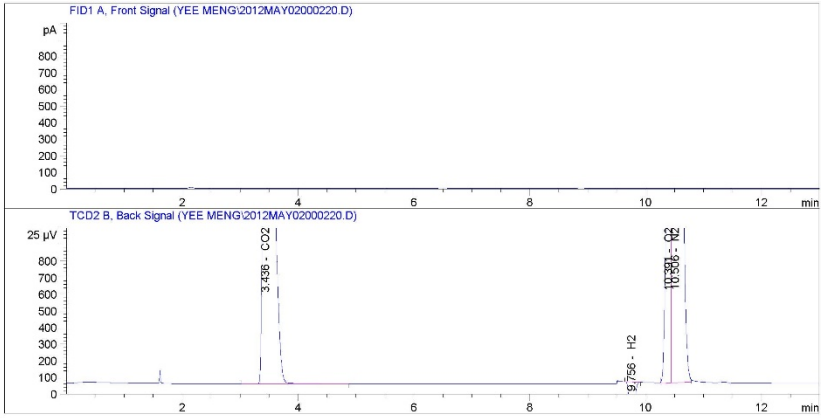
==========

Location : Vial 1

Inj Volume : Manually

Acq. Method : C:\CHEM32\1\METHODS\ANALYSIS.M
Last changed : 5/2/2012 10:53:14 PM by yee meng
Analysis Method : C:\CHEM32\1\METHODS\ANALYSIS.M
Last changed : 4/24/2014 11:20:39 AM by LUTFI
(modified after loading)
Method Info : Training

Sample Info : 48hr pH6 Rep3



==========

Normalized Percent Report

==========

Sorted By : Signal
Calib. Data Modified : 4/24/2014 11:20:43 AM
Multiplier: : 1.0000
Dilution: : 1.0000
Sample Amount: : 1.00000 [mole%] (not used in calc.)
Use Multiplier & Dilution Factor with ISTDs

Signal 1: FID1 A, Front Signal

RetTime [min]	Type	Area [pA*s]	Amt/Area	Norm %	Grp	Name
2.041		-	-	-		methane
6.326		-	-	-		ethylene
8.638		-	-	-		ethane

Data File C:\CHEM32\1\DATA\YEE MENG\2012MAY02000220.D
Sample Name: 48hr pH6 Rep3

Norm % Grp Name
0.000000

Signal 2: TCD2 B, Back Signal

RetTime [min]	Type	Area [25 µV*s]	Amt/Area	Norm %	Grp	Name
2.049		-	-	-		methane
3.436	BB	4.07435e4	3.86680e-4	9.801881		CO2
6.328		-	-	-		ethylene
8.639		-	-	-		ethane
9.756	MM N	2451.99631	6.03272e-2	73.015152		H2
10.391	MF	8819.10254	4.43881e-4	2.435514		O2
10.506	FM	4.88531e4	4.85206e-4	14.747453		N2
11.340		-	-	-		CO

Totals : 100.000000

2 Warnings or Errors :

Warning : Calibration warnings (see calibration table listing)
Warning : Calibrated compound(s) not found

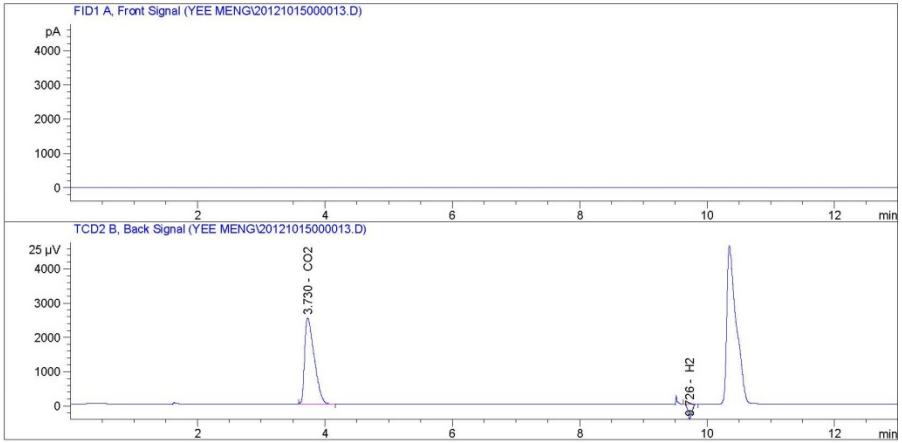
==========

*** End of Report ***

Figure A6: Chromatogram for batch fermentation at optimum conditions pH 6, 37˚C and 10 g/L glucose for 48 h. 04 May 2012 Rep 3

Data File C:\CHEM32\1\DATA\YEE MENG\20121015000013.D
Sample Name: Sludge Temp 37 48hr

```
==================================================================
Acq. Operator   : ym
Acq. Instrument : Instrument 1                    Location : Vial 1
Injection Date  : 10/15/2012 1:30:14 AM
                                                 Inj Volume : Manually
Acq. Method     : C:\CHEM32\1\METHODS\ANALYSIS.M
Last changed    : 10/15/2012 1:29:31 AM by ym
Analysis Method : C:\CHEM32\1\METHODS\SHUTDOWN.M
Last changed    : 10/15/2012 1:38:33 AM by ym
                  (modified after loading)
Method Info     : Training

Sample Info     : Sludge Temp 37 48hr Rep1
```




```
==================================================================
                      External Standard Report
==================================================================

Sorted By             :      Signal
Calib. Data Modified  :      10/15/2012 1:45:52 AM
Multiplier:                   :      1.0000
Dilution:                     :      1.0000
Sample Amount:                :      1.00000  [mole%]   (not used in calc.)
Use Multiplier & Dilution Factor with ISTDs
```

Data File C:\CHEM32\1\DATA\YEE MENG\20121015000013.D
Sample Name: Sludge Temp 37 48hr

Signal 1: FID1 A, Front Signal

RetTime [min]	Type	Area [pA*s]	Amt/Area	Amount [mole%]	Grp	Name
2.173		-	-	-		methane
6.558		-	-	-		ethylene
8.968		-	-	-		ethane
Totals :				0.00000		

Signal 2: TCD2 B, Back Signal

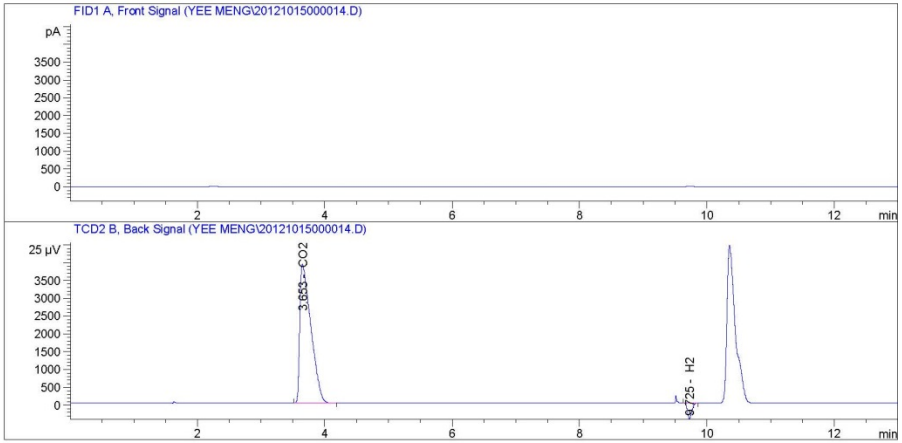
RetTime [min]	Type	Area [25 µV*s]	Amt/Area	Amount [mole%]	Grp	Name
2.075		-	-	-		methane
3.730	BB	2.58829e4	3.94268e-4	10.20480		CO2
6.425		-	-	-		ethylene
8.778		-	-	-		ethane
9.726	MM N	2266.39331	8.16850e-2	160.62482		H2
10.529		-	-	-		O2
10.718		-	-	-		N2
12.260		-	-	-		CO
Totals :				170.82962		

```
2 Warnings or Errors :

Warning : Calibration warnings (see calibration table listing)
Warning : Calibrated compound(s) not found

==================================================================
                      *** End of Report ***
```

Figure A7: Chromatogram for batch fermentation at optimum conditions pH 6, 37˚C and 10 g/L glucose for 48 h. 15 Oct 2012 Rep 1

Data File C:\CHEM32\1\DATA\YEE MENG\20121015000014.D
Sample Name: Sludge Temp 37 48hr

```
==================================================================
Acq. Operator   : ym
Acq. Instrument : Instrument 1                    Location : Vial 1
Injection Date  : 10/15/2012 1:43:59 AM
                                                 Inj Volume : Manually
Acq. Method     : C:\CHEM32\1\METHODS\ANALYSIS.M
Last changed    : 10/15/2012 1:43:21 AM by ym
Analysis Method : C:\CHEM32\1\METHODS\SHUTDOWN.M
Last changed    : 10/15/2012 1:38:33 AM by ym
                  (modified after loading)
Method Info     : Training

Sample Info     : Sludge Temp 37 48hr Rep2
```




```
==================================================================
                      External Standard Report
==================================================================

Sorted By             :      Signal
Calib. Data Modified  :      10/15/2012 1:45:52 AM
Multiplier:                   :      1.0000
Dilution:                     :      1.0000
Sample Amount:                :      1.00000  [mole%]   (not used in calc.)
Use Multiplier & Dilution Factor with ISTDs
```

Data File C:\CHEM32\1\DATA\YEE MENG\20121015000014.D
Sample Name: Sludge Temp 37 48hr

Signal 1: FID1 A, Front Signal

RetTime [min]	Type	Area [pA*s]	Amt/Area	Amount [mole%]	Grp	Name
2.173		-	-	-		methane
6.558		-	-	-		ethylene
8.968		-	-	-		ethane
Totals :				0.00000		

Signal 2: TCD2 B, Back Signal

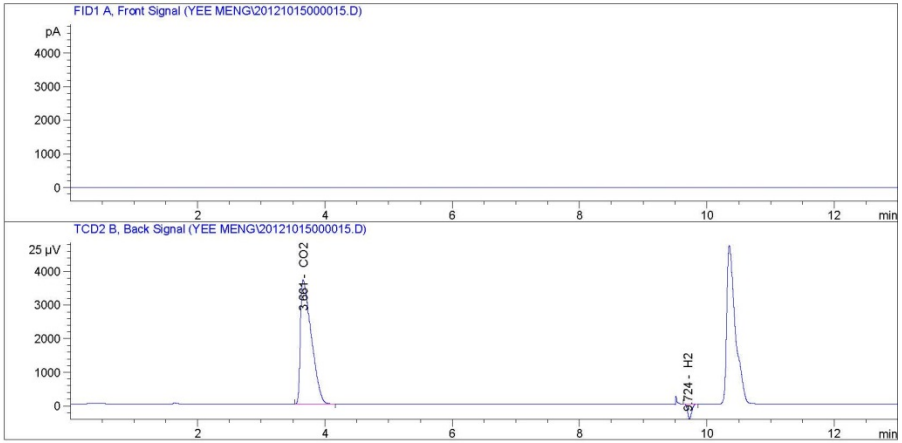
RetTime [min]	Type	Area [25 µV*s]	Amt/Area	Amount [mole%]	Grp	Name
2.075		-	-	-		methane
3.653	BB	4.67677e4	3.94268e-4	18.43899		CO2
6.425		-	-	-		ethylene
8.778		-	-	-		ethane
9.725	MM N	2188.89429	8.16850e-2	162.46281		H2
10.529		-	-	-		O2
10.718		-	-	-		N2
12.260		-	-	-		CO
Totals :				180.90180		

```
2 Warnings or Errors :

Warning : Calibration warnings (see calibration table listing)
Warning : Calibrated compound(s) not found

==================================================================
                      *** End of Report ***
```

Figure A8: Chromatogram for batch fermentation at optimum conditions pH 6, 37˚C and 10 g/L glucose for 48 h. 15 Oct 2012 Rep 2

Data File C:\CHEM32\1\DATA\YEE MENG\20121015000015.D
Sample Name: Sludge Temp 37 48hr

```
Acq. Operator   : ym
Acq. Instrument : Instrument 1                    Location : Vial 1
Injection Date  : 10/15/2012 1:57:51 AM
                                                  Inj Volume : Manually
Acq. Method     : C:\CHEM32\1\METHODS\ANALYSIS.M
Last changed    : 10/15/2012 1:57:09 AM by ym
Analysis Method : C:\CHEM32\1\METHODS\SHUTDOWN.M
Last changed    : 10/15/2012 1:38:33 AM by ym
                  (modified after loading)
Method Info     : Training

Sample Info     : Sludge Temp 37 48hr Rep3
```




```
External Standard Report

Sorted By             :      Signal
Calib. Data Modified  :      10/15/2012 1:45:52 AM
Multiplier:                   :      1.0000
Dilution:                     :      1.0000
Sample Amount:                :      1.00000  [mole%]   (not used in calc.)
Use Multiplier & Dilution Factor with ISTDs
```

Data File C:\CHEM32\1\DATA\YEE MENG\20121015000015.D
Sample Name: Sludge Temp 37 48hr

Signal 1: FID1 A, Front Signal

RetTime [min]	Type	Area [pA*s]	Amt/Area	Amount [mole%]	Grp	Name
2.173		-	-	-		methane
6.558		-	-	-		ethylene
8.968		-	-	-		ethane
Totals :				0.00000		

Signal 2: TCD2 B, Back Signal

RetTime [min]	Type	Area [25 µV*s]	Amt/Area	Amount [mole%]	Grp	Name
2.075		-	-	-		methane
3.661	BB	4.33659e4	3.94268e-4	17.09777		CO2
6.425		-	-	-		ethylene
8.778		-	-	-		ethane
9.724	MM N	2211.90857	8.16850e-2	164.34274		H2
10.529		-	-	-		O2
10.718		-	-	-		N2
12.260		-	-	-		CO
Totals :				181.44050		

```
2 Warnings or Errors :

Warning : Calibration warnings (see calibration table listing)
Warning : Calibrated compound(s) not found

                          *** End of Report ***
```


Figure A9: Chromatogram for batch fermentation at optimum conditions pH 6, 37˚C and 10 g/L glucose for 48 h. 15 Oct 2012 Rep 3

Appendix 3-1: Plot of modified Gompertz model for H_2 production from dairy wastewater

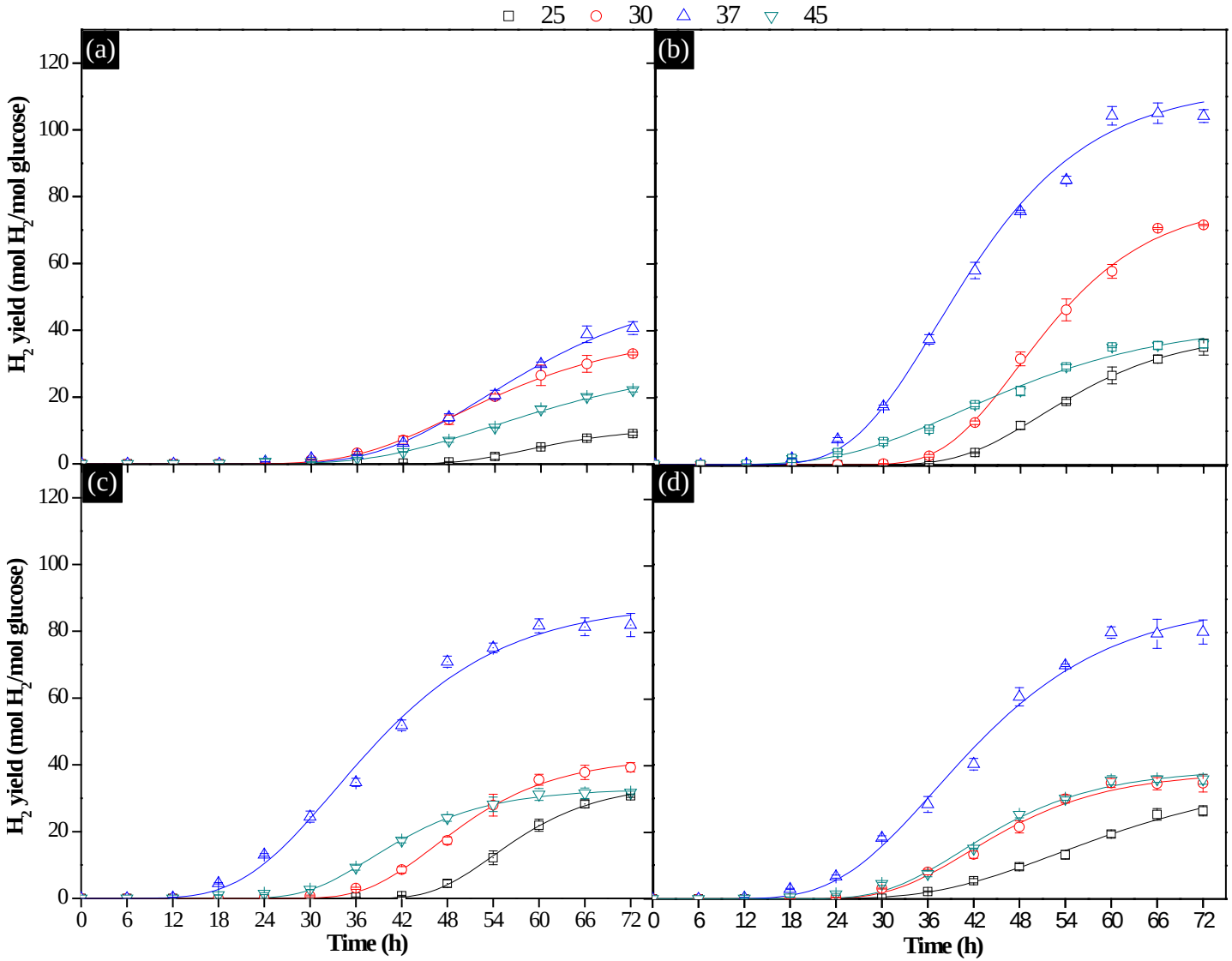


Figure A10:
Plot of modified Gompertz model for H_2 production from dairy wastewater at different pH and fermentation temperature: (a) pH 5, (b) pH 6, (c) pH 7, (b) pH8. No data from initial pH 4 and temperature 50˚C were not displayed as there were no H_2 productions detected. (Fermentation condition: dilution factor 20 % , 72 h)

Appendix 4-1: Plot of modified Gompertz model for H_2 production from isolates

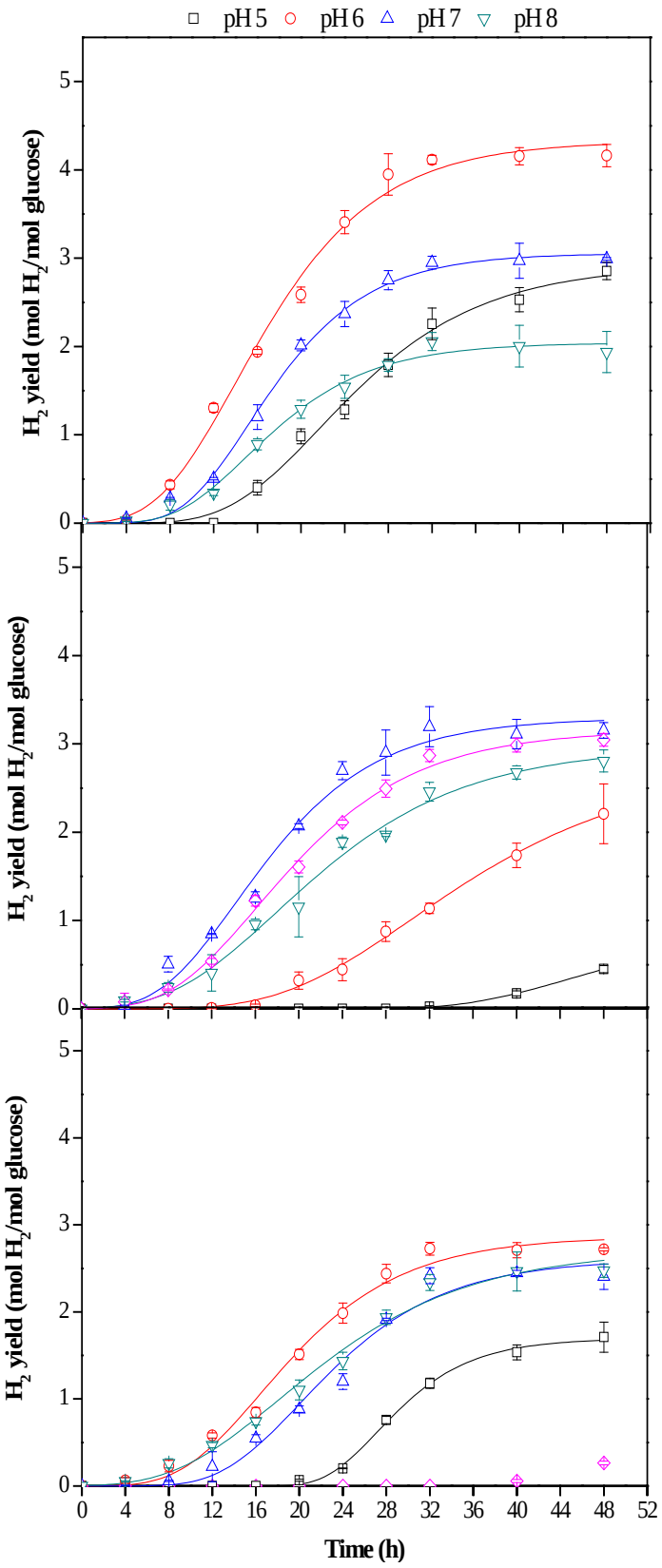


Figure A11: Plot of modified Gompertz model for H_2 production from isolates in the effect of pH: (a) C. *perfringens* strain JJC, *C. bifermentatns* strain WYM, *Clostridium* strain Ade.TY

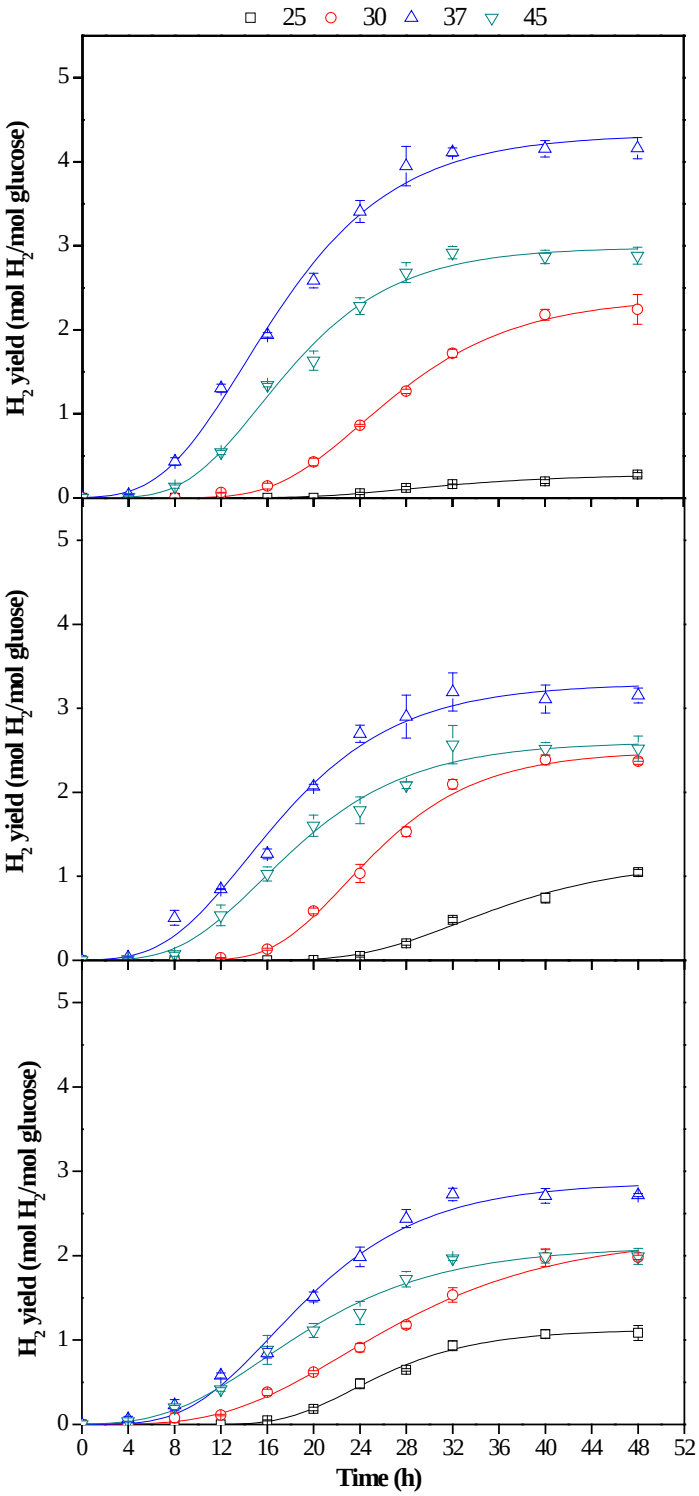


Figure A12: Plot of modified Gompertz model for H_2 production from isolates in the effect of temperature: (a) C. *perfringens* strain JJC, *C. bifermentatns* strain WYM, *Clostridium* strain Ade.TY

Appendix 5-1: Taxonomy report for H_2 producing bacteria based on 16S rRNA sequence

Taxonomy report for *Clostridium perfringens* strain JJC based on 16S rRNA sequence

```
Taxonomy Report
root .....................................  128 hits   11 orgs
. Bacteria ...............................  104 hits   10 orgs
[cellular organisms]
. . Clostridiaceae .......................   59 hits    6 orgs
[Firmicutes; Clostridia; Clostridiales]
. . . Clostridium ........................   58 hits    5 orgs
. . . . Clostridium perfringens ..........   57 hits    4 orgs
. . . . . Clostridium perfringens ATCC 13124 .    9 hits    1 orgs
. . . . . Clostridium perfringens str. 13 ....   11 hits    1 orgs
. . . . . Clostridium perfringens SM101 ......   10 hits    1 orgs
. . . . Clostridium sp. AB&J .................    1 hits    1 orgs
. . . Clostridiaceae bacterium bSSV11 ........    1 hits    1 orgs
[unclassified Clostridiaceae]
. . unclassified Bacteria (miscellaneous) ....    2 hits    2 orgs
[unclassified Bacteria]
. . . swine manure bacterium 37-4 ............    1 hits    1 orgs
. . . swine manure bacterium 37-3 ............    1 hits    1 orgs
. . environmental samples ....................   43 hits    2 orgs
. . . uncultured bacterium ...................   42 hits    1 orgs
. . . uncultured bacterium OI1612 ............    1 hits    1 orgs
. uncultured organism ........................   24 hits    1 orgs
[unclassified sequences; environmental samples]
```

Taxonomy report for *Clostridium sp.* strain Ade.TY based on 16S rRNA sequence

```
Taxonomy Report root .....................   102 hits   11 orgs
. Bacteria ...............................    84 hits   10 orgs
[cellular organisms]
. . uncultured bacterium .................    55 hits    1 orgs
[environmental samples]
. . Clostridiales ........................    29 hits    9 orgs
[Firmicutes; Clostridia]
. . . Clostridiaceae .....................    23 hits    6 orgs
. . . . Clostridium ......................    22 hits    5 orgs
. . . . . Clostridium sardiniense ........     9 hits    1 orgs
. . . . . Clostridium baratii ............     9 hits    1 orgs
. . . . . Clostridium sp. E-16 ...........     1 hits    1 orgs
. . . . . Clostridium paraputrificum .....     2 hits    1 orgs
. . . . . uncultured Clostridium sp. .....     1 hits    1 orgs
[environmental samples]
. . . . Clostridiaceae bacterium DJF_VP39k1 .     1 hits    1 orgs
[unclassified Clostridiaceae]
. . . Eubacterium ........................     6 hits    3 orgs
[Eubacteriaceae]
. . . . Eubacterium moniliforme ..........     2 hits    1 orgs
. . . . Eubacterium budayi ...............     2 hits    1 orgs
. . . . Eubacterium multiforme ...........     2 hits    1 orgs
. uncultured organism ....................    18 hits    1 orgs
[unclassified sequences; environmental samples]
```

Taxonomy report for *Clostridium bifermentans* strain WYM based on 16S rRNA sequence

```
Taxonomy Report Bacteria ..........................................
80 hits   33 orgs [root; cellular organisms]
. unclassified Bacteria (miscellaneous) ..............   14 hits   14 orgs
[unclassified Bacteria]
. . swine manure bacterium RT-1A .....................    1 hits    1 orgs
. . swine manure bacterium RT-4B .....................    1 hits    1 orgs
. . swine manure bacterium RT-5A .....................    1 hits    1 orgs
. . bacterium Te20A ..................................    1 hits    1 orgs
. . bacterium Te19A ..................................    1 hits    1 orgs
. . anaerobic bacterium B9 ...........................    1 hits    1 orgs
. . bacterium Te62A ..................................    1 hits    1 orgs
. . rumen bacterium R4-23 ............................    1 hits    1 orgs
. . rumen bacterium R2-11 ............................    1 hits    1 orgs
. . rumen bacterium R2-10 ............................    1 hits    1 orgs
. . rumen bacterium R3_91_26 .........................    1 hits    1 orgs
. . bacterium NLAE-zl-P791 ...........................    1 hits    1 orgs
. . bacterium NLAE-zl-P782 ...........................    1 hits    1 orgs
. . swine fecal bacterium RF2B-Pec1 ..................    1 hits    1 orgs
. Clostridiales ......................................   50 hits   18 orgs
[Firmicutes; Clostridia]
. . Peptostreptococcaceae ............................   28 hits    4 orgs
. . . [Clostridium] bifermentans .....................   19 hits    1 orgs
. . . [Clostridium] sordellii ........................    6 hits    1 orgs
. . . [Eubacterium] tenue ............................    2 hits    1 orgs
. . . [Clostridium] ghonii ...........................    1 hits    1 orgs
. . Clostridium ......................................   22 hits   14 orgs
[Clostridiaceae]
. . . Clostridium sp. zx5 ............................    1 hits    1 orgs
. . . Clostridium sp. Cd13 ...........................    1 hits    1 orgs
. . . Clostridium sp. EBD ............................    1 hits    1 orgs
. . . Clostridium sp. BL-21 ..........................    1 hits    1 orgs
. . . Clostridium sp. zx7 ............................    1 hits    1 orgs
. . . Clostridium sp. CS2 ............................    1 hits    1 orgs
. . . Clostridium sp. HP1 ............................    1 hits    1 orgs
. . . environmental samples ..........................   10 hits    2 orgs
. . . . uncultured Clostridium sp. ...................    9 hits    1 orgs
. . . . Clostridium sp. enrichment culture clone HT22 .    1 hits    1 orgs
. . . Clostridium sp. BS-8 ...........................    1 hits    1 orgs
. . . Clostridium sp. T7(2010) .......................    1 hits    1 orgs
. . . Clostridium sp. R1 .............................    1 hits    1 orgs
. . . Clostridium sp. HT12 ...........................    1 hits    1 orgs
. . . Clostridium sp. NB53 ...........................    1 hits    1 orgs
. uncultured bacterium ...............................   16 hits    1 orgs
[environmental samples]
```

Appendix 5-2: Multiple genome alignment

Multiple genome alignment for *Clostridium perfringens* strain JJC

	1	2	3	4
1: JJC_contig	100	95	95	88
2: Clostridium_perfringens_13	94	100	95	89
3: Clostridium_perfringens_ATCC_13124	94	95	100	88
4: Clostridium_perfringens_SM101	89	89	89	100

Multiple genome alignment for *Clostridium bifermentans* strain WYM

	1	2	3
1: C. bifermantans ATCC 19299 AVNB01	100	88	94
2: C. bifermantans ATCC 638 AVNC01	88	100	88
3: WYM_contig	95	88	100

Multiple genome alignment for *Clostridium sp.* strain Ade.TY

For multiple genome alignment for *Clostridium sp.* strain Ade.TY over 300 genome sequences was compared but only results from top 50 genome was displayed due to the constrain in space. The location of strain Ade.TY indicates by the black box. Green coloured box represents high identity score where as other yellow and orange coloured boxes represent low identity score.