Enhanced methane gas generation by reutilization of acidogenic off-gas during two-phase anaerobic digestion of food waste

Binghua Yan
Hong Kong Baptist University

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Enhanced Methane Gas Generation by Reutilization of Acidogenic Off-gas during Two-phase Anaerobic Digestion of Food Waste

YAN Binghua

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Principal Supervisor: Prof. WONG Jonathan W C

Hong Kong Baptist University

January 2015
DECLARATION

I hereby declare that this thesis represents my own work which has been done after registration for the degree of PhD at Hong Kong Baptist University, and has not been previously included in a thesis or dissertation submitted to this or any other institution for a degree, diploma or other qualifications.

Signature: __________________

Date: January 2015
ABSTRACT

Mass balance analysis of two-phase AD indicated that off-gas (H₂ and CO₂) produced in acidogenic reactor represent up to 30% of the consumed substrate and under most circumstances, this part of energy was not utilized leading to low overall energy recovery. Hence, the objective of this study was to enhance overall energy recovery during two-phase AD of food waste through reutilization of acidogenic off-gas and to further optimize the processes through manipulating the metabolic pathways and controlling acidogenic off-gas production.

In the first phase, feasibility of reutilizing acidogenic off-gas in methanogenic reactor and contribution of acidogenic off-gas to overall energy recovery was investigated. Acidogenic off-gas diversion increased the methane gas (CH₄, 0.28 L/g VSₘₐｄｄｅｄ) production up to 38.6%, of which ~8% was contributed by acidogenic off-gas. Both higher hydrolysis rate and COD production were also achieved with off-gas diversion.

Metabolic pathway determines the distributions of intermediate soluble products, which constitute the quality of acidogenic leachate. Therefore, two experiments focusing on manipulating metabolic pathways were performed. Firstly, the effects of four levels of headspace pressures, 6-12 psi (T1), ~3-6 psi (T2), ~3 psi (T3) and ambient pressure (T4) were investigated. Mixed acids metabolic pathways prevailed in all the treatments with butyrate as the single major component. Then, four different levels of H₂ partial pressure (P₇₃) were set the next experiment, self-generated P₇₃ (T1, control), 80% of H₂ (T2), 60% of H₂ (T3) and 0.04% of H₂, while the headspace pressure was kept at 3.3 psi. Typical butyrate fermentation pathways dominated in T4 whereas mixed acid fermentation pathways were prevailing in the other three treatments. Because of the improved hydrolysis/acidogenesis and higher quality of acidogenic products, overall CH₄ recovery in T4 (301.0 L/kg VSₘₐｄｄｅｄ) was 44.6% higher than the control.

In Phase III, strategies to enhance acidogenic off-gas production were investigated. First, four types of neutralization modes including daily pH adjustment of leachate to 6.0, methanogenic effluent recirculation, and initial addition of NaOH and lime separately at a dosage of 20.0 and 14.0 g/kg food waste, respectively, were investigated. Obviously, a H₂ production rate of 3.0 and 2.1 L/d with lime and NaOH addition was much higher than 0.7 and 0.4 L/d with effluent recirculation and daily
adjustment, respectively. Also, addition of alkali agents could enhance the COD leaching of food waste, especially with NaOH. A CH$_4$ production of 11.24 L/d could be attributed to both the elevated leachate quality and the acidogenic off-gas with lime addition. Another experiment investigated the effect of different carbohydrate contents in the substrates on acidogenic H$_2$ production. Anaerobic hydrolysis of wastes sourced from bakery (T1), Chinese-style restaurant (T2), western-style restaurant (T3) and wet market were performed in LBRs. Food waste collected from western-style restaurant with a carbohydrate content of 69.5% achieved the highest H$_2$ production of 61.0 L/kg VS$_{added}$. The highest specific CH$_4$ production rate at 0.42 L/gVS$_{added}$ was also achieved with western restaurant food waste.

Finally, the possible redirection of fluxes associated with shift of metabolic pathways from the experiment of PH$_2$ was proposed. Significant increase in the production of butyrate in treatment T4 with PH$_2$ of 3.3 psi $\times$ 0.04% indicated the channeling of electrons towards the production of butyrate. Dynamics of the microbial community were correlated with the distribution of metabolites. In T1 without external gas flushing, lactic acid fermentation was dominant during the initial 7-days. Accordingly, phylotypes affiliated to the genus Lactobacillus sp. were detected. A heterlatic fermentation pathway was observed in in both T2 and T4 during first four days, and thereafter the fermentation pathways shifted towards acetate and butyrate as dominant products, which were accompanied by changing the microbial community with phylotypes of Clostridium sp. and Bifidobacterium sp. becoming dominant.

To conclude, reutilization of acidogenic off-gas by diversion to methanogenic phase is a promising strategy for enhancing overall energy recovery during two-phase AD of food waste. However, improvement of the short-lived acidogenic H$_2$ production and H$_2$/CO$_2$ ratio needs further investigation.
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### ABBREVIATIONS

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<th>Full name</th>
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<tr>
<td>FW</td>
<td>Food waste</td>
<td>HRT</td>
<td>Hydraulic retention time</td>
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<td>MSW</td>
<td>Municipal solid waste</td>
<td>OLR</td>
<td>Organic loading rate</td>
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<tr>
<td>OFMSW</td>
<td>Organic fraction of msw</td>
<td>LCFAs</td>
<td>Long chain fatty acids</td>
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<td>LBR</td>
<td>Leaching bed reactor</td>
<td>VFAs</td>
<td>Volatile fatty acids</td>
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<tr>
<td>UASB</td>
<td>Upflow anaerobic sludge blanket</td>
<td>TA</td>
<td>Total alkalinity</td>
</tr>
<tr>
<td>BMP</td>
<td>Biochemical methane potential</td>
<td>CH₄</td>
<td>Methane</td>
</tr>
<tr>
<td>AR</td>
<td>Acidogenic reactor</td>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>MR</td>
<td>Methanogenic reactor</td>
<td>H₂</td>
<td>Hydrogen</td>
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<tr>
<td>AnSBR</td>
<td>Anaerobic sequencing batch reactor</td>
<td>STP</td>
<td>Standard temperature and pressure</td>
</tr>
<tr>
<td>PID</td>
<td>Proportional integral derivative</td>
<td></td>
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<tr>
<td>MFC</td>
<td>Mass flow controller</td>
<td>OWTF</td>
<td>Organic waste treatment facilities</td>
</tr>
<tr>
<td>CSTR</td>
<td>Continuous stirred tank reactor</td>
<td>EPD</td>
<td>Environmental protection department</td>
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<tr>
<td>PFOR</td>
<td>Pyruvate ferredoxin oxidoreductase</td>
<td>TSP</td>
<td>Total soluble product</td>
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<tr>
<td>SHP</td>
<td>Specific hydrogen production</td>
<td>TS</td>
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<td>DGGE</td>
<td>Denaturing gradient gel electrophoresis</td>
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CHAPTER ONE

OBJECTIVES AND OUTLINE

1.1 Background

Food waste is recognized as the single-largest component of municipal solid waste and constitutes about 30-55% in different countries (Parfitt et al., 2010; Wong et al., 2009). According to the Environmental Protection Department (HKEPD), 3,584 tons of food wastes need to be disposed of in Hong Kong each day, putting immense pressure on landfills (HKEPD, 2012). The food waste, for the most part, was disposed off in landfill during the past years, reducing life span of landfill, generating greenhouse gases and polluting groundwater (Bou-Zeid and El-Fadel, 2004). With the increasing awareness of the energy crisis and environmental sustainability, utilizing food waste for energy production is recognized as an environmentally and economically viable practice. Biological process catalyzed by microorganisms is a promising way in food waste conversion. Among various biological technologies, anaerobic digestion is known as the most feasible technology, due to its efficient conversion of organic wastes, high energy recovery, and reduced impact on the environment. Energy in terms of biogas is valuable acting as an alternative for fossil energy. Biogas yields from food waste were reported in the range of 200 - 500 L-CH₄/kgVS (Elbeshbissy et al., 2012; Kim et al., 2008).

The process of anaerobic digestion is carried out by four groups of microorganisms, i.e. hydrolytic -fermentative bacteria, acidogenic bacteria, acetogenic bacteria and methanogenic archaes (Cysneiros et al., 2011; Demirel and Scherer,
The four different groups of microorganisms are interdependent and each one plays a specific role in the degradation chain. Another characteristic of these bacteria worth to be mentioned is that they share greatly different growth rates, i.e. growth rate of acidogens ranging from 1.2 to 4.2 d\(^{-1}\), while that of acetogens and methanogens ranging from 0.74 to 1.0 d\(^{-1}\) (Arzate et al., 2014). In traditional single phase anaerobic digestion, these different groups of microorganisms shared the same condition, i.e. temperature, pH, nutrients and so on, which makes it different to optimize all the steps of the process (Cysneiros et al., 2011). One possible solution to these problems is to physically separate the process into two phases with hydrolysis and acidification occurring in one reactor, whereas acetogenesis and methanogenesis occurring in another reactor (Cysneiros et al., 2012a). During two-phase anaerobic digestion, the leachate produced during hydrolysis and acidification in the first phase, is fed to the second phase for methane production (Chynoweth et al., 2001).

Leach-bed reactor (LBR) is an efficient reactor for dry anaerobic digestion (Shewani et al., 2014). LBR reactors have been successfully applied for treating various types of solid wastes; for instance, OFMSW (Forster-Carneiro et al., 2008b; Dogan et al., 2009), fruit and vegetable wastes (Hegde and Pullammanappallil, 2007), animal manure (Demirer and Chen, 2008; Xie et al., 2011), crops (Jagadabhi et al., 2010; 2011; Nizami et al., 2011) and food wastes (Lim et al., 2014; Selvam et al., 2010; Xu et al., 2011; 2012; 2014; Yan et al., 2014a). It was reported that the average solids content of food waste in Hong Kong was about 15% (HKEPD and HKPC, 2011) and it is even higher (40.0 %) in the food waste characterized from commercial restaurants. In light of economic and operational feasibility, it is promising to choose LBR for food waste treatment in Hong Kong. Dutch scientist Lettinga and his co-
workers designed the UASB reactor in the 1970s to treat beet sugar wastewater (Lettinga et al., 2001). Since then, the application of UASB for wastewater treatment becomes popular. Today it is the most popular anaerobic design used worldwide for wastewater treatment (Li et al., 2014; Abbasi and Abbasi 2012). UASB reactors have been used to treat a variety of wastewaters such as domestic wastewater (Tawfik et al., 2008; Abbasi and Abbasi 2012), alcohol distillery wastewater (Yamada et al., 2013), berberine antibiotic wastewater (Qiu et al., 2013), saline sulfate wastewater (Li et al., 2014), etc. Application of LBR coupled to UASB or other wastewater treatment reactors for high-solid organic waste treatment is relatively new and has been successfully applied at laboratory and pilot-scale with substrates like grass silage, energy crops, crop residues and food waste (Cysneiros et al., 2008; Jagadabhi et al., 2010; Koppar and Pullammanappallil, 2008; Lehtomäki and Björnsson, 2006; Lehtomäki et al., 2008; Xu et al., 2011; 2012; 2014).

When organic matter such as food waste, sewage sludge, animal manure, and biodegradable portions of municipal solid waste undergoes decomposition in anaerobic conditions, it normally generates biogas, which consists of 40-70% methane, the rest being mostly carbon dioxide with traces of other gases (Ferrer et al., 2011; González-Fernández et al., 2011; Weiland, 2010). The derived energy from anaerobic digestion of solid organic wastes is significant to human, for it can release the pressure from exploiting the non-renewable fossil energy and simultaneously reduce greenhouse gas (GHG) emission (Abbasi et al., 2012; Weiland, 2010). Nowadays, new technologies have been developed to improve the quality of biogas and its conversion efficiency for energy. However, energy recovery efficiency reported for AD of organic solid wastes is much lower than their theoretical methane potentials (Maya-Altamira et al., 2008).
A mass balance analysis under optimized condition (Fig. 1.1, Xu et al., 2014) indicated the following conditions suitable for LBR: 20% anaerobically digested sludge as inoculum; adjusting the pH to 6.0; apply micro-aeration at 12 min/3 h (258 L-air/kg TS/d) and continuous leachate recirculation. The mass balance in Fig.1.1 indicates that both the rates of hydrolysis and methanogenesis determine the overall energy (CH₄) recovery. Under conventional operation, the loss of acidogenic biogas (H₂ and CO₂) in LBR took ~21% of the consumed substrate whereas the relatively low quality leachate fed to the UASB led to an overall 31% of CH₄ recovery. It is undesirable to scale up the technology with such a low efficiency while the emission of CO₂ would increase the greenhouse effect and reduce the carbon sequestration.

Optimized operational and environmental conditions (Jayasinghe et al., 2011; Johancen and Bakke 2006; Neves et al., 2006; Sang et al., 2008; Wang et al., 2014; Xu et al., 2011; 2012; Zhu et al., 2009a) could increase hydrolysis rate of solid waste as well as overall energy recovery from the process of anaerobic digestion. However, most of these measures, although reported to be effective to some extent, would increase the overall operational and economic input that hamper their application.

It is better to achieve the improved energy recovery through reutilization of the lost energy during AD process. The energy taken by H₂ and CO₂ in acidogenic reactor the can be as high as 30% of the consumed energy. Besides the conversion of H₂ & CO₂ to acetate by homoacetogens in acidogenic reactor, they can also be converted to CH₄ by hydrogenotrophic methanogens through 4H₂ + CO₂ → CH₄ + 2H₂O. Conversion of H₂ and CO₂ to CH₄ not only realizes the reutilization of released energy but also upgrade CH₄ once the ratio of H₂/CO₂ is above 4.0.
1.2 Research gaps

- Knowledge on feasibility of acidogenic off-gas (hydrogen and carbon dioxide) reutilization in methanogenic phase and the corresponding affecting factors is still unavailable.

- Information on both mechanism of regulation of metabolic pathways by headspace pressure (or partial pressure of H₂) and redirection of electrons is absent.

- There is few data concerning dynamic and diversity of microbes in two-phase AD system, especially the functional groups that determine the metabolic pathways prevailing in the system.

1.3 Objective and outline of the thesis

The objective of this work was to enhance overall energy recovery from two-phase anaerobic digestion of food waste through reutilization of acidogenic off-gas. Mass balance analysis of two-phase anaerobic digestion indicated that nearly 30% of consumed substrate was converted to acidogenic biogas (H₂ and CO₂) and this part of energy was usually ignored under normal operation; hence, reutilization of acidogenic

---

Fig. 1.1 Carbon balance in Optimized LBR-UASB system (Xu et al., 2014)
off-gas enables both increased energy recovery and promotion of carbon sequestration. After the selection of potential strategy, investigation of the feasibility for reutilization of acidogenic (LBR) off-gas in methanogenic reactor (UASB) is the first step. Diversion of acidogenic off-gas out of acidogenic reactor will alter the prevailing metabolic pathways during hydrolysis/acidogenesis, which could further change the concentrations and distributions of major metabolites that constitute the quality of acidogenic leachate. Quality of leachate is one key factor that determines the subsequent CH₄ generation and therefore study of measures for regulation of acidogenic metabolic pathways is one potential direction for further enhancement of energy recovery. Reutilization of acidogenic off-gas in methanogenic reactor mainly follows the reaction, 4H₂ + CO₂ → CH₄ + 2H₂O, catalyzed by hydrogenotrophic methanogens, and may give rise to new problem that a H₂ content of less than 80% in acidogenic off-gas would lead to a decrease in CH₄ content in final biogas product. To overcome the problem, enhancement of H₂ evolution especially its concentration in the mixed off-gas is another potential direction for maximising energy recovery.

Microorganisms are the main force for carrying out the anaerobic processes and a well cascade of levels of syntrophic groups is pivot to ensure the success of AD process. Therefore, investigating the diversity and distribution of microorganisms as well as functional gene analysis of dominant pathways was expected to provide more information.

The thesis is composed of the following seven sections:

**SECTION I. Background**

Chapter 1. Introduction and Objectives

Chapter 2. Literature Review
SECTION II. Methodology

Chapter 3. Research Methodology

SECTION III. Feasibility of Reutilization of Acidogenic Off-gas in Methanogenic Reactor

Chapter 4. Feasibility of Reutilization of Acidogenic Off-Gas by Diverting to Methanogenic Reactor during Two-Phase Anaerobic Digestion of Food Waste

SECTION IV. Regulation of Metabolic Pathways

Chapter 5. Enhanced Methane Generation by Regulation of Acidogenic Off-Gas during Two-Phase Anaerobic Digestion of Food Waste: Effect of Headspace Pressure in Acidogenic Reactor

Chapter 6. Regulation of Metabolic Pathways through Controlling the Partial Pressure of Hydrogen in the Acidogenic Headspace.

SECTION V. Enhanced acidogenic H₂ production

Chapter 7. Enhanced Hydrolysis and Acidogenic Hydrogen Production during Two Phase Anaerobic Digestion of Food Waste: Effect of Different Neutralization Modes

Chapter 8. Enhanced Biohydrogen Production during Two-Phase Anaerobic Digestion of Food Waste: Effect of Carbohydrate Contents in the Substrates

SECTION VI. Dynamics of microbial community and redirection of electron

Chapter 9. Dynamics of Microbial Community Associated with Regulated Metabolic Pathways and Redirection of Electron fluxes

SECTION VII. Conclusion and Recommendations for Further Research

Chapter 10. Conclusion and Recommendations for Further Research
CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction of Anaerobic Digestion

Anaerobic digestion is an effective biological process to convert complex organic matters to CH₄ and CO₂ (Grady et al., 1999); during which four groups of anaerobic microbes, i.e. hydrolytic bacteria, acidogens, acetogens and methanogens work together to degrade the organic matter. Commonly, anaerobic digestion consists of four processes (Fig. 2.1). Anaerobic hydrolysis, where hydrolysable complex particulate organic substances such as insoluble cellulose and hemicelluloses, are converted into monomers such as simple sugars, amino acids, and long-chain fatty acids; 2). Fermentation is the process after hydrolysis, where amino acids and sugars are converted to volatile fatty acids; 3). Acetogenesis, a process during which fatty acids and solvents are converted to acetate, H₂ and CO₂, direct precursors for methane gas formation; 4). Methanogenesis, this process includes hydrogenotrophic methanogenesis, where H₂ and CO₂ are converted to methane by hydrogen-utilizing methanogens and aceticlastic methanogenesis, where acetate is converted to methane by acetate-utilizing methanogens. It is worth noting that another process catalyzed by a group of homoacetogens with acetate as the sole product proceeds simultaneously with acetogenesis, named as homoacetogenesis. In a narrow sense, homoacetogenesis is the process to reduce CO₂ to acetate by H₂ (Siriwongrungson et al., 2007).

It is beneficial to apply anaerobic digestion technology for waste treatment due to the advantages of both waste disposal and energy recovery (biogas). Hence, anaerobic technologies become more and more popular in treating both liquid and solid wastes, e.g. sewage wastewater and municipal solid waste. The advantages of
anaerobic digestion of organic waste include: I. Environmental benefits, e.g. decrease in GHGs emission, reduction of pathogens and promotion of carbon sequestration; II. Economic benefits, e.g., income obtained from the processing of waste, sale of organic fertilizer, carbon credits and sale of power; and III. Energy recovery, to some extent energy exhaust from AD processes could alleviate energy crisis today.

Several groups of anaerobic microbes are involved in the complex biochemical process and each group has their distinctive characters such as diversity, growth rate, and sensitivity to change in operating conditions, e.g. oxygen level and pH. Therefore, to ensure the optimal performance of the AD system, rich experiences in operation and process monitoring are required. Better monitoring and control ensure process stability, maximum biogas production, and reduce the risk of digestion failure, which might increase the economical viability of the commercial AD industry.

![Fig. 2.1 The scheme of typical AD process](image)

**2.1.1 Hydrolysis/acidogenesis and homoacetogenesis**

Hydrolysis is the first step of anaerobic degradation and is carried out by a consortium of hydrolytic microbes through the release of extracellular enzymes. It is usually followed by formation of volatile organic acids (VFA, Demirel and Scherer, 2008). The hydrolysis rate of organic solid waste is influenced by the operational
conditions such as pH, temperature and the concentration and species of intermediate products and the properties of the substrates, e.g. nutrientional composition and particle size. The process of acidogenesis is quite important when considering the enhancement of hydrolysis. Hydrolysis is recognized as the rate-limiting step when the substrate is highly complex or cellulose-rich, such as energy crops and food waste (Kim et al., 2008). Fermentation of carbohydrates produces more H₂ than proteins and lipids (Okamoto et al., 2000), indicating that treating food waste in a LBR, H₂ production from the carbohydrates and toxicity of LCFA could prevail simultaneously. H₂, which is involved in a lot of principal biochemical reactions, is recognized as being the controlling factor of the overall performance of anaerobic hydrolysis. Low level of H₂ partial pressure was observed to be favorable for both H₂ production and H₂ – related acidogenesis (Lyberatos and Skiadas 1999; Wang et al., 2008).

Homoacetogens belong to a functional group that can heterotrophically convert H₂ and CO₂ to sole product acetate. So homoacetogens would be an ideal choice for scavenging H₂ in anaerobic digestion. In hydrolysis/acidogenesis system homoacetogens would compete with hydrogenotrophic methanogens for H₂. However, few reports on the competition between these two syntrophic microbes are available in AD. In addition, the information on their interaction with hydrolyzers, acidogens and acetogens are also lacking.

Species and distribution of intermediate products are closely related to the metabolic pathways prevailing in the reactor. An interpretation of glucose fermentation is analyzed in Fig. 2.2. Acetate is known as one of the key intermediate VFAs during the process of the anaerobic digestion (Nie et al., 2008) and also the predominant end product of the heterotrophic lifestyles of the mixed consortia that
live in the reticulo-rumen. The following acetogenic reactions indicate the fermentation of medium-chain fatty acids to acetate.

Butyrate oxidation:

\[
\text{CH}_3\text{CH}_2\text{CH}_2\text{COO}^-+2\text{H}_2\text{O} \rightarrow 2\text{CH}_3\text{COO}^-+2\text{H}_2+\text{H}^+ \quad \Delta G = 48.1 \text{ kJ/mol} \quad \text{Eq. 2.1}
\]

Propionate oxidation:

\[
\text{CH}_3\text{CH}_2\text{COO}^-+3\text{H}_2\text{O} \rightarrow \text{HCO}_3^-+\text{CH}_3\text{COO}^-+3\text{H}_2+\text{H}^+ \quad \Delta G = 76.1 \text{ kJ/mol} \quad \text{Eq. 2.2}
\]

Both of the two reactions and the reactions that involved in oxidation of LCFAs are thermodynamically or energetically unfavourable due to the high free energy requirements (Grady et al., 1999). The conversion of fermentation intermediates like butyrate, propionate, and ethanol, which are the primary substrates of the acetogenic bacteria, is only thermodynamically favorable at very low H\textsubscript{2} concentrations (Morton A. Barlaz, 1990). However, acetogens such as *Sytophobacter wolinii* (propionate degraders) and *Sytophomonos wolfei* (butyrate degraders) are sensitive to H\textsubscript{2}. Thus, the presence of homoacetogens that can consume H\textsubscript{2} through the following reaction, Eq. 2.3 is energetically favourable for the maintenance of the acidogenic process.

\[
4\text{H}_2 + 2\text{CO}_2 \rightarrow \text{acetate}^- + \text{H}^+ + 2\text{H}_2\text{O} \quad \Delta G = -95 \text{ kJ/mol} \quad \text{Eq. 2.3}
\]

2.1.2 Methanogenesis

Methanogenesis, also biomethanation is a process of methane formation, during which microbes known as methanogens play an essential role. Although methanogens may live together with hydrolytic bacteria, acidogens and acetogens, they are only identified from the domain of archaea, which are phylogenetically distinct from both bacteria and eukaryotes. Generation of CH\textsubscript{4} is as an important step of anaerobic biodegradation and it is the last step of anaerobic degradation of organic
matters. Methanogens respire under anaerobic conditions without O₂ with simple carbon compounds as substrates, e.g. CO₂, methanol, and acetate as final electron (e⁻) acceptors. The utilization of CO₂ and acetate represents two methanogenic pathways, acetoclastic and hydrogenotrophic methanogenesis:

\[
\begin{align*}
\text{CO}_2 + 4 \text{H}_2 & \rightarrow \text{CH}_4 + 2\text{H}_2\text{O} & \Delta G = -135.6 \text{ kJ/mol} & \text{Eq. 2.4} \\
\text{CH}_3\text{COOH} & \rightarrow \text{CH}_4 + \text{CO}_2 & \Delta G = -31 \text{ kJ/mol} & \text{Eq. 2.5}
\end{align*}
\]
A number of enzymes and cofactors, i.e. coenzyme M & B, methanofuran, F420 and methanopterin are involving in the complex biochemical mechanism of methanogenesis (Fig. 2.3). Studies on methanogenesis can be divided into two main directions, on the one hand, researchers are exploring different kinds of measures to
further increase CH₄ production; on the other hand, study of the diversity and dynamics of methanogenic microbes is attracting more and more interest. Measures had been reported by scholars to enhance CH₄ production include pre-treatment (Şahinkaya and Sevimli 2013; Monlau et al., 2013; Zhang et al., 2011a; Zhang et al., 2011b; Yan et al., 2013; Yao et al., 2013; Sambusiti et al., 2013; Rafique et al., 2010), co-digestion (Estevez et al., 2012; Li et al., 2013; Lim et al., 2014; Lim and Wang 2013; Regueiro et al., 2012; Riaño et al., 2011; Silvestre et al., 2014; Wang et al., 2011; Zhang et al., 2013a), nutrient addition (Nges et al., 2012; Zhang and Jahng 2012), co-harvest of H₂ and CH₄ (Luo et al., 2011; Wang et al., 2011; Cheng et al., 2012a; Cheng et al., 2012b; Lu et al., 2009), micro-aeration (Mali Sandip et al., 2012; Lim and Wang 2013), other methods (Behera et al., 2011; Adu-Gyamfi et al., 2012). Detailed review will be discussed in the following parts.
Fig. 2.3 Methanogenic, methylotrophic (broken gray arrows), hydrogenotrophic (double-lined arrows) and aceticlastic (solid arrows) pathway (Bapteste et al., 2005).

As summarized by Garrity and Holt (2001), *Methanobacteriales*, *Methanomicrobiales*, *Methanopyrales*, *Methanosarcinales* and *Methanococcales* are the five phylogenetically distant orders in the domain *archaea*. The species in all the five orders differ greatly in respect of morphological and physiological characteristics, which bring the difficult for studying the microbial community of methanogens. I. Members of order *Methanobacteriales* generally produce CH$_4$ using H$_2$ as the electron donor to reduce CO$_2$. Some species can use formate, CO, or simple alcohols as electron donors, e.g. the genus *Methanosphaera* can only reduce methanol with H$_2$. 

15
Families *Methanobacteriaceae* and *Methanothermaceae* are belong to the order of *Methanobacteriales* while the family *Methanobacteriaceae* is composed of four genera, *Methanothermobacter*, *Methanobacterium*, *Methanosphaera*, and *Methanobrevibacter*. Liu and Whitman (2008) had reported a hyperthermophilic genus, *Methanothermus*, belonging to the family *Methanothermaceae*. II. Members of the second order *Methanococcales* produce CH\(_4\) through reduction of CO\(_2\) by H\(_2\) or formate. Families *Methanocaldococcaceae* and *Methanococcaceae*, with distinct growth temperatures, are belonged to the order of *Methanococcales*. III. Similarly, the order of *Methanomicrobiales* produces CH\(_4\) from CO\(_2\) with H\(_2\) as electron donor. There are three families in the order of *Methanomicrobiales*, i.e. *Methanomicrobiaceae*, *Methanocorpusculaceae*, and *Methanospirillaceae*. IV. The order *Methanosarcinales* is distinct from the other orders for its widest range of substrate, which contains two families, *Methanosarcinaceae* and *Methanosaetaceae*. V. *Methanopyrales* is the last order; representing by only one species, *Methanopyrus kandleri*. Table 2.1 summarized the classification of methanogenic bacteria.
<table>
<thead>
<tr>
<th>Class I. Methanobacteria</th>
<th>Class II. Methanococci</th>
<th>Class III. Methanomicrobia</th>
</tr>
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<tbody>
<tr>
<td>(use $\text{H}_2/\text{CO}_2$ and formate as C source)</td>
<td>(use $\text{H}_2/\text{CO}_2$ and formate as C source)</td>
<td>(use $\text{H}_2/\text{CO}_2$ and formate as C source)</td>
</tr>
<tr>
<td>Order I. Methanobacteriales</td>
<td>Order I. Methanococcales</td>
<td>Order I. Methanomicrobiales</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Order II. Methanosarcinales</td>
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<tr>
<td></td>
<td></td>
<td>(aceto- and methylotrophic)</td>
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</table>

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<thead>
<tr>
<th>Family I. Methanobacteriaceae</th>
<th>Family I. Methanococcaceae</th>
<th>Family I. Methanomicrobiaceae</th>
<th>Family I. Methansarcinaeae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genus I. Methanobacterium</td>
<td>Genus I. Methanococcus</td>
<td>Genus I. Methanomicrobium</td>
<td>Genus I. Methanosarcina</td>
</tr>
<tr>
<td>Genus II. Methanobrevibacter</td>
<td>Genus II. Methanothermococcus</td>
<td>Genus II. Methanoculleus</td>
<td>Genus II. Methanococcoides</td>
</tr>
<tr>
<td>Genus III. Methanosphaera</td>
<td></td>
<td>Genus III. Methanofollis</td>
<td>Genus III. Methanohalobium</td>
</tr>
<tr>
<td>Genus IV. Methanothermobacter</td>
<td></td>
<td>Genus IV. Methanogenium</td>
<td>Genus IV. Methanohalophilus</td>
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<tr>
<td></td>
<td></td>
<td>Genus V. Methanolactin</td>
<td>Genus V. Methanolobus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Genus VI. Methanoplanus</td>
<td>Genus VI. Methanomethylovorans</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Genus VII. Methanimicrococcus</td>
</tr>
<tr>
<td>Family II. Methanothermaceae</td>
<td>Family II. Methanocaldococcaceae</td>
<td>Genus I. Methanococcus</td>
<td>Genus VIII. Methanosalsum</td>
</tr>
<tr>
<td>Genus I. Methanothermus</td>
<td>Genus I. Methanocaldococcus</td>
<td>Genus II. Methanotorris</td>
<td></td>
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<tr>
<td></td>
<td>Genus II. Methanothermococcus</td>
<td></td>
<td>Family II. Methanospirillaceae</td>
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<tr>
<td></td>
<td>Genus II. Methanotorris</td>
<td></td>
<td>Genus I. Methanospirillum</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>(hydrogenotrophic)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Genus I. Methanosiraeta</td>
</tr>
</tbody>
</table>
2.1.3 Two-phase anaerobic digestion

In a single-phase reactor, hydrolysis/acidogenesis and methanogenesis processes occur simultaneously (Grady et al., 1999). However, larger amount of seeding micorbes, longer solid retention time, more space for reactor construction, incomplete degradation of lipids and toxic inhibition (e.g. ammonia, LCFA) are common problems encountered in single-phase reactor. In contrast to single-phase, two-phase system is basically comprised of acidogenesis and methanogenesis (Ueno et al., 2007), i.e., hydrolysis/acidogenesis and methanogenesis are divided into in two reactors. The microorganisms were separated physically to make use of the differences in their growth kinetics. Two-phase anaerobic digestion offers considerable kinetic and process advantages over conventional single phase systems. It was demonstrated that the two-phase system could recover 3-times higher CH\textsubscript{4} from solid substrates than one-phase digestion (Lehtomaki et al., 2008). Thus, it is advantageous to apply a two-stage anaerobic process for treating complex solid wastes, e.g. food waste.

Two-phase anaerobic digestion of particulate substrates is considered as an effective strategy of anaerobic degradation, of which the rate-limiting step is hydrolysis and liquefaction. Techniques such as membrane separation, pH and kinetic control were developed to accomplish the phase separation (Fox and Pohland, 1994; Ince 1998). Two-phase process makes it possible to select and enrich different microbes in each digester by controlling the digester operating conditions independently (Shin et al., 2001).

In a parallel development, a leach bed reactor (LBR) system imitating the landfill bioreactor has been designed for dry anaerobic degradation of organic solids. In most cases, the LBRs were operated in batch modes or sequential batches with all
the steps occurred in one reactor or coupled to a second phase for high strength wastewater treatment such as UASB (Nizami et al., 2011). In case of two-phase system, LBR is the acidogenic reactor with hydrolysis and acidogenesis taking place while the second reactor is mainly responsible for treating the high-strength leachate generating from LBR. Since most of the solid is filtered by the percolation in LBR, high-rate reactors such as UASB, anaerobic filters (AF) or anaerobic membrane bioreactors can be applied in the second phase to utilize the leachate. Formation of granules and attached biofilms in high efficient reactors ensures the overall high-energy recovery (Kennedy and Lentz 2000; Dereli et al., 2014). CH₄ yields and volatile solid (VS) reduction of 0.27 to 0.39 m³ CH₄ / kg·VS added and 59-60%, respectively, were obtained in two-phase AD of grass silage in batch LBR reactor connected to anaerobic filters, in both laboratory (Cirne et al., 2007) and pilot trials (Lehtomäki and Björnsson, 2006).

Both H₂ and CH₄ can be recovered from waste materials through a two-phase AD process (Liu et al., 2006). During the two-phase process, acidogenic bacteria in the first phase convert substrates such as carbohydrates/protein/lipids to H₂, CO₂ and fatty acids. The H₂ can be harvested while the soluble intermediates (VFAs and solvents) enter the second phase where they are further converted to CH₄ and CO₂ by methanogens. Therefore, the final products of the two-phase process are H₂, CH₄ and CO₂.

UASB reactor is recognized as the most popular and robust anaerobic reactor for wastewater treatment with high efficiency. The key to the success of UASB is the establishment of a dense sludge bed in the bottom of the reactor. The sludge bed is comprised of biomass of bacteria and archea as well as the incoming suspended solids. In an UASB, the microbes in the upflow stream can naturally aggregate in
flocs and granules, which ensures good settling properties and prevent the loss of sludge from the reactor. Therefore, smaller reactor and less space is possible while, at the meantime, high quality product (CH₄) can be recovered.

**2.2 Anaerobic digestion of food waste**

Worldwide, food wastes, for the most part, are disposed in landfill. In light of limited available space for landflling and the environmental impact of the technology itself, alternative technology that can simultaneously fulfill the task of waste disposal and energy recovery is attracting the attention. The relatively high moisture content (~60%) and easily degradable nature of food waste make it an ideal substrate for anaerobic treatment.

**2.2.1 Food waste in Hong Kong**

Food waste generation in Hong Kong is approximately 3,584 tonnes per day, of which one third comes from commercial and industry (C&I) sector, and the remaining are generated from households, taking 9% and 26%, respectively of the MSW generated in Hong Kong (HKEPD 2012).

Food waste mainly contains putrescible materials that are easily biodegradable, which leads the disposal of food waste in landfill an unfavourable strategy with the risk of wasting the limited landfill space and emission of greenhouse gases such as CH₄, and odors as well as the generation of wastewater. The HKEPD is developing the Organic Waste Treatment Facilities (OWTF) to deal with the food waste problem in which biological technologies such as anaerobic digestion and composting are included.

Furthermore, food waste produced in Hong Kong is similar to that produced in other cities worldwide. The high organic content of food waste offers feasibility for
Table 2.2 Characterization of food waste

<table>
<thead>
<tr>
<th>Source</th>
<th>Characteristics</th>
<th>Country/area</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artificial</td>
<td>60.5</td>
<td>97.1</td>
<td>NA*</td>
</tr>
<tr>
<td>Cafeteria</td>
<td>78.9-80.8</td>
<td>94.8-97.8</td>
<td>10.72-11.37</td>
</tr>
<tr>
<td>Waste management centre</td>
<td>64</td>
<td>87</td>
<td>14.80</td>
</tr>
<tr>
<td>A dining hall</td>
<td>80</td>
<td>95</td>
<td>14.7</td>
</tr>
<tr>
<td>Cafeteria</td>
<td>80</td>
<td>94</td>
<td>NA*</td>
</tr>
<tr>
<td>Waste management centre</td>
<td>85</td>
<td>89</td>
<td>36.4</td>
</tr>
</tbody>
</table>

NA*—not available

2.2.2 Two-phase dry anaerobic digestion of food waste

The concept of two-phase AD was proposed by Pohland and Ghosh (1971). Two-phase design has attracted most interests since it was proposed, for the feasibility of separating acidogenic bacteria from methanogenic archaea and providing each group of microbes with different optimum environmental conditions become possible.

It is widely recognized by researchers that multi-phase system can offer better stability of operation compared to single-phase systems, especially when treating easily hydrolysable solid wastes (Ganesh et al., 2014; Shen et al., 2013). Food waste is an ideal substrate for AD; however, the system is so complicated that it difficult to use single-phase to deal with. Although, two-phase AD system is more expensive to construct and maintain, its performance and energy efficiency is much higher than single-phase reactor. For instance, compared with single-phase anaerobic co-digestion of food waste and fruit & vegetable waste (Batstone et al., 2002), two-phase system
had a 7.0-15.8% higher specific CH\textsubscript{4} recovery with a more stable operation. Similarly, compared to the single-phase system, an increase of CH\textsubscript{4} generation up to 21% was achieved by Liu et al. (2006) in a two-phase reactor when treating municipal solid waste.

Currently, anaerobic digestion of organic wastes is recognized as a reliable technology, which is confirmed by the popularity of increasing full-scale applications in Europe and other regions (Bolzonella et al., 2006; Cavinato et al., 2010). Initially, wet digestion (<10% total solids (TS) in the feed) plants were more popular; however, dry digestion (>20% TS) started to prevail since 1990 (Lissens et al., 2001). In wet digestion systems, the organic solid wastes are diluted with water to less than 15% of TS, whereas, the substrate in dry fermentation is kept at a solid content in the range of 20-40% TS. The high TS content in the substrates during dry AD makes the handling, pre-treatment and operation mode quite different from those of wet AD. However, the only necessary pre-treatment of the substrates before feeding into the reactor is to take out the coarse impurities larger than 4 cm, which leads to a much easier pre-treatment of dry AD than that of wet systems.

2.3 Energy recovery and mass balance

Both biogas and soluble solvents or fuels can be obtained as energy from AD of organic wastes. However, only energy products in terms of biogas, i.e. H\textsubscript{2}, CO\textsubscript{2} and CH\textsubscript{4} are considered as energy recovery in this study. Harvest of H\textsubscript{2} and CH\textsubscript{4} or the mixture of them from anaerobic digestion of organic wastes is attracting more research attention these years, due to the advantage of higher energy recovery efficiency. Energy recovery is one of the key issues regarding the scaling-up of anaerobic technologies for commercial practise. Therefore, it is essential to
investigate energy recovery from AD process and evaluate the feasibility for boosting up the application of the technique.

2.3.1 H₂ and CO₂ production

Hydrogen harbours high heat value (i.e. 142.4 kJ·g⁻¹) and it is an extremely clean energy as it only produces water after energy release. Also, because of its clean nature, H₂ production by anaerobic digestion of solid waste has been attracting increasing research attention. The cleavage of complex organic matter is always coupling the production of H₂ and CO₂ as the by-product. H₂ production is generally carried out by microbes that belong to the genus of Clostridium, e.g. C. butyricum (Jenol et al., 2013), C. thermolacticum (Calli et al., 2008), C. pasteurianum (Trohalaki and Pachter 2010), C. paraputrificum (Akutsu et al., 2009) and C. bifermentans (Wu et al., 2009). H₂-specific clostridia produce H₂ at the exponential phase of their growth (Kapdan and Kargi 2006). Typical biohydrogen production process during dark fermentation includes a series of reactions:

\[ \text{C}_6\text{H}_{12}\text{O}_6 \rightarrow \text{CH}_3\text{CH}_2\text{CH}_2\text{COOH} + 2\text{CO}_2 + 2\text{H}_2 \quad \text{Eq. 2.6} \]
\[ \Delta G = -257.1 \text{ kJ/mol} \]

\[ \text{C}_6\text{H}_{12}\text{O}_6 + 4\text{H}_2\text{O} + 2\text{NAD}^+ \rightarrow 2\text{CH}_3\text{COO}^- + 2\text{HCO}_3^- + 2\text{NADH} + 2\text{H}_2 + 6\text{H}^+ \quad \text{Eq. 2.7} \]
\[ \Delta G = -215.7 \text{ kJ/mol} \]

\[ \text{C}_6\text{H}_{12}\text{O}_6 + 2\text{H}_2\text{O} \rightarrow \text{CH}_3\text{CH}_2\text{CH}_2\text{COO}^- + 2\text{HCO}_3^- + 2\text{H}_2 + 3\text{H}^+ \quad \text{Eq. 2.8} \]
\[ \Delta G = -261.5 \text{ kJ/mol} \]

\[ \text{C}_6\text{H}_{12}\text{O}_6 + 2\text{H}_2\text{O} + 2\text{NADH} \rightarrow 2\text{CH}_3\text{CH}_2\text{OH} + 2\text{HCO}_3^- + 2\text{NAD}^+ + 2\text{H}_2 \quad \text{Eq. 2.9} \]
\[ \Delta G = -234.8 \text{ kJ/mol} \]

\[ \text{C}_6\text{H}_{12}\text{O}_6 \rightarrow \text{CH}_3\text{COO}^- + \text{CH}_3\text{CH}_2\text{COO}^- + \text{CO}_2 + \text{H}_2 + 2\text{H}^+ \quad \text{Eq. 2.10} \]
\[ \Delta G = -287.0 \text{ kJ/mol} \]
\[
\text{CH}_3\text{CH}_2\text{COO}^- + 3\text{H}_2\text{O} \rightarrow \text{CH}_3\text{COO}^- + \text{H}^+ + \text{HCO}_3^- + 3\text{H}_2 \\
\Delta G = 76.1 \text{ kJ/mol}
\]

\[
\text{CH}_3\text{CH}_2\text{CH}_2\text{COO}^- + 2\text{H}_2\text{O} \rightarrow 2\text{CH}_3\text{COO}^- + \text{H}^+ + 2\text{H}_2 \\
\Delta G = 48.1 \text{ kJ/mol}
\]

\[
\text{C}_6\text{H}_{12}\text{O}_6 + 2\text{H}_2\text{O} \rightarrow 2\text{CH}_3\text{COOH} + 2\text{CO}_2 + 4\text{H}_2 \\
\Delta G = -135.6 \text{ kJ/mol}
\]

\[
\text{CH}_3\text{CH}_2\text{OH} + 2\text{H}_2\text{O} \rightarrow \text{CH}_3\text{COO}^- + 2\text{H}_2 + \text{H}^+ \\
\Delta G = 9.6 \text{ kJ/mol}
\]

Besides the seeding microbes, the feasibility of \( \text{H}_2 \) production through anaerobic fermentation is influenced by operational conditions as well as the composition of substrates. Table 2.3 summarizes \( \text{H}_2 \) production under different conditions and corresponding ratios of \( \text{H}_2/\text{CO}_2 \). pH is considered as the most important environmental condition that affect \( \text{H}_2 \) production (Ramos et al., 2012; Infantes et al., 2011). pH of the medium affects \( \text{H}_2 \) yield, \( \text{H}_2 \) content in the acidogenic biogas, and distributions of intermediate organic acids as well as the specific \( \text{H}_2 \) production rate (SHPR). The optimal pH range ever reported for \( \text{H}_2 \) evolution was between 5.0 and 6.0 (Fan et al., 2004; Hwang et al., 2004; Li et al., 2008; Massanet-Nicolau et al., 2008). However, a pH range of 6.0-8.0 was also reported by other investigators (Chen et al., 2008; Collet et al., 2004; Ferchichi et al., 2005; Seol et al., 2008). But, there was a consistence of the final pH at the end of \( \text{H}_2 \) production by anaerobic digestion, from 4.0 to 4.8 (Liu and Shen 2004; Morimoto et al., 2004; Ramos et al., 2012; Yokoi et al., 2001; Zheng and Yu 2004). The decrease of pH during anaerobic fermentation is due to the generation of organic acids, which reduces the buffering capacity of the system resulting in low final pH (Ramos et al., 2012). The decrease in pH in the system could inhibit \( \text{H}_2 \) production because the \( \text{H}_2 \) -
The prevailing metabolic pathway determines the distribution and abundance of intermediates produced in the medium. Acetic, propionic and butyric acids are the major VFAs generated during anaerobic fermentation of carbohydrates. Lactic acid is the most common fatty acid that produced during the degradation of carbohydrates (Collet et al., 2004; Itoh et al., 2012). Of course, pH is always the essential factor that influences the prevailing metabolic pathways and the behaviour of acids production. Butyric acid is the dominant product in the pH range of 4.0-6.0 (Kapdan and Kargi 2006). At a pH range of 6.5 – 7.0, abundances of acetate and butyrate were almost equal (Fang and Liu 2002). However, the behaviour of ethanol production was always associated with the environmental conditions (Collet et al., 2004; Liu et al., 2003; Oh et al., 2003; Ueno et al., 2001; Yu et al., 2002; Zhang et al., 2003; Zhu et al., 2009a). Under most circumstances of the H2 production studies, CH4 was not detected, which should be attributable to the elimination of CH4 producers by lower pH condition, heat pre-treatment of seed sludge (Lin and Lay 2004; Oh et al., 2003; Yu et al., 2002) or methanogenesis inhibitor (Xu et al., 2010). However, CH4 formation might occur with long retention times of solids at mesophilic condition (Shin et al., 2004).
2.3.2 Methane gas production

Biomethane as a promising fossil fuel substitute is widely recognized and is attracting more research interests in view of its recycling, energy production and pollutants reduction characteristics. Recovery of CH$_4$ from AD is a biochemical process carried out by methanogens. There are five orders (Table 2.1) of methanogens in the nature, all of which are obligate anaerobes. Considering the substrate for methane formation, the bioprocess can be divided into two categories, hydrogenotrophic and acetoclastic methanogenesis. Usually in a bioreactor, both of the two processes exist, and the ratio between the two processes determines CH$_4$ concentration in the final biogas.

CH$_4$ concentration in the final biogas product determines the quality of the product in terms of calorific value. Regarding the chemical balance of the acetoclastic methanogenesis, 1 mol of acetate will produce 1mol of CH$_4$ and equal quantity of CO$_2$, which means the CH$_4$ content of the biogas will be 50%, when acetate is the only precursor for methane generation. However, most times the CH$_4$ content is higher than 50%, indicating the possible contribution of hydrogenotrophic methanogenesis. During hydrogenotrophic methanogenesis, H$_2$ will reduce CO$_2$ to CH$_4$, which will upgrade the biogas. There are intensive researches on upgrading biogas by using measures of separation techniques (Budzianowski 2012; Molino et al., 2013); however, the post-treatment of biogas will decrease the economic feasibility of anaerobic digestion. Then, how to improve CH$_4$ content during the biogas production will be the key point. Till now, there were few studies concerning in-situ biogas upgrading except reports by Luo and Angelidaki (2011; 2012). Luo and co-authors reported upgrading of biogas by addition of H$_2$ in-site or in a coupling reactor and they achieved satisfactory result that CH$_4$ content in the biogas can be
increased up to 95%. This gives us insight for biomethane enhancement and upgrading; however, the mechanism and reasons need to be further explored.
Table 2.3 Literatures on \( \text{H}_2 \) production and corresponding \( \text{H}_2/\text{CO}_2 \) ratio

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Reactor configuration</th>
<th>Temp (°C)</th>
<th>HRT</th>
<th>OLR (g COD/l/d)</th>
<th>( \text{H}_2 ) (%)</th>
<th>( \text{CO}_2 ) (%)</th>
<th>( \text{CH}_4 ) (%)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sugary waste water</td>
<td>5L glass vessel</td>
<td>60</td>
<td>0.5-3d</td>
<td>10.6-63.7</td>
<td>14mmol/g carbohydrate removed; 64</td>
<td>36</td>
<td>Less than 0.13%</td>
<td>Ueno et al., 1996</td>
</tr>
<tr>
<td>Artificial cellulose wastewater</td>
<td>3L reactor</td>
<td>60</td>
<td>120h</td>
<td>n.d.</td>
<td>33 for AD sludge; 50 for sludge compost</td>
<td>n.d.</td>
<td>n.d.</td>
<td>Ueno et al., 1995</td>
</tr>
<tr>
<td>Glucose medium</td>
<td>100 mL serum vials</td>
<td>35±1</td>
<td>72h</td>
<td>10 gCOD/batch</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>Chen et al., 2002</td>
</tr>
<tr>
<td>Food waste</td>
<td>715 mL serum bottle</td>
<td>35/55</td>
<td>5d</td>
<td>n.a.</td>
<td>59 for T</td>
<td>n.d.</td>
<td>n.d.</td>
<td>Shin et al., 2004</td>
</tr>
<tr>
<td>Sucrose</td>
<td>11 L CSTR</td>
<td>35±0.5</td>
<td>12h-2d</td>
<td>22.5-67.4 for ( \text{H}_2 ) reactor; 4.39-13.04 for ( \text{CH}_4 ) reactor</td>
<td>Max 57.7-60.1</td>
<td>48.8-50.2</td>
<td>65.6-73.1</td>
<td>Kyazze et al., 2007</td>
</tr>
<tr>
<td>Potato waste</td>
<td>1-5L CSTR</td>
<td>35</td>
<td>6h for ( \text{H}_2 ) stage; 30-90 h for ( \text{CH}_4 )</td>
<td>n.d.</td>
<td>45</td>
<td>n.d.</td>
<td>76</td>
<td>Zhu et al., 2008</td>
</tr>
<tr>
<td>Cornstalk</td>
<td>Serum bottle</td>
<td>55</td>
<td>n.a.</td>
<td>18.3-50.6</td>
<td>n.d.</td>
<td>30.9-67.9</td>
<td>65-67</td>
<td>Lu et al., 2009</td>
</tr>
<tr>
<td>Olive pulp</td>
<td>3 L CSTR</td>
<td>35/55</td>
<td>7.5h-30h</td>
<td>21.5-89.7 gTS/d for ( \text{H}_2 ) reactor; 3.95-15.8 for ( \text{CH}_4 )</td>
<td>26.4-34.1</td>
<td>n.d.</td>
<td>65-67</td>
<td>Koutrouli et al., 2009</td>
</tr>
</tbody>
</table>

\(^a\)Not detectable; \(^b\)Not available
Biochemical methane potential (BMP) is developed as a protocol for measurement of sample biodegradability. The CH₄ production resulting from degradation of sample is determined by subtracting the background values, obtained from seed-blanks, from overall CH₄ production. Despite, there are a mass of data of BMP values of organic solids; it is difficult for comparison. The reason is not only due to the application of different equipments, but also to the variety of operational conditions and protocols applied. For example, the mixture of inoculum-nutrients, volumes of medium and headspace, pH, headspace pressure and the detection system can all differ from one test to another. Furthermore, the results presented in variable units are another problem making the comparison very difficult. Angelidaki et al. (2009) had introduced some important experimental guidelines for the reliable and reproducible assessment of the anaerobic biodegradability of solid organic substrates.

Theoretical CH₄ production can be predicted from the chemical balance formula, described as follows:

\[
C_nH_{2a}O_bN_c + (n \frac{a}{4} - \frac{b}{2} + 3 \cdot \frac{c}{4})H_2O \rightarrow (\frac{n}{2} \cdot \frac{a}{8} + \frac{b}{4} + 3 \cdot \frac{c}{8})CO_2 + (\frac{n}{2} + \frac{a}{8} - \frac{b}{4} - 3 \cdot \frac{c}{8})CH_4 + cNH_3
\]

The specific methane yield (B) at standard temperature and pressure (STP), usually expressed as L-CH₄/g VS and calculated as,

\[
B = \frac{(\frac{n}{2} + \frac{a}{8} - \frac{b}{4} - 3 \cdot \frac{c}{8}) \cdot 22.4}{12n + a + 16b + 14c}
\]

The specific CH₄ yield of various compounds are calculated and given in Table 2.4. CH₄ yield of lipids and proteins are 1.01 and 0.50 L/g VS at STP, which are much higher than that of carbohydrate (0.42 L/g VS at STP).
Table 2.4 Theoretical methane yield for various organic compounds\textsuperscript{a}

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Composition</th>
<th>CH\textsubscript{4} yield (L/g VS, STP)</th>
<th>CH\textsubscript{4} (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrate</td>
<td>(C\textsubscript{6}H\textsubscript{10}O\textsubscript{5})\textsubscript{n}</td>
<td>0.42</td>
<td>50</td>
</tr>
<tr>
<td>Proteins</td>
<td>C\textsubscript{5}H\textsubscript{7}NO\textsubscript{2}</td>
<td>0.50</td>
<td>50</td>
</tr>
<tr>
<td>Lipids</td>
<td>C\textsubscript{57}H\textsubscript{104}O\textsubscript{6}</td>
<td>1.01</td>
<td>70</td>
</tr>
<tr>
<td>Ethanol</td>
<td>C\textsubscript{2}H\textsubscript{6}O</td>
<td>0.73</td>
<td>75</td>
</tr>
<tr>
<td>Acetate</td>
<td>C\textsubscript{2}H\textsubscript{4}O\textsubscript{2}</td>
<td>0.37</td>
<td>50</td>
</tr>
<tr>
<td>Propionate</td>
<td>C\textsubscript{3}H\textsubscript{6}O\textsubscript{2}</td>
<td>0.53</td>
<td>58</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Adapted from Angelidaki (2011).

Different substrates have different BMPs and even the same substrate will differ in BMP under different conditions. The CH\textsubscript{4} potential of waste materials usually falls in the range of 0.20 - 0.50 L CH\textsubscript{4}/g·VS. Food waste is one of the most easily biodegradable organic wastes. It is reasonable to determine BMP of food waste when exploring measures to enhance its CH\textsubscript{4} potential. The reported BMP values of food waste are summarized in Table 2.5.
Table 2.5 Biochemical methane potential of food wastes reported in literatures

<table>
<thead>
<tr>
<th>Substrate type</th>
<th>CH(_4) yield (m(^3)/kg VS)</th>
<th>CH(_4) (%)</th>
<th>Configuration</th>
<th>TS of solids (%)</th>
<th>HRT (d)</th>
<th>OLR (kg VS/m(^3)·d)</th>
<th>Temp (°C)</th>
<th>COD(_{\text{rem}}) (%)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>FW and brown water</td>
<td>0.32±0.01</td>
<td>60</td>
<td>Batch with continuous stir</td>
<td>n.d.</td>
<td>45</td>
<td>n.d.</td>
<td>35-37</td>
<td>n.d.</td>
<td>Lim and Wang 2013</td>
</tr>
<tr>
<td>Yard waste and FW</td>
<td>0.12 (with 20% fw)</td>
<td>50-70</td>
<td>Batch</td>
<td>15.2 ± 0.2</td>
<td>30</td>
<td>TS 0.0–166 g/L</td>
<td>36 ± 1</td>
<td>n.d.</td>
<td>Brown and Li 2013</td>
</tr>
<tr>
<td>Dining hall FW</td>
<td>0.39±0.10</td>
<td>n.d.</td>
<td>HR-MR(^{c})</td>
<td>89.1±5.6-106.9±3.0</td>
<td>HR, 2.87</td>
<td>HR: 36.28(^{a})</td>
<td>55</td>
<td>76.8±4.9</td>
<td>Kobayashi et al., 2012</td>
</tr>
<tr>
<td></td>
<td>0.39±0.03</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>74.1±1.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.38±0.05</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>78.3±3.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.41±0.04</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>75.7±2.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.47±0.05</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>78.7±2.8</td>
<td></td>
</tr>
<tr>
<td>Artificial FW</td>
<td>0.25</td>
<td>60-70</td>
<td>LBR-UASB</td>
<td>39.5</td>
<td>17</td>
<td>&lt;2(^{b})</td>
<td>35</td>
<td>&gt;90</td>
<td>Xu et al., 2011</td>
</tr>
<tr>
<td>Kitchen FW</td>
<td>0.40</td>
<td>62</td>
<td>900 m(^3) tank</td>
<td>27.7 &amp; 27.8</td>
<td>n.d.</td>
<td>2.5 or 2.7</td>
<td>42</td>
<td>n.d.</td>
<td>Banks et al., 2012</td>
</tr>
</tbody>
</table>

\(^{a}\) TS based; \(^{b}\) COD based; \(^{c}\) HR-MR, hydrolytic reactor, methanogenic reactor; \(^{d}\) L-CH\(_{4}\)/g-VS
| Substrate type | CH₄ yield (m³/kg VS) | CH₄ (%) | Configuration | TS of solids (%) | HRT (d) | OLR (kg VS/m³·d) | Temp (°C) | CODₗ(|rem (%) | Ref. |
|----------------|---------------------|---------|---------------|-----------------|---------|------------------|-----------|--------------|-----|
| Cafeteria FW  | 0.28 or 0.25        | 72 or 68 | 3- stage      | 12.38 or 12.86  | First 2 | Bench 110.5      | 90.6      | 90.1         | Kim et al., 2008 |
|                |                     |         |               | 2               | Second  | 63.4             | 61.8      | 35           |                  |
|                |                     |         |               | Third 12        |         | 52.8             | 54.5      | Third 50      |                  |
| FW             | 0.51                | 65.6    | Lab scale reactor | 1.8            | Batch 6 | 6.5              | 50        | 93.6         | Liu et al., 2009 |
|                | 0.50                | 67.6    |                |                 |         | 12.5             |           | 82.1         |                  |
|                | 0.52                | 66.1    |                |                 |         | 16.0             |           | 79.6         |                  |
|                | 0.25                | 63.7    |                |                 |         | 20.0             |           | 54.3         |                  |
|                | 0.24                | 56.9    |                |                 |         | 1.6              |           | 50.8         |                  |
| FW             | 20%TS: 0.49e        | 16      | Single phase 1.1 L or 5.0 L | 20% | Batch 60 | 2.5 | 55 | 49.7 | Forster-Carneiro et al., 2008a |
|                | 0.22                | n.d.    |                |                 | Batch 2 | 2.5 |                |            | 40.0 |                  |
| Vegetable market waste | 0.35 | n.d. | 100 L solid bed digester; 24.6 l UASB | 13.31 | 0.67 | 19.6 COD | 35 | 94 | Rajeshwari et al., 2001 |
2.3.3 Enhancement of CH₄ production

2.3.3.1 Inoculum

Seed microbe, decomposer of organic matter, is the decisive factor that influences the performance of anaerobic digesters and overall energy recovery in an anaerobic reactor. During a normal start-up of a batch operation, seed microbes should be added together with the substrates to initiate the anaerobic reactions. Highly active inoculum can significantly reduce the bio-stabilization time of organic solid waste and reduce the amount of inoculums required (Lopes et al., 2004). Besides the AD sludge sourced from wastewater treatment plants, some other materials from animal origins, such as rumen fluid were also applied into anaerobic digesters (Mshandete et al., 2005). Specific methanogenic activity of granular sludge showed higher activity than suspended sludge (Hashimoto, 1989; Sans et al., 1995). Similarly, LBR taking AD sludge as seed source of OFMSW digestion showed the highest CH₄ yield (0.49 L CH₄/g VS) among 7 kinds of inocula, i.e. corn silage, digestate of restaurant waste mixed with rice hulls, swine manure, cattle manure, swine with AD sludge, AD sludge (Forster-Carneiro et al., 2008b). Besides, optimization of the microorganism to feed (M/F) ratio can reduce significantly the operation time, and consequently, smaller digester volume becomes possible (Obaja et al., 2003). Optimal M/F ratio could also supplement the buffering capacity of the digester, omitting the over acidification in reactor. Alves et al. (2001) took bovine fluid as inoculum source at M/F ratios of 0.17, 0.11 and 0.05 to digest the OFMSW. Battimelli et al. (2009) adopted a range of M/F ratios (2, 1, 0.74 and 0.43) and two types of sludge inocula (granular and suspended) when measuring the BMP of kitchen wastes, in which the authors observed that the advantage of granular sludge over suspended sludge was to prevent acidification of the reactors over the range of M/F ratios. Therefore,
maintaining a suitable M/F ratio is important to achieve better performance of digesters.

2.3.3.2 pH

pH in anaerobic system is recognized as one of the critical indicators of the performance and stability of anaerobic digesters. In a single-phase operation, a well-balanced AD process, most of the intermediate products (alcohols, VFAs and LCFAs) are continuously converted into biogas (CH₄) without significant accumulation of intermediary acid, which might cause a decrease of environmental pH. Two-phase system is different from single phase operation, hydrolytic/acidogenic and methanogenic phases varied in optimum ranges of pH, and both phases need to keep optimum pH ranges to ensure a good digestion performance and reaction stability.

Changes of pH would affect a lot of complex biochemical reactions in anaerobic fermentation system, e.g. H₂ evolution. Optimal enzymatic activity of acidogens can be achieved at pH 5.0, whereas a pH maintained in the neutral range is optimal for methanogenesis. During two-phase AD, a pH range of 5.5-6.0 in acidogenic reactor is ideal for acidogenesis due to repression of methanogenic archaea, whereas a narrow pH range of 6.8-7.2 is ideal for methanogens. A pH range of 5.5-6.0 was reported to be ideal to avoid both methanogenesis and solventogenesis (Fan et al., 2006).

pH and alkalinity in anaerobic systems can be adjusted using a lot of chemicals such as sodium (bi-) carbonate, and sodium acetate (Selvam et al., 2009). The principle for supplementation of selected chemicals for pH adjustment is slowly release and avoiding of any adverse impact on the functional microbes. Till now, usage of lime for balancing acidogenic reaction or for enhancing biohydrogen production was not yet reported.
pH was reported to be an important factor that determine the anaerobic metabolic pathways in acidogenic reactor (Hwang et al., 2004). In the pH ranges of 4.0-4.5, 4.5-5.0 and 5.0-6.0, butyrate, ethanol and propionate were the main products, respectively (Hwang et al., 2004; Steinbusch et al., 2009). Acetate and butyrate production decreased while propionate production increased during the increase of pH from 5.3 to 6.0 in whey wastewater, whereas for the paper mill effluent, as pH increased from 3.5 to 6.0, butyrate and propionate production increased (Bengtsson et al., 2008). pH 5.3-5.5 for whey and pH 5.5-6.0 for the paper mill effluent was reported as the optimum pH for generation of VFA (Bengtsson et al., 2008).

2.3.3.3 Hydraulic retention time (HRT)

HRT could be influenced by several factors such as reactor design, kinetics of the reaction, operating temperature, and levels of treatment required (Han and Shin, 2004). Due to different growth and reaction rates of acidogens and methanogens, different HRTs should be applied to each group of microbes. Longer fermentation time in acidogenic reactor would induce a shift of metabolism from hydrolysis/acidogenesis to methanogenesis, which is unfavourable for the two-phase separation as well as overall energy recovery. From this point of view, short retention times are generally applied in hydrolytic-acidogenic phase, which could enable the dilution and washing out of slow-growing methanogenic microorganisms (Hawkes et al., 2007). Elefsiniotis and Oldham (1994) reported that concentration of VFA increased with a HRT of up to 12 h, whereas a decrease was observed at an HRT of 15 h during acidogenesis of primary sludge at ambient temperatures.

HRT is a critical factor for solubilisation of solid particles, due to varied hydrolysis rate among different substrates. For non-easily degradable substrate, longer HRTs have no significant improvement of the hydrolysis yields; and it was reported
that the hydrolysis degree of the feedstock was mainly affected by the OLR rather than the HRTs (De La Rubia et al., 2009). However, for the easily putrescible organic waste, shorten HRT was required to obtain the highest hydrolysis efficiency, i.e. 24 h. Moreover, because acidogenesis is the fastest step in anaerobic digestion, too long HRT may result in the decrease of acidogenic products like VFAs, due to the consumption by methanogens with slow growth rate (Guerrero et al., 1999).

### 2.3.3.4 Nutrient addition

A balanced availability of nutrients is important for the growth of the microbial biomass and the operational performance in anaerobic reactors, i.e. stability and degradation efficiency of substrates (Demirel and Scherer 2011). Trace elements such as Co, Ni, Fe, Zn, Mo and/or W are important factors that influence the activities of enzymes in methanogenic reactors (Zhang and Jahng 2012). Addition of single or combinations of trace elements could improve the rate of substrates turnover and decrease the concentration of VFAs in the system (Feng et al., 2010). Positive effects of nutrient supplementation include improved overall process performance (Banks et al., 2012; Facchin et al., 2013; Karlsson et al., 2012; Demirel and Scherer 2011; Zhang and Jahng 2012) and degradation of specific substrates such as methanol, acetate or propionate (Banks et al., 2012; Karlsson et al., 2012; Osuna et al., 2003; Worm et al., 2009).

Macro nutrients, such as P, S, Mg, K, Ca, Na, etc. are involved in the formation of cellular structure, adjusting pH and redox potential in cytoplasm, and have functions of energy transfer, protoplasm colloid control and cell permeability. Micronutrients include B, Fe, Cu, Zn, Mn, Co, Mo etc. Although, the requirements of these micronutrients are very low, they play an important role on incenting microbial activities and they are components of enzyme active centre or incentives. Typical
elemental composition food waste reported by Zhang et al. (2007), Tanaka et al. (2008) and Ibrahim et al. (2011) is present in Table 2.6. Literatures regarding enhancement of hydrolysis and methanogenesis by nutrient addition are summarized in Tables 2.7 and 2.8.

2.3.3.5 Micro-aeration

Micro-aeration has the potential to affect hydrolysis by influencing the growth of microorganisms, activities and synthesis of enzymes, and further promoting the degradation of carbohydrate and protein. This assumption is supported by the comparative studies that aimed to investigate the effect of micro-aeration on hydrolysis (Johansen and Bakke 2006; Sang et al., 2008; Zhu et al., 2009b). In addition, limiting the oxygen supplement to microaerobic levels, gasification was reduced and an accumulation of VFAs was achieved (Hasegawa et al., 2000). Pre-treatment of sludge with micro-aeration increased biogas production (1.5 times) of mesophilic anaerobic digestion (Hasegawa et al., 2000). Another advantage of micro-aeration is that the incoming oxygen can help to suppress the activity of methanogens in hydrolytic phase. Therefore, the application of micro-aeration in the hydrolysis stage may not only increase the hydrolysis efficiency but also help the separation between hydrolysis and methanogenesis, leading to higher CH₄ generation.

2.3.3.6 Pre-treatment

During anaerobic digestion, the compositions and properties of substrate are quite important in determining the performance of the digesters and the efficiency of energy recovery. Pre-treatment of substrates offers opportunities for improving the degradation efficiency due to the increased availability of substrates for microbes (Antognoni et al., 2013). Pre-treatment is recognized as one of the most popular ways to improve the availability of substrates. In the past 30 years, a great number of
scientific investigations were performed to improve the performance of AD (Hendriks and Zeeman, 2009; Neyens and Baeyens, 2003; Pilli et al., 2011; Weemaes and Verstraete, 1998; Stuckey and McCarty, 1984) in terms of both energy recovery and hydrolysis degree of solid particles. However, there was no evaluation on the application of different methods of pre-treatments as well as with respect to their effects on specific substrates. On the one hand, this is due to the lack of common/standardized protocols for the evaluation of pre-treatment efficiency (Kianmehr et al., 2010). Furthermore, under most circumstances, system boundaries vary and the focus is on specific substrates and specific options of final product based on the energy or financial aspects, which makes the results difficult to apply to other scenarios (Fdz-Polanco et al., 2008; Pickworth et al., 2006).

Table 2.6 Elemental composition of food waste\textsuperscript{a}

<table>
<thead>
<tr>
<th>Components</th>
<th>Average value</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cd</td>
<td>&lt;1 ppm</td>
<td></td>
</tr>
<tr>
<td>Cr</td>
<td>3 ± 1 ppm</td>
<td></td>
</tr>
<tr>
<td>Pb</td>
<td>4 ± 3 ppm</td>
<td></td>
</tr>
<tr>
<td>Ni</td>
<td>2 ± 1 ppm</td>
<td></td>
</tr>
<tr>
<td>C (total)</td>
<td>46.8 ± 1.2 %</td>
<td>(d.b.)</td>
</tr>
<tr>
<td>N (total)</td>
<td>3.2 ± 0.2 %</td>
<td>(d.b.)</td>
</tr>
<tr>
<td>P (total)</td>
<td>0.5 ± 0.1 %</td>
<td>(d.b.)</td>
</tr>
<tr>
<td>K</td>
<td>0.9 ± 0.1 %</td>
<td>(d.b.)</td>
</tr>
<tr>
<td>Ca (total)</td>
<td>2.2 ± 0.3 %</td>
<td>(d.b.)</td>
</tr>
<tr>
<td>Mg (total)</td>
<td>0.14 ± 0.01 %</td>
<td>(d.b.)</td>
</tr>
<tr>
<td>S (total)</td>
<td>2508 ± 87 ppm\textsuperscript{b}</td>
<td></td>
</tr>
<tr>
<td>NH\textsubscript{4}-N</td>
<td>973 ± 571 ppm</td>
<td></td>
</tr>
<tr>
<td>NO\textsubscript{3}-N</td>
<td>118 ± 80 ppm</td>
<td></td>
</tr>
<tr>
<td>Al</td>
<td>1202 ± 396 ppm</td>
<td></td>
</tr>
<tr>
<td>Fe (total)</td>
<td>766 ± 402 ppm</td>
<td></td>
</tr>
<tr>
<td>B (total)</td>
<td>12 ± 1 ppm</td>
<td></td>
</tr>
<tr>
<td>Zn (total)</td>
<td>76 ± 22 ppm</td>
<td></td>
</tr>
<tr>
<td>Mn (total)</td>
<td>60 ± 30 ppm</td>
<td></td>
</tr>
<tr>
<td>Cu (total)</td>
<td>31 ± 1 ppm</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} Adapted from Zhang R. et al. 2007, Tanaka M. et al. 2008 and Ibrahim N. et al. 2011
\textsuperscript{b} Based on wet base.
Despite, there is a large number of researches focus on the technique of pre-treatment in AD. The challenges involved in evaluation the substrates specific pre-treatment and their corresponding effects on AD process still exist.

### 2.3.3.7 Co-digestion

Measures for optimisation of anaerobic digestion focused on from pre-treating to finding suitable substrates and combining substrates (Buendía et al., 2009; Hamzawi et al., 1998; Seppälä et al., 2009). Anaerobic co-digestion (AcoD) is the process of anaerobic digestion with a mixture of two or more wastes with complementary characteristics as substrates, so that biogas production could be enhanced through their co-digestion. Thus, AcoD is not simply the digestion of a mixture of substrates (such as activated and primary sludge), or of different types of wastes in a digester, but rather substrates with complementary characteristics. If possible, to choose the best blend ratios is very important in co-digestion: a) to offer positive interactions (for instance, nutrient and moisture balance and other positive synergisms,); b) to avoid inhibition (ammonia, LCFAs); and c) to optimize CH₄ production (Mata-Alvarez et al., 2011). Thus, application of co-digestion makes the AD process become more economically feasible.

When treating wastes with high nitrogen content, inhibition of methanogenesis by ammonia becomes possible, the level of methanogenic activity decreases with increasing concentrations of ammonia (Angelidaki and Ahring, 1993; Chen et al., 2008; Fotidis et al., 2013). Therefore, one of the purposes of the co-digestion is to balance the C/N ratio, but the proper combination of several other parameters in the mixture of co-substrates, such as pH/alkalinity, macro- and micro-elements, inhibitors/toxic compounds, biodegradable organic matter, and moisture content, are also relevant (Hartmann et al., 2003). The more balanced operation achieved by co-
digestion not only enhances biogas production, but also results in a more stable operation. In addition to improved yields due to nutrient compensation (Monou et al., 2008; Cuetos et al., 2008), other advantages of AcoD include the possibility of cost-sharing, since the general infrastructures and equipment can be employed by several wastes (Macias-Corral et al., 2008).
Table 2.7 Nutrients addition for enhancement of methanogenesis

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Nutrients (mg·L⁻¹)</th>
<th>Effect</th>
<th>Reactor</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Napiergrass</td>
<td>1.6 S, 0.25 Ni, 0.19 Co, 0.30 Mo, 0.06 Se</td>
<td>CH₄ production ↑40%</td>
<td>aspirator bottles</td>
<td>Wilkie et al., 1986</td>
</tr>
<tr>
<td>Wastewaters from food-processing industry</td>
<td>40 Fe, 0.5 Ni and 0.5 Co</td>
<td>rigid granular sludge was maintained</td>
<td>UASB</td>
<td>Oleszkiewicz and Romanek 1989</td>
</tr>
<tr>
<td>Acetate, propionate, butyrate, 3:1:1 CO₂ &amp; H₂</td>
<td>trace nutrient solution</td>
<td>COD removal ↑ 28.6%</td>
<td>UASB</td>
<td>Osuna et al. 2003</td>
</tr>
<tr>
<td>Food industry waste</td>
<td>B, Mo, Ni, Se, W, Co (High, Middle, Low)</td>
<td>Se/W and a low level of Co</td>
<td>5-L glass reactors</td>
<td>Feng et al. 2010</td>
</tr>
<tr>
<td>Maize silage</td>
<td>Cu Cr Ni Zn Fe Co Mn Mo Se</td>
<td>enhanced CH₄ (860mL g⁻¹ VS) production</td>
<td>batch reactor</td>
<td>Pobeheim et al. 2010</td>
</tr>
<tr>
<td>Maize silage</td>
<td>0.6 mg kg⁻¹ Nickel, 0.05 mg kg⁻¹ cobalt</td>
<td>25% increase of CH₄</td>
<td>semi-continuous anaerobic fermentation</td>
<td>Pobeheim et al. 2011</td>
</tr>
<tr>
<td>Simple molecule wastewater</td>
<td>aMgCl₂·6H₂O (9.6), MnSO₄·H₂O (2.5), KCl (6.6), CaCl₂·2H₂O (8.2), FeCl₂·4H₂O (8.1), CoCl₂·6H₂O (1.4), NiCl₂·6H₂O (0.9), CuCl₂·2H₂O (0.05), ZnCl₂ (0.05), EDTA (1).</td>
<td>COD removal efficiency increased from 74% to 90%</td>
<td>bench-scale UASB</td>
<td>Zhang et al. 2011</td>
</tr>
<tr>
<td>Organic fraction of MSW</td>
<td>Cr, Ni, Zn, Co, Mo and W</td>
<td>Enhance biogas production</td>
<td>Mostly UASB</td>
<td>Demirel and Scherer 2011</td>
</tr>
</tbody>
</table>

a The unit is g/L
Table 2.8 Nutrients addition for enhancement of hydrolysis

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Nutrients</th>
<th>Effect</th>
<th>Reactor</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>sucrose</td>
<td>Ca, Fe, Co, and Ni</td>
<td>optimize hydrolysis and acidogenesis</td>
<td>CSTR</td>
<td>Kim et al., 2008</td>
</tr>
<tr>
<td>Energy Crops and Crop Residues</td>
<td>Fe, Co, Ni, Mo</td>
<td>Accelerate hydrolysis and acidogenesis</td>
<td>LBR-UASB</td>
<td>Pobeheim 2011</td>
</tr>
<tr>
<td>Food waste</td>
<td>Se (0.16) and Co (0.22 mg/ kg fresh food waste)</td>
<td>significant increase in process performance and operational stability</td>
<td>Batch and semi-continuous digesters</td>
<td>Banks et al., 2012</td>
</tr>
<tr>
<td>Food waste</td>
<td>Co, Fe, Mo and Ni</td>
<td>a high CH&lt;sub&gt;4&lt;/sub&gt; yield (0.35- 0.45 L CH&lt;sub&gt;4&lt;/sub&gt;/g VS&lt;sub&gt;added&lt;/sub&gt;)</td>
<td>single-stage reactor</td>
<td>Zhang and Jahng 2012</td>
</tr>
</tbody>
</table>
2.3.3.8 Reduce inhibition of methanogenesis

The process of methanogenesis is mainly influenced by environmental factors such as pH, VFAs, and ammonia (Chen et al., 2008). Optimal pH range for maximum biogas production during methanogenesis is 6.5-7.5 (Liu et al., 2008). Outside this pH range, biogas production would be reduced rapidly until totally ceased (Hwang et al., 2004). It was reported that hydrogenotrophic methanogens are less sensitive to unfavorable pH levels compared with aceticlastic methanogens (Hao et al., 2012).

High concentrations of VFA can lead to decrease of pH value, which in turn causes inhibition of methanogenesis (Wang et al., 2009). Moreover, high level of individual VFA itself can directly inhibit methanogenesis. More than 50% inhibition of methane generation occurred at concentrations above 13, 15, and 3.5 g/L of acetate, butyrate, and propionate added to granular sludge, respectively (Dogan et al., 2005). Furthermore, in the same study by Dogan et al. (2005), aceticlastic methanogenic activity was inhibited at an acetate concentration of above 4 g/L. So far, VFA was reported as process state indicator for monitoring the process (Ahring et al., 1995; Boe et al., 2007). Also, there were studies focused on toxicity levels of VFAs (Pullammanappallil et al., 2001; Nielsen et al., 2007). Although many researchers have reported the effect of VFAs on process stability and as process state indicator, the effect of exposure to acetate or other VFAs on mixed cultures in respect to metabolic pathways and microbial community composition is yet unclear.

Carbon and nitrogen are two main elements of the organic solids of food waste. Under anaerobic conditions, organic carbon could finally be degraded to CH₄ and CO₂. Organic nitrogen in proteinaceous materials is hydrolyzed to ammonia, most of which is accumulated in the fermentative reactor (Sung and Liu 2003). This
byproduct, ammonia is reported toxic to organisms because unionized ammonia has the capability to pervade through the cell membrane (Gallert and Winter 1997). Thus, anaerobic digesters used for the treatment of food waste often encounter serious ammonia inhibition due to high content of proteinaceous materials. Therefore, it is necessary to investigate the impact of ammonia on anaerobic fermentation of food waste for biohydrogen recovery.

The potential inhibitory effect of ammonia on methanogenesis is a well-known problem when digesting wastes with high nitrogen content. It has been shown that optimum values within the range of 20 - 70 were suitable carbon-to-nitrogen (C/N) ratios for the AD process (Burton and Turner 2003); however, even lower values, i.e. 12 - 16 had also been reported (Mshandete et al., 2005). The thresholds of ammonia inhibition are associated with several factors, such as temperature, substrate, co-substrates, and pH in the reactors (Chen et al., 2008; Cuetos et al., 2008).

Long chain fatty acids (LCFAs) sourced from degradation of lipid materials in solid wastes during anaerobic digestion. Palatsi et al. (2010) had observed that the addition of LCFAs caused the appearance of the lag period in the CH₄ production from acetate and in the degradation of both LCFAs and n-butyrate. The LCFAs effect on CH₄ production through hydrogenotrophic methanogenesis was not as serious as acetoclastic pathway; there was no lag phase although its rate was lowered. Besides the inhibition effect of LCFAs on acetoclastic methanogenesis, LCFAs also inhibit the process efficiency of hydrolysis/acidogenesis. It was reported that fermentation of glucose was not inhibited by LCFAs (Chaganti et al., 2012). At the initial stage, acidogenesis is normal but the consumption of acidogenic products by methanogens is inhibited and thus causing the accumulation of acidogenic products, which in turn could decrease the pH inside the reactor and inhibit further hydrolysis/acidogenesis.
Angelidaki I. and Ahring B. K. (1993) had pointed out that the inhibitory effect of LCFAs mainly caused by free fatty acids and mostly the inhibition effect is irreversible. Pre-acclimation of microorganisms seems ineffective for alleviating the inhibition of LCFAs; however, the addition of calcium chloride could reduce the inhibitory effect of LCFAs, but it did not do so after the culture had been exposed to LCFAs for more than several hours (Palatsi et al., 2010).

2.3.4 Mass balance

Mass balance analysis is a way to assess and quantify the interdependencies during anaerobic digestion process. The input materials during anaerobic digestion of food waste include substrate food waste, water, inocula, bulking agents and the facilities supplies whereas the outputs mainly contain biogas (CH₄, H₂ and CO₂), soluble products (alcohols, solvents and fatty acids) and the residue digestate. One key requirement of mass balance analysis is that all materials of importance in the individual operational unit are included. For instance, in two-phase AD, intermediate products of acidogenesis are also the substrate for methanogenesis, and they could affect the final biogas production and overall energy recovery. The overall objective of mass balance study is to develop element mass balance models for the entire waste disposal facilities including all importance elements such as C, N, P, COD, and alkalinity.

2.4 Utilization of H₂ and CO₂

H₂ and CO₂ are always the by-products during the primary fermentation of organic solid wastes. It is the fact that H₂ and CO₂ produced in acidogenic reactor comprise as high as 30% of the consumed substrates (Clark et al., 2012). Conventionally, this part of energy was ignored during two-phase anaerobic digestion process for CH₄ recovery and hence leading to the fact that although with advantages
in mass transfer and operational flexibility in two-phase configuration; the increase in CH₄ yields often only marginally higher than the single-phase reactors (Lehtomaki et al., 2008; Nizami et al., 2011; Yu et al., 2012). Therefore, it is essential to harness the energy carried by H₂ and CO₂ from acidogenic reactor to boost up the feasibility of two-phase technology.

2.4.1 Homoacetogenic pathway

Homoacetogens are a functional group that can reduce CO₂ to acetate by H₂ through the acetyl-CoA pathway. Generally, homoacetogens are known as obligatory anaerobic bacteria (Drake et al., 2002).

Fig. 2.4 The acetyl-CoA pathway

Abbreviations: e⁻, reducing equivalent; THF, tetrahydrofolate; ATP, adenosine triphosphate; [Co-protein], corrinoid protein; CoA, coenzyme A.

Fig. 2.6 Phylogenetic relationships between certain acetogens (large bold font) and their closest non-acetogenic relatives (small non-bold font) within the phylum of the Gram-positive bacteria with low G+C %
Three points are refined from the definition of homoacetogens: 1) Reduce CO$_2$ to acetyl-CoA through acetyl- CoA pathway; 2) Electron transferred and energy synthesized during acetyl- CoA pathway; 3) CO$_2$ synthesize into cellular material instead of CH$_4$. There are some confusions regarding this definition, for instance, *Thermobacteroides proteolyticus* could ferment glucose to acetate but the electron acceptor is not CO$_2$ but proton (Ollivier et al., 1985); on the other hand, *Eubacterium limosum* (Loubiere et al., 1992), *Butyribacterium methylotrophicum* (Lynd and Zekus 1983) and *Caloramator pfennigii* (Krumholz and Bryant) can reduce CO$_2$ to acetyl-CoA through acetyl- CoA pathway, but the final product is butyrate. Another important characteristic of homoacetogens is acetate not always the end products, it depends on the substrates and reaction conditions.

Homoacetogens do not belong to a phylogenetic group of closely related microbes. Although they may be grouped based on the utilization of the acetyl-CoA pathway, some other microbes can also use this metabolic pathway (Drake et al., 2008) and it is difficult for clear identification; for example, the percentage of G+C in their genomes ranges from 22% in *Clostridium ljungdahlii* to 62% in *Holophaga foetida*. Parsimony tree of 20 acetogenic bacteria is shown in Fig. 2.5. Although it is the fact that genera *Acetobacterium*, *Sporomusa* and *Moorella* are composed exclusively of homoacetogens, a lot of homoacetogens in the phylogenetic distributions are very closely related to non-acetogenic bacteria (Fig. 2.6).
Fig. 2.5 Parsimony tree of 20 genera of homoacetogens (ARB-release June 2002). Bar corresponds to 10 nucleotide substitutions per 100 sequence positions.

Generally, the habitats of homoacetogens are strictly anoxic, such as sewage sludge, marine sediments, and rumen of ruminant animals (Henderson et al. 2010). Hence, strict anaerobic condition should be created for isolation and identification of homoacetogens. The diverse habitats and understudied nature lead to the relatively scarce knowledge on homoacetogens and difficulties in isolation of these microorganisms. It is reported that homoacetogens can be distinguished from agar or Gelrite (Drake et al., 2006) because of the two aspects: they can form: (I) colored zones on the plate containing a pH indicator, e.g., bromocresol green; or (II) clear zones in the plate containing CaCO₃ (Balch et al., 1977). The presence of acetyl-CoA pathway can be considered as good evidence that the microbe is a homoacetogen and the activity of CO dehydrogenase is usually applied to assess acetyl-CoA pathway. However, the activity of CO dehydrogenase can be misleading and could not be taken as a definitive evidence that a microbe using the acetyl-CoA pathway.
Table 2.9 Typical habitats for homoacetogens

<table>
<thead>
<tr>
<th>Homoacetogen</th>
<th>Habitat</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Acetobacterium woodii</em></td>
<td>Marine sediment</td>
<td>Balch et al., 1977</td>
</tr>
<tr>
<td><em>Acetobacterium noterae</em></td>
<td>Sediment</td>
<td>Sleat et al., 1985</td>
</tr>
<tr>
<td><em>Acetobacterium fimetarium</em></td>
<td>Digested cattle manure</td>
<td>Kotsyurbenko et al., 1995</td>
</tr>
<tr>
<td><em>Acetobacterium paludosum</em></td>
<td>Fen sediment</td>
<td>Kotsyurbenko et al. 1995</td>
</tr>
<tr>
<td><em>Acetobacterium sp. LuTria3</em></td>
<td>Sewage sludge</td>
<td>Frings et al., 1994</td>
</tr>
<tr>
<td><em>Moorella thermoacetica</em></td>
<td>Horse manure</td>
<td>Fontaine et al., 1942</td>
</tr>
<tr>
<td><em>Thermoanaerobacter phaeum</em></td>
<td>Pulp waste water reactor</td>
<td>Hattori et al., 2000</td>
</tr>
<tr>
<td><em>Treponemaprimitia sp. ZAS-2</em></td>
<td>Termite, hindgut</td>
<td>Graber et al., 2004</td>
</tr>
</tbody>
</table>

Recently developed molecular methods are efficient tools for identification of microbes. However, due to the fact that homoacetogens are not a phylogenetic group and hence it is impossible to develop 16S rRNA oligonucleotide probes and primers that exclusively target homoacetogens. Oligonucleotide probes or primers targeting functional genes of unique metabolic pathways can also be used to identify homoacetogens from environmental samples. Formyl tetrahydrofolate synthetase (FTHFS) is a functional gene that catalyzes the ATP-dependent synthesis of formyltetrahydrofolate through the acetyl-CoA pathway while enzyme acetyl-CoA synthases (ACS) is the central of Wood-Ljungdahl pathway and they are highly conserved in homoacetogens. FTHFS and ACS gene based group-specific oligonucleotide probes and primers for polymerase chain reaction (PCR) amplification and quantity-PCR were developed for evaluating the occurrence of homoacetogens from environmental samples and mixed microbial consortia (Gagen et al., 2010; Henderson et al., 2010; Xu et al., 2009; Yan et al., 2014). Reported oligonucleotide probes for homoacetogens are summarized in Table 2.10.

Homoacetogens are relatively understudied distinguished group involved in the complex anaerobic digestion process. Study of homoacetogens during anaerobic digestion had intrigued some scholars’ interests. Until now, it is relatively new with
only a few publications. Production of acetate was increased by 50-80% in a system of syntrophic acetogenesis coupled with homoacetogenesis (Nie et al., 2007) This demonstration clearly indicates the possibility of exploiting the homoacetogens in the acidogenic reactors to enhance the quality of the feedstock to UASB. However, the effecting factors to the acetate production via homoacetogenesis were still unclear. In addition, the kinetics of the homoacetogenesis and their role on H₂ production need to be further investigated.

Table 2.10 Oligonucleotide probes of homoacetogens

<table>
<thead>
<tr>
<th>Probe</th>
<th>Target organism(s)</th>
<th>Sequence (5’-3’)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-F-Symn-0700-a-R-23</td>
<td><em>Syntrophospora bryantii</em>, <em>Syntrophomonas sapovorans</em></td>
<td>ACTGCGTNTTCCTCTCT</td>
<td>Hansen et al., 1999</td>
</tr>
<tr>
<td></td>
<td><em>Syntrophomonas wolfei subsp. wolfei</em></td>
<td>GATTGTA</td>
<td></td>
</tr>
<tr>
<td>S-S-S.wol-0223-a-R-19</td>
<td><em>Syntrophobacter wolini</em></td>
<td>ACGCAGACTCATCCC</td>
<td>Xing et al., 2014</td>
</tr>
<tr>
<td>S-S-MPOB-0222-a-R-19</td>
<td><em>Syntrophobacter fumaroxidans</em></td>
<td>ACGCAGGCCCATCCC</td>
<td>Xing et al., 2014</td>
</tr>
<tr>
<td></td>
<td><em>Syntrophobacter pfenigii</em></td>
<td>ACGCAGGCCCATCCC</td>
<td></td>
</tr>
<tr>
<td>FTHFs</td>
<td>homocacetogens</td>
<td>TGGGMDAARGGYRG</td>
<td>Henderson et al., 2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HBWDGGGYGG</td>
<td></td>
</tr>
</tbody>
</table>

Much higher CH₄ content (i.e. ~ 98.8%) was achieved after about 500 days’ acclimation by homoacetogen enriched substrates during lab scale anaerobic digestion in UASB (Ryan et al., 2010). It is now clear that homoacetogens are arguably the most metabolically diverse group of obligate anaerobes characterized and that they present some interesting challenges, in particular with reference to their role within the AD process. H₂-utilising homoacetogens are known to be present in mesophilic digester sludge with numbers in the range of 10⁵-10⁷ CFU/ml (Zhang and Noike, 1994), representing approximately 3-11% of total bacteria in anaerobic sludge (Wang et al., 2007). It has yet to be demonstrated whether these organisms play an autotrophic or heterotrophic role in anaerobic digestion within different temperature
ranges, although it has been shown that in certain complex microbial habitats, the metabolic interactions of homoacetogens are influenced by operational factors such as pH and temperature and are strain specific (Kotsyurbenko et al., 2001).

Demler and Weuster-Botz (2011) carried out another study regarding homoacetogens involved in AD process. The production of acetate by homoacetogen *Acetobacterium woodii* was investigated under different hydrogen partial pressures (*PH₂*) in the headspace of a batch-scale stirred-tank bioreactor. The volumetric productivity of acetate increased with increasing *PH₂* and a maximum value of 7.4 g HAc / L·day was achieved at a *PH₂* of 1.7 bar. At this *PH₂*, under the controlled pH of 7.0, a final acetate concentration of 44.0 g /L was observed after an experimental time of 11 days. Moreover, a maximum cell specific acetate productivity of 6.9 g HAc / g cdw·day was achieved under hydrogenotrophic conditions.

### 2.4.2 Hydrogenotrophic methanogenesis

Hydrogenotrophic methanogenesis is a process that converting H₂ and CO₂ to CH₄ under the catalization of hydrogenotrophic methanogens. Hydrogenotrophic methanogens are a group of achaea playing significant role during the production of CH₄. In the methanogenic reactor, the hydrogenotrophic methanogens can efficiently maintain a very low *PH₂* (< 10 Pa) necessary for the functioning of the intermediate trophic group, the syntrophic acetogens, which are responsible for the conversion of organic acid and alcohol intermediates to direct CH₄ precursors (Pauss et al., 1990).

The activities of the hydrogenotrophic methanogens are crucial for a healthy and efficient performance of AD digesters. The performances and activities of hydrogenotrophic methanogens during anaerobic conversion of simple soluble substrates, such as H₂, methanol, ethanol, acetate, and glucose have been covered and discussed in recent research activities selected here (Demirel and Scherer 2008; Costa
et al., 2013; Parshina et al., 2014; Sawayama et al., 2006; Shigematsu et al., 2006; Zhang et al., 2008). However, the role and activities of hydrogenotrophic methanogens in anaerobic degradation of complex organic compounds to CH$_4$ has not been adequately discussed, and there were fewer data available regarding this aspect (Bertin et al., 2004; Demirel and Scherer 2008; Kampmann et al., 2012; Schnürer et al., 1999). Furthermore, the amount of literature regarding this particular topic seems still lacking.

Research activities on hydrogenotrophic methanogenesis are discussed in the following text. Firstly, the performance and relative abundance of hydrogenotrophic methanogens is highly associated with the operational and environmental parameters in the system. Biogas production from the biodegradation of municipal grey waste under thermophilic and hyperthermophilic (up to 70°C) conditions was investigated by Scherer et al. (2000). In their study, the populations of hydrogenotrophic methanogens ranged from $10^8$ - $10^{10}$/g TS, and dominated by a factor of 10 to 10,000 compared with acetotrophic methanogens, which is probably due to short HRTs (e.g. 1.3 - 14.2 days) employed. The process of hydrogenotrophic methanogenesis can also be affected by the sources of seed sludges. For example, a population of up to $10^8$/gdw of hydrogenotrophic methanogens was observed from the upper layer of acidic peatlands (Cadillo-Quiroz et al., 2006). Reaction temperature is another important operational parameter that affecting hydrogenotrophic methanogenesis. Ahring et al. (2001) had investigated the performance and dynamics of microbial community in anaerobic CSTRs treating cattle manure under thermophilic temperature (55 - 65°C). Results showed that hydrogenotrophic methanogens were the only microbial group, which exhibited higher specific methanogenic activity (SMA) and unchanged MPN (most probable number) at 65°C, whereas the activities and the populations of other
methanogens were significantly reduced. It seems that hydrogenotrophic methanogens play more important role under higher operational temperature, e.g. 65°C. Furthermore, thermophilic hydrogenotrophic methanogens of the family *Methanobacteriaceae* were reported to be capable of using both H₂/CO₂ and formate as substrates (Boone et al. 1993). Researchers have also studied the influence of reactor configurations on the activities of hydrogenotrophic methanogens. Padmasiri et al. (2007) studied the performance of the start-up phase and overall AD process during anaerobic treatment of swine manure using a completely mixed anaerobic bioreactor coupled with an external ultrafiltration membrane module. Population of *Methanomicrobiales*, an order belonging to hydrogenotrophic methanogens, increased with deteriorating performance of the reactor, which indicated that the syntrophic interactions involving hydrogenotrophic methanogens remained intact regardless of the degree of shear in the bioreactor. In a recent study, lab-scale mesophilic digestion of fodder beet silage was carried out in a CSTR for CH₄ production (Klocke et al. 2007), during which an OLR of 1.2 to 2.3 kg/m³.day was applied while the HRT were ranging from 56 to 106 days. Formate / H₂ & CO₂ oxidizing *Methanobacteriales* and the H₂ & CO₂ oxidizing *Methanosarcinaceae* as well as *Methanosaetaceae* were observed in the CSTRs.

In all, the process of hydrogenotrophic methanogenesis is a relatively understudied area during anaerobic digestion. However, its role on methane recovery from organic wastes and conversion of greenhouse gas, CO₂ to useful fuel will inspire more research enthusiasm.

**2.5 Further improvement of energy recovery**

Anaerobic digestion of organic-rich material for energy recovery (CH₄) is relatively an old technology, the study of which had last for nearly a century; however
one of the key factors prevents the scaling up of this technology is low energy efficiency, especially in two-phase system. Researchers have proposed a variety of measures to improve the process efficiency during anaerobic conversion of organic materials, e.g. pH regulation and addition of bulking agent (Selvam et al., 2010; Cysneiros et al., 2012b; Xu et al., 2011), leachate and effluent recirculation (Cavinato et al., 2011; Stabnikova et al., 2008; Lü et al., 2008), and micro-aeration (Lim and Wang 2013), as well as change of reactor configurations (Cysneiros et al., 2012). Most of these strategies, although reported to be effective on enhancing the performance of hydrolysis, they might increase the overall investment of the anaerobic process and therefore makes those measures less favourable when considering their large-scale application (Carballa et al., 2011). Critical analysis of this biochemical process indicates that regulation of metabolic pathways and reutilization of the acidogenic off-gas could potential increase overall energy recovery.

2.5.1 Metabolic pathways

In biochemistry, metabolic pathways are a series of chemical reactions catalyzed by enzymes with the requirement of dietary minerals, vitamins, and other cofactors. Numerous distinct pathways co-exist within anaerobic digestion system and they are important to the performance and energy recovery of this system. Metabolic pathways are the comprehensive result of feedstock, seeds and operational conditions. The key operational factors affecting the metabolic pathways are pH, acid accumulation and headspace H₂ concentration in the acidogenic reactors. These interactions have not been adequately addressed.

In two-phase AD reactors, metabolic pathways present in acidogenic reactor play a vital role in determining overall energy recovery from the AD process. Usually,
the distributions of major soluble products (alcohols, solvents and fatty acids) reflect the prevailing metabolic pathways. Production of acetate and n-butyrate indicates mixed acids fermentation pathway while production of acetone, ethanol and n-butanol is belonging to solventogenic pathway. Acetate, ethanol, and lactate were observed as the main metabolic products during anaerobic fermentation of xylose (Kongjan et al., 2009) whereas anaerobic degradation of carbohydrates is proposed as the competition between propionic and butyric acid producing bacteria (Harper and Pohland, 1986; McCarty and Mosey, 1991). As reported by many researchers, the conversion rates of soluble metabolites to CH$_4$ varied in an order of HAc > ethanol (HEt) > butyric acid (HBu) > propionic acid (HPa) (Wang et al., 2009). Lactic acid is a reduced product, which has the potential to be converted to HPa, thus it is an undesirable intermediate fermentation product.

pH in the anaerobic reactor is an important factor affecting fermentation of organic acids, including lactic acid (Itoh et al., 2012), propionic acid (Lin et al., 2011; Khanal et al., 2004), acetic acid (Fang and Liu 2002; Lins et al., 2012), butyric acid (Wang et al., 2014), and succinic acid fermentations (Song and Lee 2006; Yan et al., 2010). In general, pH of the operational unit not only affects growth rate of microbial biomass and fermentation efficiency, but also regulates yield and purity of final product. Zhu and Yang (2004) observed that butyric acid fermentation of xylose by *C. tyrobutyricum* shifted from a predominant butyric acid production at pH 6.0 to predominant lactate and acetate production at pH 5.0. Thus, change the pH of the medium is one of the potential ways to induce a shift of metabolic pathway.

The mechanisms of acids production are a series of enzymatic reactions that regulated by operational and environmental conditions. For instance, butyrate and acetate are commonly sourced from acetyl-CoA pathway. Acetyl-CoA and butyryl-
CoA are first converted to acetyl phosphate and butyryl phosphate catalysed by corresponding enzymes of phosphotransacetylase (PTA) and phosphotransbutyrylase (PTB). The next step is converting these acyl phosphates to acetate and butyrate via the catalyzation of acetate kinase (AK) and butyrate kinase (BK). Thus, the activities of PTA, PTB, AK and BK play critical roles on the process of acetate and butyrate formation. Another example, enzyme lactate dehydrogenase (LDH) is always involved in the formation of lactic acid, which catalyzes the generation of lactic acid from pyruvate with the regeneration of NAD$^+$ and NAD-independent LDH.

Despite numerous physiological studies, it is still unclear how the metabolic switch from acid to solvent production is regulated at the molecular level. Once this metabolic transition has been initiated, most of the excreted acids are taken up and converted into solvents. A combined metagenomic approach could reveal the complete array of microbial species and functional attributes that can be used to decipher the factors affecting the generation of different acidogenic products such as fatty acids and solvents, and the overall shift in metabolic pathways under different operating conditions and reactor-environments. Understanding these microbial-metabolic interactions, which influence the metabolic pathways, is the key to further increase the CH$_4$ yield. The knowledge developed through the metagenomic analysis can be used to adjust the operating conditions and reactor environment to target specific metabolite production; thus have the potential to develop an effective AD process that have the scientific relevance in different areas of biofuel production.

In a two-phase hybrid liquid-solid AD system, the overall efficiency of the process depends mostly on the efficiency of hydrolysis-acidogenesis as well as the intermediate products of the acidogenic phase. The production of intermediate products and distribution of products could be affected by metabolic pathways
prevailing in the acidogenic reactor. Ethanol, acetate, propionate, butyrate and caproate are the most popular components found in acidogenic reactor of food waste. Ethanol can be produced from heterolactic fermentation pathway during the initial stage of anaerobic hydrolysis, accompanied by lactic acid. Acetate is not only sourced from the degradation of pyruvate through acetyl-CoA pathway directly, but also can be formed from the syntrophic oxidation of propionate and butyrate (Müller et al., 2010). Propionate is usually sourced from the conversion of lactic acid (Zhu et al., 2010). Besides the formation of butyrate from acetyl-CoA pathway, iso and normal form of butyrate can be converted to each other.

2.5.2 Acidogenic biogas production

Acidogenic biogas mainly contains H₂ and CO₂, and they are the byproducts during hydrolysis of organic substrates and the following acidogenesis. Glucose is the fundamental source unit for acidogenesis. During acidogenesis, glucose can be degraded to pyruvate via the Embden Meyerhof Parnas (EMP) pathway. Then, pyruvate would be further oxidized to acetyl-CoA with the reduction of ferredoxin (Fd). Finally, hydrogenase enzyme will catalyse the oxidation of reduced Fd, during which generates Fd and releases molecular H₂. Furthermore, oxidation of NADH, via NADH: Fd oxidoreductase (NFOR) could release electrons for the reduction of the oxidized Fd, and then the reduced Fd is oxidized with proton release for H₂ production. Therefore, H₂ production is the means through which microbes lose excess electrons, the reaction of which is reversible and highly dependent on acidogenic PH₂. Thus, it is reasonable to control the PH₂ in the liquid phase for enhancement of H₂ yield. Gas sparging is an useful strategy for decreasing PH₂ and eliminating the inhibition of acidogenesis during anaerobic digestion.
The amount and efficiency of H₂ production through anaerobic digestion could be affected by a series of factors, such as substrate type, inoculum source and abundance and environmental conditions in the reactors. Isolation of H₂ specific microbes and eliminate H₂ consumption factors is the key to increase H₂ production. Heat - treatment of inocula and substrates is the most popular way to enhance H₂ production for selecting H₂-producing community and removal of nonspore-forming H₂ consumers (Oh et al., 2003; Valdez-Vazquez et al., 2009; Nissilä et al., 2011; Bakonyi et al., 2014). Despite the advantage of heat-treatment for hydrogen, it also has adverse effect due to killing of some process-benefit microorganisms (Marone et al., 2014). BES is a kind of efficient methanogenic inhibitor and addition of proper amount to the acidogenic reactor could eliminate methanogenesis efficiently. However, adding BES to the reactor is not operational feasible when applied in scaled-up system. Some of hydrogen producing-bacteria can produce spores to survive under unfavourable conditions such as heat shock, chemical stress, pH inhibition and aeration while hydrogen consumption microbes was inhibited and accordingly increase the survival ratio of H₂-specific microbes (Massanet-Nicolau et al., 2008; Venkata et al., 2008). Acid pre-treatment is another way to enhance hydrogen production during anaerobic fermentation (Lee et al., 2009). Pre-treatment is one effective way to improve H₂ production; however, the increased complexity of operation and investment should be considered. Combining pre-treatment and operational condition optimization is a promising way to enhance H₂ hydrogen during anaerobic digestion.

PH₂ in the liquid phase is one of the key factors that affect H₂ generation. However, there were many controversial observations regarding the influence of H₂ on the anaerobic breakdown of saccharides have been reported (Liu et al., 2006; Inanc
et al., 1999). Despite the controversy, $PH_2$ do affect the process of acidogenesis that involved with $H_2$ production or consumption. Moreover, studies regarding the effects of $PH_2$ on the metabolism and the metabolic pathway of the anaerobic microbes have been discussed. $PH_2$ determines the quantitative composition of the intermediate products. Kim et al. (2006a) demonstrated that external sparging $CO_2$ to a CSTR increased the rate of butyrate production and specific $H_2$ production. The production of $H_2$ and butyrate has been considered as an obstacle for ethanol production (Liu et al., 2006) and stirring favoured $H_2$ and butyrate production, suggesting increased $PH_2$ by accumulation of $H_2$ in unstirred conditions inhibiting butyrate production.

Bothun et al. (2004) observed that under elevated headspace pressure, the decrease of theoretical maximum growth yield of microbial biomass and increase in the maintenance coefficient indicated that more cellubiose and ATP are channelled towards maintaining the cellular function. These phenomenons are partially attributed to the increasing concentrations of dissolved $H_2$ and $CO_2$ with increasing headspace pressures. Actually, the role of dissolved gas and headspace pressure effects on metabolic activity is quite complex.

Based on the pervasive effects of dissolved gases on microbial biomass growth, intermediate product generation, and selectivity of intermediate products, Jones and Greenfield (1982) suggested the potential to manipulate bio-based solvent production by regulating the concentrations of dissolved gases (from acidogenic process) or compositions of acidogenic headspace within optimal pressure.

Shift of metabolic pathways during regulation of headspace pressure are frequently related to the solubility of dissolved acidogenic gases in the digesters (Jones and Greenfield 1982), which increases with elevated headspace pressure or partial pressure of specific gas in the system. For example, the metabolic behaviours
of bacteria, fungi, and yeasts may shift at above approximately 0.01 MPa partial pressures of CO$_2$ and H$_2$ (Jones and Greenfield 1982; de Kok et al., 2013; Kraemer and Bagley 2007; Nath and Das 2004). McIntyre and McNeil (1998) observed that elevated levels of acidogenic gases, especially CO$_2$ and H$_2$ can result from hydrolysis and acidogenesis and the pressures could be amplified by increased hydrostatic pressures in large-scale digesters (0.1 MPa m$^{-1}$ depth of H$_2$O). Anaerobic digestion of organic wastes under a controlled headspace environment (both headspace pressure and PH$_2$) is important for regulation of metabolic pathways and enhancement of targeting products.

### 2.5.3 Bioefficiency of different products

CH$_3$CH$_2$OH $+$ 2H$_2$O $\rightarrow$ CH$_3$COO$^-$ $+$ 2H$_2$ $+$ H$^+$  
\[ \Delta G = 9.6 \text{ kJ/mol} \]  
(Mata-Alvarez 2003)  

CH$_3$CH$_2$COO$^-$ $+$ 3H$_2$O $\rightarrow$ CH$_3$COO$^-$ $+$ H$^+$ $+$ HCO$_3^-$ $+$ 3H$_2$  
\[ \Delta G = 76.1 \text{ kJ/mol} \]  

CH$_3$CH$_2$CH$_2$COO$^-$ $+$ 2H$_2$O $\rightarrow$ 2CH$_3$COO$+$H$^+$+2H$_2$  
\[ \Delta G = 48.1 \text{ kJ/mol} \]  

It is widely accepted that the abundances of VFAs during anaerobic digestion of organic materials decrease as the chain length increase. In addition, the presence of formic acid is transient due to its rapid conversion to other compounds (e.g. CO$_2$). Thus, it is reasonable to focus on short-chain VFAs of C2-C6 when studying bioefficiency of the acidogenic products. Ethanol is a more reduced product with the highest energy value among the acidogenic products (Pipyn and Verstraete, 1981). The conversion of ethanol to methane needs its prior oxidation to acetate and this reaction is thermodynamically unfavourable under standard conditions. In order for the conversion of ethanol to CH$_4$, it is necessary to reduce sufficiently the
concentrations of reaction products (acetate and H₂) to ensure a negative value of Gibbs free energy (ΔG). Observations of Pipyn and Verstraete (1981) indicated that it might be possible to increase CH₄ production by shifting the metabolic pathway towards ethanol and lactate (herterolactic) fermentation. However, high ethanol concentration was reported to be harmful to methanogens for inhibiting propionate-degradation (Smith and McCarty 1989). But till now the toxic limit in different system was not clear, need to be assessed in future.

Acetate is recognized as a key intermediate product in the final step of mineralization of organic materials (Scholten and Conrad 2000), production of which is generally increased along the reaction time. The production of CH₄ from acetate is an important step during AD of organic materials due to the fact that nearly 70% of the total CH₄ produced is sourced from acetate (Gujer et al., 1983; Smith and Mah. 1966). The methanization pathway from acetate can also be processed by co-function of acetate-oxiders and hydrogenotrophic methanogens (Zinder and Koch 1984). Thus, the kinetics of acetate utilization by digester sludge would depend on the predominant species in the population of aceticlastic/hydrogenotrophic methanogens.

H₂-producing acidogenic microbes always involve the process of conversion of butyrate to CH₄ because methanogens can only metabolite 1 or 2 carbon compounds. Hence the contribution of butyrate to methane is highly depends on the co-culture with methanogens and fortunately this kind of reactions are popular and much energetic efficient compared with propionate in terms of carbon recovery (Lawrence 1971). Wang et al. (1999) reported that under the same conditions, VFAs degradation rates were classified into four groups; the order being n-HBu> (HAc, n-HCa, n-HVa, i-HBu) > (HPr, i-HVa) > i-HCa. The presence of iso (n)-valeric acid, caproic acid depends on the operational conditions.
2.6 Summary

Under the increasing energy crisis worldwide, it is urgent to find sustainable alternatives to alleviate the earth’s burden. Anaerobic digestion of organic solid wastes appears to be a promising technology due to its dual effect on waste disposal and energy recovery. H₂ and CH₄ are two common energy forms recovered during anaerobic digestion. H₂ is an extremely clean energy with the only combustion product as water and more important it contains high heat value. However, the feasibility of using H₂ as a fuel is still not totally confirmed and also because of the explosive nature of H₂ itself, recovery of energy in terms of H₂ gas during anaerobic digestion is still under controversy. CH₄ is an old form of energy used by human beings. Its feasibility for household and industrial use had already been confirmed. Further, recovery of CH₄ from anaerobic digestion of organic wastes is a carbon neutral process and also it is clean. Therefore, recovery of CH₄ by anaerobic digestion of organic solid waste is attracting more research interests.

Anaerobic digestion has been considered as an energy efficiency technology; however, compare with the BMPs of organic solid wastes the energy recovery efficiency still need to be enhanced. This relatively low energy efficiency is because of the complex nature of anaerobic digestion itself, the process can be influenced by substrates types, sources of seed sludge, water regimes, reactor configurations as well as the operational parameters. Researchers had investigated measures to enhance hydrolysis and CH₄ production, e.g. phase separated instead of single-phase reactor, pH adjustment, and optimization of inoculum to substrate ratio. These optimization methods although reported to be effective, to some extent, increased the process complexity and economic input. When analysing the mass balance of a two-phase anaerobic digestion of organic solid waste, it is easy to find another great potential
(15-30% of consumed substrates) to increase energy recovery-H₂ and CO₂ produced in the acidogenic phase.

1) H₂ and CO₂ production occurred during the acidogenesis of monomers. Homoacetogenesis is a process catalysed by homoacetogens during conversion of H₂ and CO₂ to acetate. Acetate is one of the most important direct precursors for methanogens. The H₂ threshold for homoacetogens is 500 Pa, which is much higher than that for hydrogenotrophic methanogens. Hence, under enhanced H₂ production condition, homoacetogens are more easily to take their role. Once homoacetogenesis happened in acidogenic reactor, H₂ produced during organic compounds cleavage will be taken by homoacetogens as substrate to form acetate. Therefore, to establish the syntrophic relationship between homoacetogens and acetogens enables getting back some of H₂ and CO₂ produced in the acidogenic phase. However, homoacetogens have strict requirement for the operational condition and the understudied nature of this group limit their application.

2) Another way to catch back H₂ and CO₂ produced in acidogenic phase is to utilize them as substrates for hydrogenotrophic methanogens. Hydrogenotrophic methanogenesis is one of the two-methanogenic pathways taken by methanogens. Hydrogenotrophic methanogens are very efficient organisms with low H₂ utilization threshold. Diversion of H₂ and CO₂ produced in acidogenic phase to methanogenic phase for the reutilization by hydrogenotrophic methanogens is another promising strategy to enhance overall energy recovery.

3) Although utilization of H₂ and CO₂ by homoacetogens and hydrogenotrophic methanogens is promising measures for getting back energy carried by biogases, the reactions in acidogenic and methanogenic reactors are so complex that the feasibility and actual metabolic mechanisms of the two processes need further investigation.
CHAPTER THREE

RESEARCH METHODOLOGY

3.1. Reactor Design

An acidogenic LBR coupling methanogenic UASB, typical two-phase AD configuration for high-solid organic waste treatment was chosen for this study. The volume of biogas produced in LBR can be determined in terms of pressure. Flow rate of acidogenic biogas from LBR to UASB can be controlled by Mass Flow Controller (MFC, Seven Star). A computer based PID controller was applied to control the system and collect data. The schematic configuration is shown in Fig. 3.1.

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Fig. 3.1 Schematic of the experimental setup
The LBRs were made of stainless steel, and at the bottom of each reactor, perforated plate was provided for filtration of leachate. The size of LBR is 8.3 L for total volume with a working volume of 5.3 L. One thick layer of plastic beads was placed on the top of the perforated plate with stainless steel mesh to facilitate efficient production of leachate. The UASB reactors (with 10.0 L working volume) were continuously fed with artificial wastewater at OLR of 2.0 kg COD/m³·d for more than two months before starting the experiment. The composition of the artificial wastewater was (g/L): 30.96 of \( \text{C}_6\text{H}_{12}\text{O}_6 \), 19.23 of NaAc, 4.30 of \( \text{NH}_4\text{Cl} \), and 1.60 of \( \text{K}_2\text{HPO}_4 \). Batch mode operation was applied and the experimental time for each batch was 17-20 days under fixed temperature (35 ± 1 °C).

![Fig. 3.2 Picture of the experimental setup](image)

### 3.2 Substrate and inoculum

Compositions and corresponding ratios of each component of the artificial food waste are presented in Table 3.1. TS, VS, TOC and TKN of the food waste were analysed by taking a representative portion from the well-mixed food waste.
Anaerobic sludge collected from Shek Wu Hui Wastewater Treatment Plant (WWTP) in Hong Kong was used as source of seed microbes.

Table 3.1 Characterization of food waste and seed sludge

<table>
<thead>
<tr>
<th></th>
<th>Total solids (TS, %)</th>
<th>Volatile solids (VS/TS, %)</th>
<th>Total organic carbon (TOC, %)</th>
<th>Total Kjeldahl nitrogen (TKN, %)</th>
<th>Percentage (% , w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bread</td>
<td>62.6 ± 0.9</td>
<td>91.5 ± 1.1</td>
<td>56.0 ± 0.1</td>
<td>1.7 ± 0.1</td>
<td>35</td>
</tr>
<tr>
<td>Boiled rice</td>
<td>36.6 ± 0.0</td>
<td>89.8 ± 0.0</td>
<td>46.3 ± 0.6</td>
<td>1.1 ± 0.0</td>
<td>25</td>
</tr>
<tr>
<td>Cabbage</td>
<td>7.2 ± 0.0</td>
<td>91.5 ± 1.2</td>
<td>33.0 ± 1.4</td>
<td>3.0 ± 0.0</td>
<td>25</td>
</tr>
<tr>
<td>Boiled pork</td>
<td>49.3 ± 2.2</td>
<td>96.4 ± 1.3</td>
<td>52.9 ± 1.2</td>
<td>7.9 ± 0.1</td>
<td>15</td>
</tr>
<tr>
<td>Food waste</td>
<td>42.5 ± 0.8</td>
<td>90.6 ± 4.1</td>
<td>53.0 ± 2.3</td>
<td>2.1 ± 0.2</td>
<td>15</td>
</tr>
<tr>
<td>AD sludge</td>
<td>3.0 ± 0.0</td>
<td>89.7 ± 0.0</td>
<td>34.8 ± 1.0</td>
<td>2.0 ± 0.0</td>
<td></td>
</tr>
</tbody>
</table>

Initially, the LBR reactor was packed with a mixture of food waste, inoculums and bulking agents were added as designed, and then the same quantity (w/w) of tap water was added. In the LBR, the generation of leachate was occurred naturally.

3.3 Analytical Methods

There are two parts of parameters involved in the process of anaerobic digestion, environmental factor (solid, liquid and gas phases) and microbial operator (organic compound degraders).

3.3.1 Physicochemical Analyses

The total solids (TS) and volatile solids (VS) contents of the food waste, the digestate, and the seed AD sludge were determined by oven drying at 105°C for 24 h and igniting at 550 °C for 16 h in muffle furnace, respectively. A modified Walkley-Black method was used for analysis of TOC of all solid samples; whereas, TKN content was measured using digestion and spectrophotometric determination of ammonium-nitrogen (NH₄⁺-N) (Nelson and Sommers 1982). The concentrations of carbohydrate, protein and lipids were analyzed by a modified phenol - sulfuric
acid method (Masuko et al., 2005), Kjeldahl method (Salo-väänänen and Koivistoinen 1996) and ISO 6492:1999, respectively.

For the leachate collected from LBRs, parameters such as volume, pH value, COD were analyzed before filtration. Volumetric cylinder was used for measurement of leachate volume. pH of the leachate and effluent was determined directly by pH-electrode (Orion 920, Thermo). Analysis of NH₄⁺-N and TKN was following the method described by Nelson and Sommers (1982). COD was analyzed by Standard methods (APHA 2005, No. 5220). After filtration of leachate with 0.45 µm cellulose acetate membrane, soluble products (except lactic acid) were analyzed by a HP 6890 Series gas chromatograph (GC, Hewlett Packard) with injector and flame ionization detector (FID) temperatures of 250 °C. Nitrogen was used as a carrier gas with a 20 mL/min flow rate (25 psi). The oven temperature was programmed as follows: 120 °C for 5 min, increasing to 180 °C at 5 °C/min, and then constant at 180 °C for another 10 min. An Econo-Cap EC1000 (15 m × 0.53 mm × 1.20 µm) coated with 0.2 µm CP-Wax 57 CB column was used. Lactic acid was analyzed using a high performance liquid chromatography (HPLC, Waters Alliance 2695) coupled with Waters 2996 Photo Diode Array (PDA) Detector. An Ultrasphere® ODS Column C-18 column (10 µm, 25 cm × 4.6 mm i.d.), (Beckman Coulter, USA), with an injection volume of 10 µL, wavelength 210 nm, and a mobile phase of 85% 50 mM KH₂PO₄ (use phosphoric acid to adjust pH to 2.8 ) and 15% methanol, flow rate 0.7 mL/min was employed. The temperature was controlled at room temperature. Concentrations of both soluble products and lactic acid were calculated using standard curves by injecting corresponding standard solutions. The filtrate was also used for glucose analysis following the colorimetric method for reducing sugars (Dubois et al., 1956). Total soluble products (TSP) are
the sum of alcohols (ethanol, propanol and butanol), solvents (acetone) and fatty acids (lactate, acetate, propionate, \textit{iso-} and \textit{n-}butyrate, \textit{iso-} and \textit{n-}valerate and caproate).

Volume of acidogenic biogas was measured by online MFC (Seven Star) with real-time data logging (AIDCS system monitor), while methanogenic biogas production from UASB was measured by wet drum-type wet gas flow meter (BSD-0.5, Shanghai). Biogas in the headspace of the LBRs and outlets of UASBs were periodically measured using a gastight syringe (1 mL injection volume) and a gas chromatograph (HP7890) equipped with thermal conductivity detector (TCD) and PLOT-Q column (30 m × 0.53 mm × 15 µm). Argon was used as the carrier gas. Calibration was performed using external gas standards: 100% CH\textsubscript{4}, 20% CO\textsubscript{2} + 80% H\textsubscript{2}, 100% H\textsubscript{2} and 60% CH\textsubscript{4} + 30% CO\textsubscript{2} balanced in N\textsubscript{2}, injected as 1.0 mL at 1 atm.

### 3.3.2 Phylogenetic analysis

#### 3.3.2.1 DNA extraction

Total genomic DNA was extracted from seed sludge and cow manure as well as biomass obtained from the reactors at selected sampling points during the experimental period. Homogeneous leachate, 5 mL, from each LBR was centrifuged at 16,000 g for 5 min and the pellet was used for the DNA extraction. The residual pellet was washed with 1 mL of MilliQ water and centrifuged again in the same manner to ensure a maximal removal of residual leachate. DNA was extracted using QIAamp DNA Stool Mini Kit (Qiagen) following the manufacturer's protocol. DNA was purified using Wizard\textsuperscript{®} DNA Clean-Up System (Promega, USA).
Quantity of the extracted DNA was determined using Nanodrop ND-100 spectrophotometer. Further, the A260/A280 and A260/A230 ratios were also checked to assess the contamination of protein and humic acids, respectively.

3.3.2.2 Q-PCR

Real-time Quantitative PCR (Q-PCR) was adopted to quantify the microbial populations during the experimental study. Specific primers targeting desirable groups were selected to conduct Q-PCR, e.g. Primer set of 338F and 533R was selected for estimate the population of Bacterial 16S rRNA (Muyzer et al., 1993). Q-PCR was performed in 20 µL reaction volume containing 10.0 µL iQ™ SYBR® Green Supermix (2X), 1.0 µl diluted DNA template and 2.0 µl primer pair mix (10 pmol/µl each primer). The reactions were run in triplicates for each sample and on a Mx3000P QPCR System (Agilent Technologies, Santa Clara, CA, USA) with following thermal conditions: initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation (30 s at 94°C), annealing (30 s at 55°C) and extension (1 min at 72°C); and finally dissociation curve was measured by heating the samples from 55°C to 95°C in increment of 0.5°C and collect data after 10 sec for each incremental step.

3.3.2.3 Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE)

The 16S rRNA genes were amplified using PCR with the universal primers BAC338F and BAC805R. To stabilize the melting behavior of the PCR products, a 40-bp GC-clamp was attached at the 5'-end of the forward primer. PCR was conducted using PTC-200 thermal cycler (MJ Research) following thermal conditions of initial denaturation at 94 °C for 5 min; followed by 34 cycles at 95 °C for 1 min, 55 °C for 30 s and 72 °C for 1 min and final elongation at 72 °C for 10
min. The DGGE was performed using a DCode™ universal mutation detection system (Bio-Rad Laboratories, Hercules, California, USA) according to the procedure described by Muyzer et al. (1993). Briefly, 50 µL PCR product was loaded onto 8% acrylamide gel containing a 40-65% denaturant gradient, where, 100% denaturant contained 7 M urea and 40% (v/v) formamide. Electrophoresis was performed in 1×TAE buffer at a constant voltage of 70 V and a temperature of 60 °C for 13 h. After electrophoresis, the gel was stained with SYBR® Gold nucleic acid gel stain (Invitrogen™); destained in water for 1 h; and the image was captured using a digital image capture system with UV transilluminator (Bio-Rad). Computer-assisted gel image analysis was performed using the Image Lab 4.0 software (Bio-Rad). Bands of interest were excised directly from the gel using a sterile razor blade and eluted in 10 µL of nuclease-free water (Promega, Madison, WI). Similarity between bands from day 1 and 9 was analysed by Pearson correlation index using SPSS software.

3.3.2.4 Sequence and phylogenetic analysis

The PCR amplicons recovered from the DGGE gel was further amplified using the BAC338F and BAC805R primers, without the GC clamp. The PCR products were gel-purified using Wizard® SV Gel Clean-Up System (Promega, USA) according to the manufacturer’s instructions. The PCR products were ligated into the pGEM-T Easy vector (Promega, Madison, WI) and transformed into Escherichia coli JM109 super competent cells using pGEM®-T Easy Vector System II (Promega, A1380) adapting the protocol provided by the manufacturer. The plasmids were extracted from the clones after growing in LB broth and were sequenced by Beijing Genomics Institute (BGI) using T7 (5’-TAATACGACTCACTATAG-3’) and SP6 (5’-ATTTGTTGACACTATAGAAT-3’).
sequencing primers. The 16S rRNA gene sequences obtained were compared with those from the NCBI nucleotide sequence database using the BLASTN program. Multiple alignments were generated with the Clustal X program, version 1.8 and phylogenetic trees were constructed with MEGA 5.1 software on the basis of evolutionary distances that were calculated by the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method with Maximum Composite Likelihood model. Bootstrap resampling analysis was performed for 1000 replicates to estimate degrees of confidence in tree topologies.

3.3.3 Functional gene analysis

Attempts at characterizing the populations of functional groups in complex microbial ecosystems have been hindered by the phylogenetic diversity of these phenotypes. Hence, functional gene based molecular approach was chosen as an ideal way to analyse major metabolic pathways. Butyric pathway is one of the most popular metabolic pathways during AD of organic compounds and production of which can be realized through two kinds of pathways, butyryl-CoA: acetate CoA-transferase pathway and butyrate kinase pathway (Louis et al., 2004). Primer sets of BCoATscrF/R (Louis and Flint, 2007) and buk-F/R (Vital et al., 2013) as listed in Table 3.3 were chosen for identifying butyric pathway.

Primer set of HG-f/r (De Sa et al., 2011, Table 3.3) encoding enzyme hydrogenase was selected to analyze the activity of hydrogenase during the evolution of H₂ in acidogenic reactor.
### Table 3.2 Characteristics of the PCR primers and probes used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Target group</th>
<th>Sequence (5’-3’)</th>
<th>Target size</th>
<th>Annealing Temp (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>V3F</td>
<td>V3 region of Bacteria</td>
<td>CCTACGGGAGGCAGCAG</td>
<td>193</td>
<td>55</td>
<td>Muyzer et al., 1993</td>
</tr>
<tr>
<td>V3R</td>
<td></td>
<td>ATTACCGCGGCTGCTGG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BCoATscrF</td>
<td>butyryl-CoA: acetate CoA-transferase pathway</td>
<td>GCIGAICATTTCACITGGAAYWSITGGCAYATG</td>
<td>530</td>
<td>53</td>
<td>Louis and Flint, 2007</td>
</tr>
<tr>
<td>BCoATscrR</td>
<td></td>
<td>CCTGCCTTTGCAATRTCIACRAANGC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>buk-F</td>
<td>Butyrate kinase pathway</td>
<td>TGCTGTWGTGTTGAGGTYGGGA</td>
<td>500</td>
<td>64</td>
<td>Vital et al., 2013</td>
</tr>
<tr>
<td>buk-R</td>
<td></td>
<td>GCAACIGCYTTTTGATTTAATGCATGG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HG-f</td>
<td>Hydrogenase</td>
<td>AAGAAGCTTATAGAAGATCCTAA</td>
<td>250</td>
<td>58</td>
<td>De Sa et al., 2011</td>
</tr>
<tr>
<td>HG-r</td>
<td></td>
<td>GGACAACATGAGTAAACATTG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BAC338F</td>
<td>V3-V5 region of Bacteria</td>
<td>ACTCCTACGGGAGGCGAG</td>
<td>468</td>
<td>55</td>
<td>Yu et al., 2005</td>
</tr>
<tr>
<td>BAC805R</td>
<td></td>
<td>GACTACCAGGGGTATCTAATCC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T7</td>
<td>Cloning</td>
<td>AATACTTCATCAGTAG</td>
<td>-</td>
<td>55</td>
<td>Liang et al., 1993</td>
</tr>
<tr>
<td>SP6</td>
<td></td>
<td>ATTTAGGTGACACTATAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GC clamp</td>
<td>acetyl-CoA pathway</td>
<td>5’-CGCCCGCGCGCGCAGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGGACGGGGGG-3</td>
<td></td>
<td></td>
<td>Muyzer et al., 1993</td>
</tr>
</tbody>
</table>
CHAPTER FOUR

FEASIBILITY OF REUTILIZATION OF ACIDOGENIC OFF-GAS

BY DIVERTING TO METHANOGENIC REACTOR DURING

TWO-PHASE ANAEROBIC DIGESTION OF FOOD WASTE

Abstract

Recently, two-phase anaerobic digestion is gaining attention from researchers because of its increased stability as well as the great potential for specific optimization and improvement. However, the advantage of two-phase process of methane recovery over single phase is only marginal due to the unavailability of the hydrogen and carbon dioxide produced in the acidogenic reactor to the hydrogenotrophic methanogens in the methanogenic reactors. Hence, transferring the off-gas from acidogenic reactor to methanogenic reactor to provide H₂ and CO₂ for hydrogenotrophic methanogens should be a rewarding measure to improve overall energy recovery. Acidogenic off-gas transfer from acidogenic to methanogenic reactor was supposed to increase overall methane gas production. Results showed that gas transfer increased the biogas production up to 38.6%. In addition, hydrolysis rate and COD production were also achieved in the acidogenic reactor. In order to identify whether off-gas transfer or change in the acidogenic leachate quality was the major reason for the methane gas increase, another set of experiment was conducted, in which all the UASBs with and without gas transfer were fed with the same leachate. Results showed that without the contribution of leachate, there was still 8% increase
of methane gas, which should be related to off-gas transfer. Results of these two experiments indicated that increase of overall methane gas recovery by reutilization of acidogenic off-gas is a viable technique.

4.1 Introduction

Anaerobic digestion (AD) is an attractive waste treatment technology in which both waste disposal and energy recovery can be achieved. Two-phase AD is thought to be more efficient and stable than single phase and is gaining attention from researchers due to its great potential for further enhancement. With the separation of hydrolysis/acidogenesis from methanogenesis, two-phase AD facilitate the specific enrichment of acidogens and methanogens (Azbar and Speece 2001). Hydrogen and carbon dioxide are the co-products during acidogenesis and their amounts will increase with elevated biodegradation of organic wastes. Till now two-phase anaerobic digestion of organic wastes either harvests sole hydrogen /methane gas or hydrogen plus methane gas as the recovery of hydrogen. Harvesting sole energy as H₂ or CH₄ will miss potential of the other, for example, recovery of acidogenic hydrogen and methanogenic methane will decrease the overall feasibility. Considering the explosive nature of hydrogen, methane gas is considered to be the viable energy form.

Hydrogen is the by-product during hydrolysis of organic waste (C₆H₁₀O₄) and acidogenic production of butyrate and acetate. It was reported that hydrogen production during acidogenesis of food waste could reach 0.9-1.8 mol-H₂/mol-hexose with a maximum H₂ volume content of 69% (Shin et al., 2004). It is estimated that the energy H₂ carried during acidogenesis can be up to 30% of the energy recovered (Clark et al., 2012). Therefore, it is logical and profitable to harness this part of energy. Three measures can be utilized to solve this problem, firstly, retain and reduce the production of hydrogen in the acidogenic reactor; secondly, reutilization of H₂ and
simultaneously reduce CO$_2$ to acetate by homoacetogens in a coupling system (Nie et al., 2007; 2008); and thirdly, reutilization of H$_2$ and CO$_2$ in methanogenic reactor. Supplementation of hydrogen to methanogenic reactor enhanced the methane recovery as reported by Luo and Angelidaki (2012; 2013). Regarding the first method, the retention of large amounts of H$_2$ inside the reactor will inhibit the acidogenic process (Lyberatos and Skiadas 1999) and until now, there is no publication reported the reduced H$_2$ production with high degradation efficiency. Nie et al. (2007) and Ni et al. (2011) had reported the consumption of H$_2$ in a coupled homoacetogenic reactor and achieved 52% increase in acetate production. However, integrating one more reactor would increase the economic input and operational complexity of the AD process. Reutilization of H$_2$ in methanogenic reactor thus will be a desirable choice.

H$_2$ and CO$_2$ are the by-products of acidogenesis of organic compounds, which may undergo different types of metabolic pathways, i.e., ethanol, propionic and butyric-type fermentation. Ren et al. (2007) had observed that a mixed consortia fermentation often associates with more than one liquid product (acetate, propionic acid, etc.). The amount of hydrogen yield not only associated with the fermentation type, but also affect the shift of metabolic pathways in return (Kongjian et al., 2009). It has already been reported that the amount of hydrogen and in particular its partial pressure may influence the yields of the main metabolic products i.e. acetate and ethanol (Collet et al., 2005). During anaerobic acidogenesis, hydrogen often accumulates in the headspace of the reactor and hence blocks the degradation of acids (Miron et al., 2000). A relatively low H$_2$ pressure is thermodynamically favorable for biodegradation of organic acids (Nath and Das 2004). In addition, H$_2$ was recognized as being the controlling factor on regulation of anaerobic metabolic pathways (Khanal et al., 2004). Nevertheless, there was no investigation in literature regarding changes
of metabolic pathways during diversion of acidogenic off-gas to methanogenic reactor.

In this study, diversion of acidogenic off-gas from acidogenic LBR to methanogenic UASB reactor for reutilization of H₂ and CO₂ via direct hydrogenotrophic methanogenesis was investigated as a strategy for improving overall energy recovery. Addition of hydrogen to methanogenic reactor can enhance methane gas production or upgrade CH₄ content in the biogas (Luo and Angelidaki 2012; 2013) and therefore improve overall energy recovery. Therefore, the performance of the two-phase AD system and overall CH₄ recovery will be investigated to evaluate the feasibility of this strategy.

4.2 Materials and methods

4.2.1 Substrate and inoculum

Simulated food waste and anaerobically digested sludge as detailed in Chapter 3 were used as substrate and seed, respectively. The seed sludge was stored long enough (e.g. 2 months) for removing its organic matter. The selected physicochemical properties of the simulated food waste and AD sludge are presented in Table 4.1.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Food waste</th>
<th>Sludge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Solids (TS, %)</td>
<td>40.0 ± 2.5</td>
<td>4.6 ± 0.1</td>
</tr>
<tr>
<td>Volatile Solids (VS /TS, %)</td>
<td>98.0 ± 0.1</td>
<td>89.7 ± 0.0</td>
</tr>
<tr>
<td>Total organic carbon (TOC, %)</td>
<td>45.9 ± 4.4</td>
<td>8.5 ± 0.0</td>
</tr>
<tr>
<td>Total Kjeldahl nitrogen (TKN, g/kg)</td>
<td>28.8 ± 0.5</td>
<td>2.0 ± 0.0</td>
</tr>
<tr>
<td>Carbohydrate (%)</td>
<td>74.0 ± 2.0</td>
<td>ND*</td>
</tr>
<tr>
<td>Lipid (%)</td>
<td>10.0 ± 0.5</td>
<td>ND</td>
</tr>
<tr>
<td>Protein (%)</td>
<td>18.0 ± 0.2</td>
<td>ND</td>
</tr>
</tbody>
</table>

*not determined
4.2.2 Experimental set up

Two identical LBRs (LBR-gas and LBR-control) were made from 150 mm diameter steel pipe, which was capped at both ends to form a closed vessel with a 5.4-liter working volume and 2.9-liter leach bed. A stainless steel mesh was placed on the bottom of the reactor to support the food waste and a layer of glass beads was placed underneath to prevent clogging as well as solids entering into the leachate. One of the two gas outlets at the cap of the LBR was connected to the bottom inlet of upflow anaerobic sludge blanket (UASB) through a peristaltic pump. The percolation and filtration occurred naturally. Two 10-L UASB (UASB-gas and UASB-control) formed the second phase of the set ups.

Both LBRs were filled with 2.0 kg of artificial food waste which mixed with 20% (I/S, wet basis) of anaerobic digested sludge as inoculum and 10% of wood chips as bulking agent according to a previous study (Xu et al., 2012). The liquid to solid ratio during this experiment was kept at 1.0, which means 2.0 L of tap water was loaded to each LBR. One-day sampling frequency was chosen to avoid acid crisis. During each sampling, the leachate was taken out and its volume was recorded, then exactly 50% of the leachate with pH adjusted to 6.0 using 0.5 M NaHCO₃ was returned back to the LBR from the top. The remaining 50% of the leachate was fed to corresponding UASBs with 50 mL reserved for analysis. Parameters reflecting the performance, i.e. COD, VFAs, pH of LBR leachate, and the biogas compositions were analyzed to evaluate the performance of these systems.

4.2.3 Diversion of acidogenic off-gas to UASB

Biogas produced in acidogenic LBR mainly contains hydrogen and carbon dioxide, direct precursors for the hydrogenotrophic methanogenesis. To check the viability of transferring acidogenic off-gas from LBR to UASB and the effect of this
new method on the overall performance, two treatments, LBR-gas with off-gas diversion to UASB (UASB-gas) as the test and LBR-control coupled with UASB-control without off-gas transfer as control were set up (Fig. 4.1).

In order to compare the effect of acidogenic off-gas transfer on the solubilization of particulate solids and the hydrolysis rate of monomers to short-chain molecules, kinetics of COD and soluble products production was determined. First-order kinetics were used to evaluate the hydrolysis rate of food waste in LBR under different treatments. The effects of different operations on the performance of hydrolysis have traditionally been simplified to the first-order kinetics (Vavilin et al., 2008). In the First-order kinetics,

\[ \frac{dS}{dt} = -kS \]  
Eq. 4.1

\[ \frac{dP}{dt} = \partial kS \]  
Eq. 4.2

S is the volatile solids (VS) concentration of particular substrates, P represents concentration of soluble products (SP), \( k \) is the first-order hydrolysis constant (d\(^{-1}\)), and \( \partial \) (g SP/kg VS) is the conversion coefficient of substrates to SP. After integration, the product concentration is expressed as:

\[ P = P_i + \partial S_i (1 - e^{-kt}) \]  
Eq. 4.3

Where \( P_i \) and \( S_i \) are the initial concentrations of soluble products and substrates, respectively. A non-linear regression carried out using IBM SPSS Statistics 19 was used to estimate the values of constants \( k \) and \( \partial \) and their standard deviations (SD) as well as \( R^2 \) of ANOVA analysis.
4.2.4 Evaluating the contribution of acidogenic off-gas

In order to identify the sole contribution of acidogenic off-gas produced in LBR, the liquid fed to UASB should be kept the same. Similar setup used in the last experiment, LBR-gas with off-gas diversion to UASB-gas as the test while LBR-con coupled with UASB-con without off-gas transfer as control were designed. This experiment was carried out with leachate collected from both LBRs and mixed together, then divided into two identical parts and fed to the UASB of different treatments. The only difference between the test and control would be the acidogenic off-gas.

4.2.5 Mass balance analysis

Hydrogen produced in acidogenic phase is the product of degradation of VS, therefore, diverting this energy carried by H₂ and reuse it in a methanogenic reactor to produce methane could increase overall efficiency of energy recovery. Hydrogen production in acidogenic LBR was recorded by real-time Data log (AIDCS system monitor). Bioconversion of hydrogen and carbon dioxide to methane gas by hydrogenotrophic methanogens follows the equation: \( 4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \), the
reaction of which is much popular in methanogenic reactor due to its favorable energetic ($\Delta G = -135.6 \text{ kJ/mol at STP}$) nature. Then the amount of methane contributed by hydrogen reduction can be calculated from the acidogenic hydrogen production.

4.2.6 Analytical methods

Volumes of biogas from LBR and UASB were measured by MFC and wet gas flow meter, respectively; while their compositions were determined by using GC (HP7890, Agilent Technologies) as detailed in Chapter 3.

TS, VS, TOC and TKN contents of the solid food waste, digestate and seed sludge were measured following the methods described in Chapter 3, while the acidogenic leachate from LBR was analyzed for pH, COD, and soluble products.

4.3 Results and discussion

4.3.1 Enhanced hydrolysis/acidogenesis rate of food waste

Liquefaction of particulate solids to monomers and then conversion of these soluble products to VFAs, alcohols and long chain fatty acids (LCFAs), accompanying by $\text{H}_2$ and $\text{CO}_2$ occur in acidogenic LBR. Hydrolysis rate as well as the yield of soluble products determines the efficiency of this process.

The hydrolysis potential of particulate organic material can be determined by chemical oxygen demand (COD) production. As is shown in Fig. 4.2, COD generated from the LBR-gas and the LBR-control varied in daily production rate and cumulative yield. Acidogenic biogas diversion increased the production of COD in the first 3 days compared with the control. However, COD leached in LBR-control was constantly higher than that in the LBR-gas during day 4 -10, which might be due to the lower headspace pressure that did not provide enough of a driving force to remove hydrogen from the liquid, or that the partial pressures reached were not low enough to
significantly alter the thermodynamics of hydrogen evolving enzymatic reactions (Clark et al., 2012). High H₂ partial pressure was recognized as an obstacle to hydrolysis/acidogenesis during fermentation of organic compounds. The COD produced from the control decreased along the experiment as in the case of a typical batch profile (Lehtomäki et al., 2008), whereas COD production in LBR-gas experienced a short decrease on day 4 and then increased until day 14.

![Graph](image)

Fig.4.2 (a) COD of leachate collected from LBRs and of effluent from UASBs, (b) Cumulative COD production from LBRs

However, depletion of H₂ partial pressure from day 10 in LBR-gas increased the COD production and from then on the advantage of LBR-gas in COD production was significantly higher than in the control. In the LBR-gas treatment, hydrogen produced in LBR was diverted to UASB while in the LBR-control hydrogen was retained in the headspace and this high hydrogen partial pressure might have inhibited the hydrolysis and thus less COD yield was obtained in the leachate (Nie et al., 2008). In agreement with daily COD production rate, cumulative COD yields in the LBR-gas and the LBR-control were varied. A COD production of 0.61 g/g VS_{added} was achieved
in the LBR-gas reuse and there was a 27% increase compared with LBR-control treatment.

Soluble products (soluble COD) serve as precursor for the following acetogenesis and methanogenesis; amount and composition of which is highly related to the overall efficiency. First-order kinetic model was applied to analyze the hydrolysis of particulates and the production of soluble products. The fitting of correlation was evaluated with the correlation coefficient $R^2$, and the reliability of the evaluated parameters was quantitatively estimated by standard deviation (SD).

As presented in Table 4.2, the data fit first-order kinetics well with $R^2$ equal to 0.99 and acceptable SD values. A much higher hydrolysis rate of particulate organic matter was achieved in LBR-gas with a hydrolysis constant $k=0.053$ d$^{-1}$ while in the LBR-control the $k=0.042$ d$^{-1}$. Again, the higher hydrolysis rate should be related to prevention of hydrogen-based inhibition in the headspace of LBR-gas. Higher hydrogen partial pressure (e.g. > $5.7 \times 10^4$ Pa) could inhibit further H$_2$ production by different species of microorganisms (Van Niel et al., 2003).

Production of soluble products was also evaluated by first-order kinetics model and a much higher conversion co-efficient, i.e. 0.69 compared with previous report of 0.55 (Vavilin et al., 2004) was achieved. This higher conversion co-efficient on the one hand was due to the higher acidification degree (production of COD) and on the other hand should be due to the inclusion of alcohols (ethanol, i-propanol and butanol). Diversion of acidogenic headspace gas had increased the soluble product conversion co-efficient to 0.79 ± 0.009. T-test was used to analyze the significance of deviations between these two treatments, and a less than 95% significant level was observed that should be related to the complicated environmental conditions besides hydrogen pressure (Appels et al., 2008).
Table 4.2 First order kinetic parameters for hydrolysis of food waste

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hydrolysis of particulate</th>
<th>Soluble products (SP) production</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k$ (d$^{-1}$)</td>
<td>SD</td>
</tr>
<tr>
<td>LBR-gas</td>
<td>0.05</td>
<td>1.00E-03</td>
</tr>
<tr>
<td>LBR-control</td>
<td>0.04</td>
<td>4.20E-04</td>
</tr>
</tbody>
</table>

4.3.2 Changes in acidogenic metabolic pathways

![Graph](image)

Fig. 4.3 Concentration of individual soluble product (a) and the percentage of individual SP accounting for TSP (ethanol (E), acetic acid (A), propionic acid (P), butyric acid (B), n-propanol (n-P), lactic acid (L)). Error bars represent standard deviations of duplicate analysis.

Soluble products production and their speciation are closely related to the operation condition, e.g. pH and type of inoculum. As shown in Fig. 4.3, species of soluble products from both the LBR-gas and LBR-control treatments shared the same spectrums (ethanol, acetic acid, propionic acid, butyric acid, n-propanol and lactic acid); however, the abundances of the individual soluble products are different. Compared with the control, an obvious increase of butyric acid and decrease of lactic acid was observed in the LBR-gas experiment. Lactic acid is a reduced product and is
the common product during anaerobic digestion of food waste (Zhang et al., 2007). Lactic acid is produced by a consortium of *Lactobacillus* sp. belonging to facultative anaerobic or microaerophilic bacteria. A large portion of lactic acid in the control should be associated with higher hydrogen partial pressure inside the reactor whereas in LBR-gas, diversion of acidogenic off-gas had reduced this inhibitory effect caused by headspace pressure as high as 12.6 psi. Increased production of lactic acid in LBR-control may also be related to the environmental conditions, e.g. pH <4. This was confirmed by Itoh et al. (2012), who had reported selective production of lactic acid by controlling an extremely low pH (3.5). Butyric acid is a common fermentation product of butyrate type of fermentation and is also one of the favorable substrate for methane gas production (Öztürk 1991; Wang et al., 1999). The production of butyric acid from hexose often associated with hydrogen production (Hawkes et al., 2002). Diversion of acidogenic gas in the headspace would reduce hydrogen partial pressure in the headspace and thus enhanced hydrogen production, which would cause the shift of metabolic pathway towards more H₂ production fermentation pathway (Kongjan et al., 2009; Sharma and Li 2008). On the other hand, it is usually accepted, although still unclear that hydrogen accumulation, which is related to the partial pressure of hydrogen in the reactor headspace, might inhibit hydrogen producers (Wang et al., 2008). The result was consistent with the finding that the acetate could be converted to ethanol at higher hydrogen partial pressure (Oh et al., 2003). Ethanol is a reducing product and was thought to be a small contributor to hydrogen production (Hwang et al., 2004). Consequently, ethanol yields in both treatments were approximately the same in terms of concentration and percentage. Propanol is another reduced compound and could be generated through 1, 2-propanediol pathway by shunting carbon flux from glycolysis (Jain and Yan 2011). Relatively higher concentration of
propanol in the control was probably contributed by the conversion of lactic acid under the specific environmental conditions, e.g. low pH (Elferink et al., 2001). Acetate is the great contributor to hydrogen production during anaerobic fermentation, during which process the theoretical yield of hydrogen can be achieved by 4 mol $H_2$/ mol glucose (Zhang 2010).

Fig. 4.4 shows the proposed metabolic pathway during anaerobic fermentation of food waste. The abundance in the variation of different soluble products was a result of different metabolic pathways. The major components of soluble products in the gas - reuse were ethanol, acetate, propionate, n-propanol and lactic acid. And when linking the compositions of soluble metabolites with the last step, two pivotal points are pyruvic acid and acetyl-CoA, which were sourced from monomers that were hydrolyzed from food waste. Via pyruvic acid pathway, ethanol, propanol or lactic acid could be produced with the consumption of reducing equivalents, e.g. $H_2$, NADH whereas acetate and butyrate are usually considered as the end products of acetyl-CoA pathway. When available NADH in the system was surplus, end product of acetyl-CoA pathway tends to be butyrate, as the synthesis of butyrate would be accompanied by transferring NADH to NAD$^+$. It was reported that the metabolic pathway was influenced more by the on-site pH condition rather than the hydrogen partial pressure (Horiuchi et al., 2002). The consistent lower pH value in the control reactor may explain the relative higher abundance of lactic acid. Itoh et al., (2012) selectively produced lactic acid by decreasing the culture pH to as low as 3.5. Likely, the increase of butyrate in the gas-reuse experiment can be attributed to the relatively higher pH (5.0-6.0), and is consistent with Horiuchi’s (2002) study.
Fig. 4.4 Proposed metabolic pathways for soluble products production in LBR-gas. Only the key products are presented in the figure; the numbers in blue color represent the EC numbers of corresponding enzymes.

### 4.3.3 Increased methane production

Methane gas produced in UASBs was measured by the wet gas flow meter. As shown in Fig. 4.5, daily CH₄ yield exhibited a profile same as COD variation, which indicated that CH₄ production was closely related to leachate strength. In addition, the cumulative methane production also corresponds to cumulative COD feeding to methanogenic UASB. However, cumulative methane production increased 38.6% in the UASB-gas compared to the control while COD increase was only 27.0%, which means the elevated CH₄ production might also be contributed by acidogenic off-gas (H₂ and CO₂) diversion and reuse. It was reported by Luo et al. (2012) that the CH₄ production rate in treatment with H₂ addition was 22% higher than in the control that
fed only with manure; and also the CO\textsubscript{2} content in the biogas was only 15%, while it was 38% in the control. Their study showed that H\textsubscript{2} addition can both increase volume and quality of methane production and this confirmed the feasibility of acidogenic biogas reuse in methanogenic UASB.

![Fig. 4.5 Daily and cumulative methane gas production (First run)](image)

In the second run of this experiment, acidogenic leachates collected from both LBR-control and LBR-gas were mixed thoroughly and then divided equally to corresponding UASBs. As presented in Fig. 4.6, daily CH\textsubscript{4} production rates as well as the cumulative CH\textsubscript{4} production of UASB-gas were different from the control. Major differences in daily CH\textsubscript{4} production occurred during the first 12 days, which should be attributed to the relatively higher H\textsubscript{2} production rate in LBR during the initial stage of batch study. Easily biodegradable carbohydrates are the preferred substrates for H\textsubscript{2} production and degradation of carbohydrates usually occurs before protein and lipids (Miron et al., 2000). Compared with the first run, differences in cumulative CH\textsubscript{4} production between the test and the control was much smaller, because of the depletion of variations caused by COD. In the treatment with acidogenic biogas reuse,
a cumulative \( \text{CH}_4 \) production of 0.27 L/g VS \text{added} was achieved that corresponds to ~8\% (\( P<0.05 \)) increase compared with the control.

![Graph a](image)

![Graph b](image)

**Fig.4.6** Daily and cumulative \( \text{CH}_4 \) production in UASBs receiving the same leachate

### 4.3.4 Mass balance analysis of \( \text{CH}_4 \) production

Table 4.3 summarizes the general performance of this experiment in the second run. Obviously, COD production of acidogenic leachate was the largest contributor of \( \text{CH}_4 \) generation. However, there was another potential component that may contribute to \( \text{CH}_4 \) production through the reaction, \( 4\text{H}_2 + \text{CO}_2 \rightarrow \text{CH}_4 + 2\text{H}_2\text{O} \) (\( \Delta G = -135 \text{ kJ/mol} \)), metabolized by hydrogenotrophic methanogens. As the leachates fed to UASBs in the second run were the same, the difference in the \( \text{CH}_4 \) production caused by the quality and quantity of acidogenic leachate could be eliminated. As per the requirement of the hydrogenotrophic methanogenesis, cumulative \( \text{H}_2 \) production of 68.9 L in the LBR can lead to the generation of ~17.2 L \( \text{CH}_4 \) in UASB by hydrogenotrophic methanogenesis through \( 2\text{H}_2 + \text{CO}_2 \rightarrow \text{CH}_4 + 2\text{H}_2\text{O} \). Actually, the net increase of cumulative \( \text{CH}_4 \) in the test in the second run was ~20.2 L. So, the difference between the test and the control could be attributed to the reuse of

![Graph c](image)
acidogenic off-gas. Of course, still there might be some difference in metabolic efficiency in methanogenic UASBs due to the involvement of acidogenic biogas.

H₂ production in the LBR-control was accumulated in the headspace and released when during sampling, i.e. leachate collection and recycling. The hydrogen partial pressure in the headspace is known to inhibit the bacteria, especially hydrogen producers and therefore decreasing the hydrogen yields (Sharma and Li 2008; Wang et al., 2008). Therefore, accumulation of H₂ in the control further decreased the net evolution of H₂.

Table 4.3 General performance of LBR-UASB in the 2nd run

<table>
<thead>
<tr>
<th></th>
<th>Gas reuse</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cumulative COD (g/kg VS)</td>
<td>609.2 ± 17.6</td>
<td>481.6 ± 7.6</td>
</tr>
<tr>
<td>Total soluble products (g COD/L)</td>
<td>389.8 ± 8.3</td>
<td>217.9 ± 5.8</td>
</tr>
<tr>
<td>H₂ production (L/kg VS)</td>
<td>69.0 ± 4.9</td>
<td>-</td>
</tr>
<tr>
<td>VS removal efficiency (g/kg VS)</td>
<td>67.1 ± 2.8</td>
<td>54.8 ± 1.0</td>
</tr>
<tr>
<td>CH₄ gas production (L/kg VS_added)</td>
<td>269.7 ± 5.6</td>
<td>249.6 ± 7.2</td>
</tr>
<tr>
<td>CH₄ ratio (%)</td>
<td>66.0 ± 4.3</td>
<td>70.7 ± 4.7</td>
</tr>
<tr>
<td>Carbon conversion (%)</td>
<td>52.0 ± 0.1</td>
<td>45.0 ± 0.0</td>
</tr>
</tbody>
</table>

4.4 Summary

This study investigated the reutilization of acidogenic CO₂ and H₂ in the methanogenic reactor through diversion of the gases from acidogenic leach bed reactor to methanogenic UASB with the purpose of enhancing overall CH₄ recovery from food waste. Diversion of the acidogenic off-gas to UASB increased the CH₄ recovery as well as CH₄ production rate in UASB, while the changes in concentrations of CO₂ and H₂ in the acidogenic headspace of LBR exhibited a significant influence on the acidogenic metabolic pathways and also increased the COD production. Increase in methane recovery corresponds to the increased COD as
well as to the off-gas diverted to the UASB. Therefore acidogenic off-gas utilization could be a potential strategy to control the metabolic pathways in the LBR as well as to increase the methane production rate in the UASB.
CHAPTER FIVE

REGULATION OF ACIDOGENIC METABOLIC PATHWAY DURING TWO-PHASE ANAEROBIC DIGESTION OF FOOD WASTE: EFFECT OF HEADSPACE PRESSURE IN ACIDOGENIC REACTOR

Abstract

Diverting the acidogenic off-gas from acidogenic leach bed reactor (LBR) into the methanogenic reactor (UASB) increased the methane recovery that also influenced the composition of the acidogenic leachate. Headspace pressure, as affected by the diversion of LBR-off gas to the methanogenic reactor, is expected to affect the quality of the leachate produced in LBR. Therefore, this experiment aims at investigating the effect of acidogenic headspace pressure on the regulation of metabolic pathways as well as quality of leachate production in acidogenic reactor during two-phase AD of food waste. Four levels of headspace pressures were set, designated as 6-12 psi (T1), ~3-6 psi (T2), ~3 psi (T3) and ambient pressure (T4). Above this pressure the gases were diverted to the UASB employing pressure sensors. Diversion of biogas from LBR enhanced COD and soluble product generation in T2, T3 and T4 whereas, very high pressure (T1) resulted in comparatively higher lactate production and low degradation rate of proteinaceous compounds. A pressure of 3-6 psi (in T2 and T3) improved the production of COD by ~22-36%, soluble products (include VFA and solvents) by ~9-43%, VS reduction by ~14-19%, and CH₄ production by ~10-31%;
and were significantly higher than T1 reactor. Besides, acetate, butyrate and propanol production were comparatively higher in T2 and T3 reactors while lactate was higher in T1. Mixed acids fermentation prevailed in all the treatments with butyrate as the single major component and it is a good precursor for the subsequent methanogenesis. Increased H₂ production and a favourable leachate composition are found responsible for higher methane recovery.

5.1 Introduction

Two-phase anaerobic digestion (AD) offers advantages such as reactor stability, operational flexibility and higher process rate; however, methane recovery is almost similar to that of the single-phase AD. In the previous experiment, it was demonstrated that diversion the acidogenic off-gas from acidogenic leach-bed reactor (LBR) to the methanogenic reactor (UASB) increased the methane recovery and so as the composition of the acidogenic leachate (leachate quality). During acidogenic reactions, hydrogen is generated as a co-product, and the hydrogen partial pressure \( (P_{H_2}) \) influences the acidogenic reactions and the microbial community. Thus, the microbial community will be affected under varying \( PH_2 \) that can be achieved by maintaining specific headspace gas pressure.

Hydrogen and carbon dioxide are two important co-products during the primary fermentation of soluble organic substrates and these two components contribute to the pressure of the acidogenic reactor. The reactions of converting hydrolysis products to short-chain intermediates can only be processed under low \( PH_2 \) due to their unfavourable thermodynamic conditions under standard conditions. In addition, it was confirmed that high H₂ pressure would inhibit the process of hydrolysis as well as further H₂ generation (Kongjan et al., 2009; Wang et al., 2008). However, it is the fact that H₂ and CO₂ produced in acidogenic reactor comprised as
high as 30% of the consumed substrates (Clark et al., 2012), which means under the
natural process, \( \text{H}_2 \) would accumulate in the acidogenic headspace and inhibit the
process of hydrolysis/acidogenesis.

Hydrogen is an important factor that influences the metabolic pathway
occurred in acidogenic reactor. The composition of acidogenic products in terms of
leachate is affected by the balance between \( \text{H}_2 \) and reducing equivalent. Higher
hydrogen pressure in the headspace will inhibit the acetogenic biomass growth rate,
since high values inhibit the generation of propionic and butyric acids (Lyberatos and
Skiadas 1999). Vavilin et al. (1995) had demonstrated the decrease of hydrogen
pressure often coincided with the start of propionic acid production and an increase of
lactic acid concentration. Therefore, to maintaining appropriate hydrogen pressure is
the key to control the metabolic pathways towards targeted products.

Besides the effect of headspace pressure, the composition of headspace gases
also has been reported to show effect on regulating product spectrum. Researchers
have considered altering the composition of headspace gases and decreasing the
pressure of \( \text{H}_2 \) by sparging gases into the liquid phase of the reactor (Tanisho et al.,
1998). Different compositions of headspace with anaerobic microorganisms resulted
in considerable differences in the distribution of metabolized volatile fatty acids
(VFAs) (Hillman et al., 1991). Similar observations were also reported by Tanisho et
al. (1998) and Karlsson et al. (2008). However, studies with \( \text{N}_2 \)-sparging performed
by Mizuno et al. (2000) and Kim et al. (2006) did not result in significant change in
the composition of liquid end products, even when \( \text{H}_2 \) yield was increased. Headspace
gas composition can not only affect \( \text{H}_2 \) production, but also influence the overall
energy efficiency of AD system. Recently, Patra and Yu (2013) observed that the
headspace gas composition and bicarbonate concentrations in the media could affect
methane production and other characteristics of rumen fermentation in \textit{in vitro} gas production systems.

The previous experiment (Chapter 4) indicated that both the transferred off-gas and change of leachate quality contributed to the overall increase of methane recovery. However, the headspace pressure and the composition of headspace are the key factors that determine the metabolic pathways during acidogenesis. Acidogenic off-gas diversion should be related to the shift of metabolic pathway in acidogenic reactor. How this headspace pressure regulates the metabolic pathway? and which is the optimal composition of acidogenic headspace for overall energy recovery? This experiment planned to answer these two questions.

5.2 Experiment design

Four headspace pressure levels (Table 5.1) were set according to gas production levels of a preliminary experiment. The headspace pressure was controlled by online pressure sensor. The acidogenic gases beyond the set pressure point would be diverted to methanogenic UASBs.

<table>
<thead>
<tr>
<th>Set pressure (psi) a</th>
<th>Gas diversion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>12.6</td>
</tr>
<tr>
<td>T2</td>
<td>6.3</td>
</tr>
<tr>
<td>T3</td>
<td>3.3</td>
</tr>
<tr>
<td>T4</td>
<td>0</td>
</tr>
</tbody>
</table>

a the pressure is relative to atmosphere pressure

5.3 Materials and methods

5.3.1 Substrate and inoculum

Artificial food waste as detailed in Chapter 3 was used as the substrate in this experiment. The physicochemical properties of the simulated food waste are summarized in Table 5.2. Anaerobically digested sludge with 2.3% total solids (TS)
and 76% VS/TS obtained from Shek Wu Hui wastewater treatment plant was used as the seed.

Table 5.2. Selected physicochemical properties of the artificial food waste

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Food waste</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total solids (TS, %)</td>
<td>40.0 ± 2.5</td>
</tr>
<tr>
<td>Volatile solids (VS /TS, %)</td>
<td>98.0 ± 0.1</td>
</tr>
<tr>
<td>Total organic carbon (TOC, %)</td>
<td>45.9 ± 4.4</td>
</tr>
<tr>
<td>Total Kjeldahl nitrogen (TKN, g/kg)</td>
<td>28.8 ± 0.5</td>
</tr>
<tr>
<td>Carbohydrate (%)</td>
<td>69.5 ± 2.8</td>
</tr>
<tr>
<td>Lipid (%)</td>
<td>12.8 ± 2.5</td>
</tr>
<tr>
<td>Protein (%)</td>
<td>14.4 ± 1.4</td>
</tr>
</tbody>
</table>

5.3.2 Experimental set up and treatments

Leach bed reactors (LBR) with a 5.4-liter working volume and 2.9-liter leach bed volume was used as acidogenic reactor while UASB with 10-L reactor volume was used as the second phase methanogenic reactors. Both LBRs and UASBs were prepared as detailed in Chapter 3.

LBRs were filled with 2.0 kg of food waste mixed with 20% (I/S, wet basis) of inoculum and 100 g of wood chips as bulking agent according to a previous study (Xu et al., 2012). The liquid to solid ratio of 1:1 was applied to the start-up of the experiment, which means 2.0 L of water was added to each LBR. The sampling frequency was 1-day. During each sampling, the leachate was taken out and exactly 50% (v/v) of the leachate with pH adjusted to 6.0 with NaOH (0.5 mol/L) was returned back to the LBR from the top; while the remaining 50% of the leachate was fed to UASB with 50 mL reserved for chemical analysis.

5.3.3 Analyses

Physiochemical analysis of the food waste and seed sludge were performed as detailed in Chapter 3. Acidogenic leachate samples collected from LBRs were analyzed for pH, COD, soluble products, etc. according to the methods described in
Chapter 3.

Volume of acidogenic biogas in LBR was calculated by integration of real-time data of gas flow rate measured by a mass flow controller (MFC, Seven Star). The outlet acidogenic off-gas was collected using gasbag and 1-mL gas was used for composition analysis during each sampling point. Biogas from the UASB was continuously measured using a gas meter and analyzed for the methane contents using a gas chromatograph (HP7890) equipped with TCD and PLOT-Q column (30 m × 0.53 mm × 15 µm).

5.3.4 Statistical analysis

Statistical analyses were conducted using analysis of variance (ANOVA) and general linear model (GLM) procedures of SPSS Statistics v19 to evaluate the effect of regulation of acidogenic headspace pressure on the performance of the two-phase AD digesters. It was considered significantly different at P < 0.05 level.

5.4 Results and discussion

5.4.1 Pressure profile

Fig. 5.1 shows the dynamics of headspace pressures in the experiment, only data of treatments T1, T2 and T3 were given, since the headspace pressure in T4 was equivalent to ambient pressure. Although the set values in T1 was 12. 6 psi, the real pressure never reached this level while values ranging between 8 and 12 psi were observed during the first half of the digestion. Thereafter the headspace pressure gradually decreased until the end of this experiment, matching with a typical batch study. Most of the points in T2 reached the set value of 6.3 psi, while all the points in T3 could maintain the set value of 3.3 psi. The four levels of headspace pressures were set to investigate their effects on regulating metabolic pathways and quality of leachate production.
Fig. 5.1 Pressure profiles in the four treatments (the relative pressure inside T4 was equal to ambient)

5.4.2 Performance of LBR

Volumes of leachate production from all four treatments throughout the experiment were in the range of 1500-2100 mL; and the volumes increased along the experimental time (Fig. 5.2a). Varying headspace pressures led to different
leachability among treatments, e.g. the smallest volume of leachate, throughout the experiment, was observed in T1 while volumes of leachate collected in the other three treatments were almost similar. Obviously, the separation of T1 from T2, T3 and T4 indicated that acidogenic biogas release (lower absolute hydrogen pressure) was favorable for leachate production. Increasing leachability in all treatments along the time course of this experiment should be due to the hydrolysis of solid particles, and similar trends of leachability from batch study with acidogenic LBR was observed by Xu et al. (2011, 2012). Considering the cumulative leachate production, the leachability in the four treatments followed an order of T3 > T2 > T4 > T1.

Fig. 5.2 Variations of volume (a) and pH (b) of leachate collected from LBRs
pH is considered as a critical factor during the process of AD, which could influence both the microbial activity and the prevailing metabolic pathways. It is ideal for the system to buffer itself; however, the accumulation of VFAs results in decreased pH in the reactor (e.g. <4.0), which in turn would inhibit the performance of hydrolysis. Therefore, in order to prevent the possibility of process failure caused by acid crisis, the pH of the leachate was manually adjusted to 6.0 with sodium hydroxide before recycling into the LBR during each sampling. As shown in Fig. 5.2b, despite the adjustment of the pH, pH values of all reactors during the first 5 days were still around 4.0, which should be due to the rapid accumulation of fatty acids. Likely, a pH range of 3.8-4.5 during the first 8 days was observed in a previous study (Xu et al., 2012). pH in T3 treatment during the first 4 days was much higher than the other 3 treatments, probably due to inhibition of hydrolysis by higher hydrogen pressure, which led to low concentration of fatty acids (Sharma and Li 2008). Overall, the pH values are gradually increasing along the progress of the experiment, which should be attributed to the increasing buffering capacity of the systems and relatively low concentrations of fatty acids. pH of all the four treatments fell in a range of 4.0-6.0, however, during the first 10 days these values were lower than 5.0 and this may dominate the environmental conditions which influence the prevailing metabolic pathways.
Regulation of acidogenic headspace pressures led to varied profiles of COD production from the four treatments in both daily production (12.1, 26.3, 28.7 and 20.4 g/L of COD from T1-T4, respectively, leached on day 13) and cumulative yields (Fig. 5.3). The cumulative COD generation was 433.3 g/kg·VS in LBR-T1, while 586.7 g/kg·VS was achieved in LBR-T3. From day 1 to 4, COD production in T3 was much lower than all other treatments, which might be due to inhibition by the relatively higher H₂ partial pressure. Despite ~75% of headspace biogas was released from T3, large volume of acidogenic biogas production as well as higher content of H₂ (Fig. 5.7 a and b) in the biogas led to a higher H₂ partial pressure in the headspace of T3. The transient lower concentrations were observed on day 4 in T1, T2 and T3, mechanism of which is unclear. Decreasing trend of COD from day 2-10 in T4 was likely due to low activity of microorganisms inside the reactor. The growth of biomass inside the digester was closely related to the composition of headspace (Patra and Yu 2013); an ambient headspace pressure in T4 limited the availability of CO₂ for biomass. After day 4, microbes in all treatments gradually acclimated to the environment resulting in
enhanced hydrolysis and COD production. Decreasing COD generation in T1 from day 9 to the end was likely due to the inhibition by higher concentration of H\textsubscript{2} and less availability of easily degradable substrates; however, this profile was consistent with a conventional batch study (Lehtomäki et al., 2008). However, COD production in treatments with lower headspace pressures increased gradually until reaching another peak production on day 14. Obviously, the appearance of another peak in T2, T3 and T4 were the results of absence of inhibition by H\textsubscript{2} partial pressure and change in the headspace gas composition (Stams and Plugge 2009; Patra and Yu 2013). Patra and Yu (2013) reported that the composition of the headspace biogas and the equilibrium established between the dissolved CO\textsubscript{2} and the partial pressure of CO\textsubscript{2} in the headspace would affect the acidogenic gas and final methane production.

Table 5.3 Statistical analysis of COD production among the four treatments

<table>
<thead>
<tr>
<th></th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>T4</th>
</tr>
</thead>
<tbody>
<tr>
<td>T2</td>
<td>0.016\textsuperscript{a}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T3</td>
<td></td>
<td>0.12</td>
<td>0.91</td>
<td></td>
</tr>
<tr>
<td>T4</td>
<td></td>
<td>0.016</td>
<td>1.00</td>
<td>0.91</td>
</tr>
</tbody>
</table>

\textsuperscript{a} ≤ 0.05 indicates significant difference

Statistical significances among four treatments on COD production are illustrated in Table 5.3. Overall, the differences among four treatments were significant at 0.05 level. With the highest level of headspace pressure in T1, COD production behaviour was significantly different from T2, T3 and T4, especially during the later stage of the experiment. The differences among T2, T3 and T4 were not significant (T2 and T3, \(P=0.91\); T2 and T4, \(P=0.00\); T3 and T4, \(P=0.91\)), indicating the advantage of regulation of headspace pressures for hydrolysis of solid particles. Total COD production followed the order of T3 > T2 > T4 > T1. A cumulative COD
production of 0.59 g/g VS was achieved in treatment T3, which was 36% higher than the yield obtained in T1 with the highest level of headspace pressure.

Soluble nitrogen is released during the degradation of proteinaceous materials. Profiles of TKN and NH$_4^+$-N production are depicted in Fig. 5.4. TKN is the sum of NH$_4^+$-N and organic nitrogen. As presented in Fig. 5.4b, peak production of TKN in T4 was achieved on day 2 while in other three treatments, the peak values were observed between days 7 and 8. The continuous release LBR-gases kept a low pressure that shifted the peak production of TKN to an earlier stage. The production of TKN in all the treatments reached the peak concentrations within the first 3 days, which should be attributed to the leaching of readily degradable proteinaceous substrates. The easily degradable proteinaceous components of food waste could be converted into organic nitrogen and readily solubilized in the leachate during initial period of the hydrolysis (Dong et al., 2009). As is shown in Fig. 5.4a, daily NH$_4^+$-N production in T1 reached peak production on day 5 and then decreased gradually until the end of this experiment and this was in consistent with the profile of COD production. In T4, removal of 100% of headspace biogas led to more ammonia nitrogen production at the beginning of the experiment, whereas in T1 the generation of NH$_4^+$-N was lower than that in T3 despite the opposite relationship between these two treatments was observed in COD collection, indicating regulation of headspace pressure at ~3 psi could promote the degradation of protein. At the same time, the highest concentration of NH$_4^+$-N from day 11 to the end was observed in T4. Furthermore, although COD production in T4 was lower than that of T2 and T3 treatments, daily NH$_4^+$-N production in T4 was the highest among the three treatments with the lower headspace pressure, which indicated that lower headspace pressure was more favourable for the degradation of proteinaceous compounds. After the peak
values, the production of NH$_4^+$-N declined gradually in all four treatments till the end of this experiment, because the continuous dilution of protein in the remaining food waste and a relative increase of carbohydrate concentration would retard the degradation of protein (Breure et al., 1986).

Comparison of TKN with NH$_4^+$-N (Fig. 5.4a) indicated that organic nitrogen represented the dominant fraction of TKN and this was in agreement with previous report by Xu et al. (2011). Overall, the highest leaching of TKN was achieved in T2 (7.4 g/L), corresponding to 17.7% increase compared with T1. Both leaching profiles of NH$_4^+$-N and TKN demonstrated the advantages of regulation of headspace pressure at ~3 psi for protein degradation.

![Fig. 5.4 Ammonia (a) and Total Kjeldahl nitrogen (TKN) (b) leaching during acidogenesis in LBR treating food waste](image)

The concentrations of the TSP in all four treatments increased from the start-up of the experiment to days 9-14 and then decreased till the end of the experiment (Fig. 5.5). Profiles of TSP production were somewhat different from the profiles of COD, because TSP was sourced from soluble COD. Peak production of COD during
the first four days did not give corresponding TSP values, because the solubilisation degree of COD was low and most of the COD was contributed by the fine particulates leached from the LBR. Regulation of headspace pressures largely altered the profiles of TSP production. Between days 1 and 9, T1 achieved the highest TSP production than other treatments. In contrast, TSP production of T2, T3 and T4 were constantly higher than T1 after day 9. It is conceivable that regulation of headspace pressure had changed the regime of TSP production in terms of product spectrum or abundance. Cumulative production of TSP in T3 with ~ 75% of headspace pressure release achieved the highest value of 489.7 g COD/kg VS_{added} due to favourable metabolic environment. An increase of 42.8% in TSP production was achieved in T3 compared with T1 with the highest level of headspace pressure.

![Fig.5.5 Daily (a) and cumulative (b) total soluble products (TSP) production during acidogenesis in LBR treating food waste](image)

The degree of hydrolysis (%) of organic particulates from the substrates in the LBR was calculated by the production of TSP and recovery of COD (TSP/COD). As illustrated in Table 5.2, a hydrolysis degree range from 77.9% to 83.5% was achieved.
among the four LBRs. This range is slightly higher than that of 50-80% achieved in CSTR (Cavinato et al., 2011); and was significantly higher than previous reports obtained using LBRs (Xu et al., 2012). Regulation of headspace pressure at ~3 psi improved the hydrolysis degree; however, the extent was not high, roughly ~7%. It is reasonable to believe that the headspace pressure influenced the rate of hydrolysis resulting in changes in of TSP production.

5.4.3 Metabolic pathways

Concentration and composition of all soluble products including VFAs, alcohols and lactic acid were analysed and are presented in Figs. 5.6 and 5.7. Data on the concentration and composition are used to analyse the possible metabolic pathways prevail in the reactor. Production of TSP and their speciation are closely related to the operation condition, e.g. pH and H₂. As illustrated in Fig. 5.6, distribution of soluble products along the experiment showed that species of soluble products from all four treatments shared the same spectrums; however, the abundances of the individual soluble products were different. Ethanol, propanol, acetic acid, butyric acid and lactic acid were the dominant species among the measured soluble products, which is an indication of the mixed acid fermentation pathway. The reason for the existence of similar metabolic pathways in all the treatments was probably that the variations in hydrogen partial pressure in the headspace of different LBRs was not large enough to induce great differences among treatments. Despite different levels of headspace pressures were set in the four treatments, they exerted little effect on the distribution of metabolic products as reported previously (Clark et al., 2012). It is believed that the H₂ pressure rather than the headspace pressure is the key factor for regulating the metabolic pathways. The
variation in the abundance of different soluble products was a result of different metabolic pathways.

Fig. 5.6 Distribution of soluble products with different levels of headspace pressure
Fig. 5.7 Concentrations (part a) and percentages (part b) of major soluble products, (a), ethanol; (b), acetic acid; (c), butyric acid; (d), propanol; (e), lactic acid
Comparing the treatments, T2-T4 with T1, relatively larger portion of ethanol, propanol and lactate and lower percentage of acetate and butyrate were observed in T1. Coincidentally, ethanol, propanol and lactic acid are reducing compounds, production of which usually associated with the depletion of H$_2$. It was reported that under high hydrogen pressure acetate would be converted to ethanol (Steinbusch et al., 2008; Oh et al., 2003). Accumulation of H$_2$ in the headspace of T1 induced the production of reduced intermediates. Generation of acetate and butyrate is always accompanied by evolution of H$_2$ (Hawkes et al., 2002), and the relatively lower portion of these acids confirmed the inhibition of high H$_2$ partial pressure. Regulation of headspace pressure in T3 resulted in low H$_2$ partial pressure and hence a large portion of acetate and butyrate was observed. The metabolic pathway was mainly influenced by on-site pH condition (Horiuchi et al., 2002). The consistent lower pH value during the initial stage of T1, T2 and T4 reactors may explain the relative higher abundance of lactic acid. Itoh et al., (2012) selectively produced lactic acid by decreasing the culture pH to 3.5 in a chemostat culture containing mixed microbial populations for anaerobic acidogenesis. Likely, the increase of butyrate in T3 was attributed to the relatively higher pH (5.0-6.0), and this is consistent with the study of Horiuchi (2002).

Hydrogen pressure is one of the factors that affect the microbial activity (Harper and Pohland, 1986). Besides, the $P$H2 and pH, the presence of microorganism is another important factor that influences the metabolic pathways. Concentration of propanol in T4 was much higher than that in the other treatments. Propanol is a reducing compound and could be generated through 1, 2-propanediol pathway by shunting carbon flux from glycolysis (Jain and Yan 2011). Relatively higher concentration of propanol in T4 was
probably contributed by the conversion of lactic acid by *Lactobacillus buchneri* (Elferink et al., 2001) under the specific environmental conditions (e.g. pH).

### 5.4.4 Biogas production

Before each sampling of the leachate sample, biogases from both acidogenic reactor and methanogenic reactor were sampled and analyzed for their compositions. Hydrogen, the co-product during hydrolysis/acidogenesis of organic compounds, involving in many principal biochemical reactions, is recognized to exert influence on the overall scheme of waste valorization. Fig. 7.8a shows the profile of daily acidogenic biogas production in acidogenic LBRs. Easily biodegradable carbohydrates are the major substrates for H₂ production and this might explain the overall decreasing trend of biogas along the experiment. Regulation of headspace pressure led to the variations of acidogenic gas production among four treatments. In T1, biogas production was gradually increasing until the peak value on day 8 and decreasing thereafter until the end, whereas the peak production in T2, T3 and T4 were on day 6, 3 and 6, respectively. Sudden decrease of biogas on day 5 in T3 was probably caused by inhibition of high H₂ partial pressure. It was reported that high H₂ pressure was not only an obstacle for hydrolysis but also inhibit generation of H₂ (Wang et al., 2008). Hydrogen in the gas phase of the reactor determined the ratio of oxidized to reduced NAD within the bacteria, which in turn inhibited the metabolic reactions within the bacteria that were coupled to the NAD⁺→NADH redox reaction.

H₂ and CO₂ are the two major components of acidogenic biogas. The partial pressure of H₂ as well the composition of the headspace was reported to influence the gas and CH₄ production. As illustrated in Fig. 5.8 b, H₂ concentration in the biogas was
ranging from 0-60%, and changed greatly along different days of the experimental. Maintaining lower level of headspace pressure increased the concentration of H₂ in T3 and T4 on day 5 and T2-T4 on day 8 whereas constant lower concentration (<40%) of H₂ was observed in T1. Fig. 5.8 c shows the profile of the CO₂ contents in the LBR biogas. In anaerobic digestion system, headspace CO₂ and the dissolved CO₂ would build up an equilibrium that might affect the pH and gas production.

The ratios between H₂ and CO₂ are presented in Fig. 5.8 d. Although, low level of headspace pressure improved the H₂/CO₂ ratio, all values were still lower than 2.0. To maximize the economic feasibility of the anaerobic technology, increase the overall energy recovery through reutilization of the acidogenic biogas is a promising strategy. Reutilization of H₂ and CO₂ in methanogenic phase is favorable for hydrogenotrophic methanogens, a group of high efficient methane producers (Hao et al., 2011). However, the reaction \( 4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \ (\Delta G=\text{-}135 \text{ kJ/mol at STP}) \) requires a H₂/CO₂ ratio of 4, otherwise the extra CO₂ would affect the CH₄ content of the final methane gas. Therefore, there is a great importance of increasing the H₂/CO₂ ratio in the LBR biogas.
Leachates (COD) collected from LBRs were fed to UASBs to generate methane gas (recovery of carbon). CH$_4$ production was mainly due to the degradation of the soluble products of the leachates (COD) and hence the daily CH$_4$ production profile was similar to that of COD (Fig. 5.3 and Fig. 5.9a). A headspace gas pressure of about 3 psi enhanced the production of CH$_4$ and a cumulative production of 284 L/kgVS$_{added}$ was achieved in T3, corresponding to 30.8% increase compared with T1 without release of headspace pressure.
Statistical analysis of CH$_4$ production was presented in Table 5.4. The values of significant levels were generated by SPSS (v19). T3 treatment was significantly different from all other treatments. Highest overall methane production of 284 L/kgVS$_{\text{added}}$ in T3 confirmed the advantage of regulating the headspace pressure in an acidogenic reactor at a headspace pressure of ~3psi. Difference in cumulative CH$_4$ production between T1 and T2 were significant (p<0.05), while the difference between T1 and T4 was not significant. Release of 100% of acidogenic headspace in T4 did not show great advantage on hydrolysis as well as biogas production. A similar situation was reported by Clark et al. (2012), and this was probably due to that lower headspace pressure did not provide enough driving force to remove hydrogen from the liquid, or that the partial pressures reached were not low enough to significantly alter the thermodynamics of hydrogen evolving enzymes.
Table 5.4 Statistical analysis of CH$_4$ among four treatments

<table>
<thead>
<tr>
<th></th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>T4</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T2</td>
<td></td>
<td>.049</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T3</td>
<td>.000$^a$</td>
<td></td>
<td>.007</td>
<td></td>
</tr>
<tr>
<td>T4</td>
<td>1.000</td>
<td></td>
<td>.049</td>
<td>.000</td>
</tr>
</tbody>
</table>

$^a$ ≤ 0.05 indicates significant difference while ≤ 0.01 indicates highly significant difference

5.4.5 Overall performance

The performance of acidogenic-hydrolysis in LBR and overall CH$_4$ yield in UASB under different headspace pressures are presented in Table 5.5. Daily COD and total soluble products production rates were varied with different headspace pressures. Similar COD and TSP production rates were achieved in T2 and T3 with relatively low levels of headspace pressures, i.e. 6.3 and 3.3 psi, respectively. And, both of the rates achieved in T2 and T3 were higher than that in T1 with the highest headspace pressure (12.6 psi), suggesting the inhibitory effect of high headspace pressure. High headspace pressure was likely to be associated with high PH$_2$, which was recognized as the inhibitory factor for H$_2$ production and H$_2$-related acidogenesis (Tanisho et al., 1998; Wang et al., 2008). Compared with T2 and T3, production rates of COD and TSP were also lower in T4 with the ambient pressure, which was probably related to the composition (less CO$_2$ available) of the acidogenic headspace. It was reported that headspace composition of CO$_2$ was necessary for maintaining the growth of biomass inside the digester (Patra and Yu 2013).
The degree of hydrolysis in LBR was calculated based on the production of total soluble products (TSP), while the carbon recovery efficiency was calculated based on the total carbon recovered in terms of CH$_4$ divided by the total carbon of the input food waste. The degrees of hydrolysis in T2 and T3 were 81.6% and 83.5%, respectively, indicating improvement of hydrolysis at lower headspace pressures when compared with T1 and T4. Regulation of acidogenic headspace pressure at ~ 3 psi in T3 achieved the highest carbon recovery efficiency of 46.3%, which means nearly 40-50% of the input carbon was either escaped as CO$_2$ or left in the residual digestate (assuming that 10% of the digested part was used for biomass growth). Furthermore, carbon recovery efficiency could be used as a strategy to evaluate the carbon balance during two-phase AD process.

Table 5.5 General performances of four treatments

<table>
<thead>
<tr>
<th>LBR</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>T4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Set headspace pressure (psi)</td>
<td>12.6</td>
<td>6.3</td>
<td>3.3</td>
<td>0</td>
</tr>
<tr>
<td>COD production rate (g/L·d)</td>
<td>17.1 ± 7.9</td>
<td>21.40 ± 4.1</td>
<td>21.6 ± 4.6</td>
<td>19.6 ± 3.7</td>
</tr>
<tr>
<td>Cumulative COD (g/kg VS$_{added}$)</td>
<td>433.3</td>
<td>573.3</td>
<td>586.7</td>
<td>529.5</td>
</tr>
<tr>
<td>Production rate of soluble products (g COD/L·d)</td>
<td>14.0 ± 6.5</td>
<td>17.3 ± 4.6</td>
<td>17.9 ± 4.7</td>
<td>14.0 ± 3.0</td>
</tr>
<tr>
<td>Cumulative TSP (g COD/kg VS$_{added}$)</td>
<td>342.8</td>
<td>467.8</td>
<td>489.7</td>
<td>375.0</td>
</tr>
<tr>
<td>Hydrolysis degree (%)</td>
<td>79.1</td>
<td>81.6</td>
<td>83.5</td>
<td>77.9</td>
</tr>
<tr>
<td>VS removal (%)</td>
<td>49.0</td>
<td>57.4</td>
<td>60.6</td>
<td>51.3</td>
</tr>
<tr>
<td><strong>UASB</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CH$_4$ production rate (L/d)</td>
<td>7.11</td>
<td>8.87</td>
<td>9.49</td>
<td>7.98</td>
</tr>
<tr>
<td>Cumulative CH$<em>4$ (L/kg VS$</em>{added}$)</td>
<td>217.6</td>
<td>266.0</td>
<td>284.7</td>
<td>240.2</td>
</tr>
<tr>
<td>Carbon recovery (%)</td>
<td>29.5</td>
<td>39.5</td>
<td>46.3</td>
<td>30.6</td>
</tr>
</tbody>
</table>

5.5 Summary

Regulation of headspace pressure in acidogenic LBR resulted in varying levels of H$_2$ partial pressure, which is thought as one of the major factors that not only regulates the distribution of end products of the fermentative bacteria but also determines the
degradation of these fermentation products by H$_2$-producing acetogenic bacteria. Diversion of biogas from LBR enhanced the COD and TSP generation in T2, T3 and T4; whereas, very high pressure (T1) resulted in comparatively higher lactate production and low degradation rate of proteinaceous compounds. A pressure of 3-6 psi (in T2 and T3) improved the production of COD by $\sim$22-36%, soluble products (include VFA and solvents) by $\sim$9-43%, VS reduction by $\sim$14-19%, and CH$_4$ production by $\sim$10-31%; and were significantly higher than T1 reactor. Besides, acetate, butyrate and propanol production were comparatively higher in T2 and T3 reactors while lactate was higher in T1. Mixed acid fermentation prevailed in all the treatments with butyrate as the single major component and it is a good precursor for the subsequent methanogenesis. However, how to maintain a constant H$_2$ partial pressure needs further investigation.
CHAPTER SIX

REGULATION OF ACIDOGENIC METABOLIC PATHWAY
DURING TWO-PHASE ANAEROBIC DIGESTION OF FOOD WASTE: EFFECT OF HEADSPACE H₂ PARTIAL PRESSURE AND COMPOSITION

Abstract

During acidogenic reactions, hydrogen is generated as a co-product, and the hydrogen partial pressure \( (P_{H₂}) \) in the headspace of acidogenic reactor influences the acidogenic fermentation processes and \( H₂ \) evolution. Thus, the acidogenic metabolic pathways could be regulated by adjusting the \( P_{H₂} \). If the \( P_{H₂} \)-metabolite relationship is completely understood, the headspace gas pressure could be used as a tool to alter or adjust the acidogenic products highly suitable for methanogenesis in the second phase reactor. Therefore, in this study, the effects of different levels of headspace \( P_{H₂} \) on the quality of acidogenic leachate and shift of metabolic pathways, as well as \( CH₄ \) recovery in the methanogenic reactor were investigated. Four different levels of \( P_{H₂} \) were set, control without \( P_{H₂} \) regulation (T1), 80% of \( H₂ \) (T2), 50-60% of \( H₂ \) (T3) and 0.04% of \( H₂ \), while the headspace was adjusted to 3.3 psi according to the optimal condition identified in the previous experiment. In addition, \( CO₂ \) was another major component gas increasing the headspace pressure, which also led to varied compositions of headspace among four treatments. Low \( P_{H₂} \) enhanced the general performance of acidogenic reactor in terms of
COD production. In T4, a cumulative COD production of 0.8 g/g VS$_{\text{added}}$ was achieved, corresponding to 39.7% increase compared with the control (T1). A headspace gas composition of H$_2$: CO$_2$ (80:20) resulted in the solubilisation degree up to 83.9%, however, the mechanism was unclear. Although similar spectrums of soluble products were observed in all four treatments, their concentrations were different. Typical butyrate fermentation pathways dominated in T4 whereas mixed acid fermentation pathways were prevailing in the other three treatments. Because of the improved performance of hydrolysis/acidogenesis and higher quality of acidogenic products, overall CH$_4$ recovery in T4 (301.0 L/kg VS$_{\text{added}}$) was 44.6% higher than the control, whereas the values observed in T1, T2 and T3 were 208.1, 238.2 and 208.8 L/kg VS$_{\text{added}}$, respectively. Therefore, during the reutilization of the acidogenic off-gas in UASB, maintaining a low PH$_2$, e.g. 0.04% of H$_2$ facilitates achieving increased hydrolysis and methane production. Higher COD production and a favourable leachate composition are responsible for higher methane recovery.

**6.1 Introduction**

In a two-phase hybrid anaerobic solid - liquid system, the overall efficiency of the process depends mostly on the efficiency of hydrolysis-acidogenesis as well as the intermediate products of the first phase. The production of intermediate products and distribution of products could be affected by metabolic pathways dominated in the acidogenic reactor. Ethanol, acetate, propionate, butyrate and caproate are the most popular components generated in an acidogenic reactor during hydrolysis/ acidogenesis of food waste. Ethanol is a more reduced product with the highest energy value among the acidogenic products (Pipyn and Verstraete, 1981). The observation of Pipyn and
Verstraete (1981) indicated that it might be possible to increase CH₄ production by directing the metabolic pathway towards ethanol and lactic acid production. However, the conversion of ethanol to CH₄ needs its prior oxidation to acetate and this reaction is thermodynamically unfavourable under standard conditions. Moreover, high ethanol concentration was reported to be inhibitory to propionate-degradation and eventually posing toxicity to methanogenesis (Smith and McCarty 1989). But till now the toxic limit for ethanol in different systems was not clear. Ethanol production can also be inhibited by co-existing short-chain fatty acids (Zhang et al., 2010).

Acetate is recognized as a key intermediate product in the final step of mineralization of organic materials (Scholten and Conrad 2000), production of which is generally increased along the reaction time. Acetate is not only sourced from degradation of pyruvate through acetyl-CoA pathway directly, but also can be produced from the syntrophic oxidation of propionate and butyrate (Müller et al., 2010). It is widely accepted that acetate is the major intermediate during the bioconversion of organic compounds to CH₄ and CO₂, and acetate represents the substrate for ~ 70% of the total CH₄ produced during AD (Gujer et al., 1983; Smith and Mah 1966). Thus, the recovery of CH₄ from acetate is an important step in the AD process. Generally, acetate formed via fermentation of sugars and amino acids or oxidation of VFAs is converted to CH₄ and CO₂ by aceticlastic methanogens (Jones et al., 1987; Mah et al., 1978). However, methanization of acetate can also be processed by co-function of acetate-oxidizers and hydrogenotrophic methanogens (Hao et al., 2011). Thus, the kinetics of acetate utilization in methanogenic phase would depend on the predominant species in the population of aceticlastic/hydrogenotrophic methanogens.
Methanization of butyrate and propionate occurs through their oxidation to acetate by \( \text{H}_2 \)-producing acetogens; and subsequent use of acetate by aceticlastic methanogens. The acetogens require a low \( \text{PH}_2 \) and the reactions are endergonic; therefore must be syntrophically coupled with hydrogenotrophic methanogenesis. Butyrate was reported as the most favourable substrate for methanogens due to its higher efficiency relative to other VFAs (Öztürk 1991; Wang et al., 1999).

In the complex AD system, the distribution of metabolic intermediates could be affected by many factors, e.g. pH, sources of inoculum, substrate types and headspace \( \text{PH}_2 \). Here the \( \text{PH}_2 \) and the corresponding composition of headspace are discussed due to their crucial role as well as the great potential to regulate the metabolic pathways.

\( \text{PH}_2 \) in the acidogenic phase is one of the key factors affecting the metabolic pathways and \( \text{H}_2 \) production. However, many controversial observations regarding the influence of \( \text{H}_2 \) pressure on the anaerobic breakdown of saccharides has been reported (Ruzicka 1996).

The production of \( \text{H}_2 \) often involves the process of oxidation of reduced ferredoxin (Fd) and release of electrons as molecular \( \text{H}_2 \), the reaction of which is reversible and depends on the level of \( \text{PH}_2 \), suggesting that \( \text{H}_2 \) yield is significantly influenced by \( \text{PH}_2 \). Therefore, it is important to regulate \( \text{PH}_2 \) in acidogenic phase for enhancement of \( \text{H}_2 \) yield.

The effects of \( \text{PH}_2 \) on the metabolism and the fermentative pattern of the anaerobic microbes have been investigated by researchers. It was observed that \textit{Clostridium cellubioarum} could produce more \( \text{H}_2 \) when \( \text{H}_2 \) is removed by \( \text{H}_2 \)-consumers or other strategies (Chung, 1976). \( \text{PH}_2 \) determines the quantitative composition of the intermediate products. Kim et al. (2006a) demonstrated that external sparging \( \text{CO}_2 \) to a
CSTR increased the rate of butyrate production and specific H$_2$ production. The production of H$_2$ and butyrate has been considered an obstacle for ethanol production (Liu et al., 2006) and stirring the batch cultures favoured H$_2$ and butyrate production, which should be attributed to the accumulation of H$_2$ in unstirred conditions inhibiting butyrate production.

Composition of headspace gas is another potential factor affecting the performance of acidogenesis. Gas sparging was reported to be an effective strategy to enhance H$_2$ production, due to the decrease of $P_{H_2}$ by external gas sparging (Kim et al., 2006a). CO$_2$ and N$_2$ are the gases commonly selected as anaerobic sparging gas; however, the CO$_2$ was more effective than with N$_2$ in both H$_2$ and CH$_4$ production (Kim et al., 2006a; Patra and Yu 2013). One possible reason is that high CO$_2$ partial pressure had little effect on H$_2$-producing bacteria, but exhibited an inhibitory effect on other microorganisms such as acetogens and lactic acid bacteria which were competitive with H$_2$-producing bacteria (Kim et al., 2006a). Misoph and Drake (1996) had observed that under CO$_2$-limited conditions, acetogenesis of fructose yielded high levels of lactic acid as a reduced product; similarly, in the absence of supplementary CO$_2$, xylose-dependent acidogenesis yielded lactate and succinate as major reduced end products. High level of reduced products would certainly decrease the production of H$_2$.

It is logical that an equilibrium is established between the dissolved CO$_2$ in the media and the partial pressure of CO$_2$ in headspace gas (Alford, 1976), and that a higher CO$_2$ concentration in the headspace would result in a greater dissolved CO$_2$ concentration in the media. The higher concentration of CO$_2$ would stimulate the process of H$_2$-CO$_2$ based homoacetogenesis and consequently decrease the yields of H$_2$ (Park et al., 2005;
Patra and Yu 2013). Furthermore, headspace CO\textsubscript{2} composition had a marginal effect on pH and dominant metabolic pathway; however, it did pose some effect on the activity and growth of biomass (Patra and Yu 2013; Bru et al., 2012; Misoph and Drake 1996).

Therefore, this experiment aims at investigating the metabolic pathways as well as the speciations of major soluble products under the regulation of headspace \( PH_2 \). The performance of acidogenic reactor and thermodynamics of dominant intermediates as well as the overall energy recovery from the two-phase AD system was also evaluated.

6.2 Experimental design

In order to investigate the effect of headspace partial pressure of H\textsubscript{2} (\( PH_2 \)) on fermentation pattern and quality of liquid products, four levels of H\textsubscript{2} partial were set up (Table 6.1).

<table>
<thead>
<tr>
<th>H\textsubscript{2} pressure (psi)</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1 3.3</td>
<td>Optimal pressure obtained in Chapter 5</td>
</tr>
<tr>
<td>T2 3.3 \times 80%</td>
<td>Stoichiometry for CH\textsubscript{4} production ( 4H_2 + CO_2 \rightarrow CH_4 + 2H_2O )</td>
</tr>
<tr>
<td>T3 3.3 \times 60%</td>
<td>Reported threshold\textsuperscript{a} value for H\textsubscript{2} inhibition; CO\textsubscript{2} was added as balance gas</td>
</tr>
<tr>
<td>T4 3.3 \times 0.04%</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} Fukuzaki et al., 1990

6.3 Materials and methodology

6.3.1 Substrate and inoculum

Synthetic food waste prepared as previously mentioned in Chapter 3 was used as substrate. Anaerobically digested sludge with 3.2 % total solids (TS) and 53.3 % VS/TS
obtained from Shek Wu Hui wastewater treatment plant was used as seed.

6.3.2 Experimental set-up

Hybrid liquid-solid two-phase AD system, LBR-UASB (Fig. 3.1) was chosen as the configuration. Online pressure sensor was installed on the cap of each LBR and a computer with data log (AIDCS Monitor) was linked to the system for real-time record of the conditions. The temperature was kept at 35 ± 1 °C.

To ensure the hydrogen partial pressure ($P_{H_2}$) levels in different LBRs, mixed gases with designed $P_{H_2}$, i.e. 80%, 60% and 0.04% of $H_2$ balanced in $CO_2$. The pre-mixed gas containing $H_2/CO_2$ at 80/20 and 60/40 was pumped into the headspace of LBR after each sampling to a pressure of 3.3 psi. Then headspace pressure was controlled by gas-flushing at 10 mL/min during the whole experimental period. The basic operation of reactor packing, ratios of inoculum and bulking agent, water regimes and sampling frequency follow the same procedure as described in Chapter 4.

6.3.3 Analytical methods

General parameters for characterization of food waste and AD sludge, such as TS and VS, TOC and TKN were analyzed following the procedures as detailed in Chapter 3. For the leachate collected from LBRs, parameters such as volume, pH value, COD were analyzed before filtration. After filtration of leachate with 0.45 µm cellulose acetate membrane, soluble products (except lactic acid) were analyzed by a HP 6890 Series gas chromatograph (GC, Hewlett Packard) with FID while lactic acid was analyzed using a HPLC (Waters Alliance 2695) coupled with Waters 2996 Photo Diode Array (PDA) detector and equipped with an Ultrasphere® ODS Column C-18 column (10 µm, 25 cm x 4.6 mm i.d.) (Beckman Coulter, USA). The preparation of mobile phase and online
analytical method for lactic acid followed the protocols of Violeta et al. (2010). The filtrate was also used for glucose analysis following the colorimetric method for reducing sugars (Dubois et al., 1956).

Both acidogenic biogas from LBR and methanogenic biogas from UASB were collected in gasbags and a 1-mL sample drawn using pressure lock syringe and locked immediately with the locker and septum. For methanogenic biogas collection, the gasbag (large enough to avoid vacuum) was connected to the outlet of wet gas flow meter (BSD-0.5, Shang hai), which was used to record the volume of the flow through gas. Both acidogenic and methanogenic biogas was analyzed using a gas chromatograph (HP7890) equipped with TCD and PLOT-Q column (30 m × 0.53 mm × 15 µm). Temperature programs and other online analytic methods were the same as described in Chapter 3.

6.3.4 Thermodynamic analysis

The feasibility of biochemical reactions and their directions largely depend on the thermodynamic conditions. Negative values of the Gibbs free energies (ΔG) indicate the potential for the forward reaction. In this experiment, the thermodynamic feasibility of major components in soluble products, i.e. acetate and butyrate were evaluated by ΔG. ΔG of acetate and butyrate during acidogenesis were calculated from the corresponding standard Gibbs free energies (ΔG°) and the actual concentrations of reactants and products using the Nernst and Van’t Hoff equations. Values of ΔG° were calculated from the standard Gibbs energies of formation (Liu et al., 2008) using the reactions under standard ambient temperature and pressure (1 M, 105 Pa, 298.14 K, pH = 7), as shown in Table 6.2. The following assumption was made for the calculation of
acetate formation, 1) the acetate was produced mainly through acetyl-CoA pathway, 2) the β oxidation of LCFA to acetate was less and not taken into account.

Table 6.2 Standard free energy and equations of acetate and butyrate production

<table>
<thead>
<tr>
<th>Reaction</th>
<th>ΔG◦ (kJ) at 25 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>C₆H₁₂O₆ + 2H₂O → 4H₂ + 2CH₃COOH + 2CO₂</td>
<td>-206</td>
</tr>
<tr>
<td>C₆H₁₂O₆ + 2H₂O → 2H₂ + CH₃CH₂CH₂COOH + 2CO₂</td>
<td>-254</td>
</tr>
</tbody>
</table>

6.3.5 Q-PCR

Leachate and digestate samples were collected on days 3, 8 and 17 for DNA extraction. The DNA extraction procedure was referred to description in Chapter 3. To analyze the butyrate production pathway from butyryl-CoA: acetate CoA-transferase route, quantitative PCR (qPCR) assays targeting butyryl-CoA: acetate CoA-transferase genes (but) and butyrate kinase gene (buk) were conducted following the methods described by Hippe et al. (2011) and Vital et al. (2013), respectively. Degenerated primers encoding both pathways were listed in Table 3.3. The but-specific PCR primers BCoATscrF/R were used to amplify a 530-bp fragment of but, whereas the buk primers were encoding 500-bp sequence.

Q-PCR was performed using a Stratagene Mx3000p™ Real-Time PCR System. 40 ng of DNA was used as the template in a 20 μL PCR mixture containing 10 μL iQ™ SYBR® Green Supermix (Bio-Rad), 0.4 μM (final concentration in the mixture) of each primer. The thermal cycle parameters were followed the references of Hippe et al. (2011) and Vital et al. (2013). Standard curve was obtained as described in previous study (Yan et al., 2014b). All amplifications were performed in duplicate with both negative and positive controls.
6.4 Results and discussion

6.4.1. General performance of the digesters

*Leachability and pH*

Good leachability were observed in all the four treatments with leachate volumes ranging from 1710 mL to 2320 mL (Fig. 6.1a). The increasing trends of leachate production along the experimental time in all treatments should be attributable to the water release during hydrolysis of organic compounds. The best performance was observed in T4 ($PH_2$ of $3.3 \times 0.04\%$ psi), in which constant higher volume of leachate production was achieved from day 5 to end of this experiment, suggesting low $PH_2$ was favourable for leachate production. The highest volume of leachate collected from H$_2$-80% during the first 4 days indicated favourable $PH_2$; however the advantage was lost after day 5 and mechanism of which is unclear.

Fig. 6.1 Volume (a) and pH (b) of leachate collected from LBRs
During anaerobic digestion of food waste, pH is a reflection of the system’s acidity and alkalinity. As is shown in Fig. 6.1 (b), pH in all four treatments were lower than 5.0 during most of the experimental period. Although, a pH range of 4.6-5.2 was reported to be favourable for anaerobic hydrolysis (Kim et al., 2006b; Chen et al., 2007), constant low pH observed in this experiment should be attributable to the rapid accumulation of fatty acids, which would also affect the metabolic pathways and microbial activities. Differences among four treatments were not significant, and the pH were increasing along the experiment, due to the supplementation of alkali agent and dilution of fatty acids. However, the reasons for the low pH values on day 11 in all four treatments were not clear. Thereafter, the pH recovered to ~5.0 in all treatments. Headspace CO₂ composition had little effect on the variation of pH, which is consistent with previous study that pH was influenced by the concentration of bicarbonate rather than the composition of CO₂ in the headspace (Patra and Yu 2013).

![Graph](image)

**Fig. 6.2 Daily (a) and cumulative COD (b) leached from LBRs with different headspace H₂ partial pressure**
COD production represents the liquefaction of carbohydrates, proteins and lipids. As illustrated in Fig. 6.2, similar COD leaching profiles were observed among the four treatments with maximum COD concentration of 32.7-45.2 g/L was noticed on day 4. COD production during the first half of the batch experiment was much higher than the later half, which was similar to the report of Cirne et al. (2007), who observed higher solubilisation rates within the first 10 days during the digestion of sugar beet and grass/clover under low pH. Demirer and Chen (2008) also observed high initial COD during the anaerobic biogasification of undiluted dairy manure in LBRs. These COD levels, however, decreased after the peak production, probably due to conversion of the simple soluble intermediates to gaseous products, leachate production, which removed the accumulated products and water addition, which diluted the concentration of the remaining soluble products coupled with the depletion of easily biodegradable organic matter in the food waste. Comparatively, higher COD generation was achieved in Treatment 4 (PH₂ of 0.04%), indicating a H₂ partial pressure of 1.4E–3 psi is favourable for leaching of COD. The reaction rate of degradation is faster than the rate of substrate uptake in the reactor and the system cannot cope with further generation of NADH and thus inhibit the process of biohydrolysis (Ruzicka 1996). Further, higher CO₂ concentration in the headspace of T4 would increase the activity of biomass inside the reactor and thus enhance the production of COD (Misoph and Drake 1996).

Cumulative COD production in treatment with headspace PH₂ of 3.3psi ×0.04% was the highest, i.e. 0.84 g/gVS added; whereas, in the other two treatments and the control, cumulative COD production was similar, in the range of 0.60 - 0.67 g/gVS added,
suggesting that high H₂ partial in the headspace had an adverse effect on the solubilization of organic solids.

Fig. 6.3 illustrates the total soluble products (TSP leaching profiles. TSP accounted for significant fractions of the COD in all reactors, 68.1%, 83.9%, 74.4% and 68.3% in T1, T2, T3 and T4, respectively. The trend of TSP production was gradually increased until day 13; unlike previous experiment (Chapter 5) that the trend did not follow that of COD, probably due to the variation of the solubilization degree of COD. Despite peak production of COD was observed in all the treatments during the first week, gradually increased TSP concentrations were much lower than corresponding COD values. Possible explanation is that COD production was mostly contributed by leaching of fine particles and easily biodegradable carbohydrates at the starting period and this
behaviour lasted for about one week, then the TSP representation of the COD became significant.

$PH_2$ in the headspace was reported to alter the metabolic pathways (McIntyre and McNeil 1998) and change the distributions and abundances of intermediate products, such as acetate and butyrate. Lowest daily TSP production in the control should be attributed to $H_2$ pressure inhibition as well as the shift of metabolic pathway under such headspace $PH_2$, whereas recovery of the highest TSP in treatment with headspace $H_2$ pressure of $10^{-4}$ bar might due to the depletion of $H_2$ based inhibition and enhanced microbial activity under high concentration of CO$_2$. From day 3 to 9, daily generation of TSP in treatment T2 was the highest among the four treatments and this definitely contributed to the cumulative production of TSP, which might be associated with the provided headspace $H_2$ pressure; however, the mechanism of increased solubilization degree with a $H_2$ pressure as high as 80% was still unclear.

6.4.2 Metabolic pathways and distribution of soluble metabolites

Metabolic pathways were the comprehensive results of substrates, microbes and environmental conditions. As illustrated in Fig. 6.4 and Fig. 6.5, species of soluble products from the four treatments shared the same spectrums (Fig. 6.4); however, the abundances of the individual soluble products were different. Butyrate was the single largest component in all treatments, indicating butyrate type fermentation. Similarly, Zoetemeyer et al. (1982) and Cohen et al. (1979) found that the principal VFA in anaerobic acidogenic reactors was butyrate. Varying the $PH_2$ and compositions of acidogenic headspace did not significantly change the metabolic pathway, and this was in consistent with previous report that only a small increase of butyrate concentration was
observed in the N₂ sparging conditions comparing with non-sparging conditions and there was no major shift in metabolic pathways (Mizuno et al., 2000). Likely, Patra and Yu (2013) observed that profiles of VFA were not affected by headspace gas composition, except for the acetate-to-propionate ratio, which showed a quadratic relation ($P < 0.05$) with increasing concentrations of CO₂ in the headspace. And similarly, Kim et al. (2006a) achieved high H₂ evolution with butyrate as dominant product under CO₂ sparging condition. However, it is convincing that regulation of headspace pH₂ is one of the strategies to enhance biorefinery of target product from acidogenesis.

Distributions and abundances of soluble metabolites were illustrated in Figs. 6.4 and 6.5. Determination of the VFA species in the anaerobic treatment is important, since it provides important information regarding the metabolic pathway of the process. Generally, metabolic pathways prevailed in all four LBRs were similar; butyrate-type fermentation was the major pathway, taking 35-60% of the TSP generation. Degradation of butyrate for methane generation was the fastest one among volatile fatty acids (Öztürk 1991; Wang et al., 1999) and also it is advantageous in terms of carbon recovery (Equation (1)-(5)).

\[
\begin{align*}
C_6H_{12}O_6 + 2H_2O & \rightarrow CH_3(CH_2)_2COO^- + 2HCO_3^- + 2H_2 + 3H^+ & \Delta G = -254.4 \text{ kJ/mol} & \text{Eq. 6.1} \\
C_6H_{12}O_6 + 4H_2O & \rightarrow 4H_2 + 2CH_3COO^- + 2HCO_3^- + 4H^+ & \Delta G = -206.3 \text{ kJ/mol} & \text{Eq. 6.2} \\
CH_3CH_2COOH + 3H_2O & \rightarrow CH_3COOH + HCO_3^- + 3H_2 & \Delta G = 76.1 \text{ kJ/mol} & \text{Eq. 6.3} \\
\end{align*}
\]

CH₃COOH → CH₄ + CO₂
\[ \Delta G = -31.0 \text{ kJ/mol} \quad \text{Eq. 6.4} \]

\[
\text{CH}_3\text{CH}_2\text{CH}_2\text{COOH} + 2\text{H}_2\text{O} \rightarrow 2\text{H}_2 + 2\text{CH}_3\text{COOH} \\
\Delta G = 48.1 \text{ kJ/mol} \quad \text{Eq. 6.5} 
\]

Butyrate is one of the most popular intermediate products during bioconversion of organic matter to CH\(_4\) and CO\(_2\). Compared with the control, an obvious increase of butyrate production in T4 was observed, which is similar to the observation that sparging with N\(_2\) had increased the butyrate significantly (Mizuno et al., 2000). As illustrated in equation (1), H\(_2\) is the by-product during the generation of butyrate and the behaviour of butyrate metabolism would be regulated by H\(_2\) pressure. In the absence of methanogens in the LBR due to low pH, the accumulation of the H\(_2\) produced in the LBR would inhibit the process of hydrolysis and acidogenesis as reported by Ruzicka (1996) that under most circumstances the system cannot cope with exceeding concentrations of NADH and led to inhibition of the process. Decreasing abundances of butyrate in the later phase of this experiment in the control, T2 and T3 clearly indicated the inhibition effect of headspace \(PH_2\), whereas the relatively large quantity of butyrate combined with increasing abundance showed that H\(_2\) partial pressure as low as \(10^{-4}\) bar was favorable for butyrate production. The butyrate fraction dramatically increased from day 5 in all treatments except T2 with \(PH_2\) of 3.3 psi \(\times\) 80\%, however, the reason was unclear. The subsequent step is to feed these intermediate products to methanogenic reactor for CH\(_4\) generation. The degradation of butyrate, and longer chain volatile fatty acids (LCFA) involves two groups of bacteria, the obligatory hydrogen-producing acetogenic bacteria oxidizing the LCFA and the methane-producing bacteria utilizing the produced acetate and H\(_2\) (Scholten and Conrad 2000; Bryant et al., 1977; McInerney and Bryant 1981). Owing to
the unfavourable thermodynamics of fatty acid oxidation under standard conditions, the metabolism of the acetogenic bacteria demands a low PH2 and requires a H2-utilizing reaction such as hydrogenotrophic methanogenesis (Bryant 1979). The concentration of acetate and, hence, the activity of the aceticlastic methanogenic bacteria, have also been shown to influence the degradation of fatty acids, although relatively high concentrations of acetate (more than 80 mM) are needed to cause total inhibition (Kaspar and Wuhrmann 1978).

Ethanol was dominant during the initial stages of all four treatments (Figs. 6.4 and 6.5). Ethanol is a more reduced intermediate product with high energy value (Pipyn and Verstraete, 1981), production of which might be related to low pH, as observed in the early stage of this experiment. Although, ethanol is widely known as a reducing compound; it was reported that ethanol had little effect on the generation of H2 (Hwang et al., 2004), which also explain the reason for similar distribution and concentration of ethanol in all treatments under varying PH2 levels. Hydrolysis of food waste can go through an alcoholic fermentation and a subsequent conversion of ethanol into H2 and acetate before CH4 gas production starts. Observation of Pipyn and Verstraete (1981) indicated that it might be possible to increase CH4 production by directing the metabolic pathway towards ethanol and lactic acid fermentation. However, the toxic effect of ethanol towards both acidogens and methanogens was not clear.

Lactic acid is the common acidogenic product during anaerobic fermentation of organic solids under low pH (Zhang et al., 2007). Itoh et al. (2012) investigated selective production of lactic acid by decreasing culture pH to 3.5 in a chemostat culture containing mixed microbes for anaerobic acidogenesis. Lactic acid is a reduced
compound, generation of which usually accompanied by the depletion of H₂. Obviously, higher \( \text{PH}_2 \) in T1 and T3 induced the production of lactic acid while the lower amount of lactic acid observed in T2, could due to the combined effects of \( \text{PH}_2 \) and pH.

Presence of caproic acid was often associated with higher organic loading. Parawira et al. (2004) reported that the detection time of caproic acid was 9 h and 7 h after digestion with an organic loading rate of 500 g and 1000 g of potato wastes. Valeric acid and caproic acids are mainly associated with the fermentation of proteins and they could be formed via reductive deamination of single amino acids or by oxidation - reduction between pairs of amino acids via the Stickland reaction (Lata et al., 2002; McInerney 1988). Obviously, the presence of caproic acid at later stages in this experiment was not due to overloading of food waste, probably related to the headspace \( \text{PH}_2 \) as lowest amount was produced in T4 with 3.3 psi × 0.04% of H₂. Another pathway of synthesis of caproic acid is chain elongation. Agler et al. (2014) had observed that when the acetoclastic methanogenesis was inhibited by pH control, n-caproic acid was produced. Ethanol was supplemented to the system to promote chain elongation, which is a pathway in which short-chain carboxylic acids are elongated sequentially into medium-chain carboxylic acids with two-carbon units derived from ethanol. The reactor microbiome developed accordingly with the terminal process catalyzed by chain-elongating bacteria. Chain-elongation pathway for production of caproic acid might occur in this experiment; however the influencing factors were not clear.

6.4.3 Functional gene analysis

Butyrate-producing metabolic pathway is widely distributed in anaerobic digesters of organic solid wastes, especially with those pathways associated with H₂
production (Guo et al., 2014). The butyrate-producing bacteria represent a functional group rather than a phylogenetic group, within the community of anaerobes. To evaluate the dynamics of butyrate kinase and butyryl-CoA: acetate CoA-transferase genes in response to the shift of metabolic pathways under the regulation of headspace $P_{H_2}$, the abundances of both the potential functional groups were analyzed by qPCR. Primers targeting the functional genes $buk$ encoding butyrate kinase and $but$ encoding butyryl-CoA: acetate CoA transferase, enzymes unique to butyrate kinase and $but$ encoding butyryl-CoA: acetate CoA transferase pathways, respectively, were used as potential biomarkers for the butyrate-producing pathways in this study. As shown in Fig. 6.6, there was a remarkable increase in the $buk$ gene abundance in LBR-T4 and LBR-T1 of leachate samples from day 3 to day 8 (e.g. from $4.3 \times 10^3$ to $4.5 \times 10^2$ copies/mL-DNA in LBR-T4), whereas only slightly decrease in LBR-T2 and LBR-T3 were observed. The changes of $buk$ abundances of the solid digestate were not as significant as in the leachate samples (Fig. 6.6b). Generally, copy numbers of $but$ gene were much higher than $buk$ gene in both leachate and digestate samples, indicating the dominant contribution of butyryl-CoA: acetate CoA-transferase pathway to butyrate production. However, changes of $but$ abundances among treatments were not significant, except the increased abundance in 8-T4. Expression of gene $but$ in digeatate samples showed similar level with the leachate samples, but were more stable along the whole experiment.

In acidogenic reactor, the dynamics of butyrate-producing bacteria encoded by $buk$ and $but$-genes could be influenced by operational conditions and headspace $P_{H_2}$. Variations of population of butyrate-producing bacteria (butyrate kinase pathway) among the four treatments at the very beginning should be primarily due to the external sparging
of acidogenic headspaces. The lowest abundance of \textit{buk} was observed without sparging of the acidogenic headspace. It was reported by Kim et al. (2006a) that sparging the acidogenic headspace with external gas could enhance \textit{H}_2 production as well as butyrate. General decreasing trend of \textit{buk}-gene from day 8 to day 17 in each treatment should be related to the varied environmental conditions and less availability of easily degradable substrates. Hydrolysis of organic compounds will generate a series of fatty acids, which could alter the environmental conditions, e.g. lowered the pH to a range unfavorable for butyrate production. However, evidenced from the abundances of the specific genes targeting butyrate-producing pathways, butyryl-CoA: acetate CoA-transferase pathway (\textit{but}) was more dominant than butyrate kinase (\textit{buk}), which is similar to the findings of Vital et al. (2013). Increased amount of copy numbers of but of 8-T4 and 17-T4 should be related to the \textit{PH}_2 regulation at 3.3 psi \times 0.04%. Butyrate production was always linked with \textit{H}_2 evolution, and hence low level of \textit{PH}_2 was benefit for butyrate production (Guo et al., 2014). In addition, as showed in Fig. 6.6 b and d, both pathways were present at average levels of corresponding leachate samples without much change among treatments and sampling points.

In general, the abundances of the butyrate-producing pathways indicated by the q-PCR were in agreement with the pathways reflected by distribution of major soluble products (Fig. 6.5). The quantative difference between the \textit{buk} and \textit{but} targeting pathways were most likely induced by their variations of sensitivities and specificities. Furthermore, functional gene encoding but was investigate as a potential biomarker for butyrate-producing pathway; however relative information is limited and the expression
levels of functional gene might be affected by the survival conditions. Therefore, the abundances of genes might not fully indicate the density or activity of microbes.

Fig. 6.6 Functional genes analysis of butyrate-producing pathways, (a) butyrate kinase (*buk*), and (b) butyryl-CoA: acetate CoA-transferase (*but*). T1, T2, T3 and T4 represent the treatments, while 3, 8 and 17 represents the sampling points.
Fig. 6.4 Distributions of soluble products in LBRs with varying $PH_2$
Fig. 6.5 Concentration (i) and percentage (ii) of major soluble products leached from LBRs with different $PH_2$
6.4.4 Thermodynamics

Fig. 6.7 Gibbs free energies (ΔG) of acetate (a) and butyrate (b) formation from glucose calculated under the actual conditions of designed PH₂ in different LBR reactors.

Changes of Gibbs free energy during formation of acetate (Fig. 6.7 a) and butyrate (Fig. 6.7 b) from glucose during acidogenesis were illustrated in Fig. 6.6. Regarding the production of acetate, the Gibbs free energy in T4 with 10⁻⁴ bar of H₂ was maintained at about -260 kJ/mol to -250 kJ/mol, whereas values ranged from -210 kJ/mol to -170 kJ/mol in the H₂-60%, H₂-80% treatments and the control. The values of ΔG during the formation of acetate were low enough to take place in the forward direction. However, the ΔG in all four treatments increased along the experimental time, indicating decreasing favorable conditions. Highly negative ΔG was observed in T4 (H₂-10⁻⁴ bar) compared with the other three treatments, showing that low PH₂ was at least one of the factors affecting the formation of acetate. Similarly, Abreu’s et al. (2012) reported that acetate
formation was strictly related to the level of $PH_2$. Butyrate was the most abundant soluble products in the leachate. Basically, formation of butyrate under all four levels of $PH_2$ was favorable due to the low $\Delta G$ ranging from -280 kJ/mol to -220 kJ/mol as shown in Fig. 6.7 b. Likely, low $PH_2$ in T4 led to more favorable thermodynamic condition for the formation of butyrate while the forward potentials were decreasing along the time course in all treatments. Although, $H_2$-60%, $H_2$-80% and the control grouped together in Fig. 6.7 b, $H_2$-80% was a bit more favorable for butyrate production.

6.4.5 Recovery of methane gas

Methane generation was resulted from bioconversion of leachate from acidogenic reactor and hence the values were highly related to organic content (in terms of COD) and the quality of leachate. As is shown in Fig. 6.8 a the profiles of daily $CH_4$ generation matched with that of COD, e.g. peak production of COD on day 4 led to peak evolution of $CH_4$. Quality of leachate fed to methanogenic reactor is another reason causing the fluctuation of $CH_4$ production, principally due to the inhibition and energetic feasibilities of specific compounds. Butyrate was reported as the fastest precursor among propionate, acetate and valeric acid for $CH_4$ generation, whereas further utilization of acetate is usually inhibited by elevated $H_2$ pressure (Wang et al., 1999). As illustrated in Fig. 6.8 b, the variations of the methane contents among the four treatments were minor, which should be explained by the following two reasons, 1) the shifts of the metabolic pathway among four treatments were minor, and were not sufficient to cause great variations on leachate qualities; and 2) the experiment was performed in batch mode and the cumulative effect of leachate was last only for 18 days, while the UASB had capability to mask the effect caused by the differences in the leachate quality.
Cumulative CH$_4$ production, as shown in Fig. 6.8 (c), indicates that when compared with control 53% increase was achieved in T4 treatment with headspace H$_2$ pressure of 3.3 psi $\times$ 0.04%. It was still not clear why the generation of CH$_4$ in treatment T2 with 80% headspace H$_2$ pressure was higher than that in treatment with 60% H$_2$ partial pressure. T3 and the control had yielded almost similar amount of CH$_4$.

Fig. 6.8 Daily CH$_4$ (a), concentration of CH$_4$ (b) and cumulative CH$_4$ (C) in UASBs treating different food waste leachates

Besides the contribution of acidogenic leachate, methane production in methanogenic reactor was also contributed by the diverted acidogenic biogas from acidogenic reactor. Unquestionably, $PH_2$ is one of the key factors that regulate the production of H$_2$, because $PH_2$ itself determines the forward direction of the biochemical reaction for H$_2$ generation. The removal of headspace CO$_2$ should have increased the
residual NADH under low PH$_2$, which was expected to give an increased H$_2$ production (Mizuno et al., 2000). However, in this experiment, low PH$_2$ in T4 was balanced with CO$_2$, and hence no significant increase of H$_2$ was observed, suggesting that the composition of headspace also imposes important effect on performance of anaerobic digesters.

6.4.6 Overall Performance

Table 6.3 General performance of four treatments used in this experiment

<table>
<thead>
<tr>
<th>Items</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>T4</th>
</tr>
</thead>
<tbody>
<tr>
<td>PH$_2$ (psi)</td>
<td>3.3</td>
<td>3.3 × 80%</td>
<td>3.3 × 60%</td>
<td>3.3 × 0.04%</td>
</tr>
<tr>
<td>COD production rate (g/d)</td>
<td>23.1</td>
<td>25.1</td>
<td>25.6</td>
<td>32.2</td>
</tr>
<tr>
<td>Cumulative COD (g/kg VS$_{added}$)</td>
<td>601.6</td>
<td>654.3</td>
<td>668.7</td>
<td>840.5</td>
</tr>
<tr>
<td>Production rate of TSP (g COD/d)</td>
<td>15.7</td>
<td>21.1</td>
<td>19.1</td>
<td>22.0</td>
</tr>
<tr>
<td>Cumulative production of TSP (g COD/kg VS$_{added}$)</td>
<td>409.7</td>
<td>549.1</td>
<td>497.7</td>
<td>574.2</td>
</tr>
<tr>
<td>H$_2$ production rate (L/d)</td>
<td>2.1</td>
<td>3.0</td>
<td>0.7</td>
<td>0.4</td>
</tr>
<tr>
<td>Cumulative H$_2$ production (L)</td>
<td>34.8</td>
<td>50.3</td>
<td>12.2</td>
<td>6.6</td>
</tr>
<tr>
<td>CH$_4$ production rate (L/d)</td>
<td>11.6</td>
<td>13.4</td>
<td>11.8</td>
<td>16.7</td>
</tr>
<tr>
<td>Specific CH$<em>4$ (L/kg VS$</em>{added}$)</td>
<td>208.1</td>
<td>301.0</td>
<td>242.0</td>
<td>212.3</td>
</tr>
<tr>
<td>TS removal (%)</td>
<td>59.7</td>
<td>64.0</td>
<td>62.2</td>
<td>68.4</td>
</tr>
<tr>
<td>VS removal (%)</td>
<td>59.3</td>
<td>62.8</td>
<td>61.8</td>
<td>68.2</td>
</tr>
<tr>
<td>Carbon recovery (%)</td>
<td>37.9</td>
<td>41.5</td>
<td>42.1</td>
<td>40.5</td>
</tr>
</tbody>
</table>

Generally, performances of all four treatments were quite well in this experiment, both in COD production and final CH$_4$ recovery. Regulation of acidogenic headspace PH$_2$ in T2, T3 and T4 increased the production of COD and soluble products compared with the control (Table 6.3). Unexpectedly, H$_2$ production in T3 and T4 with low levels of PH$_2$ was much lower, due to the effect of headspace composition. Thus, it can be seen that H$_2$ production was not only regulated by PH$_2$ but also the composition of headspace.
However, CH$_4$ production rate of 16.72 L/d (in UASB) and also higher TS and VS removal efficiencies (in LBR) in T4 confirmed the overall advantage of low level of PH$_2$.

6.5 Conclusion

High headspace H$_2$ partial pressure of 60% (T3), 80% (T2) and self-produced H$_2$ (Control) had inhibition effect on hydrolysis in acidogenic reactor in terms of COD generation and distribution of major soluble products. Low level of PH$_2$ and high concentration of CO$_2$ in the headspace of T4 demonstrated the best performance with the most favourable metabolites and thermodynamic potential. Better leachate quality with butyrate as the dominant product was favourable to the subsequent methanogenesis, which led to the highest CH$_4$ recovery of 0.28 L/g VS$_{added}$ in T4. Therefore, manipulation of acidogenic PH$_2$ and composition of headspace is a rewarding strategy for regulation of metabolic pathways and also for enhancing the production of target compounds.
CHAPTER SEVEN

ENHANCED ACIDOGENIC HYDROGEN PRODUCTION DURING
TWO PHASE ANAEROBIC DIGESTION OF FOOD WASTE:

EFFECT OF DIFFERENT NEUTRALIZATION MODES

Abstract

A hybrid LBR-UASB solid-liquid two-phase AD system was applied in this study with food waste as substrate. Four types of neutralization modes were introduced, including daily pH adjustment of leachate to 6.0, methanogenic effluent recirculation, and initial addition of NaOH and lime separately at a dosage of 20.0 and 14.0 g/kg food waste, respectively. Results showed that addition of alkali agents could enhance the COD leaching of food waste, especially with NaOH, with a cumulative COD production of 736.6 g/kg VS$_{\text{added}}$, 45.9% higher than that of daily pH adjustment. This is probably due to the strong alkali degradation. Trends of total soluble products (TSP) did not follow the profile of COD, with the highest value of TSP in the treatment with UASB effluent recirculation, i.e. 401.3 gCOD/kg VS$_{\text{added}}$ indicating varying solubilization degrees among the four treatments. Different neutralization modes also resulted in different H$_2$ profile in terms of production rate and H$_2$ / CO$_2$ ratio. Obviously, H$_2$ production rates of 3.0 and 2.1 L/d with lime and NaOH addition were much higher than 0.7 and 0.4 L/d with effluent recirculation and daily adjustment, respectively. Furthermore, addition of alkali agents increased the ratio of H$_2$ / CO$_2$ to as high as 3.9 on day 4 whereas the highest values of
the other two treatments were lower than 1.00. Thus, it was more favorable for reutilization of acidogenic off-gas in treatments with the addition of alkaline agents. Considering the contribution of hydrogenotrophic methanogenesis, the highest CH$_4$ production of 11.24 L/d was achieved in treatment with lime addition. This together with the fact that lime is much cheaper than NaOH, lime treatment was considered the best in terms of enhanced hydrolysis as well as the feasibility for reutilizing acidogenic off-gas. However, strategies for maintaining constant high ratio of H$_2$ /to CO$_2$ need further experimentation.

7.1 Introduction

Two-phase AD (i.e. acidogenic-methanogenic) is a promising technology for generating biogases (i.e. hydrogen and methane) from concentrated organic substrates such as food wastes. If correctly operated, the first stage of these systems can achieve several objectives including hydrolysis, acidification, and hydrogen gas production. The performance of the acidogenic reactor in a two-phase system could influence the design and operation of the subsequent methanogenic reactor. Enhanced hydrolysis and acidogenesis in the first phase will reduce residence time requirements in the downstream reactor. An optimum pH range is essential for the growth of microorganisms during anaerobic digestion. Studies so far indicated that the control of pH is crucial to the production of H$_2$, because the significant effect of pH on the activity of hydrogenase enzyme (Dabrock et al., 1992) and the prevailing metabolic pathways (Khanal et al., 2004). Therefore, the adjustment of pH to an appropriate range for specific purpose is gaining attention in the area of anaerobic H$_2$ fermentation.
The performance of acidogenic digesters is known to be a function of pH (Fang and Liu, 2002). The impact of pH conditions on the rate of hydrolysis, the types and quantities of acidogenic products, and rate and extent of H₂ generation (Li and Fang, 2007). Also, pH and buffering capacity of the system will affect the activities of microbial community and salt concentration due to pH control (Jones and Woods 1989; Liu et al., 2011). It was reported that a pH range of 4.0-6.0 was favorable for hydrolysis of organic solids (Kim et al., 2006; Chen et al., 2007). Our previous study had showed that different neutralizing agents (Selvam et al., 2010) and daily adjustment to 6.0 led to better performance of anaerobic hydrolysis (Xu et al., 2011).

Hydrogen is supposed as an accompanied product during the primary fermentation of soluble organic substrates. It is the fact that H₂ and CO₂ produced in acidogenic reactor comprise as high as 30% of the consumed substrates (Clark et al., 2012). Reutilization of this part of energy carried by hydrogen possesses great potential to enhance overall energy recovery efficiency from organic solid waste. Our previous experiment indicated that both the transferred off-gas and change of leachate quality contribute to the overall increase of methane production. However, mole ratio of hydrogen to carbon dioxide is a key factor that determining the efficiency of the reutilization process. The reutilization of hydrogen by hydrogenotrophic methanogens follow the stoichiometry relations:

\[ 4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \quad \Delta G = -134 \text{ kJ/mol} \]

Off-gas tansfer should be related to the shift of metabolic pathway in acidogenic reactor. Hydrogen is also an important factor that manipulates the metabolic pathway occurred in acidogenic reactor. The composition of acidogenic products in the leaching
bed reactor is affected by the concentration of hydrogen and reducing equivalent balance. Higher hydrogen pressure in the headspace will inhibit the acetogenic growth rate, because it inhibits (thermodynamically) the generation of propionic and butyric acids (Lyberatos and Skiadas 1999). Vavilin et al. (1995) had demonstrated the decrease of hydrogen pressure often coincided with the start of propionic acid production and an increase of lactic acid concentration. So, to keep appropriate hydrogen pressure is the key to control the metabolic pathways towards the intended products.

Addition of alkali once at the beginning of this experiment simplify the operation process compared with daily adjustment. Further, the nature of alkaline reagents are different and thus they may induce different performance of the AD process. Under the environment of organic acids, NaOH react much faster than lime. However, in consideration of the practicability of application, lime is preferred due to its low cost. Methanogenic effluent recirculation is one way to control pH during biohydrogen production from organic solid waste (Chinellato et al., 2013). One of the most important challenges for sustaining hydrogen production in a reactor optimized for dark fermentation is to avoid the growth of H$_2$-consuming bacteria (Cooney et al., 2007). Due to the daily addition of mixed culture contained in the effluent, there is always a risk that unwanted archaea such as H$_2$-consuming methanogens could grow and deplete the hydrogen produced. How these neutralization modes affect the hydrolysis and hydrogen production as well as the metabolic pathway and under which mode the highest energy recovery efficiency can be achieved warrant an investigation to find the answers to the two questions.
7.2 Experimental design

In order to investigate the effect of neutralization modes on hydrolysis rate and acidogenic hydrogen production as well as the distributions of metabolic products and associated H₂ production behavior, four types of neutralization modes were adopted in the present study including one treatment each with either 20 g/kg NaOH or 14 g/kg Lime added at the beginning of the experiment, one treatment with UASB effluent recirculation and another one with adjusting the recycling LBR leachate pH daily to 6.0 (Table 7.1). Overall CH₄ recovery during two-phase AD process (the hybrid solid-liquid system) with reutilization of acidogenic off-gas was also evaluated. LBR coupled with UASB was selected as the configuration for the two-phase AD system. The size and fabrication details of both reactors were referred to previous chapter 3, so as the operational details.

Table 7.1 Experimental design

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Remark</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1 20 g/kg NaOH addition at the beginning</td>
<td>CaO (AR grade)</td>
</tr>
<tr>
<td>T2 14 g/kg Lime addition at the beginning</td>
<td>The effluent would be aerated 12 h before recirculation to eliminate the influence of methanogens</td>
</tr>
<tr>
<td>T3 UASB effluent recirculation to adjust pH daily</td>
<td></td>
</tr>
<tr>
<td>T4 pH adjust to 6.0 daily</td>
<td>The pH of the (50%) recycling LBR leachate is adjusted to 6.0 using NaOH</td>
</tr>
</tbody>
</table>

7.3 Materials and methodology

7.3.1 Substrate and seed sludge

Preparation of artificial food waste was followed the method detailed in Chapter 3. The physiochemical characters of the simulated food waste are summarized in Table 1. Anaerobically digested sludge with 2.3% total solids (TS) and 76% VS/TS obtained from Shek Wu Hui wastewater treatment plant was used as the seed.
Table 7.2 Selected physicochemical properties of the food waste.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Food waste</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Solid (TS, %)</td>
<td>40.7 ± 0.0</td>
</tr>
<tr>
<td>Volatile Solid (VS/TS, %)</td>
<td>97.8 ± 0.0</td>
</tr>
<tr>
<td>Total Organic Carbon (TOC, %)</td>
<td>47.3 ± 0.1</td>
</tr>
<tr>
<td>Total Kjeldahl Nitrogen (TKN, g/kg)</td>
<td>29.1 ± 1.8</td>
</tr>
</tbody>
</table>

### 7.3.2 Calculation of H\(_2\) production

Each acidogenic LBR was equipped with a pressure sensor for real-time determination of headspace pressure and a MFC (Seven Star, China) for both measurement and controlling the flow rate of acidogenic off-gas. The pressure sensor and the MFC were controlled by a PID controller. Data log software (AIDCS monitor) was used for recording the real-time data of headspace pressure and biogas flow rate.

Volume of biogas production in acidogenic reactor was calculated by both headspace pressure (the part retained in the headspace due to the setting of headspace pressure) and off-gas going through MFC. A representative sample of the biogas was subsequently analyzed. Sampling was conducted using special gas-tight syringe (pressure-lock gas syringes Series A-2 with matching needles; Valco Instrument Co. Inc. (VICI) Precision Sampling; Baton Rouge, LA, USA). The syringe is fitted with a push-button valve allowing for sample locking and storage. Sample was taken after measuring total gas production and assumed that gas composition would be the same in the gas released as that remaining in the headspace. H\(_2\) content in the acidogenic biogas was determined using gas chromatography (HP7890) equipped with TCD and PLOT-Q column (30 m × 0.53 mm × 15 µm). Calibration was performed using external gas standards: 100% CO\(_2\), 20% CO\(_2\) and 80% H\(_2\), and 100% H\(_2\), injected as 1.0 mL at 1 atm.

Then, H\(_2\) production could be calculated through the following equation,
\[ Y_{H2} = \sum_{k=1}^{n}(V_t \ast h_t) (L) \]  
Eq. 7.1

Where, \( Y_{H2} \) and \( V_t \) are cumulative \( H_2 \) yield and volume of daily \( H_2 \) production, respectively while \( h_t \) represents daily \( H_2 \) concentration in biogas.

### 7.3.3 Analyses

Generally, parameters of solid, liquid and gas phase will be analyzed. For the solid samples, TS, VS, TOC, and TKN content of the food waste (before and after digestion in LBR) and seed sludge were determined according to methods described in chapter 3.

Leachate samples were analyzed for volume, pH, COD and soluble products with protocols described in Chapter 3. The sum of alcohols and VFAs and lactic acid are reported as total soluble products (TSP).

Volume of biogas from the UASB was continuously measured using a wet gas flow meter (BSD-0.5, Shanghai) and its composition (\( CH_4 \), \( CO_2 \) and \( H_2 \)) was analyzed using a gas chromatograph (HP7890) equipped with TCD detector and PLOT-Q column (30 m × 0.53 mm × 15 µm). Calibration was performed using external gas standards: 100% \( CH_4 \), 20% \( CO_2 \) + 80% \( H_2 \), 100% \( H_2 \) and 60% \( CH_4 \) + 30% \( CO_2 \) balanced in \( N_2 \), injected as 1.0 mL at 1 atm.

### 7.4 Results and discussion

#### 7.4.1 Acidogenic \( H_2 \) production

Biogas produced in the acidogenic reactor mainly contains \( H_2 \) and \( CO_2 \), whereas the concentration of \( H_2 \) in the mixed biogas was affected by a series of factors, e.g. substrate type, seed microorganisms, and the prevailing metabolic pathways. Volumes and \( H_2 \) contents of acidogenic biogas production in LBRs are present in Fig. 7.1a and b.
Addition of NaOH and lime in T1 and T2, respectively led to significantly increase of H$_2$ production while daily adjustment of leachate to pH 6.0 resulted the lowest biogas production as is shown in Fig. 7.1a. Supplementation of alkali agents in T1 and T2 enhanced the biogas generation from day 3 to 5 (Fig. 7.1a) but the effects are short-lived and not able to sustain, which might be due to the decreasing pH at the later stage. Compared to the treatment with daily pH adjustment, improvement of H$_2$ production in T3 with methanogenic effluent recirculation was not as significant as that in T1 and T2. pH is always the critical factor affecting the activity of H$_2$ production and relatively low pH (as shown in Fig. 7.5) during the initial stage of this experiment may suppress the evolution of biogas in T3. Furthermore, recirculation of high quantities of methanogenic effluent could initiate methanogenesis in LBR, thus a balance between the pH and recycling UASB effluent should be maintained (Lay et al., 2011).

Daily hydrogen production was calculated based on the concentration of H$_2$ times the volume of acidogenic biogas. The profile of the concentration of H$_2$ cannot be maintained to match with the profile of biogas production. H$_2$ content in the biogas ranged from 10.4% to 93.9 %; and this range is larger than previous report (Shin et al., 2004). Despite the peak of biogas production in T1 was marginally higher than that in T2, the peak of H$_2$ content in T2 was much higher than that in T1, suggesting lime addition was better in terms of elevated H$_2$ content. It was reported that addition of lime could enhance H$_2$ production as well as the overall performance of the digester (Zhang et al., 2013b). Distributions of acidogenic end products determined the concentration of H$_2$, and active hydrogen production on day 3-5 of T1 and T2 was associated with the dominance of butyrate and acetate (Fig. 7.2). H$_2$ yield can be calculated through the equation (1),
using the data set of H$_2$ content (ht, Fig. 7.1b). According to the equation, cumulative H$_2$ productions in the four treatments were 34.8, 50.3, 12.2 and 6.6 L of T1, T2, T3 and T4, respectively. These values were converted to specific hydrogen production (SHP) as follows, 58.0, 83.9, 20.3 and 11.0 L H$_2$/ kg VS$_{\text{added}}$. T2 with addition of lime achieved the highest SHP production of 83.9 L H$_2$/ kg VS$_{\text{added}}$, which was higher than that of 66.7 L H$_2$/ kg VS$_{\text{added}}$ with previous anaerobic fermentation of food waste in CSTR by Cavinato et al. (2012). Similar range of SHP was achieved in anaerobic fermentation of cellobiose in CSTR by Kim and Kim (2013); however, the case of pure substrate was quite different from mixed wastes.

As is indicated in Fig. 7.1d, butyrate to acetate ratios (Bu/Ace) were higher than 2.0 from day 3 to day 12, except that in T3. The available H$_2$ during the fermentation was determined by the Bu/Ace ratio (Nandi and Sengupta 1998). Similar to H$_2$ production profile, Bu/Ace ratios in T1 and T2 reached the peak values on day 3-5 and then decreased along the experiment, suggesting that butyrate formation improved hydrogen production. Whereas Bu/Ace ratios in T3 exhibited a profile of lower than 2.0 during the earlier stage (day 1-8) and increased to higher than 4.0 in the latter stage (day 8-15, except day 13), which was probably related to the pH condition. Theoretically, the peak production of Bu/Ace should coincide with vigorous H$_2$ production, however, in case of the latter stage of batch study the depletion of easily biodegradable carbohydrates and relatively low activity of microbes due to toxic inhibition limited the generation of H$_2$; and this could be the possible reason for the low H$_2$ production with high Bu/Ace in the latter stage of T4. The optimal Bu/Ace ratios vary in accordance with anaerobic cultures and substrate used. The ratio between H$_2$ and CO$_2$ in the acidogenic biogas is a
controlling factor that influences the efficiency of their reutilization by hydrogenotrophic methanogens as well as the quality of final CH$_4$ product. H$_2$ and CO$_2$ can be converted to CH$_4$ through the reaction, $4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$ ($\Delta G = -134$ kJ/mol) catalyzed by hydrogenotrophic methanogens. In this reaction, the mole ratio of H$_2$ to CO$_2$ is 4.0, which means a H$_2$/CO$_2$ ratio of 4.0 is the minimum requirement for efficient reuse; however, in consideration of the efficiency of the biochemical reaction, a H$_2$/CO$_2$ ratio of $>4.0$ is preferable. The profiles of H$_2$/CO$_2$ ratios in acidogenic headspace were illustrated in Fig. 7.1c. Unexpectedly, most of the H$_2$/CO$_2$ values were lower than 4.0, except on day 3-4 in T2 the values were higher than 4.0, suggesting more favorable ratios for reutilization in methanogenic phase. Addition of NaOH and lime in T1 and T2, respectively, increased the ratio H$_2$/CO$_2$ during the first 5 days and decreased afterwards, which was probably related to the enhanced H$_2$ production during the starting period. In T3 and T4, the ratios of H$_2$/CO$_2$ were constantly lower than 1.0, indicating unfavorable condition for reutilization through hydrogenotrophic methanogenesis. Therefore, further exploration to improve the H$_2$/CO$_2$ ratio is necessary for enhancing the energy recovery of two-phase AD and improving quality of final CH$_4$ product.
Fig. 7.1 Acidogenic biogas production in LBRs, volume of biogas production (a), \( \text{H}_2 \) content in acidogenic biogas (b), \( \text{H}_2/\text{CO}_2 \) ratio (c), and butyrate to acetate ratio (d)
7.4.2 Distribution of soluble products

In this part, effects of neutralization modes on distributions of soluble products and shift of metabolic pathways were going to be interpreted. Organic compounds were converted to H$_2$, soluble fermentation products, and biomass. Ethanol, propanol, acetate, butyrate and lactate were the five dominant components of the TSPs, and their distributions and concentrations are presented in Figs. 7.2 and 7.3, respectively. Soluble products production and their speciation are closely related to the operation condition, e.g. pH. Results showed that soluble products species from the four treatments shared the same spectrums, however, the abundances of the individual soluble products were different. Comparing with methanogenic effluent recirculation and daily adjustment, an obvious increase of lactic acid and decrease of butyrate acid of the treatments with NaOH and CaO addition was noted. The abundance variation of different soluble products was a result of different metabolic pathways. It had been reported that the metabolic pathway was influenced more by on-site pH condition rather than the $PH_2$ (Horiuchi et al., 2002). The constant lower pH value in treatments T1 and T2 during the later stage of the batch may explain the relative higher abundance of lactic acid. Lactic acid is a reduced intermediate, yield of which often associated with low pH (Silva and Yang, 1995; Itoh et al., 2012). Likely, the increase of butyrate acid in the T3 was attributed to the relatively higher pH (5.0-6.0), and this is in agreement with Horiuchi et al. (2002) study. Reduced abundance of butyrate in T1 should be due to the inhibition of low pH, similar to that observed by Ntaikou et al. (2010).
Fig. 7.2 Compositions of soluble products in the four acidogenic reactors during the time course
Besides the differences in lactic acid and butyric acid, differences exist also in the propanol and ethanol production among the four treatments. Ethanol was produced simultaneously with acetate through ethanol - acetate pathway and this usually associated with H₂ production (Hwang et al., 2004). Propanol is usually sourced from lactic acid (Jain and Yan 2011). Propanol and ethanol are reducing compounds, production of which will be accompanied by hydrogen depletion (Lay et al., 1999; Noike and Mizuno 2000). Higher propanol and ethanol production in T3 and T4 were consistent with less hydrogen production in the two treatments, especially in T4. The shift in acid-producing pathway to solvent-producing pathway was probably due to changes of microbial activities induced by the on-site pH conditions, as reported previous during biohydrogen production from OFMSW (Lay et al., 1999).

Acetate and butyrate were the major volatile fatty acids generated during H₂ fermentation (Junghare et al., 2012), indicating a mixed acid fermentation type.

\[ C_6H_{12}O_6 \rightarrow CH_3 (CH_2)_2COOH + 2H_2 + 2CO_2 \] Eq. 7.2

\[ C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 4H_2 + 2CO_2 \] Eq. 7.3

Similar results were also reported by Lee et al. (2012), in which butyrate and acetate were the two primary soluble metabolites, accounting for 85–99% of total soluble microbial products.

In T1, from Day 8, the pH was less than or equal to 4.0 that promoted the lactate production from the butyrate and acetate (mixed acid fermentation). The reactor is heading towards the heterotrophic lactic acid fermentation (products are lactic acid, ethanol and CO₂ in equimolar ratio) while retaining part of the mixed acid fermentation. Typical ethanol fermentation may also exist during days 6-10. Different regimes can be
observed along the experiment, (1) Day 3-4: Mixed acid fermentation resulted in more H₂ production as by-product, and the main products were butyrate and acetate; (2) Days 5-7: Ethanol fermentation and mixed acid fermentation was equally dominated, resulting in butyrate, acetate and ethanol as dominant products; and (3) Days 8-17: Lactic acid (heterotrophic) fermentation was dominant. Thus, one-off NaOH addition is not sufficient to sustain the pH buffering in a batch acidogenic digestion.

The profile of T2 (Fig. 7.2) is similar to the NaOH with minor variation in the concentrations of different compounds. This means, at the early stage when pH is within the range of 5.5-6.5, the metabolism is oriented towards mixed-acid fermentation resulting in H₂ production. When the pH drops below this range other fermentation pathways comes into play. From day 3 to 5, mixed acid fermentation dominated which resulted in more H₂ production as by-product (day 3-4) with butyrate and acetate as the main products. However, increase in ethanol and (or) lactate from day 6 to 12 indicates the onset of heterolactic fermentation. During days 13-17, homolactic fermentation dominated over heterotrophic fermentation; whereas mixed acid fermentation still exist in less dominant condition.

As for T3, the pH during days 3-10 was ~3.9-4.6 resulting in higher lactate production, which also reduced the dominance of the mixed acid fermentation. Considering the ethanol concentration, heterolactic fermentation could be the pathway prevailed during this period. During days 11-17, the pH fluctuated between 6.2 and 5.6 and resulted in higher production of butyrate and some acetate (mixed-acid fermentation). However, the concentration of butyrate and acetate depends on the type of organism dominates in this situation. Increase in caproic acid production during days 15-17
indicated that increased oil/lipid degradation and/or less efficient conversion of caproic acid to acetate (less efficient β-oxidation). Appearance of propanol (days 7-17) is an indication of channeling electrons towards the solvent production indicating the lactate production during days 2-8 is due to the heterolactic fermentation (products ethanol and lactate in equimolar ratio) and existence of propanol producing organisms. Regarding the H₂ production in T3, it is different from the T1 and T2 that no sharp peak was observed; however, higher quantities were observed during the later phase of the digestion. In T4, the pH was between 5.0 and 5.2 during days 9-11; while in other days it was around 4.0 resulting in significant lactate production throughout the experiment. This very clearly indicates that when the pH is >5.0, a butyrate type fermentation can be induced.

Mixed acid fermentation released more H₂ as the by-product. Peak production of H₂ on days 3 and 4 coincided with higher butyrate and acetate during days 2-4 in T1 and T2. A similar dominance of mixed acid fermentation was observed in Days 9-17 of T3 and Days 9-11 of T4; however, such peak H₂ production (Fig. 7.1 a and b) was not observed in T1 and T2. The following reasons are proposed to explain this phenomenon. (1) The microbes were already selected (by the existing low pH and other factors) and the hydrogen producers lost their dominance within a few days of their operation. A typical situation was evident when the system is designed to produce H₂ in other studies and it was very hard to (achieve) retain the activity of the H₂ producers through specific inoculum or heat treated/air-dried inoculum (Oh et al., 2003). (2) The existence of fermentation products acidifies the environment. Unless the lactate production is stopped, only a minimal acetate and butyrate is observed (T3); which means, the H₂ produced is consumed during the lactate fermentation leading to low H₂ yield. (3) Higher propanol
and ethanol production in T3 and T4 were consistent with less hydrogen production in the two treatments, especially in T4.

The ratio of oxidized to reduced NAD within the bacteria is determined by H₂ content in the headspace of the reactor. Therefore, H₂ generation activity in the reactor would in turn regulate the metabolic reactions within the bacteria that were coupled to the NAD⁺ - NADH redox reaction. Hence, H₂ evolution was closely related to the metabolic pathways in the reactor and further regulates the overall performance of anaerobic digester.
Fig. 7.3 Concentrations of major soluble products with four neutralization strategies, (a) NaOH and (b) Lime addition at the beginning; (c) Methanogenic effluent recirculation; (d) Daily adjustment of pH to 6.0.
7.4.3 General performance of the digesters

Reactions associated with production and consumption of H$_2$ in acidogenic reactor are quite complex while pH seems to be a critical factor in controlling the metabolic pathways. Therefore investigating a suitable neutralizing method that can generate high H$_2$ is advantageous. In this part, parameters indicating the general performance of acidogenic reactors such as leachability (volume of leachate production), pH and consumption of alkali agents, production of COD and total soluble products (TSP) as well as the overall CH$_4$ recovery are evaluated. As expected, the changes on hydrolysis behavior, raised by different neutralization modes, affected the product yield of acidogenic fermentations.

![Graph showing leachate volume in LBRs with different neutralization strategies](image)

Fig. 7.4 Volume of leachate in LBRs having different neutralization strategy

Volume of leachate produced in all the treatments were increasing along the process, ranging from 1400-2400 mL (Fig. 7.4); and the increasing volume was due to the release of water during hydrolysis of food waste. Sudden decrease of leachate production from day 4 to day 7 in treatment T1 was because of clogging. The burst of NaOH
degradation increased the density of food waste and decreased hydraulic conductivity, which resulted in the poor leaching performance. It was reported that NaOH increased the density of food waste and decreased hydraulic conductivity that resulted in poor leaching performance (Staub et al., 2009). After day 7 the effect of NaOH decreased and the leachate production recovered.

From day 3 to the end of this experiment, pH of all the treatments were between 4.0 and 6.0; however during the first 8 days these values were lower than 5.0 and this might have influenced prevailing metabolic pathways in T1 and T2. The decrease of pH was caused by rapid generation of fatty acids and changes of pH could further affect the metabolic pathways in the reactors. Methanogenic effluent recirculation provided better buffering capacity in T3 and led to a higher pH range, i.e. 4.6-5.5 during days 8-17, compared with the other treatments, whereas constant low pH of < 5.2 was observed in T4 with daily adjustment.
In order to maintain an optimum pH range for hydrolytic bacteria and acidogens, addition of neutralizing agent is necessary. In this study, treatments T1, T2 and T4 were supplemented with extra alkali for buffering the acidogenic process while methanogenic effluent recirculation was practiced in T3. Cumulatively, 40.6 g NaOH (for 2 kg FW) was consumed in the whole experiment of T4 treatment, which is the highest compared with T1 and T2. Despite, the highest amount of alkali reagent was used in T4; the buffering capacity was not as good as in the other treatments, indicating the inefficiency of the strategy of daily adjustment. Considering the future large-scale application, the economic viability is of great importance. Lime is much cheaper than NaOH and also has more OH\(^{-}\) equivalent/mol while recirculation of methanogenic effluent can not only save the investment for alkali reagent but also reduce the volume of water addition. However, the final decision should be based on the net energy recovery from the system.

![Graph](image-url)

Fig. 7.6 Daily (a) and cumulative (b) COD production in LBRs having different neutralization strategy
As illustrated in Fig. 7.6, COD leaching profiles varied greatly among the four treatments. Addition of NaOH in T1 led to the burst of COD production from day 3 to day 8, i.e. to a peak value of 118.1 ± 4.5 g/L on day 6, which should be due to the strong alkalinity of NaOH that enhanced the cleavage of organic polymers. Meanwhile the addition of lime in T2 had increased the COD leaching from day 4 to day 8 compared with T3 and T4; however, the extent of increase was much smaller than that of T1 treatment. Reaction with lime was much slower due to its low soluble nature and also it is not as strong as NaOH. Decrease of COD production during the latter half of this experiment should be related to the depletion of buffering capacity as well as the lack of decreasing the easily available organic matter. Methanogenic effluent recirculation improved COD leaching compared with T4 due both to the pH buffering and nutrient supplementation. It seems daily adjust of leachate to pH 6.0 with 0.5 mol/L NaOH was not enough to relieve the acid crisis at the beginning of this experiment, and the increase of COD from day 8 to day 14 might due to the acclimation of microbes. Cumulative COD was consistent with daily COD production, following the order of T1>T2>T3>T4.

Daily and cumulative production profiles of total soluble products (TSP, sum of acetone, ethanol, 1-propanol, 1-butanol, acetate, propionate, iso-butyrate, butyrate, isovalerate, valerate, caproate acid and lactate) are presented in Fig. 7.7. After a sharp drop on day 2, TSP production profiles of T1, T2 and T3 from day 4 to day 9 were similar to COD profiles. However, the peak production is bit delayed compare to COD production because of the fact that TSPs represents the soluble COD. Despite the higher values of TSP yields in treatment T1 and T2, cumulative TSP production in T3 was the highest, which should be related to the missing determination of some intermediates. Addition of
NaOH and CaO at the beginning led to different metabolic pathways, which may induce production of compounds that was not included in TSP analysis. Despite the higher values of TSP yields in treatment T1 and T2, cumulative TSP production in T3 was the highest, which should be related to the higher particulate nature of COD in T1 and T2.

Fig. 7.7 Daily (a) and cumulative (b) soluble products yields during the time course

CH₄ was the final target product of the two-phase system. Profiles of biogas production in methanogenic UASBs were illustrated in Fig. 7.8. Daily CH₄ yield exhibited a profile similar to that of COD profiles, which indicated that CH₄ production was closely related to COD loading. Despite the highest daily COD leached in T1, CH₄ generation in T1 was similar to that of T2, indicating poor leachate quality. CH₄ contents of the four treatments are almost the same, around 70%, indicating headspace gas transfer had little effect on CH₄ content in biogas and the H₂/CO₂ ratio was high enough to fix extra CO₂. Besides the contribution of H₂ from LBR, CH₄ recovery in T1, T2 and T3
were all similar; however, adding the contribution of H₂, lime addition treatment gave the highest CH₄ recovery (P <0.05).

Fig. 7.8 Biogas production in UASB: (a) Daily CH₄, (b) Content of CH₄ in the biogas and (c) Cumulative CH₄ production

7.4.4 Overall performance

Although, addition of NaOH could significantly improve the COD production, the quality of soluble products and H₂ production was not as good as the lime treatment, especially when considering the overall CH₄ production. UASB effluent recirculation resulted in the best performance in terms of solubilization of COD, however, H₂ production was negligible and hence overall energy recovery was marginally lower than
lime treatment. Daily pH adjustment was not good enough to provide a good environment for hydrolysis and H₂ production resulting in the lowest CH₄ recovery among the treatments. NaOH supplementation (T1) increased the VS reduction in LBR by 29% while both NaOH and lime addition enhanced the H₂ production significantly compared with daily adjustment of pH to 6.0. As presented in Table 7.3, CH₄ recovery in T1-T3 was almost similar and was higher by ~28% compared with daily adjustment.

Table 7.3 Summary of the performance of LBR-UASB

<table>
<thead>
<tr>
<th>Items</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>T4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutralization mode</td>
<td>NaOH addition</td>
<td>Lime addition</td>
<td>Effluent recirculation</td>
<td>Daily adjustment</td>
</tr>
<tr>
<td>COD production rate (g/d)</td>
<td>30.3</td>
<td>24.6</td>
<td>24.0</td>
<td>20.8</td>
</tr>
<tr>
<td>Cumulative COD (g COD/kg VS&lt;sub&gt;added&lt;/sub&gt;)</td>
<td>736.6</td>
<td>605.7</td>
<td>581.6</td>
<td>504.9</td>
</tr>
<tr>
<td>Production rate of TSP (g COD/d)</td>
<td>12.0</td>
<td>11.9</td>
<td>16.5</td>
<td>12.7</td>
</tr>
<tr>
<td>Cumulative soluble products (g COD/kg VS&lt;sub&gt;added&lt;/sub&gt;)</td>
<td>290.71</td>
<td>288.3</td>
<td>401.3</td>
<td>307.7</td>
</tr>
<tr>
<td>H₂ production rate (L/d)</td>
<td>2.1</td>
<td>3.0</td>
<td>0.7</td>
<td>0.4</td>
</tr>
<tr>
<td>Cumulative H₂ production (L)</td>
<td>34.8</td>
<td>50.3</td>
<td>12.2</td>
<td>6.6</td>
</tr>
<tr>
<td>CH₄ production rate (L/d)</td>
<td>10.9</td>
<td>11.2</td>
<td>10.7</td>
<td>8.8</td>
</tr>
<tr>
<td>Specific CH₄ production (L/kg VS&lt;sub&gt;added&lt;/sub&gt;)</td>
<td>303.9</td>
<td>314.6</td>
<td>298.6</td>
<td>245.0</td>
</tr>
<tr>
<td>TS removal (%)</td>
<td>68.5</td>
<td>62.5</td>
<td>62.6</td>
<td>53.3</td>
</tr>
<tr>
<td>VS removal (%)</td>
<td>68.3</td>
<td>62.3</td>
<td>62.4</td>
<td>53.0</td>
</tr>
<tr>
<td>Carbon recovery (%)</td>
<td>37.9</td>
<td>41.5</td>
<td>42.1</td>
<td>40.5</td>
</tr>
<tr>
<td>TOC removal (%)</td>
<td>72.1</td>
<td>66.6</td>
<td>65.9</td>
<td>56.4</td>
</tr>
</tbody>
</table>

7.5 Conclusion

Addition of alkali agents (NaOH and CaO) at the beginning performed better in terms of substrate hydrolysis and hydrogen production, especially to provide a higher H₂/CO ratio. However, considering the net energy output and the potential impact on the performance of the methanogenic reactor, lime addition was the best for energy recovery.
Lime and NaOH can be applied to accelerate the acidogenesis with very short period of high H$_2$ production (reaching 80-90%) with a H$_2$/CO$_2$ ratio reaching 3.85. However, this production could not be sustained with one-off application. Therefore, a strict pH control is required. Further investigation on how to sustain the buffering capacity for hydrolysis and H$_2$ production is necessary.
CHAPTER EIGHT

ENHANCED BIOHYDROGEN PRODUCTION DURING TWO-PHASE ANAEROBIC DIGESTION OF FOOD WASTE: EFFECT OF CARBOHYDRATE CONTENTS IN THE SUBSTRATE

Abstract
Anaerobic digestion (AD) of food waste is more rewarding for the concomitant energy recovery from the waste digestion. Two phase AD offers more advantages over traditional single phase AD. However, the low efficiency of energy conversion is always the bottleneck. From the mass balance point, off-gas energy loss (~20%) from the first stage leads to the lower energy efficiency. Therefore, acidogenic off-gas reutilization is a promising solution to increase energy efficiency. Anaerobic hydrolysis of food wastes sourced from bakery (T1), Chinese restaurant (T2), western-style restaurant (T3), and wet market (T4) were performed in a hydrolytic-acidogenic leach bed reactor (LBR). The headspace of each LBR had an outlet linked to the bottom of the corresponding UASBs for acidogenic off-gas transfer. H₂ generation was closely related to the composition of substrate. In T3, food waste collected from western-style restaurant with a composition of carbohydrate: protein: lipid at 69.5: 14.4: 12.8 achieved the highest H₂ production of 61.0 L/kg VSadded. Ethanol, acetate and butyrate were the common fermentation products of the four treatments and variations in the production and speciation of the soluble products were closely related to the composition of substrates when the operational conditions were similar. Fermentation pathways with dominance of butyrate and acetate were
favourable for H\textsubscript{2} production and subsequent methanogenesis. High volatile solids (VS) conversion efficiency (76.7\%) with western-style restaurant food waste was observed whereas VS reduction efficiency for the other three types of food wastes ranged from 37\% to 55\%. High COD production efficiency, i.e. 0.65 gCOD /gVS\textsubscript{added} and the solubilization degree (total soluble products/COD) as high as 83\% was achieved in T3 with western-style restaurant food waste. High specific CH\textsubscript{4} production rate was achieved (0.42 L/gVS\textsubscript{added}) with western-style restaurant food waste.

8.1 Introduction

Food waste in Hong Kong is the single largest component of municipal solid waste with a daily generation of 3,337 tonnes per day (HKEPD 2014). Anaerobic digestion is a promising technology for the treatment of organic solid waste. Two-phase AD offers more advantages over traditional single-phase AD. However, the low efficiency of energy conversion is always the bottleneck. Mass balance analysis of hybrid anaerobic liquid-solid bioreactors indicated that H\textsubscript{2} and CO\textsubscript{2} generation in the acidogenic reactor accounted for ~20\% of the consumed substrate (Xu et al., 2014) and mostly this part of energy carried by H\textsubscript{2} was lost due to reactor operation/handling and hence decrease the overall energy recovery from the bioreactor. Under conventional operation, acidogenic biogas (mainly H\textsubscript{2} and CO\textsubscript{2}) was retained in the headspace between the sampling points and this elevated H\textsubscript{2} pressure would therefore inhibit further yield of H\textsubscript{2} as well as hydrolysis of organic compounds involved (Sharma and Li 2008; Wang et al., 2008). Therefore, reutilization of acidogenic biogas to improve overall energy recovery is a promising strategy, which can simultaneously alleviate the inhibition caused by high H\textsubscript{2} pressure. In the previous chapters, regulations of headspace pressure and H\textsubscript{2} partial
pressure as well as the corresponding composition of headspace were studied and the optimized headspace pressure was proposed. However, the ratio between H₂ and CO₂ was too low for efficient reuse of acidogenic gas, because reutilization needs H₂/CO₂ ratio of at least 4.0 (H₂ + CO₂ → CH₄ + 2H₂O, ΔG = -134 kJ/mol), otherwise there will be extra CO₂, that would affect the quality of final biogas product. Therefore, strategy to improve the H₂/CO₂ ratio is critical for efficient reuse of acidogenic biogas.

Carbohydrates, the large components of food waste, are widely accepted to be easily and rapidly converted to simple monomers and subsequently converted to VFAs via hydrolysis (Viéitez et al., 2000). Degradation of carbohydrate usually linked with the higher production of H₂. Hydrogen-producing microorganisms oxidize reduced ferrodoxin (Fd₉) to produce molecular hydrogen catalysed by hydrogenase enzymes. Both the generation of H₂ and the H₂/CO₂ ratio in the acidogenic biogas could be affected by fermentation pathways and the availability of Fd₉ (Rydzak et al., 2014). These metabolic pathways are the result of a multitude of factors, including composition of the substrate especially the carbohydrate, levels of H₂ partial pressure in the headspace and pH of the digesters. It was reported that supplementary carbohydrate could enhance the biohydrogen production from protein-rich sludge (Feng et al., 2009). Similarly, co-digestion of lipid-rich or protein-rich substrate with carbohydrates for H₂ production was widely adopted by researchers (Fountoulakis and Manios 2009; Sreela-Or et al., 2011; Kim et al., 2012; Wang et al., 2013).

Therefore, the objective of this study is to investigate the effect of substrate composition especially the content of carbohydrate on acidogenic H₂ production. Four types of food wastes collected from bakery shop, western restaurant, Chinese restaurant
and vegetable market were used as substrates. Headspace pressure regulation and reutilization of acidogenic biogas was integrated to study the metabolic behaviour and biohydrogen generation. Overall performance of the reactors and energy recovery was also evaluated.

8.2 Experimental design

Four types of food wastes collected from different sources (bakery, Chinese-style restaurant, Western-style restaurant and wet market), with different elemental compositions were used in this experiment. These food wastes were acidified in leachbed reactor (LBR) and the leachates produced were utilized in UASB to produce CH₄ as presented in Chapter 4. In addition, the acidogenic off-gas was also transferred to the UASB. As identified in Chapter 5, a headspace pressure of 3.3 psi was applied in LBR. The reactor performance and the CH₄ recovery was investigated with a focus on acidogenic H₂ production behaviour.

8.3 Materials and methods

8.3.1 Food waste and seed sludge

Bakery waste was collected from Hong Kong Baptist University (HKBU) student Hall canteen, while Chinese-style restaurant and western-style restaurant of HKBU were the sources of food wastes. Vegetable wastes were collected from Lok Fu wet market, Hong Kong. In order to achieve representative components, the food wastes were collected for a period of one week on daily basis (stored in 4 °C cold lab) and mixed together. Before feeding to the reactors, bones, shells and other indigestible components were removed and large size components were cut evenly into small particles with a particle size of <6 mm. Relevant physicochemical characteristics of the four food wastes
are presented in Table 8.2. Anaerobically digested sludge with 2.2% total solids (TS) and 83% VS/TS obtained from Shek Wu Hui WWTP was used as the seed sludge.

### 8.3.2 Experimental set up and treatments

Mesophilic digestion controlled at 35 ± 1°C in a batch mode of operation was used in this experiment as practiced in previous experiments. The configurations of acidogenic LBR and UASB were the same as described in previous Chapters with headspace pressure maintained at 3.3 psi. Four identical LBRs were filled with 2.0 kg of food waste with seeding of 20% (I/S, wet basis) AD sludge and ~10% of wood chips as bulking agent according to a previous study (Xu et al., 2012). Initially, equal volume of tap water was added to reach a liquid to solid ratio of 1.0, which means 2.0 L of tap water was added to each LBR. Lime was supplemented at the initial stage with a dosage of 14 g/kg food waste. Once a day removal of acidogenic leachates and leachate sampling was performed to avoid acid crisis in the LBR. During each sampling, the leachate was taken out and 50% of the leachate was recycled back to the LBR from the top after mixing with equal volume of water; while the remaining 50% of the leachate was fed to UASB while 50 mL was reserved for analysis.

### 8.3.3 Calculation of H₂ production

As described in the Chapter 4, the acidogenic LBRs were equipped with pressure sensor for real-time measurement of headspace pressure and MFC (Seven Star, China) for both measurement and controlling the flow rate of acidogenic biogas. Both the pressure sensor and MFC were controlled by PID controller; and data monitoring software (AIDCS monitor) was used to record the real-time data of headspace pressure and biogas flow rate. Volume of biogas production in acidogenic reactor was calculated by both...
headspace pressure (the part retained in the headspace was calculated by State Equation of Ideal Gas) and off-gas going through MFC. Concentration of H$_2$ and CO$_2$ in the acidogenic biogas was measured by gas chromatography (HP7890) equipped with TCD detector and PLOT-Q column (30 m × 0.53 mm × 15 µm). Calibration was performed using external gas standards: 100% CO$_2$, 20% CO$_2$ and 80% H$_2$, and 100% H$_2$, injected as 1.0 mL at 1 atm.

Volume of H$_2$ production could be calculated through the following equation,

\[
Y_{H2} = \sum_{k=1}^{n} (Vt \times ht) (L)
\]

(Eq. 8.1)

Where, \( Y_{H2} \) and \( Vt \) are cumulative H$_2$ yield and volume of daily H$_2$ production, respectively while \( ht \) represents daily H$_2$ concentration in biogas.

### 8.3.4 Activity of hydrogenase

Expression level of hydrogenase was used as an indicator for evaluation of H$_2$ production potential of an acidogenic system. The expression of hydrogenase gene was analyzed using Q-PCR runs. The reaction system (20 mL total volume) was performed as aforementioned with 10 ng of each genomic-DNA as template. The thermal cycle parameters were as follows: initial denaturation at 95 °C for 10 min, then denaturation at 95 °C for 15 S, annealing 58 °C for 1 min and extension at 72 °C for 30 S (40 cycles) with dissociation curve included. The expression levels were determined by using external standard curve based on Ct values. The result of dissociation curve showed that the amplifications were performed well.

### 8.3.5 Analyses

Methods for analysis of the solid substrates and digestates, liquid leachates and both acidogenic and methanogenic biogas were referred to Chapter 3.
8.4 Results and analysis

8.4.1 Characterization of food waste and acidogenic H₂ production

Characteristics of food wastes collected from four different sources (bakery shop, Chinese restaurant, Western restaurant and vegetable market) in Hong Kong were analyzed and are presented in Table 8.1. These four types of food wastes varied greatly in terms of carbohydrate, protein and lipids. Moisture contents (MC) of these food wastes were in the range reported in the literature (Kim et al., 2004; Zhang et al., 2007) except that of bakery waste (with a MC of ~49%), which was seldom studied as individual waste previously. The carbohydrates, proteins and lipids in the food wastes are closely related to the sources of food wastes. For example, bakery waste contained 72.27% carbohydrate due to the large amount of bread in the waste stream while 52.8 and 69.5 % of carbohydrate was measures in food wastes collected from Chinese-style and Western-style restaurants, respectively. It was reported that hydrolysis was found to be the rate-limiting step for the conversion of carbohydrates (Miron et al., 2000). The contents of protein in the four wastes were ranging from 9.5% to 20.0%. Proteins can be hydrolysed to amino acids and further degraded to VFAs with or without H₂ generation (McInerney and Zehnder, 1988). Whereas, 55.40% of lipid was observed in the waste from wet market waste, in which a lot of meat was disposed together with vegetables. Carbohydrates are easily degradable components and were the major source of H₂ production (Viéitez et al., 2000; Fang and Liu 2002).

Degradation of lipids from LCFA to acetate or propionate via β oxidation was closely related to the levels of H₂ pressure, because β oxidation is thermodynamically unfavourable under standard conditions (Cirne et al., 2007). β oxidation can only occur at low H₂ partial pressure, which can be reached by the presence of H₂-consumers or can be
externally controlled. Under acidogenic conditions, acidification was the rate-limiting step for conversion of lipids, while both hydrolysis and acidification were limiting for the conversion of proteins (Miron et al., 2000).

Table 8.1 Characterization of food waste

<table>
<thead>
<tr>
<th>Food waste</th>
<th>Bakery waste</th>
<th>Chinese-style food waste</th>
<th>Western-style food waste</th>
<th>Wet market waste</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>4.8</td>
<td>4.0</td>
<td>4.3</td>
<td>4.7</td>
</tr>
<tr>
<td>TS (%)</td>
<td>51.5</td>
<td>24.5</td>
<td>22.0</td>
<td>27.7</td>
</tr>
<tr>
<td>VS/TS (%)</td>
<td>97.1</td>
<td>96.7</td>
<td>96.5</td>
<td>97.1</td>
</tr>
<tr>
<td>TOC (%)</td>
<td>40.2</td>
<td>40.8</td>
<td>37.4</td>
<td>42.6</td>
</tr>
<tr>
<td>TKN (%)</td>
<td>2.1</td>
<td>3.2</td>
<td>2.3</td>
<td>1.5</td>
</tr>
<tr>
<td>Carbohydrate (%)</td>
<td>72.3</td>
<td>52.8</td>
<td>69.5</td>
<td>32.7</td>
</tr>
<tr>
<td>Protein (%)</td>
<td>13.4</td>
<td>20.0</td>
<td>14.4</td>
<td>9.5</td>
</tr>
<tr>
<td>Lipid (%)</td>
<td>11.4</td>
<td>24.2</td>
<td>12.8</td>
<td>55.4</td>
</tr>
</tbody>
</table>

H₂ and CO₂ are the two major components in acidogenic biogas. Amount of acidogenic biogas produced in the reactor builds up the headspace pressure. Volumes and H₂ contents of acidogenic biogas produced in four LBRs were present in Fig. 8.1a and b. Similar to the observation in Chapter 7, supplementation of lime at the initial stage triggered the evolution of acidogenic biogas in T1, T2 and T3, while very low level of biogas was produced in T4 with waste from wet market (Fig. 8.1a). Without lag phase, acidogenic biogas production in T1 with bakery waste reached the highest peak on day 2, and after then decreased along the experiment. The short-lived peak production of biogas in T1 was likely due to the reduction in buffering capacity of the substrates leading to low pH (as presented in Fig. 8.4b) as well as the shift of metabolic pathways (Venkata Mohan et al., 2007; Wang et al., 2007). High concentration of carbohydrates and low MC in T1 led to a highest organic loading rate (OLR), which induced rapid accumulation of fatty acids and decreased the pH. Biogas production in both T2 and T3 showed a lag phase of
one day and then reached the peak production on day 2-3, and decreased from day 4. Peak production of biogas in T2 was lower than that in T1 and T3, which correlates with the carbohydrate content of corresponding food wastes. i.e. T1 (72.3%) > T3 (69.5%) > T2 (52.8%), suggesting H$_2$ was mainly sourced from the degradation of carbohydrates (Fang and Liu 2002). Compared with T1, T2 and T3, biogas production in T4 was constantly low and the peak production was delayed to day 4-5, indicating poor performance of the acidogenic reactor. It was reported that degradation of lipids was quite difficult due to the unfavourable thermodynamic potential and was assumed not to be degraded in the acidification phase (Miron et al., 2000).

![Graphs showing biogas production, H$_2$ and CO$_2$ content, and H$_2$/CO$_2$ ratio](image_url)

**Fig. 8.1** Volume of acidogenic biogas produced (a), concentration of H$_2$ (b), concentration of CO$_2$ (c) and H$_2$/CO$_2$ ratio of the off-gas (d) in LBRs treating different food wastes
H₂ content in the acidogenic biogas was another important factor highly depend on the type of substrate and metabolic pathways. As presented in Fig. 8.1b, H₂ content in the biogas ranged from 8.1% to 74.5%; and this range is wider than previous report of 41-59% (Li and Chen 2007). Increased concentration of H₂ during the initial stage was likely due to the addition of lime, which could not only enhance the production of H₂ but also improve the performance of the digesters (Zhang et al., 2013b). Similar to the profile of biogas production, a lag phase was observed in T2, T3 and T4 for H₂ production; whereas the content of H₂ in T1 reached the peak on the first day. Apparently, the variations of H₂ concentrations among four treatments were related to the compositions of substrates. Despite different peaks of acidogenic biogas production in T1-T3, the peaks of H₂ contents were similar, suggesting similar metabolic reactions in the earlier stage of experiment (Fig. 8.2). Distributions of acidogenic end products determined the concentration of H₂, and active H₂ production during days 1-4 of T1, T2 and T3 reactors coincided with the dominance of butyrate and acetate (Fig. 8.2). H₂ yield, calculated through Eq. 8.1, using the data of H₂ content (ht, Fig. 8.1b) showed cumulative H₂ productions of 23.2 L, 22.5 L, 29.9 L and 5.3 L in T1, T2, T3 and T4 reactors, respectively. These values are equivalent to 24.5, 46.0, 61.0 and 11.3 L H₂/ kg VS_add specific hydrogen production (SHP) in T1-T4 reactors, respectively. T3 with the western-style food waste showed the highest SHP production of 61.0 L H₂/ kg VS_add, which was comparable to that of 66.7 L H₂/ kg VS_add reported with anaerobic fermentation of food waste in CSTR by Cavinato et al. (2012). Most of the H₂ produced in acidogenic reactor (except the part retained in the headspace due to the pressure setting) was diverted to methanogenic UASBs for utilization.
Variations of CO₂ and H₂ constitute the dynamics of the headspace in acidogenic reactor which affect the H₂ production as well as metabolic pathways (Patra and Yu 2013; Park et al., 2005). Concentrations of CO₂ in all four treatments were above 30% except the initial 3-4 days (Fig. 8.1c). During the first 2 days of T1, the environmental conditions was favourable for H₂ generation and therefore CO₂ concentration was low, but afterwards the content of CO₂ increased, due to the inhibition of H₂ production and shift of metabolic pathways. Lag phases were observed in T2-T4, which led to low levels of both H₂ and CO₂ on day one. Low levels of CO₂ during day 2-3 in T2 and T3 should be similarly attributable to high H₂ contents.

When considering the reutilization of acidogenic biogas in methanogenic phase through hydrogenotrophic methanogenesis, H₂/CO₂ ratio of the biogas is an important parameter affecting the efficiency of the reutilization as well as the composition of biogas. At least a H₂/CO₂ of 4.0 is needed to enable the hydrogenotrophic methanogenesis to reduce CO₂ to CH₄. The H₂/CO₂ ratios observed in all four reactors were lower than 4.0 (Fig. 8.1d), suggesting unfavourable ratios for reutilization in methanogenic phase. Therefore, further exploration is necessary to increase the ratio of H₂/CO₂.

The profiles of the activities of hydrogenase are presented in Fig. 8.2. Hydrogenase plays an important role during H₂ generation, which would catalyse the reduction of proton to form H₂ (2H⁺ + 2e⁻ → H₂) (Hallenbeck and Benemann 2002). Hence, the expression levels of hydrogenase can be used as an indicator of H₂ production in the reactor. As indicated in Fig. 8.2, the expression levels of hydrogenase in leachate samples were decreasing from day 3 to day 17, and a lowest level was observed in T4 with
substrates sourced from wet market waste in all measuring stages (days 3, 8 and 17), indicating a poor performance of H₂ evolution. On day 3, higher expression levels of hydrogenase in T1, T2 and T3 were achieved and this is likely due to the larger fractions of carbohydrate contents in corresponding wastes. The decrease of hydrogenase expression level in T1 was more obvious than in T2 and T3, which might be related to the inhibition by low pH. Accompanied by the decreasing availability of easily degradable carbohydrates and accumulation of toxic compounds, lowest expression levels of hydrogenase were observed on day 17. Generally, the expression levels of hydrogenase in the digestate samples were lower than that in the leachate samples. And the variations among treatments were not as large as in leachate samples; however, the expression level in T4 was always the lowest.

Fig. 8.2 Activity of hydrogenase, (a) of leachate samples and (b) solid digestate; 3, 8 and 17 represents the sampling points

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8.4.2 Distributions of soluble products

Speciation of soluble products showed distinct variation with the function of substrate composition (Figs. 8.3 and 8.4). Generally, soluble products from the four treatments shared almost the same spectrums except that no lactic acid was detected in T4 treatment. However, the abundances of the individual soluble product are different. Ethanol, acetate and butyrate were the common intermediates in all four treatments, while propionate and caproic acid were dominant in T4 and high concentrations of propanol was observed in T1. Large amount of carbohydrates in T1 with bakery waste induced the production of lactic acid. Similarly, lactic acid was observed as the major fermentation product during anaerobic digestion of food waste (Zhang et al., 2007a). Production of lactic acid was usually associated with low pH and that agreed with constant low pH observed in T1 (Fig. 8.5). Selectively production of lactic acid by decreasing the culture pH to 3.5 was demonstrated by Itoh et al. (2012). Large amount of carbohydrate of the bread and relatively low pH in T1 triggered the generation of propanol also, production of which was probably due to the inhibition of high H2 pressure. It was reported that propanol can be produced through 1, 2-propanediol pathway by shunting carbon flux from glycolysis under suitable environmental conditions (Jain and Yan 2011). Ethanol is another reduced fermentation product, could be generated through acetyl-CoA pathway accompanied by depletion of H2 (Hwang et al., 2004). Acetate and butyrate can also be yielded from the branch of acetyl-CoA and both reactions can proceed with the evolution of H2. However, great portion of lactic acid together with ethanol and propanol evidenced the inhibition by H2.
Different from T1, butyrate and acetate were dominant in T2 with small portions of ethanol, propionate and caproic acid. Degradation of 1 mol hexose to acetate or butyrate was about to generate 4 or 2 mol of H$_2$, respectively. The dominance of butyrate and acetate was the index of H$_2$ production and this was in consistent with the H$_2$ recovery result in Fig. 8.1. Besides the effect of H$_2$ pressure, pH is always the critical factor determining the distribution of soluble intermediates as well as metabolic pathways. A pH range of 5.0-6.0 was reported to be favourable for butyrate production (Horiuchi et al., 2002), which is evident in T2. Till now, the mechanism of caproic acid production was still unclear; however, two strategies of caproic acid production were proposed in the literatures: (1) higher organic load oftentimes lead to the production of caproic acid (Parawira et al., 2004); (2) n-caproic acid synthesis by chain elongation (Agler et al., 2014).

Similar to T2, soluble fermentation intermediates in T3 were mainly composed of butyrate, acetate and ethanol, indicating a mixed acid fermentation type. Again, propionate and caproic acid were detected in smaller amount. Dominance of butyrate and acetate during the initial stage (days 2-4) was associated with vigorous H$_2$ production and later the involvement of reduced compounds and the inhibition of H$_2$-producing microbes under unfavourable conditions (pH and depletion of easily degradable carbohydrates) led to decreased H$_2$ production. Propionate is an undesirable intermediate due to its toxicity to common anaerobes and degradation of which is thermodynamically unfavourable with $\Delta G = +76.1$ kJ/mol (Amani et al., 2011; Dhaked et al., 2003; Schink and Stams 2006).

Distribution of fermentation soluble products in T4 was completely different from the other three treatments. No lactic acid was detected whereas relatively large portion of propionic acid was observed from day 4 to the end of this experiment. Obviously, this
should be due to the high lipid contents in the wet market waste as it was reported that lipid could be degraded through mixed acid pathway with the ratio of acetic acid and propionic acid around 1 (Oh et al., 2010). Large portion of propionic acid may be the reason for the poor performance of T4 as propionic acid was thought to be inhibitory the AD process (Gourdon and Vermande 1987). Presence of significant quantities of caproic acid might be related to the decrease of ethanol, which can serve as the 2-C unit for the chain elongation during caproate formation.
Fig. 8.3 Distributions of soluble products in LBRs T1-T4 with food waste collected from different sources.
Fig. 8.4 Concentration (i) and percentage (ii) of major TSP leached from LBRs loading with different food wastes
8.4.3 Performance of LBR-UASB

In this section, parameters indicating the general performance of acidogenic reactors such as leachability (volume of leachate production), pH, production of COD and TSP, and the overall CH\(_4\) recovery are discussed. As expected, the changes on hydrolysis behaviour, triggered by varied compositions of substrates, affected the product yield of acidogenic fermentations.

![Graph showing volume and pH of leachate collected from LBRs]

Fig. 8.5 Volume (a) and pH (b) of leachate collected from LBRs

Volumes of leachate produced in the four treatments with different substrates are depicted in Fig. 8.5a. The profiles can be grouped into two: T1 with a range of 700-1800 mL and T2-T4 with a range of 2300 – 3100 mL, indicating that the leachability of food waste was significantly affected by composition of the substrate. Comparing with T2-T4, less leachate was produced in T1, which was likely due to the lowest moisture content of the bakery waste. The increasing the volume of leachate production along the experimental period was due to the hydrolytic reactions. Overall, leachate production in all four treatments was increasing along the process.
pH is always the critical parameter in anaerobic digestion system. As presented in Table 8.1, pH of food waste is relatively acidic in nature. While different food waste differs in pH when fresh, upon slight decomposition, pH falls, most likely exacerbated by the production of acid from acidogens. Hence, the addition of sufficient alkali, 14 g lime/kg-food waste in these LBRs is required. Alternately, organic loading should be regulated carefully and sufficient dilutions are needed to ensure a healthy microbial population and hydrolysis.

![Fig. 8.6 Daily (a) and cumulative COD (b) leached from LBRs treating food wastes from different sources](image)

As illustrated in Fig. 8.5, from day four to the end of this experiment, pH of all the four treatments were between 4.0 and 6.0, coinciding with the reported favourable range for anaerobic hydrolysis (Chen et al., 2007). However, pH was constantly lower than 5.0 with bakery waste and this might have dominated the environmental conditions, and subsequently the metabolic pathways in T1. Constant low pH in T1 should be related to high percentage of carbohydrates, as carbohydrates are much easier to be degraded to volatile fatty acids (carbohydrate is a superior short-term fuel.
for organisms because they are simpler to metabolize than lipid). Digestion of lipid rich food waste collected from wet market showed the highest pH during the whole process due to the slow degradation of the lipid in the meat and the cellulosic polymers in the vegetables resulting in less quantities of volatile fatty acids.

COD production represents the liquefaction of carbohydrates, proteins and lipids. As is shown in Fig. 8.6, both daily and cumulative COD leaching profiles varied greatly among the four treatments. The burst of COD production from days 1 to 7 in T1, i.e. $119.2 \pm 4.5$ g/L on day 3 (Fig. 8.6a) should be related to the strong alkalinity of lime that enhanced the cleavage of easily degradable carbohydrates. However, the extent of decrease in COD production was high, e.g. from $119.2 \pm 4.5$ to $15.2 \pm 0.3$ g/L, which might be attributable to the phenomenon of carbon repression encountered during the complex anaerobic metabolism (Venkata Mohan et al., 2007). Product inhibition could be a significant factor that affected the hydrolysis in this conditions as observed by Ginkel and Logan (2005). Besides, the less moisture content should result in less dilution of the COD generated leading to high concentrations compares with other three treatments. Obviously, COD leaching in the other three treatments were much lower than that of T1 treatment. Daily COD production in T2-T4 were much lower than that in T1, and variations among them were not significant, probably due to the relatively low OLRs and less amounts of easily degradable components. It was reported that carbohydrate was much easier to be degraded followed by protein, and lipid (Miron et al., 2000). The theory was confirmed in T4; more than 55% of lipid content led to the constant lower COD leaching of wet market waste. Decrease of COD production in all four treatments during the latter half of the experiment should be related to the reduction in buffering capacity as well as the lack of easily degradable organic fractions. Small increase of
COD from day 8 to day 13 in T1 might due to the acclimation of microbes to the acidic environment. Because of variations of moisture contents among the four treatments, profiles of cumulative COD production were not consistent with that of daily COD. Highest value of cumulative COD production was achieved in T3 with food waste collected from western-style restaurant, followed by T1, T2 and T4. Degradation of lipid was quite difficult (Cirne et al., 2007) and thus the lowest COD was leached from food waste sourced from wet market.

Fig. 8.7 Daily (a) and cumulative TSP (b) leached from LBRs treating food wastes from difference sources

Daily and cumulative production profiles of TSP are presented in Fig. 8.7. The trend of TSP production was similar to COD profiles; however, the peak production is a bit delayed compared to COD production because of the fact that the TSP was sourced from solubilisation of COD. Despite the big difference in daily COD production profile between T1 and the other treatments, the differences of TSP production were not as large as COD, which indicated that solid particles
(particulates) were not solubilized thoroughly in T1. Despite the highest values of TSP yields in T1 treatment, cumulative TSP production was highest in T3 that is consistent with COD leaching profile. Further, higher cumulative TSP in T2 and T4 indicated higher solubilization degree of COD and this can facilitate the utilization of soluble products in the subsequent methanogenic reactor.

Fig. 8.8 Daily CH\(_4\) production (a), concentration of CH\(_4\) in biogas (b) and cumulative CH\(_4\) production (C) in UASBs treating LBR leachates obtained from food wastes of different sources

Methane gas produced in UASBs was measured using wet gas flow meter and the concentration of CH\(_4\) was analyzed using GC. As is shown in Fig. 8.8, daily CH\(_4\) yield exhibited a profile similar to that of LBR TSP production, indicating that CH\(_4\) production was closely related to leachate quality. Extremely large volume of CH\(_4\) production on day 2 of T1 and T3 treatments should be attributable to the contribution of H\(_2\) as evidenced from Fig. 8.1. Large portion of lactic acid in the leachate of T1
treatment probably caused the inhibition on methanogenesis. In addition, the significant fraction of particulates in the COD (as indicated by the TSP/COD ratio) of T1 reactor should have delayed the CH₄ production after the initial burst of CH₄ production eventually reducing the cumulative CH₄ production. Constant low rate of CH₄ production in T4 was closely related to the high percentage of lipid. Lipids were assumed not to be degraded in acidogenic phase and their degradation products, LCFAs, were reported to inhibit methanogenesis severely (Cirne et al., 2007) resulting in eventual reduction of overall methane recovery. In addition, when these LCFAs are transferred to the UASB, they must be oxidised to VFA by the acetogenic bacteria to acetate before used up for CH₄ production. Therefore, the CH₄ production efficiency was lowered.

The conversion of H₂ and CO₂ to CH₄ requires a ratio of 4; however, as evidenced from Fig. 8.1 none of the four treatments had reached the ratio of 4. Therefore, the CO₂ transferred from acidogenic reactor might not be totally converted and thus this may led a lower CH₄ concentration in the biogas.

Table 8.2 Overall performances of the LBRs and UASBs in the experiment

<table>
<thead>
<tr>
<th></th>
<th>Bakery waste</th>
<th>Chinese-style food waste</th>
<th>Western-style food waste</th>
<th>Wet market waste</th>
</tr>
</thead>
<tbody>
<tr>
<td>COD production (g/kg VS&lt;sub&gt;added&lt;/sub&gt;)</td>
<td>565.0</td>
<td>456.4</td>
<td>653.24</td>
<td>374.64</td>
</tr>
<tr>
<td>TSP production (g COD/kg VS&lt;sub&gt;added&lt;/sub&gt;)</td>
<td>268.4</td>
<td>329.14</td>
<td>544.54</td>
<td>288.24</td>
</tr>
<tr>
<td>H₂ production (L)</td>
<td>23.2</td>
<td>22.5</td>
<td>29.9</td>
<td>5.3</td>
</tr>
<tr>
<td>Specific H₂ production (L/gVS)</td>
<td>24.5</td>
<td>46.0</td>
<td>61.0</td>
<td>11.3</td>
</tr>
<tr>
<td>CH₄ production (L)</td>
<td>198.3</td>
<td>110.7</td>
<td>174.3</td>
<td>23.2</td>
</tr>
<tr>
<td>Specific CH₄ production (L/gVS)</td>
<td>0.20</td>
<td>0.23</td>
<td>0.42</td>
<td>0.05</td>
</tr>
<tr>
<td>VS Removal (%)</td>
<td>37.2</td>
<td>55.5</td>
<td>76.7</td>
<td>54.1</td>
</tr>
<tr>
<td>Carbohydrate removal (%)</td>
<td>47.6</td>
<td>92.6</td>
<td>73.0</td>
<td>95.0</td>
</tr>
<tr>
<td>Protein removal (%)</td>
<td>12.1</td>
<td>54.4</td>
<td>14.9</td>
<td>81.3</td>
</tr>
<tr>
<td>Lipid removal (%)</td>
<td>0.7</td>
<td>60.5</td>
<td>4.1</td>
<td>29.1</td>
</tr>
</tbody>
</table>
8.5 Conclusion

$H_2$ production was closely related to the composition of substrate; especially the content of carbohydrate and other environmental factors such as OLR, pH and $H_2$ level in the headspace. Highest $H_2$ recovery of 61.0 L/kg VS$_{added}$ was achieved in T3 with food waste from western-style restaurant. Ethanol, acetate, butyrate and lactic acid were the four dominant components of the total soluble products (TSPs); and dominance of acetate and butyrate was associated with higher production of $H_2$. High volatile solids (VS) conversion efficiency (76.7%) in LBR was observed with western-style restaurant food waste when compared with 37-55% VS removal achieved from the other three food wastes. In addition, high COD production efficiency, i.e. 0.65 gCOD /gVS$_{added}$ together with the solubilization degree (TSP/COD) as high as 83% and high specific methane production rate (0.42 L/gVS$_{added}$) indicate that a composition of carbohydrate: protein: lipid at 69.5: 14.4: 12.8 facilitated the most favourable metabolic pathway with butyrate and acetate as the dominant products.
CHAPTER NINE

CONTROLLING THE ACIDOGENIC METABOLIC PATHWAYS

TOWARDS THE PRODUCTION OF TARGET PRODUCTS

Abstract

During acidogenic reactions, hydrogen is generated as a co-product. Hydrogen partial pressure \( (P_{H_2}) \) in the headspace of acidogenic reactor regulates the acidogenic reactions and the dynamics of microbial community while the acidogenic metabolic pathway is the result of channeling electrons towards fermentation end products. Thus, the diversity of microbial community would change in response to varying \( P_{H_2} \). If the microbial community-metabolite relationship is completely understood, the headspace gas pressure could be used as a tool to alter or adjust the acidogenic products highly suitable for methanogenesis in the second phase reactor. Therefore, this study aims at investigating the flow of electron flux that led to the predominant metabolic pathway and the dynamics of microbial community during regulation of \( P_{H_2} \). Four different levels of \( P_{H_2} \) were set, control without \( P_{H_2} \) regulation (T1), 80\% of \( H_2 \) (T2), 50-60\% of \( H_2 \) (T3) and 0.04\% of \( H_2 \), while the headspace pressure was adjusted to 3.3 psi as identified in Chapter 5. During the regulation of \( P_{H_2} \), \( CO_2 \) was used for balancing and reaching the designed \( P_{H_2} \), which also led to varied compositions of headspace among four treatments. Significant increase in the production of butyrate in T4 indicated that the channeling of electrons towards the production of butyrate while in the other three treatments the electrons shunted
towards mixed acids. Dynamics of the microbial community, monitored by fingerprinting of bacterial 16S rRNA genes were correlated with the distribution of metabolites. In T1 treatment, lactic acid fermentation dominated. Accordingly, phylotypes affiliated to the genus *Lactobacillus* sp. were detected. In T2 and T4, the fermentation pathways shifted towards acetate and butyrate as dominant products, which were accompanied by increased off-gas production in acidogenic reactor and changing the microbial community with phylotypes of *Clostridium* sp. and *Bifidobacterium* sp. becoming dominant.

### 9.1 Introduction

It is well known that H$_2$ accumulation in both liquid and gaseous phases poses inhibitory effect on microbial growth and H$_2$ evolution in acidogenic reactor (Ciranna et al., 2012; Kengen et al., 1996; Schröder et al., 1994; Soboh et al., 2004; van Niel et al., 2003). The liquid phase of acidogenic reactor can be easily supersaturated with H$_2$ due to liquid-to-gas mass transfer limitations and this seems to be inevitable during batch fermentation of organic solids. Under these conditions, generation of H$_2$ becomes thermodynamically unfavorable and consequently the disposal of accumulated reducing equivalents in the cell is channeled towards the production of more reduced metabolites, such as lactate, ethanol, acetone, butanol, or alanine (Verhaart et al., 2010). Due to the changes in distribution of metabolites, production of H$_2$ and ATP decrease. Maintaining acidogenic headspace $P_{H_2}$ at a low level could enhance H$_2$ production and shunt the electrons towards non-reduced products (Ciranna et al., 2014).

Butyrate, as a common acidogenic metabolite, was reported to be effective for the subsequent methanogenesis (Öztürk 1991; Wang et al., 1999). However, butyrate yield in acidogenic reactor was regulated by the growth condition of biomass and
levels of ATP and NADH, and generation of butyrate can meet both energy requirement and consumption of NADH (Girbal and Soucaille 1998; Macfarlane and Macfarlane 2003). Once the NADH is consumed, it is not available to convert acetoacetyl-CoA to butyryl-CoA, thus butyrate production would be reduced (Macfarlane and Macfarlane 2003). Furthermore, the presence of branched metabolic pathway that leads to the formation of ethanol and lactic acid redirect carbon and electrons away from butyrate and H₂. Optimization of butyrate production, through either manipulation of acidogenic conditions or metabolic engineering, requires a thorough understanding of the route and yield of the end products.

The fermentative pathways branching from pyruvate to various end products have been tentatively elucidated (Rydzak et al., 2009; Zhu and Yang 2004). Through these researches, the flow of carbon is quite clear. Basically, pyruvate can be converted into (i) CO₂, (ii) formate and acetyl-CoA, or (iii) lactate, reduced ferredoxin (Fd), and acetyl-CoA using pyruvate:ferredoxin oxidoreductase (PFOR), pyruvate:formate lyase (PFL), and lactate dehydrogenase (LDH), respectively. Acetyl-CoA is an important metabolite, which can be further catalyzed into (i) ethanol using either acetaldehyde dehydrogenase (AldH) and alcohol dehydrogenase (ADH) or a bifunctional acetaldehyde/alcohol dehydrogenase (AdhE) or into (ii) acetate using either phosphotransacetylase (PTA) and acetate kinase (ACK) or acetate thiokinase (ATK). However, the redirection of electrons in an acidogenic reactor under varying pH₂ is not clear and is the focus of this experiment.

In AD system, microbes play a pivotal role on the production of metabolites and channeling of electrons. Investigation of microbial community in anaerobic digesters has gained attention from researchers because understanding of microbial behavior is essential to improve the process performance (Rincón et al., 2013). Recent
developments in molecular techniques have provided useful tools to analyze microbial communities in different digester configurations (Heeg et al., 2014; Lee et al., 2009a; Rivière et al., 2009). However, information on monitoring acidogenic communities under regulated headspace $PH_2$ in an LBR of a two-phase system is lacking (Liu et al., 2002; Ueno et al., 2007). Monitoring the dynamics of microbial community in a two-phase AD process can provide valuable information that can be used to optimize conditions for efficient breakdown of wastes (Shin et al., 2010).

Therefore, this study aims at investigating the electron flux and dynamics of microbial communities in LBR with varying headspace $PH_2$, to reveal the possible mechanism and to evaluate the potential of $PH_2$ as a tool to alter or adjust the acidogenic products highly suitable for methanogenesis in the second phase reactor.

9.2 Materials and methods

9.2.1 Experimental setup and operation

This experiment is the continuation of Chapter 6; four levels of $PH_2$ were set up in acidogenic reactors (T1-control, T2-$H_2$-80%, T3-$H_2$-60% and T4-$H_2$-0.04%). Hybrid liquid-solid two-phase AD system including LBR coupled with UASB (LBR-UASB, Fig. 3.1) was chosen as the reactor configuration. Online pressure sensor was installed on the cap of each LBR and an online monitoring system with data log (AIDCS Monitor) was linked to the operational system for real-time record of the conditions. The whole experiment was conducted with constant temperature of 35°C.

To ensure the different hydrogen partial pressure ($PH_2$) levels in LBRs, mixed gases with designed $PH_2$, i.e. 80%, 60% and 0.04 % of $H_2$ balanced in $CO_2$ were flushed through the headspace of corresponding LBRs during the whole experimental period. The basic operation of reactor packing, ratios of inoculum and bulking agent
as well as the water regimes, sampling frequency follow the same pattern as described in Chapter 5.

### 9.2.2 End product analysis

Acidogenic end products mainly contain alcohols (ethanol, propanol and butanol), fatty acids (lactate, acetate, propionate, iso-butyrate, butyrate, iso-valerate, valerate and caproate) and acetone. After filtration of the leachate using 0.45 µm cellulose acetate membrane, acidogenic end products (except lactic acid) in the filtrate were analyzed using a HP 6890 Series gas chromatograph (GC, Hewlett Packard) with FID detector, while lactic acid was analyzed using Waters HPLC equipped with an Ultrasphere® ODS Column C-18 column (10 µm, 25 cm x 4.6 mm i.d.), (Beckman Coulter, USA). The preparation of mobile phase and analytical conditions were similar to that reported by Violeta et al. (2010). Concentrations of end products were calculated using standard curves obtained using corresponding standard solutions.

Redox values of end products, calculated as the number of oxygen atoms less one half the number of H₂ in each compound (Moat et al., 2002), were used to determine if all electrons were accounted for.

Elemental biomass composition (in mM) was calculated from protein content using a molecular weight of 131 g·mol⁻¹, corresponding to the average composition of cell material (empirical formula, C₆H₉O₃N) based on a stoichiometric conversion of substrate into cell material (Mosey 1983). The barometric pressure was taken into account during calculation of gas measurements.
9.2.3 Microbial community analysis

9.2.3.1 DNA extraction

Total genomic DNA was extracted from seed sludge and biomass obtained from the leachate and digestate samples at selected sampling points. Details of the extraction procedure were the same as described in Chapter 3.

9.2.3.2 DGGE and phylogenetic analysis

DNA extraction methods and procedures for PCR and DGGE were referred to Chapter 3.

9.2.3.3 Ordination

Principal component analysis (PCA) offers the visualization of high-dimensional distribution of datasets by plotting the strongest structure into reduced dimensions. PCA is an useful analytical method for gathering datasets of morphological, physiological and reactor performance information (Costa et al., 2009). In this study, the abundance values of microbial biomass quantified by Image Lab v4.1 (Bio-Rad) were used to generate data metrics of species information inside acidogenic reactors while the environmental datasets were comprised of pH, COD, TSP, ethanol, acetate, butyrate, caproate and lactate in the leachate as well as H$_2$ yield and pressure of H$_2$ (P$_{H_2}$) in the headspace. Software Canoco was chosen to evaluate the correlation between species and process performance in the joint plots.

9.2.3.4 Diversity index

In order to evaluate the bacterial diversity inside the acidogenic reactor, species diversity indexes were applied. The bacterial diversity among samples was evaluated using DGGE band profiles through the Shannon-Weaver diversity index (H’) (Keylock 2005), Berger-Parker index (d), Brillouin Index and Simpson’s index (Hill et al., 2003). The Shannon-Weaver diversity index was calculated as follows: H’
$= -\sum_p\ln(p_i)$, where the summation was overall unique bands i, and $p_i$ was the relative abundance of band i. The abundance of a particular band was determined using the pixel intensity in Image Lab v4.1.

9.3 Results and discussion

9.3.1 End-product analysis

Compared with the control, regulation of acidogenic headspace $PH_2$ in T4 at $3.3 \times 0.04\%$ psi increased the production of butyrate and $CO_2$ by as much as 97% and 73% while caproic acid decreased by 78 % (Fig. 9.1). Enhanced butyrate production should be attributable to the shift of metabolic pathway under low $PH_2$ and this is consistent with previous reports by Zoetemeyer et al. (1982) and Cohen et al. (1979) that butyrate-type fermentation was prevailing under low $PH_2$. $CO_2$ is generated during the degradation of pyruvate catalyzed by pyruvate:ferredoxin oxidoreductase (PFOR). Increased $CO_2$ evolution might due to the vigorous respiration of microbes inside the acidogenic reactor and also the regulation of headspace $PH_2$ which led to a headspace gas composition of >90% of $CO_2$ that triggered the activity of PFOR. Caproic acid was usually formed by chain-elongation of 2-C short-chain metabolites, but need specific environmental conditions (Agler et al., 2014). Significant increase of caproic acid in the control was probably due to the inhibition by high $PH_2$. Generation of $H_2$ was probably through reoxidization of additional reduced Fd generated via PFOR in response to pyruvate degradation. The increase in flux through butyrate-producing pathways results in increased ATP production, which is reflected by higher biomass yields in T4.
Acetate is an oxidized compound while ethanol has an opposite redox potential; however, both of them were generated from the branch of acetyl-CoA from pyruvate. The minor advantage of acetate and ethanol production in T4 implies the improved performance of acetyl-CoA pathway due to the regulation of headspace $P_{\text{H}_2}$. Lactic acid is sourced directly from pyruvate catalyzed by using lactate dehydrogenase (LDH), production of which was commonly controlled by operational conditions such as pH (Itoh et al., 2012). Almost the same amount of lactic acid recovery in the control and T4 suggested the presence of similar acidic conditions (pH 3.8-5.2).

9.3.2 Dynamics of microbial community

Considering the process of hydrolysis and acidogenesis phases under the regulation of headspace $P_{\text{H}_2}$, analysis of the dynamics of microbial community in the digesters is important and could reflect the state of performance. DGGE and subsequent phylogenetic analysis were conducted to characterize the structures of
micr
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communit
ing the
acidogenic reactor of the two-phase AD system (LBR-
UASB). Phylogenetic affiliations of the genomic sequences from DGGE bands were
determined by blast against the database of GenBank (National Center for
Biotechnology Information, NCBI). Neighbor-joining trees showing the phylogenetic
identities of the 16S rRNA gene fragments were also constructed (Figs. 9.3 and 9.4).
Fig. 9.2 illustrates the DGGE profiles of the 16S rDNA amplified from leachate and
digestate samples of the LBRs T1-T4 on day 1, 8 and 17. Each band on the DGGE
profile represents a specific bacterial species in the microbial community. The relative
abundance of the corresponding microbial species was indentified by the intensity of
the band. A total 60 bands (31 from the leachate and 29 from the LBR digestate) were
excised and used for identification (Fig. 9.2). The bacterial community in leachates of
all four treatments mainly consisted of the phylotypes Lactobacillus, Weissella, Alpha
Proteobacteria, Clostridium and Bifidobacterium (Fig.9.2a and 9.3); while the
bacterial species determined in the digestate contained representatives from the
following bacterial divisions: Lactobacillus, Spirochaetes, and Firmicutes excluding
Lactobacillus. Most of the bands resolved in the DGGE belonged to the phylum of
Firmicutes, especially the species of Lactobacillus, even 15 bands out of 29 isolated
from digestate affiliated with the phylotype of Lactobacillus.

Overall, bacterial band patterns in the seed sludge, leachate, and digestate
were distinct. The phylogenetic affiliations of the bacterial community in leachate
converged within three phyla, Alpha Proteobacteria, Actinobacteria (Bifidobacterium)
and Firmicutes (Lactobacillus, Weissella and Clostridium) (Fig. 9.3); however, the
species composition of the bacterial community shifted clearly between day 1 (1-T1,
1-T2, 1-T3 and 1-T4) and day 8 (8-T1, 8-T2, 8-T3, and 8-T4), between day 8 and day
17 (17-T1, 17-T2, 17-T3, and 17-T4), and among the four treatments within each
stage of the batch study (Fig. 9.2). Bands L10 and L11 were the two dominant bands along the whole experiment (Fig. 9.2a). Both of them were closely (99%) related to *Lactobacillus plantarum* (KJ152777.1), a gram-positive-aerotolerant bacterium that utilize various carbohydrates to form lactic acids, they also had the capability to liquefy polymers (Lamia and Moktar 2003). Species of *Lactobacillus* are a group of highly competitive bacteria and their constant presence in all reactors during all stages verifies this point.
Fig. 9.2 DGGE profiles of bacterial 16S rDNA extracted from the food waste leachate (a) and digestate (b) in the LBRs with different levels of headspace $P_{H_2}$, 1, 8 and 17 above the gel profile indicate the sampling points.
The intensity of band L16 was also dominant in day 1 (T1, T2, T3 and T4), and it showed 99% sequence similarity with *Weissella cibaria*. It was reported that *W. cibaria* was responsible for lactic acid production, a primary product at the earlier phase of hydrolysis/acidogenesis and they disappeared after the initial stage of the batch study (Ye et al., 2007). Bands L18, L20 and L21 were closely (99-100%) matched with *Clostridium* sp. (bands L18 and L21) and *Clostridium tyrobutyricum* (band L20), common H₂ production and acid-forming anaerobes (Chen et al., 2005). The presence of band L18 on day 8 and band L20 on day 17 were responsible for the behavior of H₂ production in corresponding treatment. However, band L21 was more distinct in lanes 8-T3 and 8-T4, suggesting the involvement of more H₂-producing bacteria and this was in agreement with higher H₂ recovery from the two reactors. Because of the largely changed composition of headspace (>90% of CO₂) in T4 did not induce the presence of band L21. Although the mechanism is unclear, CO₂ in the headspace of acidogenic reactor was considered as an obstacle of H₂ evolution, and removal of which has been developed as a strategy to increase H₂ production (Park et al., 2005). Bands L22 and L24 were affiliated to *Lactobacillus satsumensis* and *Lactobacillus helveticus* with sequence similarity of 98% and 99%, respectively. These two species are homofermentative, producing lactic acid as the major fermentative product (Tango and Ghaly, 1999; Endo and Okada, 2005). With high similarity, bands L26 and L27 were affiliated to *Acetobacter peroxydans* and uncultured *Prevotella* sp., respectively. *Bifidobacterium* sp. are strong degraders of organic compounds (Yue et al., 2012) and the presence of which may indicate better hydrolysis performance, bands L28 (17-T2, T3 and T4) and L31 (8-T2) were closely related to this genus. Generally, regulation of headspace PH₂ shifted the microbial community towards higher hydrolysis rate (T4) and H₂ production (T2 and T3).
The bacterial community in the digestate of the four treatments is illustrated in Figs. 9.2b and 9.4. Nearly half of the bands excised from DGGE (8.2b) were belonged to the genera of *Lactobacillus* sp., lactic acid producing bacteria, and commonly their presence would associate with low pH in the environment. Itoh et al. (2012) reported the measure to induce lactic acid production with low pH of < 4.0. Bands 22 and 23 were affiliated to *Spirochaetes* bacterium, species of which was not present in the leachate samples. *Spirochaetes* is a phylum of distinctive diderm bacteria, most of which have long, helically coiled (corkscrew-shaped) cells and may be suitable in the habitat of solid or slurry. Besides, species of *Spirochaetes* are expected to retrieve energy from the fermentation process (Delbes et al., 2000). DGGE bands belonged to Firmicutes other than *Lactobacillus* were also observed from the digestate, e.g. bands S1, S2, S3, S16, S19 and S24.

As evidenced from the above description, the experimental stages as well as the regulation of acidogenic headspace $PH_2$ had shifted the microbial community. Composition and abundances of microbial species in the leachate samples were largely different from that of digestate. In both leachate and digestate samples, the *Lactobacillus* sp. were the most abundant group, which was related to the low pH in both liquid and solid phases. Regulation of acidogenic headspace $PH_2$ and corresponding changes of headspace compositions triggered the activity of H$_2$-producing *Clostridium* sp. and strong organic compounds-degraders in T2, T3 and T4.
Fig. 9.3. Phylogenetic tree for 16S rDNA sequences obtained from leachate of LBR-T1, LBR-T2, LBR-T3, and LBR-T4 on day 1, 8 and 17. The evolutionary distances were computed using the Maximum composite likelihood method (Kemenas and Notredame 2009) and are in the units of the number of base substitutions per site. LBRL refers to the bands isolated from the DGGE gel of leachate samples.

208
Lactobacillus

- LBRS17
- Uncultured Bacteroidetes bacterium clone RSg13-32 (AB603818.1)
- LBRS22
- Uncultured Spirochaetes bacterium (KF356039.1)
- LBRS23
- Uncultured Spirochaetes bacterium clone SWHR3 (JQ346773.1)
- LBRS10
- Uncultured bacterium clone G250WV301AQI00 (KF336857.1)
- Uncultured Deltaproteobacteria bacterium (CU918113.1)
- LBRS25
- LBRS16
- Uncultured Clostridia bacterium clone G4 (EU551099.1)
- LBRS24
- Planococcus sp. B-QPkG8 (EU710708.1)
- LBRS1
- Uncultured bacterium clone 7N227hL54 (KJ853500.1)
- LBRS2
- Uncultured Bacilli bacterium clone MS005A1 F10 (EF705763.1)
- LBRS3
- Uncultured bacterium clone 13LEC04 (FJ163865.1)
- Uncultured Propionivibrio sp. clone RUGL1-569 (GQ420977.1)
- LBRS26
- Bifidobacterium thermophilum RBL67 strain RBL67 (NR 102973.1)
- LBRS27
Fig. 9.4 Phylogenetic tree for 16S rDNA sequences obtained from digestate (a and b) in LBR-T1, LBR-T2, LBR-T3, and LBR-T4 on day 8 and 17. LBRS refers to the bands isolated from DGGE gel of LBR digestate (solid) samples.
Diversity indexes in terms of Shannon-Weaver diversity index (H’), Berger-Parker index (d), Brillouin Index and Simpson’s index were calculated according to the intensities of major bands isolated from DGGE profiles (Tables 9.1 and 9.2). Generally, overall species richness estimated by the number of major bands present in DGGE profiles on day 1 and 17 were more diverse than that of day 8; and within the same sampling stage, regulation of acidogenic headspace \( PH_2 \) in treatments T2, T3 and T4 had induced to more diverse microbial communities. As presented in Table 9.1, Shannon-Weaver diversity index, H’ for the bacterial community of leachate samples on day 1 were lower than that of the later stages, i.e. day 8 and 17, ranging from 1.73-2.53. Regulation of \( PH_2 \) during the initial stage (day 1) led to relatively lower H’ in T2 and T3; however, when combined with the dominance index (D), the advantages were clear (0.39 and 0.42 in T2 and T3 while 0.30 in T1). For the Brillouin Index (E), the results of bacterial diversity were similar to H’. The results of bacterial diversity in terms of Simpson’s index were similar to that of dominance index (D).

### Table 9.1 Bacterial diversity index in leachates collected from LBRs with varying \( PH_2 \)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>No. of taxon</th>
<th>H</th>
<th>D</th>
<th>E</th>
<th>Simpson's Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-T1</td>
<td>15</td>
<td>1.87</td>
<td>0.30</td>
<td>1.59</td>
<td>0.18</td>
</tr>
<tr>
<td>1-T2</td>
<td>20</td>
<td>1.73</td>
<td>0.39</td>
<td>1.47</td>
<td>0.22</td>
</tr>
<tr>
<td>1-T3</td>
<td>21</td>
<td>1.76</td>
<td>0.42</td>
<td>1.46</td>
<td>0.24</td>
</tr>
<tr>
<td>1-T4</td>
<td>17</td>
<td>1.93</td>
<td>0.30</td>
<td>1.67</td>
<td>0.18</td>
</tr>
<tr>
<td>8-T1</td>
<td>18</td>
<td>2.04</td>
<td>0.26</td>
<td>1.89</td>
<td>0.15</td>
</tr>
<tr>
<td>8-T2</td>
<td>17</td>
<td>1.99</td>
<td>0.24</td>
<td>1.80</td>
<td>0.15</td>
</tr>
<tr>
<td>8-T3</td>
<td>14</td>
<td>2.00</td>
<td>0.28</td>
<td>1.81</td>
<td>0.15</td>
</tr>
<tr>
<td>8-T4</td>
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<td>1.85</td>
<td>0.36</td>
<td>1.54</td>
<td>0.19</td>
</tr>
<tr>
<td>17-T1</td>
<td>16</td>
<td>1.87</td>
<td>0.25</td>
<td>1.72</td>
<td>0.16</td>
</tr>
<tr>
<td>17-T2</td>
<td>19</td>
<td>2.42</td>
<td>0.21</td>
<td>2.14</td>
<td>0.10</td>
</tr>
<tr>
<td>17-T3</td>
<td>19</td>
<td>2.23</td>
<td>0.20</td>
<td>2.04</td>
<td>0.12</td>
</tr>
<tr>
<td>17-T4</td>
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<td>2.53</td>
<td>0.15</td>
<td>2.20</td>
<td>0.08</td>
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</tbody>
</table>

H-Shannon Diversity Index; D- Berger-Parker Index; E-Brillouin Index
Bacterial diversity indexes of digestate samples are depicted in Table 9.2. Similar to the trends of H' in leachate samples, the advantage of PH2 regulation was more obvious in T2, T3 and T4 compared with T1 in both day 8 and day 17 of the experiment. Trends of evenness index of Brillouin Index were same as that of H', whereas the dominance index and Simpson’s index did not totally follow the same trends, e.g. both index values of T4 on day 1 and T2 & T4 on day 17 were lower than the others. Overall, the microbial diversity indexes showed that regulation of headspace PH2 improved diversity of the bacterial community.

Table 9.2 Bacterial diversity index in digestates of LBRs with varying PH2

<table>
<thead>
<tr>
<th>Treatments</th>
<th>No. of taxon</th>
<th>Bacterial diversity index</th>
<th>Simpson's Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-T1</td>
<td>16</td>
<td>H 1.51 D 0.51 E 1.28</td>
<td>0.32</td>
</tr>
<tr>
<td>8-T2</td>
<td>15</td>
<td>1.52 0.50 1.36</td>
<td>0.31</td>
</tr>
<tr>
<td>8-T3</td>
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<td>1.62 0.57 1.33</td>
<td>0.34</td>
</tr>
<tr>
<td>8-T4</td>
<td>16</td>
<td>1.66 0.49 1.47</td>
<td>0.28</td>
</tr>
<tr>
<td>17-T1</td>
<td>11</td>
<td>1.60 0.48 1.38</td>
<td>0.29</td>
</tr>
<tr>
<td>17-T2</td>
<td>17</td>
<td>2.19 0.21 1.94</td>
<td>0.13</td>
</tr>
<tr>
<td>17-T3</td>
<td>15</td>
<td>1.73 0.37 1.51</td>
<td>0.22</td>
</tr>
<tr>
<td>17-T4</td>
<td>18</td>
<td>2.12 0.21 1.87</td>
<td>0.13</td>
</tr>
</tbody>
</table>

H-Shannon Diversity Index; D- Berger-Parker Index; E-Brillouin Index
9.3.3 Ordination analysis

Fig. 9.5 Ordination plots of leachate samples from LBRs with different PH$_2$: species (a), samples (b), species and samples (c), species and environmental factors (d). The species data (band intensity) were generated by Image Lab v4.1 (Bio-Rad) while the environmental data of pH, H$_2$ production, PH$_2$, COD and TSP production, and concentrations of ethanol, acetate, lactic acid, butyrate, caproic acid were used to generate the data matrices for the performance.
PCA is widely known as a useful technique to visualize the relationships between species communities and environmental conditions with the advantage of effective reduction of the multi-dimensional space into a few components. In this study, score plots of microbial species and leachate samples from four treatments with different $PH_2$ (Figs. 9.4 a and b), as well as joint plots of species and samples (Fig. 9.4c), species and environmental factors (Fig. 9.4 d) were analyzed.

PCA is very useful in grouping samples according to characterizations of samples (Abouelwafa et al., 2008). In the score plots of the first and second PCs (Fig.9.4 a and b), the distributions of species and samples were grouped according to the reaction stages as well as the influence of headspace $PH_2$. The combined results of batch reaction stages and headspace $PH_2$ led to the groups of 1-T1, 1-T2 and 1-T3 samples of day 1 in the second quadrant, 8-T1, 8-T3 and 8-T4 samples of day 8, 17-T1, 17-T2, and 17-T4 samples of day 17 whereas samples 1-T1, 8-T2 and 17-T3 were grouped in together in the third quadrant. The regrouped distributions of samples other than the phased stages of the batch experiment should be due to the regulation of headspace $PH_2$. Despite sample 1-T4 occurred in the first stage of the batch study, it grouped with samples from the second and third stages with the regulation of $PH_2$.

In the joint plots of samples and species as well as environmental factors plus species, the influence of each variable could be visualized by the weighted variables, and respective loading maps (Figs. 9.4 e and d). The joint plots allow to identify the variables that are the most important for the differences observed among samples. The species that dominated in closely related samples were clear, e.g. species 7 ($Weissella cibaria$), 9 ($Weissella confusa$) and 16 ($Weissella cibaria$) were grouped with samples 1-T1, 1-T2 and 1-T3. $Weissella$ sp. that accounted for lactic acid production, which is in agreement with the characteristic of the initial stage that easily degradable
carbohydrates in the food waste coupled with relatively low pH led to the generation of lactic acid (Zhang et al., 2007). Sample 1-T4 was closely grouped with species 30 (Uncultured Ruminococcaceae bacterium) and 31 (Bifidobacterium animalis), which are strong organic matter degraders (Yue et al., 2012) while samples 8-T2 and 17-T3 grouped with species 28 (Bifidobacterium thermophilum) and 20 (Clostridium tyrobutyricum). Clostridium tyrobutyricum was a common H₂ production bacterium in anaerobic digestion system (Jo et al., 2008). It is logical to link the major functional species with the performance of the reactors. Most of the environmental factors were grouped in the second quadrant, suggesting the major difference in reactor performance was caused during the initial stage of experiment. Obviously, regroup of samples 1-T4, 8-T2 and 17-T3 was attributable to PH₂ regulation as well as concentrations of ethanol and lactic acid. H₂ production grouped in the last quadrant with samples of day 17.

PCA analysis of the solid digestate was illustrated in Fig. 9.6. Groups of species, samples of different treatments & stages and environmental factors were present in Figs. 9.6 a, b and c, respectively, while the joint plot of species and environmental factors was interpreted in Fig. 9.6 d. Different from the distribution of leachate samples, sample 8-T1 was separated from 8-T2, 8-T3 and 8-T4 with regulations of PH₂ while 17-T1 & 17-T3 and 17-T2 & 17-T4 were grouped separately. Separation of 8-T1 (control) from 8-T2, 8-T3 and 8-T4 verified the effective role of PH₂ regulation. In the later stage, 17-T1 was grouped with 17-T3 (H₂-60%) probably due to similar headspace compositions were achieved in both reactors. In the joint plot H₂ production and PH₂ were grouped with species 22 (Uncultured Spirochaetes bacterium) and 15 (Lactobacillus plantarum) while
concentration of butyrate was grouped with species 16 (Uncultured Clostridia bacterium) and 17 (Uncultured Bacteroidetes bacterium).

Fig. 9.6 Ordination plots of digestate samples from LBRs with different PH2: species (a), samples (b), environmental factors (c), species and environmental factors (d). The species data were generated by Image Lab v4.1 (Bio-Rad) while the environmental data of pH, H2 production, PH2, COD and TSP production, and concentrations of ethanol, acetate, lactic acid, butyrate, caproic acid were used to generate the data matrices for the performance.
In all, the ordination of the species and operational factors was closely related to the regulation of headsace $PH_2$ and corresponding changes of composition. Low level of $PH_2$ was grouped with $H_2$-specific microbes and strong organic degraders, and also related to high $H_2$ evolution and the generation of acetate and butyrate.

**9.3.4 Redirection of carbon and electron flux**

It is critical to understand the factors that dictate the distribution of carbon and electron fluxes towards end metabolites during the optimization of two-phase AD of food waste. In this experiment, proposed metabolic pathways and redirection of electron fluxes were elucidated by measuring various end products in response to different $PH_2$.

Considering the complex oxidative-reductive relationships in acidogenic reactor, functional enzymes that catalyzed the metabolic pathways were quite difficult to measure. Therefore, in the absence of specific enzymatic data, the following assumptions were made to analyze the possible redirection of electron flux: (i) $CO_2$ production is equal to reduced Fd production catalyzed by PFOR; (ii) NADH production is constant during glycolysis; and (iii) NAD(P)H consumption/production can be determined from lactate, ethanol, and $H_2$ production measurements (Rydzak et al., 2014). In this experiment, the control treatment (T1) and T4 with $PH_2$ as low as 3.3 psi $\times$0.04% were selected to analyze the possible redirection of electrons and get insight for metabolic manipulation. The left panels of Fig. 9.7 a and b illustrate how flux through pathways leading to major acidogenic end products (lactate, butyrate, acetate, and caproate) are redirected in response to regulation of headspace $PH_2$. The right panels demonstrate how electrons can flow between Fd, NADH, NADPH, and $H_2$ using the “malate shunt” (Fig. 9.7 a and b). Note that electron carriers coupling
Regulation of acidogenic headspace $PH_2$ in T4 resulted in increased production of butyrate, acetate and CO$_2$ and decreased production of caproic acid (Figs. 9.1 and 9.7). Using the canonical pathway, additional reduced Fd produced by metabolism of pyruvate into acetyl-CoA via PFOR can be converted into H$_2$ using Fd dependent H$_2$ase (Fig. 9.7a). However, given that the production of reduced Fd is greater than the H$_2$ production (by 110 and 230 mM in the control and T4, respectively), excess reduced Fd produced must be utilized by alternative pathways. This can be accomplished by NFOR. Electrons transferred from NADH to NADP$^+$, which can subsequently be used for biomass or amino acid yield were expected. Similarly, Rydzak et al. (2009) observed this phenomenon in *Clostridium thermocellum*. Transhydrogenation can be accomplished by increasing flux either through the malate branch (Fig. 9.7; black integers) or via NfnAB, which simultaneously uses reduced Fd and NADH to produce double NADPH (Fig. 9.7a; white integers).
Fig. 9.7 Proposed redirection of carbon and electron fluxes in response to (a) no headspace $\text{PH}_2$ regulation and (b) regulation of headspace $\text{PH}_2$ at 3.3 psi $\times$ 0.04%. Carbon and electron fluxes based on changes in measured end products (shown in gray boxes) are depicted in the left panel. End-product redox values are provided in...
superscript next to the corresponding end product. Right panel depicts predicted changes in the electron flux using only a NFOR and Fd-dependent H2ase (black integers in boxes) or NFOR, Fd-dependent H2ase, and NfnAB (white integers in boxes). Note that proposed electron flow through the malate shunt and toward biomass production is also indicated in the right panel. Positive values indicate increased flux while negative values indicate decreased flux in the direction depicted. Solid lines reflect carbon and electron fluxes, and broken lines reflect electron flux only. Blue numbers in the bracket were EC numbers of functional enzymes.

It is important to stress that the anaerobic environmental conditions and in turn the steady state intracellular redox potentials (i.e., ratios of NADH/NAD\(^+\), Fd\(_{\text{red}}$/Fd\(_{\text{ox}}\), and NADPH/NADH) affect the direction of electron flux greatly. For example, under standard condition the conversion of glucose to butyrate is thermodynamically favorable ($\Delta G = -254$ kJ/mol) via acetyl-CoA pathway. However, given high headspace $P_{H_2}$, the production of butyrate became comparatively less favorable ($\Delta G = -246$ kJ/mol). Consequently, the evolution of $H_2$ associated with butyrate production would become thermodynamically less favorable. Similarly, Buckel and Thauer (2013) reported that $H_2$ production was highly favorable (-37 kJ/mol) when the $H_2$ partial pressure was around 10 pa ($1.45 \times 10^{-3}$ psi) under anaerobic conditions. Therefore, elucidation of electron flux and accurate measurement of steady state redox potentials may bring forth alternative metabolic engineering strategies to redirect carbon and electron fluxes toward desired pathways and target end products (Veit et al., 2008; Cho et al., 2011).

**Summary**

Distribution of metabolic end products and proposed redirection of electrons are the ways to interpret the mechanism of enhanced performance of acidogenic rectors.
under the regulations of headspace $PH_2$. Both headspace $PH_2$ regulation and the gas composition influenced the performance of digesters. Regulation of headspace $PH_2$ at low levels altered the microbial community resulting in increased dominance of strong organic compounds degraders ($Bifidobacterium$ sp.) and H$_2$-producing microbes ($Clostridium$ sp.) in T4 and T2-T3, respectively. Microbial diversity index analysis also confirmed the advantage of $PH_2$ regulation. The ordination of the microbial data, and factors representing the performance of digesters further offered clear map of interactions among the microbial species and operational conditions (especially $PH_2$).
CHAPTER TEN

CONCLUSIONS AND RECOMMENDATIONS FOR FURTHER RESEARCH

10.1. Introduction

Anaerobic digestion of food waste is a promising strategy for both waste disposal and energy recovery, especially under the situation of energy crisis. Energy efficiency is the key issue during the scaling up of AD technology to commercial practice. Hence, overall objective of this study is to enhance energy recovery using two-phase AD.

Two-phase AD is gaining attention due to the fact that it can offer more stable operation process and is easy to optimize acidogenesis and methanogenesis separately. However, the energy efficiency of two-phase AD system was only marginally higher or even equal to single phase that do not justify the addition of one more reactor, which limits industrial application. Therefore, increasing the energy recovery is the key solution. Researchers have proposed various measures to enhance hydrolysis rates of organic particles; however, most of them were focused on the optimizations of the operational and environmental conditions (Lü et al., 2008; Stabnikova et al., 2008; Selvam et al., 2010; Xu et al., 2011), modification of reactor configurations or sources of seed sludge (Mshandete et al., 2005; Yan et al., 2014a), optimization of the composition of substrates (co-digestion) (Li et al., 2013; Silvestre et al., 2014), addition of nutrient elements (Hu et al., 2008; Zhang et al., 2012), and addition of specific enzymes (Jayasinghe et al., 2011), etc. Most of these methods,
although reported to be effective on improving hydrolysis of food waste, they could increase the overall investment that may limit their application (Carballa et al., 2011). Hence, an integrated approach that exploits the reactor operation as well as the microbes is more appropriate and economical.

Based on the mass balance analysis of previous two-phase AD process (Clark et al., 2012; Xu et al., 2014), ~30% of the consumed substrates was converted to acidogenic biogas (H₂ and CO₂), which was not effectively utilized under normal operation. Conversion of this part of energy to final product is promising and rewarding. Hydrogenotrophic methanogens in the methanogenic reactor can make use of these H₂ to reduce CO₂ to CH₄ thus requiring only to divert the H₂/CO₂ to the methanogenic reactor. Diversion of acidogenic off-gas out of acidogenic reactor would alter the headspace condition inside the acidogenic reactor, which will further cause shift in metabolic pathways in the system and consequently the composition of quality of leachate produced. It is necessary to assess the shift of metabolic pathways under acidogenic off-gas diversion and evaluate the leachate qualities. Besides, the efficiency of reutilization depends on H₂ to CO₂ ratio in the acidogenic off-gas, having the potential to influence the methane content of the biogas produced. Therefore increasing the H₂ to CO₂ ratio in the acidogenic off-gas is a potential strategy to upgrade biogas.

The efficiency of second-phase methanogenesis is highly dependent on the performance and intermediate products of hydrolysis. High hydrolysis rate of the substrate in the acidogenic reactor fulfills sufficient substrate for the subsequent methanogenesis. However, it is not just the quantity while the composition of the leachates could affect the efficiency of the methanogenic reactor. Ethanol, acetate, propionate, butyrate, lactate and caproate are the common compounds generated in an
acidogenic reactor during hydrolysis/ acidogenesis of food waste. Ethanol is a more reduced product with the highest energy value among the acidogenic products (Pipyn and Verstraete, 1981). The observation of Pipyn and Verstraete (1981) indicated that it might be possible to increase CH$_4$ production by directing the metabolic pathway towards ethanol and lactic acid production. However, the conversion of ethanol to CH$_4$ needs its prior oxidation to acetate and this reaction is thermodynamically unfavourable under standard conditions. Moreover, high ethanol concentration was reported to be inhibitory to propionate-degradation and eventually posing toxicity to methanogenesis (Smith and McCarty 1989). But till now the toxic limit of ethanol in different systems was not adequately defined. Ethanol production can also be inhibited by co-existing short-chain fatty acids (Zhang et al., 2010). Acetate is recognized as a key intermediate product in the final step of mineralization of organic materials (Scholten and Conrad 2000), production of which is generally increased along the reaction time. Acetate is not only sourced from degradation of pyruvate through acetyl-CoA pathway directly, but also can be produced formed from the syntrophic oxidation of propionate and butyrate (Müller et al., 2010).

It is widely known that acetate is the major intermediate during the bioconversion of organic compounds to CH$_4$ and CO$_2$, and acetate represents the substrate for ~ 70% of the total methane produced during anaerobic digestion (Gujer et al., 1983; Smith and Mah. 1966). Thus, the recovery of CH$_4$ from acetate is an important step in the anaerobic digestion process. Generally, acetate formed via fermentation of sugars and amino acids or via $\beta$-oxidation of LCFA is converted to CH$_4$ and CO$_2$ through aceticlastic methanogenesis (Jones et al., 1987; Mah et al., 1978; Zehnder et al., 1980). However, methanization of acetate can also be processed by co-function of acetate-oxidizers and hydrogenotrophic methanogens (Hao et al.,
Thus, the kinetics of acetate utilization in methanogenic phase would depend on the predominant species in the population of aceticlastic/hydrogenotrophic methanogens. Butyrate is one of the most popular acidogenic products, and formation of which usually generates H\textsubscript{2}. Methanization of butyrate and propionate occurs through their oxidation to acetate by H\textsubscript{2}-producing acetogens; and subsequent use of acetate by aceticlastic methanogens. The acetogens require a low $PH\textsubscript{2}$ and the reactions are endergonic; therefore must be syntrophically coupled with hydrogenotrophic methanogenesis. Butyrate was reported as the most favourable substrate for methanogens due to its higher efficiency relative to other VFAs (Öztürk 1991; Wang et al., 1999).

AD is a biochemical process carried out by a consortium of anaerobic microbes and the efficiency depends on the balance among these different groups of microorganism. Therefore, any change in the operation would affect the dynamics and diversity of the microbial community, and exploiting them beneficially is the key to achieve an efficient process.

With these backgrounds, a series experiments was carried out in four phases. In the first phase, feasibility of diversion of acidogenic off-gas from acidogenic LBR to methanogenic UASB was confirmed and also the contribution of acidogenic off-gas to overall energy recovery was demonstrated. Experimental details and results of Phase I was presented in Chapter 4. Phase II aimed to optimize energy recovery by regulating the metabolic pathways. Two experiments were conducted in this phase, i.e. the acidogenesis was performed with different headspace pressure and partial pressure of H\textsubscript{2} ($PH\textsubscript{2}$) as detailed in Chapters 5 and 6. In order to further increase H\textsubscript{2}/CO\textsubscript{2} ratio in acidogenic off-gas, different neutralization modes and compositions of substrates were investigated in Phase III and these parts were covered by Chapters
7 and 8. Finally, dynamics of microbial community and proposed redirection of electron flux associated with prevailing metabolic pathways were analyzed in the Phase IV (Chapter 9).

10.2 Feasibility of diversion of acidogenic (LBR) off-gas to methanogenic UASB for reutilization

The first experiment was to check the feasibility of reutilization of acidogenic off-gas through diversion to methanogenic UASB, and the second one was aimed at revealing the contribution of acidogenic off-gas and the improved leachate quality on enhancing the CH$_4$ recovery.

Compared with the control (without reutilization of acidogenic off-gas), diversion of acidogenic off-gas from LBR to UASB increased methane (CH$_4$, 0.28 L/g VS$_{added}$) production up to 38.6%. Besides, diversion of acidogenic off-gas improved the hydrolysis constant and COD production to 0.053 d$^{-1}$ and 0.61 g/g VS$_{added}$ compared with 0.042 d$^{-1}$ and 0.48 g/g VS$_{added}$ in the control, respectively. The increased production of CH$_4$ was probably contributed by both the improved quality of leachate and the reutilization of acidogenic off-gas. In the second experiment, LBR-control and LBR-gas were operated similar to the first experiment, whereas leachate fed to both UASB-control and UASB-gas was the same in composition, leaving reutilization of acidogenic off-gas as the single factor influencing the UASB performance. Deducting of the contribution of leachate, there was ~8% increase of methane gas, which should be contributed to reutilization of acidogenic off-gas. Experimental results from this phase confirmed the feasibility of reutilization of acidogenic off-gas in methanogenic reactor (UASB).
10.3 Optimization through manipulation of metabolic pathways in acidogenic reactor

Metabolic pathway determines the distributions of intermediate soluble products and the quality of acidogenic leachate. Therefore, in this phase two experiments focusing on manipulating metabolic pathways were performed. In Chapter 5, four levels of headspace pressures were applied: 6-12 psi (T1), ~3-6 psi (T2), ~3 psi (T3) and ambient pressure (T4). Mixed acids fermentation was observed in all the treatments with butyrate as the single largest component, which is an efficient precursor for the subsequent methanogenesis. Despite similar metabolic pathways, the distributions of soluble products were varied with different headspace pressures. Acetate, butyrate and propanol production were comparatively higher in T2 and T3 reactors while lactate was higher in T1. Regulation of headspace pressures at lower levels led to enhanced COD and soluble product generation in T2, T3 and T4 reactors; whereas, very high pressure in T1 resulted in comparatively higher lactate production and lower protein hydrolysis. A pressure of 3-6 psi (in T2 and T3) improved the production of COD by ~22-36%, soluble products (include VFA and solvents) by ~9-43%, VS reduction by ~14-19%, and CH₄ production by ~10-31%; and were significantly higher than T1 and T4 reactors. Then, four different levels of \( PH₂ \) in the LBR headspace to regulate the metabolic pathways were investigated in Chapter 6: control with naturally generated \( PH₂ \) (T1), whereas in other three treatments, a headspace pressure of 3.3 psi was maintained as identified in Chapter 5 while the \( H₂ \) contents were varied as 80% (T2), 60% (T3) and 0.04% (T4). Typical butyrate fermentation pathways dominated in T4 whereas mixed acid fermentation pathways prevailed in the other three treatments. Low \( PH₂ \) enhanced the general performance of acidogenic reactor in terms of COD production. In T4, a cumulative
COD production of 0.84 g/g VS$_{\text{added}}$ was achieved, corresponding to 39.7% increase compared with the control (T1). Because of the improved performance of hydrolysis/acidogenesis and higher quality of acidogenic products, overall CH$_4$ recovery in T4 (301 L/kg VS$_{\text{added}}$) was 44.6% higher than the control, whereas the values observed in T1, T2 and T3 were 208.1, 238.2 and 208.8 L/kg VS$_{\text{added}}$, respectively. Therefore, during the reutilization of the acidogenic off-gas in UASB, maintaining a low PH$_2$, e.g. 0.04% of H$_2$ facilitates achieving increased hydrolysis and methane production.

### 10.4. Enhanced CH$_4$ recovery through controlling acidogenic biogas production

Acidogenic biogas contributed to overall energy recovery and hence strategies to enhance acidogenic biogas production were investigated in this phase. Firstly, four types of neutralization modes were investigated in Chapter 7, including daily pH adjustment of recycling LBR leachate to 6.0 (T4), methanogenic effluent recirculation (T3), and initial addition of NaOH (T1) and lime (T2) separately at a dosage of 20 and 14 g/kg food waste, respectively. Different neutralization modes had resulted in different H$_2$ profile in terms of production rate and H$_2$/CO$_2$ ratio. Obviously, H$_2$ production rates of 3.0 and 2.1 L/d with lime and NaOH addition were much higher than 0.7 and 0.4 L/d observed with effluent recirculation and daily pH adjustment, respectively. Furthermore, addition of alkali agents increased the ratio of H$_2$/CO$_2$ to $>4.0$ on day 3 whereas the highest values of the other two treatments were lower than 1.0. Thus, addition of alkaline agents was more favourable when reutilization of acidogenic off-gas is applied. Addition of alkali agents also enhanced the COD production, especially in T1 with a cumulative COD production of 736.6 g/kg VS$_{\text{added}}$, corresponding to 45.9% increase compared with that of daily pH adjustment.
Considering the contribution of reutilization of acidogenic off-gas, the highest CH$_4$ production of 11.2 L/d was achieved in treatment with lime addition. This together with the fact that lime is much cheaper than NaOH, lime treatment was considered the best in terms of enhanced hydrolysis as well as the feasibility for reutilizing acidogenic off-gas. In Chapter 8, the effect of different carbohydrate contents in the substrate on acidogenic H$_2$ production was studied. Anaerobic hydrolysis of organic wastes sourced from bakery (T1), Chinese-style restaurant (T2), western-style restaurant (T3), and wet market (T4) were performed in LBRs. H$_2$ generation was closely related to the composition of substrate. In T3, a composition of carbohydrate: protein: lipid at 69.5: 14.4: 12.8 achieved the highest H$_2$ production of 61.0 L/kg VS$_{\text{added}}$. Ethanol, acetate and butyrate were the common fermentation products of the four treatments and variations in the production and speciation of the soluble products were closely related to the composition of substrates when the operational conditions were similar. Fermentation pathways with dominance of butyrate and acetate were favourable for H$_2$ production and subsequent methanogenesis. High volatile solids (VS) removal efficiency (76.7%) was observed with western-style restaurant food waste; whereas, it ranged from 37% to 55% in the other treatments. High cumulative COD production, i.e. 0.65 gCOD/gVS$_{\text{added}}$, solubilization degree as high as 83% and the highest specific methane production rate at 0.42 L/gVS$_{\text{added}}$ was achieved with western-style restaurant food waste.

**10.5 Dynamics of microbial community and proposed redirection of electron flux**

In phase IV, the dynamics of microbial community and possible redirection of electron flux associated with shift of metabolic pathways was proposed based on the second experiment of Phase III. Significant increase in the production of butyrate in
treatment with $PH_2$ of 3.3 psi × 0.04% indicated the channelling of electrons towards the production of butyrate while in the other three treatments the electrons shunted towards mixed acid fermentation. Dynamics of the microbial community, monitored by fingerprinting of bacterial 16S rRNA genes were correlated with the distribution of metabolites. In T1, $PH_2$ of 3.3 psi without external gas flushing, lactic acid fermentation was dominant during the initial 7-days. Accordingly, phylotypes affiliated to the genus *Lactobacillus* sp. were detected. A heterolactic fermentation pathway was observed in both T2 ($PH_2$ of 3.3 psi × 80%) and T4 ($PH_2$ of 3.3 psi × 0.04%) during the first four days, and thereafter the fermentation pathways shifted to pathways with acetate and butyrate as the dominant products. The pathways dominated by acetate and butyrate were accompanied by increased acidogenic biogas production and changing the microbial community with phylotypes of *Clostridium* sp. and *Bifidobacterium* sp. becoming dominant.

10.6 Recommendations for Further Research

The ultimate objective of this study is to support scaled-up application of two-phase AD of solid waste and to solve the mounting solid waste (food waste) problem with efficient energy recovery. Reutilization of acidogenic (LBR) off-gas in methanogenic (UASB) reactor was effective enabling both increased overall energy (CH$_4$) recovery and achieving carbon sequestration. Methane generation as high as 0.42 L-CH$_4$/ g VS$_{added}$ was achieved with food waste collected from western-style restaurant under the diversion of acidogenic off-gas and lime addition as neutralization reagent. Regulation of metabolic pathways in acidogenic reactor by controlling headspace pressure and partial pressure of H$_2$ ($PH_2$) indicated that a moderate level of headspace pressure (3.3 psi) and a low $PH_2$ (3.3 psi ×0.04%) was favourable for hydrolysis/acidogenensis that increased the overall energy recovery
from 0.25 \( \text{L-CH}_4/\text{g VS}_{\text{added}} \) to 0.32 \( \text{L-CH}_4/\text{g VS}_{\text{added}} \) with butyrate pathway prevailing in the reactors. Enhanced \( \text{H}_2 \) production was achieved through selection of neutralization modes and carbohydrate composition of substrate. In this study, the effort was focused on the performance and how to exploit the existing potential of acidogenic phase during two-phase AD; and hence studies regarding the performance of methanogenic reactor were not carried out systematically. Although the methanogenic reactor was a focus of many studies, its dynamism and efficiency under a two-phase systems were not investigated adequately and need to be further explored. Besides, I also believe that researches conducted in methanogenic phase under the scheme of acidogenic off-gas diversion would definitely give more insights for scaling-up this technique.

Furthermore, regulation of metabolic pathways to obtain target acidogenic products is really an amazing research area; and therefore further investigation of this topic will give more information as well as insights regarding energy recovery (or high value-added products) from organic waste. The metabolic pathways occurred in the reactors are the results of shunting electron flux, and hence study of metabolic engineering regarding the redirection of electron flux is an interesting research area.

The main advantage of two-phase systems is not its putative higher reaction rate, but rather a greater biological reliability for waste disposal, and therefore a thorough investigation of the functional microbes by using metagenomic techniques in the two-phase system could add more knowledge for further application of this technology.
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