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UNIVERSITY**

**EFFECTS OF MICROBIAL COMMUNITY, LIPIDS
AND PRETREATMENT ON THE PERFORMANCE
OF TWO-PHASE ANAEROBIC DIGESTION**

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**SCHOOL OF CIVIL & ENVIRONMENTAL ENGINEERING
2009**

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Effects of Microbial Community, Lipids and Pretreatment on the Performance of the Two-Phase Anaerobic Digestion

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ABSTRACT

Compared to conventional anaerobic digestion (AD), two-phase AD strategy, in which acidogenesis and methanogenesis reactions can be optimized separately, offers a robust and flexible means to better control the process of decomposing organic solid waste to renewable energy at an upgraded level. In recent years, a modified two-phase AD system, known as the hybrid anaerobic solid-liquid (HASL) system, was successfully developed by the Nanyang Technological University (NTU). The HASL system is characterized by recirculating alkaline methanogenesis phase broth to buffer the quick food waste acidity that is often encountered in the acidogenesis phase, so as to realize a self-relief of pH inhibition with no need for external chemical addition. Albeit the HASL system has demonstrated excellent capacity in overcoming the inherent pH issue in conventional food waste AD, information about its potential in mineralizing the recalcitrant food waste components is very limited. Technically, food waste is a mixture of organic substances with various biodegradabilities. The recalcitrant fraction tends to pass through the AD system without sufficient degradation. Some hydrolyzed products from food waste may even be toxic to microorganisms responsible for AD. Meanwhile, the efficiency of the hydrolysis, the rate-limiting step in AD, also determines the ultimate methane yield in the overall process. Thus, enhancement of hydrolysis is critical to improve the overall performance. For these regards, this thesis is focused on the influence of the food waste recalcitrant fraction on the performance of the HASL and the enhancement of hydrolysis by thermal and freeze/thaw pretreatments, with molecular biotechnology as an insightful tool.

In the first part of the study, the HASL was started up by enriching slow growing methanogens in the form of anaerobic granules in the methanogenic reactor. The successful startup in this step will ensure methanogenesis reaction will not be a rate limiting step in subsequent food waste AD studies. Fluorescence *in situ* hybridization (FISH) technique was employed in conjunction with a confocal laser scanning microscopes (CLSM) to visualize the methanogens community in

anaerobic granules. The amount of methanogens was also quantified by an innovative molecular tool as specific binding of Arc915 probe, i.e. specific binding of Arc915 probe was in linear function with the concentration of methanogens population by enumeration in anaerobic sludge when it was between 0.7 and 14.0 mg mL⁻¹. The consistency of this new method was high with a low VOC of 5-19%, which was necessary for the parameter measurements of microbial growth and activity and thus provided a better means for subsequent microbial studies in the two-phase AD.

In the second part of the study, the effect of lipids, the major recalcitrant food waste component, on the HASL system performance was investigated by digesting food waste with different percentages of lipids. It was shown that the lipid content has a two-faced effect on the HASL system. According to present studies, the positive effect of lipids dominated AD, with a greater than 88% COD removal and an 18% increase of methanogens growth rate when its contents were controlled within the range of 20-30%. Lipids content higher than 40% would be unfavorable for two-phase AD due to the toxicity of the long chain fatty acids produced by hydrolysis of lipids, which is reflected by lower COD removal and slower methanogens growth rate.

The potential role of food waste pretreatment in the HASL performance enhancement was studied by the thermal and freeze/thaw methods in the third and fourth parts, respectively. The optimal temperature and duration in both pretreatments for a defined food waste was determined in a series of batch experiments and verified in the subsequent HASL performance study. It was found that both thermal and freeze/thaw pretreatments enhanced the performance of the HASL system in terms of higher and faster SCOD, VFA and methane production, which reduced the time needed to produce the same quantity of methane, up to 25%-48% with thermal pretreatment and around 42% with freeze/thaw pretreatment.

The positive effect of thermal pretreatment on the HASL system can also be ascribed to the superior microbial community being successfully cultivated

separately in the two-phase reactors. Polymerase chain reaction and the denaturing gradient gel electrophoresis (PCR- DGGE) technique showed that the number and types of dominant species in the acidification phase changed dramatically with the extent of thermal pretreatment, answering the reason why better hydrolysis and acidification results were obtained with pretreated food waste.

Finally, the stability, toxicity and fertility of the HASL residue were testified through a series of tests. Results showed that the residue had positive effects on cucumber growth. It had shown fertility and non-toxicity from 1% to 3% extract concentration, and seed germination was improved significantly at 0.5 - 1 g L⁻¹ extract concentration. However, the residue from the HASL system was still unstable because of a relatively high C/N ratio and consequently could not be applied directly as fertilizer without any further treatment.

Keywords: anaerobic digestion (AD), F₄₂₀, FISH, food waste, freeze/thaw, hybrid anaerobic solid-liquid (HASL) system, lipids, methanogens, PCR-DGGE, thermal pretreatment

PUBLICATIONS

Journal papers:

1. **X.Y. Liu**, H.B. Ding, S. Sreeramachandran, O. Stabnikova and J.Y. Wang (2008). Enhancement of food waste digestion in the hybrid anaerobic solid-liquid system. *Water Science and Technology* 57 (9): 1369-1373.
2. H.B. Ding, **X.Y. Liu**, O. Stabnikova and J.Y. Wang (2008). Effect of protein on biohydrogen production from starch of food waste. *Water Science and Technology* 57 (7): 1031-1036.
3. Stabnikova O., **Liu X.Y.** and Wang J.Y. (2008) Digestion of frozen/thawed food waste in the hybrid anaerobic solid-liquid system. *Waste Management* 28 (9), 1654-1659.
4. Stabnikova O., **Liu X.Y.**, Wang J.Y. and Ivanov V. (2006) Quantification of methanogens by fluorescence *in situ* hybridization with oligonucleotide probe. *Applied Microbiology and Biotechnology* 73(2): 696-702.
5. Wang J.Y., **Liu X.Y.**, Kao J.C.M. and Stabnikova O. (2006) Digestion of pre-treated food waste in hybrid anaerobic solid-liquid (HASL) system. *Journal of Chemical Technology and Biotechnology* 81(3): 345-351.
6. Stabnikova O., Ang S.S., **Liu X.Y.**, Ivanov V., Tay J.H. and Wang J.Y. (2005) The use of hybrid anaerobic solid-liquid (HASL) system for the treatment of lipid-containing food waste. *Journal of Chemical Technology and Biotechnology* 80(4): 455-461.

Conference papers:

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2. **Liu X.Y.**, Stabnikova O. and Wang J.Y. (2006). Enhancement of food waste anaerobic digestion in hybrid anaerobic solid-liquid (HASL) system. *Water and Environmental Management Series*, 265-270. *The 3rd IWA Young Researchers Conference*. 24-26th May 2006, Singapore.

LIST OF ABBREVIATIONS

AD	Anaerobic Digestion
APHA	American Public Health Association
CFU	Colony Forming Units
CLSM	Confocal Laser Scanning Microscope
COF	Commercial Organic Fertilizer
COV	Coefficient of Variation
CSTR	Completely Stirred Tank Reactor
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic Acid
FISH	Fluorescence <i>in situ</i> Hybridization
GC	Gas Chromatography
GI	Germination Index
HPLC	High Performance Liquid Chromatography
MSW	Municipal Solid Waste
OFMSW	Organic Fraction of Municipal Solid Waste
PCR	Polymerase Chain Reaction
Ra	Acidogenic Reactor
rDNA	Ribosome Deoxyribonucleic Acid
RNA	Ribonucleic Acid
Rm	Methanogenic Reactor
SCOD	Soluble Chemical Oxygen Demand (mg L ⁻¹)
SEM	Scanning Electron Microscope
SMA	Specific Methanogenic Activity
TOC	Total Organic Carbon (mg L ⁻¹)
TS	Total Solids (g)
TSS	Total Suspended Solid (mg L ⁻¹)
UASB	Upflow Anaerobic Sludge Blanket
VFA	Volatile Fatty Acids
VS	Volatile Solids (% TS)
VSS	Volatile Suspended Solids (mg L ⁻¹)

LIST OF SYMBOLS

α	Biodegradation of organic matter (%)
A	Area (mm^2)
B	Specific binding of Arc915 probe (pmol mL^{-1})
C	Specific heat capacity ($\text{J g}^{-1} \text{ } ^\circ\text{C}$)
D	Dilution factor
d	Equivalent weight of $\text{CO}_2\text{-C}$ in NaOH ($d = 6$);
E	Experiment
F	Fluorescence
f	Mass unit, fraction of VS (g g^{-1} TS)
L	Root length (mm)
M	Mass (g)
n	Normality of standardized HCl ($\text{mol}_\text{C} \text{ L}^{-1}$)
N	Concentration of bacteria cells (cells mL^{-1})
No	Cells number
P	Concentration of the added labeled probe (pmol mL^{-1})
Q	Energy (kJ)
R	CO_2 evolution rate ($\text{mg CO}_2\text{-C g}^{-1} \text{ VS d}^{-1}$)
T	Tube
t	Time
μ	Specific growth rate of methanogens (d^{-1})
V	Volume (mL)
w	Moist weight of the sample in container (g)

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CHAPTER 1

INTRODUCTION

1.1 Background

The renewable energy generating from anaerobic digestion (AD) process serves as an economical and environmentally friendly means for food waste mineralization and stabilization (Cecchi et al. 1992b; Edelman et al. 2000). Acidogens and methanogens are the two groups of microorganisms responsible for converting food waste to acids and then to methane during the two-step conversion process. Because of their dissimilar requirements in favorable cultivation condition, a two-phase AD strategy was developed to separately cultivate these two groups of microorganisms in two individually optimized reactors in a series, so as to achieve a maximized system of stability and efficiency (Pohland and Ghosh 1971; Cohen et al. 1982; Ince 1998; Kim et al. 2000; Shin et al. 2001b). However, the rapid sourness in the acidogenesis step has always been a major obstacle for food waste AD due to its ready acidification nature and the fact that accumulated VFA directly inhibits methanogenic activity, resulting in system failure (Mata-Aalvarez et al. 1992; Edelman et al. 2000). In order to overcome this technical barrier, a modified two-phase AD process, known as the hybrid anaerobic solid-liquid (HASL) system, was developed in Singapore to realize a self-pH adjustment in the acidogenesis phase by recirculating the alkaline broth from methanogenesis phase. This optimized pH control strategy thus provides stable acidification products as resources for methanogenic growth and subsequent methane gas production. So far, the HASL system has demonstrated advantages over the conventional two-phase AD process for food waste AD in terms of (1) upgraded acidification efficiency; (2) excessive methane production; (3) free of feedstock pH control; and (4) no need of mixing mechanics (Wang et al. 2002 and 2005; Xu et al. 2002).

Due to technical drawback, slow growth rate of microorganisms, the difficulty in isolating anaerobes and the complexity of the bio-conversion processes, the two-phase AD was not fully understood. Since acidogenic bacteria and methanogenic bacteria differ widely in terms of physiology, nutritional needs, growth kinetics and sensitivity, it is a must to monitor the microbial communities in two-phase AD for successful phase separation and excellent system performance. Meanwhile, as the rate-limiting step in AD, hydrolysis can be enhanced to improve the overall efficiency by various pretreatment methods, i.e. mechanical, biological and physico-chemical methods. Technically, food waste is actually a mixture of organic matters with different properties. Recalcitrant components such as lipids may not experience sufficient mineralization while the readily biodegradable fractions show toxicity from long chain fatty acids produced in the course of lipids hydrolysis. Currently, research works about the effects of these critical factors on the two-phase AD and the mechanisms behind them are very limited (Alves *et al.*, 2001). For this regard, this thesis is focusing on the effects of the recalcitrant fraction of food waste on the HASL performance and the possible means available to minimize its negative effects while maximizing its positive effects by thermal and freeze/thaw pretreatments. It is expected that the information derived from this study would provide extensive knowledge on the potential of two-phase AD in converting various types of food waste to bioenergy. The research objectives and scopes are outlined to offer a systematic approach to reach the goal of this thesis.

1.2 OBJECTIVES AND SCOPES

In order to give insight into the potential of two-phase AD in handling food waste, the effect of lipids content on the performance of the HASL system and the possible pretreatment methods to enhance the HASL's capacity in relation to changes in the microbial community are chosen as the scope of this study. Accordingly, the following specific objectives are proposed:

- To identify the characteristics of the microbial community in the HASL system.

To develop a new method, specific binding of Arc915 probe for methanogens quantification during the cultivation of Rm, compared to direct count and auto-fluorescence of co-enzyme F₄₂₀. The typical accuracy for the new method and the relationship between different methods were also investigated. Meanwhile, the distribution of bacteria and methanogens in granule was observed by fluorescence *in situ* hybridization and confocal laser scanning microscopes (FISH-CLSM).

- To evaluate the effect of lipids content in food waste on the performance of the HASL system.

Four identical HASL reactors were operated to investigate the effect of lipids content in food waste on anaerobic digestion in terms of pH, SCOD, VFA and methane production. Meanwhile, the number and activity of methanogens in Rm was estimated by direct count and auto-fluorescence of co-enzyme F₄₂₀.

- To investigate the effects of thermal and freeze/thaw pretreatment of food waste on the HASL system.

The favorite temperature and duration for thermal and freeze/thaw pretreatment of food waste was optimized in a series of batch experiments and applied in the HASL operation. The effect of thermal and freeze/thaw pretreatment of food waste was evaluated in terms of pH, SCOD, VFA and methane production. The number and activity of methanogens in Rm was estimated by direct count, auto-fluorescence of co-enzyme F₄₂₀ and specific binding of Arc915 probe. The correlation among of them was evaluated using statistics study. Meanwhile, the dominant species was investigated

using PCR-DGGE techniques. And the energy consumption for pre-treatment process was calculated.

- To analyze the stability, toxicity and fertility of the HASL residue

The characteristics of residue were tested for future application as fertilizer, including nutrition, toxicity, stability and fertility. Meanwhile, the effect of residue and commercial organic fertilizer (COF) on plant growth was compared.

1.3 ORGANIZATION OF THE THESIS

This thesis contains 7 chapters. Chapter 1 is a brief introduction on the research, and Chapter 2 looks into the relevant literature. The effect of the microbial community on the performance of two-phase AD and different methods for the quantification of microorganisms are investigated in Chapter 3. Chapter 4 looks into the effect of lipids content in food waste on the performance of the HASL system. Chapters 5 and 6 demonstrate that thermal and freeze/thaw pretreatment can enhance food waste digestion in the HASL system in terms of higher and faster COD, VFA and methane production. The stability, toxicity and fertility of residue produced in the HASL system was also tested for application as fertilizer. Conclusions and recommendations are made in Chapter 7.

CHAPTER 2

LITERATURE REVIEW

In the last century, industrialization and urbanization have led to a rising trend in waste output. In Singapore, a total of 460,000 tonnes of waste were disposed of in 1970, while a total of 2.5 million tonnes in 2007 (NEA 2007). The amount of waste disposed of increased six times over a 33-year span which means that a new incineration plant is required every 5-7 years and a new landfill is needed every 25-30 years. The fast growth in solid waste generation places considerable demand on waste management and the environment.

According to information from the National Environment Agency of Singapore in 2007 (Table 2-1), an enormous amount of 5.6 million tonnes of wastes were produced with a total of 0.56 million tonnes in food waste. Almost all of this food waste was disposed of, contributing to around 20% of the total waste disposal of 2.55 million tonnes. Among the different types of waste, the recycle rate of food waste was only 9%, which is much lower than the overall recycling rate of 54%. Therefore, there is a big potential for food waste to be recycled or recovered.

Food waste comes from residential premises, markets, food retail outlets, schools, trade premises, food process factories, etc. The composition of food waste may vary depending on location, season, economical conditions, business type and food type. For example, the waste from meat processing plants contains high fat and protein content which are difficult to degrade, while that from the canning industry contains high concentrations of sugars and starches which are easily degradable. But generally, food waste is free of metals, pesticides and other toxic substances and has low inorganic

solids content. Food waste is a highly biodegradable organic fraction of municipal solid waste (OFMSW) and is the major source of odor emanation, leachate, toxic gas emission and groundwater contamination as it contains high volatility (VS/TS) (0.8-0.9), which is the ratio of volatile solids (VS) to total solids (TS), and high moisture content (75-85%) (Kim et al. 2004a).

Table 2-1 Waste statistics and recycling rate of Singapore in 2007

Waste type	Waste output (tonne)	Waste disposed of (tonne)	Waste recycled (tonne)	Recycling rate (%)
Food waste	558,900	507,700	51,200	9
Paper/Cardboard	1,202,900	583,900	619,000	51
Plastics	659,800	584,800	75,000	11
Construction Debris	778,300	19,000	759,300	98
Wood/Timber*	246,200	118,400	127,800	52
Horticultural Waste*	224,600	133,500	91,100	41
Ferrous Metals	736,500	68,500	668,000	91
Non-ferrous Metals	92,300	16,700	75,600	82
Used Slag	527,200	7,200	520,000	99
Sludge	124,600	124,600	0	0
Glass	65,300	59,500	5,800	9
Textile/Leather	110,100	104,100	6,000	5
Scrap Tyres	25,500	3,500	22,000	86
Others (stones, ceramics & rubber)	248,600	234,600	14,000	6
Total:	5,600,800	2,566,000	3,034,800	54

* Includes 70,000 tonnes used as fuel in cogen plant

At present, about 90% of waste is incinerated and the remaining non-incinerable waste is landfilled in Singapore. Although incineration can reduce the volume of waste significantly, landfill is still needed for the non-incinerable waste and incineration ash

which is the residue of the incineration process. Incineration and landfill are not sustainable solutions to managing waste and the environment because of the escalation of waste output and costly waste disposal facilities. The most important thing is that waste also imposes harmful effects on the environment and humans by polluting water, air and land. In order to save our living environment and conserve natural resources, it is an obligation to minimize waste output and to recycle as much as possible.

In this chapter, a brief review on waste characteristics, management and disposal, the history, development and key affecting factors in AD, and some recent research on AD such as the HASL system are presented.

2.1 Waste characteristics, management and disposal

Waste is defined as something that is rejected because it is deemed to be worthless or unneeded. However, discarded waste materials are often reusable and may be considered as a resource in another setting with a proper management because of their intrinsic properties (Tchobanoglous et al. 1993).

2.1.1 Waste characteristics

Municipal solid waste (MSW) is typically composed of (1) digestible organic fraction e.g. kitchen scraps, food residue, food processing waste, grass cuttings, etc., (2) low or non-digestible organic matter such as coarser wood, paper and cardboard. These are lignocelluloses which are not readily degradable solely with anaerobic treatment, and (3) inert fraction such as stones, glass, sand, metal, etc. which are suitable to recycle as construction materials.

The organic fraction of MSW (OFMSW) is typically a non-homogeneous substrate and its characteristics determine the biogas potential and biodegradability in AD treatment along with the system configurations and operational conditions. The largest

determining factor for waste characterization is the collection system. Source sorting of MSW generally provides a higher quality OFMSW in terms of smaller quantities of non-biodegradable contaminants like plastics, metals and glass. While mechanical sorting of MSW always produces a more contaminated stream, which leads to persistent handling problems and lower acceptability of the end product used as fertilizer on agricultural land (Castillo et al., 2006; Hartmann and Ahring, 2006).

2.1.2 Waste management

Waste management has the dual function of waste disposal and resource recovery which aims to manage waste in a manner that meets public health and environmental concerns and the public's desire to reuse and recycle waste materials.

A complete waste management system encompasses collection, transfer, resource recovery, recycling and treatment. The primary target of waste management is to protect the health of the population, promote environmental quality, develop sustainability and provide support to economic productivity (Henry et al. 2006). To meet these goals, sustainable solid waste management systems should include returning recoverable and recycled waste material into the life cycle (a reusable resource) by adopting the waste management hierarchy, i.e. the 3Rs - reduction, reuse and recycling. The 3Rs will change wasteful consumption patterns into sustainable ways to save natural resources and diminish environmental pollution. For instance, recycling of paper reduces air pollution by 74% and water pollution by 35%. Each tonne of paper recycled could also save 17 small trees (NEA 2007).

So far, centralized waste management systems are used by most countries. However, according to estimates from the World Resources Institute, many local authorities in developing countries spend over 30% of their budgets on refuse collection and disposal but can only collect at most 50-70% of waste generated (Matrix 1993). Most of them do not meet environmentally safe waste disposal levels because of a lack of sanitary

landfills. In the context of public service reforms, decentralization is regarded as an important means to achieve improved efficiency and quality of services. Decentralization, as an alternative political and administrative strategy, is a new concept for waste management which can reduce the cost for waste collection and storage, transfer and transport (Lane 2003; Henry et al. 2006). New roles and responsibilities are emerging and being aimed at promoting development and improving waste management.

2.1.3 Waste disposal

After collection and separation of solid wastes, reusable materials are recycled and the remaining waste is disposed of. There are three main methods for waste treatment and disposal, landfill, incineration and bio-conversion.

Landfill

Historically, landfill has been the most economical and environmentally acceptable method for waste disposal. Even with the implementation of waste reduction, recycling and transformation technologies, disposal of residue solid waste in landfill still remains an important component of an integrated waste management strategy because of its great capability, low cost, convenient operation and compatibility with different wastes. To minimize public health and environmental impacts, traditional landfills are modified into sanitary landfills or landfill bioreactors with specific engineered designed and operation (Pohland and Alyousfi 1994; Bendz et al. 1997; Reinhart 1998; Warith 2002).

Although sanitary landfills are designed carefully based on soil, climate, geologic and hydrological conditions and strict restrictions, there are some concerns, including: (1) odor problems because of the landfill gases with toxic compounds which may be released to the surrounding environment; (2) enhanced greenhouse effect due to the release of CH₄ and CO₂; (3) explosion risks due to methane; (4) land consumer because

landfill is a big land consumer and is difficult for remediation; and (5) leachate which is an inevitable consequence of the waste degradation in landfills. Among them, leachate is the most important issue related to landfills, because leachate is a complicated and toxic which varies highly depending on composition (Khoury et al. 2000; El-Fadel et al. 2002), climate condition (Tatsi and Zouboulis 2002; Al-Yaqout and Hamoda 2003) and different season (Akesson and Nilsson 1997). Although leachate are collected and transferred to treatment plants, contamination of groundwater, soil and surface water may still happen (Mikac et al. 1998).

Incineration

Incineration is the oxidative degradation of waste at extremely high temperature and is mainly used in the land-scarce and developed countries like Singapore, Japan, Sweden, Norway, Netherlands and Denmark due to its compact process but extremely costly facilities. Incineration is an effective waste disposal method with a high volume reduction of around 90%. In some cases, thermal and electricity energy can be co-generated when special energy recovery techniques are used. However, there are still some big problems: (1) air pollution which is mainly caused by particulate matter, nitrogen oxides (NO_x), sulphur dioxide (SO_2), carbon monoxide (CO), dioxins and furans; (2) ash disposal because the non-incinerable waste and the remaining ash and slag still need to be disposed of in landfills; and (3) nutrient biomass loss because most organic waste is destroyed during the process.

Bio-conversion

A biological conversion process converts organic matter in waste to stable end products. Aerobic composting and AD are two main biological processes for the transformation of OFMSW. The relative advantages of these two processes are summarized in Table 2-2.

Table 2-2 Comparison of anaerobic digestion and aerobic composting for processing the OFMSW (Tchobanoglous et al. 1993)

Characteristic	Aerobic composting	Anaerobic digestion
Energy	Net energy consumer	Net energy producer
End products	Humus, CO ₂ , H ₂ O	Digested slurry, CO ₂ , CH ₄
Volume reduction	Up to 50%	Up to 50%
Processing time	20 - 30 days	20 - 40 days
Temperatures	50 - 70 °C	Ambient
Odour	Problem for open system	Less problem due to closed system
Humus content	Abundant	Less than composted product
Nitrogen loss	Low to high	Low to medium

Aerobic composting is the most commonly used biological process for the conversion of OFMSW to a stable humus-like material known as compost. It aims to reduce volume and produce compost. Aerobic composting offers the advantage of relatively simple operation and can significantly reduce the volume of OFMSW. However, this process is a net energy consumer because oxygen must be supplied for waste transformation.

AD is the intra-molecular oxidation-reduction of an organic molecule or oxidation by external electron acceptors to produce sludge, carbon dioxide and methane. Its primary goal is energy recovery in the form of methane gas and thus is a net energy producer. Its secondary goal is volume reduction and waste stabilization (to be used as fertilizer).

Nowadays, about 80% of global energy requirements are met by fossil fuels. The perspective of petroleum depletion increases worldwide interest in alternative sources of energy. Biogas generated from AD can be used to produce heat and energy or as an alternative fuel for vehicles. The combustion of biogas does not contribute to the enhanced greenhouse effect as it is considered to be a part of the natural carbon cycle of the earth. Finally, it is a renewable energy that can be easily stored, compared to

solar and wind power. The residue of AD can also be used for soil conditioning and as organic fertilizers (Wang et al. 2003) which recycle the nutrient into the life cycle. Finally, AD adapts to decentralized waste management systems which is a new trend for promoting development and improving efficiency of waste management.

On the basis of ecological reason, economic considerations as well as the condition of high energy demand and low energy resources, AD is within the philosophy of sustainability and appears to be the best solution for solid waste management (Cecchi et al. 1993; Edelmann et al. 2000) and will become considerably more important in the future.

2.2 Anaerobic digestion (AD)

AD is a biological process in which organic material is broken down, dissolved and converted into substrate which can be absorbed by specialized bacteria. As a result, complex carbohydrates, lipids, fibers and proteins are converted into simpler compounds (e.g. sugars, glycerin and fatty acids, and amino-acids), and finally biogas (a mixture of methane and carbon dioxide) and a stable biomass will be delivered as end products. AD normally produces ten times less refractory biomass than aerobic treatment. Meanwhile, biogas produced in AD has been promoted as a part of the solution to energy problems, thereby lessening waste biomass disposal requirements and the financial burden associated with disposal considerably.

2.2.1 History and development

AD dates as far back as the 10th century when the Assyrians used it to heat bath water. It was historically insignificant before reappearing in Europe in the 17th century when it was determined that decayed organic matter produced flammable gases, and was again used to heat water (Mahony et al., 2002). The first full scale AD was used to treat wastewater in the city of Exeter, UK in the 1890s. From then on, it continued to be

widely used to stabilize sewage sludge, as it is today. Due to solid settling and scum formation, there were always significant operational problems and frequent system disturbances which limited the AD application until the 20th century (Stronach 1986; Lusk 1998).

The development of microbiology in the 1930s allowed scientists to identify that the gas production depended on anaerobic bacteria. Subsequent experiments were carried out to determine the optimal environmental conditions for gas production. As knowledge on AD expanded, it was employed to treat farm wastes, wastewater, industrial organics and MSW. The energy crises in the 1970s prompted American research into alternative energy strategies and AD was one such option. This push resulted in the first farm digester built in America in 1970 where the biogas could be used for heat and power (Lusk 1999). At present, development continues on farms as well as wastewater treatment plants, where anaerobic processes and subsequent gas recovery are an industrially-mature technology.

Biodegradable solid waste should be treated to reduce their environmental impact and to recover energy while massive disposal methods (e.g. landfill and incineration) are avoided. However, the treatment of solid waste using AD adds several new challenges because of the variety in the feedstock and space limitations. The OFMSW may contain agricultural, food, yard waste or paper in varying concentrations, sizes and composition. Furthermore, MSW is contaminated with non-organics, such as glass and metal, and therefore requires pretreatment to separate the feedstock (Bilzonella et al. 2005; Castillo et al. 2006). Though the ideal waste stream for an AD plant would be source-separated organics, there is always a small degree of contamination that must be handled on site.

Despite these challenges, European nations have led the way in expanding AD to be a significant part of OFMSW management. Over 50 plants were operated to treat MSW either alone or with sewage sludge in Germany, Denmark, France, Spain, Austria,

Holland, England, Belgium and other European nations, where environmental legislation concerning waste disposal is stringent.

The current designs of AD systems reflect the need for shorter hydraulic retention times, higher retention of biomass, smaller reactor volume, higher loading rates, etc. Future development of AD as a MSW management strategy depends on several parameters ranging from environmental concerns to economic considerations including increased process efficiency, reduced digester operation costs, higher and more stable gas production, recovery of marketable co-products, expanding markets for energy, co-products and final solids uses, and competitive economics compared to composting, landfill, or incineration. It seems that AD systems will continue to play a major role in decomposing OFMSW.

2.2.2 Biochemical and microbiological principles of AD

AD is the biological conversion of organic matter into biogas (mainly methane and carbon dioxide) in the absence of oxygen which consists of a series of biological processes and involves several groups of microorganisms, mostly bacteria (Archer and Kirsop 1990). Generally, three main groups of microorganisms are distinguished: the fermentative bacteria, the acetogens and the methanogens. Each of them has a specific role in the overall process, which can be described as follows: (1) hydrolysis and liquefaction, (2) acidogenesis, and (3) methanogenesis. An overall scheme of AD is given in Figure 2-1.

1: Hydrolytic and non hydrolytic fermentative bacteria

2: Syntrophic acetogens

3: Homoacetogens

4: Hydrogenotrophic methanogens (i) and acetoclastic methanogens (ii)

Figure 2-1 Bioconversion of complex organic material in AD

Hydrolysis and liquefaction

Hydrolysis and liquefaction are the first and a necessary step in AD of MSW in which the large, complex and insoluble organics breakdown into their component units, i.e. proteins are degraded to amino acids; carbohydrates into soluble sugars; and lipids to long chain fatty acids and glycerin. Only in water soluble state, can they be transported and assimilated into microbial cells and metabolized (Schieder et al. 2000), and hence, the first step is also known as solubilization.

The hydrolysis process is mediated by extra-cellular enzymes such as cellulase, protease and lipase secreted by bacteria. It is often the slowest and is the rate-limiting step in AD, especially for digesting high solid content substrates. Only after hydrolysis has taken place, can the acid-forming bacteria ferment the subunits to a group of extra-cellular intermediates, including volatile fatty acids, hydrogen and carbon dioxide which eventually give rise to the methanogenic precursors. Therefore, the efficiency of the hydrolysis step also determines the ultimate methane yield in the overall process.

Acidogenesis

The acidogenesis process involves acid-forming fermentation, hydrogen production and an acetogenic (acetic acid-forming) step. Once complex organics are hydrolyzed, acidogenic bacteria convert sugars, amino acids and fatty acids into smaller organic acids, hydrogen and carbon dioxide. These products vary highly with the types of bacteria as well as with environmental conditions.

Hydrogen is produced by the acidogenic bacteria including hydrogen-producing acetogenic bacteria. These microorganisms are related and can tolerate a wide range of environmental conditions. Under standard conditions, the presence of hydrogen in the solution inhibits oxidation, therefore hydrogen-producing bacteria are required to endure the conversion of all acids (Novaes, 1986; Parkin and Owen, 1986). Under

conditions of electron or hydrogen accumulation, numerous other fermentation products may be formed, including propionate, butyrate, lactate and alcohols, as a way of removing electrons or hydrogen.

Longer chain organic acids (propionate and above) will be accumulated when the rate of hydrolysis and fermentation exceeds the rate of acetogenic conversion of fermentation intermediates into acetate and formate. This is usually because methanogens cannot consume formate at the rate at which it is produced. Therefore, the overall fermentation is dependent on a delicate balance between activities of bacteria which form organic acids, carbon dioxide and formate and methanogens which utilize these substrates. Because organic acids are not the major intermediates in balanced methane fermentation, they are not metabolized after formation in imbalanced conditions until a population of bacteria capable of their metabolism can be developed. The growth rate of propionate-utilizing bacteria is slow in comparison with other organisms. Thus this process often requires several weeks.

The volatile fatty acids (VFA) are converted through acetogenesis into the main substrates for methanogenesis: acetate, hydrogen and carbon dioxide. In addition, there is also the other group of bacteria called homoacetogens which form acetate through reverse reactions with hydrogen and carbon dioxide. The chemical reactions involved in the process are listed below.

Methanogenesis

The formation of methane, which is the ultimate product in AD, occurs by two major routes. One is from acetate; the other is from the reduction of carbon dioxide by hydrogen. Under most circumstances in treating solid wastes, acetate is a common end-product of acidogenesis. This is fortunate because acetate is a favorite substance for methanogens and can easily be converted into methane in the methanogenesis phase. The overall chemical reactions are given below:

Depending on the substrate utilized, methanogens are categorized into hydrogenotrophic (hydrogen-utilizing chemolithotrophs) and acetoclastic (acetate-splitting) methanogens. About 60-75% of methane is produced from acetate splitting and the remaining from hydrogen reduction in anaerobic digesters (Smith 1966; Mackie and Bryant 1981; Adrianus et al. 1994).

Bacteria and archaea in AD

The consortia of microorganisms involved in the overall conversion of complex organic matter to methane begins with bacteria that hydrolyze complex organic matter –such as carbohydrates, proteins, and fats – into simple carbohydrates, amino acid, and fatty acids. The simple carbohydrates and acids are then utilized to obtain energy for growth by fermentation bacteria, producing organic acids and hydrogen as the dominant intermediate products. The organic acids are then partially oxidized by other fermenting bacteria, which producing additional hydrogen and acetic acid. Hydrogen and acetic acid are the main substrates used by archaea methanogens, which convert them into methane. Hydrogen (H_2) is used as an electron donor, which dioxide as an electron acceptor to form methane from the methyl group and carbon dioxide from the

carbonyl group in a fermentation reaction. The complex and close community interaction of many prokaryotic organisms from two entirely different biological kingdoms--Bacteria and Archaea--in this widely prevalent natural process are truly amazing. The Phylogenetic tree of life is shown in Figure 2-2

Figure 2-2 Phylogenetic tree of life (Madigan et al. 2000)

Bacteria are microscopic organisms whose single cells do not have a membrane-bounded nucleus or other membrane-bounded organelles like mitochondria and chloroplasts. Another group of microbes, the archaea, meet these criteria but are so different from the bacteria in other ways that they must have had a long, independent evolutionary history since close to the dawn of life.

Bacteria are maligned because of the human and animal disease they cause. However, some, like the actinomycetes, produce antibiotics such as streptomycin and nocardicin. Others have different functions. They live symbiotically in the guts of animals

(including humans) or elsewhere in their bodies, or on the roots of certain plants, converting nitrogen into a usable form. They are seen everywhere. For instance, bacteria give the tangy taste in yogurt and the sour in sourdough bread. Bacteria are responsible for the break down of dead organic matter. They are also an important base of the food web in many environments. Life on earth may be complex but it appears that this was the basis of all early life. The oldest fossils known, nearly 3.5 billion years old, are fossils of bacteria-like organisms.

The community of bacteria responsible for acid production may include facultative anaerobic bacteria, strict anaerobic bacteria or both (e.g. *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Lactobacillus* and *Streptococcus*). Acetogenic bacteria such as *Syntrobacter wolini* and *Syntrophomonas wolfei* convert volatile fatty acids (VFA) (e.g. propionic acid and butyric acid) and alcohol into acetate, hydrogen and carbon dioxide, which are used in methanogenesis.

Archaea are microbes and most live in extreme environments. Those that do are called extremophiles. Other Archaea species are not extremophiles and live in ordinary temperatures and salinities. When these microscopic organisms were first discovered in 1977, they were considered bacteria. It became apparent however, that they were not when their ribosomal RNA was sequenced. The sequencing showed that they were not closely related to the bacteria but were instead more closely related to the eukaryotes. Archaea are a much different and simpler form of life. They may also be the oldest form of life on Earth. Because they requires neither the sunlight for photosynthesis as do plants, nor oxygen as to animals. Archaea absorbs CO₂, N₂, or H₂S and gives off methane gas as a waste product the same way humans breathe in oxygen and breathe out carbon dioxide. Some methanogenic Archaea are summarized in Table 2-3.

Table 2-3 Characteristics of methanogenic Archaea^a (Madigan et al. 2003)

Genus	Morphology	Number of species	Substrates for methanogenesis	DNA (mol % GC)
Methanobacteriales				
<i>Methanobacterium</i>	Long rods	8	H ₂ +CO ₂ , formate	30-55
<i>Methanobrevibacter</i>	Short rods	7	H ₂ +CO ₂ , formate	27-31
<i>Methanosphaera</i>	Cocci	2	Methanol + H ₂ (both needed)	23-26
<i>Methanothermus</i>	Rods	2	H ₂ +CO ₂ ; and also reduce S ⁰ ; hyperthermophiles	33
<i>Methanothermobacter</i>	Rods	6	H ₂ +CO ₂ , formate, thermophiles	32-61
Methanococcales				
<i>Methanococcus</i>	Irregular cocci	3	H ₂ +CO ₂ , pyruvate+ CO ₂ , formate	29-35
<i>Methanothermococcus</i>	Cocci	1	H ₂ +CO ₂ , formate	31-34
<i>Methanocaldococcus</i>	Cocci	4	H ₂ +CO ₂	31-33
<i>Methanotorris</i>	Cocci	1	H ₂ +CO ₂	31
Methanomicrobiales				
<i>Methanomicrobium</i>	Short rods	1	H ₂ +CO ₂ , formate	49
<i>Methanogenium</i>	Irregular cocci	4	H ₂ +CO ₂ , formate	47-52
<i>Methanospirillum</i>	Spirilla	1	H ₂ +CO ₂ , formate	45-50
<i>Methanoplanus</i>	Plate-shaped cells---- occurring as thin plates with sharp edges	3	H ₂ +CO ₂ , formate	39-50

Continue to next page

Genus	Morphology	Number of species	Substrates for methanogenesis	DNA (mol % GC)
<i>Methanocorpusculum</i>	Irregular cocci	4	H ₂ +CO ₂ , formate, alcohols	48-52
<i>Methanoculleus</i>	Irregular cocci	6	H ₂ +CO ₂ , alcohols, formate	49-61
<i>Methanofollis</i>	Irregular cocci	2	H ₂ +CO ₂ , formate	54-60
<i>Methanolacinia</i>	Irregular rods	1	H ₂ +CO ₂ , alcohols	38-45
Methanosarcinales				
<i>Methanosarcina</i>	Large irregular cocci in packets	5	H ₂ +CO ₂ , methanol, methylamines, acetate	36-43
<i>Methanolobus</i>	Irregular cocci in aggregates	5	Methanol, methylamines	39-46
<i>Methanohalobium</i>	Irregular cocci	1	Methanol, methylamines, halophilic	37
<i>Methanococcoides</i>	Irregular cocci	2	Methanol, methylamines	42
<i>Methanohalophilus</i>	Irregular cocci	3	Methanol, methylamines, methyl sulfides,	39-41
<i>Methanosaeta</i>	Long rods to filaments	2	Acetate	52-61
<i>Methanosalsum</i>	Irregular cocci	1	Methanol, methylamines, dimethyl/sulfides	38-40
Methanopyrales				
<i>Methanopyrus</i>	Rods in chains	1	H ₂ +CO ₂ ; hyperthermophile, growth at 110°C	60

^aTaxonomic orders are listed in bold.

Thermodynamics and kinetics are crucial to the mixed microbial community involved in methane fermentation. Both must be understood if one is to appreciate this process in its various complexities. From an operational standpoint, the overall complexity of the process can be simplified into a few principles that can be applied readily. For instance, the process can be broken into two basic steps: (1) hydrolysis and fermentation of complex organic matter into simple organic acid and hydrogen, and (2) the conversion of the organic acid and hydrogen into methane.

The microorganisms involved in the first step grow relatively rapidly, because the fermentation reactions give a greater energy yield than the reaction that lead to methane formation. For this reason, the methanogens are more slowly growing and tend to be rate-limiting in the process. This is generalization is true with domestic wastewater organic matter, municipal sludge, and most industrial wastewater. However, with certain organic matter, for example the anaerobic decomposition of lignocellulosic material such as grasses, agricultural crop residues, or newsprint, the hydrolysis step may be very slow and rate-limiting.

Due to the difficulty in isolating anaerobes and the complexity of the bio-conversion processes, there are many affecting factors in AD that still remain unsolved, i.e. active agent/catalysts, microorganisms, pathway of bio-conversion, nutrient supplement and co-digestion, etc. (Hansen and Cheong 2007).

2.3 Key affecting factors in AD for solid waste

The completion of the AD process requires complex interactions of several varieties of anaerobic bacteria that must be in equilibrium in order to remain stable for the digester. AD is affected by many environmental factors, and changes in operational conditions can disturb the equilibrium and result in accumulation of intermediaries that may inhibit the overall process or even shut it down. Several parameters within the anaerobic digester affect the physical environment, the efficiency of digestion and biogas production potential. Since AD is a multi-parameter-controlled process and each individual parameter can affect the overall system either through its own

effect or through the interaction with other parameters. The key affecting factors in AD for solid waste are described below.

2.3.1 pH

The pH varies in response to biological conversions during the different processes in AD. At a low total alkalinity of waste, a stable pH indicates system equilibrium and digester stability. A falling pH can point toward acid accumulation and digester instability. In general, the acid-producing bacteria tolerate a low pH and have an optimal pH of 5.0 to 6.0. However, most methane-producing bacteria can only function optimally in a very narrow pH range of 6.7 to 7.4 (Elefsiniotis and Oldham 1994c; Bitton 1999).

In a conventional anaerobic system, the pH is typically maintained within methanogenic limitations to prevent the predominance of acid-forming bacteria, which will cause acid accumulation and lower pH of below 5.0, a level lethal to methanogens. That is great potential for system failure. Many studies report that the pH required in anaerobic digester for good performance and stability is in the range of 6.5-7.5, although stable operation has been observed out of this range. The anaerobic process may fail if the pH is close to 6.

AD can operate over a wide range of volatile acid concentrations if proper control is maintained (Anderson and Yang 1992; Parkin and Owen 1986). On the other hand, prolific methanogenesis may result in a higher concentration of ammonia, increasing the pH to above 8.0, and will impede acidogenesis (Lusk 1999). Under normal conditions, this pH reduction is buffered by the bicarbonate produced by methanogens. Under adverse environmental conditions, the buffering capacity within the system can be upset, eventually stopping the methane production. Therefore, excess alkalinity or ability to control pH must be available to avoid acid accumulation.

Maintaining pH is especially delicate in the start-up stage because organic waste must undergo acid forming stages before methanogenesis. To raise the pH during the early stages or when the pH drops during operation, a buffer must be added to keep bicarbonate alkalinity as high as possible in order to maintain the pH high enough for methanogens to survive.

The common materials used as buffer to increase the alkalinity are lime, sodium hydroxide, ammonia, ammonium bicarbonate, soda ash and sodium bicarbonate. The first three are the least expensive (Anderson and Yang 1992; Parkin and Owen 1986). An advantage of adding these alkalis is that they induce swelling of particulate organics, making the cellular substances more susceptible to enzymatic attack (Vlyssides and Karlis 2004). In some case, automatic pH control is considered more economical than adding alkalis in a random manner because fewer chemicals are consumed.

2.3.2 Temperature

AD is strongly affected by temperature and can be grouped based on the temperature: psychrophilic (0-20°C), mesophilic (20-42°C) and thermophilic (42-75°C). Anaerobic bacteria resist temperature changes. In the mesophilic range, the activity and growth rate of bacteria decrease by 50% for each 10°C drop, while the overall process kinetics doubled for every 10°C increase. Despite high hydrolytic activity at higher temperatures, the activity of other groups of bacteria, such as acetate degrading bacteria, decrease when temperature increases to more than 60°C due to the increased VFA concentration which causes a rapid drop off in microbial activity.

The optimum temperature for mesophilic digestion is 35°C at which bacteria are more robust and can tolerate greater changes in the environmental parameters. This stability makes the mesophilic AD more popular but requires longer retention times. Thermophilic digestion allows higher loading rates and achieves a higher rate of pathogen destruction as well as a higher degradation of the substrate and smaller

digester size at lower capital cost (Mackie and Bryant 1995). However, it is more sensitive to toxins and smaller fluctuations in the environment and it takes longer time to establish thermophilic population. Another disadvantage is more energy required to heat the feedstock to reactor temperature which makes it less attractive from an energy point of view (Parkin and Owen 1986; van Lier et al. 1996). Over the past 15 years, however, more and more biogas plants have been established and operated under thermophilic conditions since it offers a higher reaction rate and a more profitable process with a lower retention time.

When compared with the high-solids digestion of OFMSW and the co-digestion of OFMSW with sewage sludge, it was found that the biogas production at thermophilic conditions, with a hydraulic retention time (HRT) of 12 days, was almost double that at mesophilic conditions with a HRT of 15 days. This surplus in biogas production was enough to compensate for the additional energy consumption required to heat the digester. The change from mesophilic to thermophilic conditions was achieved over 2 months without digester instability (Cecchi et al. 1991 and 1992a; Hansen et al. 2006). Meanwhile, thermophilic digestion leads to a better pathogens reduction of the waste material than mesophilic digestion. Fecal coliforms could not be detected in the effluent from the thermophilic DRANCO process whereas the original waste contained 3,103 CFU/g TS (CFU: colony forming units) (Six and De Baere 1992). Scherer et al. (2000) also studied the difference between single-stage and multi-stage reactors operated under thermophilic and hyperthermophilic conditions. Besides a higher pathogens reduction, VS reduction was also higher in a hyperthermophilic (60-70°C) first-stage and a thermophilic (55°C) second-stage system compared to a conventional thermophilic system.

The effect of temperature on acidogenesis at an HRT of 30 hours was also tested at 22°C, 30°C and 35°C using mixed industrial wastewater and primary sludge as feedstock. The net VFA production went up by 15% when temperature increased from 22°C to 30°C but decreased by 23% at 35°C. As the VS destruction was fairly constant, it can be suggested that there is an optimum at around 30°C for this

particular biomass and wastewater composition (Banerjee et al. 1998). However, other studies showed 45°C was the optimum temperature for pre-acidification of coffee waste with acetic acid and n-butyric acid production (Kozuchowska and Evison 1995). Rapid temperature drops also affected starch degradation significantly at a temperature level between 15°C and 30°C (Cha and Noike 1997).

Compared to methanogenesis, the effect of temperature on the acidogenesis is not so significant. Because there are always some bacteria that have their optimum within the range concerned among the mixed population. However, methanogenesis can only be undertaken by a few microorganisms, thus, it is much more sensitive to a temperature shift (Mudrak and Kunst 1986; Kim et al. 2006). Although the reaction rate and the pathogens reduction in the thermophilic level are much faster and higher than those in the mesophilic level, the fact is that the methane requirements to maintain thermophilic temperatures are excessive and uneconomical. In addition, when the reactor temperature is below 30°C, the activity of methanogens is seriously reduced. These are the main reasons why mesophilic AD is more popular and must be operated at a temperature of 30 to 35°C for successful functioning.

Recently, attention has been given to the impact of low temperature on AD. Results showed that COD removal efficiency can be as high as 70 to 90 % in an upflow anaerobic sludge blanket (UASB) reactor operated at 11°C with a HRT of 6 hour (Singh and Viraraghavan 2003). In addition, anaerobic migrating blanket reactors (AMBRs) have also been successfully applied to treat low-strength wastewaters at low temperatures (Angenent et al. 2001). Clearly, anaerobic granular sludge systems are suitable for the treatment of municipal wastewater at low and moderate temperatures.

2.3.3 Characteristics of feedstock

The performance of AD on household waste has been extensively studied on a laboratory scale to investigate the effect of quality of waste, waste collection and

composition, the moisture content, the C/N ratio and particle size (Peres et al. 1992; Chynoweth et al. 1993; Mata-Alvarez et al. 1993; Owens and Chynoweth 1993; Speece et al. 1997; Saint-Joly et al. 2000). Compared to fruit and vegetable wastes, lipids and protein are more difficult to digest because the hydrolysis constant of lipids and protein range from 0.005 to 0.075 while the value of this in normal food waste is 0.4 (Mata-Alvarez et al. 2000). Lipids content of 40% diminished the VFA production in the acidogenic reactor as well as biogas production and methanogens in methanogenic reactor due to potential inhibitors (Mtz-Viturtia and Mata-Alvarez 1996; Pavan et al. 2000; Stabnikova et al. 2005).

Particle size is an important parameter in AD. The reduction of particle size increases specific surface available to the medium, improves accessibility of the substrate for enzymes and leads to speeding up the rate of AD and biogas production. Two effects of particle size reduction have been reported.

Firstly, if the substrate has a high fibre content and low degradability, their comminution leads to improved gas production. For agricultural and forest residuals, appreciable positive effect on biogas production was obtained when they were shredded to particles with sizes ranging from 0.088 to 0.40 mm (Sharma et al. 1988). In general, the smaller the size of the fibre, the higher the biogas potential. The best results showed an increase of about 20% with fibre smaller than 0.35 mm.

Secondly, size reduction can lead to more rapid digestion. When average particle size increased from 1.02 to 2.14 mm, the maximum substrate utilization rate coefficient (k_{HA}) in the Valentini model decreased from 0.0033 h^{-1} to 0.0015 h^{-1} (Kim et al. 2000; Mata-Alvarez et al. 2000). In an industrial trial in Switzerland, fruit and vegetable wastes were chopped and then reduced to sizes of $1 \pm 2 \text{ mm}$ in order to obtain a homogeneous suspension with the primary sludge. The results showed an acceleration of the digestion process as well as an increase in the degree of anaerobic digestion of the sludge. These findings corroborate with former studies (Oleszkiewicz and PoggiVaraldo 1997). In addition, the particle size of the organic waste has an influence on its dewaterability after co-digestion.

The carbon/nitrogen (C/N) ratio is used to measure the relative amounts of organic carbon and nitrogen present in the feedstock. A C/N ratio of 20-30 is considered to be optimum for an anaerobic digester, based on biodegradable organic carbon. If the C/N ratio of OFMSW is very high, the waste used as the single substrate will be deficient in nitrogen, which is needed for the buildup of bacterial communities. As a result, the biogas production will decrease. If the C/N ratio is very low, nitrogen will be liberated and accumulate in the form of ammonia. This will increase the pH and a pH value higher than 8.5 will start to show a toxic effect on the methanogenic bacterial communities (Hartmann and Ahring 2006; Van Opstal 2006).

The C/N ratio varies significantly depending on the material composition. For example, the average C/N ratio of food and yard waste is below 20 while it is 24 for animal manure, nearly 70 for rice straw, more than 100 for mixed paper and more than 200 for sawdust, because proteins such as meats are high in nitrogen while paper products and plant materials contain a high percentage of carbon (Kayhanian and Rich 1995). To maintain the C/N ratio at an acceptable level, materials with high C/N ratio can be mixed with those with a low C/N ratio. For example, organic solid waste can be mixed with municipal sewage, biosolids or animal manure. Co-digestion with nutrient-rich organic wastes like manure would be another option to overcome nutrient deficiency (Hartmann and Ahring 2005).

2.3.4 Co-digestion

Co-digestion is an effective option to improve biogas yield in AD for solid wastes due to positive synergisms established in the digestion medium and the supply of missing nutrients by the co-substrates. In addition, economic advantages derived from the sharing of equipment are quite significant. Sometimes, the use of a co-substrate can also help establish the required moisture contents of the feedstock. Other advantages are the easier handling of mixed wastes, the use of common access facilities and the known effect of economies of scale (Mata-Alvarez et al. 2000). Mata-Alvarez and Cecchi (1990) referred to the co-digestion of OFMSW

with sewage sludge in existing digesters because both wastes are produced in large quantities and in many places, and much research has focused on these examples.

Since the late 1980s, co-digestion of manure and industrial organic wastes has been widespread in around 20 centralized co-digestion plants in Denmark (Denmark Environment Agency 1995). From then on, there has been ongoing research. For instance, Kubler et al. (1999) tested different co-substrates for OFMSW. The tests carried out in an industrial facility showed surplus energy production and no negative impact on digester performance. Sosnowski et al (2003) fed bioreactors with three different kinds of feedstock: sewage sludge, OFMSW and a mixture of sewage sludge (75% vol.) and OFMSW (25% vol.). The addition of the OFMSW to the sewage sludge improved the C/N ratio from 9:1 to 14:1 and the cumulative biogas production of the mixtures increased with increasing proportions of the OFMSW (Sosnowski et al. 2003). Co-digestion is also used in many plants for starting up digesters (Griffin et al. 1998).

2.3.5 Mixing/agitation

The way in which materials flow impacts the degree of contact of the substrate with resident bacterial communities and therefore how quickly it is digested. Mixing in the anaerobic digester helps to distribute organisms uniformly throughout the mixture and to transfer heat. The importance of adequate mixing is considered, to encourage distribution of enzymes and microorganisms throughout the digester. Furthermore, agitation aids in particle size reduction as digestion progresses and in the removal of gas from the mixture (Karim et al. 2005; Vavilin and Angelidaki 2005).

In the earliest AD systems, such as covered lagoons, the feedstock simply sits in a large bath without mixing. Improvements on the AD system focused on (1) changing the way materials flow such as in complete mix digesters and plug-flow digesters. Some digesters have interior walls that require a longer distance traveled for the waste, thereby increasing mixing. And (2) changing the way materials are

mixed such as by mechanical mixers, by recirculation of digester contents or biogas produced using pump. Mechanical mixers inside digesters are less common because maintenance is somewhat difficult, while biogas is bubbled through the digester as an inexpensive way to promote movement. Mixing can also be achieved through waste recirculation. The continuous recirculation of digested waste through the heat exchangers not only improves mixing but also ensures proper temperature control. When part of the digested waste is fed into the incoming fresh waste, the bacterial mass comes in contact with the fresh waste and waste movement increases, preventing the buildup of a scum layer (Lissens et al. 2001; Stroot et al. 2001; Karim et al. 2005).

Karim et al (2004, 2005a, 2005b and 2005c) investigated the effect of mixing by biogas recirculation, impeller and slurry recirculation on the AD of animal waster. The main conclusions were (1) unmixed and mixed digesters performed quite similarly when fed with 5% manure slurry; (2) in the digesters fed with 10% manure slurry, the biogas production increased by 29%, 22% and 15% when mixed by slurry recirculation, impeller mixing and biogas recirculation, respectively, compared to the unmixed digester; and (3) mixing using biogas recirculation system was ineffective for 15% manure slurry feed.

The intensity and duration of mixing are also important and are dependent on the content of the digestion mixture (Stroot et al. 2001; Kim et al. 2002). It has been demonstrated that gentle or slow mixing may improve anaerobic digester performance due to process stability (Chen et al. 1990; Vavilin et al. 2004). Over-frequent or excessive mixing can disrupt the anaerobic microbes.

2.3.6 Organic loading rate (OLR)

The organic loading rate (OLR) is the most meaningful parameter for describing the feed rate and the degree of starvation of the microorganisms in an AD system, typically expressed as weight of organic matter (VS or COD) per volume of the reactor per day. This parameter is used as an index of the stress imposed on the

microbial population and affects the amount of total biogas, methane production, COD stabilization and alkalinity (Lettinga 1995; Speece 1996).

A higher OLR demands more bacteria to sustain fast microbial growth, which may cause the anaerobic consortium system to crash if it is not prepared or result in excessive VFA production in the digester with a consequent decrease in pH. This will affect the methanogens and lead to system failure. Low biogas production and a lower pH are early indicators of organic overloading. If there is a significant rise in volatile acids, this normally requires that the OLR be reduced. On the other hand, a low OLR could not provide a sufficient substrate which subjects microorganisms to nutrient starvation and makes the digester unnecessarily large (Bitton 1999).

The maximum OLR for an anaerobic digester depends on a number of parameters such as reactor design, wastewater characteristics, the ability of the biomass to settle activity, etc. Speece (1996) reported several factors which control organic loading rates: (1) concentration of viable biosolids which can be retained in the anaerobic reactor; (2) mass transfer between the incoming wastewater and the retained biomass; (3) biomass proximity for the metabolism of hydrogen intermediate; (4) temperature and pH within the reactor; (5) level of toxicity; and (6) reactor configuration and presence of staging.

It was reported that during the start-up phase of a cross-flow ultra-filtration membrane anaerobic reactor system, the acetoclastic capacity of the reactor sludge was found to be low, which resulted in a poor COD removal efficiency (about 14%) at an OLR of $1 \text{ kg COD m}^{-3} \text{ d}^{-1}$. When the OLR was maintained at approximately $0.7 \text{ kg COD m}^{-3} \text{ d}^{-1}$ in the next two weeks of operation, the COD removal efficiency increased to over 65% (Ince et al. 1995). The same phenomenon has also been found in a study of fluidized bed reactors with sepiolite immobilized biomass at 35°C, which was used to treat wastewaters from the washing of olives prior to oil production, the COD and volatile fatty acid (VFA) concentration were increased in parallel with the increase of OLR (Borja et al. 1998). Others experiments were conducted to determine the influence of OLR on the performance of attached-film

anaerobic digesters under low temperature conditions (10°C). Methane yield increased as OLR was reduced, apparently because of continued digestion of solids that had accumulated at higher OLR. After the OLR was reduced to its lowest value (0.01 kg VS m⁻³ d⁻¹), yield began decreasing as stable operation was approached (Vartak et al. 1999).

2.3.7 Solid retention time (SRT)

Solids retention time (SRT), so-called sludge age, is one of the most important design parameters. In a completely mixed digester system, all the contents in the system have the same biomass retention time which is governed by the replication time of the slowest growing organisms. Below this value, the system fails due to the washout of the slowest growing organisms that is necessary to the anaerobic process (Droste 1997; Parkin and Owen 1986).

The effect of SRT on the acid-phase anaerobic digestion of primary sludge was conducted using a completely mixed reactor (CMR) and an UASB reactor. It showed that C2 to C5 VFA were the predominant compounds formed. At a constant HRT of 12 hours, a longer SRT from 10 to 20 days resulted in a slight increase in VFA production in both systems, but at a shorter SRT (5 days), a drastic drop in acid production was observed. In addition, the percent distribution of VFA was to some extent affected by the variation in SRT. On the other hand, organic matter degradation appeared to be independent of SRT, at least in the range investigated. However, the percent SCOD in the form of VFA increased steadily with increasing SRT, approaching 90% in 20 days (Elefsiniotis and Oldham 1994a). Meanwhile, the degree of organic substrate degradation primarily depended on SRT for complex substrate such as meat extract and municipal primary sludge (Dinopoulou et al. 1988; Elefsiniotis and Oldham 1994b).

A new study on reducing SRT focused on the following areas: (1) to separate the acidogenesis and methanogenesis into individual reactors so that the bacteria population in each reactor is optimized for its purpose, i.e. two-phase AD; (2) to

alternate the mixing flow patterns to improve circulation within the digester; (3) to introduce a surface or combination of surfaces to the reactor on which the anaerobic bacteria can live longer, reducing the microbial population wash out with the effluent and (4) to use pretreatment of the organic waste to increase digester efficiency (Marjoleine et al. 1998; Lissens et al. 2001; van Lier et al. 2001; Libanio et al. 2003; Mata-Alvarez et al. 2000; Nguyen et al. 2007).

2.3.8 Hydraulic retention time (HRT)

The hydraulic retention time (HRT) is determined by the average time it takes for organic material to digest and is used to measure the rate of substrate flow into and out of a reactor. HRT is in the range of 14-30 days for most dry (solids content is above 20%) anaerobic processes and can be as low as 3 days for wet (solids content is below 20%) anaerobic processes. The optimal values vary significantly with temperature, characteristics of solid waste and the specific technology. Therefore, for a specific anaerobic digester, the HRT may change from day to day or from season to season.

Effects of HRT on the performance of AD have been evaluated in many studies. The mesophilic chemostat digester for primary sludge was operated satisfactorily at a HRT of 10-24 hours (Ghosh et al. 1975). However, for diluted primary sludge and a mixture of diluted primary sludge and starch-rich industrial wastewater, the maximum VFA and SCOD concentrations and specific production rate were gained at relatively longer HRT (30 hours) (Maharaj and Elefsiniotis 2001). It also showed that the net VFA production and COD solubilization increased significantly with an increasing HRT from 18 hours to 30 hours (Banerjee et al. 1998).

A shorter HRT will reduce the size of the digester, leading to a higher production rate per reactor volume unit, but a lower overall degradation. These two effects have to be balanced in the design of the full-scale anaerobic digester. One method generally accepted for minimizing HRT is mixing in the digester. The other method is the recirculation of water and biogas in the digester to keep materials moving.

This will ensure that bacterial populations have rapid access to as many digestible surfaces as possible and that environmental characteristics are consistent throughout the digester (Lin et al. 1997; Vlyssides and Karlis 2004).

2.3.9 Pretreatment of feedstock

Various pretreatment processes are used to improve the performance of AD treating solid waste based on the waste characteristics and the effects they have on AD. There is an obvious link between successful pretreatment and improved biogas yield. By means of efficient pretreatment, the suspended solids become more accessible for the anaerobic microbial consortium, improving hydrolysis and liquefaction which is often the rate-limiting step in AD (Shin et al. 2001a).

The most promising pretreatment method is source separation which provides an immediate clean waste stream ready to digest. Other pretreatment methods can enhance the biodegradability of a particular substrate mainly by improving the accessibility of the substrate for enzymes (Vavilin et al. 2002; Vavilin and Angelidaki 2005). These pretreatment methods can be grouped as mechanical, biological and physico-chemical methods (Mata-Alvarez et al. 2000; van Lier et al. 2001) and are described as following:

Mechanical methods

The representative mechanical pretreatment methods are the disintegration and grinding of solid particles which reduce the particle size and solid content of the feedstock. As a result, the amount of soluble organics increases in the influent. Shredding, pulping or crushing of the waste allows bacteria access to a greater surface area and therefore reduces the retention time required for the treatment. Diluting the waste with water also allows the bacteria to move more freely inside the digester (Mata-Alvarez et al., 2000).

Mechanical reduction of particle size increases the specific surface area of organic waste, which results in the speeding up of the rate of AD and biogas production (Sharma et al. 1988; Mata-Alvarez et al. 2000). However, when particle size is small enough, further shredding is not necessary as there is no significant hydrolysis enhancement. For example, to obtain appreciable positive effects on biogas production from agricultural and forest residuals, they must be shredded to particles of sizes from 0.088 to 0.40 mm (Sharma et al. 1988). Meanwhile, mechanical mixing can only be used at low solid content. When solid content is more than 30%, the blender is easily spoilt and can be difficult to repair during operation.

Engelhart et al. (1999) studied the effects of mechanical disintegration by a high-pressure homogenizer on anaerobic biodegradability of sewage sludge. The degradation of soluble proteins and carbohydrates showed that a slowly degradable fraction of carbohydrates was released through disintegration. Meanwhile, a 25% increase in VS reduction was achieved. Hartmann et al. (1999) also found an increase of up to 25% in biogas from fibers in manure feedstock, after pretreatment in a macerator before digestion.

In mechanical pretreatment methods, the energy necessary for the destruction of cell membranes is supplied in the form of pressure, translational or rotational energy. The cells are broken up, such that the substances contained within are set free without any chemical changes or denaturing. Mechanical treatment methods like maceration and ultrasonification have also shown promising results in overcoming the hydrolysis stage. During ultrasonification, extreme conditions are created to cause cell disruption or lysis. When the cells are disrupted, intracellular organics are released, thus enhancing the digestion process. The physical action produced by the cavitation bubbles can reduce the food waste particle size which will increase the number of active sites available for microbial action in the subsequent stages.

Biological methods

Enzymatic and microbial pretreatment methods are very promising. The effect of enzymes on degradation of high-molecular weight proteins, carbohydrates and lipids can be improved by biological slimes and gels and the delivery capacity of water. In these investigations, pretreatment was only carried out on the effect of enzyme on carbohydrate. Enzymatic hydrolysis of cellulose with immobilized cellulose was reported by Kumakura (1997). Microbial solubilization and liquidization of food waste at 35°C in an aerated reactor with supplied water and activated sludge added as inoculum was also proposed to reduce organic loading for further biological treatment (Gonzales et al. 2005).

Physico-chemical methods

Chemical treatments such as the ozone method have been effective in reducing COD and making the compounds more biodegradable. Another form of chemical treatment is the use of an alkaline agent such as NaOH at room temperature. It was reported by Lin et al. (1997) that an increase of 20% in COD degradation was obtained in an anaerobic digester with an HRT of 20 days using NaOH solubilization. The alkaline wet oxidation for pretreatment of wheat straw was also reported by Klinke et al. (2002).

Thermal pretreatment was also used for the solubilization of food waste. Acid hydrolysis of cellulose and hemicellulose under high temperature and pressure was proposed as a promising pretreatment method by Mosier et al. (2005). It was reported that pyrolysis of cellulose at temperatures around 400°C can produce pyrolysate containing levoglucosan (Zhuang et al. 2001). The treatment of food wastes with temperatures from 160 to 200°C and pressure of 4 MPa for 60 minutes was recommended to transform organic solid materials of food waste to liquid hydrolyzate, which could be used for AD directly or after separation of the solid fraction (Tsukahara et al. 1999; Schieder et al. 2000). Use of this hydrolyzate increased the rates of the anaerobic process and biogas production in comparison to

the digestion rate of crushed food waste. Biogas production was 500 L kg^{-1} and 470 L kg^{-1} with added COD, and 850 L kg^{-1} and 780 L kg^{-1} with dry organic matter for pre-treated and raw food waste, respectively (Schieder et al. 2000).

All these methods result in particle size reduction and enhance solubilization of solid waste because biopolymers are depolymerized into shorter chains. Therefore, pretreatment of food waste is useful to enhance anaerobic digestion of waste. However, a biological solubilization of food waste requires a special aerated reactor equipped with a strong stirrer (Gonzales et al. 2005). Both chemical and biological methods require a long time to become stable. Amongst the pretreatment methods, heating of food waste appears to be the simplest pretreatment method (Schieder et al. 2000). The main limitation for thermal pretreatment of food waste is the energy cost. To cover these expenses, biogas produced could partially be used for the combustion and thermal pretreatment of food waste.

2.3.10 Inoculation

The quality and quantity of inoculum is critical to the performance and stability of AD. Theoretically any medium containing the proper bacterial flora can be used as inoculum for AD. Potential seed sludge includes manure, fresh water sediments, septic tank sludge, digested sewage sludge and surplus sludge from anaerobic treatment plants.

In a conventional, completely stirred tank reactor (CSTR), the ratio of inoculum to feed (on a VS basis) is typically greater than 10. Low inoculum levels may lead to imbalance due to the more rapid growth rate of acid-forming bacteria (compared to methanogens) and depression of pH. Depending on the buffering capacity (alkalinity), a digester may be able to recover from low inoculum rates. However, inoculum may become imbalanced when exposed to toxic substances or environmental stress factors for which they are not acclimated.

2.3.11 Process configurations

AD can be operated with different process: such as wet or dry process type, with one stage or several stages and in batch, semi-continuous or continuous mode (Chynoweth et al. 1992; Speece et al. 1997; Wang et al. 2005b). Yeoh (1997) studied AD of cane-molasses stillage in a thermophilic two-phase system in comparison with the single-phase process. The methane content in biogas generated from the two-phase process was significantly higher by about 17% than that from the single-phase process. The mean overall methane yield was found to be 0.168 L CH₄ g⁻¹ COD (added) or 0.292 L CH₄ g⁻¹ VS (added) from two-phase methanogenesis, compared to 0.055 L CH₄ g⁻¹ COD (added) or 0.082 L CH₄ g⁻¹ VS (added) from single-phase fermentation. Azbar and Speece (2001) studied three anaerobic process configurations - namely, two-phase duel sludge (TPDS), two-stage single sludge (TSSS), and single-stage for effluent COD concentration. The same temperature, SRT and substrate were applied in all experiments. In every case, TPDS and TSSS configurations outperformed the single stage significantly.

2.3.12 Inhibition and toxicity

The feedstock to the reactor may contain some compounds which inhibit or are toxic for the anaerobic bacteria even at low concentrations. On the other hand, low concentrations of these compounds often have a stimulating effect on the digestion process. Both individual and combined inhibitions are responsible for the occasional failure of anaerobic digesters. The following are some common toxicant causing problems in AD systems.

Heavy metal

Heavy metals such as copper, nickel, chromium, zinc and lead are essential for bacterial growth in small quantities, but they will have a toxic effect in higher quantities.

The inhibitory and toxic effects of heavy metals on performance in the acidogenic phase were evaluated in the batch experiments. The limiting concentrations were within the ranges of 1-10 mg L⁻¹ for copper and 5-40 mg L⁻¹ for zinc, respectively, which indicated that copper was more toxic than zinc for VFA producing organisms (Yenigun et al. 1996). In acidogenic experiments using serum bottle assay, chromium was found to be less toxic than copper and zinc. Recently, it was reported that at a dosage of over 20 mg mL⁻¹, cadmium inhibited acidogenesis, while at a dosage of over 5 mg mL⁻¹, chromium (Cr³⁺) exhibited severe inhibition on acidogenesis, suggesting that chromium (Cr³⁺) was more toxic to acidogenic bacteria than cadmium (Yu and Fang 2001).

The effects of heavy metals on performance in the methanogenic phase were investigated in various studies. The results showed that VFA-degrading methanogens were heavily affected by cadmium and copper followed by lead and nickel (Lin 1992). In chemostat experiments, a free cadmium level of 0.1 mg mL⁻¹ was determined as the safe limit for acetate-utilizing methanogens (Bhattacharya et al. 1995).

Ammonia-nitrogen

Ammonia is usually formed in anaerobic processes, as a result of mineralization of organic nitrogen in wastes rich in protein or urea. The excess ammonia-nitrogen in the fermentation medium could cause an inhibitory effect in three different ways: (1) free ammonia, which is more toxic for anaerobic microbial communities than the ammonium ion; (2) amination of α -ketoglutaric acid by ammonia-nitrogen coupled with rapid disappearance of α -ketoglutaric acid from the metabolic pool of the tricarboxylic acid cycle, which could cause difficulties in the metabolism of organic compounds; and (3) buildup of ammonia-nitrogen, which may result in undetectable VFA because ammonia will keep the pH above 8 (Krylova et al. 1997; Sterling et al. 2001).

In order to investigate the effect of ammonia concentration on glucose degradation and on the acetoclastic methanogenic reaction, the batch experiments were performed at various ammonia concentrations. As the concentration of ammonia nitrogen increased from 740 to 3500 mg N L⁻¹, the glucose degradation rate decreased significantly. This result was fairly similar to the decrease in carbohydrate removal efficiencies in continuous experiments. Therefore, it seems that the accumulation of ammonia had an inhibitory effect on the glycolytic pathway via which glucose hydrolyzed from carbohydrates was degraded. However, methane production rates from acetic acid were almost identical at ammonia concentrations of 750, 3400 and 4400 mg N L⁻¹, respectively. It was clear that the acetoclastic methanogenic bacteria had resistance to the high ammonia concentrations. These results showed that with sufficient time, the methanogenic population of the bacterial community will acclimate to high ammonia-nitrogen concentrations (Fujishima et al. 2000).

In fact, the problem of ammonia inhibition has been addressed by many authors (Angelidaki and Ahring 1993; De Baere et al. 1984; Hansen et al. 1998). Relative concentrations of dissolved ammonia and ionized ammonium are dictated by the system pH. At high pH values, the unionized form of N-ammonia dominates, and this form is more inhibitive than the ionic form (Hobson et al. 1981). However, dissolved ammonia and ionized ammonium are measured together and most of the papers report this total amount as the ammonia concentration responsible for inhibition.

Sulfide and sulfate

Sulfide toxicity is a common problem with organic waste containing high concentrations of sulfate. Sulfate is used primarily as an electron acceptor in organic waste treatment and is converted to sulfide. Sulfide in complexes with heavy metals such as iron, zinc or copper, is not toxic. It is the soluble form, primarily un-ionized hydrogen sulfide that is most inhibitory.

Sulfide concentrations of between 50 and 80 mg L⁻¹ did not have any adverse effect on methane production in a methanogenic fluidized bed reactor (Yoda et al. 1987). Moreover, sulfide addition before exposure to copper can significantly reduce the recovery time and is more beneficial than sulfide addition after copper spiking in an acetate-fed methanogenic reactor (Jin et al. 1998).

Sulphate is another industry pollutant and inhibitor of the anaerobic process. The inhibition of hydrogen sulphide has been widely reported in literature, which is a major end product of sulphate reduction when wastewaters containing high levels of sulphates are subjected to AD. Sulfate could be reduced in the acidogenic reactor without adversely affecting the normal acidogenesis process when the operating parameters, especially pH is carefully controlled (OchiengOtieno 1996). Thus phase separation could enable wider application to the treatment of industrial effluents, especially those with high levels of sulfate, which were previously considered unsuitable for anaerobic treatment.

Cation toxicity

Sodium, potassium, calcium, magnesium, ammonium and sulfur can stimulate bacterial growth at low concentrations, but become inhibitory at high concentrations. The concentrations of cations reported to be inhibitory cations to anaerobic microorganisms are summarized in Table 2-4 (Parkin and Owen 1986).

Table 2-4 Inhibitory concentrations of cations to anaerobic microorganisms

Cation	Moderately inhibitory (mg L ⁻¹)	Strongly inhibitory (mg L ⁻¹)
Sodium (Na)	3,500 - 5,500	8,000
Potassium (K)	2,500 - 4,500	12,000
Calcium (Ca)	2,500 - 4,500	8,000
Magnesium (Mg)	1,000 - 1,500	3,000

When sodium hydroxide or other sodium-containing bases were added into AD to control VFA accumulation and maintain pH, the high salt concentration could affect the activity of microorganisms and interfere with their metabolism. Acclimation is a factor that could affect the characteristics of sodium inhibition. e. g. adaptation of methanogens to high sodium concentrations over prolonged times which could increase the sodium tolerance of these methanogens. Another phenomenon associated with sodium toxicity is the antagonistic effect. Here, if one cation such as sodium is present at an inhibitory concentration, this inhibition might be relieved when another cation such as potassium is added. With stimulatory concentrations of the various cations present, it helps reduce the extent of inhibition caused by individual cations present at a moderately inhibitory concentration (Feijoo et al. 1995; Soto et al. 1993).

Others

Besides the above mentioned, detergents such as soap, antibiotics and organic solvents also inhibit the anaerobic bacteria. The methane production rate decreased progressively with an increasing concentration of phenolic compounds, because the acetate-utilizing methanogens were inhibited by phenols (Wang et al. 1991). In order to treat acrylic wastes in a methanogenic chemostat system, it was necessary to acclimatize both acetate and propionate utilizers. Despite the acclimatization, the acrylic acid loading rate was limited to about $0.35 \text{ g g}^{-1} \text{ VSS} \cdot \text{d}$ (Qu and Bhattacharya 1996). For acetate-utilizing methanogens, 10 mg L^{-1} of nitrobenzene seemed to have no effect on total biogas production, while both 20 mg L^{-1} and 30 mg L^{-1} caused reversible inhibition in the methanogenic phase (Bhattacharya et al. 1996). However, only 10 mg L^{-1} formaldehyde inhibited acetate utilization completely in the reactor (Qu and Bhattacharya 1997). Other studies also showed the effect of high sodium chloride and ammonia chloride salt level on methanogenic association (De Baere et al. 1984).

2.4 The hybrid anaerobic solid-liquid (HASL) system

In conventional AD, both acidogenesis and methanogenesis occur in a single reactor system. The acidogenesis will cause a consequential decrease in pH due to the production of VFA and other intermediates. This unfavorable environment will slow down the methanogen's activity because the optimum pH for methanogens is at neutral pH levels (Table 2-3). Because the acid-forming and the methane-forming microorganisms differ widely in terms of physiology, nutritional requirement, growth kinetics and sensitivity to environmental conditions, it takes quite a long time to get a delicate balance between these two groups of organism in one reactor.

In order to overcome this problem, Pohland and Ghosh (1971) first proposed the physical separation of the acid-former and the methane-former in two separated reactors where optimum environmental conditions for each group of microorganism would be provided to enhance the overall process stability and control.

Over the last 30 years, an improved two-phase AD became more popular, in which one phase is responsible for acid formation and another optimized for only methane production. In each of these two phases, carried out in different reactors, optimum environmental conditions for each group of microorganism can be maintained (Cohen et al. 1982; Anderson et al. 1994; Ince 1998; Shin et al. 2001b).

2.4.1 Introduction of the HASL system

The hybrid anaerobic solid-liquid (HASL) system, a modified two-phase anaerobic digester, has been successfully developed for food waste digestion in Singapore. It includes an acidogenic reactor (R_a) to treat solid food waste and a modified UASB as the methanogenic reactor (R_m) to treat leachate from R_a . The leachate from R_a is 5 times diluted with the effluent from R_m . This mixture is fed into the R_m as a new influent. The effluent from R_m is divided into two streams, S1 and S2, and the initial flow rate ratio of S1 and S2 is 1:4. S1 flows to the R_a , while S2 is used to dilute the leachate from R_a and pump it back into the R_m . After the HASL set up,

the flow rates of S1 and S2 may change according to the COD concentrations in the leachate from Ra and the effluent from Rm in order to keep a COD concentration of less than $5,000 \text{ mg L}^{-1}$ and an OLR of less than $10 \text{ g COD L}^{-1} \text{ d}^{-1}$ as the optimal conditions in Rm. (Teo et al. 2000; Wang et al. 2002; Xu et al. 2002a). The schematic diagrams of the HASL system are shown in Figure 2-3.

The Ra and Rm in the lab-scale HASL system are cylinders which are made of 5 mm thick transparent perspex. The working volume of Ra is 5 L with an inner diameter of 150 mm and a height of 350 mm. The working volume of Rm is 5 L with an inner diameter of 120 mm and a height of 600 mm. To prevent the plugging problem, a removable steel sieve with 1 mm diameter holes is installed at the bottom of Ra. Peristaltic pumps (Masterflex, Cole-Parmer Instrument Company, USA) are used for influent filling and effluent withdrawing. The HASL system are operated at a constant temperature room at $35 \pm 1^\circ\text{C}$.

Figure 2-3 Schematic diagram of the HASL system

2.4.2 Inoculation in the HASL system

The seed sludge for Ra is collected from an anaerobic digester at the Ulu Pandan Water Reclamation Plant in Singapore, which will provide the microorganisms necessary for effective hydrolysis and acidification in Ra.

Three L of mature sludge granules adapted to high VFA wastewater (pH 5.5-6) are collected from a 10 L UASB reactor and are added to the Rm for its cultivation. The TS and VS of the anaerobic granules were $26.04 \pm 2.1 \text{ g L}^{-1}$ and $17.82 \pm 1.8 \text{ g L}^{-1}$, respectively. Prior to the HASL operation, the Rm is fed with $5000 \text{ mg COD L}^{-1}$ of synthetic wastewater as substrate with an OLR of $10 \text{ g COD L}^{-1} \text{ d}$ and an HRT of 12 hours. A small case of synthetic wastewater is prepared daily, stored at 4°C and supplied to reactors using peristaltic pumps. Chemical composition of the synthetic wastewater used is shown in Table 2-5.

Table 2-5 Chemical composition of the synthetic wastewater

Macro-nutrients	Concentration (mg L^{-1})	Micro-nutrient	Concentration (mg L^{-1})
Glucose	3400	H_3BO_3	0.1
Peptone	1000	ZnCl_2	0.1
Beef extract	700	$\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$	0.1
NH_4Cl	800	$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	0.1
K_2HPO_4	200	$(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$	0.1
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	48	AlCl_3	0.1
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	54	$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.1
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	40	NiCl_2	0.1
NaHCO_3	3000	1 mL micronutrients for 1 L substrate	

An effluent containing less than $500 \text{ mg COD L}^{-1}$ (COD removal efficiency is more than 90%) and an average methane content of biogas exceeding 75% indicates that the Rm is in an active state and ready for connection with the Ra to start the HASL system.

2.4.3 Research and development of the HASL system

The HASL system is newly developed for food waste minimization and energy recovery, thus most research on the HASL system focus on system optimization and efficiency improvement on a lab scale with batch processes or semi-continuous processes.

The HASL system has proven its high efficiency in the treatment of solid food waste. A TOC removal efficiency of 77-80%, a VS removal efficiency of 56-60% and a COD reduction rate of 79-82% was achieved in a batch experiment. Some 99% of the biogas yield with high methane content (68-70%) from the Rm demonstrated that the HASL system is effective and efficient for the conversion of food waste to biogas, which favors the application of the HASL system to practical solid waste management.

Besides higher efficiency of methane production, another advantage of the HASL system is small volume of discharge from the system due to recirculation. Leachate recirculation increases the digestion rate by means of increasing moisture moving through the digestion system and accelerating the stabilization of waste. Separating anaerobic process into two phases, i.e. acidification and methanogenesis phases, facilitates the optimal growth for non-methanogenic and methanogenic bacteria. Removal of VFAs in an active Rm is a built-in mechanism for prevention of system instability. Dilution of VFAs prior to their entry into the Rm makes pH controlling unnecessary. The flow rates of two streams (S1 and S2) of Rm effluent can be adjusted to supply Rm with an optimal OLR. All of these make the HASL system highly efficient, highly flexible and highly stable.

The features of the HASL system are summarized as: (1) effective acidification by leachate recirculation; (2) washout of VFAs by circulating the effluent from Rm into Ra; (3) efficient methanogenesis in an UASB reactor; (4) built-in mechanism to prevent system instability by VFA removal in an active Rm; (5) no pH control due

to the leachate dilution prior to entering into the Rm; and (6) no solid mixing, no excess fresh water addition and no wastewater production.

Leachate recirculation within Ra is a fast way for effective acidification of food waste. Comparison among the single stage, single stage with leachate recirculation and the HASL system was also conducted. The following conclusions were reached: (1) No methane was detected in the single stage with or without leachate recirculation during the 60 days operation. The acidification products, mainly VFAs, greatly inhibited the methane fermentation; (2) The HASL system was more efficient in the conversion of food waste into methane. All methane was only produced from the Rm with an average methane content of 71%.

The lab scale HASL system was also operated for 36 days in a semi-continuous mode. This study showed (1) the effective acidification in a semi-liquid recycle reactor and the high-efficiency methanogenesis in a UASB reactor; (2) that the use of a UASB reactor enhanced the methanogenesis process and increased the methane content in biogas production; (3) during the first 23 days of operation, the leachate from Ra with a TVFA of 9,500-11,500 mg L⁻¹ and a COD of 8,000-11,800 mg L⁻¹ was efficiently treated in the UASB reactor, with about 88.5% of TVFA removal and 85.7% of COD removal; (4) 99% of the total methane was generated from the Rm with average methane content of 71%. This will be beneficial to energy recovery; and (5) at the end of operation, 78% VS removal and 0.29 L g⁻¹ VS of methane production were obtained.

All the above studies have been focusing on the effect of the operation conditions on the HASL process. Relatively little knowledge has been acquired with regards to the structure and function of the microbial populations within the process. Therefore, understanding the distribution of microbial communities could be a key to opening the black box of the two-phase anaerobic food waste treatment process.

To date, molecular microbiological assay, such as polymerase chain reaction (PCR), fluorescent *in situ* hybridization (FISH), denaturing gradient gel electrophoresis

(DGGE), etc., have been widely used to investigate microbial communities and rapidly provide an image of the structure of an ecosystem. It's expected that the two-phase anaerobic food waste treatment process would be further enhanced after the distribution of microbial population is better understood. This will enhance the performance of the HASL system and deliver it to industry for conversion of organic waste into biogas as alternative energy.

The HASL system appears to be technically feasible at its current state of development, and was used in this study to investigate the effect of pretreatment, lipids content of food waste and microbial community on the performance of two-phase AD.

CHAPTER 3

EFFECT OF MICROBIAL COMMUNITY AND QUANTIFICATION OF METHANOGENS IN THE HASL SYSTEM

3.1 Introduction

As reviewed in Chapter 2, methanogens play a key role in producing methane in AD. They are essential components in different anaerobic ecosystems including wetlands, marine and fresh-water sediments, marshes, intestinal tracts and rumen of animals, and anaerobic reactors for organic waste treatment. To study and monitor natural anaerobic ecosystems, as well as anaerobic engineering processes, an accurate and quick method for methanogen quantification should be used. Direct count of auto-fluorescent methanogens using fluorescent microscopy (Ince and Ince 2000; Solera et al. 2001), indirect solid-medium plate counting method employing an anaerobic chamber for cultivation of methanogens (Solera et al. 2001; Uyanik et al. 2002), and most probable number (MPN) method are commonly used for quantification of methanogens (Wu et al. 1987; Agrawal et al. 1997; Skillman et al. 2004).

Direct count of auto-fluorescent methanogens, which is based on auto-fluorescence of cell coenzyme F_{420} by their blue-green auto-fluorescence under UV light, is frequently used in AD studies (Ince and Ince 2000; Solera et al. 2001). However, this method can only detect methanogens with high content of coenzyme F_{420} . Therefore, the cells of acetoclastic methanogens cannot be counted under fluorescent microscope since they belong to the genus *Methanosaeta* and have lower content of F_{420} than that in hydrogenotrophic methanogens (Kamagata and Mikami 1991). Another problem of auto-fluorescent cells count is the aggregates

formation of methanogenic cells, which is typical for the species from genus *Methanosarcina* (Mayerhofer et al. 1992).

The methods which are based on the cultivation of methanogens in liquid (e.g. MPN method) or solid (e.g. plate counting method) media require special laboratory equipment and technique and can only evaluate viable methanogens. This quantification process requires between one month (Agrawal et al. 1997; Skillman et al. 2004) to three months of incubation (Ahring et al. 2001; Svenning et al. 2003) to count the visible colony-forming units of methanogens due to the slow growth of Archaea.

Molecular biology method for determination of methanogens by real-time polymerase chain reaction (PCR) using broad-range (universal) probes and primers can also be used for the quantification of methanogens (Nadkarni et al. 2002; Sawayama et al. 2004). However, this method needs to be proven suitable for quantification of methanogens in complex microbial community (Zoetendal et al. 2004).

There are some known biochemical methods for the quantification of methanogens which are based on detection and measurement of several potential biomarkers present mainly in the biomass of methanogens. One method is the determination of coenzyme F₄₂₀ (7, 8-didemethyl-8-hydroxy-5-deazaflavin electron transfer coenzyme) by fluorescence/luminescence spectrometer (Heine-Dobbernack et al. 1988; Stabnikova et al. 2005). However, this method has the following disadvantages (1) intracellular content of coenzyme F₄₂₀ varies widely among methanogens (DiMarco et al. 1990); (2) it is low in some methanogenic species; and (3) it has been found in non-methanogenic Archaea (Lin and White 1986). Another biochemical method is the determination of thiol coenzyme M (CoM) (2-mercaptoethanesulfonic acid) by high-performance liquid chromatography (HPLC). However, the content of CoM varies with the growth phase of methanogens and it is not accurate in samples after freezing (Elias et al. 1999).

One of the modern methods used to study ecology of complex microbial communities is whole cell FISH with rRNA-targeted fluorescent oligonucleotide probes (Amann et al. 1995; Amann and Kuhl 1998). The whole cell FISH is usually detected by confocal laser scanning microscopy (CLSM) (Tagawa et al. 2000), but it is complicated due to the variability of the microscopic images and the parameters of CLSM. Information on relative abundance of the rRNAs of the target microbial groups can be obtained by hybridization of ^{32}P -labeled oligonucleotide probes with extracted RNA (Raskin et al. 1994a, b; Hansen et al. 1999). The disadvantages of this method may include the extraction of RNA and the use of radioactive labels. Application of 16S rRNA-targeted oligonucleotide probe was used for whole-cell FISH to quantify the ratio of Archaea and Bacteria in anaerobic microbial community (Tay et al. 2001). Further development of this method was the discrimination between specific (hybridization) and non-specific binding of the probe using a fluorescence spectrometer (Ivanov et al. 2003). The comparisons of different techniques are given in Table 3-1.

Evaluation of this fluorescence spectrometry method for the determination of methanogenic cells, based on the measurement of specific and non-specific binding of 16S rRNA-targeted oligonucleotide probe Arc915 and enumeration of auto-fluorescent cells of methanogens, is discussed in this chapter. The method was tested to study the microbial community of the Rm in the HASL system (Wang et al. 2005a; Stabnikova et al. 2005 and 2006). The distribution of different microbial communities in the granules in Rm was also investigated in this study.

3.2 Materials and methods

3.2.1 Experimental set-up

Food waste was shredded and fed to Ra when Rm was in an active state and ready for connection with Ra. The lab-scale HASL system was operated at a constant temperature room at $35 \pm 1^\circ\text{C}$. The system configuration, inoculums, start-up and operation can be referred to Section 2.4.1 and 2.4.2.

Table 3-1 Comparison of different methods for quantification of methanogens

Techniques		Advantages/Applicability		Disadvantages
Direct count of methanogens	auto-fluorescent	Fast enumeration of methanogens		Not applicable for cell aggregates and Cells of <i>Methanosaeta</i>
Determination of specific coenzymes		Detection of specific groups of methanogens		Content of coenzymes is varied depending on species growth conditions
Most probable number (MPN) method		Verification of the results produced by other methods		Long term cultivation and strict anaerobic condition
Cultivation on solid media		Verification of the results produced by other methods		Long term cultivation and strict anaerobic condition
Real-time (quantitative) Polymerase Chain Reaction (PCR)		Microbial suspension with low concentration of methanogens		Probably, not suitable for dense suspensions of methanogens
FISH with rRNA-targeted fluorescent ligonucleotide probes detected by CSLM		Analysis of biofilm and suspended cell aggregate structures		Not quantitative but qualitative detection of methanogenic cells
discrimination between specific and non-specific binding of the rRNA- targeted oligonucleotide probe using labeled and non-labeled probes by fluorescence spectrometer after FISH		Quantitative determination of methanogens in attached or suspended microbial aggregates such as biofilm and granules		Cannot be used for analysis of diluted suspensions of methanogens

3.2.2 Sampling

Thirty nine samples were taken from the middle port of the Rm at a distance of 250 mm from the bottom of the reactor. Anaerobic granules were suspended in a layer from 0 to 150 mm from the bottom of the Rm. Therefore, only changes in the suspended methanogens were taken into account. Anaerobic sludge, collected from an anaerobic digester at the Ulu Pandan Water Reclamation Plant, was used to evaluate sensitivity and accuracy of the monitoring methods.

The samples were fixed in 3% paraformaldehyde in phosphate buffered saline (PBS) immediately after sampling, and stored at 2°C for no longer than 2 hours. All enumeration experiment and FISH must be completed as soon as possible (Stabnikova O. 2006).

3.2.3 Analytical methods

TSS, VSS and SCOD

Total suspended solids (TSS), volatile suspended solids (VSS) and soluble chemical oxygen demand (SCOD) were measured in the well-mixed samples by standard methods (APHA 1998).

Biogas and methane production

Biogas production was monitored daily by a wet gas meter (Ritter TG 05, Bochum, Germany), while the methane content in biogas composition was analyzed by gas chromatography (GC) (HP5890A, HACH, Avondale, PA, USA) which was equipped with a thermal conductivity detector (TCD) and a stainless-steel column packed with Hayesep Q (80/100 mesh). The operating temperatures of the injector, detector and column were kept at 100°C, 200°C and 50°C, respectively. Helium was used as carrier gas at a flow rate of 40 mL min⁻¹.

Enumeration of auto-fluorescent methanogens

Enumeration of auto-fluorescent cells of methanogens was performed using an epifluorescence microscope BX-FLA-3 (Olympus, Tokyo, Japan) at 900-fold magnification under UV wavelengths of 330 to 385 nm (Solera et al. 2001; Stabnikova et al. 2005). The samples from the Rm were diluted with phosphate buffered saline (PBS), pH 7.2, by five to ten times, and treated on a Mini-BeadBeaterTM (Biospec Products, Bartlesville, OK, USA) for 3 min at 4200 oscillations/min and filtrated through a 35 µm cell strainer cap (Falcon, Becton Dickinson, Franklin Lakes, NJ, USA). 5 µL filtrate was spread on a Helber Bacteria Thomas Ruling counting chamber (Weber Scientific International, West Sussex, England) with a area of 10×10 mm and a depth of 20 µm. The concentrations of auto-fluorescent cells of methanogens in the samples were counted by blue-green auto-fluorescence under UV light (Figure 3-1) in six fields of vision for each slide, and each sample was done in triplicate.

Figure 3-1 Microphotograph of methanogens with blue fluorescence

The concentration of methanogens was calculated by the following equation:

$$N = \frac{N_o \times A_s}{A_N \times V} \times D \quad (3-1)$$

where N is the concentration of methanogen (cells mL^{-1}), N_0 is the cells number counted in the field of vision, A_s is the area of sample distributed on the slide ($A_s=100 \text{ mm}^2$), A_N is the area of the counted cells occupied under microscopy ($A_N=3.63 \times 10^{-2} \text{ mm}^2$), V is the volume of sample spread on the slide ($V=5 \mu\text{L}$) and D is the final dilution factor.

The specific growth rate of methanogens using direct count (μ) was estimated by the following equation:

$$\mu = \frac{\ln N - \ln N_0}{t - t_0} \quad (3-2)$$

where N_0 and N are the initial and final concentrations of methanogens at time t_0 and t , respectively.

Quantification of methanogens by fluorescence in situ hybridization (FISH)

The main principle of this measurement is the determination of specific binding (hybridization) of the 16S rRNA-targeted probe using the difference between total binding and non-specific binding of the probe. Non-specific binding of the labeled probe was determined in the presence of the unlabeled probe, which competitively inhibits binding of the labeled probe (hybridization), but does not inhibit non-specific binding. This principle was examined using fluorescence spectrometry measurements (Ivanov et al. 2003) and flow cytometry enumerations (Joachimsthal et al. 2003 and 2004). Quantification of intact 16S rRNAs in the samples from Rm were performed using FISH with labeled by Cy5TM water-soluble cyanine dye and unlabeled 16S rRNA-targeted oligonucleotide probe Arc915 measured by fluorescence spectrometry.

The Arc915 probe, with an aminolinker at the 5'-end, was synthesized by the Synthegen company (Houston, TX, USA). It had the sequence of 5'-GTGCTCCCCCGCCAATTCCT-3', and was known as S-D-Arch-0915-a-A-20. The probe was labeled by Cy5TM water-soluble cyanine dye. The fluorescence of Cy5TM was measured at emission of 648 nm using synchronous fluorescence spectra,

produced with a fluorescence/luminescence spectrometer LS-50B (Perkin-Elmer, Wellesley, MA, USA). Optical slits were set at 10 nm for excitation and emission wavelengths (Raskin et al. 1994a, b and 1995; Alm et al. 1996; Sorensen et al. 1997; Sekiguchi et al. 1999). The concentrations of the labeled probe after FISH in all samples were determined. The labeled probe was added before FISH in the experiment and after FISH in the control. FISH was performed at 50°C for 2 hours. These conditions were established as optimal ones for labeled probe binding (Tay et al. 2001; Ivanov et al. 2003). The concentration of the unlabeled probe was 10 times higher than the concentration of labeled probe to ensure strong competitive inhibition of labeled probe hybridization by unlabeled probe.

Four samples with volume 1 mL (V) were used for analyses. Each sample was added to 1 mL of hybridization buffer (0.9 mol L⁻¹ NaCl, 0.01% sodium dodecyl sulphate, 20 mmol L⁻¹ Tris-HCl, pH 7.2) with 2 mL of formamide in the tubes labeled from T₁ to T₄. Labeled Arc915 probe (10 µL) with known final concentration (P) ranging from 5 to 15 pmol mL⁻¹ was added to tube T₁. Unlabeled Arc915 probe (10 µL) and 10 µL of labeled Arc915 probe were added to tube T₂. No probe was added to tubes T₃ and T₄. All the four tubes were placed in a hybridization chamber (Lab-Line Instruments Inc., IL, USA) with a rotational speed of 6 rev per min for 2 hours at 50°C. After incubation, the suspension was centrifuged at 11,000g for 15 min in an Eppendorf 5415C centrifuge (Eppendorf AG, Hamburg, Germany). The supernatant of tube T₁ was used for the measurement of the probe fluorescence remaining in solution (fluorescence F₁). The supernatant of tube T₂ was used for the determination of non-specific binding (fluorescence F₂). An aliquot (10 µL) of labeled Arc915 was added to the supernatant from tube T₃ immediately before the fluorescence measurement (fluorescence F₃). This was the control to counter the effect of substances that were extracted from the substrate during incubation and might interfere with the probe fluorescence. The supernatant of tube T₄ was used for the self-fluorescence of the substances extracted from the sample (fluorescence F₄) during FISH.

An example of the curves of synchronous fluorescence emission spectra, which were used for the calculation of specific binding of Arc915 probe, is shown in Figure 3-2. Auto-fluorescence of microbial biomass at 420 nm was significantly lower than fluorescence of the applied probe. Fluorescence F_4 was used for the measurement of the auto-fluorescence of the substances extracted from the sample during FISH. Fluorescence F_4 was subtracted from initial probe fluorescence F_3 .

The specific binding of Arc915 probe (B) was determined by the following equation:

$$B = \frac{P}{V} \times \frac{(F_2 - F_1)}{(F_3 - F_4)} \quad (3-3)$$

where P is the concentration of the added labeled probe in tubes T_1 , T_2 , and T_3 ; V is the volume of the sample (mL); F_1 , F_2 , F_3 and F_4 are the values of fluorescence of the supernatants from tubes T_1 , T_2 , T_3 and T_4 , respectively.

Figure 3-2 Synchronous fluorescence emission spectra with peaks F_1 , F_2 , F_3 and F_4 in the supernatants from tubes T_1 , T_2 , T_3 and T_4 , respectively

The specific growth rate of methanogens using specific binding of Arc915 probe (μ_B) was calculated by the following equation:

$$\mu_B = \frac{\ln B - \ln B_0}{t - t_0} \quad (3-4)$$

where B_0 and B are the initial and final specific bindings of Arc915 probe (pmol mL⁻¹) at time t_0 and t , respectively.

Statistical analysis

The statistical analysis was carried out with Microsoft program. Coefficient of variation (COV) was used as an index to evaluate the consistency of the series of independent measurements. Meanwhile, the FISH method was calibrated by using enumeration method. And validation and sensitivity test for the FISH method was also conducted.

Fixation and cryosectioning of anaerobic granules

Intact anaerobic granules harvested at the exponential phase were washed in 1×PBS fixed with freshly prepared paraformaldehyde (PFA) (Sigma, USA) solution (4%, wt/vol) at 4°C for 6 hours, and then stored in 1×PBS: ethanol (1:1) at -20°C (Amann et al., 1995).

The fixed granules were embedded for cryosectioning in an optimum cutting temperature (OCT) compound (Miles, Elkhart, USA) by immersion. Embedded samples were frozen overnight at -20°C, after which 50 µm sections were cut on a cryomicrotome (CM3050S, Leica, German) and mounted to a gelatin-coated (0.1% gelatin and 0.01% chromium potassium sulfate) microscopic slide. The OCT compound was removed by immersion in Milli-Q water. The granule sections were then dehydrated by sequential immersion of the slides for 3 minutes in 50%, 80% and 98% ethanol, and air-dried before being used for FISH.

Fluorescence in situ Hybridization - Confocal Laser Scanning Microscopy (FISH-CLSM).

The following 16S rRNA-targeted oligonucleotide probes were used: (1) Arc915 probe with sequence 5'-GTGCTCCCCCGCCAATTCCT-3' labelled with CY5TM; (2) EUB338 probe with sequence 5'-GCTGCCTCCCGTAGGAGT-3' labelled with CY3 (Manz et al. 1992); and (3) Arc915 probe without fluorescein labelling as competitive probe (Ivanov and Tay 2003). The final concentration of unlabeled probe Arc915 in the hybridization solution was 20-fold higher than the concentration of the corresponding labeled probe. The unlabeled probe was used to completely inhibit the hybridization of the labeled probe with the target (Ivanov and Tay 2003).

Hybridization conditions were optimized for probe Arc915 and probe Eub338 as follows. Firstly, the hybridization temperature was set at 46°C. Hybridization stringency conditions were improved empirically by increasing the formamide concentration by 5% step increments from 5% to 40% in hybridization buffer (0.9 M NaCl, 0.01% sodium dodecyl sulfate, 20 mM Tris-HCl, pH7.2) (Manz et al. 1992). The mixture was placed in 2-mL polypropylene microtubes in the hybridization incubator with a rotational speed of 6 rpm for 4 hours. Optimal stringencies to enhance the specificity of the designed probe were adjusted by hybridization against cells of reference strains using different formamide concentrations. Hybridization was performed for 3 hours at 48°C in an isotonically equilibrated humid chamber; followed by a 30-min washing step at 46°C in washing buffer (0.9 M NaCl, 0.01% sodium dodecyl sulfate, 20 mM Tris-HCl, pH7.2) in a 2-mL polyethylene tube.

Hybridization for the pre-treated slides of microbial granules was performed with optimized formamide concentration for 3 hours at 48°C in an isotonically equilibrated humid chamber, followed by a 30-min washing step at 46°C. For combinations of probes with different optimal hybridization stringencies, two hybridizations were done successively. The first hybridization was achieved with the probe which required higher formamide concentration, and a second hybridization at a lower stringency.

The granule images were obtained by a Fluoview300 confocal laser scanning microscope (CLSM) (Olympus, Japan). Green and red fluorescence were excited by a 10 mW argon laser at 488 nm, separated with a 570 nm splitting filter and detected in channel 1 with a 510 nm long pass filter and in channel 2 with a 660 nm band pass filter.

3.3 Results and discussion

3.3.1 Method validation and sensitivity test

Anaerobic sludge taken from an anaerobic digester for activated sludge digestion was diluted to different concentrations to determine sensitivity and consistency of these FISH method for quantification of methanogens. It can be seen in Table 3-2 that the specific binding of the Arc915 probe was a linear function of biomass concentration when it was between 0.7 and 14.0 mg mL⁻¹ with the COV of 5-19% in this region. This high consistency with low VOC is necessary for the parameter measurements of microbial growth and activity, since variation of conditions and processes can affect the parameter itself and its measurement.

Table 3-2 Sensitivity and consistency of the anaerobic sludge analysis using specific binding of Arc915 probe (average and standard deviations for 6 determinations was shown).

Concentration of biomass (mg VSS mL ⁻¹)	Probe binding (pmol mg ⁻¹ VSS)	Coefficient of variation (COV) (%)
0.14	n.d.	n.d.
0.28	n.d.	n.d.
0.7	0.434 ± 0.083	19
1.4	0.383 ± 0.032	8
2.8	0.394 ± 0.029	7
7	0.401 ± 0.047	12
14	0.370 ± 0.016	5

Note: n.d. means not detectable

Direct cell count of auto-fluorescent methanogens was used to calibrate the measurement of specific binding of oligonucleotide Arc915 probe with 16S rRNAs and they have been compared in Figure 3-3. Equation of linear correlation between specific binding of Arc915 probe (Y, pmol mL⁻¹) and the concentration of auto-fluorescent methanogens by enumeration method (X, cells · 10⁶ mL⁻¹) was as follows:

$$Y = 7.3X + 11.4 \quad (3-5)$$

This correlation was strong with a linear correlation coefficient of 0.95. Therefore, it can be used for the quantification of the concentration of auto-fluorescent methanogens by measurement of oligonucleotide Arc915 probe specific binding instead of enumeration method using the following equation:

$$X = (0.12Y - 1.00) \times 10^6 \quad (3-6)$$

Figure 3-3 Correlation between specific binding of Arc915 probe and concentration of auto-fluorescent methanogens

The average specific binding of Arc915 probe was calculated in different periods of batch anaerobic culture and was 13.4 ± 0.5 amol (10^{-18} moles) per cell of auto-fluorescent methanogen for whole process. Different periods were characterized by

specific growth rate shown in Table 3-3 as the log-phase (with highest values of growth rate), the stationary phase (with lowest growth rate close to zero), and the lysis phase (with negative values of specific growth rate). The average specific binding of Arc915 probe was 14.3, 13.3 and 12.9 amol (10^{-18} mole) per cell in the log phase, the stationary phase, and the lysis phase, respectively.

Table 3-3 Specific growth rate of methanogens

Time (day)	Phase of growth	Specific growth rate (d^{-1}) determined by	
		direct cell count (μ)	specific binding of Arc915 probe (μ_B)
0-3	Lag-phase	0.016	0.015
3-5	Log-phase	0.116	0.120
5-7	Stationary phase	0.085	0.113
7-9	Lysis phase	-0.050	-0.070
9-11	Lysis phase	-0.105	-0.110
11-13	Lysis phase	-0.095	-0.105
13-15	Lysis phase	-0.145	- 0.150

The growth curves of methanogens followed a typical pattern of growth in a batch culture. The maxima of methanogenic cells and rRNAs concentrations on the 7th day of cultivation were detected by both direct count and specific binding of Arc915 probe methods. Specific growth rates of methanogens, calculated by these two methods, were the same for the periods from the beginning of anaerobic cultivation to the 5th day and from the 7th day to the 15th day (Table 3-3). However, these parameter values were different during the period from the 5th day to the 7th day of cultivation, 0.113 d^{-1} and 0.085 d^{-1} for the probe binding method and for the direct count method, respectively. This difference might have been caused by increase of rRNA content in methanogenic cells during intensive growth in the log phase which was from the 3rd day to the 5th day. The similar value of specific growth rate, 0.2 d^{-1} , was observed for a microbial community in an anaerobic digester (Pollard and Greenfield 1997). The maximum specific growth rates from 0.24 d^{-1} to 0.28 d^{-1} were found for the mesophilic acetoclastic methanogen

Methanosarcinales concilii (Boone et al. 2001), and from 0.15 d^{-1} and 0.19 d^{-1} for anaerobic culture enriched with *Methanobacterium thermoautotrophicum* (Stams et al. 1992).

The quantity of the Arc915 probe changed from 12.9×10^{-18} to 14.3×10^{-18} mole per cell depending on growth phase, but these changes were just 10.4% from the mean value. It is a well-known linear correlation between the rRNA content and specific growth rate for fast growing prokaryotic cells. In this experiment, the specific binding of rRNA-targeted Arc915 probe per cell depended, but not significantly, on the specific growth rate. Apparently, rRNA content changed insignificantly in slow growing methanogens, because linear correlation between specific binding of Arc915 probe and the concentration of auto-fluorescent methanogenic cells was strong with a correlation coefficient of 0.95. Therefore, specific binding of Arc915 probe can be used for the comparative estimation of methanogens if the density of anaerobic sludge is between 1.4 and 14.0 mg mL^{-1} . The COV of the measurements in this region was from 5 to 12%. This high consistency with low VOC is necessary for the parameter measurements of microbial growth and activity, since variation of conditions and processes can affect the parameter itself and its measurement. Therefore, there is major applicability of the described technique in quantitative determination of methanogens in attached or suspended microbial aggregates.

3.3.2 Estimation of growth rate of methanogens by specific binding of Arc915 probe

Concentration of methanogens shown in Figure 3-4 was estimated by specific binding of Arc915 probe and direct count of auto-fluorescent methanogens. Concentration of organics in the influent used for feeding Rm, estimated by COD, changed during the experiment from 8.23 g COD L^{-1} (at the beginning of the process) to $21.16\text{ g COD L}^{-1}$ (on the 7th day) and 1.43 g COD L^{-1} (on the 15th day). Daily methane production increased from 0.85 L on the first day to 6.7 L on the 6th day and then decreased constantly to 0.13 L at the end of the experiment. Methanogen concentrations were maxima during the period from the 5th to the 7th

day, which were from 2.0×10^6 to 2.1×10^6 cells mL^{-1} estimated by direct count, and from 28.6 to 29.4 pmol mL^{-1} estimated by specific binding of Arc915 probe, respectively. The specific growth rates of methanogens, calculated by these two methods, are shown in Table 3-3. The maximum specific growth rate was 0.12 d^{-1} when methanogens grew during the first 7 days of cultivation. After that, the biomass of methanogens was increasingly lysed with rates from -0.05 to -0.15 d^{-1} . Lysis of methanogens coincided with the decrease of daily methane production rates.

Figure 3-4 Dynamics of methanogens concentration in Rm estimated by specific binding of Arc915 probe (●) and direct count (◆). It is also shown daily (▲) and accumulative (Δ) methane production; dynamics of SCOD concentration (■) in the influent fed the Rm and (□) in the effluent from Rm

3.3.3 Distribution of methanogens in anaerobic granules

FISH-CLSM was used to elucidate the abundance and spatial distribution of bacteria and methanogens in the anaerobic granules. The oligonucleotide probe of Eub338 labeled with CY3 was designed to target the 16S rRNA gene of bacteria, while the oligonucleotide Arc915 probe labeled with CY5TM was designed to target the 16S rRNA gene of methanogens. They were used in this study to track the fate and spatial distribution of bacteria and methanogens during formation of anaerobic granules.

The samples were taken before inoculation and after experiment completion and were named as initial and final granules with a diameter of 100-300 μm and >500 μm , respectively. Anaerobic bacteria and methanogens were visualized as a 100- μm -thick layer located at a depth of 100 μm below the surface of the initial granules and 200 μm below the surface of final granules. Three of each granule sample was selected and there was a similar trend for the abundance and spatial distribution of bacteria and methanogens. The clearest image was selected and shown in Figure 3-5. If there was no probe conferred fluorescence, it indicated no hybridizing of probe with target bacteria or methanogens, which means there was no bacteria or methanogens existed.

It is obvious in Figure 3-5 that final granule contained abundant microbial communities due to addition of carbon and nutrient sources for 15 days. And they were densely distributed in the outer shell since there was more nutrient available surrounding the surface of final granule (Figure 3-5c & d). However, since the initial granule was under the starvation condition, the outer shell became loose, while granule core part still remained intact for survival (Figure 3-5a & b).

In the initial granule, bacteria that hybridized with the EUB338 probe were distributed densely in the central region (Figure 3-5a). The substrate is the limiting factor under such starvation condition, so the bacteria on the surface may lose their functions or even die. However, in the central part, the bacteria still can utilize

substrate surrounding them and survive. If the starvation time extended, all bacteria will died eventually and the granule become smaller and smaller until disappear.

Figure 3-5 Distribution of bacteria and methanogens within a cross-section through anaerobic granules observed by CLSM. (a). Initial granule hybridized with Cy3 labelled probe EUB338, (b) Initial granules hybridized with Cy5 labelled probe Arc915; (c) Final granule hybridized with Cy3 labelled probe EUB338; (d) Final granules hybridized with Cy5 labelled probe Arc915; scale bar —: 100 μm .

The case is totally different for final granules which just experienced an active methanogenesis process with abundant nutrients under favorite conditions, so bacteria that hybridized with the EUB338 probe were distributed across the entire granule section (Figure 3-5c). In this case, bacterial growth rate instead of substrate is the rate limiting factor. Due to the mass gradient from high to low along the radius to center direction, accordingly a high to low bacterial growth rate was formed in this direction. Thus the final granules consisted of a dense layer of bacterial cells, surrounding a less dense central region (Figure 3-5c).

Methanogen distribution had the similar trend as bacteria distribution both in the initial and final granules. For the initial granules with diameters of 100 to 300 μm , methanogens in the central part were affected lightly by substrate shortage compared to that in the outer layer. Sequentially, more methanogens survived in the central part than in the outer layer (Figure 3-5b). The same phenomenon, methanogens only found in the core was also observed by Santegoeds et al. (1999) when he used microsensor and molecular analyses to study the distribution of sulfate-reducing and methanogenic bacteria in anaerobic aggregates. For the final granules with diameters greater than 500 μm , there is a high to low bacterial growth rate along the radius to center direction due to the mass gradient. Accordingly, the final granules consisted of a dense outer layer of methanogens, surrounding a less dense central region (Figure 3-5d). When the operation time is long enough, the core of granule becomes loose and will break eventually.

3.4 Conclusion

This study showed that the specific binding of Arc915 probe could be used for the comparative estimation of methanogens during anaerobic digestion of organic waste, i.e. specific binding of Arc915 probe was a linear function of anaerobic sludge concentration when it was between 0.7 and 14.0 mg mL^{-1} . The COV of the measurements in this region was from 5 to 19%. The high consistency with low VOC is necessary for the parameter measurements of microbial growth and activity.

Meanwhile, the abundance and spatial distribution of methanogens in the anaerobic granules were observed by FISH-CLSM. Both methanogens and bacteria distributed in the central part of the granules with diameters of 100 to 300 μm , but in the outer layers of the granules with diameter greater than 500 μm . In this way, methanogens can survive and maintain a high activity for methanogenesis.

CHAPTER 4

EFFECT OF LIPIDS ON FOOD WASTE DIGESTION IN THE HASL SYSTEM

4.1 Introduction

As mentioned in Chapter 2, the efficiency of AD is dependent on the composition of the substrate. Lipids (characterized as oil, grease and fat), which are present in high quantity in Asian food, are potential inhibitors of methane production (Forster et al. 1992). It is well known that fat and vegetable oil are biodegraded to glycerol and long-chain fatty acids (LCFA), followed by subsequent β -oxidation of LCFA. LCFA can inhibit their β -oxidation (Hanaki et al. 1981). LCFA are slowly degraded under anaerobic conditions and the millimolar concentration of LCFA can inhibit the growth of anaerobic microorganisms, including acetogenic bacteria and methanogens (Roy et al. 1985; Rinzema et al. 1994; Chu et al. 1997; Hwu et al. 1997; Ivanov et al. 2002). The main mechanism of inhibition is adsorption of surface-active LCFA on cell membrane following dissipation of membrane potential ensuring transport of nutrients from medium to cells. However, energetic value of lipids is twice that of carbohydrates (Fernandes et al. 1988). Therefore, theoretically, the presence of lipids in anaerobically treated matter can increase biogas production.

It was shown that a two-phase AD system was more efficient than a single-stage process for the treatment of lipid-containing wastewater due to prevention of methanogen inhibition by removal of lipids in the acidogenic reactor. Methane production ($\text{mL CH}_4 \text{ g}^{-1} \text{ COD}$) from cafeteria waste containing 30% lipids, was higher in the two-phase system than in the single-stage system (Hanaki et al. 1990). Kim et al. (2004b) also reported that a two-phase anaerobic system was able to treat synthetic LCFA-containing wastewater with higher loading rate than a single-stage

system. The two-phase system was successfully used for the treatment of such fat-containing solid waste like slaughtered chickens (Chen and Shyu 1998; Salminen et al. 2000).

The aim of this study was (1) to evaluate the feasibility of using the HASL system to treat food waste with high content of lipids; and (2) to investigate the effect of lipids content in food waste on the performance of two-phase AD using the HASL system.

4.2 Materials and methods

4.2.1 Feedstock and anaerobic sludge

Food waste used in the experiments was collected from a canteen on the university's campus and sorted in terms of vegetable roots, fruits peels, cooked rice and noodles. They were shredded into particles with an average size of 6.0 mm in a Robot-Coupe Shredder (CL50 Ultra, France). After weighting separately, they were mixed completely. Composition of the food waste mixture is shown in Table 4-1. Lipids were prepared by melting the internal fat of a hog that was solidified at room temperature.

Table 4-1 Composition of food waste mixture (mean values $\pm$ standard deviations)

Food waste	Composition of mixture (% of wet weight)	Content (%)	
		TS (%)	VS (%TS)
Vegetable roots	50	5.5 $\pm$ 0.5	60.2 $\pm$ 1.0
Orange peels	20	43.7 $\pm$ 0.8	89.6 $\pm$ 1.2
Cooked rice	15	26.7 $\pm$ 0.3	94.8 $\pm$ 0.8
Cooked noodles	15	42.5 $\pm$ 1.1	89.6 $\pm$ 0.9

Anaerobic sludge, used as inoculum in the acidogenic reactor (Ra), was collected from an anaerobic digester at the Ulu Pandan Water Reclamation Plant which was

designed for domestic wastewater treatment. The concentrations of TSS and VSS in this sludge were $3.79 \pm 0.12 \text{ g L}^{-1}$ and $2.71 \pm 0.08 \text{ g L}^{-1}$, respectively.

4.2.2 Experimental set-up

The experiments were carried out simultaneously in four identical HASL systems operated in a constant temperature room at $35 \pm 1^\circ\text{C}$ for two weeks. The system configuration, inoculums, start-up and operation can be referred to Section 2.4.1 and 2.4.2.

Food waste, 800 g, was placed in each Ra. Lipids were added to the reactors as follows (w/w of total solids in food waste): 0 (control), 20% (E1), 30% (E2) and 40% (E3), respectively.

4.2.3 Analytical methods

pH, TS, VS, TSS, VSS and SCOD

The pH value was measured using a pH meter (CORNING 145, England). Total solid (TS), volatile solid (VS), TSS, VSS and SCOD were measured in the well-mixed samples in triplicates by standard methods (APHA 1998).

Carbon and nitrogen

The content of carbon and nitrogen in the food waste mixture was determined using an Elemental Analyzer CHNS/O 2400 (Perkin Elmer, USA). Samples were oven-dried at 105°C for 24 hour, then ground into powder with particle size less than 2 mm, 1 mg of this powder was sealed for analysis.

Biogas and methane production

This method has been detailed in Chapter 3.

Volatile fatty acids (VFA)

For the determination of volatile fatty acids (VFA), samples were filtrated through Whatman 0.2 μm nitrocellulose membrane filters and then analyzed using high performance liquid chromatography (HPLC) (Perkin Elmer, Series 200, Norwalk, CT, USA). The HPLC was equipped with a 220 mm $\times$ 4.6 mm polypore H column and a 210 nm UV detector. The mobile phase was 0.005 N H_2SO_4 with a flow rate of 0.15 mL min⁻¹.

Contents of lipids determination

Content of lipids in the initial food waste and in the residue after 14 days of anaerobic digestion was determined by extraction into a Soxhlet apparatus (Hwang et al. 2002). 10 g of dry sample was placed in a cellulose thimble 30 $\times$ 100 mm (Whatman International Ltd, UK), which was fitted to a distillation flask containing 200 mL of diethyl ether. To prevent the boiling liquid from bumping, 5-6 anti bumping granules from fused alumina were placed in the distillation flask. After extraction for 24 hours at 38°C, the solvent was evaporated at 80°C for 12 hours until the difference between two consecutive results was smaller than 2 mg.

Enumeration of total bacterial cells and methanogenic cells by direct count

Enumeration of total bacterial cells and methanogenic cells was performed by epifluorescence microscope BX-FLA-3 (Olympus, Tokyo, Japan) at 900-fold magnification under UV wavelength from 330 to 385 nm (Ivanov et al. 2004; Solera et al. 2001). The samples were treated on a Mini-BeadBeaterTM (Biospec, USA) for 3 min at 4200 oscillations per min and filtrated through a 35 μm cell strainer cap (Becton Dickinson, USA) to remove the particles larger than 35 μm . The suspension was diluted with a phosphate-buffered saline solution (PBS) with pH of 7.2. The suspension was placed in a 0.02 mm depth Helber Bacteria Thomas Ruling counting chamber (Weber Scientific International, England).

The methanogens were determined directly by their blue auto-fluorescence under UV light. However, the total bacterial cell was counted after staining with 4',6-diamidino-2-phenylindole (DAPI) (Sigma) at final concentration of $0.1 \mu\text{g mL}^{-1}$. After 7 minutes of incubation (Kepner and Pratt 1994), the excess of stain was removed by washing three times with PBS. The suspension was placed in a 0.02 mm depth Helber Bacteria Thomas Ruling counting chamber (Weber Scientific International, West Sussex, England). The DAPI-stained cells were visualized by blue fluorescence of DAPI under UV light using an epifluorescent microscope.

The concentration of bacteria was calculated by the equation below:

$$N = \frac{N_o \times A_s}{A_N \times V} \times D \quad (4-1)$$

where N is the concentration of bacteria (cells mL^{-1}), N_o is the cells number counted in the field of vision, A_s is the area of sample distributed on the slide ($A_s=100 \text{ mm}^2$), A_N is the area of the counted cells occupied under microscopy ($A_N=3.63 \times 10^{-2} \text{ mm}^2$), V is the volume of sample spread on the slide ($V=5 \mu\text{L}$) and D is the final dilution factor. Bacterial cell count was done in 6 fields of vision for every sample.

The specific growth rate of microbial cells determined by direct count (μ) was estimated by using the following equation:

$$\mu = \frac{\ln N - \ln N_0}{t - t_0} \quad (4-2)$$

where N_0 and N are the initial and final concentration of cells at time t_0 and t, respectively.

Measurement of auto-fluorescence of coenzyme F_{420}

F_{420} , a 7, 8-didemethyl-8-hydroxy-5-deazaflavin electron transfer coenzyme, is an essential component of methanogenic biomass. The auto-fluorescence of coenzyme F_{420} , can be used as a relative parameter of the concentration of methanogens (Ivanov et al. 2004). The sample taken from the Rm was treated in a Mini-

BeadBeater™ (Biospec Products, USA) for 100 sec at 500 oscillations per min. The suspension was filtered through a 35 µm cell strainer cap (Becton Dickinson, USA) to remove particles larger than 35 µm. Then the suspension was put into 3 ml fluorescence quartz cuvette with 1 cm path length. F_{420} fluorescence is the emission intensity of an emission wavelength of 420nm and was measured under a synchronous scan regime using a Fluorescence/Luminescence Spectrometer LS-50B (Perkin Elmer, Wellesley, MA, USA). The difference between excitation and emission wavelengths was 20 nm. The excitation wavelength was changed from 360 nm to 480 nm and the emission wavelength was synchronously changed from 380 nm to 500 nm (Stabnikova et al. 2005; Wang et al. 2006). The fluorescence emission spectrum of coenzyme F_{420} under fluorescence spectrometer is shown in Figure 4-1.

Figure 4-1 Fluorescence emission spectrum of coenzyme F_{420}

The specific growth rate (μ_F) of methanogens was estimated by the equation below:

$$\mu_F = \frac{\ln F - \ln F_0}{t - t_0} \quad (4-3)$$

where F_0 and F are the initial and final fluorescence of coenzyme F_{420} at time t_0 and t , respectively.

Evaluation of food waste biodegradation

The percentage of biodegradation of organic matter (α) was estimated by the following equation:

$$a = \left(1 - \frac{VS_2 \times (1 - VS_1)}{VS_1 \times (1 - VS_2)} \right) \times 100\% \quad (4-4)$$

where VS_1 and VS_2 are the initial and final amounts of volatile solids in the organic matter, respectively. This equation is based on the assumption that inorganic matter remains unchanged during the biodegradation of food waste (Stabnikova et al. 2005).

4.3 Results and discussion**4.3.1 Changes of pH, VFA and SCOD in the leachate from Ra**

Change of pH in the Ra is correlated with the different phases of the acidogenesis process, while the pH in the Rm indicates the stability of the anaerobic system during methanogenesis. Normally, for a batch HASL system, pH in the leachate from Ra drops rapidly to 5.0 and then increases to the neutral pH (Xu et al. 2002; Wang et al. 2003). The pH changes in the leachate from Ra in the control and the experiments are shown in Figure 4-2.

During the first three days of the start-up period, rapid pH decrease was observed in the leachate from all four Ra (Figure 4-1). The lowest pH of 5.7 was reached in the control, which indicates that acidogenesis was more active when food waste did not contain lipids. The presence of 20% to 40% lipids lowered pH value to 6.3 - 6.5. After dropping, the pH increased to 8.2, 8.4, 8.5 and 8.6 in the control, E1, E2 and E3, respectively at the end of the experiments.

Although there was no big difference in pH measurement under different lipids content, the similar trend is obvious. Due to the low lipid removal rate shown in

Table 4-2, only small part of lipids was degraded. Accordingly the effect of different lipids content was not so significant.

Figure 4-2 pH in the leachate from Ra in the control and the experiments

Figure 4-3 illustrates the influence of different content of lipids on VFA production in the Ra. It is consistent with the pH data (Figure 4-2), the TVFA also had peak around 2-3 days. The maximum VFA concentrations were $1,724 \text{ mg L}^{-1}$ and $1,610 \text{ mg L}^{-1}$, in the control and E1 on the 2nd day, $1,597 \text{ mg L}^{-1}$ and $1,449 \text{ mg L}^{-1}$ in E2 and E3 on the 3rd day, respectively. After that, the TVFA decreased consistently to the end of food waste bioconversion. During the two weeks of operation, the total VFA productions in Ra were 7.1 g, 7.3 g, 7.4 g, and 6.4 g in the control, E1, E2 and E3, respectively.

The SCODs of the leachate from Ra shown in Figure 4-4 is consistent with the pH data (Figure 4-2) and VFA data (Figure 4-3). The SCOD also had peak around 2-3 days. The maximum SCOD concentrations were reached on the 2nd day, and were $36,290 \text{ mg L}^{-1}$, $34,770 \text{ mg L}^{-1}$, $33,250 \text{ mg L}^{-1}$ and $38,380 \text{ mg L}^{-1}$ in the control, E1, E2 and E3, respectively. After the biomass growth phases, the SCOD decreased consistently to the end of food waste bioconversion.

Figure 4-3 TVFA in the leachate from Ra in the control and the experiments

Figure 4-4 SCOD in the leachate from Ra in the control and the experiments

4.3.2 Changes of pH and SCOD in the effluent from Rm

Although there was some fluctuation for pH measurement, the pH of the effluents from Rm were in a narrow range of 7.8 to 8.7 (Figure 4-5). The relatively constant pH value indicates operational stability in Rm (a modified UASB) and in the HASL systems. The data also meant different lipids content did not affect pH in Rm.

Figure 4-5 pH in the effluent from Rm in the control and the experiments

It can be seen in Figure 4-6 that the maximum SCOD concentrations in the effluent from Rm were $4,000 \text{ mg L}^{-1}$ on the 3rd day in the control, E1 and E2, but increased to $5,000 \text{ mg L}^{-1}$ on the 4th day in E3. Since most organic matters were converted into methane in Rm by methanogens, the SCOD concentrations in the effluent from Rm were much lower than that in leachate from Ra. The overall SCOD removal rates were in the range of 87.5% to 88.9% for the control, E1 and E2, but dropped to 79% in E3. It meant high lipids content (40%) had negative effect in SCOD removal.

Figure 4-6 SCOD in the effluent from Rm in the control and the experiments

4.3.3 Biogas production in the control and the experiments

The biogas production from Rm in the control and the experiments is shown in Figure 4-7. The total biogas production increased in comparison with the control when lipids were added to food waste in the quantity of 20% to 30%. It was probably due to the higher biogas production potential of lipids compared to carbohydrates. However, when content of lipids was increased to 40%, the accumulative biogas production was the same as that in the control (Table 4-2). The methane content in biogas was 78%, 80%, 83% and 70% in the control, E1, E2 and E3, respectively.

During the first 9 days, methane production is higher in control followed by E1, E2 and E3, which probably caused by slow degradation of lipids in experiments. At the end of experiments, more lipids in E1 and E2 were degraded to available substrate for methanogenesis resulting greater methanogenic activities and higher methane production due to high energetic value of lipids. While there is no lipid

was graded in E3, the total available substrate are in the same quantity in control and E3. So the same quantity methane was produced in control and E3.

Figure 4-7 Biogas production from Rm in the control and the experiments

4.3.4 Comparison of anaerobic digestion of food waste with different lipids content in the HASL system

The influence of lipids content in the food waste on anaerobic digestion in the HASL system is shown in Table 4-2. The VS and lipids removal efficiencies negatively correlated with the presence of 20% to 40% lipids in the food waste. VFA production in Ra treated food waste with lipids content 0%, 20%, 30% and 40%, had the same trend and were 7.1 g, 7.3 g, 7.4 g and 6.4 g, respectively (Figure 4-3). The COD removal efficiencies were in the same range of 87.5% to 88.9% for Rm in the control and in the experiment treated food waste with addition of 20% to 30% of lipids, but dropped to 79% when lipids content was increased to 40% (Figure 4-6). The percentage of lipids removal decreased with the increase of their initial content (Table 4-2).

Table 4-2 Influence of lipids on food waste digestion in the HASL system

Experiment	VS removed		Lipids removed		Biogas produced	
	(g)	(%)	(g)	(%)	(L)	(L g ⁻¹ VS)
Control	104.3	85	nd*	nd	33.7	0.32
E1	99.2	71	6.2	23.8	36.0	0.36
E2	92.0	63	5.8	14.8	38.3	0.42
E3	85.7	55	2.6	5.0	33.7	0.39

*nd – not determined

Biogas production was increased in E1 and E2 with addition of 20% to 30% of lipids, but further increase in lipids content in food waste diminished its positive effect on AD. Hypothetically, the presence of lipids in low quantity (20%-30%) enhanced biogas production in the HASL system due to high energetic value of lipids, but the higher content of lipids (40%) diminished this positive effect due to inhibition of methanogenic activity.

Chemical composition of the residue after anaerobic digestion is shown in Table 4-3. The presence of lipids in treated food waste increased the content of organic matter (VS) and carbon, and decreased the content of nitrogen in the residue after anaerobic digestion. C/N ratio of the residue increased from 13.6 in the control to 15.9, 19.5 and 24.3 in E1, E2 and E3, respectively. Because of high C/N ratio, additional composting or intensive aerobic treatment is needed for bioconversion of this residue into fertilizer or soil amendment (Sharma et al. 1997; Stabnikova et al. 2005).

Table 4-3 Characteristics of the initial material and residuals after anaerobic digestion of food waste with different lipids content in the HASL system (mean values $\pm$ standard deviations are shown)

Parameters	Control			E1		E2		E3	
	Initial	Final		Initial	Final	Initial	Final	Initial	Final
TS, %	17.0 $\pm$ 0.3	8.8 $\pm$ 0.1		18.5 $\pm$ 0.2	13.1 $\pm$ 0.1	18.9 $\pm$ 0.3	13.9 $\pm$ 0.3	19.4 $\pm$ 0.4	12.0 $\pm$ 1.4
VS, % of TS	90.9 $\pm$ 0.6	60.2 $\pm$ 0.8		91.2 $\pm$ 0.7	74.9 $\pm$ 0.1	92.1 $\pm$ 0.7	81.2 $\pm$ 0.8	94.3 $\pm$ 0.2	88.3 $\pm$ 1.4
Lipids, % of TS	2.5 $\pm$ 0.6	5.5 $\pm$ 0.8		12.1 $\pm$ 0.2	24.9 $\pm$ 0.5	18.2 $\pm$ 0.1	33.1 $\pm$ 0.1	22.1 $\pm$ 0.8	37.1 $\pm$ 0.5
C, % of TS	nd*	49.8 $\pm$ 1.2		nd	51.2 $\pm$ 1.2	nd	52.7 $\pm$ 0.4	nd	57.1 $\pm$ 0.3
N, % of TS	nd	3.7 $\pm$ 0.3		nd	3.2 $\pm$ 0.3	nd	2.8 $\pm$ 0.01	nd	2.4 $\pm$ 0.1
C/N	nd	13.6		nd	15.9	nd	19.5	nd	24.3

*nd – not determined

4.3.5 Estimation of methanogenic activity by F_{420} fluorescence

F_{420} fluorescence was used to estimate the methanogenic activity in Rm and is shown in Figure 4-8. The F_{420} fluorescence had the typical shape of a batch growth curve, with a lag phase (adaptation phase), a log phase, a stationary phase, and a death phase. The presence of lipids in food waste diminished the rate of F_{420} fluorescence, and increased and enlarged the period of adaptation. Decrease of F_{420} fluorescence was observed during the first 5 days in E3. The presence of lipids slowed down the growth of methanogens and a long adaptation period was needed to start up their activity. However, high fluorescence was observed on the 8th day in E1 and E2 with 20% to 30% of lipids, meanwhile the fluorescence in E3 only reached its initial value on the 14th day. The maximum growth rates of methanogens estimated by equation (4-3) were 0.2 d^{-1} , 0.11 d^{-1} , 0.05 d^{-1} and 0.03 d^{-1} in the control, E1, E2 and E3, respectively.

Figure 4-8 Fluorescence of F_{420} in the control and the experiments

4.3.6 Estimation of methanogens by direct count

The concentrations of total bacteria and methanogens in Rm estimated by direct count are shown in Figures 4-9 and 4-10. They have a similar and typical shape of a batch culture growth curve and no phase of adaptation was observed.

Figure 4-9 Concentrations of total bacteria in the control and the experiments

Figure 4-10 Concentrations of methanogens in the control and the experiments

Figure 4-11 shows the percentage of methanogens over total bacteria, which were in the range of 11 - 22%. During the first 5 days, the percentage of methanogens over total bacteria increased slightly to 15 - 22% since it is in biomass growth phase, and then slowly diminished to 11 - 21% at the end of the anaerobic process due to substrate shortage.

Figure 4-11 Percentage of methanogens in the control and the experiments

According to Figures 4-9, 4-10 and 4-11, the presence of lipids enhanced the growth of total bacteria and methanogens in comparison with the control. However, the maximum cell concentrations for both total bacteria and methanogens were highest in E1 with 20% lipids. It is supposed that the presence of lipids enhanced the energetic value of leachate from Ra, which caused the increase of total bacteria and methanogens in Rm. The growth curves of E2 and E3 did not differ much. However, in E3 with lipids content of 40%, the growth curves were almost the same as in the control with no lipids. The maximum growth rates estimated by Equation 4-2 were 0.33 d^{-1} , 0.39 d^{-1} , 0.29 d^{-1} and 0.26 d^{-1} for the control, E1, E2 and E3, respectively. Based on this data, the content of lipids in Ra higher than 20% would inhibit bacterial growth in Rm.

The maximum growth rates of methanogens estimated by direct count and by F_{420} fluorescence are summarized in Table 4-4. The differences between them can be explained by direct count showing both alive and dead cells, but F_{420} fluorescence is closely related to the viable of methanogens. According to F_{420} measurement, the presence of lipids had more negative effects on methanogens than that determined by direct count.

Table 4-4 The maximum growth rate of methanogens estimated by direct count and by F_{420} fluorescence

Experiments	The maximum growth rate of methanogens (d^{-1}) determined by	
	direct count (μ)	F_{420} fluorescence (μ_F)
Control (no lipids)	0.33	0.2
E1 (20% lipids)	0.39	0.11
E2 (30% lipids)	0.29	0.05
E3 (40% lipids)	0.26	0.03

The presence of lipids in food waste had two main effects on the HASL system: it (1) enhanced biogas production and (2) inhibited the growth of methanogens. The first effect was prevalent when the lipids content was 20%. It was balanced by the second effect when the lipids content was 30%. The negative effect dominated when the lipids content was 40% due to the toxicity of long chain fatty acid produced by hydrolysis of lipids. Cirne et al. (2007) also observed a stronger inhibition in AD for higher lipids contents addition (31%, 40% and 47%). When studying the effect of addition of lipase on enzymatic hydrolysis of lipids, the enzyme appeared to enhance the hydrolysis, but the intermediates produced caused inhibition of the later steps in the degradation process. Since the VFA profiles presented similar trends for the different concentrations of lipid tested, the major obstacle to methane production was believed to be the long-chain fatty acids (LCFA) formation.

4.4 Conclusion

This study showed that (1) the HASL system can be used for the treatment of food waste containing 20-40% of lipids and (2) that lipids content is a key parameter which greatly affects food waste digestion in the HASL system. Food waste with high lipids content would result in poor digestion, even though the biogas production is higher. According to the results obtained, the presence of lipids enhanced the biogas production but could also inhibit the growth of methanogens. The positive effects of lipids on AD dominated when the content of lipids were in the range of 20-30%. Relatively high lipids content, e.g. 40%, could worsen VFA production in Ra and inhibit the growth of methanogens and biogas production in Rm. It appears that the role of food waste composition, i.e. lipids content should be taken into account seriously in the design and operation for a full-scale HASL system.

CHAPTER 5

EFFECT OF THERMAL PRETREATMENT ON FOOD WASTE DIGESTION IN THE BATCH EXPERIMENTS AND IN THE HASL SYSTEM

5.1 Introduction

As mentioned in Chapter 2, as the simplest pretreatment method, heating of food waste has been used to enhance hydrolysis and liquefaction of organic waste, the rate-limiting step in the anaerobic process.

Heat can be used to disrupt hydrogen bonds and non-polar hydrophobic interactions. This occurs because heat increases the kinetic energy of molecules and causes the molecules to vibrate so rapidly and violently that the bonds are disrupted. For example, proteins present in the cells are denatured at high temperature. As temperature increases, the polypeptide chain present in protein start to unfold and lose its 3-D structure. This results in a less complex protein structure which can be readily broken down into peptides and amino acids in the later stages.

Since temperature plays a major role in hydrolysis, it is necessary to select an optimum temperature for pretreatment. It has been proven that thermal pretreatment of sludge at elevated temperature (100-275°C) significantly increases the disintegration and solubilization of sludge solids and thus improves sludge stabilization (Müller 1998). The advantages of operating at high temperature are: (1) Enhanced removal of organic matter which in turn leads to an increased production of biogas; (2) Increased hydrolysis rate; (3) Shorter hydraulic retention time; and (4) Pathogens present can be destroyed by the high temperature. However, operating at such a high temperature requires a large amount of energy and is difficult to operate.

Food wastes contain more than 80% of water. If the temperature is greater than 100°C, most of the energy consumption will be used for heating the water. Water will evaporates and the amount of water for AD will decrease. Thus extra water needs to be added into the system. Since overheating is not economical, the pretreatment should be conducted at low temperature (less than 100°C) where it will be more cost-efficient.

Since both temperature and duration will affect the hydrolysis, the optimum combination of temperature and duration will be determined in the batch experiments. The thermal pretreatment method was then applied to enhance hydrolysis in the HASL system.

5.2 Material and methods

5.2.1 Optimization of temperature and duration in the batch experiments

Food waste

Food waste was collected from Canteen A on campus. After sorting in terms of rice, bread, noodles, vegetables and fruits, they were shredded into particles with average size 6.0 mm in a Robot-Coupe Shredder (CL50 Ultra, France). After weighing individually and mixing completely, the composition of the food waste was recorded and is shown in Table 5-1.

Experimental Set-up

The food waste mixture was blended into puree form using a Braun Food Blender. 50 mL plastic tubes were used to contain the food waste puree. Four different water bath machines were set at 35°C, 50°C, 70°C and 95°C, respectively. While the six tubes were placed in each water bath machine, temperatures in each tube were being monitored. Once the temperature of the food waste inside the tube had reached the desired set point temperature, it was recorded as time zero. Then different tubes

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were removed from the water bath at 30, 60, 90, 120, 150 and 180 minutes and cooled down at room temperature for about 3 hours.

Table 5-1 Composition of food waste mixture in the batch experiments

Food waste	Percentage (% of wet weight)	TS (%)	VS (% of TS)
Cooked rice and bread	10	33.91 ± 0.52	98.69 ± 0.15
Cooked noodles	10	41.84 ± 0.11	98.47 ± 0.98
Vegetables	40	5.05 ± 0.13	88.62 ± 0.84
Fruits peels	40	19.82 ± 0.09	95.16 ± 0.24
Mixed food waste		17.52 ± 1.72	95.88 ± 1.27

Sampling

All samples were measured for pH immediately after cooling, followed by centrifuging at 3000 rpm for 30 minutes to separate the supernatant from the residue. The supernatant was then extracted and tested for COD, TOC, VFA, protein and sugar, while the residue was measured for TSS and VSS.

5.2.2 Digestion of thermally pre-treated food waste in the HASL system

Food waste

The food waste is identical to that in section 5.2.1 except for a slight difference in composition as shown in Table 5-2, due to different collection times. The content of TS and VS were 16.3% and 88.0% in the mixed waste. The contents of nitrogen and phosphorus were 2.2% and 0.1% of TS, respectively. The theoretical COD of raw food waste was 126.17 g COD (i.e. 1.1 g COD g⁻¹VS × 114.7 g VS).

Table 5-2 Composition of food waste in the HASL system

Food waste	Percentage (% of wet weight)	TS (%)	VS (% TS)
Vegetables	50	6.6± 0.7	71.0 ± 0.9
Fruits	20	23.9 ± 1.1	97.3± 1.4
Cooked rice	15	9.5 ± 0.5	56.3 ± 1
Cooked noodles	15	45.3 ± 0.8	96.2 ± 1.1

Sampling

The leachate was taken from Ra to test for pH, COD and VFA. The effluent from Rm was collected to measure for pH and COD. The liquid taken from the middle port in Rm was used in a cells enumeration test. The biogas produced from Rm was monitored daily. Only the leachate from Ra and liquid from Rm collected on the 3rd day were used for testing the dominant microbial community.

5.2.3 Analysis methods

TS, VS, TSS, VSS, pH, SCOD, VFA, biogas production and methane content, microbial enumeration, coenzyme F₄₂₀, specific binding of Arc915 probe and statistical analysis are identical to that mentioned in Chapter 4.

Total Organic Carbon (TOC)

A sample was diluted and filtered using a 0.45 µm filter paper, then analyzed using a TOC analyzer. (TOC-Vcsh, Shimadzu)

Protein Test

3 mL of Bradford reagent was added to 0.1 mL of a diluted sample. After complete mixing, the sample was left at room temperature for 20-30 minutes. The absorbance was measured at 595 nm using a spectrophotometer (UV-1201 V Shimadzu, Japan).

The testing must be and was finished within 60 minutes after the addition of the Bradford reagent to avoid the negative effects of long reaction time.

Sugar Test

0.5 mL of phenol and 2.5 mL of concentrated sulphuric acid were added to 0.5 mL of a diluted sample and mixed completely in a thick-walled Pyrex tube. The sample was allowed to stand for 10 minutes before being placed them into a water bath at 25°C for 15 minutes. The sugar value was measured at 488 nm using a spectrophotometer (UV-1201 V Shimadzu, Japan).

Scanning electron microscope (SEM)

The structure of the initial and pre-heated food waste was observed with a scanning electron microscope (SEM) (Stereosan 420, Leica Cambridge Instruments). The food waste sample was gently washed with a phosphate buffer solution and then fixed with 4 % paraformaldehyde for 4 hours. The fixed sample was dehydrated by a successive gradient of 25, 50, 75, 80, 90, 95 and 100% ethanol solutions. The dehydrated sample was finally dried in a critical point dryer (E3000, VG Mircotech, England), coated and was ready for observation in the SEM.

DNA extraction

The leachate taken from Ra on the 4th day was centrifuged at 3000 rpm for 30 minutes to separate the supernatant from the solid. The solid was used for DNA extraction. A DNA sample was extracted using a phenol-chloroform procedure. A 100-200 mg sample (wet weight) was suspended in 800 µL TE buffer (pH8) (910 mM Tris-HCL pH8, 1 mM EDTA) and placed into a 2 mL BeadBeater tube containing 800 ng baked glass beads. 50 µL of 20% sodium dodecyl sulfate phenol (pH8) was added into tube. The tube was run in the BeatBeater for 3 minutes and placed in a 60°C water bath for 5 minutes. The aqueous layer was removed from the BeadBeater tube after spinning for 10 minutes and was sequentially extracted with

saturated phenol (pH8) once, saturated phenol-chloroform (4:1(v/v)) twice and chloroform twice. The extracted aqueous solution was precipitated in ten volumes of 20% sodium acetate and 2 volumes of 95% ethanol overnight at -20°C. The precipitates were spun for 10 minutes in a micro-centrifuge with the removal of the supernatant. The pellet was then washed with 80% ethanol twice, dried in a vacuum micro-centrifuge, suspended in 100 µL Q-H₂O and frozen at -20°C. The concentration of the extracted DNA was measured using a UV-vis wave scan spectrophotometer at a wavelength of 260 nm.

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

The hot start, touchdown polymerase chain reaction (PCR) was performed with a mastercycler gradient (5331, Eppendorf, Germany). HPLC purified PCR primers P3 forward with 40 bases of a GC clamp, 5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3', and reverse primer P2, 5'-ATT ACC GCG GCT GCT GG-3' were used to amplify the V3 region of the bacterial 16S rRNA gene corresponding to positions 341 and 534 in the *Escherichia coli* sequence (Muyzer et al. 1993).

Amplification was performed with a Mastercycler gradient (Eppendorf AG, Hamburg, Germany) using a 50 µL (total volume) mixture containing 1.25 U of Taq polymerase (Promega, WI. USA), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.75 mM MgCl₂, 200 µM deoxynucleotide triphosphate (dNTP), 25 pmol of each primer, and 1 µL of DNA solution (20 ng µL⁻¹) extracted from the sample. The final PCR program for the hot start, touchdown PCR was performed at 94°C for 4 min for denaturation, followed by 25 cycles of 15-second denaturing at 94°C, 30-second annealing at 65°C to 55°C, (decrease of 1°C per cycle), 30-second elongating at 72°C, and 5-minute elongation at 72°C.

After the PCR amplification, 5 µL of aliquots were subjected to agarose gel electrophoresis with 1% agarose gel. The PCR-amplified DNA fragments were

separated by denaturing gradient gel electrophoresis (DGGE) with a DCode universal mutation detection system (Bio-Rad Laboratories, Richmond, CA, USA) as described previously (Jiang et al. 2004; Yu and Morrison 2004). Twenty microliters of PCR amplicon from DNA extracted from the sample were resolved on polyacrylamide gels (37.5:1) containing a 30-70% gradient of denaturants (100% denaturants consisting of 40% (V/V) formamide and 7 M urea) for V3 region amplicons. The gel was placed in a Tris-Acetate-EDTA (TAE) buffer and run at 82 V at 60°C for 15 hours for all amplicons. After electrophoresis, the gel was stained with ethidium bromide for 30 min, then viewed and photographed with an EDAS 290 gel imaging system (Kodak, NY, USA).

5.3 Results

The batch experiments were conducted to evaluate the effect of temperature and duration on the food waste pretreatment, then the optimized temperature and duration was applied in the HASL system to compare with the thermal pretreatment at 150°C for 1 hour. Results in the batch experiments and in the HASL system will be discussed individually.

5.3.1 Optimization of temperature and duration in the batch experiments

pH

Figure 5-1 shows the pH variations at different temperatures with different durations. Although the pH did not vary much at the same temperatures, which meant that duration did not affect pH values significantly at the same temperatures, the pH values at high temperature (50- 95°C) were much higher than that at 35°C, which were above 5.05 and below 4.8, respectively. All pH values were in the range of 4.6 and 5.3, which is acceptable for acidogenic bacteria. Therefore, in terms of pH, pretreatment at high temperature made pH a little bit higher and duration did not affect the pH as much.

Figure 5-1 pH at different temperatures with different durations

TSS & VSS reduction

It can be seen from Figure 5-2 that the TSS reduction had similar trends at different temperatures. Since the thermal pretreatment solubilized part of the suspended solids (SS) to dissolved solids (DS), the TSS in residue will decrease. The TSS reduced more after pretreatment at high temperatures (50-95°C) compared to 35°C, which was in the range of 14-26% at 50-95°C and below 8% at 35°C, respectively. Beyond 120 minutes, the TSS reductions in residue remained consistent without much fluctuation, which indicated operation time can not make solubilization enhanced any more at that temperature. The same trend was also observed in VSS reduction (Figure 5-3) due to the solubilization during the thermal pretreatment. Although the highest VS reduction of 0.84% happened at 95°C and 60 minutes, the net change of VSS is very limited in a batch process. That is why the VSS reduction in residue was less than 1%.

Based on TSS and VSS analysis, the optimum temperature of 95°C with duration of 60 minutes was selected.

Figure 5-2 TSS reduction in residue at different temperatures with different durations

Figure 5-3 VSS reduction in residue at different temperatures with different durations

SCOD

SCOD represents indirectly the amount of organic matter in the solution. It was observed in Figure 5-4 that pretreatment at 95°C produced the highest SCOD with an average value of 64.2 g L^{-1} , and it increased with duration time. The peak SCOD of 61.65 g L^{-1} was achieved at 120 minutes for 70°C. However the sharp peaks observed at 60 minutes for 35°C and 50°C were 51.9 g L^{-1} and 55 g L^{-1} , respectively. It could be an experimental error at these two data points since SCOD should be increased with time extended. The optimum temperature of 95°C with duration of 180 minutes was selected based on hydrolysis rate. The energy efficiency and other considerations were not considered.

Figure 5-4 SCOD at different temperatures with different durations

Total organic carbon (TOC)

Figure 5-5 shows that pretreatment at 95°C produced the highest TOC with an average value of 229.2 g L^{-1} , and it increased during the first 30 minutes and then fluctuated. The peak TOCs observed at 120 minutes for 50°C and 70°C were 208.9 g L^{-1} and 219.4 g L^{-1} , respectively. However the highest TOC of 201.9 g L^{-1} was

achieved at 150 minutes for 35°C. Based on TOC analysis, the optimum temperature of 95°C with duration of 30 minutes was selected because the highest amount of TOC was present at that point.

Figure 5-5 TOC at different temperatures with different durations

VFA

VFA and its dominant composition were illustrated in Figure 5-6 and Figure 5-7. The highest TVFA was presented at 95°C with an average value of 21.3 mM, and it decreased with extended duration time. The peak TVFAs observed at 60 minutes for 50°C and 95°C were 14.4 mM and 24.8 mM, respectively. However, the peak values were achieved at 90 minutes for 35°C and 70°C, which were 17.7 mM and 16.5 mM, respectively. The dominant VFA composition was formic acid, acetic acid, acetic acid and propionic acid after pretreatment at 35°C, 50°C, 70°C and 95°C, respectively. The percentage of the dominant VFA composition in TVFA was plotted in Figure 5-7.

Figure 5-6 TVFA at different temperatures with different durations

Figure 5-7 percentage of dominant VFA at different temperatures with different durations

Different durations did not affect the percentage of dominant VFA with pretreatment at 35°C and 70°C, which was always kept at 100%. However, different

durations had a big effect on the dominant VFA with pretreatment at 95°C. The percentage of propionic acid decreased to 40% between 60 to 120 minutes and remained at 100% at other times. The shift may be caused by propionic acid type fermentation or hydrogen builds up.

If propionic acid is dominant, the fermentation process is called propionic acid type fermentation, in which propionic acid converts to methane and accumulates, resulting in excess acidification and causing the process unsuccessful (Hanaki et al., 1994a & b). So propionic acid type fermentation, even propionic acid, should always be avoided. On the other hand, if hydrogen builds up, higher molecular weight acids such as butyric and propionic acids are accumulated in the system (Grady 1999). Thus, it is important to remove excess hydrogen from the system.

Only acetic acid is the favorite VFA for methanogens, and was also the dominant VFA after pretreatment at 50°C and 70°C. Meanwhile, the highest TVFA appeared at 90 minutes with pretreatment at 70°C. Therefore, Based on VFA analysis, pre-heating food waste at 70°C for 90 minutes would be considered.

Protein

It can be seen from Figure 5-8 that all the proteins release had a similar trend at different temperatures which was a sharp increase in the initial 30-60 minutes, then a decrease at 90 minutes. Beyond 90 minutes, it maintained a steady state with 0.37 mg L⁻¹ at 95°C, 0.38 mg L⁻¹ at 70°C, 0.40 mg L⁻¹ at 50°C, and 0.44 mg L⁻¹ at 35°C, respectively. The highest protein levels were achieved at 60 minutes for 35°C and 50°C, 0.49 mg L⁻¹ and 450 mg L⁻¹, respectively. However, the peak values appeared at 30 minutes for 70°C and 95°C, 440 mg L⁻¹ and 400 mg L⁻¹, respectively. This shows that protein levels decreased in a short time at high temperatures due to denaturation.

Figure 5-8 Protein at different temperatures with different durations

Sugar

Sugar increased with temperature increase, there was a similar trend at different temperatures, which was a sharp increase during the first 30 minutes, then kept at a constant level. The average sugar concentrations were 3231 g L⁻¹, 3413 g L⁻¹, 3702 g L⁻¹ and 3791 g L⁻¹ after pretreatment at 35°C, 50°C, 70°C and 95°C, respectively. This shows that sugar increased in a short time and more complex compounds were broken into carbohydrates at high temperatures.

Figure 5-9 Sugar at different temperatures with different durations

Both temperature and duration affected the properties of food waste after thermal pretreatment. Among all four selected temperatures, most parameters after thermal pretreatment at 95°C were more positive compared to the other three temperatures due to more energy input, including pH, TS, VS, SCOD, TOC, TVFA, sugar, etc.

Because VFA and its composition are the most important parameters in AD, and acetic acid is the favorite VFA for methanogens in the following process, the optimized temperature and duration was selected based on the dominant VFA followed by other parameters.

It was shown in Figure 5-7 that acetic acid was the dominant VFA after pretreatment at 50°C and 70°C, and its percentage was not affected by duration time at 70°C, meanwhile the efficiency at 70°C was much higher than at 50°C, such as in terms of TS, VS, SCOD, TOC, TVFA and sugar. Other parameters such as pH and protein did not show too much difference between 50°C and 70°C.

Although different duration times did not show any significant effect on the dominant VFA, it made a big difference in other parameters. The highest TSS reduction, VSS reduction SCOD and TOC values were all achieved at 120 minutes, meanwhile other parameters such as TVFA, pH, protein and sugar did not show too much difference at different duration times.

Based on the above results, thermal pretreatment of food waste at 70°C for 120 minutes would be recommended for the HASL system in the following experiments. Other thermal pretreatments at high temperature, i.e. 150°C for 60 minutes as recommended by Schieder et al. (2000) were also conducted, while fresh food waste without any pretreatment was used as the control (C).

5.3.2 Digestion of thermally pre-treated food waste in the HASL system

Structure of food waste

The structures of vegetable roots before and after thermal pretreatment are shown in Figure 5-10. Thermal pretreatment was able to improve the structure of food waste, i.e. increased porosity, decreased bulk density, thickness and volume of the material. It can be seen in Figure 5-10. The fresh vegetable roots had a tight and firm structure which became loose after thermal pretreatment, and made more surface ready for bacteria to attach to and made hydrolysis easy and fast. After heating at 150°C for 60 minutes, cavities appeared and the structure of the vegetable roots was broken.

Figure 5-10 SEM images of fresh (a), pre-treated at 70°C (b) and 150°C (c) vegetable roots

Performance of the HASL operation

The variation of pH in the leachate from Ra in the control and the experiments are shown in Figure 5-11. The lowest pH observed was 4.8 on the 5th day, 5.7 on the 3rd day and 6.2 on the 2nd day in C, E1 and E2, respectively, which matched the variation of VFA concentration shown in Figure 5-12. As reported by Stabnikova et al. (2005), the pH in the leachate from Ra usually dropped rapidly to about 5.0, probably due to lysis of cells and VFA production, and then slowly increased to neutral pH, probably due to hydrolysis of proteins and buffering of pH by released amino acids. Therefore, lower pH values in the control in comparison with the experiments can be explained as more complete hydrolysis of proteins after thermal pretreatment of food waste.

Figure 5-11 pH in the leachate from Ra in the HASL system

Figure 5-12 shows that the initial VFA concentrations in the leachate from Ra were 1382 mg L⁻¹, 2578 mg L⁻¹ and 3008 mg L⁻¹ in C, E1 and E2, respectively, which indicated the intensification of the acidification process due to thermal pretreatment of food waste in E1 and E2. The highest VFA concentrations were 12470 mg L⁻¹ on

the 4th day in C, 17818 mg L⁻¹ on the 4th day in E1, and 19795 mg L⁻¹ on the 3rd day in E2, respectively. The peak VFA values were higher and were reached faster in E1 and E2 compared with the control, which means that thermal pretreatment enhanced the acidification process in food waste digestion. The overall VFA removal efficiency in the HASL system was 82.5%, 87.6% and 94.5% in C, E1 and E2, respectively. As a result, the VFA concentration in the effluent from Rm was much lower than that in leachate from Ra.

Figure 5-12 TVFA in the HASL system

Figure 5-13 shows the variation of SCOD in the leachate from Ra and in the effluent from Rm in the control and the experiments, which experienced almost the same trend as the VFA (Figure 5-12). The initial SCODs in the leachate from Ra were 7221 mg L⁻¹, 8544 mg L⁻¹ and 13325 mg L⁻¹ in C, E1 and E2, respectively, which indicated more soluble organic matter was available in E1 and E2 because of the intensification of the hydrolysis process due to thermal pretreatment of food waste.

Figure 5-13 SCOD in the HASL system

The highest SCODs in the leachate from Ra were 17575 mg L^{-1} on the 4th day in C, 19980 mg L^{-1} on the 3rd day in E1, and 24235 mg L^{-1} on the 2nd day in E2, respectively. The peak SCOD values were higher and were reached faster in E1 and E2 in comparison with the control, which meant that the thermal pretreatment enhanced the hydrolysis process in food waste digestion during the HASL operation. Although, the average rate of SCOD production increased only by 1.4% and 5.5% in E1 and E2 compared to the control, the maximum rate of SCOD production increased by 15% and 82% in E1 and E2 in comparison with the control (Table 5-3). Since most organic matters were converted into methane in Rm by methanogens, the overall COD removal efficiency in the HASL system was 78.2%, 80.3% and 82.2% in C, E1 and E2, respectively. As a result, the SCOD concentrations in the effluent from Rm were much lower than that in leachate from Ra.

Figure 5-14 shows the accumulative methane production, which was 26 L in 11 days in C, 26.7 L in 10 days in E1, and 29 L in 9 days in E2, respectively. The

maximum rate of methane production was 4.2 L d^{-1} in C, while it was 5.5 L d^{-1} and 7.1 L d^{-1} in E1 and E2, respectively. The same volume of methane could be produced in 12 days in C, 8 days in E1 and 6 days in E2, which indicated that thermal pretreatment of food waste improved the methane production and diminished time needed to produce the same quantity of methane. Meanwhile, methane production, in terms of L g^{-1} VS removed, increased by 4.0% and 7.1 % in E1 and E2 compared to the control (Table 5-3). A similar increase of 8.9% was observed by Schieder et al (2000).

Figure 5-14 Accumulative methane production in the HASL system

Methanogens monitoring in Rm

For deeper understanding of the improvement in mechanisms of food waste anaerobic digestion, the estimation of methanogenic population in the methanogenic reactor was carried out using three different methods: direct count, fluorescence of coenzyme F_{420} and specific binding of Arc915 probe, which are detailed in Chapters 3 and 4.

Figure 5-15 and Figure 5-16 show the concentrations of total bacteria and methanogens in Rm. Both of them increase and were higher in E1 and E2 than in the control. For example, the maximum concentrations of total bacterial were 2.1×10^8 cell mL⁻¹, 2.4×10^8 cell mL⁻¹ and 2.7×10^8 cell mL⁻¹ after 9 days in C, E1 and E2, respectively (Figure 5-15). The maximum concentrations of methanogens were 2.3×10^7 cell mL⁻¹ on the 11th day in C, 3.8×10^7 cell mL⁻¹ on the 9th day in E1, and 4.3×10^7 cell mL⁻¹ on the 7th day in E2, respectively (Figure 5-16). Therefore, it is conclude that thermal pretreatment increased the yield of total bacterial and methanogens in Rm.

The methanogens percentage in total bacterial ranged between 10-12 % in the control, it increased from 14% and 12% to 18% and 17% in E1 and E2, respectively (Figure 5-17). It is shown that thermal pretreatment increased the methanogens in Rm, which ensured higher methanogenic activity and higher methane production in E1 and E2 compared to the control (Figure 5-14 and Table 5-3).

Figure 5-15 Concentrations of total bacteria in Rm

Figure 5-16 Concentrations of methanogens in Rm

Figure 5-17 Percentage of methanogens in Rm

It can be seen in Figure 5-18, although the F_{420} fluorescence was highest in E2 followed by E1 and C, they had the same trends and were similar to the changes of methanogens concentration estimated by direct count (Figure 5-16). The linear

correlation between F_{420} fluorescence and methanogens concentration estimated by direct count (Figure 5-19) was statistically reliable with a coefficient of 0.69.

Figure 5-18 Fluorescence of F_{420} in Rm

Figure 5-19 Correlation between F_{420} fluorescence and methanogens concentration

It can be seen from Figure 5-20 that the specific binding of Arc915 probe was much higher in E1 and E2 than in the control. It had the same trend as the direct count (Figure 5-16) and the F_{420} fluorescence (Figure 5-18).

Figure 5-20 Specific binding of Arc915 probe in Rm

PCR-DGGE analysis

Since the hydrolysis and acidification in Ra were most active during the first 3 days, the leachate from Ra and liquid from Rm collected on the 3rd day were used in PCR-DGGE test. The initial inoculated activated sludge was also used to compare different dominant bacterial species and also the shift of dominant bacterial species in the HASL system.

The DGGE profiles of 16S rRNA gene fragments after the PCR amplification of the V3 region of the nucleic acids from different samples were shown in different lane in Figure 5-21. The dominant species in different samples were illustrated by the well-marked brightness band in the different lanes. For example, there are 7 notable

bands in lane 4, which identified 4 kinds of dominant species in the inoculated activated sludge.

Figure 5-21 DGGE analysis of initial sludge, leachate from Ra and liquid from Rm

In the leachate from Ra, there are four dominant species in E1 and E2, which appeared at the same position. However, there are three dominant species in the control and they appeared at different positions. The results demonstrated that thermal pretreatment of food waste would change the dominant species in Ra in terms of number and types. In the liquid from Rm, although the dominant species in the control and E1 were similar, the dominant species in E1 and E2 were more complex than in the control in terms of number and types.

The shifting of the dominant species in the HASL system was also displayed obviously in Figure 5-21. Because the dominant species in Ra and Rm were acidogenic bacteria and methanogens, respectively, the different number of bands appeared at different positions.

5.4 Discussion

5.4.1 Effect of thermal pretreatment on food waste digestion in the HASL system

The effect of thermal pretreatment on the stoichiometric parameters and kinetic parameters for food waste digestion in the HASL system is summarized in Table 5-3. The thermal pretreatment of food waste changed the stoichiometric parameters slightly, but changed the maxima of these parameters significantly, as well as the kinetic parameters. In summary, the average COD production rate increased by 1.4% and 5.5% in E1 and E2 in comparison with the control, while maximum COD production rate increased by 15% and 82% in E1 and E2 in comparison with the control. Meanwhile, the average methane production rates were almost the same in the control, E1 and E2, while the maximum methane production rates were 4.2 L d⁻¹ in the control, 5.5 L d⁻¹ in E1, and 7.1 L d⁻¹ in E2, respectively.

Thermal pretreatment of food waste enhanced hydrolysis and acidification in Ra and also methanogenesis in Rm in terms of higher and faster COD, VFA and methane production and diminished time needed to produce the same quantity of methane. The most effective method was pre-heated food waste at 150°C for 60

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minutes followed by 70°C for 120 minute, which diminished the time to produce the same quantity of methane by 48% and 25%, respectively and increased the methane production by 7.3% and 19.7%, respectively. The additional cooling time should be considered in practice.

Table 5-3 Influence of thermal pretreatment on food waste anaerobic digestion in the HASL system

Parameters	Control	E1	E2
Stoichiometric parameters			
Total COD produced in Ra (g)	86.6	87.6	90.9
Maximum COD concentration (g L ⁻¹)	17.6	20.0	24.2
Total VFA produced in Ra (g)	67.9	55.5	48.4
Maximum VFA concentration (g L ⁻¹)	12.5	17.8	19.8
Ratio of VFA/COD (%)	78.4	63.3	53.2
Lowest pH	4.78	5.74	6.23
Overall VS removal efficiency (%)	79.9	81.6	83.4
Overall COD removal efficiency (%)	78.2	80.3	82.2
Residue (g)	390	347	330
Total biogas production (L)	37.2	37.5	40.3
Total methane production (L)	26.0	26.7	29.1
Average methane content in biogas (%)	70.0	71.2	72.0
Kinetic parameters			
Average COD production rate (g d ⁻¹)	7.2	7.30	7.6
Maximum COD production rate (g d ⁻¹)	6.0	6.9	10.9
Average VFA production rate (g d ⁻¹)	5.7	4.6	4.0
Maximum VFA production rate (g d ⁻¹)	6.1	7.2	12.3
Maximum methane production rate (L d ⁻¹)	4.2	5.5	7.1
Maximum rate of pH drop (d ⁻¹)	1.3	1.5	1.7
Methane production (L g ⁻¹ VS removed)	0.28	0.29	0.30

5.4.2 Energy consumption for thermal pretreatment

Thermal pretreatment of food waste facilitated the hydrolysis and acidogenic phase in terms of higher and faster SCOD and VFA concentration, and enhanced the energy recovery from food waste in terms of methane production. However, external energy was required to implement pretreatment processes. Since the energy for running the HASL system is same, only the theoretical energy required for thermal pretreatment was considered. An energy balance between the energy required by the pretreatments and increased energy output was made.

To calculate the energy required to increase temperature of food waste from 25°C to 70°C or 150°C, it was assumed that there was no heat loss or evaporation. The energy for heating of a suspension of 800 g of food waste in 1000 mL of water (Q_t), used for anaerobic digestion in present study, was calculated by equation:

$$Q_t = C_{\text{organic}} M_{\text{organic}} \Delta t + C_{\text{water}} \times M_{\text{water}} \times \Delta t \quad (5-1)$$

where C_{organic} is the specific heat capacity of organic matter, ($C_{\text{organic}} = 0.89 \text{ J g}^{-1} \text{ }^\circ\text{C}$); C_{water} is the specific heat capacity of water, ($C_{\text{water}} = 4.18 \text{ J g}^{-1} \text{ }^\circ\text{C}$); M_{organic} is the mass of organic matter in 800 g of food waste with a content of TS 18.6%, ($M_{\text{organic}} = 148.72 \text{ g}$); M_{water} is the mass of water in 800 g of food waste, 651.28 g; 1000 mL is the mass of water added to prepare the food waste suspension, ($M_{\text{water}} = 1651.28 \text{ g}$); Δt is the difference between the final temperature, 70°C or 150°C, and the initial temperature, 25°C.

The theoretical energy for heating food waste to 70°C was calculated as follows:

$$\begin{aligned} Q_{70} &= 0.89 \text{ J g}^{-1} \text{ }^\circ\text{C} \times 148.72 \text{ g} \times (70 - 25)^\circ\text{C} \\ &\quad + 4.18 \text{ J g}^{-1} \text{ }^\circ\text{C} \times (651.28 + 1000) \text{ g} \times (70 - 25)^\circ\text{C} \\ &= 316.56 \text{ kJ} \end{aligned}$$

The theoretical energy for heating food waste to 150°C was calculated as follows:

$$\begin{aligned} Q_{150} &= 0.89 \text{ J g}^{-1} \text{ }^\circ\text{C} \times 148.72 \text{ g} \times (150 - 25)^\circ\text{C} \\ &\quad + 4.18 \text{ J g}^{-1} \text{ }^\circ\text{C} \times (651.28 + 1000) \text{ g} \times (150 - 25)^\circ\text{C} \\ &= 879.33 \text{ kJ} \end{aligned}$$

If some portion of water evaporated during thermal pretreatment, the calculated value will be even higher because of partial liquid–gas phase transition.

The energy of methane released by the digestion of 800 g of food waste was estimated to be 1273 kJ. The increased energy was 297 kJ and 385 kJ when food waste was subjected to pretreatment at 70°C and 150°C, respectively. It seems to indicate that there is no net energy generation when employing pretreatment procedures as compared to the control tests. In addition, it should be realized that the actual energy required by those pretreatments could be much greater than the calculated theoretical value since thermodynamic efficiency was not considered. However, optimization of pretreatment procedures might improve the efficiency of energy recovery. In fact, an external energy input of 522.5 kJ or 310.6 kJ which was required to heat 1000 mL water from 25°C to 70°C or 150°C was likely to be saved if only food waste was heated during the thermal pretreatment procedure.

Based on the results of methanogens and methane production, the specific activities of methanogens for biogas production were 2.2×10^{-11} , 2.4×10^{-11} and 3.0×10^{-11} L per cell in the control, E1 and E2, respectively during the first 3 days of operation. However, the values dropped rapidly to 1.9×10^{-11} , 1.5×10^{-11} and 1.0×10^{-11} L per cell in the control, E1 and E2, respectively during the 3 to 5 day operation, and to 1.3×10^{-11} , 0.5×10^{-11} and 0.06×10^{-11} L per cell in the control, E1 and E2, respectively during the 5 to 7 day operation, which was due to faster depletion of nutrients in Rm in E1 and E2 (Figure 5-12 and Figure 5-13).

Based on the PCR-DGGE analysis, thermal pretreatment of food waste did change the number and type of the dominant species in Ra. That is the main reason for faster and more complete hydrolysis and acidification process in Ra with thermal pretreatment.

5.5 Conclusion

As the simplest methods, thermal pretreatment of food waste can enhance the hydrolysis, the rate-limiting step in AD. The favorite temperature and duration was 70°C for 120 minutes which optimized in the batch experiments and applied in the HASL operation. This study shows that thermal pretreatment of food waste facilitated the hydrolytic and acidogenic processes in Ra and increased the content and activity of methanogens in Rm, which enhanced anaerobic digestion of food waste in the HASL system in terms of higher and faster SCOD, VFA and methane production. The most effective method was pre-heated food waste at 150°C for 60 minutes followed by 70°C for 120 minute, which increase the methane production by 7.3% and 19.7%, respectively, compared to anaerobic digestion of fresh food waste without any pretreatment.

Meanwhile, the dominant species in Ra and Rm were totally different which illustrated the HASL system was a successful two-phase AD process. The number and types of the dominant species in Ra change dramatically by thermal pretreatment that is why faster and more complete hydrolysis and acidification process in Ra with thermal pretreatment.

CHAPTER 6

EFFECT OF FREEZE/THAW PRETREATMENT ON FOOD WASTE DIGESTION IN THE BATCH EXPERIMENTS AND IN THE HASL SYSTEM

6.1 Introduction

As mentioned in Chapter 5, hydrolysis is the rate-limiting step in AD. There are many known pretreatment methods that enhance hydrolysis. The combined effects of temperature and duration on anaerobic digestion of food waste have been discussed in Chapter 5. This chapter is a follow-up of the study presented in Chapter 5, and looks into another pretreatment method, namely freeze/thaw of food waste. It should be pointed out that the effects of freeze/thaw pretreatment of food waste in AD are still unknown.

6.1.1 Effect of freeze/thaw pretreatment

As liquefaction (solubilization) and hydrolysis of solid wastes are the rate-limiting steps in AD (Shin et al. 2001a), an increase of these processing rates should lead to better performance of food waste anaerobic digestion in the HASL system. To promote solubilization of organic matter and release of intracellular material from plant cells, cell disruption can be used (Mata-Alvarez et al. 2000).

Cell disruption is used to improve anaerobic digestion of activated sludge. As a result of cell disruption, intracellular and cell wall polymers, including polysaccharides,

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proteins, lipids and other macromolecules, are released into the surrounding medium. Consequently, cell disruption will enhance anaerobic fermentation of activated sludge and increases biogas generation (Bien et al. 2004). For microbial or plant cell disruption, different disintegration methods such as mechanical (e.g., sonication), chemical (e.g., alkali treatment), osmotic (e.g., NaCl treatment), thermal (e.g., heat treatment), and biological (e.g., treatment by enzymes) were proposed (Erdincclerm and Vesilind 2000; Mata-Alvarez et al. 2000). Cell disruption can be achieved also by freezing at low temperatures due to intracellular ice crystal formation, which causes damage to cell membranes (Webb et al. 1996; Thomashow 1998).

During freezing, water present in the cell starts to turn into ice and the cell volume increases. When the cell is thawed to room temperature, the sudden change in temperature will cause the cell to burst and release intracellular organics. Since organic material is produced readily for the hydrolytic bacteria, hydrolysis can be carried out more easily.

Currently, freeze/thaw pretreatment is being studied for dewatering of activated sludge. Freeze/thaw was studied as a waste treatment method for concentration and volume reduction of contaminated waste concentrates that are derived from the use of membrane technology in the treatment of high strength kraft pulp mill effluents (Facey et al. 2001). Not many studies have been conducted on the effects of freeze/thaw pretreatment on food waste.

6.1.2 Application of residue

As discussed in Chapter 5, by using different pretreatment methods on food waste in the HASL system, the biogas production increased and the operation time was shortened. Meanwhile, the food waste bioconversion was enhanced significantly. However, no matter how good the HASL system is, there will still be some residue, which can not be digested, left.

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The products of aerobic composting of food waste can be directly used as soil fertilizers, while the residue of conventional AD of food waste needs further treatment to improve stability and reduce toxicity before use. For example, the residue after hydrogen fermentation in BIOCELL was post-treated by Korean researchers (Han and Shin, 2004) in the same reactors without transfer. After post-treatment, moisture content decreased to 55.8% and VS reduction increased to 72.5%. The remaining acids in the residue were volatilized so that the pH would increase to 7. The characteristics of the output after post-treatment met the Korean regulation on compost and could be used as a soil amendment.

Although these researchers suggested that the residue after further treatment could be used as a soil amendment, there is little information available regarding the fertility of residue after AD. Meanwhile, the condition of residue from the HASL system is still unknown, which could greatly affect the residue-fertilizer conversion by shortening the treatment time and reducing the treatment cost.

The aim of the research was (1) to optimize the freeze/thaw cycle for food waste pretreatment in the batch experiments; (2) to evaluate the effects of frozen/thawed food waste on its anaerobic digestion in the HASL system, and (3) to investigate the feasibility of direct application of residue from the HASL system as organic fertilizer.

6.2 Materials and methods

6.2.1 Optimization of temperature and freeze/thaw cycle in the batch experiments

Food waste

The collection, preparation and composition of food waste was exactly the same as that detailed in Section 5.2.1

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Experimental Set-up

50mL plastic tubes were used to contain the food waste puree. Three different refrigerators were chosen at different set point temperatures of 4°C, -20°C and -80°C, which are the typical temperatures for storing, freezing and deep freezing. Room temperature of 25°C was selected as the control temperature. Four tubes were placed in each refrigerator while one was set aside at room temperature as the control. The four different tubes represent the different cycles of freezing and thawing which are shown in Table 6-1.

Table 6-1 Different cycles of freezing and thawing

Experiment	Duration for freezing and thawing cycle
E1	12-hour Freezing, 6-hour thawing at room temperature.
E2	24-hour Freezing, 12-hour thawing at room temperature.
E3	12-hour Freezing, 6-hour thawing at room temperature. Repeat once.
E4	24-hour Freezing, 12-hour thawing at room temperature. Repeat once.

Sampling

All samples are measured for pH immediately after freeze/thaw cycles, followed by centrifuging at 3000rpm for 30 minutes to separate the supernatant from the residue. The supernatant is then extracted and tested for COD, TOC, VFA, protein and sugar, while the residue is measured for TS and VS.

6.2.2 Digestion of frozen/thawed food waste in the HASL system

Food waste

The collection and preparation of food waste were exactly the same as that detailed in section 5.2.2, except for the different composition shown in Table 6-2.

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Table 6-2 Composition of food waste in the HASL system

Food Waste	Percentage (% of wet weight)	TS (%)	VS (% TS)
Vegetable roots	50	5.05±0.13	63.00±0.84
Orange peels	20	21.28±0.09	95.06±0.24
Cooked Rice	15	33.91±0.52	98.69±0.05
Cooked noodles	15	44.82±0.98	98.47±0.11
Fresh wastes mixture		18.59±1.72	92.93±1.24
Frozen/thawed waste		17.90±0.65	94.55±2.53

Experimental Set-up

The experiments were carried out simultaneously in two identical HASL systems (Figure 2-3) and operated at 35±1°C as described (Stabnikova et al. 2005). Reactor configurations and the other operation were kept exactly the same as those used in Section 5.2.2, except that the frozen/thawed food wastes which are frozen for 24 hours at -20°C and thawed for 12 hours at room temperature were used in the experiment.

Sampling

Leachate was taken from Ra to test for pH, COD and VFA. Effluent from Rm was collected to measure for pH and COD. The biogas produced from Rm was monitored daily. All wet residue samples were taken immediately when all experiments were finished. After the CO₂ evolution test, other parameters were measured.

6.2.3 Analysis methods

The methods for analyzing TS, VS, CHNS, pH, SCOD, VFA, TOC, Protein, Sugar, biogas production, methane content and SEM are detailed in Section 3.2.3, Section 4.2.3 and Section 5.2.3.

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Element and heavy metal content

Determination of element (Na, Mg, Ca, K and P) and heavy metal (Cd, Cr, Cu, Ni, Pb and Zn) content was carried out according to the modified Test Methods for Evaluating Solid Waste (SW-846) (U.S. EPA, 1999).

Samples were oven-dried at 105°C for 24 hours, and then grounded into powder with particle size of less than 2 mm. 0.2 g of dried sample and 6mL HNO₃ were added to each holder for complete digestion in a microwave reaction system (Perkin Elmer Anton Paar, USA). The solution was diluted using deionised water and measured using an Inductively Coupled Plasma (ICP) emission spectrometer (Perkin Elmer Optima 2000DV, USA). All heavy metal and element content were calculated and reported on dry weight basis.

Carbon dioxide (CO₂) evolution test

Residue taken from Ra was left to pre-incubate at room temperature (25±1°C) for 48 hours, which allowed microorganisms to adapt to the mesophilic environment in which the test would be conducted. 25 g of the pre-incubated sample were transferred into an incubation vessel containing 25 mL of 1M NaOH. A control was also set up in which only NaOH was in the vessel. All vessels were sealed and kept at 35°C for 4 days. The amount of CO₂ absorbed by NaOH was determined daily by back titration of the residual NaOH with 1M HCl.

The stability index was evaluated according to the CO₂ evolution rate (R), in mg CO₂-C g⁻¹ VS d⁻¹, as being very stable (R < 2), stable (R < 4) or unstable (R > 4) (United States Department of Agriculture and the United States Composting Council, 2001a).

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CO₂ evolution rate (R) was calculated by the following equation:

$$R = \frac{(V_1 - V_2) \times n \times d}{w \times f} \quad (6-1)$$

Where Q is the CO₂ evolution rate (mg CO₂-C g⁻¹ VS d⁻¹); V₁ is the volume of standardized HCl used in back titration (mL); V₂ is the volume of standardized HCl used in sample titration (mL); n is the normality of standardized HCl (mol_C L⁻¹); d is the equivalent weight of CO₂-C in NaOH (d = 6); w is the moist weight of the sample in container (g), and f is the mass unit, fraction of VS (g g⁻¹ TS)

Germination test

The effect of organic fertilizer on seed germination was determined by the modified germination and root elongation test (United States Department of Agriculture and the United States Composting Council, 2001b).

The extracts from organic fertilizers for this test were prepared by the following procedure. The fertilizer was suspended in distilled water at a 1:10 ratio (w/v) and incubated in an orbital shaker (ORBIT) at 100 rpm for 3 hours. The extract was separated from the solids by centrifugating at 3300 rpm for 10 minutes followed by filtration through a 0.45 µm membrane filter. Ten pieces of cucumber seeds were placed on a 9-cm-diameter paper (Whatman No. 3 disc) inside a Petri dish which contained 10 mL of the water extracts at various concentrations (0.5 g L⁻¹, 1 g L⁻¹, 2 g L⁻¹, 5 g L⁻¹ and 10 g L⁻¹). Distilled water was used as the control. Petri dishes were placed in a naturally lit area, not in direct sunlight to avoid rapid evaporation. After 5 days, the numbers of germinated seeds were counted and root lengths were measured as shown in Figure 6-1 and Figure 6-2.

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Figure 6-1 Cucumber seeds germination test

Figure 6-2 Measurement of cucumber root length in germination test

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Germination tests were carried out to determine whether the residue was toxic to the germination of seeds. In order to minimize the bias due to different seed types and weather condition, Germination Index (GI) was used as a combination indicator of root growth responses to various extract concentration, which was calculated according to the following equation (Zuconni et al. 1981)

$$GI = \frac{N_s \times N_w}{L_s \times L_w} \times 100\% \quad (6-2)$$

Where N_s is the number of germinated seeds in the sample; N_w is the number of germinated seeds in distilled water (control); L_s is the average root length of seeds in the sample (mm) and L_w is the average root length of seeds in distilled water (control) (mm). A higher GI (>100%) indicates growth stimulation (Keeling et al. 1994); while a lower GI (<100%) implies phyto-toxicity.

Plantation test

A plantation test was carried out to investigate the effect of the HASL residue on direct application as organic fertilizer on plant development and yield. Kang Kong, a common local vegetable which has characteristics that resemble that of grass, will be used as a representative plant from an agricultural and horticultural point of view.

Subsoil used in this test was collected from a garden on campus. pH of the subsoil was 7.33, which was considered suitable for plant growth and no adjustment was made. Dry residue powders were thoroughly mixed with subsoil to make 1%, 2% and 3% (dry weight / wet weight) mixtures. A pot with only subsoil was used as a negative control. Equivalent N content of COF-subsoil mixtures were also made as a positive control. Each mixture was incubated for 1 week before seeding.

The test was performed in ceramic pots (18 cm in diameter and 40 cm in height) with 3 kg of fresh subsoil. These pots, sheltered from direct sunlight and rainfall, were situated outdoors and watered daily as required. Three seeds were placed in every pot. The

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growth of the plants were observed and measured over a period of 3 weeks. At harvesting, the plants (both above and below ground parts) were carefully removed from the soil and rinsed with distilled water. The length of roots and shoots were recorded. The plants were then dried at 105°C for 24 hour and the oven dry weight was measured after cooling down at room temperature.

Figure 6-3 Kang Kong in plantation test

Figure 6-4 Length measurement of Kang Kong with residue in plantation test

Figure 6-5 Length measurement of Kang Kong with COF in plantation test

6.3 Results

The results for optimization in the batch experiments, operation in the HASL system and residue application will be discussed individually.

6.3.1 Optimization of freeze/thaw cycle in the batch experiments

pH

Figure 6-6 shows the pH variation in the batch experiments. The same pattern was observed for all experiments, and all pH values were in the range of 3.73 and 4.39. This meant that the different freeze/thaw cycles and different temperatures did not affect pH significantly. The peak pH in all experiments were achieved at -20°C, followed by -80°C, 4°C and 25°C. Since a pH level of less than 4 would pose a problem to the acidogenic bacteria in the later stages, thus -20°C and -80°C will be considered as the temperatures for pretreatment.

Figure 6-6 pH with different freeze/thaw cycles

TSS & VSS reduction

As shown in Figure 6-7 and Figure 6-8, the TSS reduction and VSS reduction in residue maintained the same trend with a peak value at -20°C in all four experiments. At -20°C , the highest peak value was achieved in E4, followed by E3, E2 and E1, which meant longer freeze/thaw time and more freeze/thaw cycles solubilize more suspend solids to dissolved solids. Since the freeze/thaw pretreatment can enhance hydrolysis, the TSS in residue was reduced up to 14%. However, the net change of VSS is very limited in a batch process, so the VSS in residue did not change with only 0.6% reduction. Based on TSS and VSS analysis, E4 at -20°C was the most effective in all experiments.

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Figure 6-7 TSS reduction in residue with different freeze/thaw cycles

Figure 6-8 VSS reduction in residue with different freeze/thaw cycles

SCOD & TOC

SCOD represents indirectly the amount of organic matter in the solution. It can be seen in Figure 6-9, the changes in SCOD experienced the same trend with a peak value at -20°C in all experiments. At -20°C , the highest SCOD concentration of 40.08 g L^{-1} was achieved in E4, followed by 38.5 g L^{-1} in E3, 38.11 g L^{-1} in E2 and 37.72 g L^{-1} in E1, which meant longer freeze/thaw time and more freeze/thaw cycles enhanced hydrolysis and produced more soluble matter.

Figure 6-9 SCOD with different freeze/thaw cycles

Since TOC and SCOD are all related to organic matter, the variations of TOC shown in Figure 6-10 are almost the same as SCOD (Figure 6-9), and E4 at -20°C was the most effective in all experiments.

Figure 6-10 TOC with different freeze/thaw cycles

VFA

It can be observed in Figure 6-11 that TVFA was affected heavily by different freeze/thaw cycles and different temperatures, because the TVFA concentrations in E1 and E2 with one cycle were 10-50 times higher than those in E3 and E4 with two cycles, although higher TVFA was achieved at high temperatures in E1 and E2 with one cycle, which meant that freeze (-20°C) and deep freeze (-80°C) weakened acidification of food waste. The TVFA did not change much in E3 and E4 with two cycles, which meant more freeze/thaw cycles will inhibit acidification of food waste.

Figure 6-11 TVFA with different freeze/thaw cycles

Figure 6-12 Percentage of acetic acid with different freeze/thaw cycles

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Since acetic acid is the favorite substance for methanogens, the percentage of acetic acid over TVFA was plotted in Figure 6-12. The acetic acid content in E2 and E3 was not much different at different temperatures since the total time for freezing and thawing are the same in these two experiments. While in E1 and E4, the acetic acid content at -20°C and -80°C, ranging between 89-100% was two times higher than that at 4°C and 25°C, ranging between 40-66%. This meant freeze (-20°C) and deep freeze (-80°C) improved acetic acid content.

Based on TVFA and acetic acid content, E2 at -20°C will be the most suitable cycle for freeze/thaw pretreatment due to the high total VFA production, high ratio of acetic acid and short cycle duration.

Protein & Sugar

Figure 6-13 shows that protein concentrations in E1 and E2 with one cycle ranged between 54-77 mg L⁻¹ which was 4-7 times higher than those in E3 and E4 with two cycles. Thus it is deduced that protein concentration is affected heavily by different freeze/thaw cycles. Although the peak protein values were achieved at -20°C, they did not vary too much at -80°C, 4°C and 25°C, which meant that temperature between -80-25°C will not affect protein release from food waste.

Unlike protein, sugar is denser and was affected by both freeze/thaw cycle and temperature. The same trend is observed in Figure 6-14. The peak value in all experiments occurred at -20°C, which meant normal freeze (at -20°C) enhances sugar release from food waste compared to deep freeze (at -80°C), storing (at 4°C) and at room temperature (25°C). During the same freezing at -20°C, the highest sugar concentration was 3009 g L⁻¹ in E4, followed by 2823 g L⁻¹ in E3, 2661 g L⁻¹ in E2 and 2452 g L⁻¹ in E1. Therefore more freeze/thaw cycles and longer freeze/thaw time improved sugar production, which makes hydrolysis easier.

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Figure 6-13 Protein with different freeze/thaw cycles

Figure 6-14 Sugar with different freeze/thaw cycles

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Both temperature and freeze/thaw cycle play an important role in affecting the amount of organic matter available for hydrolysis. Among all four selected temperatures, all parameters at -20°C were almost the same or more positive compared to other three temperatures. Therefore deep freeze at -80°C is not necessary for freeze/thaw pretreatment of food waste since it will incur more energy costs. Normal freeze at -20°C is good enough for freeze/thaw pretreatment of food waste to enhance hydrolysis.

For freezing at -20°C , different durations and cycles made a big difference. In terms of pH, TS, VS, SCOD, TOC and sugar, E4 was slightly more positive than E3, E2 and E1, by 10-40%. However E2 and E1 were much more positive than E3 and E4, by 4-50 times in terms of TVFA and protein. Compared to protein which is not the main component in food waste, VFA and its composition are more important parameters in AD. Although the acetic acid ratio was the same in E1 and E2, the total VFA in E2 was higher than that in E1 by 18.2%.

Based on the above results, E2 at -20°C would be recommended for freeze/thaw pretreatment of food waste, (i.e. freezing at -20°C for 24 hours and thawing at room temperature for 12 hours). To evaluate the effect of freeze/thaw pretreatment on food waste anaerobic digestion in the HASL system, frozen/thawed food waste was placed in Ra in the experimental HASL system, while fresh food waste was used as the control.

6.3.2 Digestion of frozen/thawed food waste in the HASL system

Structure of food waste

The structure of initial and frozen/thawed food waste was observed by SEM and is shown in Figure 6-15. The freeze /thaw pretreatment increased porosity and decreased bulk density, thickness and volume of the food waste. After the pretreatment, cavities appeared, and the structure of vegetable roots became loose, which made hydrolysis breakdown fast and easy.

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a

b

Figure 6-15 SEM images of fresh (a) and frozen/thawed (b) vegetable roots

pH

The pH variation in two different reactors in the HASL system were monitored daily and plotted in Figure 6-16. The pH in Rm was relatively constant in the range of 7.1 to 8, which indicated a good stability of Rm during operation. The pH in Rm had the expected trend of a drop in pH followed by rising to neutral values.

The lowest pH in the leachate from Ra was 4.6 on the 3rd day and 4.8 on the 1st day in the control and the experiment, respectively. The pH dropped faster in the experiment,

which meant that VFAs formed rapidly at the beginning and demonstrated a higher rate of hydrolysis of frozen/thawed food waste.

Figure 6-16 pH in the HASL system

TVFA

The high rate of hydrolysis resulted in a rapid rise of TVFA in the leachate from Ra in the experiment. The peak of TVFA concentration was higher and was reached in a shorter time in the experiment than in the control (Figure 6-17), 17.0 g L^{-1} on the 1st day and 11.7 g L^{-1} on the 3rd day, respectively. Because of the conversion of VFA into biogas by methanogens, the remaining VFA in the effluent from Rm was in a lower range of 150 mg L^{-1} to 532 mg L^{-1} , which demonstrated high efficiency of Rm and also led to an increase of pH to neutral values (Figure 6-16).

Figure 6-17 TVFA in the HASL system

SCOD

It can be seen from Figure 6-18 that the initial SCOD concentration in Ra was almost twice in the experiment than that in the control, which meant that freeze/thaw pretreatment made more organic matter available for hydrolysis and acidification. The highest SCOD concentrations in the leachate from Ra were 18.9 g L^{-1} on the 1st day in the experiment and 16.9 g L^{-1} on the 3rd day in the control, respectively. Due to methanogenesis, the remaining SCOD in the effluent from Rm was much lower and only ranged between 558 mg L^{-1} and 2556 mg L^{-1} .

Figure 6-18 SCOD in the HASL system

Methane production

The total methane production in the experiment increased by 8.5% from that in the control, they were 34.4 L and 31.7 L, respectively. The maximum methane production rates in the experiment and the control were 7.6 L d^{-1} and 5.4 L d^{-1} , respectively. The same volume of methane was produced in 12 days in the control and only 7 days in the experiment (Figure 6-19 and Table 6-3). Thus the methane production was increase by 8.4% and the operational time needed to produce the same quantity of methane diminished by 42% with freeze/thaw pretreatment. The additional thawing time should be considered in practice

Figure 6-19 Accumulative methane production in the HASL system

Based on the above results, the effects of freeze/thaw pretreatment on the performance of the HASL system for food waste digestion were recorded and are summarized in Table 6-3. Although during the 12-day operation, the average value of all parameters did not differ much in the control and the experiment HASL system. For example, the average rates of SCOD, TVFA and methane production in the experiment increased by 4%, 9% and 9% in comparison with the control, the maximum rates of SCOD, TVFA and methane production increased by 20%, 235% and 41%, 6.3 g d^{-1} , 4.6 g d^{-1} , 5.4 L d^{-1} in the control to 7.3 g d^{-1} , 15.4 g d^{-1} and 7.6 L d^{-1} , respectively.

Table 6-3 Effects of freeze/thaw pretreatment on food waste anaerobic digestion in the HASL system

Parameters	Process duration (days)			
	Control		Experiment	
	7	12	7	12
SCOD produced in Ra (g)	93.6 ± 3.7	115.0 ± 4.8	103 ± 4.4	119.6 ± 5.9
Maximum SCOD concentration (g L ⁻¹)	16.9 ± 0.3	16.9 ± 0.3	18.9 ± 0.5	18.9 ± 0.5
Average rate of SCOD production (g d ⁻¹)	13.4	8.8	14.7	9.2
TVFA produced in Ra (g)	85.0 ± 2.6	102.7 ± 3.3	98.5 ± 4.7	112.8 ± 4.9
Maximum TVFA concentration (g L ⁻¹)	11.7 ± 0.6	9.5 ± 0.5	17.0 ± 0.9	11.1 ± 0.6
Average rate of TVFA production (g d ⁻¹)	12.1	8.6	14.1	9.4
TVFA/SCOD ratio (%)	63.5	60.7	64.2	63.7
Total methane production (L)	25.7	31.7	31.8	34.4
Methane yield (L g ⁻¹ VS removed)	nd*	0.3	nd*	0.32
Average methane content (%)	68 ± 2.6	65.8 ± 3.7	69.2 ± 2.5	66.1 ± 4.4
Average rate of methane production (L d ⁻¹)	3.7	2.6	4.5	2.9
Maximum rate of methane production (L d ⁻¹)	5.4	5.4	7.6	7.6

nd* means not determined

Total VS removal was 77.2% and 80.1% in the control and the experiment. Methane yield, in terms of L g⁻¹ VS removed, increased by 6.7 % in the experiment in comparison with that in the control. A similar increase of 7.1% was observed when food waste pre-treated at 150°C for 60 minutes was used in the HASL system (Wang et al, 2006). Thus, successive freeze/thaw pretreatment of food waste changed the stoichiometric parameters slightly, but significantly changed the maxima of these parameters as well as kinetic parameters.

It can be seen in Table 6-3, the parameters such as SCOD, TVFA and methane production were almost the same for 12 days in the control and for 7 days in the experiment. Meanwhile, average methane content in biogas was even higher, 66% in the control and 69 % in the experiment. Therefore, use of freeze/thaw pretreatment gives opportunity to diminish operation time by 42%.

The following additional considerations were taken into account. To prevent the decay of collected waste, formation of nuisance odor as well as the production of the spores and toxins of micromycetes in places of waste collection, the collected food waste could be stored either outdoors in winter or in an industrial refrigerator in summer in countries with moderate climates. It can be accumulated in an industrial refrigerator in tropical countries to avoid decay of waste. Freezing and thawing of food waste followed by centrifuging is considered a safe and energy-saving method for the treatment of food waste and is expected to be applicable for recycling, transportation and incineration (Shimiya and Niyawaki 2006). Freezing in a refrigerator, followed by thawing before AD, is not only an essential procedure for food waste management but is also a useful pretreatment procedure as shown in the present study.

6.3.3 Application of residue as bio-fertilizer

There were 7 tests in total, performed on residue taken from the control (R_C) and the frozen/thawed experiment (R_{FT}).

Biological stability of residue

Biological stability is an important factor to decide the feasibility of residue to be used as fertilizer. Compared to the standard value of $4 \text{ mg CO}_2\text{-C g}^{-1}\text{VS d}^{-1}$, a larger CO_2 evolution rate would indicate that the residue was more unstable, which meant that biodegradation was still in progress and potentially unknown chemicals may be produced and affect the properties of fertilizer. The CO_2 evolution rates of residue collected in the HASL system are shown in Table 6-4. Although all residue were unstable, the stability increased when freeze/thaw pretreatment was applied.

Table 6-4 CO_2 Evolution Rates of residue

Sample	CO_2 Evolution Rate ($\text{mg CO}_2\text{-C g}^{-1}\text{VS d}^{-1}$)	Stability grade
Food waste	27.56	Extremely unstable
R_C	9.51	Unstable
R_{FT}	4.96	Slightly unstable

TS & VS

Table 6-5 shows the TS and VS values in residue which were used in the CO_2 evolution rate tests to calculate the stabilities index. Both TS and VS had the same trend, and decreased when freeze/thaw pretreatment was applied. This shows that the more TS and VS were consumed during AD with pretreatment.

Table 6-5 TS and VS content of residue

Residue	TS (g g^{-1})	VS ($\text{g g}^{-1}\text{TS}$)
R_C	0.099	0.711
R_{FT}	0.066	0.522

The results agree with the changes in the stabilities index. When freeze/thaw pretreatment was applied in the HASL operation, the residue would become more

stable because the stabilization speed became faster and the absolute VS reduction increased more.

CHNS content and C/N ratio

N is possibly the most important element required for plant growth. It is an essential component of proteins, which takes up most of the dry weight of plant and animal cells. It is available to plants in the forms of ammonia (NH_4^+) and nitrate (NO_3^-). Potassium nitrate (KNO_3), ammonium nitrate (NH_4NO_3) and calcium nitrate ($\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$) are most commonly used. These compounds provide the plant inorganic nitrogen to synthesize complex organic molecules. Ammonium is mainly stored in the roots as organic nitrogen. Nitrate can be transported through the xylem to other parts of the plant, where it participates in the nitrogen assimilation. Nitrate can be stored in the vacuoles of the cell and fulfils an important function in the osmo-regulation and anion-cation balance of the plant.

The purpose of determining CHNS contents in residue was to obtain nitrogen content and C/N ratio. Nitrogen content gives the nitrogen fertility of the residue as organic fertilizer and was used in calculating the relative amount of commercial organic fertilizer (COF) to be applied in the plantation test as positive control. Table 6-6 presents the CHNS content and C/N ratio in residue. A COF named Sheep Organic Fertilizer (Brilliant supplies and trading, Malaysia) was tested as a reference.

Table 6-6 CHNS content and C/N ratio in residue

Sample	C (%)	H (%)	N (%)	S (%)	C/N (%)
R _C	27.43±1.04	3.62±0.04	6.87±1.25	0.87±0.06	3.99
R _{FT}	38.20±5.08	9.10±1.17	7.92±0.96	1.07±0.13	4.82
COF	37.62±5.38	9.17±1.85	2.49±0.56	1.13±0.18	15.11

C/N ratio best represents the nitrogen-richness of organic materials. The COF used in agriculture usually have a C/N ratio of around 20 and behave microbiologically

stable without any degradation. The C/N ratio ranged from 3.99 to 4.82, suggesting a lower limit for stable organic fertilizer. Further treatment of the residue was necessary to reduce the instability caused by unbalanced C/N ratio because excessive organic nitrogen can be lost in the form of free NH_3 gas.

Element content

Element content indicates the chemical compositions or nutrients that fertilizers can provide to help in the plant growth. Besides nitrogen and carbon, phosphorus (P) and potassium (K) are very essential to plant growth as well.

P is taken up by the plant roots as $\text{H}_2\text{PO}_4^{-1}$, HPO_4^{-2} or HPO^{-3} , depending on the pH of the soil. P is present in all living cells and is used by plants and animals to form nucleic acids (DNA and RNA). The highly energetic pyrophosphate bond of phosphorous is very important for the energy metabolism of the cells.

K is a cofactor for several important enzyme activities and the potassium ion is present in the highest concentration, 100-200 mM in the cytoplasm and 20-200 mM in the chloroplasts. Potassium salts have an important function in the osmotic regulation of the cell. In the cell extension and other process regulation by the turgor, the K^+ serves as a counteracting ion for soluble (in) organic ions to maintain a pH between 7 and 8, which is the ideal acidity for most enzymes. The osmotic pressure of the cytoplasm is also mainly regulated by K^+ .

Element contents of initial food waste, sludge and residue were determined and compared in Figure 6-20. The sludge used to provide bacteria for AD had a high P content of 22000 mg kg^{-1} . However, P in the residue dropped to nearly half because of dissolution into the leachate in Ra during the digestion process.

Figure 6-20 Element content in initial food waste, sludge and residue

Heavy metal

Heavy metal content in waste-originated fertilizer is a big concern as it may pollute the soil and plants produced. If the heavy metal content is too high, the residue will have deleterious effects on plants even when other parameters such as C/N ratio, N, K and P are satisfied. Therefore, it is very important to keep heavy metal content below the minimum level for organic fertilizers. The U.S. standard of heavy metal limits for bio-solids land application are shown in Table 6-7, and the comparison with the heavy metal content in sludge and residue is shown in Figure 6-21.

Table 6-7 Limits for bio-solid land application (US EPA)

Element limits (mg kg ⁻¹ dry weight)						
Element	Cd	Ni	Pb	Cr	Cu	Zn
value	39	420	300	1200	1500	2800

Figure 6-21 Comparison of heavy metal in sludge and residue with standard limit

It was found that heavy metal content in both sludge and residue were far below the U.S. standard limit, which suggests that the residue would not be very harmful to the plants even in direct application. However, the actual situation depends on many factors such as the concentration of residue in soil and the type or growing stage of plants. It should also be noted that the U.S. and most European countries have heavy metal limits either as guidelines or regulations. The heavy metal standards can differ widely in various countries. The U.S. standard was chosen in this report because the criteria are comprehensive. Since most heavy metals were mainly from sludge. One way to control the heavy metal content in the residue is to reduce the sludge input in digestion.

Germination test

It can be seen from Figure 6-22 that all R_{FT} GI values were higher than in R_C, which means residue from the freeze/thaw pretreatment experiment is less toxic to seed germination. Compared to distilled water, GI dropped with the increase of extract

concentration. The maximum GI of 164% (R_C) and 178% (R_{FT}) occurred at concentration 0.5 g L^{-1} followed by 121% (R_C) and 136% (R_{FT}) at concentration 1 g L^{-1} . When the extract concentration was high enough and reached toxic levels, germination was inhibited. For example, the GI was only 35% (R_C) and 39% (R_{FT}) at concentration 10 g L^{-1} . Therefore, the most suitable extract concentration for cucumber germination was reduced to less than 1 g L^{-1} .

Figure 6-22 Germination index of residue

Plantation Test

It can be seen in Figure 6-23 that R_{FT} illustrated the most positive effect on the root length of cucumber followed by R_C and COF. The longest roots of 78mm, 85mm and 61mm occurred with 1% of R_C , R_{FT} and COF. Although the root length was maintained constant with different COF concentrations, a decreasing trend in root lengths was observed with increased percentages of residue, probably due to the toxicity in residue from the HASL system at high concentrations.

Figure 6-23 Comparison of root length in plantation test

It can be seen in Figure 6-24 that unlike the root length, COF had the most positive effect on the stem length of cucumber followed by R_C and R_{FT} although stem length increased with extended concentration of residue and COF. When comparing the same concentrations, cucumber grew much better with COF in terms of stem length because of the higher P content in COF.

It can be seen in Figure 6-25 that the oven-dry weight of cucumbers increased dramatically with extended percentages of residue and COF, and COF had a slightly heavy effect on the oven-dry weight of cucumber, but there was no big difference between R_C and R_{FT} in the effect of the oven-dry weight of cucumber.

In general, there was an increasing trend in plant growth with the increase of either residue or COF concentration compared to the negative control. The application of the HASL residue was proven to have positive effects on plant growth. However, there was a significant difference between residue and COF as their effect was concerned.

Figure 6-24 Comparison of stem length in plantation test

Figure 6-25 Comparison of oven-dry weight in plantation test

6.4 Discussion

6.4.1 Energy consumption for freeze/thaw pretreatment

Freeze/thaw pretreatment enhanced the energy recovery from food waste in terms of methane production, and could be considered an alternative method for thermal pretreatment of food waste before AD. However, external energy is required to implement these pretreatment processes. Since the energy for running the HASL system is same, only the theoretical energy required for freeze/thaw pretreatment was considered. An energy balance between the energy required by the pretreatments and increased energy output was made.

Assume there is no phase change for food waste during freezing, and water is in form of ice at below 0°C. The energy for freezing of 800 g of food waste was calculated by following equation:

$$Q_f = C_{organic} M_{organic} \Delta t_1 + C_{water} \times M_{water} \times \Delta t_1 + C_{ice} M_{ice} \Delta t_2 + Lf_{water} M_{wai} \quad (6-2)$$

where C_{ice} is the specific heat capacity of ice, ($C_{ice} = 2.06 \text{ J g}^{-1} \text{ } ^\circ\text{C}$); M_{ice} is the mass of ice equal to the mass of water in food waste; Lf_{water} is the latent heat of fusion, ($Lf_{water} = 334.4 \text{ J g}^{-1} \text{ water}$); Δt_1 is the difference between the initial temperature (25°C) and the final temperature (0°C); Δt_2 is the difference between the initial temperature (0°C) and the final temperature (-20°C).

The theoretical energy for freezing food waste to -20°C was calculated as follows:

$$\begin{aligned} Q_f &= 0.89 \text{ J g}^{-1} \text{ } ^\circ\text{C} \times 148.72 \text{ g} \times (25 + 20) ^\circ\text{C} \\ &\quad + 4.18 \text{ J g}^{-1} \text{ } ^\circ\text{C} \times 651.28 \text{ g} \times (25 - 0) ^\circ\text{C} \\ &\quad + 2.06 \text{ J g}^{-1} \text{ } ^\circ\text{C} \times 651.28 \text{ g} \times (0 + 20) ^\circ\text{C} \\ &\quad + 334.4 \text{ J g}^{-1} \times 651.28 \text{ g} \\ &= 318.64 \text{ kJ} \end{aligned}$$

The energy of methane released by the digestion of 800 g of food waste was estimated to be 1273 kJ. The increase in energy was 302 kJ when food waste was subjected to the freeze/thaw pretreatment. It indicates that there is no net energy generation when employing pretreatment procedures compared to the control tests. In addition, it should be realized that the actual energy required by the pretreatment could be much greater than the calculated theoretical value since thermodynamic efficiency was not considered. For example, the thermodynamic efficiency of the refrigeration cycle, which is proportional to the temperature difference, i.e. the energy required to cool a sample from 25°C to -20°C is much greater than $C_p \times (25 - (-20))$ as assumed. However, optimization of pretreatment procedures might improve the efficiency of energy recovery.

6.4.2 Stability, toxicity and fertility of residue from the HASL system

The germination test showed that the HASL residue was able to provide the required nutrients and had positive effects at concentrations of 0.5 g L⁻¹ and 1 g L⁻¹. However, extrapolation of presented germination tests to diverse agriculture is still inadequate because different seeds will react differently to the same extract concentrations.

Although the application of the HASL residue was proven to have positive effects in the plant growth test compared to the negative control, it cannot be applied directly because it was still unstable with a low C/N ratio. Moreover, the concentration of the residue was in a low range of 1-3%. The effect of higher residue content on plant growth is not clear.

6.5 Conclusion

This study showed that freeze/thaw pretreatment of food waste facilitated the hydrolytic and fermentation processes in Ra and ensured faster supply of nutrients in Rm in the HASL system in terms of higher and faster SCOD, VFA and methane production. The favorite temperature and duration was freezing at -20°C for 24

hours and thawing at room temperature for 12 hours, which optimized in the batch experiments and applied in the HASL operation. The freeze/thaw pretreatment enhanced the methane production and was comparable with thermal pretreatment at 70°C for 120 minutes and at 150°C for 60 minutes. The time needed to produce the same quantity methane was diminished by 42% in experiments with freeze/thaw pretreatment, while the methane production with freeze/thaw pretreatment was increase by 8.4%. The additional thawing time should be considered in practice.

Meanwhile, this study showed that the residue from the HASL system had positive effects in cucumber germination and plantation tests compared to the negative control. For example, it had shown fertility and non-toxicity from 1% to 3% concentrations, and GI improved significantly at 0.5 g L⁻¹ and 1 g L⁻¹ extract concentrations. It also demonstrated that freeze/thaw pretreatment of food waste could improve the prosperities of the residue, but the residue from the HASL system was still unstable with a low C/N ratio and could not be applied directly without any further treatment.

CHAPTER 7

CONCLUSIONS AND RECOMMENDATIONS

7.1 Conclusions

The HASL system successfully achieved a two-phase microbial community separation reflected by the dominance of the slow growing methanogens in the methanogenic reactor, as indicated from a newly developed Arc915 probe specific binding genetic method.

A successful retention of methanogens population is crucial to keep the HASL system from methanogenesis rate-limitation. The specific binding of Arc915 probe is shown to be able to serve as a straightforward genetic tool to monitor the abundance of the methanogens community, i.e. the specific binding of Arc915 probe was in linear function to the anaerobic sludge concentration when it was between 0.7 and 14.0 mg mL⁻¹ with the COV of 5-19% in this region. This high consistency with low COV is necessary for the parameter measurements of microbial growth and activity. Results show that the methanogens were enriched in the methanogenic reactor in the HASL system with a 28.6 to 29.4 pmol mL⁻¹ concentration.

Lipids content in food waste has a two-faced effect on the performance of the HASL system

Due to the low degradability but high energetic value property of lipids, only controlling its content within an appropriate range will bring incremental productivity to the HASL performance; otherwise, the digester will be subjected to inhibition incurred failure. The positive effect of lipids on food waste anaerobic digestion dominated when the content of lipids was increased by 20-30%, indicated by the COD removal rate increase of 13% and the methane production increase of

7-15%. While, it was found that lipids content addition greater than 40% by weight was harmful for the HASL performance since the growth of methanogens was inhibited by the long-chain fatty acid in this case, shown by the COD removal rate drop of 13% and the maximum growth rate of methanogens reduced by 21-85%. Apparently, the amount of lipids content in food is crucial and therefore should be taken into serious concern in the application of the HASL system.

Thermal pretreatment is proved to be a simple and effective means to enhance food waste digestion in the HASL system

As a simple and straightforward approach, thermal pretreatment demonstrated apparent favorable effect on the HASL performance. Methane production rates increased of 7.3% and 19.7%, and the time needed to produce the same quantity methane diminished by 25% and 48% when applied to thermal pretreatment at 150°C for 60 minutes and 70°C for 120 minute, respectively. The dramatic number and types of dominant species change in the HASL systems with and without thermal pretreatment offers an explanation for why the hydrolysis and acidification process was more complete and faster with thermal pretreatment. It is clear from this study that thermal pretreatment has facilitated the hydrolytic and acidogenic processes in the acidogenic reactor and increased the number and activity of methanogens in the methanogenic reactor, which in turn enhanced anaerobic digestion of food waste in the HASL system in terms of higher and faster SCOD, VFA and methane production.

Freeze/thaw pretreatment is a novel method for food waste digestion enhancement in the HASL system

Based on the food cells wall disruption mechanism in the process of freezing and thawing, total methane production from the HASL system was observed to increase by 8.4% and the time needed to produce the same quantity methane was reduced by 42%. Obviously, freeze/thaw disrupted cell walls facilitated the acidification

process, generating a faster supply of resources to power methane production in the methanogenic reactor.

Residue from the HASL system cannot be applied directly

It was showed that the residue from the HASL system had positive effects in cucumber germination and plantation tests compared to the negative control. For example, it had shown fertility and non-toxicity from 1% to 3% extract concentrations, and GI was improved significantly at 0.5 g L⁻¹ and 1 g L⁻¹ extract concentrations. Meanwhile, it also demonstrated that freeze/thaw pretreatment of food waste would improve the quality of residue with less toxicity and higher stability, but the residue form the HASL system was still unstable with a low C/N ratio and could not be applied directly without any further treatment.

7.2 Recommendations for future study

Although the HASL system has demonstrated excellent capacity for food waste AD over its conventional counterpart, results shown from this thesis study indicated that further research efforts are still needed to improve the process stability and efficiency from the viewpoint of feedstock property effect. Accordingly, the following future works are recommended:

1. In view of the two-faced effect of lipids content on the HASL performance, a co-digestion strategy might be taken in the further study to control lipids level in an appropriate range by mixing different lipids content containing food waste based on results obtained from an inventory study. This approach would offer a direct solution to the lipids inhibitory problem. A recipe model can be developed to estimate the best proportional composition of different carbon, nitrogen and lipids content feedstock to reach a balanced feedstock ingredient combination within the capacity of the HASL system.

2. A lipase based biological pretreatment method is an optional alternative to the physicochemical approaches used in this study. Lipase is a water-soluble enzyme produced from fungi and bacteria to catalyze the hydrolysis of ester bonds in water-insoluble lipids. Therefore, the biological lipids hydrolysis process might be combined with the acidogenesis process into consolidated lipids tolerable to the HASL system. The realization of such an approach may offer a once and for all solution to the lipids problem in food waste digestion.
3. An inventory study is desirable to investigate the components of organic carbon from the HASL residual, which might possibly be dominated by lignocelluloses materials brought with plant roots. The further reduction of this portion organic carbon can be achieved with the help of pretreatment or subsequent composting before application on soil. Better operational strategy should be taken into account to maximize organic carbon acidification in the first phase through approaches such as introduction and retention of high performance microbial community in catabolic phase should be considered.

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