Optimizing the anaerobic co-digestion of brown
water and food waste for clean energy recovery,
by phase separation and microaerotion :
laboratory reactor performance and microbial
community profiling

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**OPTIMIZING THE ANAEROBIC CO-DIGESTION OF
BROWN WATER AND FOOD WASTE FOR CLEAN
ENERGY RECOVERY, BY PHASE SEPARATION AND
MICROAERATION: LABORATORY REACTOR
PERFORMANCE AND MICROBIAL COMMUNITY
PROFILING**

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SCHOOL OF CIVIL AND ENVIRONMENTAL ENGINEERING

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About thesis

This dissertation compares the performance of single- and two-phase anaerobic digesters treating brown water and food waste as well as the effects of micro-aeration on the anaerobic co-digestion process. The microbial diversity of reactors, subjected to both strictly anaerobic and micro-aerobic conditions were also investigated. Results published in manuscripts listed below are included in this thesis:

- I. **Lim, J.W.**, Chiam, J.A., Wang, J.-Y., 2014. Microbial community structure reveals how microaeration improves fermentation during anaerobic co-digestion of brown water and food waste. *Bioresource Technology* 171, 132-138. (Reported in Chapter 8)
- II. **Lim, J.W.**, Chen, C.-L., Ho, J.R.I, Wang, J.-Y., 2013. Study of microbial community and biodegradation efficiency for single- and two-phase anaerobic co-digestion of brown water and food waste. *Bioresource Technology* 147, 193-201. (Reported in Chapter 6)
- III. **Lim, J.W.** and Wang, J.-Y., 2013. Enhanced hydrolysis and methane yield by applying microaeration pretreatment to the anaerobic co-digestion of brown water and food waste. *Waste Management* 33 (4), 813-819. (Reported in Chapter 7)
- IV. Rajagopal, R., **Lim, J.W.**, Mao, Y., Chen, C.L., Wang, J.-Y., 2013. Anaerobic co-digestion of source-segregated brown water (feces without urine) and food waste. *Science of The Total Environment* 443, 877-886. (Reported in Chapter 5)

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- I. **Lim, J.W.**, Chen, C.-L., Wang, J.-Y., 2013. Effects of microaeration pretreatment on the anaerobic co-digestion of brown water and food waste. Proceedings of 13th World Congress on Anaerobic Digestion, Santiago de Compostela, Spain, 25-28 June. (Reported in Chapter 7)
- II. **Lim, J.W.**, Chen, C.-L., Ho, J.R.I, Wang, J.-Y., 2013. Microbial population under steady-state conditions in the anaerobic co-digestion of brown water and food waste. Proceedings of the International Conference on Solid Waste 2013: Innovation in Technology and Management, Hong Kong SAR, P.R. China, 5-9 May. pp.347-350. (Reported in Chapter 6)
- III. **Lim, J.W.**, Rajinikanth, R., Mao, Y., Ho, I., Ahamed, A., Wang, J.Y., 2012. Anaerobic Co-digestion of Brown Water with Food waste in Decentralized Source-Separation-Based Sanitation Concepts. Proceedings of ORBIT 2012, Rennes, France, 12-14 June. Section 1 pp.9-15. ISBN 3-935974-36-1. (Reported in Chapter 5)
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Executive Summary

Brown water (BW) and food waste (FW) are wastes generated in the households that are high in organic content and therefore suitable as substrates for the anaerobic digestion process to recover biogas as a form of energy. In recent years, there has been on-going research on the anaerobic co-digestion of black water and FW. However, there has been limited research on the source separation between feces and urine, and its subsequent resource recovery approach. The separation of feces and urine would be important as this facilitates the recovery of nutrients (N, P, K) from urine and also reduces the inhibition effects of ammonia on the anaerobic digestion process. The objective of the research was to determine the reactor configuration and operating parameters suitable for efficient energy recovery from the anaerobic co-digestion of BW and FW. This was achieved through biochemical methane potential (BMP) tests, 5 L and 30 L reactor performance studies and microbial community profiling for the anaerobic co-digestion of BW and FW. The effect of microaeration on the co-digestion system was also investigated by carrying out BMP tests, fed-batch reactor studies and microbial analysis.

The preliminary study in chapter 4 compared the BMP for the anaerobic digestion of BW, FW and FW/BW mixtures. This study demonstrated the advantages of co-digestion, in terms of higher methane yield and organic matter removal efficiencies, as compared to the anaerobic digestion of the substrates individually.

Optimization of the co-digestion process in three different configurations – two-phase continuously stirred tank reactor (CSTR), single-stage CSTR and single-stage sequencing batch reactor (SBR) was reported in Chapter 5. Phase separation resulted in more stability and SBR was able to retain solids more efficiently than CSTR.

Chapter 6 discussed the difference in reactor performance between single- and two-phase CSTR by comparing their bacterial and archaeal

community structures through the construction of 16S rRNA gene clone libraries. The unexpected predominance of aerobic bacteria species – *Acetobacter peroxydans* in the acidogenic reactor suggested the reactor might be unknowingly exposed to partial aeration. Nevertheless, the high degree of acidogenesis observed in the acidogenic reactor showed that the fermentation process was not inhibited when exposed to partial aeration.

In view of the tendency for unavoidable oxygen loading, as well as the benefits of microaeration in terms of enhanced fermentation, Chapter 7 investigated the role of microaeration pretreatment on the degradation of BW with FW in BMP tests. Microaeration was shown to enhance methane yield through higher COD solubilization and greater VFA accumulation.

Chapter 8 showed that the fermentation of BW and FW under microaeration conditions gave rise to a significantly more diverse bacterial community structure and higher proportion of bacterial clones affiliated to the phylum *Firmicutes*. The acidogenic reactor was therefore able to metabolize a greater variety of substrates and had higher hydrolysis rates as compared to the anaerobic reactor.

Co-digestion of brown water and food waste plays an important role in the proposed decentralized waste to resources system. However, there has been limited research on the source separation between feces and urine, and its subsequent resource recovery approach. This doctoral research has demonstrated the feasibility of brown water and food waste co-digestion. On top of that, the co-digestion process was also shown to be optimized with phase separation and the sequencing batch reactor mode of operation. Comprehension of microbial community and its function is necessary to potentially improve the efficiency and process stability of anaerobic digesters. Insights gained from the laboratory-scale reactor performance and microbiology studies conducted during this doctoral research could help to aid the selection of seeding sludge for rapid startup of source-separation-based anaerobic digestion systems in future applications.

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Abbreviations

AC: accumulation reactor system

AD: anaerobic digestion

AN: anaerobic conditon

ASBR: anaerobic sequencing batch reactor

ATP: adenosine triphosphate

BOD: biochemical oxygen demand

BMP: biochemical methane potential

BW: brown water

C: carbon

C:N: carbon to nitrogen ratio

CH₄: methane

CM: cow manure

CO₂: carbon dioxide

COD: chemical oxygen demand

CSTR: continuously stirred tank reactor

DUR² System: Decentralized Urban Resource Recovery System

EGSB: expanded granular sludge bed

FW: food waste

H₂: hydrogen

H₂S: hydrogen sulfide

H-Ac: acetic acid

H-Pr: propionic acid

H-Bu: butyric acid

H-Va: valeric acid

H-Ca: caproic acid

H-He: heptanoic acid

HRT: hydraulic retention time

LBR: leach bed reactor

LCA: life cycle assessment

LCFA: long chain fatty acids

MA: microaerobic condition

N: nitrogen

NPK: nitrogen, phosphorus, potassium

SBR: sequencing batch reactor

SCFAs: short chain fatty acids

SRT: solid retention time

TN: total nitrogen

TOC: total organic carbon

TS: total solids

UASB_{ST}: upflow anaerobic sludge blanket septic tank

VFA: volatile fatty acids

VS: volatile solids

Chapter 1

1. Introduction

1.1 Background

1.1.1 Centralized sanitation systems

Sanitation issues are important because poor sanitation brings serious health risks and directly affects our quality of life. During the nineteenth century, it was common practice for citizens to collect human excreta from their residences for disposal or storage as fertilizer for agricultural use. However, these actions exposed the citizens to significant public health risks, such as parasite and bacterial infections. To minimize the exposure of sewage to citizens, centralized off-site sanitation concepts and aerobic wastewater treatment systems were developed since the 1930s, to flush away human excreta from households as quickly as possible.

Due to the ease of operation and proven concepts, centralized off-site aerobic processes are the core and conventional wastewater treatment system adopted by many countries today (Lettinga, 2010). Although this system has successfully minimized the exposure of wastewater to citizens, it consumes large amounts of potable water and energy for the extensive transportation and aerobic treatment of sewage, respectively. Moreover, by the time the sewage reaches the wastewater treatment plant, its diluted nature greatly reduces the potential for resource and energy recovery. Although costly, aerobic wastewater treatment and centralized off-site sanitation systems were observed to be preferred by the public sector. Nonetheless, this trend might change with rising concerns over the energy crisis and scarcity of clean water.

The current energy mix that fuels the global economy is mainly composed of fossil sources such as crude oil, coal and natural gas. However, fossil fuels are finite resources and the world is consuming nearly four times more oil than it is finding, leading to a situation that is ultimately unsustainable

(Salameh, 2003). In contrast, renewable energy sources are able to generate electricity without depending on finite reserves of fossil fuels. Therefore, a transition from fossil fuels to renewable energy sources is inevitable in bridging the energy gap and creating a sustainable future energy supply. Biogas is an example of renewable energy with a fuel value of approximately 5,850 kg-cal/m³ that offers a partial solution to two pressing problems — the environmental crisis and the energy shortage.

Water is essential for life and the presence of clean water is a core issue for public health and economic growth. The rapid population growth along with an increasing water pollution and scarcity calls for action on a global level. Since 1980, the United Nations (UN) have addressed the world wide water problem, with the first International Water Decade (1981-1990) and now the second Water Decade “Water for Life” (2005-2015). The targets to halve the proportion of people without access to clean water and adequate sanitation until 2015 (Millennium Development Goal 7) were set in 2000 and 2002 by the UN. Although many efforts have been made to meet these targets, there were still 1.6 billion people without access to safe sanitation in 2006 (WHO and Unicef, 2006).

With rising concerns over the energy crisis and scarcity of clean water, there is a need to come up with more sustainable alternatives to the centralized off-site sanitation concepts and aerobic wastewater treatment systems.

1.1.2 Rationale behind source separation concepts

The traditional household sanitation concept is heavily dependent on centralized and aerobic wastewater treatment systems. These are "end-of-pipe" technologies which would not be a long-term solution to the growing volumes of wastewater and rapidly depleting availability of fossil fuels and clean water. Scientists and politicians in several European countries have been actively exploring the feasibility of adopting decentralized systems as a sustainable alternative to the current centralized and aerobic wastewater treatment systems (Fang, 2011).

In contrast to centralized systems, decentralized systems focus on source separation of wastewater flows and organic waste on a household level, followed by an appropriate treatment or reuse of each stream at or near the point of waste generation. If there are no nearby treatment facilities, the different fractions of the wastewater can also be transported to different centralized points for further treatment and reuse (Tchobanoglous, 1996). Decentralized systems therefore enable closing of water and substance loops which will minimize the use of clean water in wastewater collection and transport.

Figure 1-1 shows the concept of "Communities as Renewable Resource Recovery Centers", which is part of the Competitive Research Programme (CRP) project "Sustainable Urban Waste Management for 2020" funded by the National Research Foundation, Singapore. Similar projects on the decentralized "waste to resources" concept were implemented in Germany and the Netherlands, which will be described in greater detail in Chapter 2.

Table 1-1 shows that wastewater generated on the household level can be separated and described as black, grey, yellow and brown water. Household wastewater is a mixture of wastewater from several sources – toilet, bath, kitchen and wash. Black water comprises of human faeces (brown water) and urine (yellow water) while grey water refers to wastewater from washing activities.

Table 1-1: The wastewater palette (definition of wastewater fractions from households)

Type	Content
Classic	Toilet, bath, kitchen, wash
Black	Toilet
Grey	Bath, kitchen, wash
Yellow	Urine
Brown	Faeces

Table 1-2 illustrates the components (i.e., BOD, COD, nitrogen, phosphorus and potassium) present in different types of domestic wastewater. Black water was found to contain most of the nutrients (nitrogen, phosphorus and potassium), around half of the domestic COD load and the major part of the pathogens (Otterpohl, 2002; Vinneras et al., 2006).

However, the source of most nutrients in black water was reported to be contributed by urine (Sundberg, 1995) while majority of BOD and COD were present in faeces. Hence, the separate collection of urine and faeces (by using a no-mix toilet) would facilitate the recovery of nutrients and biogas from yellow and brown water, respectively.

Figure 1-2 shows the vacuum source separation toilet designed by Residues and Resource Reclamation Centre (R3C) for the CRP project as illustrated in Figure 1-1. The no-mix toilet is similar in appearance to a cistern flush toilet except for the diversion in the bowl into two sections. Urine is collected in the front section while faeces are collected at the back. Yellow water is flushed with 0.3-L water and collected through a separate pipe into a storage tank while brown water is flushed with 1-L to 2-L of water and collected into another tank before possible treatment in decentralized anaerobic digesters.

Table 1-2: Typical pollution loads in domestic wastewater (kg per person per year). (Henze, 1997; Sundberg, 1995).

Type	BOD	COD	Nitrogen	Phosphorus	Potassium	Volume (L/p/d)
Classic	22	47	5.1	0.9	1.6	140
Black	9	27	4.4	0.7	1.3	40
Grey	13	20	0.7	0.2	0.3	100
Yellow	1.8	5.5	4.0	0.5	0.9	n.a.
Brown	7.3	22	0.4	0.2	0.4	n.a.

n.a.: data not available

Figure 1-1: CRP Project: Communities as Renewable Resource Recovery Centers, adapted from Competitive Research Program Project: Sustainable Urban Waste Management for 2020 (Wang, 2013).

Figure 1-2: Source separation toilet bowl for use in decentralized "waste to resources" concept (Wang et al., 2014).

Source separation toilets are usually designed with low flushing water consumption to achieve a low dilution of brown water while still able to transport waste efficiently away from households. Hence, other than maximizing the potential of full resource recovery, the separate collection of yellow and brown water via a separation toilet also significantly reduces the consumption of flushing water (most of the time potable water). This new/alternative approach to sanitation and wastewater management is also known as a resource conservation and recovery system since it reduces household water consumption, encourages the recycling of nutrients for agriculture, as well as generates a source of renewable energy. Similar systems have been developed and implemented in different projects in recent years, and would be described in detail in the following sub-section 1.1.4.

Due to economic reasons, it may not be possible for many countries to provide sewerage facilities for populations in rural and peri-urban areas, either now or in the near future. Thus, the focus of the field of wastewater management should change from the construction and management of regional sewerage systems to the construction of decentralized wastewater treatment and resource conservation and recovery facilities (El-Gohary, 2001). Decentralized resource conservation and recovery systems coupled with anaerobic digestion supplemented with adequate complementary methods would offer a sustainable alternative to centralized off-site sanitation concepts and aerobic wastewater treatment systems.

1.1.3 Role of anaerobic digestion in treatment of concentrated wastewaters

Anaerobic digestion can play a key role in the treatment of concentrated wastewaters in decentralized resource conservation and recovery systems. Anaerobic digestion has been widely applied in the treatment of organic wastewaters due to its high degree of waste stabilization and methane generation. Methane is a potentially valuable by-product that can be used as a fuel in producing heat and electricity, and the significance of this advantage is increasingly being emphasized in our energy conscious society. Having a fuel value of approximately 5,850 kg-cal/m³, biogas has been widely used in heating digesters and gas engines.

Anaerobic digestion is a treatment that scores considerably better than other treatments in terms of contribution to global warming. Aerobic treatment of organic waste releases extensive emission of undesired volatiles such as ketones, aldehydes, ammonia and even methane. The landfill of organic waste releases landfill gas that can be extracted, but only partially (maximum 60%) recovered. Thus, considerable methane emissions to the atmosphere would still occur, contributing to global warming. In contrast, all gases are contained during anaerobic treatment and, via the use of the biogas, incinerated.

Anaerobic digestion is one of the most sustainable treatment methods in the mineralization of organic matter due to the production of biogas. Thus, anaerobic digestion has the potential to play a major role in closing the water, raw materials, and nutrient cycles in decentralized resource conservation and recovery systems. With suitable pre- and post treatment systems, the role of anaerobic digestion in closed circuit and/ or side stream treatment was predicted to increase in the near future (van Lier et al., 2001).

1.1.4 Developments in source separation concepts

During the late 1970s and 1980s in Sweden, composting and urine-separating toilets were developed and installed in many experimental houses called 'ecological villages' to separate urine from faeces directly at source.

The collected urine was diluted with water and used as agricultural fertilizer while the faeces were stored in composting chambers. Later on, Zeeman et al. (2000) demonstrated the separation of different wastewater streams and their treatments with the aim of energy production and nutrients reuse. Toilet systems generally generate three different wastewater streams - yellow water, brown water and black water. To improve the potential of energy recovery, food waste was subsequently added to domestic sewage to create high-strength domestic wastewater streams, which were then treated in anaerobic digesters.

In Europe, anaerobic digestion of black water has already been applied in several pilot projects to demonstrate its feasibility and its potential within resource management sanitation concepts. Although source separation concepts are still in the developing stage, several pilot projects have demonstrated their potential to save water, recover energy, and recover nutrients from brown water and urine.

1.2 Limitations of earlier research work

As discussed, several projects on decentralized resource conservation and recovery systems have been developed and implemented in Europe. Benefits of such systems included the reduction of household water consumption and energy recovery from the anaerobic digestion of black water with and without food waste (Wendland et al., 2007; Kujawa-Roeleveld et al., 2005; Zeeman et al., 2008). However, research on source separation between feces and urine, and its subsequent resource recovery approach is limited.

Other than the recovery of nutrients, the separate treatment of yellow and brown water is expected to be advantageous because high amounts of ammonia present in urine might inhibit the anaerobic digestion process. Moreover, decentralized resource conservation and recovery systems were mostly implemented in rural and peri-urban locations, and such research in the urban context has been scarce. With increasing population and concerns over water and energy shortage, the onsite decentralised and anaerobic

treatment of brown water and food waste in urban cities might be a more sustainable alternative to the conventional, centralized waste management systems.

Similar to many other countries, wastewaters in Singapore are currently being transported long distances to centralized plants for treatment, which consumes too much unnecessary energy and water. An alternative approach would be to treat concentrated wastewaters in decentralized systems. An ongoing project with the objective of converting community wastes (brown water and food waste) to energy is carried out by R3C of Nanyang Technological University (NTU), Singapore.

This is part of the research program to develop a practical and alternative option for sustainable urban waste management not only for Singapore but also numerous other cities in urban setting and developing countries. This would be achieved by the creation of 'Communities as renewable resource recovery centers' (Figure 1-1). Other than significantly reducing daily household flush water consumption, the source separating toilet using 0.3-L of water per urine flush (yellow water) and 1 to 2-L water per feces flush (brown water) produces lowly diluted brown water that facilitates the subsequent energy recovery process. Yellow water is considered a good source of plant-available nitrogen and phosphorus that could be treated and used as fertilizer and soil amendments (Heinonen-Tanski and van Wijk-Sijbesma, 2005). Other than improving the potential of energy recovery, the addition of food waste as a co-substrate would also help to divert food waste from incineration plants and also raise the current food waste recycling rate of 12% in Singapore.

1.3 Objective and scopes of the research

The objectives of the research were to determine the reactor operation parameters suitable for efficient anaerobic co-digestion of brown water and food waste; and to study the effect of different operation parameters on the microbial population in the biomass. This research will seek to optimize the

anaerobic co-digestion process of brown water and food waste for clean energy recovery as well as waste minimization.

The scopes of the research were divided into separate studies and included:

- Study 1 – to compare the biochemical methane potential between the mono-digestion of brown water, mono-digestion of food waste and the co-digestion of brown water and food waste (Chapter 4)
- Study 2 – to optimize the operating parameters and reactor configurations of 5- and 30-L digesters treating brown water and food waste (Chapter 5)
- Study 3 – to compare the microbial community of single- and two-phase continuously stirred tank reactors treating brown water and food waste (Chapter 6)
- Study 4 – to investigate the role of microaeration pretreatment on the anaerobic co-digestion of brown water and food waste (Chapter 7)
- Study 5 – to understand how microaeration improves fermentation in anaerobic digestion through process performance and microbial community profiling (Chapter 8)

1.4 Structure of the report

The report is in fulfillment of the requirements for the degree of Doctor of Philosophy and will cover studies 1, 2, 3, 4 and 5 in Chapters 4, 5, 6, 7 and 8, respectively. The structure of the report is as presented in Figure 1-7.

Chapter 2 presents a literature review on several aspects of the anaerobic digestion process and the anaerobic treatment of black/ brown water in decentralized resource conservation and recovery concepts in recent years. Chapter 3 describes the materials and analytical methods used for experimental work presented in Chapters 4 to 8. Chapter 9 shows the conclusion for the report and describes the proposed future studies to extend the doctoral research.

Figure 1-3: Schematic of the report structure. ⁴ Chapter 4, ⁵ Chapter 5, ⁶ Chapter 6, ⁷ Chapter 7, ⁸ Chapter 8.

Chapter 2

2. Literature Review

2.1 Developments of anaerobic technology for production of energy from organic waste

The first anaerobic digestion plant was operated in Bombay, India in 1859 and by 1895, biogas was recovered from a sewage treatment facility and used to fuel street lamps in Exeter, England. In the 1930s, Buserl and coworkers identified anaerobic bacteria and the conditions that promoted methane production. As the understanding of the anaerobic digestion process and its benefits improved, closed tanks, heating and mixing systems were used to optimize the anaerobic digestion process. Regardless of improvements, anaerobic digestion suffered from the development of aerobic treatment and low costs of coal or petroleum (Monnet, 2003). While anaerobic digestion was used only for the treatment of wastewater sludge digestion, countries such as India and China embraced the technology for energy and sanitation purposes. The anaerobic treatment of human excreta, animal manure and kitchen residues in small, home-made and decentralized dome-shaped anaerobic digesters was commonly adopted by rural households in countries like India and China since the 1950s. The capacities of biogas plants for individual households ranged between 2 m³ to 6 m³ while that at the community level was at least 25 m³ (Raha et al., 2014).

With recent concerns over higher energy prices and increasingly stringent environmental regulations, European countries have actively explored the field of sustainable sanitation in recent years. Case studies of sustainable sanitation projects implemented in Asian and European countries would be discussed in greater detail in the following sub-sections.

2.1.1 India

The Indian Government has initiated a number of different programmes to replace biomass cooking with alternative cleaner fuels such as biogas. These programmes include the National Project on Biogas Development in

1981; the National Programme for Improved Chulhas (cookstoves), NPIC in 1986; and the National Biomass Cook Stove Initiative in 2009 (Raha et al., 2014). The National Project on Biogas Development was set up in 1981 for the promotion of biogas plants using cattle dung and other biomass waste to generate methane for household cooking and lighting (Bond and Templeton, 2011). The National Biogas and Manure Management Programme (NBMMP) was started in 2005 as a result of the merger of the National Project on Biogas Development and a manure management initiative. The objectives of such initiatives were to promote clean and efficient energy cooking for poorer sections of the country. On top of that, the new NBMMP scheme aimed to encourage people in rural areas to adopt biogas technologies to meet their household cooking and lighting needs (Raha et al., 2014). NBMMP household plants were designed to be multifunctional and to achieve the following targets:

- reduce dependency on LPG and kerosene for cooking and lighting purposes
- produce waste digestate fertilizer to reduce dependency on chemical fertilizers
- preserve forests by removing the need for use of firewood
- improve sanitation in villages by linking sanitary toilets with biogas plants

National programs on biogas development were launched in the 1980s which led to the rapid construction of digesters in India. The Ministry of Energy supported the implementation of both family and community sized biogas digesters by developing guidelines and allocating budgets for training and subsidies. Great emphasis has been given on implementation, training and monitoring.

Small biogas digesters serving one or a small number of households have thus become more and more popular. Their main goal is to produce biogas and provide the family with energy mainly for cooking. Up to now, the program has supported the building of 3.1 million plants in the country (Ndzana, 2004). An increasing number of public toilets have been installed in India as well.

Around 100 large scale plants are in operation treating black water from public pour-flush toilets in biogas plants.

2.1.2 China

Due to the lack of power stations or coal pits in certain areas, large numbers of biogas plants were built in China, resulting in China being one of the leading countries in biogas technology in Asia. In 1975, a big Chinese campaign “biogas for every household” led to the construction of about 1.6 million household biogas plants per year (Marchaim, 1992). Today there are more than 5 million family sized plants of 6, 8 and 10 m³ in operation in China that feed on animal waste (pig and cattle manure, and poultry), human excreta and organic waste such as food waste. Constructed with bricks and cement, the household digesters are usually constructed as fixed dome plants and operated as an accumulation digester (AC). It is generally underground and shaped as an egg, where the headspace at the top is for gas collection.

In many Chinese regions, forest wood is increasingly used for cooking purposes causing environmental problems like deforestation and soil erosion. The restrictions by the government on tree felling and wood cutting to preserve the forest led to an on-going interest in biogas (Knecht, 2006). For example, the project of 1,300 biogas plants in Shanxi was awarded in 2006 with a sustainability award (Ashden, 2006). In the framework of increasing energy demand in China, the Ministry of Agriculture is now addressing the importance of biogas again and wants to support the connection of households to biogas plants further. There is also a growing interest in sustainable aspects such as water saving, and sanitation projects involving vacuum toilets with black water digestion was gradually implemented (Zhang, 2008).

2.1.3 Japan

The treatment of night soil using anaerobic digestion in Japan was recommended by the Economic Stabilization Agency in the 1950s (Figure 2-1). During the period of 1953-1975, anaerobic digestion was one of the major methods for treating night soil in Japan. The Hokko plant in Hokkaido is an

example that began treating 90 m³.d⁻¹ night soil produced from Sapporo. The process consisted of a two-stage mesophilic anaerobic digester coupled to a trickling filter (Inoue, 2009).

Subsequently, the tightening of water quality standards required the BOD/N ratio of the effluent to be kept below 3. As a result, most plants began to employ biological nitrogen removal processes for the treatment of night soil instead of anaerobic digestion. In recent years, new technologies including high-rate denitrification and membrane bioreactors have been developed and applied in many plants. Now, the prevalence of the sewer system has resulted in a decrease in the treatment of stored night soil by anaerobic digestion, as shown in Figure 2-1 (Li and Kobayashi, 2011).

2.1.4 Ecological housing estate, Lübeck-Flintenbreite, Germany

The separation of different wastewater streams and their treatments with the aim of energy production and nutrients reuse was demonstrated in year 2002 within a housing estate for about 400 inhabitants in the pilot project Flintenbreite in Lübeck, Germany (Figure 2-2). The settlement is situated to the west of Lübeck and covers an area of 5.6 ha, out of which 2.1 ha are left as natural green space. Pictures of the site layout and the settlement are shown in Figures 11-1, 11-2 and 11-3 in Appendix. With no connection to the central sewerage system, this ecological settlement demonstrates a working example of the concept of sustainable sanitation.

The wastewater is collected and treated in an internal cycle. The concept comprises vacuum toilets with subsequent pasteurization and anaerobic digestion of black water together with food waste in a semi-centralized biogas plant and finally recycling of the digested anaerobic effluent in agriculture. Grey water is treated in vertical flow constructed wetlands and locally infiltrated into the soil, together with the rainwater collected from roofs and sealed areas.

Figure 2-1: History of night soil treatment in Japan (Li and Kobayashi, 2011).

Investment cost for this sanitation system was reported to be approximately 40% higher than for the common wastewater system. However, operation costs were estimated to be 25% less than that in conventional settlements. The energy balance of sustainable sanitation systems was predicted to be positive due to biogas utilization and the substitution of industrial fertilizer.

2.1.5 KREIS, Hamburg, Germany

KREIS is a research project in Germany subsidized by the Federal Ministry of Education and Research (BMBF). KREIS stands for the linking of regenerative energy production to innovative wastewater engineering in cities in order to generate electricity and heat from biogas.

Construction works started in October 2013 and is expected to complete in 2016. The Jenfelder Au Quarter project is situated on a former military site in Hamburg and has a total area of 45 ha. 720 accommodation units as well as the necessary social, cultural and commercial infrastructure will be constructed to house about 2,000 inhabitants (KREIS, 2014).

Figure 2-2: Scheme of the pilot project Flintenbreite in Lübeck (Wendland and Oldenburg, 2003).

Designed with high energy standards, the Jenfelder Au Quarter will have an innovative integrated energy production and drainage system for the purposes of demonstration and scientific analysis. The technology is based upon the HAMBURG WATER Cycle®, under which toilet water (black water) and other domestic sewage (grey water) are separated (Figure 2-3).

The grey water is treated in an energy-saving, decentralised system before flowing into the receiving water. The black water (faeces, urine and flush water) is collected separately via vacuum toilets and then treated anaerobically together with organic waste (co-substrates). The biogas produced in this process is converted into power and heat in a block heating plant. The residual substances (the digestates) are processed into high-quality soil conditioners and fertilizers. These products and the energy generated are designated for priority use inside the Jenfelder Au quarter. The aim was to optimize the utilization of thermal and electrical energy via model-

based control and regulation. In this project, energy is produced not only from biogas but also from geothermal and solar heat.

2.1.6 Sneek, the Netherlands

The Netherlands Institute of Ecology (NIOO-KNAW) started research programs on source separation since 1999. Their research focused on (1) Vacuum collection and transport to concentrate black water, (2) Anaerobic digestion for energy recovery at local scale and (3) Use of remaining product as fertilizer.

After years of research, a black water treatment system serving a housing estate of 32 houses in Sneek was implemented in 2006 (Figure 2-4). The system was provided with a collection, transport and anaerobic treatment system for black water from vacuum toilets. Inhabitants produced an average of 7-L/p/d concentrated black water and utilization of such vacuum toilets saved 30 to 42-L/p/d of flushing water. Black water and food waste were treated at 25°C in two UASB septic tanks of 6 m³ each. With a liquid retention time of 2 days, gas production was approximately 11-m³ CH₄/cap.year. Influent COD was 8-10-g/L while effluent COD was 2-3-g/L. The effluent of the UASB was subjected to a post treatment where residual COD is removed while NH₄ and phosphate were recovered (Zeeman et al., 2008). The technical feasibility of UASB septic tank system for black water was previously demonstrated in laboratory scale by Kujawa-Roeleveld et al. (2005). More pictures of the pilot project Sneek is shown in Figures 11-4, 11-5 and 11-6 in Appendix.

Anaerobic digestion (AD) refers to the fermentation process that produces biogas (composed of mainly methane and carbon dioxide) from the degradation of organic material. AD processes are very effective in removing biodegradable organic compounds and can occur at almost any place where redox potential is low (i.e., in the absence of oxygen). In contrast to aerobic treatment in the activated sludge process, the amount of excess sludge produced by anaerobic digestion is very small and well stabilized. Moreover, useful energy in the form of biogas is produced instead of energy consumed.

Figure 2-5 compares the fate of carbon and energy in both aerobic and anaerobic wastewater treatment assuming that the oxidation of 1 kg-COD requires 1 kWh of aeration energy (Henze, 2008). Other than being energy intensive, aerobic treatments tend to have high operational costs due to the large amount of sludge produced from the COD converted. The new sludge requires further treatment, normally AD, before it is disposed off or incinerated. Due to rising concerns over energy and water shortage, it is not surprising that anaerobic process has evolved into a competitive waste treatment technology.

Figure 2-3: The HamburgWaterCycle implemented in the KREIS project (KREIS, 2014).

Figure 2-4: Black water treatment system in Sneek (Pereira, 2014).

2.2 Definition and environmental benefits of anaerobic digestion

Anaerobic digestion (AD) refers to the fermentation process that produces biogas (composed of mainly methane and carbon dioxide) from the degradation of organic material. AD processes are very effective in removing biodegradable organic compounds and can occur at almost any place where redox potential is low (i.e., in the absence of oxygen). In contrast to aerobic treatment in the activated sludge process, the amount of excess sludge produced by anaerobic digestion is very small and well stabilized. Moreover, useful energy in the form of biogas is produced instead of energy consumed.

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2.3 Microbiology of anaerobic digestion process

2.3.1 Overview

AD is a complex degradation pathway that consists of several sequential and parallel steps involving biochemical and physico-chemical processes (Batstone et al., 2002). During AD, the transformation of complex high-molecular-weight organic compounds can be generally expressed by Equation 2.1.

Figure 2-5: Fate of carbon and energy in aerobic and anaerobic wastewater treatment, adapted from Henze (2008).

As shown in Figure 2-6, the bio-conversion of complex high-molecular-weight organic compounds into biogas is the result of complex interactions among different microorganisms: (1) fermentative bacteria, (2) hydrogen-producing acetogenic bacteria, (3) hydrogen-consuming acetogenic bacteria, (4) carbon dioxide-reducing methanogens, and (5) aceticlastic methanogens. The digestion process may be subdivided into four steps: (1) hydrolysis; (2) acidogenesis; (3) acetogenesis; and (4) methanogenesis.

2.3.2 Hydrolysis and acidogenesis (Fermentative bacteria)

Hydrolysis is usually the first step during the AD of complex substrates. During hydrolysis, particulate organic matter which are too large to cross the bacterial cell barrier will be converted into smaller molecules which are readily accessible for the acidogenic bacteria. As shown in Figure 2-6, enzymes excreted by fermentative bacteria (also known as exo-enzymes) hydrolyze proteins into amino acids, polysaccharide into simple sugars and lipids into long chain fatty acids (LCFA) during the enzymatic hydrolysis process.

During the acidogenesis step, the hydrolysis products (amino acids, simple sugars, LCFAs), which are relatively small soluble compounds, are diffused inside the bacterial cells through the cell membrane and subsequently fermented or anaerobically oxidized. Fermentative acidogenic bacteria refer to acid-forming bacteria (e.g. *Clostridium*, *Bacteroids*, *Peptostreptococcus*, *Eubacterium*, and *Lactobacillus*). They convert sugars, amino acids and fatty acids to organic acids (e.g. acetic, propionic, formic, lactic, butyric or succinic acids), alcohols and ketones (e.g. ethanol, methanol, glycerol, acetane), CO_2 and H_2 (Wong, 2007). If methanogenesis is inhibited and H_2 accumulates, more reduced products such as propionate, butyrate and lactate are likely to appear.

Figure 2-6: Overview of the degradation pathway during anaerobic digestion. Adapted from Wong (2007)

2.3.3 Acetogenesis (Hydrogen producing acetogenic and homoacetogenic bacteria)

Acetogenic bacteria are acetate and hydrogen-producing bacteria which convert fatty acids (e.g. propionic acid and butyric acid) and alcohols into acetate, hydrogen and carbon dioxide. They include the syntrophs like *Syntrophomonas*, *Syntrophobacter* and *Acetobacter*.

Ethanol, propionic acid and butyric acid are converted to acetic acid by acetogenic bacteria via the reactions shown in Equations 2.2 to 2.4. The production of acetate or certain other fatty acids is energetically advantageous because it allows the organism to make ATP by substrate-level phosphorylation.

Homoacetogens are a group of strictly anaerobic prokaryotes that use CO_2 as an electron acceptor in energy metabolism. Homoacetogens are involved in the reduction of CO_2 via the acetyl-CoA pathway, where one molecule of CO_2 is either reduced to the methyl group of acetate or reduced to the carbonyl group. H_2 is the major electron donor for both acetogenic bacteria and homoacetogens (Wong, 2007).

2.3.4 Methanogenesis (Methanogenic bacteria)

Methanogens are a group of strictly anaerobic Archaea which carry out methanogenesis to produce CH_4 . CH_4 production can occur via the cleavage of two acetic acid molecules to generate CO_2 and CH_4 , or by reduction of CO_2 with H_2 . Due to limited H_2 available, acetic acid is the major precursor of CH_4 (about 70% of the COD flux). It is interesting to note that there is only COD conversion and no COD destruction during the AD process. COD removal takes place owing to the fact that the end product of the reaction chain, CH_4 , is gaseous and highly insoluble in water.

2.4 Pretreatment

Hydrolysis is usually the rate limiting step in the AD of suspended matter and organic solids. High hydrolysis efficiencies are significant for the anaerobic treatment of solid wastes, manure, and domestic sewage, as they have a high concentration of suspended solids. An efficient pretreatment increases the contact of suspended substrate with the anaerobic bacteria, resulting in the optimization of the methanogenic potential of the waste. Due to improved accessibility of the substrate for enzymes, the biodegradability of the waste is enhanced. This can be achieved by several pretreatment methods:

- Mechanical – the disintegration of solid particulates that releases cell compounds as well as creates new surface where biodegradation take place (Dohnyos and Zbransk, 1991; Doheanyos et al., 1997; Eastman and Ferguson, 1981; Tiehm et al., 1997)
- Ultrasonic – a kind of mechanical pretreatment that disrupts the physical, chemical and biological properties of the particulate organic matter (Pilli et al., 2011; Mukherjee and Levine, 1992)
- Chemical – the destruction of complex organic compounds through the addition of strong mineral acids or alkalis (Demirel and Yenigun, 2002)
- Thermal – accelerating the solubilization of particulate organic matter into less complex molecules (Haug et al., 1983; McCarty et al., 1977)
- Enzymatic – a promising method for the future for specific substrates such as cellulose and lignin (Hakulinen, 1988; Lagerkvist and Chen, 1993; Knapp and Howell, 1978)

2.5 Anaerobic reactor configurations

Anaerobic bacteria are generally slow growing and therefore maintenance of a high sludge retention time (SRT) is of great importance in the practical application of AD processes. The septic tank was one of the first conventional anaerobic digesters used in the 1890s to retain solids in sewage. Subsequently in 1905, the Imhoff tank was developed in Germany.

It was only until the 1930s that the anaerobic digesters were started to be mixed and heated to improve the digestion of solids in the sewage.

As shown in Figure 2-7, the main difference between conventional AD and high-rate AD systems lie in the mixing and the way microorganisms are retained in the bioreactor. In conventional AD systems, the contents are stratified while the contents are homogeneous in high-rate AD systems as a result of mixing. Due to the increased contact between wastewater and anaerobic bacteria in mixed systems, the hydraulic retention time (HRT) for high-rate AD systems are significantly lower than that of conventional AD systems.

Several high-rate anaerobic reactor configurations have been developed to accomplish higher treatment efficiency and reliability associated with long SRT and efficient biomass retention methods. These high-rate reactors include: 1) Continuously stirred tank reactor (CSTR); 2) Anaerobic sequencing batch reactor (ASBR); 3) Upflow anaerobic sludge blanket (UASB) reactor; 4) Anaerobic baffled reactor (ABR); 5) Leach bed reactor (LBR), 6) Expanded granular sludge bed (EGSB) reactor, etc. In this thesis, the CSTR and ASBR will be described in greater detail.

2.5.1 Continuously stirred tank reactor (CSTR)

The CSTR is a completely mixed reactor with no solids recycle in which the SRT equals the HRT. Wastewater and anaerobic bacteria are mixed together and allowed to react. When the organic pollutant is reduced to desired levels, the treated wastewater is then removed. The CSTR can be operated in either batch or continuous mode and depends on the continuous growth of new biomass to replace that lost in the effluent. Due to the slow growing methanogens and to prevent wash-out of microorganisms in effluent, at least 10 days of SRT and HRT are required. This results in the requirement of a large volume reactor. Therefore, CSTR is more suitable for the treatment of sludge (Figure 2-8) and wastewaters containing high organic matter content rather than the treatment of industrial wastewaters.

2.5.2 Anaerobic sequencing batch reactor (SBR)

Anaerobic batch reactors have also been studied extensively as an alternative to continuous systems. In a SBR, there is no continuous flow of wastewater entering or leaving the reactor. Instead, the cycle consists of four stages: 1) a flow entering the reactor, followed by 2) treatment, 3) settling and 4) decant.

Figure 2-7: Conventional vs. high-rate AD systems (Marmara Universitesi).

Figure 2-8: Treatment of sludge in egg-shaped CSTR anaerobic digester (Marmara Universitesi)

Batch operation was reported to generate effluent with better quality control due to its improved retention of biological solids and process control. Unlike continuous systems, the reactor draw for batch operation can be made only when the standard emission has been attained. On top of that, the intermittent operation of the batch reactor results in high initial substrate concentration and thus high biogas production. The low substrate concentration at the end of the operation cycle also enables efficient separation of the liquid (supernatant) from the solid (sludge) phase. The advantages of anaerobic SBR include no need for additional biomass settling stage or solids recycle, and short operational cycle times if biomass granulation is achieved.

Granular sludge is usually required to allow for efficient liquid and solid separation. Otherwise, a significant time is required for the settling of biomass from the treated wastewater. As a result, reactor volume requirement for anaerobic SBRs could be higher than that for CSTRs. Figure 2-9 shows the four steps during the operation of the anaerobic SBR (Zaiat et al., 2001; Wong, 2007).

1) Feeding of liquid influent

Depending on the influent flow rates, batch reactors could either be operated as batch or fed-batch systems. Longer feeding time leads to lower substrate concentrations inside the reactor, hence avoiding initial organic overloads.

2) Anaerobic biological reaction

This is usually the longest part of the operational cycle when biodegradation of organic matter takes place. Continuous or intermittent mixing occurs during this step to ensure sufficient contact between the substrate and microorganisms. Mechanical agitation and recycle of the gas generated in the reactor are the two commonly adopted methods in the operation of SBR. As shown in Figure 2-9, the contents in the anaerobic SBR are only completely mixed during the reaction step. This is significantly

different from the CSTR where it is assumed that complete mixing occurs instantaneously and uniformly throughout the reactor as inflow and outflow takes place simultaneously.

3) Biomass sedimentation

Solid-liquid separation takes place by gravitational force during the settling step. The time required for this step is dependent on the settling characteristics of biomass and may vary from some minutes to several hours. The time should be chosen so as to obtain a clarified effluent and an increasing biomass concentration in the sludge bed. Biomass growth as granules was reported to be necessary to provide high cellular retention in the SBR, since this improves separation of the liquid from the solid phase. After the reaction step, substrate concentration in the SBR is low and a low F/M ratio is known to improve the settling properties of biomass.

4) Effluent discharge

After the quality of the supernatant reaches the desired level, the effluent is withdrawn from the SBR from above the sludge blanket. It is usually done at a slow rate to minimize disturbance of the settled solids. During this step, poorly settled biomass is washed out of the system while the self-immobilized sludge is retained in the SBR.

Figure 2-9: The four steps during operation of anaerobic sequencing batch reactor (Marmara Universitesi)

SBR is a type of high rate methanogenic reactor process developed by Dague et al. (1992). The maintenance of a high active biomass concentration inside the reactor gives SBRs some advantages over conventional continuous-flow reactors, such as better organic matter removal efficiency, higher sludge retention time, efficient operating control and high organic matter removal efficiency (Dague et al., 1992).

The sedimentation stage is directly dependent on the formation of biomass with good settling characteristics, as in granular form, avoiding losses of the metabolic adapted sludge during discharging of the treated effluent. The use of inert supports for cell's adhesion and/ or immobilization within a SBR is considered to be a promising method to improve the retention of solids and eliminate the uncertainty about biomass granulation. Inert support material was used to promote biomass attachment and thus eliminating the settling step during the cited operational cycles, as commonly seen in SBR, and also consequently increasing contact for biological reaction. This idea was firstly proposed by Ratusznei et al. (2000).

2.6 Anaerobic digestion of food waste and factors affecting the process

With economic development and population growth, the increasing amount of food waste (FW) generated worldwide is a growing concern. According to US EPA, FW generated in the United States increased from 34.3 million tons in 2009 to more than 36 million tons in 2012 (US EPA, 2014). As shown in Figure 2-10, China has surpassed the United States as the world's largest waste generator in 2010 and during the same year, China produced nearly 90 million tons of FW (Zhang et al., 2014). Another study revealed that British households generate approximately 1.8 million tons of FW yearly (WRAP, 2009) while in Singapore, the annual FW generation increased from 558,900 tons in 2007 to 605,800 tons in 2011 and to 703,200 tons in 2013 (Singapore National Environment Agency, 2014).

Figure 2-10: The amount of FW generated in some countries in 2010 (Zhang et al., 2014)

2.6.1 Characteristics of food waste

According to Table 2-1, the total (TS) and volatile solid (VS) contents of FW from different countries ranged between 70 g/L to 309 g/L and from 66 g/L to 264 g/L, respectively. Due to the high moisture content and high biodegradability of FW, the disposal of FW has caused severe environmental pollution in many countries (Zhang et al., 2014). FW has been mostly disposed in landfill and incineration plants or used for aerobic composting. However, the high organic content of FW led to uncontrolled emission of landfill gas which poses serious environmental problems. Furthermore, incineration of FW is energy intensive due to its high moisture content.

In view of rising costs for waste disposal as well as depleting energy resources, the AD of FW was found to be a more sustainable treatment method due to the high degree of waste stabilization and methane generation. Using a life cycle assessment (LCA), Evangelisti et al. (2014) compared three treatments for organic fractions of MSW in the London area: (1) landfill with electricity production; (2) incineration with steam recovery for combined heat and power (CHP) and (3) anaerobic digestion with energy recovery as CHP.

LCA results revealed that AD emerged as the best treatment option for organic wastes.

As shown in Table 2-2, FW contains both macromolecular organic matter as well as various trace elements. The anaerobic digestion of FW is governed by different key parameters such as VFA, pH, ammonia nutrients, trace elements, and others. A good nutrient and trace element balance as well as a stable environment are essential for microbial growth. It is therefore very important to maintain the key parameters within the appropriate range for long term operation of anaerobic digestion.

2.6.2 VFA and pH

Volatile fatty acids (VFAs) are the main intermediate products during AD of organic wastes and they include acetic acid (H-Ac), propionic acid (H-Pr), butyric acid (H-Bu) and valeric acid (H-Va). VFAs are an important intermediate in the anaerobic process as they are ultimately transformed into biogas (CH_4 and CO_2). However, the accumulation of VFAs at high organic loading will result in the decrease of pH and eventually the failure of AD systems. Previous research demonstrated that a H-Pr to H-Ac ratio exceeding 1.4 or the concentration of H-Ac exceeding 0.8 g/L could lead to AD failure (Buyukkamaci et al., 2004; Pullmmanappallil et al., 2001; Hill et al., 1987). Therefore, the H-Pr to H-Ac ratio could be utilized as an indicator for digestion imbalance.

pH of the AD system is also determined by VFAs. Different species of anaerobic bacteria grow at different pH ranges. Fermentative bacteria require pH range of 4.0 – 8.5 while methanogens only grow in a limiting range of 6.5 – 7.2. Appels et al (2011) and Horiuchi et al. (1999) found that the main VFAs were H-Ac and H-Bu at low pH, while H-Ac and H-Pr were dominant at pH 8.0.

Table 2-1: Characteristics of food waste (TS, VS, C/N ratio) from literature

Waste	TS (g/L)	VS (g/L)	VS/TS (%)	C/N ratio	Country	Source
Kitchen waste	169	150	89	NA	Kenya	Georgiadis (2013)
Food waste	231	210	90	24.5	China	Zhang et al. (2013a)
Food waste	181	171	94	13.2	Korea	Zhang et al. (2011)
Food waste	309	264	85	14.8	US	Zhang et al. (2007)
Dining hall	200	190	95	14.7	Korea	Han and Shin (2004)
Dining hall	70	66	94	18.3	Korea	Shin et al. (2004)
Fruit and vegetable markets, household and juice centers	150	134	89	36.4	India	Rao and Singh (2004)
Mixed municipal sources	260	234	90	NA	Australia	Steffen et al. (1998)
Mixed municipal sources	100	80	80	NA	Germany	Nordberg and Edstrom (1997)

NA: not available

2.6.3 Souring of anaerobic reactors

Acidogenesis is the most rapid conversion step in the anaerobic food chain. The ΔG° of acidifying reactions is highest of all anaerobic conversions, resulting in 10- to 20-fold higher bacterial growth rates, and 5-fold higher bacterial yields and conversion rate compared to methanogens. For that reason, anaerobic reactors are subjected to souring i.e. a sudden pH drop, when reactors are overloaded or perturbed by toxic compounds. Once alkalinity is consumed by the produced acids the pH starts to drop, resulting in a higher concentration of non-dissociated VFAs, leading to a more severe inhibition of methanogens. The latter, obviously leads to an even quicker accumulation of VFAs and subsequent pH drop. The fact that acidifiers are active even at low pH (4), means the reactor souring to pH 4 to 5 can and will occur when the methanogenic capacity of the system is trespassed (Henze, 2008). Therefore, effluents of overloaded or perturbed anaerobic reactors often contain these more reduced intermediate products.

Table 2-2: Characteristics of FW (marcomolecular organic matter and trace elements) reported in literature (Zhang et al., 2014)

Parameters	Units	Zhang et al. (2011)	Zhang et al. (2013a)	Zhang et al. (2007)	Li et al. (2010)
Country		Korea	China	US	China
pH		6.5	4.2	NA	NA
Carbohydrate	% d.b.	61.9	NA	NA	55.2
Protein	% d.b.	NA	NA	NA	15
Fat	% d.b.	23.3	NA	NA	23.9
Oil	% d.b.	NA	4.6	NA	NA
S	ppm, w.b.	0.33	NA	2508	8.6
P	ppm, w.b.	1.49	NA	NA	88
Na	ppm, w.b.	0.84	3.45	NA	2.24
K	ppm, w.b.	0.3	2.30	0.90	NA
Ca	ppm, w.b.	0.07	0.4	2.16	2.44
Mg	ppm, w.b.	0.03	0.16	0.14	NA
Fe	ppm, w.b.	3.17	100	766	NA
Cu	ppm, w.b.	3.06	NA	31	NA
Zn	ppm, w.b.	8.27	160	76	NA
Al	ppm, w.b.	4.31	NA	1202	NA
Mn	ppm, w.b.	0.96	110	60	NA
Cr	ppm, w.b.	0.17	NA	<1	NA
Ni	ppm, w.b.	0.19	NA	2	NA

NA: not available

2.6.4 Ammonia

Ammonia is formed during the biodegradation process of protein or other nitrogen-rich organic substrates, and mainly exist in the form of ammonium (NH_4^+) and free ammonia (NH_3). While ammonia is required in small concentrations for bacterial growth, high concentrations of ammonia could be inhibitory to the AD process.

On top of the nutritional needs for bacterial growth, ammonia was also shown to enhance the buffer capacity of the AD process. As shown in Equations 2.5 to 2.7, VFAs formed during the digestion process could be neutralized by ammonia (Zhang et al., 2013a; Wang et al., 2012; Wang et al., 2013; Banks and Humphreys, 1998).

where C_xH_yCOOH represent the VFAs. High organic loading rate (OLR) leading to VFA accumulation might risk digestion failure due to souring of the reactor. The presence of ammonia could react with these VFAs to avoid inhibition from VFAs and allow the conversion of VFAs to biogas. Despite its buffer capacity, ammonia was proven to be an inhibitor to lots of bacteria at high concentrations (Appels et al., 2011; Chen et al., 2008). Free ammonia can diffuse through cell membrane and further hinder cell functioning through the disruption of potassium and proton balance inside the cell (Zhang et al., 2014).

2.6.5 Long chain fatty acids

FW is a lipid-rich resource in which the lipid concentration is about 5.0 g/L (Zhang et al., 2013a). Long chain fatty acids (LCFA) are intermediate by-products of the lipid degradation process and are mainly composed of oleic acid (C18:1), linoleic acid (C18:2) and palmitoleic acid (C16:0) (Palatsi et al., 2012; Zonta et al., 2013). LCFA biodegradation process is considered to be the rate-limiting step of AD process and higher concentration of LCFA was shown to result in AD failure (Oh and Martin, 2010; Neves et al., 2006).

LCFAs could adsorb onto the cell wall and membrane of microbial flocs floating on the surface which affects the metabolic processes of transportation and therefore inhibit the activity of anaerobic microorganisms (Palatsi et al., 2009). To overcome the inhibition from LCFA, strategies for recovering inhibition caused by LCFAs have been studied by many researchers. Palatsi et al. (2009) found that increasing the biomass/LCFA ratio through dilution with active inoculum as well as adsorbing the LCFA and reducing the bio-available LCFA concentration through addition of adsorbents were effective recovery approaches. Discontinuous feeding was also shown to enhance the

activity of the anaerobic community and the efficient transformation of lipid-rich effluents (Zhang et al., 2014).

2.6.6 Metal elements

As shown in Table 2-2, FW also contains certain light metal ions (Na, K, Mg, Ca, Al) and heavy metal ions (Cr, Co, Cu, Zn, Ni, etc.). These metal elements are required by anaerobic bacteria because these cations play an important role in enzyme synthesis as well as maintaining enzyme activities. According to Facchin et al. (2013), specific trace metals such as Co, Ni, W, Se or Mo are important cofactors in enzymes involved in the biochemistry of methane formation and therefore important for stable operation of the AD process. However, high concentrations of these metal ions are inhibitory to the anaerobic microorganisms.

The concentrations of heavy metal elements in FW were always insufficient while the light metal elements such as sodium and potassium were generally at high concentrations (Zhang et al., 2014). To enhance the performance of AD, addition of metal elements or metal-rich organic substrates has been studied by many researchers. Zhang et al. (2011) showed that trace elements played an important role in improving the anaerobic co-digestion of FW with piggy wastewater. Zhang and Jahng (2012) found that the stabilization of anaerobic system with trace elements addition was obviously enhanced in comparison with the single digestion of FW. Banks et al. (2012) reported that selenium is essential for both propionate oxidation and syntrophic hydrogenotrophic methanogenesis. Selenium supplementation allows digestion to proceed at substantially higher organic loading rates. The above literature indicated that the addition of metal elements or metal-rich organic substrates is an effective approach for improving the performance of AD, and measures should be taken to avoid the inhibition from sodium and potassium.

2.7 Anaerobic co-digestion

Anaerobic co-digestion refers to the simultaneous digestion of two or more substrates. It is regarded as a feasible option to overcome the

drawbacks of mono-digestion and to improve plant's economic feasibility due to higher methane production. In addition, co-digestion allows the treatment of waste that cannot be successfully treated alone, such as wastes rich in lipids or protein content. By supplying missing nutrients, co-digestion of different waste improves the carbon and nutrient balance, thus enhancing the AD process and biogas yields (Parawira et al., 2004; Mata- Alvarez et al., 2000). The treatment of more than one substrate in a single digester was also shown to establish positive synergism (Di Palma et al., 1999).

Research on co-digestion is ongoing and some examples are the co-digestion of OFMSW with sewage sludge (Edelmann et al., 1999; Rintala and Jarvinen, 1996; Batstone et al., 2002) and the 20 centralized co-digestion plants treating manure and industrial organic wastes in Denmark since the late 1980s (Danish Energy Agency, 1995). Anaerobic co-digestion publications have experienced a dramatic increase in the last few years. As shown in Figure 2-7, 50% of the overall papers on co-digestion have been published between 2012 and 2013, whereas 75% of them have been published in the period 2009-2013 (Mata-Alvarez et al., 2014).

AD is a biochemical process and the microorganisms involved require a small yet sufficient amount of nutrients in order to grow their biomass (Metcalf and Eddy 2003). Nitrogen (N) is an important nutrient for the AD process as it enables the bacteria to produce appropriate enzymes for the utilization of carbon (C). A balance between the two elements is required for the optimization of the AD process. Bacteria cannot utilize carbon when C/N ratio is too high and if the C/N ratio is too low, the anaerobic digestion process is inhibited due to high levels of ammonia-nitrogen.

Table 2-3: Improved performance due to co-digestion of FW with other organic substrates (Zhang et al., 2014)

Feedstock	Source of FW	Result of co-digestion	Influencing factor	Reference
FW + CM	Beijing University Canteen, China	Improve methane yield and system stability	High buffering capacity and trace elements supplement	Zhang et al. (2013b)
FW + CM	University Canteen, Northern Spain	Improve biogas production	High buffering capacity from ammonia	Marañón et al. (2012)
FW + CM	Waste Management Company, Northern California	Improve methane yield	Nutrient balance	El-Mashad and Zhang (2010)
FW + CM + card packaging	Household, UK	Allow higher organic loadings and more stable process	Trace elements supplement	Zhang et al. (2012)
FW + piggery wastewater	Restaurant in Myongi University, Korea	Improve biogas productivity and process stability	Trace elements supplement	Zhang et al. (2011)
FW + sewage sludge	Dinning Hall of an Academic Institute, Korea	Allow higher organic loading rate	High buffering capacity from ammonia	Kim et al. (2011)
FW + green waste	Restaurant, National Chiao Tung University, Taiwan	Improve VS reduction	C/N ratio	Kumar et al. (2010)

FW: food waste;

CM: cow manure

Figure 2-11: Evolution of number of papers published with the words co-digestion or co-digestion in its title (Mata-Alvarez et al., 2014).

According to literature search, several authors concluded the optimal C/N ratio for their AD studies to be between 20:1 and 30:1 (Parkin and Owen, 1986; Monnet, 2003; Yen and Brune, 2007). For C/N ratios below 20, an imbalance between C and N requirements for the anaerobic bacterial community will lead to N release in the form of ammonia during the AD process. High levels of ammonia-N are inhibitory to methanogenic bacteria and will result in VFA accumulation within a digester (Ward et al., 2014; Ehimen et al., 2009). Though ammonia-N and VFA are important

intermediates in AD, they can also be detrimental to the AD process when allowed to accumulate.

On the other hand, high C/N ratio will lead to insufficient amounts of total ammonia-N for the cellular needs of the anaerobic microorganisms. It has been shown that a minimum concentration of 50 to 200 mg/L of N as ammonia is essential for the requirements of the bacteria community associated with AD (Parkin and Owen, 1986; Chen et al., 2008).

FW is a promising feedstock for AD due to its high potential for methane production. However, the AD process could be inhibited due to nutrient imbalance (i.e., insufficient trace elements (Zn, Fe, Mo, etc.) and excessive Na or K, C/N ratio outside the optimum range and high concentrations of lipids for the long-term operation of FW single digestion. As shown in Table 2-3, anaerobic co-digestion of FW with other organic wastes improved biogas production and methane yield by improving the buffer capacity caused by higher ammonia from the co-substrates, the optimum C/N ratio in the anaerobic reactor, and the supplement of trace elements.

The C/N ratio of FW was reported to be between 14.7 and 36.4 (Zhang et al., 2007). To overcome the inhibition problems brought about by FW single digestion, several researchers have investigated co-digestion, where FW has been co-digested with other waste streams or biomass such as pig manure, cow manure and sewage sludge (Zhang et al., 2011; Zhang et al., 2013; Li et al., 2010). As shown in Table 2-3, the co-digestion of two substrates may improve C/N ratio and VFA/alkalinity ratios and attenuate unfavourable ratios in a single substrate (Ward et al., 2014).

The selection of co-substrates previously focused on mixing substrates which favor synergisms (positive interactions), i.e. macro- and micronutrient equilibrium, moisture balance, dilute inhibitory compounds, optimize methane production and improve digestate quality. Under these circumstances, $1 + 1 > 2$ may be achieved and co-digestion produced more methane than the addition of the methane produced in both single digestions.

In recent years, the transport cost of the co-substrate from the generation point to the AD plant has also become one of the more important selection criteria. For instance, Hammes et al. (1999) reported that the combined treatment of black water with other types of human-generated solid wastes in thermophilic AD systems provided the possibility of integrating and simplifying domestic waste management as well as producing biogas and residues that can either be used for agricultural purposes or be further treated through processes such as incineration (Hammes et al., 1999).

AD has been applied widely and can be considered as a mature technology. Nonetheless, some drawbacks related to the anaerobic digestion of single substrates (mono-digestion) have been reported. For example, black water/ brown water are characterized by low organic loads and have high N concentrations that may inhibit methanogens. On the other hand, FW has higher C:N ratio, insufficient trace elements and high concentrations of lipids. The anaerobic co-digestion of brown water/ black water and FW could lead to the following advantages:

- Enhancement of the biogas yield due to synergistic effects and therefore financial benefits for the plant operator.
- Easier handling in the digestion process since food waste (mainly solid) are converted into pumpable slurries when mixed with liquid brown/ black water
- Improved NPK ratio by mixing brown water and food waste could enhance the value of the digestate as a fertilizer.

Co-digestion of FW with other substrates could enhance the biodegradation of LCFA as well as the methane yield. In addition, co-digestion could also improve the buffer capacity and result in increased acceptable organic loadings in comparison with single digestion.

2.8 Developments in anaerobic co-digestion of brown water and food waste

Among wastes generated within the household, food waste has the highest organic content, followed by brown/ black water. As discussed earlier in Section 1.1.2, the toilet collection system will affect the composition and concentration of the brown/ black water to be treated. According to literature, the concentration of black water collected from vacuum toilets ranged between 8.7 g-COD/L to 12.3 g-COD/L (de Graaff et al., 2010; Elmitwalli et al., 2006a), while that of brown water was around 38.8 g-COD/L (Wendland et al., 2007). Table 2-4 compares the results of anaerobic digestion/ co-digestion of black water, brown water and food waste, obtained from available literature papers. They demonstrated that the co-digestion of brown/ black water with food waste gave rise to synergistic effects, which could be mainly attributed to increased organic strength of the waste mixture.

According to various sources, the concentration of black water (from vacuum toilets) and food waste mixtures ranged between 12.0 g-COD/L and 19.2 g-COD/L. The concentration of brown water and food waste mixtures is higher and ranged between 31.8 g-COD/L and 66 g-COD/L (Kujawa-Roeleveld et al., 2003) (Kujawa-Roeleveld et al., 2006).

In Table 2-4, all studies that compared the mono-digestion of black water and co-digestion of black water and food waste found that co-digestion led to higher COD removal efficiencies and/ or higher methane production. Kujawa et al. (2005) showed that COD removal efficiency increased from 74% to 82% while Wendland (2007) reported an increase in COD removal efficiency from 61% to 71% and increase in CH₄ production from 10 L/p/d to 27 L/p/d when food waste was added to the digestion of black water. This production rate was similar to that of 26.5 L-CH₄/p/d reported by (Kujawa-Roeleveld et al., 2003), who also added food waste to black water in an accumulation reactor operated at 20°C. In another study by Luostarinen and Rintala (2007), CH₄ production from black water and food waste was 6.7 L/kg-VS as compared to 4.9 L/kg-VS from the AD of black water only.

Among the limited studies on the co-digestion of brown water and food waste, all found that brown water as co-substrate achieved better results as compared to black water. Kujawa et al. (2003) found that when food waste was added to brown water instead of black water, both CH₄ production and COD removal efficiency increased. In an accumulation reactor (1,200 L) operated at 20°C, methanization and CH₄ production increased from 34% to 61% and from 26.5 L-CH₄/p/d to 50.8 L-CH₄/p/d respectively. On top of that, soluble COD removal rate also increased from 74% to 80% (Table 2-5).

Similarly, Elmitwalli et al. (2006a) showed that soluble COD removal rate increased from 60% to 85% and anaerobic biodegradability increased from 81% to 96% when food waste was added to brown water instead of black water. On top of that, hydrolysis constant of 0.11/d and 0.095/day were reported for (food waste + black water) and (food waste + brown water), respectively.

2.8.1 Reactor configuration

As shown in Table 2-4, co-digestion of black water and food waste was experimented in various anaerobic reactors such as the continuously stirred tank reactor (CSTR), the upflow anaerobic sludge bed (UASB), the UASB-septic tank and the accumulation (AC) reactor. These reactors were mostly connected to a vacuum toilet to facilitate the collection of black water (Wendland et al., 2007; de Graaff et al., 2010). According to Kujawa-Roeleveld et al. (2000), high-strength domestic wastewaters that contain faeces, urine and food waste are more suitable to be treated in anaerobic treatment systems without sludge retention such as CSTR and AC systems. The following sections will elaborate more on the three different reactor configurations.

Table 2-4: Summary of literature studies on the anaerobic digestion of black water and/ or food waste

Substrate	Reactor	Reactor Vol. (L)	Temp. (°C)	OLR (gCOD/L/d)	HRT (days)	Total COD RE (%)	VSS RE (%)	Methane production	% Methane	Reference
Black water + Food waste	Accumulation systems	1200	20	-	-	83	-	26.5L/p/d	70	Kujawa-Roeleveld et al. (2003)
Black water	UASB septic tank	200	25	0.42	29	74	-	-	-	Kujawa-Roeleveld et al. (2005)
Black water + Food waste				-	29	82				
Black water + Food waste	Accumulation systems	1220	20	-	20	59	-	-	70	Elmitwalli et al. (2006a)
Black water	UASB septic tank	200	20-25	0.33	27-29	78	-	22L/p/d	66	Kujawa-Roeleveld and Zeeman (2006)
Black water + Food waste	Accumulation systems	1000		0.3	115-150	75	-	31L/p/d	66	
Black water	CSTR	10	37	0.5	20	61±12	51±19	10L/p/d	76	Wendland et al. (2007)
Black water + Food waste				1.0	20	71±13	65±20	27L/p/d	65	
				1.5	15	75±7	69±12	28L/p/d	65	
				2.0	10	50±15	51±21	21L/p/d	65	
Black water	Two-phased UASB septic tank	3-12	20	-	2.9	90	-	4.9L/kg VS	-	Luostarinen and Rintala (2007)
Black water + Food waste				-	2.9-3.4	89	-	6.7L/kg VS		
Black water	Batch test	0.35	35	-	30	-	-	295mL/g VS	79	Georgiadis (2013)

RE: removal efficiency

Table 2-5: Efficiency of anaerobic digestion of black water/ brown water and food waste in different reactor configurations

Reactor	Black water			Black water + Food waste			Brown water + Food waste			References
	Total COD removal	Soluble COD removal	CH ₄ production	Total COD removal	Soluble COD removal	CH ₄ production	Total COD removal	Soluble COD removal	CH ₄ production	
CSTR	61%	N.D.	10 L/p/d	71%	N.D.	27 L/p/d	N.D.	N.D.	N.D.	Wendland et al. (2007)
UASB	75%	56%	10 L/p/d (1.8 m ³ /m ³ BW)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	de Graaff et al. (2010)
UASB_{ST}	74%	N.D.	N.D.	82%	15%	N.D.	N.D.	N.D.	N.D.	Kujawa et al. (2005)
Accumulation Tank	N.D.	N.D.	N.D.	>83%	74%	26.5 L/p/d	>23	80	50.8 L/p/d	Kujawa et al. (2003)

Wendland et al. (2007) demonstrated the AD of black water in CSTR with a volume of 10 L at 37°C for a period of 413 days. The headspace with a volume of 1.5 L was connected to a gas meter that measured the amount of biogas generated by displacement, thus enabling the standard volume of biogas to be calculated continuously. As vacuum toilets were used, a small but high strength flow of about 5 L/cap/day of homogeneous black water was generated. FW collected from a student restaurant contained a mixture of meat, vegetables, salad, pasta, bread and potatoes, and was ground into particles of 2 mm diameter. The homogeneous mixture of FW was then stored at 6°C. The feedstock for co-digestion was 40 g-FW per litre of black water added.

As shown in Table 2-4, the addition of FW in the AD of black water improved the performance of CSTR. At 20 days HRT, total COD removal increased from 61% to 71%, particulate COD removal increased from 53% to 67% and VSS removal increased from 51% to 65%. On top of that, CH₄ yield was enhanced from 10 L-CH₄/cap/day to 27 L-CH₄/cap/day. Wendland et al. (2007) also found that the performance of co-digestion in CSTR was optimized at HRT of 15 days. Total COD removal of 75%, VSS removal of 69% and CH₄ yield of 28 L-CH₄/cap/day were achieved during this time. The same study also showed that COD and VSS removal rates as well as CH₄ yield increased as HRT was decreased from 20 to 15 d. However, as HRT was further decreased to 10 d, the performance of CSTR in terms of COD removal and CH₄ yield dropped.

2.8.3 Upflow anaerobic sludge blanket (UASB) reactors and UASB-septic tanks

According to Lettinga (2001), the most applied high-rate anaerobic system used to treat industrial and domestic sewage is the UASB reactor. The UASB-septic tank is designed for both the accumulation and stabilization of sludge (Elmitwalli et al., 2003). The traditional UASB reactor does not have sufficient reactor space to accumulate the sludge and thus the sludge is removed more regularly. The difference between the UASB-septic tank and

the conventional septic-tank system lies in the upflow mode during the operation of UASB-septic tanks. This improves both the removal of suspended solids and the biological conversion of dissolved organic components (Elmitwalli et al., 2006b). Several research results have shown that the treatment of black water using UASB-septic tanks were very effective, achieving a total COD removal rate of 61% – 78% (Elmitwalli et al., 2003; Elmitwalli et al., 2006b; Wendland et al., 2007; Luostarinen and Rintala, 2005; Kujawa-Roeleveld et al., 2005)

The feasibility of using a UASB reactor for the anaerobic treatment of black water was reported by de Graaff et al. (2010). 78% total COD removal and 10 L-CH₄/p/d were achieved using a UASB reactor with a volume of 63 L/p and HRT of 8.7 days at 25°C. With that amount of CH₄ produced, 56 MJ/p/y of energy was supplied as electricity while 84 MJ/p/y was supplied as heat. In comparison to the CSTR (Wendland et al., 2007), accumulation reactor (Kujawa-Roeleveld et al., 2006) and UASB-septic tank (Kujawa-Roeleveld et al., 2005), the reactor volume of the UASB reactor is more than two times smaller than any of the other reactor systems. The reactor volumes are 140 L/p, 1000 L/p and 200 L/p for CSTR, AC system and UASB-septic tank, respectively. de Graaff et al. (2010) believes that a smaller reactor volume would be an important consideration if a large scale system is to be applied at a location where space might be limited, and hence recommended the use of UASB reactors over the others.

The sludge removal period in a UASB/ UASB-septic tank is the most important parameter in the determination of its HRT (Elmitwalli et al., 2006b), and hence the reactor volume. Thus, the volume of a UASB reactor is dependent on the minimum SRT needed for methanization to occur and stabilization of the sludge (Zeeman and Lettinga, 1999). The sludge bed in the UASB/ UASB-septic tank occupies a maximum of 65% of the reactor height (Elmitwalli et al., 2006b) and 70% – 80% removal efficiencies of particulates in these reactors were reported (Lettinga, 2001). A UASB-septic tank has a long SRT (and hence a larger volume) with a relatively short HRT

because the biomass can be retained by an internal gas/ sludge/ liquid separation system.

Zeeman et al. (2008) compared the treatment of black water using both the UASB and UASB-septic tank operated at 25°C. The UASB had a reactor volume of 50 L, HRT of 8.3 days and was inoculated with 20 L anaerobic sludge. The UASB-septic tank had a reactor volume of 200 L, HRT of 29 days and was not inoculated with sludge. CH₄ production increased from 6 L/d for UASB-septic tank to 14 L/d for UASB reactors.

2.8.4 Accumulation systems

In an accumulation system, influent is being fed continuously without any production of effluent. Instead, the effluent is emptied after the reactor is filled up (every 100 to 200 days), hence resulting in large reactor volumes required (1,000 to 1,200 L). Because of this, it was recommended to use the accumulation reactor only for very concentrated waste such as brown water and food waste so as to minimize the reactor volume. After removing the effluent, a portion of the sludge will be left behind as inoculum for the next cycle. The co-digestion of black water or brown water with food waste in accumulation systems at 20°C have been reported previously (Kujawa-Roeleveld et al., 2003; Elmitwalli et al., 2006a; Kujawa-Roeleveld et al., 2006).

2.8.5 Comparison of the different types of reactors

The removal efficiencies of organic macro-pollutants in domestic wastewater and their methane production rates are dependent on many factors such as operational temperature, HRT, SRT, inoculum source and concentration of waste. Table 2-5 shows that AD of black water in UASB and UASB_{ST} achieved higher COD removal as compared to CSTR. In spite of this, both CSTR and UASB produced 10 L-CH₄/p/d. The performances of UASB_{ST} and accumulation tank were similar for the co-digestion of black water and food waste. Both achieved COD removal of 82% – 83%. On the other hand, both CSTR and AC achieved approximately 27 L-CH₄/p/d even though the COD removal rate for CSTR was lower at 71%. Although the UASB seemed

to be more effective in the conversion of COD into CH₄ as compared to CSTR, and UASB require significantly smaller operational volume as compared to accumulation tanks, the choice of reactor is still dependent on the local situation, especially on the type of toilet used (which determines the concentration of wastewater), space allocation and frequency of emptying the contents.

The CSTR is a continuously fed tank containing a mixture of the treated medium and bacteria that have the same retention times (i.e. HRT equals SRT). Its HRT lies between 15 and 30 days and has a reactor volume of around 10L. The co-digestion of highly concentrated brown water and solid fraction of kitchen refuse will most probably result in surplus energy generated. Since a small volume of effluent is produced, the cost of transporting them to agriculture fields is reduced.

UASB or UASB_{ST} provides high removal efficiency of organic matter as compared to the conventional septic tanks used commonly to treat whole wastewater in the past. In UASB reactors, a dense sludge bed (made up of inert suspended solids from the influent and produced biomass) would form at the bottom and this is where all biological processes occur. Although not complete, a high removal of dissolved and particulate organic matter and total COD is achieved. However, high concentrations of pathogens are not inactivated and effluents require post treatment processes to meet the WHO standards for irrigation or fertilization. The volume of UASB reactor is smaller than that of UASB_{ST} because of shorter SRT. UASB may be preferred over UASB_{ST} reactor because of its long SRT at relatively short HRT (de Graaff et al., 2010), thus reducing reactor volume.

Accumulation reactors are easy to maintain and operate and are suitable for countries with seasonal changes as the reactors can act as a pre-treatment step during the colder months, which will enhance the degradation of organic pollutants during the hotter months (at approximately 25°C). In addition, digested medium treated in accumulation reactors tend to be highly stabilized and most pathogens would be inactivated by the end of the

operational period because of the longer retention times. Hence, effluent may be reused directly for soil conditioning and fertilization without any post treatments. However, due to the long retention times, larger reactor tanks ($1.4 - 1.6 \text{ m}^3/\text{p}$) are required for 6 months storage and this may pose as a problem in space-limited places like Singapore. One way to minimize the volume (to $0.3 - 0.5 \text{ m}^3/\text{p}$) is to use vacuum separation toilets so that highly concentrated media such as brown water and food waste is treated instead. Accumulation systems are recommended for situations where the digested sludge can be applied in nearby areas as fertilizers.

AD of domestic waste streams is of great interest because this process converts oxygen demanding and odourous organic matter to methane, a renewable source of energy. In addition, the sludge produced is high in nitrogen and phosphorus which are valuable fertilizer components. Since the removal efficiency of suspended matter during AD is usually high, the presence of little suspended matter in effluent enables the use of small bore sewer system for transportation to post-treatments systems. This helps to reduce the construction and maintenance costs of the collection system by 30 – 65% (Ahring and Angelidaki, 1992).

Studies on the co-digestion of black water (wastewater containing feces and urine, collected from vacuum toilets) and food waste showed positive synergistic effects of co-digestion in the form of increased methane production and COD removal rates (Wendland et al., 2007; Kujawa and Zeeman, 2006). When food waste was added to brown water (wastewater containing feces only) instead of black water, both methanization and methane production further increased from 34% to 61% and from 26.5 to 50.8L $\text{CH}_4/\text{p}/\text{d}$, respectively (Kujawa et al., 2003). Other than being more diluted, black water also contains high amounts of ammonia, due to the presence of urine, which might inhibit the AD process. Hence, the co-digestion of brown water and food waste would be preferred for more efficient AD processes.

2.9 Two-phase anaerobic digestion process

In conventional applications, AD processes usually occur in a single reactor system. However, some of such systems gradually gave rise to problems related to reactor instability because the acid-forming and methane-forming microorganisms, which differ widely in terms of physiology, nutritional needs and sensitivity to environmental conditions, were kept together in the same reactor system (Ghosh et al., 1985). The physical separation of acid-formers and methane-formers in two separate reactors was first proposed by Pohland and Ghosh (1971). Such systems provided optimum environmental conditions for each group of organisms and thus led to enhanced stability and control of the overall process. As shown in Figure 2-12, two-phase AD systems employ separate reactors for acidogenesis and methanogenesis. These two reactors are connected in series to allow each phase to be optimized independently since the microorganisms concerned have different nutritional requirements, pH optima, growth and nutrient uptake kinetics and tolerances to environmental stress factors. The advantages of two-phase AD systems include:

- Improvement in process control
- Disposal of excess fast growing acidogenic sludge without any loss of slow growing methanogens
- Degradation and attenuation of toxic materials in the first phase (protects the sensitive methanogens)
- Precise pH-control in each reactor
- Higher CH₄ content in biogas from methanogenic phase
- Increased loading rate possible for methanogenic stage
- Balancing tanks in existing treatment plants might be readily converted to acidification tanks

The disadvantages of two-phase AD systems include:

- Possible disruption of syntrophic relationships
- High sludge accumulation in the first phase

- Lack of process experience and therefore more difficult to operate
- Difficulty in maintaining a balanced segregation of the phases

The advantages of phase separation were reported in several studies. Yeoh (1997) showed that the bench-scale two-phase study on soft-drink waste showed higher methane production and COD removal efficiency attained at a shorter HRT and higher loadings with respect to conventional single-phase high-rate digestion (Yeoh, 1997). In another study by Fadel (2001), the laboratory-scale two-phase system treatment of cane-molasses alcohol stillage achieved COD reduction of more than 65% and produced a methane yield three times higher than conventional single-phase systems (Fadel, 2001).

Lipid degradation was also found to be higher in a two-phase AD process as compared to a corresponding single-phase system. In addition, the acid reactor containing unsaturated long-chain fatty acids was found to prevent lipid inhibition in a two-phase AD process (Hanaki et al, 1990; Jeyaseelan and Matsuo, 1995; Komatsu et al., 1991).

Figure 2-12: Schematic of two-phase anaerobic digestion process (Marmara Universitesi)

The type of end products depends on the conditions in the reactor medium. If H_2 is effectively removed by H_2 scavenging organisms such as methanogens, acetate will be the main end product in the methanogenesis reactor. However, if methanogenesis is inhibited and H_2 accumulates, more reduced products such as propionate and butyrate are likely to remain in the methanogenesis reactor. Therefore, reactors designed as acidifying reactors in an anaerobic two-step process often contain these reduced intermediate products (Henze, 2008).

Currently, most (95%) of the European full-scale plants are operated as single-stage systems. However, Zhang et al. (2014) compared the criteria for one-stage versus two-stage AD plants and showed that AD plants treating high solid content feedstocks, e.g. FW are more suitable for two-phase systems (Table 2-6).

Table 2-6: Comparison of different digester configurations for high solid content feedstock (Zhang et al., 2014)

Criteria	One-stage versus two-stage AD		Batch vs continuous AD	
	One-stage	Two-stage	Batch	Continuous
Biogas production	Irregular and discontinuous	Higher and stable	Irregular and discontinuous	Higher and stable
Solid content (%)	10 – 40	2 – 40	25 – 40	2 – 15
Relative cost	Low	High	Low	High
VS destruction	Low to high	High	40 – 70%	40 – 75%
HRT (days)	10 – 60	10 – 15	30 – 60	30 – 60
OLR (kg VS/m ³ day)	0.7 – 15	10 – 15 in second stage	12 – 15	0.7 – 1.4

2.10 Molecular biology methods in anaerobic digestion

The anaerobic wastewater treatment technology did not take off for decades since its introduction in the 1860s and the biological reactor has been considered as a “black box” due to the lack of appropriate microbiological techniques. Identification of microorganisms by conventional microbiological techniques, such as plate count or Most-Probable Number (MPN) counting (Nielsen et al., 2009), require the isolation of pure cultures followed by laborious experiments for characterizing their physiological, biochemical, and morphological properties (Raoa et al., 2000).

These culture-dependent methods suffer from severe limitations that may lead to serious misinformation. First, only a small fraction of microorganisms may be cultivable, as the artificial growth media may not be a good simulation of the environment in the anaerobic reactors. Second, many microorganisms require syntrophic interactions with others, and thus cannot be cultured individually (Wagner et al., 1993). Third, some microorganisms share similar physiological, biochemical and/ or morphological characteristics, and thus cannot be distinguished from one another accordingly. As a result, many estimated that no more than 1% of the existing bacteria had been isolated and characterized (Amann et al., 1995), hence unable to reflect the composition and diversity of a microbial community. According to DSMZ (2012), about 7000 bacterial species have been described, which is several orders of magnitude lower than the real number (Amann et al., 1995).

Molecular biology techniques, based on the RNA of the small ribosomal subunit (16S rRNA for prokaryotes), allow the identification of specific populations of microorganisms in their native habitat without the need to isolate them. The development of molecular methods led to insights in microbial species involved in anaerobic degradation which aided in the optimization of new generation reactors to maintain reactor stability and maximize methane production.

Following great advances in the recent years, nucleic acid-based molecular methods are able to identify microorganisms by the DNA

sequences of their genes, e.g., ribosomal RNA (rRNA) genes, which are used as biomarkers. With application of nucleic acid-based molecular techniques, it has been found that microbial communities in the anaerobic process are highly diverse, and the interactions among different species and regulation of metabolic functions mostly remain unknown. As mentioned before, the anaerobic degradation of organic matter is carried out syntrophically by microbial communities consisting of both *Bacteria* and *Archaea* species.

Nucleic acid-based molecular methods are better choices for the analysis of microbial communities in anaerobic reactors. Common nucleic acid-based methods include cloning and sequencing for characterization, quantitative real-time polymerase chain (qRT-PCR) for quantification, fluorescence *in situ* hybridization (FISH) for visualization, denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP) for screening, typing, monitoring, and stable isotope probing (SIP) and microautoradiography-FISH to link the microbial identity with the specific functions (Fang, 2011). This review will be limited to the discussion of three techniques most widely used for the characterization of microbial communities in anaerobic digesters – cloning of 16S rDNA, denaturant gradient gel electrophoresis (DGGE) and Fluorescent *in situ* hybridization (FISH).

2.10.1 Cloning of 16S rRNA and construction of gene clone library

The cloning and sequencing of the gene coding for 16S rRNA is the most widely used molecular technique in the field of microbial ecology. The cloning procedure for studying a microbial community is outlined in Figure 2-13 (Sanz and Köchling, 2007). The work cycle is as follows: (A) direct nucleic acid extraction, without the need for previous isolation of microorganisms; (B) amplification of the genes that code for 16S rRNA by polymerase chain reaction (PCR), commonly using universal primers for bacteria or archaea, resulting in a mixture of rDNA copies corresponding to the microorganisms present in the sample; (C) cloning of the PCR products obtained into a suitable high copy number plasmid and transformation of competent *E. coli*

cells with this vector; (D) selection of transformed clones with an indicator contained in the plasmid; (E) extraction of plasmid DNA; (F) sequencing of the cloned gene, creating a clone library; (G) determination of the phylogenetic affiliation of the cloned sequence with the help of dedicated computer programs.

Cloning and the creation of rRNA gene libraries can identify an extensive share of the microbial species living in a sample and yield far more exact phylogenetic information than other molecular techniques such as DGGE and FISH. But cloning techniques are less popular in research of AD because such a complete picture of a bacterial habitat is extremely time consuming and laborious to obtain.

Cloning is also commonly used as a tool for designing new specific primers and gene probes for detection and/ or quantification of microorganisms. Crocetti et al. (2000) extracted genomic microbial DNA from a SBR, cloned the bacterial 16S rDNA and identified *Rhodocyclus* sp. And *Propionibacter pelophilus* as the microorganisms responsible for the polyphosphate accumulation taking place in the reactor. The authors then designed probes for these species that could establish a correlation between phosphorous removal and the number of hybridized cells in different sludges. Beer et al. (2004) designed new probes for *in situ* hybridization with information provided by 16S rDNA gene library sequences and DGGE analysis.

2.10.2 Denaturant Gradient Gel Electrophoresis (DGGE)

DGGE is based on the mobility of different nucleic acid sequences on a gel of denatured DNA-fragments. Band patterns directly reflecting the genetic biodiversity of the sample would be generated and the number of bands corresponds to the number of dominant species. Coupled with sequencing and phylogenetic analysis of the bands, this method can give a good overview of the composition of a given microbial community.

As shown in Figure 2-14, the work cycle is as follows: (A) DNA is extracted from the original sample, in this case as granular sludge from a UASB reactor; (B) the 16S rRNA gene is partially amplified by PCR usually with universal primers to give a mixture of DNA fragments, all of the same length. Each of the different product DNA molecules resulting from this step represents a different microorganism; (C) the DNA mixture is then separated by denaturant gradient electrophoresis on an acrylamide gel with an increasing urea/formamide gradient. The DNA molecules migrate towards the positive pole and come to a halt on the gel on reaching their corresponding denaturant force I , which depends on the DNA sequence. Every band on the gel corresponds to a different microorganism in the sample, providing sufficient information for many requirements; (D) the bands can be cut from the gel and the DNA extracted and sequenced; I comparison of the sequences with a 16S rDNA database helps to determine the phylogenetic affiliation of the microorganism. The most important application of DGGE is monitoring dynamic changes in microbial communities, especially when many samples have to be processed.

Figure 2-13: Outline of the cloning procedure for studying a microbial community (Sanz and Köchling, 2007)

Figure 2-14: Schematic representation of DGGE (Sanj and Köchling, 2007).

As compared to cloning, DGGE allows rapid and simple analysis of a large number of samples to obtain an overview of the dominant species of an ecosystem. However, as the sequences of the bands obtained from a gel correspond to short DNA fragments (200-600 bp), they are less useful for designing new specific primers and probes as compared to the cloning of the whole 16S rRNA gene.

2.10.3 Fluorescence *in situ* Hybridization (FISH)

FISH is a visualization technique based on microscopic examination of a given species or groups of bacteria after staining cells using specific fluorogenic oligonucleotide probes which bind RNA molecules in the cells (Amann et al., 1990). This technique has become the method of choice for reliable and rapid identification of microorganisms in environmental samples since DeLong et al. (1989) reported the suitability of fluorescently labeled rRNA-targeted oligonucleotide probes as phylogenetic stains for cultivation-independent identification of microorganisms.

As a method without DNA extraction and PCR, FISH is an excellent means to overcome problems associated with PCR-based molecular methods, such as DGGE, T-RFLP, cloning, and sequencing. FISH probes are

short sequences of DNA (about 16-20 nucleotides) labeled with one or two fluorescent dyes. The probe may specifically hybridize *in situ* with 16S rRNA, 23S rRNA, or mRNA in cells according to DNA-RNA complementary matching. A list of existing probes commonly used in anaerobic studies is available at ProbeBase (2012) while ARB/ SILVA is a good tool to perform online analysis and design when designing new probes (2012).

As shown in Figure 2-15, the *in situ* hybridization process can be divided into four stages: (A) sampling and immediate fixation in formaldehyde to preserve the integrity of the cells, especially the ribosomes; (B) hybridization with a specific probe, labeled with a fluorescent dye at its 5' –end and matched with a sequence of the 16S rRNA; (C) counterstaining with a universal marker (DAPI, which attaches non-specifically to DNA molecules) or another more general probe, labeled with a different fluorescent dye; (D) visualization via fluorescence microscopy.

Direct quantification is possible by manual counting of hybridized cells (epifluorescence and laser confocal microscope) or by image analysis of digital photos (both microscopes) or automated counting with a flow cytometer. The ratio of cells that have hybridized with the specific probe to the total cell count with DAPI or a more general probe gives the percentage of bacteria present in the sample, based on the total number of cells or on the group that was chosen with the general probe (e.g. bacteria or archaea).

Other than visualization, FISH is also commonly used for microbial quantification, by either determining the fluorescence-emitting area or counting the number of individual cells. However, the latter is only suitable for homogeneous and evenly distributed microbial samples. The bacteria count per region of the microscopic grid should lie between 30 and 150. Between 10 and 20 regions should be counted to ensure statistically significant cell counts. Non-ideal samples and fluorescent background (a common phenomenon with environmental and sludge samples) can make cell counting by fluorescence microscopy a tedious and time consuming process that can

be influenced by the judgment of the operator and his or her experience (Sanz and Köchling, 2007).

FISH is an easy and fast technique that allows the direct visualization of non-cultured microorganisms. In contrast to conventional techniques, such as most probable number and plate counts, or other molecular techniques, quantification of specific microbial groups is possible with FISH. However, the quantification process can be tedious and subjective for manual counting, or complex and expensive for image analysis. Furthermore, some previous knowledge of the expected microorganisms in the sample is often required to apply FISH successfully. To target a particular species, a specific probe must be ready or its 16S rRNA sequence must be available.

2.10.4 Comparison between gene library, DGGE and FISH

Table 2-8 summarizes the molecular techniques and their possible fields of application within the area of AD. Lapara et al. (2000) pointed out that both PCR-derived methods (DGGE and cloning) can result in the detection of similar diversity patterns of bacterial populations in an ecosystem, but they do not give a valid estimate of the relative species distribution. This suggests one or both methods are not suitable for determining total biodiversity, a parameter that depends on both the number of different species and their relative abundance and distribution.

Figure 2-15: Schematic representation of FISH (Sanz and Köchling, 2007).

In situ hybridization with fluorescently labeled probes can overcome the problems associated with PCR-based methods: localization of the microorganisms to be examined *in situ* (topology) and its quantification. The techniques are not mutually exclusive and should be considered complementary. In an ideal situation, the methods required for a given task are used simultaneously.

Table 2-7: Comparison of the main molecular biology techniques and their application in bioreactors for anaerobic digestion (Sanz and Köchling, 2007)

	Gene library	DGGE	FISH
Required skill level in molecular biology	High	Medium	Low
Specific facilities	PCR	PCR, DGGE	Fluorescence microscope
Requires sequencing	Yes	Yes (conditional, band pattern could be sufficient)	No
Quantitative	No	No	Yes
Time demands	High	Medium (low if sequences are not required)	Low
Phylogenetic precision	High	Medium	Low/medium
Structural analysis (3D-architecture)	No	No	Yes (requires confocal laser microscopy)
Sample throughput	Low	High	High
Apt for monitoring bioreactors	No	No/yes (only if band pattern shows obvious differences)	Yes, easy to standardize process for indicator species
Applicability in AD	Low	Medium	High

PCR: polymerase chain reaction; DGGE: denaturant gradient gel electrophoresis;

FISH: fluorescent *in situ* hybridization

Chapter 3

3. Materials and Methodology

3.1 Substrate and inoculum

The feed for the reactors in this study consisted of a mixture of food waste (FW) and brown water (BW) in the ratio of 150 g/ 2L. FW was collected once in a week from one of the canteens at NTU campus, where the majority of the waste came from Chinese, Indian, Indonesian and Malay food stalls. It was a mixture of meat, rice, noodles, vegetables and salad. After removing bones and non-food materials, the FW was then blended into a slurry form by a food blender to promote homogeneity of the substrate as well as disintegration of particulate organics. The blended FW was then mixed well, and stored in a refrigerator at 4°C. BW refers to fecal waste without urine. It was collected from a specially designed source-separation toilet located in our laboratory, where urine and feces was collected in separate tanks. BW used in this study was mixed with 2 L of flush water, collected once in a week and stored in a refrigerator at 4°C. Inoculation for the anaerobic digestion studies used seed sludge collected from an anaerobic digester at the Ulu Pandan sewage treatment plant, Singapore. Before use, the sludge was stored at 35°C for one week to remove any remaining biodegradable fraction of the sludge and was sieved through a 1 mm mesh to remove any large particulates.

3.2 Analytical procedures

pH values were measured using a compact titrator (Mettler Toledo) equipped with a pH probe (Mettler Toledo Dgi 115-SC). Alkalinity, total (TS) and volatile (VS) solids were analyzed according to the Standard Methods (APHA, 1998). Total and soluble chemical oxygen demand (COD) measurements were made using COD digestion vials (Hach Chemical) and a spectrophotometer (DR/2800, Hach). Soluble COD measurements were made using the supernatant of samples after centrifugation (KUBOTA 3700, Japan) at 10,000 rpm for 15 min. The determination of volatile fatty acids (VFAs) was carried out using a gas chromatograph (Agilent Technologies

7890A, USA), equipped with a flame ionization detector (FID) and a DB-FFAP (Agilent Technologies, USA) column (30 m x 0.32 mm x 0.50 μ m) and the samples were filtered through Membrane Solutions 0.45 μ m cellulose acetate membrane filters.

Total biogas production was measured using the following methods. In Chapter 4, methane production was monitored online periodically using the Automatic Methane Potential Test System (AMPTS) (Bioprocess Control, Sweden). Total biogas production for Chapters 5 and 6 was monitored daily using a mass flow meter (McMillan Company, Model 50D-3E). The batch study in Chapter 7 used the syringe method to measure biogas production. For Chapter 8, biogas was collected and stored in a Tedlar gas sampling bag (Sigma-Aldrich, USA) and its volume was monitored daily using a rotary displacement meter.

The composition of biogas (i.e., methane, carbon dioxide and nitrogen contents) was analyzed by gas chromatograph (Agilent Technologies 7890 A, USA) equipped with a thermal conductivity detector (TCD). Carbon, hydrogen, nitrogen and sulfur were measured using an elemental analyzer (Vario EL Cube), and aqueous TOC and IC was measured using a TOC analyzer (TOC-V CSH, Shimadzu, Japan).

3.3 Calculation

For batch studies in Chapters 4 and 7, methane production was determined by subtracting the methane produced by inoculum from that produced in the reactors containing substrate and inoculum. Methane yield refers to the amount of methane produced per unit of volatile solids contained in the feedstock. Cumulative methane yield was calculated as the sum of methane produced throughout the study period and expressed as litres per gram of VS of substrate fed to the reactors. The composition of each SCFA was calculated by taking the ratio of individual SCFA concentration over that of TVFA.

3.4 DNA extraction and PCR purification

Genomic DNA from sludge samples was extracted using chemical lysis and phenol-chloroform-isoamyl alcohol (25:24:1, v:v:v) purification protocol (Amann et al., 1995). The forward primers used to amplify 16S rRNA gene from the total-community DNA, targeting total prokaryotes, Bacteria and Archaea were forward primers 530F (5'-GTGCCAGC(A/C)GCCGCGG-3'), 8F (5'-AGAGTTTGATYMTGGCTC-3') and Ar1F (5'-TCYGKTTGATCCYGSCRGAG-3'), respectively. The reverse primer used was 1490R (5'-GGTTACCTTGTACGACTT-3'). The thermal program used for amplification of 16S rRNA gene was as follows: hotstart 94°C for 3 min, 30 cycles of denaturation (30 s at 94°C), annealing (30 s at 54°C) and extension (45 s at 72°C) and a final extension at 72°C for 5 min.

3.5 Quantification of purified PCR products

Following PCR purification, an aliquot of the DNA was run on an agarose gel alongside a DNA ladder to confirm that a purified 16S rDNA product was recovered successfully. After a DNA band of the expected size was observed, a UV/ visible spectrophotometer known as a NanoDrop (Thermo Scientific) was used to quantify the purified PCR products. Optical density (OD) measurements at wavelengths of 260 and 280 nm were measured by the NanoDrop to determine an appropriate dilution of the DNA sample. One absorbance unit at a wavelength (λ) of 260 nm is equal to a DNA concentration of 50 $\mu\text{g/ml}$. The DNA concentration was therefore determined through OD_{260} with the following equation:

$$\frac{50 \mu\text{g/ml}}{1 \text{ Å}} = \frac{x \mu\text{g/ml}}{\text{Measured } \text{OD}_{260}}$$

DNA purity was calculated by dividing the OD_{260} by the OD_{280} . For pure DNA, the $\text{OD}_{260}/\text{OD}_{280}$ ratio should be between 1.7 and 1.8. Ratios lower than 1.7 indicate protein contamination in the sample and ratios higher than 1.8 indicate RNA contamination. A ratio of 2 indicates pure RNA (Held, 2001).

3.6 Construction of 16S rRNA gene clone libraries

TOPO TA cloning kit (Invitrogen, CA) was used for clone library construction according to the manufacturer's instructions. In Chapter 6, approximately 100 and 50 clones were randomly selected from R_A , R_M and R_S for the members in the domain Bacteria (amplified by primer set 530F and 1490R), and Archaea (amplified by primer set Ar1F and 1490R), respectively. In Chapter 8, 100 clones were randomly selected for members in the domain Bacteria (amplified by primer set 8F and 1490R). The amplified DNA insert was then PCR amplified with a vector-specific primer set (i.e., M13F and M13R). The sequence for M13F was 5'-GTA AAA CGA CGG CCA G-3' and that of M13R was 5'-CAG GAA ACA GCT ATG AC-3'. Restriction fragment length polymorphism (RFLP) was used to screen the 16S rRNA gene fragments to further remove the possible redundant clones. The M13-PCR products were separately digested to completion with tetramer restriction enzymes *MspI* and *RsaI* (New England BioLabs, UK), and separated by electrophoresis in a 3% agarose gel. Gels were visualised using the FireReader gel documentation (UVItec, Cambridge, UK) after staining with Gelred (Invitrogen, CA). Unique RFLP patterns were defined as a unique sequence type of operational taxonomy unit (OTU).

3.7 Sequence analysis

The 16S rRNA gene of the representative clones with different RFLP patterns were sequenced, by Axil Scientific Sequencing (Singapore), to determine their phylogenetic affiliation. Nearly full-length 16S rRNA gene sequences of representative clones were compared to available rRNA gene sequences in GenBank using the NCBI BLAST program. Chimeric artifacts were determined using DECIPHER (Wright *et al.*, 2012) and phylogenetic trees were constructed with MEGA5 program using the remaining clone sequences after removing the chimeric sequences. The Jukes-Cantor correction was used for distance matrix analyses and the trees were constructed using the Neighbor-joining method. Archaeal and bacterial 16S rRNA partial sequences obtained for Chapter 6 were deposited in the nucleotide Genbank database, under the accession numbers: KF169842 –

KF169904. The bacterial 16S rRNA partial sequences for Chapter 8 were deposited in the nucleotide Genbank database, under the accession numbers: KJ907449 – KJ907466.

3.8 Fluorescence in situ hybridization (FISH)

In Chapter 6, sludge samples from R_A, R_M and R_S were collected towards the end of the operational period (on day 150) for FISH analyses. The sludge samples were pretreated according to the protocol by Amann et al. (1995), and fixed overnight with 4% paraformaldehyde solution at 4°C. Hybridization was carried out at 46°C for 3 hours with hybridization buffer containing 5 ng μL^{-1} of specific fluorescent probe. Two oligonucleotide probes, EUBmix (i.e., EUB338, EUB338-II, EUB338-III) and ARC915, were used to target the members of Bacteria and Archaea, respectively (Amann et al., 1995; Daims et al., 1999). The sequence for EUBmix was 5'-GCT GCC TCC CGT AGG AGT-3' and that of ARC915 was 5'-GTG CTC CCC CGC CAA TTC CT-3'. FISH hybridization was performed with 35% formamide concentration for both probes (EUBmix and ARC915) in the hybridization buffer. An Olympus BX53 epifluorescence microscope equipped with a cooled CCD camera DP72 with a 100 W halogen bulb and fluorescence filter sets (U-FGW and U-FF-Cy5) under $\times 100$ objective lens (Olympus, Japan) was used to capture FISH-stained images.

Chapter 4

4. Characteristics and bio-chemical methane potential of brown water and food waste

Publication: Rajagopal, R., Lim, J. W., Mao, Y., Chen, C.-L., Wang, J.-Y. (2013). Anaerobic co-digestion of source segregated brown water (feces-without-urine) and food waste: For Singapore context. *Science of the Total Environment* 443, 877-886.

4.1 Introduction

As discussed earlier in Section 1.1.2, the decentralized sanitation system focuses on source separation of wastewater flows and organic waste on a household level, followed by appropriate treatments of each stream in decentralized or semi-centralized systems, and consequent reuse of water and nutrients (previously shown in Figure 1-1). Among the different wastewaters, brown water (BW) is the main source of water pollution in terms of organic matter and pathogens. Another source of waste high in organic content is food waste (FW). Since BW and FW are high in organic matter, they are ideal substrates for anaerobic digestion – a process known to be highly efficient in harnessing the untapped renewable energy potential of organic waste by converting the biodegradable fraction of biomass into high calorific gases, producing energy from biomass.

As shown in Tables 2-4 and 2-5, the addition of FW to black/ brown water demonstrated positive synergistic effects during their anaerobic digestion. The positive synergistic effect was observed in terms of increased methane production and increased removal efficiency of total COD (Kujawa et al., 2003; Kujawa et al., 2005; Kujawa and Zeeman, 2006; Wendland et al., 2007).

According to a life cycle assessment (LCA) study conducted in Singapore, the amount of FW generated by one individual was estimated to be 150 g per person per day, while that of BW was approximately 2 L per person per day. This is similar to another LCA report by Remy (2010) which suggested a generation of 160 g of FW per person per day. This preliminary study would

investigate the synergy between BW and FW in terms of CH₄ production; and subsequently compared with that of FW and BW as a sole substrate. The objective of this batch study was to evaluate the technical feasibility of anaerobic co-digestion of BW and FW by comparing the biochemical methane potential of BW, FW and the 150g-FW/2L-BW mixture, over a period of 30 days. The characterization of BW, FW and their mixture in terms of pH, alkalinity, TS, VS, COD and VFA was also carried out. Other than determining the biodegradability of substrates, the results for this study would serve as a useful reference for future larger-scale studies.

4.2 Experimental set-up

Batch reactors were operated using the Automatic Methane Potential Test System (AMPTS) (Bioprocess Control, Sweden) with a total volume of 0.5 L and working volume of 0.4 L. Three series of experiments were conducted in parallel with different substrates: (a) BW, (b) FW and (c) 150g-FW/ 2L-BW. All sets of experiments were topped up with tap water to achieve an organic load of 1.5g/L, and then seeded with sludge in a ratio of 50:50 (v/v). The contents in the digesters were flushed with N₂ to provide anaerobic conditions and incubated at 35°C with intermittent mixing (mixing time: 10 min ON/ 10 min OFF) at 100 rpm using an overhead mechanical stirrer. The digesters were fed once at the beginning, with the methane production and its flow rate being monitored on-line with real-time data logging throughout the study of 30 days. All experiments were conducted in duplicates. The contents in the digesters were characterized at the beginning and end of the study. The contents were measured for their pH, TS, VS, VFA and COD.

4.3 Results and discussion

4.3.1 Characteristics of BW, FW and their mixture

The characterization of sludge, BW, FW and 150g-FW/2L-BW mixture are shown in Table 4-1. Due to the complex nature of FW, its characteristics could be dependent on the geographical location of analysis carried out.

Table 4-1: Characteristics of starting material for batch study

Parameter	Units	Sludge	Brown Water (BW)	Food waste (FW)	150g-FW/2L-BW
pH	-	6.90 ± 0.06	7.23 ± 0.04	4.40 ± 0.15	6.46 ± 0.15
Alkalinity	[mg CaCO ₃ /L]	-	837 ± 3	774 ± 1	746 ± 64
TS	[g/kg]	17.83 ± 0.04	14.1 ± 0.2	295.0 ± 1.5	14.8 ± 0.3
VS	[g/kg]	12.52 ± 0.05	9.8 ± 0.2	280.0 ± 1.5	10.9 ± 0.3
VS/TS	[%]	70	70	95	74
Total COD	[g COD /L]	14.71 ± 0.07	15.68 ± 0.28	394 ± 14	16.40 ± 0.45
Soluble COD	[g COD /L]	-	0.65 ± 0.13	13.02 ± 4.36	7.16 ± 0.20
VFA-COD	[g COD /L]	-	0.46 ± 0.07	-	0.98 ± 0.03
TOC	[g/kg]	-	417.27 ± 6.24	402.47 ± 5.56	-
TN	[g/kg]	-	7.19 ± 2.15	1.50 ± 1.02	-
C	%	41.44 ± 1.40	-	-	45.03 ± 0.59
N	%	3.29 ± 0.08	-	-	3.93 ± 0.20

Nevertheless the characteristics of FW used in this study are similar to those available in the literature (Table 2-1).

The co-substrate brown water contains bacteria, digestion liquids and enterocytes. Approximately one-third of faeces consists of food remains, one-third is intestinal bacteria and one-third is from the intestine itself (enterocytes and liquids). As a result, the composition of faeces does not vary much in relation to the eating pattern (Roeleveld, 2001). The weight of faeces is basically determined by non-biodegradable fibre (for example, from bread and other grain products, vegetables, potatoes and fruits). According to various researchers (Table 4-2), the dry weight of faeces can vary between 70 and 200 g/person/day (g/p/d) while the average frequency of excretion oscillates around 1 time/person/day (1/p/d).

According to Gaillard (2002), the concentration of raw faeces was around 550 g-COD/L, and that of brown water collected from a vacuum toilet was 90 g-COD/L. In this study, brown water was collected from a separation toilet using 2L flushing water, and had an average concentration of 16 g-COD/L.

Table 4-2: Total and dry weight of faeces and the average frequency of passing stools

Reference	Total weight of faeces (g/p/d)	Dry weight of faeces (g/p/d)	Frequency (1/p/d)
Bingham (1979)	70-140	19-38	NA
Cummings et al. (1992)	106	NA	NA
Glatz and Katan (1993)	170	44.2	1.2
Cummings et al. (1996)	138	34	0.9
Belderok et al. (1987)	100-200	NA	NA

NA: not available

4.3.2 Biochemical methane potential

The biochemical methane potentials of BW, FW and the 150 g-FW/2L-BW mixture were evaluated in batch tests over 30 days. Each of the substrates were mixed with tap water to 1.5gVS/L and then seeded with inoculum. All the experiments performed well without long start up times or inhibition phenomena and the final methane yields of BW, FW and 150g-FW/2L-BW were 0.30, 0.42 and 0.77L-CH₄/g-VS fed, respectively (Figure 4-1). It was also observed that 95% of the final methane yields in all experiments were attained within the first 15 days of the study.

On top of higher methane yield, the co-digestion of BW and FW also led to higher removal efficiencies for TS, VS, total COD, soluble COD and VFA. The TS removal efficiencies for BW, FW and 150g-FW/2L-BW were 8%, 29% and 24%, respectively and their VS removal efficiencies were 10%, 38% and 32%, respectively. The total COD removal efficiencies for BW, FW and 150g-FW/2L-BW were 7%, 18% and 21%, respectively and their soluble COD removal efficiencies were 39%, 62% and 95%, respectively. VFA levels measured at the end of the batch study were 369 ± 43, 460 ± 24 and 298 ± 81 mg-COD/L for BW, FW and 150g-FW/2L-BW, respectively.

As shown in Table 4-1, BW is of lower organic strength as compared to FW. Since the amount of biodegradable organic matter in FW was higher than that in BW, the biochemical methane potential of FW was expected to be higher than that of BW. As shown in Figure 4-1, the methane yield for FW was 29% higher than that of BW.

The benefits of adding FW as co-substrate was clearly illustrated by the more than 2-fold increase in methane yield and approximately 3-fold increase in TS, VS, total COD and soluble COD removal efficiencies observed for 150g-FW/2L-BW as compared to BW. The addition of FW as co-substrate not only reduced the start-up time (Figure 4-1), but also improved both the methane production rate and final methane yield.

Figure 4-1: Methane yield for –●– BW, –○– FW, and –■– 150-gFW/2L-BW during their biochemical methane potential batch studies

This is similar to a previous study where the addition of FW increased the methane yield of black water, collected from vacuum toilets. Kujawa-Roeleveld and Zeeman (2006) also showed that methane yield increased from 240 to 270 L-CH₄/kg-COD when FW was added as co-substrate.

4.3.3 Hydrolysis and acidification

The hydrolysate of the 150g-FW/2L-BW mixture was analyzed on days 1, 2 and 4 of the batch study. As shown in Figure 4-2, the soluble COD level almost doubled after 1-day of fermentation. After which, the soluble COD level for 150g-FW/2L-BW mixture did not differ significantly for the next 3 days. On the other hand, VFA levels increased 4-, 7- and 5-folds after 1-, 2- and 4- day of fermentation, respectively (Figure 4-2).

During the AD process, influent COD are being converted to CH₄-COD, effluent COD and sludge or biomass COD. Effluent COD can be differentiated into VFA and non VFA-COD, where VFA-COD is the intermediate COD during the conversion of influent COD to CH₄-COD.

Figure 4-2: Characteristics of 150g-FW/2L-BW hydrolysate

As shown in Figure 4-2, soluble COD levels did not change significantly between days 1 and 4 of the study. However, VFA-COD levels increased by 4- to 7-folds during the same period. Non VFA-COD accounted for about 80% of soluble COD at the start of the study and it dropped to 50, 24 and 35% after days 1, 2 and 4 of the study, respectively. While significant soluble COD production did not take place, significant conversion of non VFA-COD to VFA-COD occurred during the first 4 days of this study.

4.3.4 Composition of VFA

In this study, SCFAs i.e. acetate (C2), propionate (C3), butyrate (C4), iso-butyrate (iC4), valerate (C5), iso-valerate (iC5), caproate (C6) and heptanoate (C7) were analyzed. As shown in Figure 4-3, butyrate accounted for more than 50% of TVFA on days 1, 2 and 4 of the study. Acetate, which is the direct source of food for methanogens, represented only around 20%, while propionate accounted for about 5% of TVFA during the first 4 days of the study. The dominance of acetate and butyrate during fermentation in anaerobic digestion of FW was also reported by Xu et al., (2014a). Butyric acid and acetic acid are known to be better precursors of methane production, especially acetic acid. According to Xu et al. (2014a), conversion of organic

matters into acetic acid and butyric acid in the hydrolytic-acidogenic stage will improve the overall energy yield of the anaerobic digestion process.

Figure 4-3: VFA composition of 150g-FW/2L-BW hydrolysate, where C-2 (acetic acid); C3 (propionic acid); C4 (butyric acid); C5 (valeric acid); C-6 (caproic acid); C-7 (heptanoic acid)

4.4 Conclusion

Other than the 2-fold increase in methane yield, the addition of FW to the anaerobic digestion of BW also led to about 3-fold increase in TS, VS, total COD and soluble COD removal efficiencies. Although the batch study was conducted over 30 days, 95% of the final methane yields in all experiments were attained by day-10 of the study. The VFA composition analysis showed that butyrate accounted for more than 50% while that of acetate was only around 20% of TVFA in the hydrolysate during the first four days of the study. Other than improving the potential of energy recovery, the addition of FW as a co-substrate would also help to raise the current FW recycling rate of 12% in Singapore.

This study showed that the co-digestion experiment achieved shorter lag time, higher removal efficiencies and higher methane potential as compared

to the mono-digestion experiments. These indicate the complementary characteristics of co-substrates BW and FW. Anaerobic biodegradability was shown to be higher for the 150g-FW/2L-BW mixture in terms of higher organic matter removal and higher methane production. Parts of this study have been published (Rajinikanth et al., 2013).

4.5 Significance of study

This study demonstrated the advantages of anaerobic co-digestion, in terms of increased organic removal efficiencies and enhanced methane yield. The final methane yields and organic matter removal efficiencies observed in this study would serve as a reference for subsequent reactor studies using FW/BW mixture as feeding material. As there were no significant increases in soluble COD and VFA levels after day 4 of the study, a HRT of 4 days would be used for the initial operation of the acidogenic reactor for two-phase digesters in subsequent digester studies. The HRT of digesters would start from 20 days and then gradually reduced to 10 days since 95% of the final methane yields were attained by day-10 in this study.

Chapter 5

5. Optimization of brown water and food waste co-digestion in two-phase continuous stirred tank reactor (CSTR), single-stage CSTR and single-stage sequencing batch reactor (SBR)

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5.1. Introduction

The preliminary BMP study reported in Chapter 4 demonstrated the benefits for co-digestion of brown water (BW) and food waste (FW). On top of higher degree of conversion from organic matter into biogas, co-digestion of BW and FW shorten start-up time as well as overall digestion time. These benefits will most likely give rise to higher production of renewable energy and lower the requirement of reactor volume and land. In this chapter, the performance study of 150g-FW/2L-BW mixture was investigated in 5-L reactors. Three different reactor configurations – (1) two-phase continuously stirred tank reactor (CSTR); (2) single-stage CSTR; and (3) single-stage sequencing batch reactor (SBR) were run in parallel for 110 days.

Anaerobic digestion (AD) of organic matter is carried out syntrophically by microbial communities consisting of both bacterial and archaeal species. The degradation may be divided into three steps. During the first step, hydrolytic bacteria degrade polymeric organic matter into monomers, such as sugar and amino acid, which are further degraded in the second step by acetogenic bacteria into volatile fatty acids (VFAs), such as acetate. In the last step, methanogens produce biogas mainly from formate, hydrogen and acetate.

In conventional applications, AD processes usually occur in a single reactor system. However, acid- and methane-forming microorganisms have very different nutritional needs. When kept together in a single reactor system, some of such systems gradually gave rise to reactor instability

problems (Boe and Angelidaki, 2009). The physical separation of acid- and methane-forming microorganisms in different reactors was first proposed by Poland and Ghosh (1971). Such systems provided optimum environmental conditions for each group of organisms and thus led to enhanced stability and control of the overall process.

Single-phase anaerobic systems, in which all three reactions of hydrolysis, acetogenesis and methanogenesis take place simultaneously in a single reactor have been the preferred reactor design for the majority of waste. However, the operation of such systems for waste with large biodegradable organic content such as FW becomes difficult as this type of waste undergoes rapid acidification resulting in the inhibition of methanogenic activity. Two-phase systems, in contrast, have the advantage of buffering the OLR in the first stage, allowing a more constant feeding rate to the methanogenic second stage. Since the 150g-FW/2L-BW mixture has a relatively high solid content of 15 g/L (Table 4-1), a two-phase system might be more suitable since simultaneous liquefaction along with acidification in the first phase would enhance hydrolysis.

The three reactor designs investigated in this study differed mainly in the way microorganisms were retained in the bioreactor and in the phase separation of acidogenic process from the methanogenic process. Given the growing demand for energy recovery and efficient disposal of solid waste, such research – comparison of anaerobic co-digestion efficiency among these three reactor designs will be useful.

The objective of this study was to conduct, in parallel, the anaerobic co-digestion of BW and FW in three different reactor designs (i.e., two-phase CSTR, single-stage CSTR and single-stage SBR). The other objective was to identify the key operating conditions to optimize process performance. These would facilitate the selection of the most suitable reactor configuration for the co-digestion of BW and FW in future larger scale applications (i.e., 30-L laboratory reactors and pilot-scale plants). This chapter will report the

performance of the three different 5-L reactor systems as well as that of a two-phase 30-L reactor system.

5.2. Experimental set-up

5.2.1. 5-L laboratory scale reactors

Anaerobic co-digestion of the 150g-FW/2L-BW mixture was carried out in three different reactor configurations in parallel. As shown in Figure 5-1, the different reactors consisted of (a) two-phase CSTR; (b) single-stage CSTR; and (c) single-stage SBR. Two-phase CSTR comprised of acidogenesis reactor, R_A (1.2 L working volume) and methanogenesis reactor, R_M (4.1 L working volume). Both single-stage CSTR (R_S) and SBR (R_{SBR}) had working volume of 5.3 L each. R_A , R_S and R_{SBR} were fed daily with freshly prepared 150g-FW/2L-BW mixture while R_M was fed with the effluent from the acidogenesis reactor (R_A). R_A , R_M and R_S were operated with continuously mixing (mixing time: 5 min ON/5 min OFF) at 80 rpm using an overhead mechanical stirrer while R_{SBR} was operated in cycles, such that each 24 h cycle consisted of filling (1 h), mixing (20 h), settling (2 h), drawing (30 min) and idling (30 min) phases.

Figure 5-1: Schematic of experimental set-up for (a) two-phase CSTR, (b) single-stage CSTR and (c) single-stage SBR

All the reactors were operated inside a temperature-controlled room at 33°C and inoculated with anaerobic sludge (50% by volume) from an anaerobic digester treating municipal wastewater. The anaerobic sludge had a concentration of 17.8 g TS/L and 12.5 g VS/L. The 150g-FW/2L-BW mixture and effluents from R_A, R_M, R_S and R_{SBR} were collected and analyzed for their pH, TS, VS, COD and VFA weekly while biogas production from R_M, R_S and R_{SBR} were measured daily. The analysis were carried out according to Chapter 3 of this thesis.

5.2.2. 30-L laboratory scale reactor

After comparing the performance of the three 5-L reactor systems, the one with higher conversion of organic matter to biogas was selected for the scale-up to 30-L reactor. In the second part of this chapter, a two-phase CSTR/SBR configuration was chosen for the co-digestion of the FW/BW mixture in 30-L reactor system. It consisted of an acidogenesis reactor operated as CSTR (7-L) and methanogenesis reactor operated as SBR (23-L). HRT of the acidogenesis reactor was maintained at 5 days while that of methanogenesis reactor was at 25 days for the initial 75 days of operation. The operating conditions are identical to that of the 5-L reactors.

5.3. Results and discussion

5.3.1. Performance of 5-L reactors

The feed mixture was prepared and fed daily to the reactors in a fed-batch mode. The characteristics of the 150g-FW/2L-BW mixture is as shown in Table 5-1. All the reactors were operated in parallel and had an initial organic loading rate (OLR) of about 1 g-COD/L.d. The anaerobic co-digestion of BW and FW in 5-L reactors was monitored for 110 days with HRT ranging between 24 to 16 days (Figure 5-2). R_A and R_M of the two-phase CSTR started operation at a HRT of 4 and 20 days, respectively while both R_S and R_{SBR} started with HRT of 20 days. In addition to the comparison of reactor performance between two-phase and single-phase systems in CSTRs (Figures 5-1a and 5-1b), the performance of CSTR was also compared to that of SBR (Figures 5-1b and 5-1c).

Table 5-1: Characterization of 150g FW/ 2L BW mixture (feedstock for 5-L reactors operated from Jun 29 to Oct 17 2011)

Parameter	Unit	Range	Average with standard deviation
pH	–	5.5 – 7.0	6.18 ± 0.55
Alkalinity	mg CaCO ₃ /L	491 – 518	505 ± 19
TS	g/kg	12.7 – 24.8	17.8 ± 3.6
VS	g/kg	11.4 – 23.9	16.5 ± 3.5
TSS	g/L	3.0 – 10.7	7.3 ± 2.6
VSS	g/L	2.9 – 10.4	7.1 ± 2.6
Total COD	g/L	20.7 – 54.1	33.7 ± 10.4
Soluble COD	g-COD/L	8.1 – 17.4	11.9 ± 3.7
TVFA	mg-COD/L	872 – 2,743	$1,827 \pm 488$

Figure 5-2: HRT of 5-Lreactors

5.3.1.1. Degree of hydrolysis and acidification for R_A

The COD solubilization and degree of acidification of the 150g-FW/2L-BW mixture were monitored in the acid reactor of the two-phase CSTR. No pretreatment or control techniques were applied to R_A for preventing methanogenesis during this period. As shown in Figure 5-3, R_A had an average pH of 4.2 ± 0.1 throughout the study period.

Figure 5-3: pH profile of ■ 150g-FW/2L-BW, □ R_A, ● R_M, ○ R_S and ▲ R_{SBR}.

Hydrolysis is the solubilization of particulate organic fraction in the feed mixture and can be represented by changes in soluble COD levels (Figure 5-4a). Acidification is the process of metabolizing hydrolyzed organics into organic acids and can be represented by the changes in levels of TVFA. The degree of acidification was therefore expressed in terms of TVFA/ soluble COD ratio.

As shown in Figures 5-4b and 5-4c, there was a 4 fold increase in TVFA production ($1,827 \pm 488$ mg-COD/L for feed and $8,095 \pm 1,540$ mg-COD/L for R_A) and 5 fold increase in the degree of acidification ($15 \pm 5\%$ for feed and $70 \pm 16\%$ for R_A) during the hydrolysis and acidification period in R_A. As shown in Figure 5-3d, more than 70% of TVFA in R_A comprised of acetic acid (H-Ac), propionic acid (H-Pr) and butyric acid (H-Bu). H-Bu was the dominant VFA throughout the study where it accounted for more than 40% of TVFAs and proportions of H-Ac and H-Pr were on average 15% each. The dominance of H-Bu was also observed in Chapter 4 of this thesis as well as in other studies that utilized food waste as substrate for anaerobic digestion (Xu et al., 2014a; Han and Shin, 2004).

Figure 5-4: Profile of R_A in terms of levels of (a) soluble COD, (b) TVFAs, (c) degree of acidification, and (d) VFA composition

According to Xu et al. (2014a), H-Bu and H-Ac are regarded as preferred precursors for CH₄ production. Therefore, conversion of organic matter into H-Ac and H-Bu in the hydrolytic-acidogenic stage will improve the overall energy yield and increase the process efficiency. In this study, the 4 to 5 folds increase in TVFA levels and degree of acidification (TVFA/ soluble COD ratio) but low proportion of H-Ac also suggested that the activities of acidogens but not those of acetogens were high in the acidogenic reactor of the two-phase system.

5.3.1.2. Organic matter removal in R_M , R_S and R_{SBR}

In order to compare the performance of three different configurations, the two-phase ($R_A + R_M$) CSTR, single-stage CSTR (R_S) and single-stage SBR (R_{SBR}) were operated in parallel at almost similar operating conditions. Sufficiently long HRT and an appropriate choice of inoculum at the start-up of reactor operation allowed for a high-stabilization of treated effluent of up to 90%. Figure 5-5 shows the overview of the reactors' performance in terms of total COD, soluble COD, TS and VS removal efficiencies. During the first 40 days of the study, the effluent quality in terms of organic fraction was almost similar in all the three different configurations. As shown in Table 5-2, average soluble COD removal efficiencies ranged between 88% to 93% while TS and VS removal were between 44% to 59% and 54% to 68%, respectively.

5.3.1.2.1. Response of R_M at different HRTs

From 41 d onwards, the HRT of R_M was reduced from 20 to 16 days (41 – 60 d). According to Table 5-2, there was a slight decrease in TS and VS but increase in total COD and soluble COD removal rates as HRT was reduced. TS and VS removal efficiencies dropped from 59% to 57% and 68% to 64%, respectively. On the other hand, total and soluble COD removal rates increased slightly from 64% to 74% and 89% to 96%, respectively during this period. pH of R_M also decreased slightly from 7.14 to 6.93. In view of the slightly higher washout of biomass from R_M at HRT of 16d, the HRT was increased slightly to 17d for the study period 61 – 90 days. However, total and soluble COD removal rates unexpectedly dropped drastically from 74% to

62% and 96% to 73%, respectively despite improvement in biomass retention and organic matter degradation (61 – 90 d). Therefore, the HRT for R_M was adjusted back to 20 d from 91 d onwards. At a longer HRT of 20 d, TS and VS removal efficiencies increased significantly from 58% to 70% and 64% to 77%, respectively. However total and soluble COD removal rates continued to decrease despite a longer HRT cycle.

Anaerobic reactions are highly pH dependent and the optimal pH range for methanogenesis was suggested to be from 6.8 to 7.2. According to Figure 5-3, the pH of R_M was maintained within the optimal pH range until day 74 of operation (i.e., during HRT of 16d). After that, its pH dropped below 6.7, and did not recover until the end of the study.

The reduction in soluble COD removal was observed together with an increase in the accumulation of TVFA from 637 ± 571 mg-COD/L to $1,163 \pm 394$ mg-COD/L to $2,623 \pm 1,194$ mg-COD/L to $5,121 \pm 632$ mg-COD/L for days 0-40, 41-60, 61-90 and 91-110 of the study respectively (Figure 5-6a).

Table 5-2: Average values for removal efficiencies by R_M , R_S and R_{SBR}

	Time	HRT (d)	pH	Removal efficiency (%)			
				TS	VS	Total COD	Soluble COD
R_M	0-40 d	20	7.14	59.43	68.06	64.31	88.72
	41-60 d	16	6.93	56.72	63.93	74.22	96.41
	61-90 d	17	6.77	58.06	64.18	61.5	72.52
	91-110 d	20	5.94	70.38	77.13	42.9	42.5
R_S	0-40 d	20	7.11	43.56	54.26	48.13	92.69
	41-60 d	16	6.62	44.54	52.84	64.69	95.77
	61-90 d	20	5.78	48.84	57.62	51.64	70.5
	91-110 d	20	6.74	60.85	69.71	47.34	55.14
R_{SBR}	0-40 d	20	7.05	52.9	63.24	64.46	92.28
	41-60 d	16	6.85	64.06	71.12	70.55	95.75
	61-90 d	16	6.82	60.17	65.55	67.46	91.19
	91-110 d	16	6.86	59.29	58.87	65.86	88.53

Figure 5-5: Removal efficiencies of (a) total COD, (b) soluble COD, (c) TS and (d) VS for two-phase CSTR, single stage CSTR and single-stage SBR

Figure 5-6: (a) TVFA levels, and VFA composition for (b) two-phase CSTR, (c) single stage CSTR, and (d) single stage SBR

At HRT of 20 d (during 0 – 40 d), all the SCFAs produced from R_A were consumed by the methanogens present in R_M . In particular, H-Bu removal was almost complete at this HRT, and no inhibitory effects of this acid were observed in R_M . However, as HRT was further decreased from 20 to 16 d, H-Pr started to accumulate in this reactor. H-Pr levels increased from 75 ± 71 to 643 ± 72 to $2,311 \pm 6$ mg-COD/L for days 0-40, 41-90 and 91-110 of this study, respectively. The proportion of H-Pr in R_M also increased from $11 \pm 5\%$ to $27 \pm 14\%$ to $47 \pm 7\%$ for the periods stated above (Figure 5-6b). This led to the increase in TVFA levels observed for R_M .

As expected, H-Pr exhibited more severe inhibition than H-Bu and H-Ac in R_M . Although majority of TVFA existed as H-Bu in R_A , it was degraded more competently than the others because of its high energy gain via degradation. On the other hand, H-Ac removal was also high since H-Ac is regarded as an efficient substrate for methane production due to its one-step degradation (Wong et al., 2008). The excess of VFA built up in R_M caused a drop in pH and inhibition of the methanogenic process. H-Pr accumulation occurs commonly when the reactor is overloaded and its removal is difficult due to a positive Gibbs free energy change for H-Pr conversion which is thermodynamically infeasible. Hence to improve the process stability of R_M , HRT was increased back to 20 d. Despite the improved retention of biomass and higher degradation of organic matter at 20 d HRT, H-Pr continued to accumulate in R_M .

5.3.1.2.2. Response of R_s at different HRTs

According to Table 5-2, TS, VS, total COD and soluble COD increased as HRT was reduced from 20 to 16 d. However, pH started to drop due to VFA accumulation in R_s . VFA levels increased from 303 ± 182 mg-COD/L to 710 ± 417 mg-COD/L for days 0-40 and 41-60 of the study, respectively (Figure 5-6a). Therefore, HRT for R_s was adjusted back to 20 d from 61 d onwards. Similar to R_M , TS and VS removal efficiency increased but total and soluble COD removal rates continued to decrease at longer HRT of 20 d.

The low TVFA values of R_s during the first 2 cycles of 20 d HRT (0-40d) indicated a good balance between acidogens and methanogens in the reactor and its buffering capacity was able to neutralize any acid accumulation. However, the stability of the reactor dropped after HRT was reduced to 16 d as shown by the increased levels of TVFA. Similar to the two-phase CSTR, the increase in TVFA levels for single-stage CSTR could be attributed to the increase of H-Pr levels from 44 ± 26 mg-COD/L to 537 ± 503 mg-COD/L to $1,464 \pm 61$ mg-COD/L for days 0-40, 41-90 and 91-110 of the study, respectively (Figure 5-6c). In addition, the proportion of H-Pr in TVFA increased from $15 \pm 9\%$ to $24 \pm 12\%$ to $50 \pm 7\%$ for the periods stated above. Therefore, to improve the process stability of R_s , HRT was maintained at 20 d for this reactor until the end of the study. Although the pH recovered to above 6.6 (from 91 d onwards), soluble COD and TVFA levels still remained high.

5.3.1.2.3. Response of R_{SBR} at different HRTs

According to Figure 5-3, the pH of R_{SBR} maintained within the optimal pH range of above 6.7 throughout the whole study (at both HRTs of 20 d and 16 d). According to Table 5-2, the soluble COD removal efficiency of R_{SBR} remained at high levels and varied slightly from 92% to 96% to 91% to 89% for days 0-40, 41-60, 61-90 and 91-110 of the study, respectively. Similarly, there was a small variation in TS and VS removal efficiencies for R_{SBR} throughout the study. TS and VS removal efficiencies ranged between 53% to 64% and 59% to 71%, respectively.

There was a slight variation in TVFA levels from 782 ± 571 mg-COD/L to 424 ± 186 mg-COD/L to 567 ± 309 mg-COD/L to 321 ± 80 mg-COD/L for days 0-40, 41-60, 61-90 and 91-110 of the study, respectively (Figure 5-6a). In contrast to R_M and R_s , the H-Pr levels in R_{SBR} remained low at 80 ± 42 mg-COD/L, 87 ± 62 mg-COD/L and 58 ± 52 mg-COD/L for days 0-40, 41-90 and 91-110 of the study, respectively. This corresponded to the proportion of H-Pr being $13 \pm 9\%$, $16 \pm 5\%$ and $18 \pm 13\%$ for the periods stated above.

5.3.1.2.4. Comparison between R_M and R_S

A similar trend was observed for both R_M and R_S as HRT was reduced from 20 d to 16 d and then adjusted back to 20 d. Firstly, VFA started to accumulate within R_M and R_S from 41 d onwards, leading to increased levels of COD. The subsequent increasing of HRT managed to improve biomass retention within the reactors as well as organic matter removal. However, COD and VFA levels continued to accumulate in both R_M and R_S despite a longer HRT.

The performance of the two-phase CSTR system was superior to that of single-phase CSTR throughout the study period. The average removal efficiencies for TS were 60% and 47% (Figure 5-5a), VS were 68% and 60% (Figure 5-5b), total COD were 61% and 52% (Figure 5-4a), and soluble COD were 77% and 80% (Figure 5-4b) for two-phase and single-phase CSTRs, respectively. Throughout the study, the TVFA levels of two-phase CSTR were higher than that of single-phase CSTR (Figure 5-6a). This could be attributed to the enhanced acidification in R_A due to the physical separation of acid- and methane-forming microorganisms in the two-phase CSTR.

This study has also shown that the two-phase CSTR demonstrated better stability and control of the overall AD process as compared to the single-stage CSTR. At HRT of 16 d, both two-phase and single-stage CSTR systems showed signs of reactor instability. However, this was observed first in the single-stage CSTR followed by two-phase CSTR.

5.3.1.2.5. Comparison between CSTR and SBR

As shown in Table 5-2, single-stage SBR showed better reactor performance than single-stage CSTR in terms of higher total COD, soluble COD, TS and VS removal rates at HRT of 16 days. SBR displayed very stable reactor performance as its soluble COD removal efficiencies were at least 80%, and the TVFA level was maintained at low levels of less than 800 mg-COD/L. According to Figure 5-6, TVFA levels for R_S and R_{SBR} were maintained at low levels for the first 50 d. After that, TVFA levels for R_S started to increase and by the end of the study, R_S had TVFA levels of 3,147 mg-

COD/L. On the other hand, R_{SBR} managed to maintain low TVFA levels of less than 800 mg-COD/L throughout the study of 110 d. As shown in Figure 5-6c, the increase in TVFA levels for R_s after 50 d was mainly contributed by the accumulation of H-Pr in the reactor. H-Pr was reported to be inhibitory to AD systems due to the difficulty in degradation. On the other hand, the percentage of all individual VFAs did not exceed 35% and no H-Pr accumulation was observed in R_{SBR} .

Although R_{SBR} displayed very stable performance, the removal efficiencies of TS, VS, total COD and soluble COD showed signs of decreasing towards the end of the study (Table 5-2). As compared to R_{SBR} , the VS removal efficiency of R_s was higher by approximately 10%. Nevertheless, other parameters such as TS, total COD, soluble COD and VFA levels were still lower in R_{SBR} as compared to R_s .

Within the study period of 110 days, the single-stage SBR was observed to be superior to the single-stage CSTR. This could be attributed to the maintenance of a high active biomass concentration inside R_{SBR} that enabled effective removal of organic matter thus enhancing reactor stability. However, a longer period of observation is required to conclude if the long-term operation of SBR will be superior to that of CSTR.

Table 5-3: Overall comparison of removal rates and biogas yield in 5-L and 30-L reactors

	Removal Rates		TS (%)	VS (%)	Biogas (L/kg- VS _{fed})
	Total COD (%)	Soluble COD (%)			
5-L Two-phase CSTR	61 ± 17	77 ± 21	60 ± 10	67 ± 10	589
5-L Single-stage CSTR	52 ± 14	80 ± 18	47 ± 10	57 ± 9	551
5-L Single-stage SBR	67 ± 15	92 ± 5	58 ± 13	65 ± 13	510
30-L Two-phase CSTR/SBR	84 ± 17	93 ± 5	66 ± 24	75 ± 20	560 ± 219

The biogas yield of 0.5-0.6 m³/kg VS added was recorded during this study (Table 5-3). Methane content of 60-65% was observed in the total biogas. The first part of this study demonstrated the benefits of phase separation as well as operating in SBR configuration. Thus, the two-phase CSTR/ SBR configuration was selected for the scale-up to 30-L digester.

5.3.2. Performance of 30-L digesters

Based on the performance of 5-L reactors in the earlier part of this study, the two-phase CSTR/SBR configuration was maintained for the scale-up to 30-L reactor system. This 30-L system was operated for 370 d (Dec 07th 2011 to Dec 31st 2012) at different HRTs (30 – 16 d). As shown in Figure 5-7a, the acidogenesis reactor (R1) was operated as CSTR at HRTs of 5 – 3 d while the methanogenesis reactor (R2) was operated as SBR at HRTs of 25 – 13 d.

5.3.2.1. Degree of hydrolysis and acidification for 30-L reactor

The 150g-FW/2L-BW mixture had an average pH of 6.01 ± 0.56 , TVFA level of $1,242 \pm 762$ mg-COD/L and soluble COD level of 10.44 ± 7.01 g-COD/L. After hydrolysis and acidification in the first phase, R1 had average pH level of 4.09 ± 0.27 , TVFA level of $5,459 \pm 3,687$ mg-COD/L and soluble COD level of 13.25 ± 8.47 g-COD/L (Figures 5-7b, c and d). After fermentation in the acidogenesis reactor, soluble COD levels increased by 30%, and TVFA levels by more than 300%. This indicated that acidification took place to a larger extent as compared to hydrolysis and there was a large conversion of non VFA-COD to VFA-COD in R1.

The main acidification products were H-Ac (33% of TVFAs), H-Bu (29% of TVFAs), H-Va (27% of TVFAs) and H-Pr (8% of TVFAs) comprising 97% of the TVFAs. As shown in Table 5-4, TVFA levels increased from 4,702 mg-COD/L to 7,584 mg-COD/L as HRT changed from 5 d (0 – 49 d) to 4 d (121 – 180 d), respectively. When HRT was further reduced to 3 d, a drop in TVFA levels to 6,166 mg-COD/L was observed. TVFA levels continued to decrease to 4,305 mg-COD/L despite a longer HRT of 4 d (292 – 311 d).

5.3.2.2. Organic matter removal for 30-L reactor

As shown in Figure 5-7c, VFAs produced in R1 were mostly degraded by the methanogens in R2 at all the studied HRTs. Therefore, low levels of VFA and soluble COD were maintained throughout the study. The average removal efficiencies for TS, VS, total COD and soluble COD were $66 \pm 24\%$, $75 \pm 20\%$, $84 \pm 17\%$ and $93 \pm 5\%$, respectively (Figure 5-8a). Average levels of pH, soluble COD, TVFA and biogas yield were 7.00 ± 0.18 , 0.78 ± 0.48 g/L, 480 ± 498 mg-COD/L and 560 ± 219 mL/g-VS.d, respectively. The methane composition of the biogas collected ranged from 55% to 70%. These average values were exclusive of the first 25 d, which were considered the adaptation period for the reactors.

Table 5-4: TVFA and pH levels of 30-L two-phase CSTR/SBR system

Duration		0 – 49	50 –	92 –	121 –	247 –	292 –
(d)			75	114	180	291	311
HRT	(R1, R2)	5, 25	5, 20	4, 18	4, 16	3, 13	4, 16
R1	TVFA	4,702 $\pm$	6,478	N.D.	7,584 $\pm$	6,166 $\pm$	4,305 $\pm$
		2,793	$\pm$ 149		6,035	5,589	2,508
R2	(mg-COD/L)	99 $\pm$ 61	835 $\pm$	N.D.	234 $\pm$	723 $\pm$	720 $\pm$
			226		159	864	312
R1	pH	4.31 $\pm$	4.06 $\pm$	4.08 $\pm$	4.16 $\pm$	3.88 $\pm$	4.20 $\pm$
		0.70	0.11	0.16	0.41	0.29	0.34
R2		6.94 $\pm$	6.86 $\pm$	7.16 $\pm$	6.98 $\pm$	6.94 $\pm$	7.21 $\pm$
		0.11	0.22	0.09	0.19	0.14	0.06

N.D.: not determined

According to Table 5-5, R2 consistently achieved high removal rates for TS, VS, total COD and soluble COD (at least 80%) from the start of the study until 211 d. From 212 d onwards, TS, VS and total COD levels started to increase while soluble COD and VFA levels continued to remain low. The increasing levels of TS, VS and total COD measured in the R2 effluent could be attributed to the washout of biomass. High degradation of soluble COD and VFA despite biomass washout from 212 d onwards indicated that there

was still sufficient active biomass retained in R2 to carry out the anaerobic degradation process. An attempt to decant the biomass in R2 on 280 d did not help to improve the biomass retention ability of the SBR. As shown in Table 5-5, Removal efficiencies of TS, VS and total COD continued to decrease from 280 d to the end of the study.

5.3.3. Overall comparison

The second part of this study successfully demonstrated the scale-up from 5-L to 30-L digester by adopting a two-phase CSTR/ SBR configuration, in terms of improved organic matter removal efficiencies (Table 5-2). This study confirmed the ability of SBR to retain active biomass in the reactor for effective degradation of organic matter for up to 211 days. During this 211 days, sedimentation of biomass within the SBR occurred naturally and led to the discharge of cleaner AD effluent (i.e., effluent free of suspended solids) as compared to the CSTR. On top of that, the SBR had shorter mixing time and therefore will lead to reduced operating costs.

Although the SBR showed very consistently good performance for the first 211 days, operation of the SBR beyond 211 days led to biomass washout, and the effluent had higher solids content as compared to the CSTR. This could be attributed to the accumulation of solids in the SBR and washout of biomass started to occur as the height of sludge bed in the SBR exceeded the level of effluent discharge. However, it was clear that biomass washout from the SBR did not lead to a significant reduction in the soluble COD removal efficiency, indicating that sufficient active biomass was still retained in the SBR to carry out the anaerobic degradation process. Both the 5-L and 30-L SBR showed that the ability of the SBR to degrade soluble COD was high despite biomass washout.

This study demonstrated that the continuous operation of the SBR will eventually lead to biomass washout due to solids accumulation. Although this study showed that soluble COD and VFA levels remained low despite biomass washout, a longer term observation is still required to determine the consequences of biomass washout by the SBR. In addition, the SBR lacks

well-defined and established methodologies and operating techniques since its research and development are very recent.

Figure 5-7: (a) HRT; (b) pH; (c) VFA levels and (d) soluble COD levels of $\circ$ 150g-FW/2L-BW, $\blacksquare$ R1 and $\square$ R2

Figure 5-8: (a) –□– Total COD, –■– soluble COD, –○– TS and –●– VS removal efficiencies and (b) –▲– biogas yield for R2

Several fundamental features and technological aspects such as the occurrence of dead zones, high settle time, solids wash-out, slow start-up period, inhibition due to organic overloading and poor knowledge of agitation and feed strategy remain to be investigated. The lack of process experience in decanting dead biomass or re-lacitrant biomass and difficulty in maintaining active biomass in SBR are the challenges that need to be solved in order to adopt the SBR system effectively. In comparison, the CSTR is advantages in terms of long-term stability and easy of operation. Furthermore, Kujawa-Roeleveld et al. (2000) reported that high-strength domestic wastewaters that contain faeces, urine and food waste are more suitable to be treated in anaerobic treatment systems without sludge retention such as CSTR systems.

Table 5-5: Performance (removal efficiencies) of R2 for the 30-L two-phase CSTR/SBR system

			92 –	121 –	212 –		292 –	
Duration (d)	26 – 49	50 – 91	120	211	279	280-291	329	330 – 367
HRT	25	20	18	16	13	13	16	16
TS removal (%)	66 ± 26	87 ± 5	N.D.	83 ± 6	71 ± 17	68 ± 5	63 ± 6	59 ± 7
VS removal (%)	74 ± 20	95 ± 3	N.D.	93 ± 2	76 ± 17	73 ± 4	70 ± 6	66 ± 6
Total COD removal (%)	76 ± 22	87 ± 20	96 ± 1	95 ± 1	83 ± 8	71 ± 1	71 ± 17	63 ± 14
Soluble COD removal (%)	92 ± 6	92 ± 3	92 ± 3	89 ± 5	94 ± 3	92 ± 2	90 ± 3	90 ± 3
Biogas yield (L/kg-VS _{fed})	271 ± 59	622 ± 237	442 ± 91	N.D.	747 ± 191	N.D.	623 ± 135	707 ± 93

N.D. not determined

5.4. Conclusion

The first part of this study discussed the operation of 5-L digesters for the initial 110 days and found that SBR was better at retaining solids than CSTR and the two-phase system was less prone to VFA inhibition. The second part of this study was a scale-up from 5 L to 30 L digesters. The scale-up effect included higher organic matter removal efficiencies and longer sludge retention time due to the coupling of a CSTR acidogenesis reactor to a SBR methanogenesis reactor. This study also demonstrated that the continuous operation of the SBR will eventually lead to clogging from solids accumulation. Although this study showed that soluble COD and VFA levels remained low despite biomass washout, a longer term observation is still required to determine the consequences of biomass washout by the SBR.

Chapter 6

6. Study of microbial community and biodegradation efficiency for single- and two-phase anaerobic co-digestion of brown water and food waste

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6.1. Introduction

Studies on bacterial and methanogenic archaeal community structures in anaerobic digesters treating food waste (FW) have been reported recently (Ike et al., 2010; Wang et al., 2010). However, the understanding of microbial aspects for co-digestion of brown water (BW) and FW is still limited due to the lack of references on this topic. Comprehension of microbial community and its function is necessary to improve the efficiency and process stability of anaerobic digesters. 16S rRNA cloning and sequencing is the well known method used to characterize microbial community in an anaerobic reactor while fluorescent *in situ* hybridization (FISH) is a useful method to verify cloning findings and to visualize the different cells in anaerobic sludge. Therefore, these two methods were employed in the current study to determine the microbial populations of anaerobic digesters treating BW and FW.

The objective of this work was to study the microbial community and reactor performance for the anaerobic co-digestion of BW and FW in single- and two-phase continuously stirred tank reactors (CSTRs). Insights gained from this study would enhance the understanding of microorganisms involved in the anaerobic co-digestion of BW and FW as well as the complex biochemical interactions that promote digester stability and performance. This information could help to optimize existing processes and also aid the selection of seeding sludge for rapid startup in future applications.

6.2. Experimental set-up

The feed for this study consisted of a mixture of 300 g blended FW and 2 L BW, and had an average pH of 6.23 ± 0.07 . The characteristics of the feed are as shown in Table 6-1. Anaerobic co-digestion of BW and FW was performed in laboratory scale (5 L) single- and two-phase CSTRs. The co-substrates were prepared daily and fed to the reactors, which included the acidogenic (R_A) and methanogenic (R_M) reactors of the two-phase CSTR system and the single-phase CSTR (R_S), in batch mode. The working volumes of R_A , R_M and R_S were 1.2 L, 4.1 L and 5.3 L, respectively, and the contents were mixed continuously (mixing time: 5 min ON followed by 5 min OFF) at 80 rpm by an overhead mechanical stirrer. R_A , R_M and R_S were initially inoculated with mesophilic anaerobic sludge collected from a local wastewater treatment plant (Ulu Pandan Water Reclamation Plant, Singapore).

The single and two-phase CSTR systems were operated in parallel for 150 days at 35°C with hydraulic retention time (HRT) as shown in Table 6-1. HRT was reduced by adding increased volumes of the 300g-FW/2L-BW mixture into the reactors of fixed working volumes. The organic loading rate (OLR) was maintained at around 0.5 to 0.8 g-VS L⁻¹ d⁻¹ in this study. Both the single- and two-phase CSTRs were operated in the same way and had the same overall reactor working volume of 5.3 L. Both R_A and R_S were fed with the 300 g-FW/ 2 L-BW mixture prepared daily while R_M was fed with the acidified effluent from R_A during the study.

The reactor performances for R_A , R_M , and R_S were monitored weekly, while their sludge samples were collected once on 150 d for microbial community characterization – 16S rRNA cloning and sequencing as well as FISH. Archaeal and bacterial 16S rRNA partial sequences obtained in this study were deposited in the nucleotide Genbank database, under the accession numbers: KF169842 – KF169904.

Table 6-1: Operational conditions and reactor performance

Period	1 (Days 0-55)				2 (Days 56-109)				3 (Days 110-150)			
	Feed ^a	R _A	R _M	R _S	Feed ^a	R _A	R _M	R _S	Feed ^a	R _A	R _M	R _S
HRT ^b [d]		10	30	40		10	30	40		6	29	35
Biogas Production [L g-VS ⁻¹ d ⁻¹]	n.d.	n.d.	1.91±0.37	0.96±0.28	n.d.	n.d.	1.15±0.38	0.62±0.27	n.d.	n.d.	1.57±0.61	0.65±0.20
TS [g L ⁻¹]	30.40±9.99	23.17±5.92	7.93±0.50	4.75±2.28	23.30±6.25	12.21±2.36	4.83±1.57	7.76±2.45	19.62±10.37	11.14±1.69	4.04±0.93	7.93±1.13
TS removal [%]	n.d.	n.d.	72.3±8.74	83.61±8.26	n.d.	n.d.	76.07±15.98	63.67±18.25	n.d.	n.d.	76.54±8.99	53.05±17.07
VS [g L ⁻¹]	26.21±4.80	21.56±5.60	5.39±0.43	3.21±2.02	22.07±5.99	11.37±2.95	3.27±1.40	5.76±2.11	18.29±10.28	9.63±1.59	2.51±0.84	5.87±0.79
VS removal [%]	n.d.	n.d.	79.25±4.40	85.40±9.20	n.d.	n.d.	76.07±15.98	63.67±18.25	n.d.	n.d.	81.42±6.09	56.50±6.05
Soluble COD [g L ⁻¹]	15.47±6.71	19.01±12.63	0.70±0.32	0.65±0.22	13.01±3.26	15.39±3.75	1.04±1.02	0.53±0.09	8.41±1.36	18.60±1.11	0.81±0.40	0.49±0.04
Soluble COD removal [%]	n.d.	n.d.	94.84±2.34	95.10±2.62	n.d.	n.d.	94.02±2.31	95.59±1.28	n.d.	n.d.	91.79±3.30	94.19±0.74
TVFA [mg-COD L ⁻¹]	815±669	3532±2853	62±18	78±37	2001±658	14173±3368	801±256	481±195	1234±569	19367±7799	1146±401	215±36

^a: 300 g food waste + 2 L brown water

^b: Hydraulic retention time

n.d.: not determined.

6.3. Results and discussion

6.3.1. Reactor performance

The experimental results of this study were categorized into three periods as shown in Table 6-1. The average biogas productions for R_M and R_S throughout the study were 1.54 and $0.74 \text{ L g-VS}^{-1} \text{ d}^{-1}$, and their average CH_4 concentrations were 60% and 50%, respectively. With no pH control in any of the reactors, the pH levels in the three reactors were stable throughout the study. R_A , R_M and R_S had average pH levels of 3.72 ± 0.35 , 6.98 ± 0.14 and 6.98 ± 0.15 , respectively. The pH of R_M and R_S were not significantly different and were in the suggested optimal pH range suitable for methanogenesis to take place.

The overall average concentration of total volatile fatty acid (TVFA) for R_A was $11,115 \pm 7,074 \text{ mg-COD L}^{-1}$, representing an average of 9 fold increase in TVFA production as compared to the feed. With average soluble COD values of 12.93 ± 5.67 and $17.38 \pm 7.66 \text{ g L}^{-1}$, the degree of acidification (proportion of VFA in soluble COD) for the feed and R_A effluent were 11% and 79%, respectively. This translated to a 7 fold increase in the degree of acidification after treatment in R_A of the two-phase system. Almost 80% of TVFA in R_A comprised of acetic acid (H-Ac), propionic acid (H-Pr) and butyric acid (H-Bu). H-Ac was the dominant VFA throughout the study where it accounted for 40% of TVFA. The percentages of H-Pr and H-Bu in R_A were on average 17% and 23%, respectively. The high degree of acidification as well as conversion of longer chain VFA to acetate suggested that the activities of acidogens and acetogens were high in R_A .

In R_M , VFAs fed from R_A were mostly consumed and their total content in R_M was reduced to an average amount of $618 \text{ mg-COD L}^{-1}$. H-Ac was present in highest concentrations (average of $101 \text{ mg-COD L}^{-1}$) as compared to H-Pr (average of 85 mg-COD L^{-1}) and H-Bu (average of $114 \text{ mg-COD L}^{-1}$). The levels of TVFA in R_M were approximately 60% and five times higher than that in R_S for periods 2 and 3 of the study, respectively. R_M had an average NH_4^+

concentration of 689 mg L⁻¹, which was almost 2 times higher than that in R_S (366 mg L⁻¹). Despite the higher levels of TVFA and NH₄⁺ in R_M, there were no significant differences in the reductions of total and soluble COD between R_M and R_S. Both achieved at least 74% total COD and 91% soluble COD removal rates. In addition, the higher levels of TVFA and NH₄⁺ did not lead to any observed inhibition effects by the two-phase system. As shown in Table 6-1, the average TS and VS removal efficiencies for R_M did not vary much throughout the study. On the contrary, TS removal efficiency for R_S dropped from 84 to 53%, while its VS removal efficiency dropped from 85 to 57% as reactor operation proceeded from period 1 to 3. Overall, the performance of two-phase CSTR system (i.e., R_A and R_M) was better than that of single-phase CSTR (i.e., R_S) in terms of higher biogas production, methane composition as well as solids reduction.

6.3.2. Microbial community characterization

Cloning and subsequent phylogenetic analysis were carried out to characterize the microbial community structures in both single- and two-phase CSTRs. Primer set 530F and 1490R were initially used to amplify the prokaryotic sequences from the extracted DNA. However, all the clones sequenced were affiliated within the domain Bacteria, indicating that bacterial cells were dominant in R_A, R_M and R_S. Similar findings were also reported previously by Tang et al. (2004). Therefore, an additional set of Archaeal primers Ar1F and 1490R was used in this study to construct the archaeal rRNA clone libraries for R_M and R_S. Neighbor-joining trees showing the phylogenetic identities of the 16S rRNA gene fragments were constructed and are shown in Figures 6-1, 6-2, 6-3 and 6-4.

6.3.2.1. Bacterial community in acidogenic reactor of two-phase CSTR (R_A)

As shown in Figure 6-1, the bacterial community structure of R_A was exclusively composed of the phyla *Firmicutes* and *Proteobacteria*. In total, 7 bacterial operational taxonomic units (OTUs) were identified. The most detected OTU (BFABac_040), representing 49% of the total clones, was affiliated to

Acetobacter peroxydans strain LMG 1633 (AJ419836) with 99% similarity. BFABac_001 was the second most detected I accounting for 36% of the clones. Together with BFABac_009 and BFABac_111, the second predominant group was affiliated with *Lactobacillus amylovorus* GRL 1112 (NR_075048) with 99% similarity. BFABac_137 (1% of total clone) was affiliated with *Lactobacillus fermentum* strains IFO 3956 (NR_075033) and JCM 8596 (AB690185). The two remaining OTUs (BFABac_065 and BFABac_058) each corresponded to 1% of the total clone and were closely related to uncultured *Clostridium* species (HQ183766) and *Desulfobulbus propionicus* DSM 2032 (NR_074930), respectively.

6.3.2.2. Bacterial community in methanogenic reactor of two-phase CSTR (R_M)

The bacterial community in R_M was found to be more diverse and a total of 28 bacterial I were identified and classified into 13 different phyla. Figure 6-2 shows that the dominant I included members affiliated within four different phyla: *Bacteroidetes*, *Chloroflexi*, *Proteobacteria* and *Firmicutes* in proportions of 40%, 13%, 10% and 8% of the bacterial clones, respectively. Within the 28 I, 5 were classified as *Bacteroidetes*, 5 as *Chloroflexi*, 5 as *Proteobacteria* and 4 as *Firmicutes*.

Figure 6-1: Phylogenetic tree of 16S rRNA gene sequences constructed for bacterial clones from R_A. The 16S rRNA gene sequence of *Sulfolobus acidocaldarius* (NR_043400) was used as the outgroup.

Figure 6-2: Phylogenetic tree of 16S rRNA gene sequences constructed for bacterial clones from R_M. The 16S rRNA gene sequence of *Sulfolobus acidocaldarius* (NR_043400) was used as the outgroup.

As shown in Figure 6-2, the closest matches for bacterial clones were mostly detected from food-processing, toluene-degrading and sulfate-rich wastewaters, human faeces, human oral cavities, animal waste treatment and biogas plants, which were all related to anaerobic fermentation.

6.3.2.3. Bacterial community in single-phase CSTR (R_s)

The bacterial community in R_s was slightly less diverse as compared to R_M where a total of 17 bacterial I were identified, which could be classified into 8 different phyla. Figure 6-3 shows that the bacterial I were mainly affiliated with *Fusobacteria*, *Bacteroidetes*, *Proteobacteria* and *Chloroflexi* in proportions of 51%, 32%, 4% and 4% of the bacterial clones, respectively. Within the 17 I, 2 were classified as *Fusobacteria*, 6 as *Bacteroidetes*, 3 as *Proteobacteria* and 2 as *Chloroflexi*. BFSBac_073, the predominant I in R_s, accounted for 35% of total bacterial count and was affiliated with uncultured *Fusobacterium* species (FM242289). The closest matches for bacterial clones, as shown in Figure 6-3, were detected from sources similar to that of R_M.

6.3.2.4. Overview of bacterial communities in R_A, R_M and R_s

The reactors in this study were fed daily with mixtures of BW and FW. Therefore, their bacterial community structures showed a close relationship to human sources such as gastrointestinal tract, oral cavity and faeces. *Bacteroidetes* and *Firmicutes* are known to be dominant phyla present in the human gastrointestinal tract and adult faecal microbiota (Harmsen et al., 2002) while *Fusobacteria* was isolated from human oral cavities (Bennett and Eley, 1993). However, analysis of bacterial communities in this study demonstrated clear differences in both dominant groups and phylogenetic distribution between single- and two-phase CSTRs.

Lactobacillus species and *Acetobacter peroxydans* LMG 1633 represented the exclusive dominant phylogenetic group (close to 98% by cloning analysis) in R_A, suggesting a major impact of these bacteria on the solubilization and acidification of BW and FW, at short retention time of 6 to 10 days and acidic pH conditions. In this study, *Lactobacillus* species was predominant in R_A where the pH was around 4. However, it was not detected in R_M and R_s where the pH was around 7. This is in agreement with other studies reporting that the presence and dominance of *Lactobacillus* was

dependent on pH values. According to Ye et al. (2007), *Lactobacillus* grew intensively at pH 4 to 6, but slowly at pH 7 to 8.

The predominant *Lactobacillus* species in R_A, *Lactobacillus amylovorus*, is a lactate-producing organism possessing amylolytic activity. Therefore it is able to metabolize starch directly to produce lactate and small amounts of acetate (Nakamura, 1981; Zhang and Cheryan, 1991). The other predominant species in R_A – *Acetobacter peroxydans* was reported to contain enzymes for the oxidation of lactate, pyruvate, ethanol and acetaldehyde to acetate, through the transfer of electrons to oxygen. De Ley and Schel (De Ley and Schel, 1959) showed that lactate was oxidized to pyruvate followed by slow oxidation of pyruvate to acetate. Therefore, the co-existence of *Lactobacillus* species and *Acetobacter peroxydans* possibly resulted in the high levels of solubilization and acidification observed in R_A.

All the reactors in this study were designed to operate under completely anaerobic conditions. Therefore, the predominance of an obligately aerobic bacteria – *Acetobacter peroxydans* in R_A was unexpected. *Acetobacter peroxydans* was able to co-exist with *Lactobacillus* species in R_A since the latter is an aero-tolerant anaerobic bacteria. The exact experimental condition (i.e., anaerobic, microaerobic or aerobic) of R_A was not determined by analytical methods such as levels of dissolved oxygen or oxidation reduction potential. Nonetheless, the predominance and co-existence of obligately aerobic *Acetobacter peroxydans* and aero-tolerant anaerobic *Lactobacillus* species suggested R_A was unintentionally operated at microaerobic conditions. The operation of R_A under completely aerobic conditions was highly unlikely since the concentrations of TVFAs and soluble COD in the effluent of R_A were higher than that in the feed mixture (Table 6-1). However, further investigations are required to verify that the predominance of *Acetobacter peroxydans* was due to the unintended operation at microaerobic conditions.

The predominant bacterial group present in R_M was *Bacteroidetes* (40% by cloning analysis), which is a major microbial component of anaerobic reactors. Another bacterial group present in R_M was *Firmicutes* (8% by cloning analysis), which are known to produce cellulases, lipases, proteases and other extracellular enzymes (Levén et al., 2007). Therefore, the presence of *Firmicutes* reflects the ability of digesters to metabolize a variety of substrates including protein, lipids, lignin, cellulose, sugars and amino acids, which are commonly found in food waste.

For R_S , the predominant bacterial group present was *Fusobacterium* species (51% by cloning analysis), which are obligate anaerobic gram-negative bacilli found in large numbers in the mouth. It was reported that *Fusobacterium* species weakly ferment simple sugars to produce large amounts of n-butyric acid (Bennett and Eley, 1993).

In comparison to R_M , the distribution of bacteria within the phylum *Fusobacteria* was higher, that of *Bacteroidetes* was lower, and *Firmicutes* was absent in R_S . Since *Firmicutes* contain extracellular enzymes that carry out the solubilization of brown water and food waste, its absence could play a part in the poorer performance of R_S , in terms of lower solids reduction. In addition, the lack of *Firmicutes* could also suggest that there were large amounts of long chain fatty acids (LCFAs) in R_S since LCFAs were reported to inhibit gram-positive bacteria such as *Clostridia* (Galbraith and Miller, 1973).

There is a lack in studies on the microbial diversity of anaerobic digesters treating BW or mixtures of BW and FW. On the other hand, several studies had reported the predominance of *Lactobacillus* species in the fermentation of FW (Wang et al., 2005; Ye et al., 2007), and that of *Lactobacillus amylovorus*-related species in the first-stage reactor of a two-stage anaerobic digestion system treating FW (Shin et al., 2010). However, the diversity of *Lactobacillus* species in R_A was lower as compared to the above three references. In addition, none of the references reported the predominance of

Acetobacter peroydans, which represented 49% of total clone count in R_A. Therefore, the predominance of *Acetobacter peroxydans* was likely due to the unique operation (i.e. unintended microaerobic conditions) of R_A in this study. Shin *et al.* (2010) also showed that the second-stage reactor consisted of members affiliated within four different phyla, *Firmicutes*, *Proteobacteria*, *Spirochaetes*, and *Bacteroidetes*. This is similar to the bacterial community structure of R_M in this study. Comparisons with existing literature showed that the bacterial diversity of reactors treating BW and FW were similar to those treating FW only. They also suggested that the predominance of *Lactobacillus* species in R_A was largely due to the nature of FW.

Figure 6-3: Phylogenetic tree of 16S rRNA gene sequences constructed for bacterial clones from R_s. The 16S rRNA gene sequence of *Sulfolobus acidocaldarius* (NR_043400) was used as the outgroup.

6.3.2.5. Archaeal community in R_M and R_S

As shown in Figures 6-4a and b, the diversity of archaeal clones in R_M and R_S was limited to members of two orders: *Methanosarcinales* and *Methanomicrobiales* with proportions of 69% and 30%, respectively for R_M and 86% and 14%, respectively for R_S. This indicated that methanogenesis took place preferentially via acetoclastic metabolism for both single- and two-phase CSTRs. For R_M, the most detected archaeal I (BFMArc_004), representing 48% of the total clones, and the second dominant I (BFMArc_039), representing 19% of total clones, were closely related to *Methanosaeta* species (AB479394) isolated from beer brewery effluent with 99% similarity. 30% of total archaeal count was composed of various species and clones within the genus *Methanoculleus*. They included BFMArc_134, BFMArc_103 and BFMArc_183 representing 21%, 7% and 2% of total archaeal clones, respectively.

The predominant archaeal I for R_S was BFSArc_019, which represented 74% of total count, and had the same closest match as BFMArc_004, the predominant archaeal I in R_M. The second dominant I (BFSArc_061), representing 14% of total clones, was affiliated with *Methanoculleus* species dm2 (AJ550158). The remaining 12% of archaeal clones were closely related to *Methanosaeta* species.

Methanogens can be categorized into two major groups according to the substrate they utilize. Acetoclastic methanogens consume acetate while hydrogenotrophic methanogens consume hydrogen and formate for growth. Analysis of archaeal communities in this study showed similar dominant groups and phylogenetic distribution in single- and two-phase CSTRs. As shown in Figures 6-4a and b, *Methanosarcinales* and *Methanomicrobiales*, which are acetoclastic and hydrogenotrophic methanogens, respectively were found in both R_M and R_S.

Figure 4: Phylogenetic tree of 16S rRNA gene sequences constructed for archaeal clones from (a) R_M and (b) R_S. The 16S rRNA gene sequence of *Methanopyrus kandleri* (M59932) was used as the outgroup in both (a) and (b).

The order *Methanosarcinales* consist of genus *Methanosarcina* and *Methanosaeta*. In this study, only *Methanosaeta* species were detected and they formed the predominant archaeal group in both R_M (76% by cloning analysis) and R_S (86% by cloning analysis). The analysis of archaeal communities in this study well agreed with a review of archaeal populations in anaerobic digesters where *Methanosarcinales* was reported to constitute more than 29% of the sequences in all the studies, where sequences affiliated with *Methanosaeta* species were most frequently retrieved (Sekiguchi and Kamagata, 2004). The same study also found that the hydrogenotrophic pathway is commonly represented by *Methanomicrobiales* with proportions in a range of 1-29%.

As compared to *Methanosarcina* species, *Methanosaeta* species have higher substrate (i.e., acetate) affinity as well as lower maximum specific growth rate ($\mu_{\max}$) of 0.20 d⁻¹ and half-saturation constant (K_s) of 10-50 mg-COD L⁻¹ ((Vrieze et al., 2012). According to Vrieze et al. (2012), *Methanosaeta* dominated at acetate concentrations not exceeding 100-150 mg-COD L⁻¹, whereas *Methanosarcina* became dominant at acetate concentrations above 250-500 mg-COD L⁻¹. In this study, the acetate concentrations in both R_M and R_S were low, with average levels of 101 and 46 mg-COD L⁻¹, respectively. This correlated with the dominance of *Methanosaeta* species, which are capable of scavenging acetate at low acetate concentrations. The results in this study were in agreement with earlier studies reporting the dominance of *Methanosaeta* in well-operated mesophilic methanogenic systems with low effluent soluble COD (Raskin et al., 1994; Ariesyady et al., 2007). *Methanosaeta* was also shown to dominate reactors underfed with FW (Williams et al., 2013). Hence, the predominance of *Methanosaeta* and absence of *Methanosarcina* species suggested that R_M was at steady-state but working at less than optimum OLRs. The OLR could be further increased to improve biogas production.

The analysis of archaeal communities in this study revealed that higher levels of hydrogen-utilizing methanogens were present in R_M (30%) than in R_S (14%). This could be attributed to the feed of R_M, which contained higher levels of solubilized organic matter as compared to that of R_S. Therefore, the greater extent of fatty acid fermentation in R_M possibly led to increased hydrogen production which encouraged the growth of more hydrogen-utilizing methanogens. Lerm et al. (2012) also detected a shift of hydrogenotrophic methanogens due to increased VFA concentrations.

It was reported earlier that syntrophic degradation of H-Pr and H-Bu is thermodynamically favorable, only when the hydrogen partial pressure is low enough ($<10^{-4}$ atm) (Lowe et al., 1993; McCarty and Smith, 1986). Therefore, well established hydrogenotrophic methanogens possibly allowed the syntrophic VFA oxidizers to grow more quickly, and to degrade H-Pr more

rapidly, resulting in a more rapidly stabilizing digester (McMahon et al., 2001). The more diverse bacterial community in R_M (28 I) as compared to R_S (17 I) was also suggested to be due to the higher prevalence of hydrogen-utilizing methanogens in R_M . Similar findings were reported by St-Pierre and Wright (2013) where digesters with more hydrogenotrophic methanogens were shown to support a greater level of phylogenetic diversity as compared to digesters predominated by acetotrophic methanogens.

6.3.3. FISH analyses

The FISH analyses of bacterial (green) and methanogenic archaeal (red) populations in R_A , R_M and R_S can be found in Figure 6-5. No methanogens and only bacterial cells of fat and thin rods were detected in R_A . This well agreed with the predominance of *Lactobacillus* species and *Acetobacter peroxydans* in the reactor, by cloning analysis, as shown earlier in section 6.3.2.1. A mixed structure of bacterial and methanogenic archaeal populations was observed in R_M and R_S as shown in Figure 6-5b and c, respectively. High bacterial diversity with different morphologies of small rods, fat rods, ovals, cocci, and thin filaments were detected in R_M and R_S . Bamboo-shaped *Methanosaeta*-like and coccus-shaped *Methanoculleus*-like populations were also observed in R_M and R_S . The morphologies of cells in R_A , R_M and R_S detected by FISH were consistent with the cloning and sequencing results.

6.3.4. Relationship between reactor performance and microbial community structure

Although the effluent from R_M of the two-phase CSTR contained higher levels of TVFA and soluble COD, it produced an average of 23% more methane as compared to the single-phase CSTR. The main reason for higher methane production could be due to the greater extent of solubilization (represented by the reduction of solids) and acidification in the two-phase CSTR.

Figure 6-4: FISH analyses of bacterial (green) and archaeal (red) populations in (a) R_A , (b) R_M and (c) R_S .

Through the determination of microbial diversity in the reactors, the greater degree of solubilization observed in the two-phase CSTR was deduced to be attributed to the predominance and presence of *Firmicutes* in R_A and R_M , respectively, and the lack of such bacteria in R_S . The unique operation (i.e., unintended microaerobic conditions) in R_A possibly led to the co-existence of *Lactobacillus amylovorus* and *Acetobacter peroxydans*, which could have enhanced the acidification process in the two-phase CSTR. An earlier study showed that microaeration resulted in greater degree of hydrolysis and acidification in anaerobic digesters (Lim and Wang, 2013). In addition, R_M had higher prevalence of hydrogen-utilizing methanogens as compared to R_S . The syntrophic association between hydrogenotrophic methanogens and fatty acid-oxidizing syntrophic bacterium could have also contributed to higher degree of acidification in the two-phase CSTR.

Methanosaeta species dominated the archaeal populations of R_M and R_S and this correlated to low levels of acetate in their effluent. The greater distribution of hydrogen-utilizing *Methanoculleus* species in R_M , and that of acetate-utilizing *Methanosaeta* species in R_S possibly led to the lower levels of acetate observed in R_S as compared to R_M . The higher prevalence of hydrogenotrophic methanogens likely gave rise to increased bacterial diversity in R_M . As a result, R_M became more resistant towards process disturbance and had shorter recovery times from shocks.

6.4. Conclusion

The differences in biodegradation efficiencies for single- and two-phase CSTRs could be explained by their microbial community structures. Better reactor performance of two-phase CSTR could be due to the presence of *Firmicutes* as well as greater bacterial diversity and proportion of hydrogenotrophic methanogens. The predominance of *Lactobacillus*, *Acetobacter peroxydans* and *Methanosaeta*, as well as possible syntrophic interactions between fermentative bacteria and hydrogenotrophic methanogens played an important role in maximizing BW and FW

decomposition. The determination of microorganisms involved in the co-digestion process would enhance the understanding of the complex biochemical interactions that promote digester stability and performance.

6.5. Significance of study

Insights gained from this study would enhance the understanding of microorganisms involved in the anaerobic co-digestion of BW and FW as well as the complex biochemical interactions that promote digester stability and performance. This information could help to optimize existing processes and also aid the selection of seeding sludge for rapid startup in future applications.

Chapter 7

7. Microaeration pretreatment batch study

Publication: Lim, J. W., Wang, J.-Y. (2013). Enhanced hydrolysis and methane yield by applying microaeration pretreatment to the anaerobic co-digestion of brown water and food waste. *Waste Management* 33, 813-819.

7.1 Introduction

The first two steps of the AD process – hydrolysis and acidification involve the solubilization of complex particulate organic compounds into simple soluble compounds such as volatile fatty acids (VFAs). They are followed by the acetogenesis step which converts the VFAs to acetate and hydrogen gas that would in turn be consumed by methanogens to produce methane in the final step of the AD process (Batstone et al., 2002). As hydrolysis is usually the rate-limiting step for substrates in particulate form, pretreatments that enhance hydrolysis efficiencies are commonly employed to improve the overall AD process. In order for AD systems to be applicable to land scarce urban cities, there is a need for compact anaerobic digesters. This could be partly achieved by shortening the hydraulic retention time of the AD process by employing suitable pretreatment methods.

Several methods such as mechanical, ultrasonic, thermal, chemical, and biological pretreatments were shown to improve methane production in anaerobic digesters (Dhar et al., 2012; Vavouraki et al., 2013; Benabdallah El-Hadj et al., 2007). However, pretreatments often lead to increased capital costs due to the additional energy or chemicals required (Bordeleau and Droste, 2011). In some cases, the additional methane produced due to pretreatments was insufficient to recover the additional costs (Tartakovsky et al., 2011; Liu et al., 2008) thus rendering the process not feasible financially.

Chapter 6 suggested that the acidogenic reactor (R_A) of the two-phase CSTR was possibly unintentionally operated at microaerobic conditions. This resulted in the predominance and co-existence of *Lactobacillus amylovorus* and *Acetobacter peroxydans* in R_A , which enhanced the acidogenic stage of

AD. In this chapter, the effectiveness of applying microaeration to the co-digestion of brown water (BW) and food waste (FW) was investigated in batch test.

The term “microaeration” was defined by Botheju and Bakke (2011) as the introduction of small amounts of oxygen into an anaerobic biochemical process to enable both anaerobic and aerobic biological activities to occur within a single bioreactor. Microaeration has been used conventionally for the desulphurization of biogas (Fdz-Polanco et al., 2009), and recently it was shown to be an alternative pretreatment to enhance hydrolysis of complex organic matter.

Although oxygen is known to induce inhibitory effects on methanogens which are strict anaerobes, advantages of limited aeration in AD systems have been reported. Earlier studies have shown that limited aeration could reduce formation of toxic metabolites such as lactic acid and ethanol (Zeng and Deckwer, 1996) as well as promote the synthesis of certain lipids required for the stability of anaerobes cell membrane (Ghaly and El-Taweel, 1995). Limited aeration was also shown to enhance the hydrolysis of carbohydrates and proteins (Johansen and Bakke, 2006) as well as the activities of cellulase and protease hydrolytic enzymes (Charles et al., 2009).

Recent studies that discussed the effects of microaeration on AD processes are summarized in Table 1. Some of them reported a positive impact on hydrolysis efficiency (Johansen and Bakke, 2006); (Zhu et al., 2009); (Jagadabhi et al., 2010) and a shorter lag-phase time achieved with microaeration (Díaz et al., 2011a). Díaz et al. (2011a) found that limited oxygen supply to anaerobic digesters did not inhibit methane production and other studies reported that microaeration improved methanogenic activity (Zitomer and Shrout, 1998) and methane yield (Tartakovsky et al., 2011; Botheju et al., 2010; Nguyen et al., 2007). As shown in Table 1, Botheju et al. (2010) reported a 30 – 55% increase in methane yield due to improved solubilization of starch, while another study conducted by Tartakovsky et al. (2011) showed a 26% increase in methane yield when oxygen produced from

the electrolysis of water was applied to the anaerobic digestion of a concentrated stock solution of synthetic wastewater. The enhanced methane production was due to both increased hydrolysis and the conversion of electrolytic hydrogen to methane by hydrogenotrophic methanogens.

However, enhanced hydrolysis under high oxygen levels did not translate to higher methane potential for studies by Zhu et al. (2009) and Johansen and Bakke (2006). In another study, limited aeration brought about a 20% higher methane production but the washout of methanogens and accumulation of substrate were observed as oxygen flux was further increased (Gerritse et al., 1990). Excessive aeration was found to decrease the methane potential of AD systems due to higher aerobic respiration and lower anaerobic fermentation leading to increased biomass and CO₂ generation but lower VFAs and soluble COD levels. In addition, facultative organisms have higher growth rates and would out-compete strict anaerobes under high oxygen levels due to substrate competition. Although the introduction of oxygen could influence a range of reactions in AD systems, Joss et al. (1999) reported that microaeration in AD systems did not affect other biochemical interactions significantly other than aerobic respiration and carbon dioxide generation.

Previous studies on the effects of microaeration in AD systems showed that it led to improved hydrolysis for substrates with complex organic matter, such as sludge, starch, grass-silage and cellulose. However, the effects of microaeration pretreatment on the hydrolysis of substrates with higher biodegradability, such as BW and FW, have yet to be reported. Due to the lack of consistent microaeration intensities, previous studies were not comparable and thus inconclusive in proving the effectiveness of microaeration to the overall AD process. It is also unclear if microaeration will impact biochemical processes other than hydrolysis in the AD process. The aim of this study was to investigate the effect of microaeration pretreatment on the degradation of BW and FW in batch tests. The effects of microaeration on the hydrolysis, acetogenesis and methanogenesis steps would be studied by comparing the hydrolysate from reactors pretreated with microaeration and

the hydrolysate from the anaerobic control reactors during the 4-day pretreatment. This would be followed by the monitoring of methane production from both reactors over the next 40 days. The influence of inoculum on the effects of microaeration was also investigated.

7.2 Experimental set-up

The substrate for this batch study consisted of a mixture of 150 g-FW and 2 L-BW. Batch reactors were operated using 0.25 L continuously stirred reactors with a working volume of 0.2 L. Two series of experiments were conducted in parallel. The first one consisted of the substrate seeded with sludge in a ratio of 50:50 (v/v), giving rise to a $VS_{\text{inoculum}}/VS_{\text{substrate}}$ ratio of 0.5. The other series consisted of only the substrate, which was seeded with sludge after the 4-day pretreatment. The contents in the digesters were flushed with N_2 to provide anaerobic conditions and incubated at 35°C with mixing at 100 rpm. The digesters were fed once at the beginning with the biogas being monitored, and hydrolysate/ digestate extracted regularly using a syringe. The microaeration pretreatment was applied from the start for 4 consecutive days where 37.5 mL O_2/L_R -day was added to the liquid once daily. Due to the relatively low $VS_{\text{inoculum}}/VS_{\text{substrate}}$ ratio of 0.5, biogas production was observed over 40 days in this study. Biogas production as well as the quality of the digestate were monitored throughout the 40 days of batch study. The performance of the digesters pretreated with microaeration was compared to a control with no pretreatment. All experiments were conducted in duplicates.

Table 7-1: Summary of literature carried out on the effects of microaeration on the anaerobic digestion process at mesophilic conditions (35-37°C)

Material	Reactor Scale	Reactor Volume	Aeration Intensity	Effect on hydrolysis efficiency	Effect on degree of acidogenesis	Effect on methane yield	Reference
Primary Sludge	Batch (4 days)	0.5L	33.3L air/kg.TS/d	50-60% increase	-	Negative impact	Johansen and Bakke (2006)
Vegetable and Flower Waste	Batch (5 days)	1.4L	74, 147, 442, 1768L air/kg.TS/d	Positive impact	Negative impact	-	Zhu et al. (2009)
Starch	Batch (17 days)	0.1L	16% of feed COD. Air added once at beginning	-	-	30-55% increase	Botheju et al. (2010)
Grass-silage	Batch, Leach Bed Reactor (60 days)	1.0L	2.5L air (1L/min)	Positive impact	4-fold increase	-	Jagadabhi et al. (2010)
Cellulose	Batch (19 days)	2.1L	0.01L O ₂ /d	Positive impact (shorter lag-phase time)	-	No impact	Diaz et al. (2011a)
Synthetic Wastewater	Batch UASB Reactor, HRT=6hrs	0.5L	3.24×10 ⁻⁴ mL O ₂ /min, from the electrolysis of water	-	-	26% increase	Tartakovsky et al. (2011)
Brown Water and Food Waste	Batch (45 days)	0.25L	0.0375L O ₂ /L _{reactor} /d	Positive impact	Positive impact	10-21% increase	This Study

Biogas production and its composition were monitored every 2-7 days. The extracted hydrolysate/ digestate was measured for its pH, oxidation-reduction potential (ORP), VFA and chemical oxygen demand (COD). Total solids (TS) and volatile solids (VS) were measured only at the beginning and end of the study.

7.3 Results and discussion

The effect of applying microaeration pretreatment to the co-digestion of BW and FW was evaluated in batch reactors. The 4-day microaeration pretreatment was applied to reactors (I) containing substrate inoculated with sludge, and (II) containing substrate only. Sludge was added to reactor (II) after the 4-day pretreatment, and methane production from both reactors were monitored over the next 40 days under completely anaerobic conditions. The hydrolysate and methane yields of reactors with 4-day microaeration pretreatment (MA) were compared to a control that was operated under fully anaerobic conditions throughout the study (AN).

7.3.1 Reactor (I): pretreatment applied to substrate inoculated with sludge

The soluble COD levels of hydrolysate from MA were 6.62 ± 0.01 g/L, 7.00 ± 0.08 g/L, 8.24 ± 0.06 g/L and 6.88 ± 0.06 g/L, while those from AN were 6.00 ± 1.00 g/L, 5.42 ± 0.08 g/L, 5.68 ± 0.03 g/L and 3.06 ± 0.04 g/L after 1-, 2-, 3- and 4-day pretreatment, respectively. This corresponded to 9%, 23%, 31% and 56% higher solubilization for MA after 1-, 2-, 3- and 4-day pretreatment, respectively. The ORP was used to monitor the level of oxygen in the reactors since it could sense the dosing of oxygen even at a level well beyond the detection limit of commercially available oxygen probe. ORP varies between -200 and -300mV in fully anaerobic systems, and increases to above 50mV in aerobic systems (Wall et al., 2008). The ORP of MA ranged between -220 and -278mV throughout the 4-day microaeration pretreatment. This was similar to the ORP of AN (-253 to -270mV), indicating that the oxygen added to MA (37.5 mL-O₂/L_R-d) was consumed by the facultative

microorganisms and both reactors had anaerobic zones where fermentation could occur.

In this study, VFAs i.e. acetic acid (H-Ac), propionic acid (H-Pr), butyric acid (H-Bu), iso-butyric acid (i-H-Bu), valeric acid (H-Va), iso-valeric acid (i-H-Va), caproic acid (H-Ca), iso-caproic acid (i-H-Ca) and heptanoic acid (H-Hp) were analyzed daily during the 4-day pretreatment. The concentrations of VFAs in the hydrolysate after 1-, 2-, 3- and 4-day pretreatment are shown in Figure 7-1(a). The total VFA production for MA was $2,320 \pm 70$ mg-COD/L, $5,900 \pm 1,280$ mg-COD/L, $7,060 \pm 370$ mg-COD/L and $5,570 \pm 70$ mg-COD/L after 1-, 2-, 3- and 4-day pretreatment, respectively. This was 2%, 76%, 35% and 330% higher than that of AN on the respective days of pretreatment.

The compositions of H-Ac and H-Pr for MA were on average 10% higher and 12% lower, respectively than that of AN during the 4-day pretreatment, while the compositions of the remaining SCFAs in the hydrolysate of MA and AN were not significantly different. This suggested that microaeration enhanced the breakdown or oxidation of H-Pr to H-Ac. H-Pr degrading organisms are known to be the slowest growing and the most sensitive acetogenic organisms present in most anaerobic digester ecosystems (Gujer et al., 1995). Thus, the accumulation of H-Pr in reactors often leads to inhibition and reduced organic removals. On the other hand, H-Ac is considered to be a direct substrate for methanogens. The reduced levels of H-Pr and increased production of H-Ac after pretreatment meant a more efficient AD process.

Figure 7-1: Composition of volatile fatty acids for hydrolysate of (a) Reactor I and (b) Reactor II during 4-day pretreatment

The cumulative biogas yields of MA and AN were 530 ± 36 L/kg VS_{fed} and 431 ± 13 L/kg VS_{fed} , respectively (Figure 7-2a). The composition of biogas was tested for H_2 , CH_4 and CO_2 contents. H_2 was present in either small amounts (less than 2% of biogas volume) or not detected in both MA and AN

in this study. As shown in Figure 7-3(a), CH₄ yield of the pretreated substrate (MA) after first day of pretreatment was 14 ± 1 L/kgVS_{fed}, which was 34% higher than the control (AN). For subsequent days, CH₄ yield of MA was inhibited due to pH drop (Figure 7-4a) caused by high degree of COD solubilization and VFA accumulation. After 25 d, CH₄ yield of MA surpassed that of AN, resulting in the cumulative CH₄ yield for MA to be 318 ± 8 L/kgVS_{fed}, while that of AN was 258 ± 15 L/kgVS_{fed}. The improvement in CH₄ yield for MA was due to enhanced solubilization and VFA accumulation after microaeration pretreatment.

Similar to the higher methane yield of MA observed after the first day of pretreatment in this study, Díaz et al. (2011a) also found a shorter lag phase with the microaerobic assays for the anaerobic digestion of cellulose. The accelerated solubilization of cellulose in the very early stages of the digestion led to higher methane production during the first few days. However, the final methane production of cellulose after 19 days was not significantly different between the anaerobic and microaerobic assays. In contrast to the findings of Díaz et al. (2011a), the final methane yield in this study was 21% higher for the pretreated substrates due to the higher degree of solubilization and acidification.

7.3.2 Reactor (II): pretreatment applied to substrate not inoculated with sludge

The soluble COD levels for the hydrolysate in MA were 8.02 ± 0.31 g/L, 8.50 ± 0.00 g/L, 8.01 ± 0.04 g/L and 8.76 ± 0.06 g/L while that for AN were 13.50 ± 0.25 g/L, 14.20 ± 1.13 g/L, 13.63 ± 0.04 g/L and 13.79 ± 0.07 g/L after 1-, 2-, 3- and 4-day pretreatment, respectively. The ORP of MA ranged between -17 and -67mV throughout the 4-day microaeration pretreatment. This was similar to the ORP of AN (-20 to -64mV), indicating that the oxygen added to MA ($37.5 \text{ mL-O}_2/\text{LR-d}$) was consumed by the facultative microorganisms and both reactors had anaerobic zones where fermentation could occur.

Figure 7-2: Average cumulative biogas yield of —■— MA and —□— AN for (a) Reactor I and (b) Reactor II throughout the entire experiment duration

Figure 7-3: Average cumulative methane yield of —■— MA and —□— AN for (a) Reactor I and (b) Reactor II throughout the entire experiment duration

The concentrations of SCFAs in the hydrolysate after 1-, 2-, 3- and 4-day pretreatment are shown in Figure 7-1b. The total VFA production for MA was $2,700 \pm 220$ mg-COD/L, $2,840 \pm 120$ mg-COD/L, $3,520 \pm 550$ mg-COD/L and $3,570 \pm 480$ mg-COD/L after 1-, 2-, 3- and 4-day pretreatment, respectively. This was 17%, 22%, 39% and 78% higher than that of AN on the respective days of pretreatment. The composition of H-Ac for MA was on average 10%

higher than that of AN during the 4-day pretreatment. Similar to the observations for reactor (I), the application of microaeration pretreatment enhanced the conversion of other SCFAs to H-Ac for reactor (II).

It was also observed that the extent of VS degradation was 10% higher for MA than for AN, suggesting that the application of microaerobic conditions could have led to the production of exoenzymes by aerobic microorganisms, which degraded compounds that were otherwise resistant to degradation under fully anaerobic conditions. In an earlier study, Hasegawa and Katsura (1999) also found that microaerobic conditions stimulated the production of exoenzymes by aerobic microorganisms.

Figure 7-4: Average pH levels of —■— MA and —□— AN for (a) Reactor I and (b) Reactor II throughout the entire experiment duration.

As shown in Figure 7-4b, the pH of pretreated substrate (3.0 to 3.5) was slightly lower than that of the control during pretreatment, reflecting the higher VFA accumulation due to microaeration. After 4 days, the substrate was seeded with sludge and a rise in pH to levels above 6.5 was observed. Subsequently, the pH of both MA and AN were not significantly different.

Figure 7-2b shows the cumulative biogas yields and composition of biogas for MA and AN. The cumulative biogas yield of MA and AN were 456 ± 23 L/kg VS_{fed} and 385 ± 9 L/kg VS_{fed}, respectively. H₂ was present in negligible amounts (less than 2% of biogas volume) while the average CH₄ and CO₂ contents for MA was 64% and 36%, and that for AN was 62% and 38%, respectively.

Figure 7-3b shows that the CH₄ yields of MA and AN were not significantly different for the first 24 days, at 185 ± 7 L/kgVS_{fed} and 175 ± 3 L/kgVS_{fed}, respectively. However, the final cumulative CH₄ yield of MA was 256 ± 9 L/kgVS_{fed}, while that for AN was 233 ± 17 L/kgVS_{fed}. This 10% improvement in final CH₄ yield for MA could be due to its 10% higher VS degradation. This could mean that microaeration promoted degradation of slowly biodegradable compounds which were otherwise resistant to degradation under fully anaerobic conditions.

7.3.3 Comparison between reactors (I) and (II)

As described in sections 7.3.1 and 7.3.2, the enhancements of COD solubilization and acidification after microaeration pretreatment were more significant for the 150g-FW/2L-BW mixture when inoculated with sludge. This led to an improvement in CH₄ yield by 21% for reactors (I) and 10% for reactors (II). The application of microaeration to the inoculum could have enhanced the production and activities of hydrolytic and acidogenic bacteria present in it, hence giving rise to higher hydrolysis and acidification efficiencies. Botheju et al. (2010) also found that the nature of inoculum played a significant role on the level of oxygen induced enhancement of CH₄ yield. In contrary to the negative perception of inhibitory and toxic effects of

oxygen on methanogens, the application of microaerobic conditions to the inoculum did not cause any inhibition effects to the AD process.

In both reactors, the composition of H-Ac in the hydrolysate was 10% higher for MA than for AN, indicating improved conversion of SCFAs to H-Ac. Another common observation for both reactors (I) and (II) was the pH drop induced by microaeration pretreatment. As shown in Figure 7-4, pH levels of MA were in general lower than that of AN during the 4-day microaeration pretreatment period due to enhanced COD solubilisation and VFA accumulation. The lower pH of MA also led to inhibition of CH₄ yields for the initial 15 days of batch study (Figure 7-3a). Hence, another potential advantage of applying microaeration pretreatment would be to control CH₄ formation in the pretreatment stage of AD.

7.3.4 Final discussion

Hydrolysis is the breakdown and solubilization of particulate matter by extracellular enzymes excreted by hydrolytic microorganisms. Because of the higher hydrolytic enzyme production, hydrolysis efficiency is known to be higher under aerobic as compared to anaerobic conditions (Gujer et al., 1995; Johansen and Bakke, 2006). The intensity of microaeration determines both the hydrolysis efficiency and the degree of acidification, thus affecting the methane yield of the AD process. At a microaeration intensity of 33.3L air/kg.TS/d, Johansen and Bakke (2006) found a 50-60% increase in hydrolysis efficiency but a negative impact on methane yield during the anaerobic digestion of primary sludge. Similarly, Zhu et al. (2009) found a positive impact on hydrolysis efficiency but a negative impact on acidogenesis when a microaeration intensity of 74L air/kg.TS/d was applied to the anaerobic digestion of vegetable and flower waste. At these microaeration intensities, the extra solubilized organic matter was converted into CO₂ and new biomass by the aerobic respiration of facultative microorganisms, leaving less substrate for methanogenesis. To ensure the conversion of extra solubilized organic matter into methane, an increase in the degree of COD solubilization and the accumulation of VFAs must be achieved.

At a microaeration intensity of 37.5 mL-O₂/ L_R-d, enhanced solubilization of organic matter without the oxygen-related inhibition of acidogenesis was achieved in this study. The accumulation of VFA achieved with microaeration pretreatment was also observed previously by Hasegawa et al. (2000), who found that the thermophilic pretreatment of sludge under microaerobic conditions led to VFA accumulation, and Jagadabhi et al. (2010), who found a 4-fold increase in VFA levels upon inducing microaerobic conditions (2.5 L air at 1L/min) to the fermentation of grass-silage.

In this study, the enhanced solubilization and acidification achieved with microaeration pretreatment at 37.5 mL-O₂/ L_R-d resulted in 21% and 10% higher CH₄ production for the anaerobic co-digestion of BW and FW in reactors (I) and (II), respectively. However, the improvement in CH₄ yield by microaeration pretreatment was only observed after 25 d. This was due to the inhibition of methanogenesis at low pH levels caused by enhanced hydrolysis and acidification in MA. The enhancement of CH₄ yield by microaeration pretreatment could also be observed earlier if the VS_{inoculum}/VS_{substrate} ratio was higher.

Unlike other pretreatment methods that improve the overall AD process by increasing the hydrolysis efficiency, this study showed that microaeration pretreatment could also enhance the conversion of other SCFAs to H-Ac, and no inhibition of methanogenesis was observed. Microaeration pretreatment does not require the addition of chemicals but only a limited supply of air. Thus, it could be a cost effective pretreatment since anaerobic digesters are inevitably subjected to varying levels of limited and unintended aeration due to activities such as feeding and effluent removal. As pointed out by Botheju et al. (2010), the optimum oxygenation level for maximum methane yield of an AD system could depend on various factors such as the reactor biomass concentration and feed composition. Hence, one oxygen supply rate that worked well in one AD system may not do so in others. We could thus rely on ORP readings to control the amount of oxygen entering the AD systems. ORP sensors could be integrated in anaerobic digesters to monitor or control the

intensity of applied microaeration, so as to avoid aerobic respiration and damage to those critical strict anaerobic microorganisms due to excess oxygen. However, further investigations are still required to develop a suitable operating system for the application of microaeration pretreatment in AD systems.

7.4 Conclusion

This study demonstrated the benefits of adding small amounts of oxygen in AD systems. The results concluded that microaeration is an effective pretreatment for the anaerobic co-digestion of BW and FW. Besides increased solubilization and acidification efficiencies, microaeration enhanced the breakdown of other SCFAs to H-Ac. The application of microaeration pretreatment was also suggested to stimulate the production of exoenzymes that carried out the degradation of slowly biodegradable compounds which were otherwise resistant to degradation under fully anaerobic conditions. The more significant enhancement in solubilization and acidification efficiencies observed for the inoculated substrate after pretreatment suggested that the nature of inoculum influenced the effects of microaeration, likely through the higher production and activities of hydrolytic and acidogenic bacteria present in the inoculum under microaerobic conditions. The microaeration pretreatment was sufficient to increase the degree of solubilization but not enough for the subsequent VFA oxidation, thus resulting in higher methane yields. The accumulated VFA achieved with microaeration pretreatment resulted in 21% and 10% higher CH₄ yield when pretreatment was applied to inoculated substrates, and substrates without inoculum, respectively.

7.5 Significance of study

An alternative to current centralized sanitation systems is the treatment of different waste streams, separated at source, in a decentralized system. This system recovers energy and nutrients from the AD of waste high in organic content, such as wastewater containing feces (BW) and FW. Decentralized anaerobic digesters need to be compact in order to be

applicable to land scarce urban cities. This could be partly achieved by shortening the hydraulic retention time of the AD process by employing suitable pretreatment methods. Microaeration is a possible pretreatment method and several studies have shown that it enhanced the hydrolysis of substrates with complex organic matter. However, the effects of microaeration pretreatment on substrates with higher biodegradability such as BW and FW have yet to be reported. This study explored the role of microaeration pretreatment in the anaerobic co-digestion of BW with FW. Enhanced hydrolysis was observed which was in agreement with previous studies. In addition, this study showed that microaeration pretreatment led to greater VFA accumulation and conversion of other SCFAs to H-Ac. It also revealed that the nature of inoculum influenced the effects of microaeration as a 21% increase in CH₄ yield was observed when pretreatment was applied to inoculated substrates.

Chapter 8

8. Microbial community structure reveals how microaeration improves fermentation during anaerobic co-digestion of brown water and food waste

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8.1. Introduction

Anaerobic digestion (AD) refers to the fermentation process that produces biogas (composed of mainly methane and carbon dioxide) from the degradation of organic material. Due to the production of useful energy in the form of biogas, AD has been widely applied for the treatment of organic waste such as brown water (BW) and food waste (FW) (Rajagopal et al., 2013; Curry and Pillay, 2012; Zeeman et al., 2008). AD is a complex degradation pathway that proceeds in four successive stages, namely hydrolysis, acidogenesis, acetogenesis and methanogenesis. Acid-forming bacteria carry out hydrolysis, acidogenesis and acetogenesis, while methane-forming archaea produce biogas during methanogenesis. Due to the different nutritional needs of the microorganisms involved in each stage, the physical separation of acid- and methane-forming microorganisms in two separate reactors was first proposed by Pohland and Ghosh (1971) to provide optimum environmental conditions for each group of microorganisms. Recent studies have indeed shown that two-phase systems could lead to enhanced stability and control of the overall AD process (Nizami and Murphy, 2011; Demirel and Yenigun, 2002; Lim et al., 2013).

Though the AD technology existed for more than 100 years, there are still unresolved challenges faced by AD operators, thus limiting the implementation of more AD plants. In view of current global concerns over environmental sustainability, AD is regarded as a promising process due to its potential in renewable energy generation and waste stabilization.

However, one of the main problems faced by AD operators is its inherent instability since AD is alarmingly sensitive to changes in operation and feed conditions. Accidental or unavoidable oxygen loading is one aspect of this problem. Theoretically, the AD process will be inhibited when exposed to oxygen. However, several studies have shown that partial aeration did not cause any inhibition, but enhanced AD performance instead (Botheju and Bakke, 2011). According to Botheju and Bakke (2011), the presence of oxygen led to a higher yield and population of facultative acidogens and therefore higher amount of enzymes excreted. It was hypothesized that more acidogenic biomass leads to more hydrolysis, since hydrolysis is carried out by the extracellular enzymes excreted by acidogens.

The term microaeration refers to the controlled introduction of small amounts of oxygen into an anaerobic biochemical process to enable both anaerobic and aerobic biological activities to occur within a single bioreactor. The term microaeration will be used in this study to describe the conditions of adding oxygen to the acidogenic reactor. The study by Rolfe et al. (1978) was among the first few that investigated the oxygen tolerance level of anaerobic bacteria. Zitomer and Shrout (1998) subsequently reported that oxygen addition did not inhibit the growth of methanogens, but increased their initial activity. More recently, several studies reported advantages of microaeration in terms of higher degree of solubilization and acidification of organic matter (Lim and Wang, 2013; Mshandete et al., 2005; Díaz et al., 2011a; Jagadabhi et al., 2010; Díaz et al., 2010). Therefore, microaeration has been regarded as a potential pre-treatment method for improving the hydrolysis stage during the AD process. Another reported benefit of microaeration was the cleaning of biogas by removing more than 99% hydrogen sulfide (H₂S) (Ikbal et al., 2003; Díaz et al., 2011b; Tang et al., 2004). As compared to the other chemical and physical pre-treatment methods or biological processes for the desulphurization of biogas, microaeration of AD system has a relatively smaller footprint and require lower investment costs as well as small modification to the existing process (Ramos et al., 2014).

An earlier study on the anaerobic co-digestion of BW and FW (Lim et al., 2013) reported the unexpected predominance of aerobic bacteria species – *Acetobacter peroxydans* in the acidogenic reactor of a two-phase continuously stirred tank reactor (CSTR). As the acidogenic reactor was operated under anaerobic conditions, the predominance of an aerobic bacteria species suggested the reactor might be unknowingly exposed to partial aeration. Despite the predominance of *Acetobacter peroxydans*, the AD system achieved high degrees of COD solubilization and VFA production. This study illustrated that in case of accidental or unavoidable oxygen loading, the fermentation process of AD was not compromised.

In view of the tendency for accidental or unavoidable oxygen loading, as well as the benefit of microaeration in terms of enhanced fermentation and desulphurization of biogas, it is important to understand how AD systems respond to small amounts of oxygen stress. Determining the microbial diversity of reactors will provide more insights on the changes in the biochemical processes due to microaeration conditions. However, information on the microbiology of anaerobic digesters operated under microaeration conditions is limited (Ramos et al., 2014; Zhou et al., 2007; Tang et al., 2004).

Tang et al. (2004) reported that microaeration led to a decrease in *Methanosarcina* and increase in *Methanoculleus* populations while Zhou et al. (2007) found that limited aeration caused the predominant microorganisms to change from rod-shape to cocci-shaped methanogens in the UASB reactor. The DGGE analysis carried out by Ramos et al. (2014) showed that oxygen affected the richness, evenness and structure of the bacterial and the archaeal communities in the long term. These studies mainly discussed the effect of microaeration on the archaeal populations and very little is known about the bacterial community shifts due to oxygen. Since one of the main benefits of microaeration was reported to be enhanced hydrolysis, it is essential to have a more detailed understanding of how microaeration affects the bacterial population. Therefore, the objective of this study was to investigate how microaeration affected the fermentation process in the

anaerobic co-digestion of BW and FW. This aim was achieved by determining the bacterial community structure of the acidogenic reactor of a two-phase CSTR and correlating the microbial structure to the reactor's performance.

8.2. Experimental set-up

The feed for this study consisted of a mixture of 150 g blended food waste (FW) and 2 L brown water (BW), with an average pH of 5.96 ± 0.22 . The co-substrates were prepared and fed daily to the acidogenic reactor of a two-phase CSTR system, in batch mode. The working volume of the reactor was 3 L and the contents were mixed continuously at 80 rpm by an overhead mechanical stirrer. The reactor was initially inoculated with sludge collected from another acidogenic reactor treating BW and FW at 35°C for more than one year. With hydraulic retention time (HRT) of 4 days, the organic loading rate (OLR) for the acidogenic reactor was maintained at 5.15 ± 0.44 g-VS/L/d in this study. As shown in Table 1, the study consisted of three operating conditions. The reactor was operated under anaerobic conditions (AN) from week 1 to 6, low microaeration conditions (MA1) from week 7 to 13, and higher microaeration conditions (MA2) from week 14 to 20. Oxygen was added to the liquid part of the reactor at a rate of 3 mL/min daily one to two hours after feeding.

To further understand the system, biomass were collected towards the end of each condition of the study for microbial community characterization. Genomic DNA in the biomass samples were extracted using chemical lysis and phenol-chloroform-isoamyl alcohol (25:24:1, v:v:v) purification protocol. Primer set 8F (5'-AGAGTTTGATYMTGGCTC-3') and 1490R (5'-GGTTACCTTGTTACGACTT-3') was used to amplify Bacterial 16S rRNA gene from the total-community DNA. The thermal program used for amplification of 16S rRNA gene was as follows: hotstart 94°C for 3 min, 30 cycles of denaturation (30 s at 94°C), annealing (30 s at 54°C), extension (45 s at 72°C) and a final extension at 72°C for 5 min.

Molecular cloning and sequencing was used to determine the microbial consortium in the reactor. TOPO TA cloning kit (Invitrogen, CA) was used for clone library construction according to the manufacturer's instructions. Approximately 100 clones were randomly selected for members in the domain Bacteria (amplified by primer set 8F and 1490R). The amplified DNA insert was then PCR amplified with a vector-specific primer set (i.e., M13F and M13R). The 16S rRNA gene fragments were screened by restriction fragment length polymorphism (RFLP) to further remove the possible redundant clones. This was followed by the separate digestion of M13-PCR products with tetramer restriction enzymes *MspI* and *RsaI* (New England BioLabs, UK). Electrophoresis was carried out to separate the digestion products in a 3% agarose gel. The gels were then visualized using the FireReader gel documentation (UVIttec, Cambridge, UK) after staining with Gelred (Invitrogen, CA). Unique RFLP patterns were defined as a unique sequence type of operational taxonomy unit (I).

The 16S rRNA genes of the representative clones with different RFLP patterns were sequenced, by Axil Scientific Sequencing (Singapore), to determine their phylogenetic affiliation. Nearly full-length 16S rRNA gene sequences of representative clones were compared to available rRNA gene sequences in GenBank using the NCBI BLAST program. Chimeric artifacts were determined using DECIPHER (Wright et al., 2012) and phylogenetic trees were constructed with MEGA5 program using the remaining 61 bacterial clone sequences after removing chimeric sequences. The Jukes-Cantor correction was used for distance matrix analyses and the trees were constructed using the Neighbor-joining method. Bacterial 16S rRNA partial sequences obtained in this study were deposited in the nucleotide Genbank database, under the accession numbers: KJ907449 – KJ907466.

8.3. Results and discussion

8.3.1. Oxidation reduction potential (ORP) levels

ORP level was measured daily to monitor the level of oxidants in the reactor. In fully anaerobic systems, ORP values vary between -200 and -300 mV while in aerobic systems, ORP increases to above 50 mV. (Henze, 2008) As shown in Figure 8-1a, ORP levels dropped to negative values within 1-2 weeks from the start of the study. Throughout the AN condition, the average ORP levels decreased from 226 mV (in week 1) to -168 mV (in week 6). According to Xu et al. (2014), the optimum ORP range for acid fermentation was between -100 mV to -250 mV. In this study, the acidogenic reactor reached redox conditions favoring fermentation within 2 weeks from the start of the study.

Operation changed from anaerobic (AN) to microaeration (MA1) conditions during week 7, where 5 mL-O₂/L_R/d was added to the acidogenic reactor. Due to the introduction of oxygen into the acidogenic reactor, ORP levels became less negative. Although ORP values were still within the negative range, the rising ORP levels indicated that the microorganisms did not manage to adapt well to the sudden supply of oxidants. ORP levels only recovered to between -157 mV to -255 mV (i.e., the range favorable for fermentation) towards the end of MA1 (in week 12). This could be attributed to the acclimatization of microbial community towards microaeration conditions in the acidogenic reactor. The ORP trend suggested that the growth of microorganisms able to survive oxidative stress were stimulated during MA1, which led to the lowering of ORP levels towards the end of MA1.

The oxygen dosage increased from 5 mL-O₂/L_R/d to 7 mL-O₂/L_R/d from week 14 onwards (MA2 conditions). Due to the higher oxidant level present in the acidogenic reactor, ORP levels increased slightly but quickly stabilized at levels between -117 mV to -256 mV after one week.

Figure 8-1: Performance of acidogenic reactor: (a) —□— ORP, (b) —●— $\text{NH}_3\text{-N}$, (c) —■— pH, (d) —○— Biogas volume and —△— % H_2

This suggested that the bacterial groups cultivated during MA1 were better able to tolerate or consume additional oxygen added and adapted to

higher levels of oxidants quickly. According to Table 8-1, the average ORP levels during AN, MA1 and MA2 conditions were -97 mV, -100 mV and -172 mV, respectively. Although oxygen was added to the acidogenic reactor during MA1 and MA2, the average ORP values during each period became increasingly negative with higher levels of oxygen added. After an adaptation period of approximately six weeks (during weeks 7 to 12), the bacterial population in the acidogenic reactor was able to effectively consume oxygen and maintain a reducing environment such that fermentation could occur despite higher amounts of oxygen introduced.

8.3.2. NH₃-N, pH and biogas production

According to Table 8-1, the average NH₃-N levels during AN, MA1 and MA2 were 96 mg/L, 110 mg/L and 99 mg/L, respectively. As shown in Figure 8-1b, microaeration led to increasing NH₃-N levels, and were highest during weeks 7 and 15 – the first one to two weeks after introducing MA1 and MA2 conditions, respectively.

During anaerobic degradation, proteins are solubilized into amino acids, which are then degraded into NH₃ and VFAs such as acetic acid (H-Ac), propionic acid (H-Pr) and butyric acid (H-Bu). Therefore, the spikes in NH₃-N levels observed during increasing dosage of oxidants suggested that microaeration led to a greater extent of proteins degradation. NH₃-N levels were highest during week 7 (158 mg/L) and week 15 (125 mg/L) but quickly returned to lower levels after one and two weeks of MA1 and MA2, respectively. The drop in NH₃-N levels during weeks 8 and 16 could mean the microorganisms quickly adapted to increased levels of oxidants.

pH levels ranged between 3.5 to 4.6 in this study (Figure 8-1c). As shown in Table 8-1, pH levels increased from 4.00 to 4.12 and dropped from 4.12 to 4.07 as conditions changed from AN to MA1 and from MA1 to MA2, respectively. On top of that, Figures 1b and 1c show that pH levels followed the same trend as NH₃-N levels. pH levels rose with increasing levels of NH₃-N and pH levels dropped as NH₃-N levels decreased.

Table 8-1: Average values of parameters for acidogenic reactor during AN, MA1 and MA2 conditions

Condition	Week	pH	ORP (mV)	NH ₃ -N (mg/L)	TVFA (mg-COD/L)	H-Ac	H-Pr	H-Bu	SCOD (mg/L)
AN	1-6	4.00	-97	96	7,789	1,560	918	2,440	17,105
MA1 (5 mL- O ₂ /L _R /d)	7-13	4.12	-100	110	11,14	1,608	1,715	4,199	17,529
MA2 (7 mL- O ₂ /L _R /d)	14-20	4.07	-172	99	10,28	1,337	1,541	4,482	17,775

According to the Stickland reaction, NH₃ is released during the acidogenic conversion of amino acids. Since NH₃ is a weak base that will partially react with water to produce an equilibrium concentration of hydroxyl anion (OH⁻), increased levels of NH₃-N led to a pH rise. Biogas produced from the fermentation process was measured daily. As shown in Figure 8-1d, the biogas production and its composition of H₂ also followed the same trend as pH.

8.3.3. Soluble COD and VFA

According to Table 8-1, the average levels of soluble COD and TVFA were higher during MA1 and MA2, as compared to AN conditions. This indicated that microaeration conditions led to a greater extent of solubilization and acidification as compared to anaerobic conditions. The increase in TVFA levels could also be attributed to the enhanced degradation of protein since the degradation of proteins produces VFAs such as H-Ac, H-Pr and H-Bu. In addition, Ding (2010) also found that protein degradation promoted the activity of acidogenic microorganisms by providing readily available organic nitrogen in the form of soluble protein and amino acids. Therefore, enhanced protein degradation during MA conditions contributed to the rise in VFA production.

Among the VFAs produced, 60% were composed of H-Ac and H-Bu, where H-Bu accounted for up to 42%. In this study, the change in TVFA levels is mainly influenced by H-Bu levels. As shown in Figure 8-2, the increase and decrease in TVFA levels follow the same trend as that of H-Bu levels. High concentrations of H-Ac and H-Bu was also reported in other studies that utilized food waste as substrate for anaerobic digestion (Xu et al., 2014; Han and Shin, 2004). According to Xu et al. (2014), H-Bu and H-Ac are regarded as preferred precursors for CH₄ production. Therefore, conversion of organic matter into H-Ac and H-Bu in the hydrolytic-acidogenic stage will improve the overall energy yield and increase the process efficiency.

With higher dosage of oxidants, pH, TVFA and H-Bu levels increased. On the other hand, H-Ac levels increased during MA1 but decreased during MA2. As shown in Figure 8-2, H-Ac levels dropped as H-Bu levels increased. While % H-Ac of TVFA dropped from 38% to 16% to 14%, % H-Bu increased from 22% to 37% to 42% during AN, MA1 and MA2 conditions, respectively. This suggested that microaeration not only accelerated fermentation, but also had an impact on the fermentation product patterns (i.e., shifted the metabolism from H-Ac production to H-Bu production).

The high level of H-Bu reported in this study was likely due to the presence of butyrate-producing bacteria. H-Bu production generally proceeds via two distinct pathways, via butyrate kinase or butyryl coenzyme A (CoA)-acetyl CoA transferase (Pryde et al., 2002; Duncan et al., 2002). The study by Pryde et al. (2002) reported that the butyryl CoA-acetyl CoA transferase pathway was more dominant in the human bacterial flora. Another study also found that 50% of the butyrate-producing isolates were net H-Ac consumers during growth, suggesting the preference of the butyryl CoA-acetyl CoA transferase pathway (Morrison et al., 2006). This was similar to the results reported in a mixed human faecal incubation study which found that a significant amount of carbon in H-Bu was contributed by free H-Ac (Barcenilla et al., 2000). Since the butyryl CoA-acetyl CoA transferase pathway is more dominant in the human bacterial flora, the high levels of H-Bu and low levels

of H-Ac during microaeration conditions was likely due to the metabolism of H-Ac by the butyrate-producing bacteria, for the synthesis of H-Bu.

It was also observed that H-Ac levels decreased whenever H₂ production increased. During acetogenesis, hydrogen-producing acetogenic bacteria convert higher VFAs such as H-Pr and H-Bu into H₂, CO₂ and H-Ac. H₂ production by acetogens is generally energetically unfavorable due to high free energy requirements. Without the combination of H₂-consuming bacteria in the acidogenic reactor, conditions were unfavorable for the production of H-Ac, therefore contributing to the drop in H-Ac levels.

Figure 8-2: Performance of acidogenic reactor: –△– SCOD, –□–TVFA, –○– H-Ac, –●–H-Pr and –▲– H-Bu

8.3.4. Microbial community characterization

8.3.4.1. Overview

As shown in Figure 8-3, the bacterial community structure of the acidogenic reactor was composed of phyla *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Actinobacteria* in proportions of 72%, 24%, 2% and 2% of the bacterial clones, respectively. In total, 18 bacterial operational taxonomic units (I) were identified. Within the 18 I, 13 were classified as *Firmicutes*, 3 as *Bacteroidetes*, 1 as *Proteobacteria* and 1 as *Actinobacteria*. The most detected I (R1Bac_19), representing 33% of the total clones, was affiliated to *Megasphaera* species NMBHI-10 (HM990965) with 99% similarity. R1Bac_18 was the second most detected I accounting for 18% of the clones and was closely related to uncultured *Prevotella* species (DQ168844). The third most detected I was R1Bac_51 (10% of the bacterial clones) and was affiliated to *Lactobacillus amylovorus* GRL 1112 (NR_075048). R1Bac_19 and R1Bac_51 both belonged to phylum *Firmicutes*, which is known to produce cellulases, lipases, proteases and other extracellular enzymes (Levén et al., 2007). Therefore the predominance of *Firmicutes* (72% by cloning analysis) reflected the ability of the acidogenic reactor to metabolize a variety of substrates including protein, lipids, lignin, cellulose, sugars and amino acids, which are commonly found in FW.

The predominance of *Firmicutes* was also reported in several studies that investigated the AD of FW (Tang et al., 2004; Lim et al., 2013; Shin et al., 2010). The next predominant phylum *Bacteroidetes* (24% by cloning analysis) represents a group of fermentative microorganism and is also a major microbial component of anaerobic reactors. In addition, *Bacteroidetes* is known to be proteolytic and play important roles in stabilizing semi-solid waste (Rivière et al., 2009; Kindaichi et al., 2004).

Figure 8-3: Phylogenetic tree of 16S rRNA gene sequences constructed for bacterial clones from acidogenic reactor of two-phase CSTR. The 16S rRNA gene sequence of *Sulfolobus acidocaldarius* (NR_043400) was used as the outgroup

8.3.4.2. Dominant bacterial species

Bacteria within the phyla *Firmicutes* and *Bacteroidetes* represented the exclusive dominant phylogenetic group (96% by cloning analysis) in the acidogenic reactor, suggesting a major impact of these bacteria on the fermentation of BW and FW under microaerobic conditions. R1Bac_19 (33% of total clones) is closely related to the *Megasphaera* species NMBHI-10, whose genome was reported to code for enzymes essential for carbohydrate fermentation to yield H₂, CO₂, and VFAs such as H-Bu, H-Pr and H-Ac (Shetty et al., 2013).

The co-culture of lactic acid producing bacteria and *Megasphaera elsdenii* was previously shown to stimulate H-Bu production (Tsukahara et al., 2006). In this study, R1Bac_51 (10% of total bacterial clones) was affiliated to *Lactobacillus amylovorus* – a lactate-producing bacteria possessing amylolytic activity. Therefore, the high levels of H-Bu in this study could also be due to the co-existence of *Lactobacillus amylovorus* and *Megasphaera* sp.. In addition, butyryl-CoA dehydrogenase – the enzyme involved in the butyryl CoA-acetyl CoA transferase pathway was present in the genome of *Megasphaera* sp. NMBHI-10. In the butyryl CoA-acetyl CoA transferase pathway, H-Ac is metabolized by butyrate-producing bacteria for the synthesis of H-Bu. Therefore, the predominance of *Megasphaera* sp. Would explain the increasing levels of H-Bu and decreasing levels of H-Ac observed during microaerobic conditions in this study.

On top of carbohydrate metabolism, the genome of *Megasphaera* sp. NMBHI-10 also had several mechanisms for protection against oxidative stress. The presence of enzymes capable of regulating oxidative stress thus allowed the survival of *Megasphaera* sp. Under microaerobic conditions. The ability of *Megasphaera* species to survive under oxidative stress and ferment soluble COD into H-Bu correlated well to the negative ORP levels, increasing levels of soluble COD, rising H-Bu levels and falling H-Ac levels observed during microaerobic conditions.

The second predominant group – R1Bac_18 (18%) was closely related to uncultured *Prevotella* sp. (DQ168844). *Prevotella* belongs to phylum *Bacteroidetes* and is a group of asaccharolytic, anaerobic and proteolytic bacteria. Under anaerobic conditions, asaccharolytic bacteria ferment nitrogenous compounds into organic acids and ammonia while proteolytic bacteria degrade nitrogenous compounds into small peptides and amino acids (Takahashi, 2003). During amino acid degradation, *Prevotella* sp. Are capable of producing organic acids and ammonia. The base produced contributed to the acid-neutralizing activity and played a role in maintaining pH in the reactor. The predominance of R1Bac_18, which was affiliated to

uncultured *Prevotella* sp., would explain the higher levels of NH₃-N observed during microaerobic conditions. The capability of *Prevotella* to neutralize pH and produce organic acids by nitrogenous metabolism would also account for the increasing pH and TVFA levels.

8.3.4.3. Effect of microaeration on bacteria involved in fermentation of brown water and food waste

An earlier study investigated the microbial community for the anaerobic co-digestion of BW and FW in two-phase CSTR (Lim et al., 2013). As shown in Table 8-2, there were clear differences in phylogenetic distribution between reactors operated under microaerobic and anaerobic conditions. In comparison to the reactor operated under anaerobic conditions, the bacterial community of the acidogenic reactor operated under microaerobic conditions (in this study) was significantly more diverse.

A total of 18 bacterial I (classified into 4 phyla) were identified for microaerobic conditions whereas only 7 bacterial I (classified into 2 phyla) were identified for anaerobic conditions. In particular, the distribution of bacteria within the phylum *Firmicutes* was higher and that of *Proteobacteria* was lower in the microaerobic reactor. Since *Firmicutes* are an important group of fermentative bacteria that produces extracellular enzymes, the greater distribution of *Firmicutes* in the microaeration reactor led to a higher degree of hydrolysis during microaeration as compared to anaerobic conditions. A comparison of the bacterial communities in this study and that of Lim et al. (2013) showed that the presence of oxygen in the anaerobic digester caused the bacterial community structure to change quite significantly. Microaeration conditions also led to the development of a more diverse bacterial population that will likely enable the acidogenic reactor to metabolize a greater variety of substrates.

A study by Tang et al. (2004) investigated the microbial communities of thermophilic digesters treating synthetic FW under anaerobic and microaeration conditions. Similar to this study, the study by Tang et al.

Table 8-2: Comparison of bacterial community in acidogenic reactor operated under microaerobic and anaerobic conditions

	With microaeration (This study)	No microaeration (Lim et al., 2013)
Bacterial I	18	7
Phylum	4	2
<i>Firmicutes</i>	72%	58%
<i>Proteobacteria</i>	2%	42%
<i>Bacterioidetes</i>	24%	0%
<i>Actinobacteria</i>	2%	0%

(2004) found that microorganisms affiliated with the phylum *Firmicutes* were dominant, independent of the aeration conditions. However, Tang et al. (2004) reported that microaeration did not cause a dramatic shift in the structure of microbial community. On the contrary, this study showed that microaeration has led to obvious effects on the bacterial diversity in the mesophilic digester treating BW and FW. Although *Firmicutes* was predominant in both microaeration conditions (72%) and anaerobic conditions (58%), both the diversity and composition of *Firmicutes* were significantly higher in the microaeration as compared to anaerobic reactor.

8.4. Conclusion

The bacterial population for microaeration reactor was composed of phyla *Firmicutes*, *Bacterioidetes*, *Proteobacteria* and *Actinobacteria* in proportions of 72%, 24%, 2% and 2% of the bacterial clones, respectively. Many of the bacterial species possessed the ability to consume oxygen and maintain a reducing environment such that fermentation could occur despite higher amounts of oxygen introduced. As compared to anaerobic conditions, microaeration led to a significantly more diverse bacterial community. There was a greater distribution of *Firmicutes*, which enabled the acidogenic reactor to metabolize a greater variety of substrates, giving rise to enhanced COD solubilization and VFA production under microaeration conditions.

Chapter 9

9. Conclusion

In this research, the anaerobic co-digestion of BW and FW was investigated through biochemical methane potential (BMP) tests in Chapter 4, 5L and 30L reactors performance studies in Chapters 5 and 6 as well as microbiology studies in Chapter 6. On top of that, the role of microaeration pretreatment on the co-digestion system was investigated in BMP tests (Chapter 7) and through performance and microbial studies (Chapter 8). This research showed that the co-digestion of brown water and food waste was optimized through phase separation and operation in a sequencing batch reactor. The microaeration studies demonstrated the positive effects of introducing small and controlled amounts of oxygen to the anaerobic digestion system to enhance the fermentation processes. Reactor performance studies and microbial profiling conducted in this research provided insights to how phase separation and microaeration enhanced biogas production from the co-digestion of brown water and food waste. Knowledge gained from this research could help to optimize existing anaerobic digestion processes and aid the selection of seeding sludge for rapid startup of source-separation-based anaerobic digestion systems in future applications.

Chapter 10

10. Reference list

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11. Appendices

Figure 11-1: Building site of ecological housing estate at Lübeck-Flintenbreite, Germany (Otterwasser GmbH, 2009)

Figure 11-2: The three water systems of pilot project 'Flintenbreite' (Otterwasser GmbH, 2009).

Figure 11-3: Vacuum toilets with a very low water consumption of 0.7 to 1.2-L per flush (Otterwasser GmbH, 2009).

Figure 11-4: Illustration of UASB septic tanks used to treat black water collected from 32 houses in Sneek development. Biogas is collected in a gas bag on the roof (Pereira, 2014).

Figure 11-5: Vacuum toilet collection and transport system used for Sneek pilot project (Pereira, 2014).

Figure 11-6: Prototype vacuum kitchen grinder used for Sneek pilot project (Pereira, 2014).