Optimizing two phase thermophilic anaerobic digestion utilizing food waste for production of biogas

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NANYANG
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**OPTIMIZING TWO PHASE THERMOPHILIC ANAEROBIC
DIGESTION UTILIZING FOOD WASTE FOR
PRODUCTION OF BIOGAS**

MENON AJAY SHIVADASAN

**SCHOOL OF CIVIL AND ENVIRONMENTAL
ENGINEERING**

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OPTIMIZING TWO PHASE THERMOPHILIC ANAEROBIC DIGESTION UTILIZING FOOD WASTE FOR PRODUCTION OF BIOGAS

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About the Thesis

This dissertation investigates the optimization of two phase thermophilic anaerobic digestion utilizing food waste for the production of biogas and in addition studies the microbial dynamics of the process due to the actions of various pretreatments and micronutrient supplementation.

The results of the follow manuscripts and IPs listed below are included in this thesis:

Publications

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Executive Summary

Food waste (FW) forms 10% of the total solid waste production in Singapore and ranks amongst the least recycled solid wastes. Due to its high water content and high biodegradability, it is not a good candidate for disposal via incineration. One of the most efficient methods for processing food waste is anaerobic digestion (AD), as it removes a large portion of its biodegradable solids and most importantly, it treats this waste as a resource by producing biogas. However, there are a few shortcomings of the conventional (i.e., mesophilic) AD process, most notably, its (comparatively) long processing time. One way of overcoming this is using a thermophilic AD which operates at 55°C. At this temperature, the faster hydrolysis rates combined with the natural higher activity of biological processes lead to a process that is faster with an ability to produce more biogas than mesophilic AD. However there is still great scope for optimizing it in certain critical areas such as the start-up time of the process, enhancing stability by reducing build-up of the products of the hydrolysis step and studying its nutritional requirements and microbial community.

The objective of this Ph.D. research was to optimize the thermophilic AD process for utilizing FW as a source of biogas by optimizing the process. This was done by dividing the AD process into two phases- an acidogenic phase and a methanogenic phase and improving both phases separately. The acidogenic phase was enhanced by optimizing a pretreatment regime for increasing substrate solubilization as well as hydrogen production. The methanogenic phase was optimised by designing a micronutrient supplement that increased the productivity and stability and productivity of the methanogenic community. In addition the microbial community dynamics were also studied.

In Chapter 3 describes studies on the hydrolysis of food waste in thermophilic conditions under various pretreatment. It was concluded that alkaline, thermal and ultrasonic pretreatment had major potential of enhancing substrate solubilization. A method for acclimatizing mesophilic sludge for thermophilic AD was also described. Lastly, a long term comparison of mesophilic and two phase thermophilic AD that showed the superiority of thermophilic AD in terms of productivity.

Chapter 4 details the optimization of various pretreatment methods for improving the acidogenic phase. Two pretreatment regimes were identified to have the best performance- thermal pretreatment, which maximised solubilization and a combination of alkaline and ultrasonication, while maximised hydrogen productivity and production rate. In Chapter 5 these regimes were tested in both long term continuous single stage and two phase thermophilic AD. It was found that thermal pretreatment had no impact on the productivity of the acidogenic phase but the combination of alkaline and ultrasonication greatly improved the hydrogen and VFA production. The study of the changes in the bacterial community showed that this was due to the massive enrichment of a high hydrogen producing species, *Thermoanaerobacterium thermosaccharolyticum* due the alkaline process.

In Chapter 7, the formulation of a novel micronutrient supplement containing calcium, magnesium, cobalt and nickel was described. Batch studies showed that this supplement contained optimised concentrations of each element and could potentially increase biogas production as well as decrease process time. In Chapter 8 this supplement was tested in long term 2 phase thermophilic AD. It was found that not only could the supplement improve the productivity of the process, it could also help it recover for acidification and stabilise it. The microbial dynamics study showed that this was due the

microbial supplement greatly increasing the functional diversity and redundancy of the bacterial community, thus making the community more robust and resistant to changes.

Thermophilic AD shows great potential as a solution to the connected problems of waste disposal and energy recovery. However there had been limited research on its application for treating FW. This doctoral research has demonstrated the effectiveness of two phase thermophilic AD at converting food waste into hydrogen and methane. It produced two separate methods of enhancing hydrogen and methane production in novel ways. In addition, the bacterial communities in the acidogenic and methanogenic phases and their dynamics caused by pretreatment and micronutrient addition were studied. The new insights gained from these studies shed new light on the microbial functionality in thermophilic AD and can help in understanding the microbial enrichment of this process as well as provide scope for commercial applications for the use of food waste as a source of energy.

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List of acronyms and abbreviations

MSW	Municipal Solid Waste
AD	Anaerobic Digestion
COD	Chemical Oxygen Demand
CSTR	Continuously Stirred Tank Reactor
HRT	Hydraulic Retention Time
OLR	Organic Loading Rate
FW	Food Waste
VFA	Volatile Fatty Acids
LCFA	Long Chain Fatty Acids
UASB	Upflow Anaerobic Sludge Blanket
OFMSW	Organic Fraction of Municipal Solid Waste
BMW	Biodegradable Municipal Waste
DGGE	Denaturing Gradient Gel Electrophoresis
FISH	fluorescence in situ hybridization
PCR	polymerase chain reaction
WAS	Waste Activated Sludge
TS	Total Solids
VS	Volatile Solids
TKN	Total Kjeldahl Nitrogen
SRT	Solids Retention Time
RSM	Response surface methodology
AMPTS	Automatic Methane Potential Test System
ANOVA	Analysis of variance

CHAPTER 1

INTRODUCTION

1.1 Background

Singapore generated 7.5 million tonnes of municipal solid waste (MSW) in 2014, of which 4.5 million tonnes were recycled while the remaining 3 million tonnes were disposed of in the incineration plants and eventually in the landfill (NEA, 2014). This was a staggering number for a city of almost 5.5 million inhabitants that was rapidly running out of land and was already resource-poor. Current projections show that MSW would be quadrupled in the next two decades due to population increase and economic development. The disposal of MSW was a major issue, thus new treatment methods need to be developed, considering that incineration had its limitations and Singapore had no spare land for landfilling.

Paper/cardboard, ferrous materials, plastic, construction debris, and food waste (FW) are the most common types of MSW generated in Singapore, each accounting 10% or more to the total waste generation. As most of construction debris and ferrous materials are recycled or reused (>95%), food waste, paper and cardboard and plastics are considered the ‘Big Three’ problematic wastes. Of these, food waste is the least recycled waste, in spite of its being a very energy-rich waste which can be used for energy production.

The quote “waste was simply a misplaced resource” was very much applicable to food waste. Due to its high biodegradability and nutrient content, FW can be used as source for energy production. Anaerobic digestion (AD) had become a well-established method for this purpose (Appels et al., 2011). AD is a proven and robust process with great potential in treating high moisture containing biomass, producing biofuel and

applicability on a wide range of modes (Park et al., 2008). AD plants have been identified as promising waste disposal/energy production solutions in several European countries like Germany, Sweden and Switzerland. Many different bio-chemical reactions take place in AD process, all of which have different optimum conditions (Romano et al., 2009). Hydrolysis and solubilization phase, which was the rate limiting step in AD (Jeihanipour et al., 2011), was used to produce short chain fatty acids and hydrogen, and provides substrate to the downstream methanogenic phase (Palmowski et al., 2000). Thus, AD can be used to treat food waste as well as produce energy in the form of biogas.

However, there are a few shortcomings in AD process. The most conventional AD method was in a single stage at 35 °C (mesophilic temperature). While this was a stable process, the biogas productivity and organic loading utilization rate was rather low (De Baere, 2006). An alternative method to improve the productivity of AD process was to conduct it in thermophilic conditions (55 °C). This enhances hydrolysis and solubilization of the substrate, which was the rate limiting step in AD process (Climent et al., 2007). At this temperature, the metabolic rates are improved increasing the rate of substrate utilization and product formation. Thus, the rate and productivity of AD are greatly improved. However, this method had shortcomings of its own. The thermophilic AD process was not as stable as the mesophilic AD because the microbial consortium was less diverse (Alvarez and Lidé, 2008; Lettinga, 2004; Massé et al., 2003). The high temperatures involved in the process means the microbial cells have a high turnover rate, making it susceptible to collapse if the cells are not being replaced by newer ones. There was also the issue of overloading the process with intermediate products such as volatile fatty acids (VFAs), which would inhibit the subsequent methanogenesis phase. This was a particularly important matter with thermophilic AD as hydrolysis and

acidogenesis are greatly enhanced at high temperatures, leading to a much faster production of VFAs. If the methanogens cannot utilize the VFAs at the rate as they are produced, acidification can be established breaking down the entire process.

One of the ways to tackle the overloading issue was separating the process into two phases, a hydrolysis/acidogenesis phase and a methanogenic phase. By phase separation, optimal environments for both acidogens and methanogens can be established. The acidogenic phase acts as precursor to the methanogenic phase by partially converting the complex food waste into more desirable compounds for the downstream anaerobic process.

It had been reported that FW pretreatment could enhance the AD process (Lagerkvist and Morgan-Sagastume, 2012). However, very few studies have been conducted on the pretreatment of food waste in thermophilic AD. Therefore, investigating the enhancement the acidogenic phase of FW by the application of pretreatment techniques like alkaline, ultrasonication and thermal. Statistical model was used to optimize the different pretreatment techniques and determine the optimal parameters in relation to solubility, increasing VFA and hydrogen production. In addition, the optimized conditions were applied in single stage and two phase thermophilic AD process, and the effect on biogas (methane) production was also determined.

Micronutrients (or trace metals) are necessary for many of the metabolic processes of all microorganisms (Gronauer et al., 2009; Lebuhn et al., 2008). In most cases, these elements are not present in optimal concentrations, thus, the metabolic processes are undergone at limited rates or even fail (Madigan et al., 2003, Zandvoort et al., 2006). It was essential to determine the optimal amounts of the micronutrients as in excess quantities could increase the cost and cause toxicity in the overall process. This a major

gap in literature as there are no such studies done on finding the optimal concentrations of various elements in thermophilic AD (Schattauer et al., 2011). Thus, a ‘Micronutrient Cocktail’ was developed which contained the essential micronutrients in their optimal and bioavailable concentrations that could enhance the biogas production and stability of thermophilic AD process.

1.2 Objectives of the project

The purpose of this PhD work was to enhance the production of biogas from the thermophilic anaerobic digestion of food waste. This was done by separating the acidogenic and methanogenic phases of the AD system and enhancing them separately, the former by developing an optimised pretreatment regime which led to higher solubilization, VFA and hydrogen production; and the latter by development of a ‘microbial cocktail’, which improved methane production and solubility of the process by micronutrient supplementation. Thus, this thesis was dedicated to producing a highly productive and efficient process for waste disposal and energy production ability.

1.3 Organisation of the thesis

This thesis consists of the following chapters. Each chapter elaborates a specific area of the thesis as discussed in the objectives section.

Chapter 2: Literature review

This chapter presents the current state-of-the art of thermophilic anaerobic digestion process, with its current developments and limitations. A detailed survey of pretreatment methods and their application to food waste treatment was done. Lastly, a review of the

role of micronutrients in AD and its use in enhancing biogas production was done. The conflicting findings of various studies are also discussed.

Chapter 3: Basic studies

Preliminary data on thermophilic AD are presented in this chapter. Firstly, a method for conversion of mesophilic sludge into thermophilic inoculum was derived. The characteristics of FW are studied and basic batch studies are done to determine to optimal organic loading rate (OLR), HRT and substrate-to-inoculum ratio for further studies. A comparison of hydrolysis under mesophilic and thermophilic conditions was done and a long-term study for comparing the performance of mesophilic and thermophilic AD in single stage and two phase continuous stirred tank reactor (CSTR) reactor was carried out.

Chapter 4: Effect of pretreatment techniques on food waste solubilization and biogas production during thermophilic batch anaerobic digestion

In this chapter, three pretreatment methods (alkaline, thermal and ultrasonic) were studied in batch mode. The parameters of each method were optimised and the best method was selected on the basis of substrate solubilization, VFA production, process time and biogas production.

Chapter 5: Chapter 5: Effect of pretreatment techniques on long term two phase and single stage thermophilic batch anaerobic digestion and bacterial community dynamics

The optimised thermal and alkaline pretreatment methods were used in a long term study of thermophilic AD in single stage and two phase CSTR. The AD performance was evaluated on the basis of solid destruction, chemical oxygen demand (COD)

destruction and biogas production. The impact of the pre-treatments on the AD process was studied. A microbial community dynamics study was done to investigate the impact of the pretreatments on the bacterial communities of the acidogenic reactor and the single stage reactor.

Chapter 6: Optimization of micronutrient supplement for enhancing biogas production from food waste in two-phase thermophilic anaerobic digestion

In this chapter, a model based statistical technique (Response Surface Methodology) was used to determine the optimal concentrations of basic micronutrients for enhancing thermophilic AD of food waste. Batch experiments were done to study the impact of various combinations of the micronutrients and the final cocktail formation on methane production.

Chapter 7: Influence of micronutrient supplementation on long term continuous two-phase AD system and effect on the bacterial community dynamics

Here, a long term 2-phase AD study was carried out to evaluate the effect of the micronutrient cocktail. The effect of the cocktail on the stability of the process, solids and COD destruction and biogas production was studied. The impact of the addition of the micronutrient on the bacterial community of the thermophilic reactor was also studied.

Chapter 8: Conclusions and Recommendations

The last chapter lists the major findings of the study and draws conclusions from the results discussed in the previous chapters. An alternative thermophilic AD system for treatment of food waste was proposed. In addition, recommendations for additional

investigations on use of micronutrients and thermophilic AD are suggested based on this thesis' findings.

CHAPTER 2

LITERATURE REVIEW

In the year 2011, Singapore, a small city-state, had to dispose of 2.8 million tonnes of solid wastes (Table 2.1). In terms of total waste production, it almost equals the neighbouring, vastly larger and more populated country of Malaysia (UNEP, 2001). The disposal of this waste presents a major challenge for the land and resource poor country. The three biggest types of waste are food waste, paper and cardboard and plastics which are mostly produced in the form of domestic refuse from residences, food centres, markets and commercial premises, which account for 48.8% of the refuse and industrial premises and institutions account for the other 51.2%.

2.1 Food Waste Generation and Disposal in Singapore

Table 2.1 gives the details of waste production and disposal of the major types of solid wastes in Singapore. 'Waste disposed' refers to the amount of waste disposed via landfills or incineration, 'Total waste output' refers to the total waste generated that year while 'Total waste recycled' was the amount of the produced waste that was reused or converted into other usable form and hence, does not need to be disposed. While food waste accounts for 10% of the total waste generated in Singapore, it forms 20% of the disposed waste due to extremely low recycling rate. Hence, the overwhelming majority of it was disposed of by incineration.

Table 2.1: Waste Statistics and Recycling Rate for 2011 (NEA, 2014)

Waste Type	Waste Disposed of (tonne)	Total Waste Recycled (tonne)	Total Waste Output (tonne)	Recycling Rate (%)
Construction Debris	12,600	1,191,100	1,203,700	99%
Used Slag	5,700	335,900	341,600	98%
Ferrous Metal	67,600	1,171,600	1,239,200	95%
Non-ferrous Metals	14,500	102,800	117,300	88%
Scrap Tyres	3,700	18,300	22,000	83%
Wood/Timber	97,400	176,500	273,900	64%
Paper/Cardboard	603,200	765,000	1,368,200	56%
Horticultural Waste	149,800	89,000	238,800	37%
Glass	51,400	21,400	72,800	29%
Textile/Leather	113,700	17,300	131,000	13%
Plastics	656,000	77,000	733,000	11%
Food waste	605,800	69,700	675,500	10%
Others (stones, ceramics and rubber)	325,200	3,200	328,400	1%
Sludge	152,900	0	152,900	0%
Total	2,859,500	4,038,800	6,898,300	59%

In general, the characteristics of the food waste generated from most domestic and institutional sources are:

- Rich in degradable organic matter such as proteins, carbohydrates and lipids
- High BOD and COD
- High moisture content
- Free from metals, pesticides and other toxic substances
- Low in inorganic solids
- Shows high variability in quality based on seasons and locations

- Due to rapid growth in processed food industry, there was an increase in the production of liquid food waste (dilute streams of proteins, sugars, starches and fats) (Hua, 2008)

As seen from Table 2.1, FW was the one of least recycled major waste type in Singapore, proportion-wise. The main route for food waste disposal in Singapore was incineration. Singapore had one landfilling site, the off-shore Semakau Landfill facility which only accepts inert, inorganic wastes due to the environmental concerns. Hence no FW was only sent for landfilling after being incinerated along with all other types of combustible wastes (Tan and Khoo, 2006). . Incineration was a technology that involves combustion and conversion of waste into heat and energy and results in extreme volume minimization of the waste (McDougall and Hruska, 2000), which makes it a viable technology for places with limited land area like Singapore.

However, incineration had its disadvantages as it leads to air and soil pollution (Morselli et al, 2002). In addition, the high moisture content of FW renders it an unsuitable fuel for such a process. Hence, there was a need for developing alternative methods for disposing FW.. One such alternative was diverting this waste to Anaerobic Digestion plants. Till recently, a Singapore based company, IUT Pte Ltd., used to dispose of FW using anaerobic digestion (AD). The main product of AD, biogas was used to generate energy that was fed to the national grid and the residues were diverted to a composting process where microorganisms were used to break down the residues in the presence of oxygen, thus avoiding the production of methane. The bio-compost material can be used as a replacement of mineral fertilizers. This leads to carbon dioxide savings which can be achieved by the avoided production of the mineral fertilizers (Schleiss et al., 2008).

Thus, this process had two major benefits: reducing the amount of FW entering the incineration plants as well as earning carbon credits.

To emphasise the great potential of the AD process, a Life Cycle Assessment study (Khoo et al, 2010) made the following observations with regards to the necessity and feasibility of its use in processing FW in Singapore:

- AD was the best process for disposing FW due to the lowest energy requirements, emissions and CO₂ savings from the bio compost production.
- The energy generation from AD could potentially be 2 entire orders of magnitude more than the maximum possible for the incineration plants and aerobic composting.
- Operating the AD plant at full capacity was Singapore's best bet at achieving the target of recycling 30% its food waste by 2012 (MEWR, 2008) and could potentially surpass it by reaching 47%

Hence, it was important to develop AD as an efficient way to manage Singapore organic wastes.

2.2 The Anaerobic Digestion Process

2.2.1 Microbial Steps in the AD process

Anaerobic digestion consists of 4 different steps that are carried out by different microbial populations, in a mixed consortium of species carrying out different roles. This section provides a brief overview of the different steps of the process.

2.2.1.1 Hydrolysis

In hydrolysis, complex insoluble organic material was solubilized by extracellular enzymes excreted by hydrolytic microorganisms. This was an essential first step to allow the transport of macromolecules into microbial cells for subsequent steps to take place. Hydrolysis was usually the rate-limiting step in the process that deals with biodegradation of complex particulate substrates, (Sanders, 2001), and therefore determines the solids retention time in the reactor (Veeken et al., 2000).

2.2.1.2 Fermentation / Acidogenesis

In acidogenesis, soluble organic components are converted into organic acids (volatile and long chain fatty acids), alcohols, hydrogen and carbon dioxide. The generation of volatile fatty acids (VFA) was understood to be the fastest step in the process, and VFA are rapidly produced from soluble substrates (Mata-Alvarez, 2003).

2.2.1.3 Acetogenesis

In acetogenesis, the products of the acidogenesis stage are converted primarily into acetic, propionic and butyric acids, hydrogen and carbon dioxide. Acetate was the most common precursor in methanogenesis. The production of acetate from hydrogen and carbon dioxide was called homoautotrophic acetogenesis (Madigan et al., 2003).

2.2.1.4 Methanogenesis

Methanogenesis in AD happens the conversion of acetate and carbon dioxide/hydrogen into methane. Other substrates such as formic acid and methanol can also act as precursors. It was carried out by methanogenic archaea. The various species have different ranges of substrate utilisation ability; for example *Methanobrevibacter*

arboriphilus uses only H₂ /CO₂ (autotrophic methanogenesis); species in the *Methanosaeta* genus use only acetate; *Methanospirillum hungatei* and *Methanobacterium formicicum* can use hydrogen and formate; while *Methanosarcina* species can produce methane from a wide variety of pathways, including one that utilize acetate, hydrogen/carbon dioxide, methanol and other simple organic compounds (Stams et al., 2006). Methanogens have a lower growth rate than acidogens and acetogens (Angelidaki et al., 1999) and are therefore slower to react to changes in operational conditions and are more sensitive to unfavourable conditions in the system than the bacterial populations.

2.2.2 Nutrient Requirements for the Biological Process

The nutrients of the microbial community in anaerobic digestion can broadly be divided into two groups: Micro- and Macro-nutrients (Speece, 1996).

All living organisms require carbon, hydrogen, oxygen and nitrogen as the basic building blocks for synthesis of monomers and macromolecules. These are termed ‘macronutrients’ as they are required in large amounts.

Table 2.2 Macronutrients required for AD (adapted from Madigan et al, 2003)

Macronutrient	Role in Microbial Cells
Phosphorus (P)	Nucleic acids and phospholipids
Sulphur (S)	Amino acids cysteine and methionine, some vitamins including thiamine, biotin, and lipoic acid; also found in coenzyme A, important electron carrier for cellular energy-yielding reactions including homoacetogenic acetate production
Potassium (K)	Several enzymes, including those involved in protein synthesis
Magnesium (Mg)	Stabilises ribosomes, cell membranes, nucleic acids; required for activity of some enzymes
Sodium (Na)	Not required by all organisms; stabilises cell wall and plays role in regulating diffusion of solutes in/out of cells

Calcium (Ca)	Not required by all organisms; stabilises cell wall and plays role in regulating diffusion of solutes in/out of cells. Also important in heat stability of spores.
Iron (Fe)	Cytochromes and iron-sulphur proteins active in electron transport for cellular respiration.

As most micronutrients are metals, the terms ‘trace metals’ and micronutrients are used interchangeably. High doses of heavy metals have a toxic effect, due to disruption of enzyme structure and activity (Oleskiewicz and Sharma, 1990). They are essential only in small concentrations. The importance of certain metals for maintaining a stable anaerobic digestion process had been well studied (Florencio et al., 1994; Speece, 1996, Zandvoort et al., 2002; Zandvoort et al., 2005). Iron had been listed as a macronutrient in Table 2.2 as it was needed in high concentrations in cells, but as it was a metal, it was also commonly grouped with the trace elements.

A summary of the roles of some important micronutrients required for fermentation adapted from (Schattauer, Abdoun, et al., 2008) was given in Table 2.3.

Table 2.2 Micronutrients required for AD

Micronutrient	Cellular function	Role in Methanogenesis
Cobalt (Co)	Vitamin B12; <u>transcarboxylase</u>	<u>Methlytransferase</u> , propionic acid bacteria
Copper (Cu)	Respiration, cytochrome c oxidase; photosynthesis, superoxide <u>dismutases</u>	Inhibition of <u>hydrogenotrophic</u> activities of methanogens
Manganese (Mn)	Activator of many enzymes; some superoxide <u>dismutases</u>	
Molybdenum (Mo)	<u>Flavin</u> -containing enzymes, <u>nitrogenase</u> , <u>nitrate reductase</u> , <u>sulfite oxidase</u> , some <u>formate dehydrogenases</u>	<u>Formate-dehydrogenase</u> , <u>formyl-MF-dehydrogenase</u>
Nickel (Ni)	Most <u>hydrogenases</u> ; coenzyme F430 in methanogens, carbon monoxide dehydrogenase (CODH), urease	CODH, <u>methylreductase</u> , <u>hydrogenases</u>
Iron (Fe)	Cytochromes; catalases; peroxidases; iron-sulphur proteins; <u>oxygenases</u> ; <u>nitrogenases</u>	CODH, <u>formyl-MF-dehydrogenase</u> , ACS, <u>hydrogenases</u>
Selenium (Se)	<u>Formate dehydrogenase</u> ; some <u>hydrogenases</u> , <u>senocysteine</u> (amino acid)	CODH, <u>formyl-MF-dehydrogenase</u> , ACS, <u>hydrogenases</u> , <u>Formate-dehydrogenase</u>
Zinc (Zn)	Carbonic anhydrase; alcohol dehydrogenase; RNA and DNA polymerases, many DNA-binding proteins	Methyl-coenzyme M formation

As shown in Table 2.3, trace elements or micronutrients are essential for a variety of physical processes in microorganisms that take part in anaerobic digestion. Cobalt was a key part of vitamin B12, which was involved in the activity of methyl transferase, required for the transfer of methyl groups from compounds including methanol, in methanogenesis (Madigan et al., 2003). Cobalt also forms a part of carbon monoxide dehydrogenase (CODH), which plays an essential role in methanogenesis from acetate (Madigan et al., 2003, Zandvoort et al., 2006). Cobalt was also involved acetatogenesis from methanol by acetogens as this also requires methyl transferase. Iron was part of cytochromes and ferredoxin in methylotrophic methanogens, and as iron-sulphur proteins in some enzymes (Takashima and Speece, 1990). CODH also requires nickel,

which was also found in the cofactor coenzyme F430 (Takashima and Speece, 1990). Coenzyme F430 was a porphyrinoid (Shima et al., 2002) that plays a role in autotrophic methanogenesis, making nickel especially important when H_2 and CO_2 are the sole sources of energy. Nickel and/or iron was at the catalytic centre of most hydrogenases (Shima et al., 2002) and may be involved in membrane stability (Jarrell and Sprott, 1982). Zinc was present in hydrogenase, CO dehydrogenase, formate dehydrogenase, and superoxide dismutase (Takashima and Speece, 1990). Selenium and molybdenum are important for the functioning of many dehydrogenases which are essential for production of H_2 and CO and for acting as transmembrane anchors. Although copper was essential for many cellular respiratory functions, it actively inhibits hydrogenotrophic activities of methanogens (Karri et al., 2006), and hence it was important to monitor its concentrations.

A short review of important findings concerning the addition of micronutrients to AD processes was given in Table 2.4 below.

Apart from these, two other elements that might have a high impact on AD process are calcium and magnesium. Magnesium ions been known to stimulate the production of single cells (Harris, 1987; Xun et al., 1988; Schmidt and Ahring, 1993). These single cells, which are necessary for catabolizing acetic acid, are highly susceptible to lysis and their loss was important factor in the loss of acetoclastic activity in anaerobic reactors. This problem was heightened in thermophilic reactors as cell lysis was accelerated here. Therefore, the addition of Mg should theoretically improve the process by increasing the replacement rates of these single cells.

Long chain fatty acids (LCFAs) are a major inhibitor of biogas production (Luostarinen et al., 2009; Kuang et al., 2006; Miron et al., 2000). These are a cause of major concern

when biogas plants are operated at high OLR and can cause reactor failure by overacidification. Calcium addition can address this problem as it forms insoluble salts with LCFAs and stops them from interfering with the process (Kleyböcker, 2012).

Therefore, strictly speaking, even though these two elements are not micronutrients, it was important to study the effect of their addition on thermophilic AD as a part of this project.

Table 2.3 A short review of important findings concerning the addition of micronutrients to AD processes

Study	Micronutrients	Substrate	Effect	Observation
Zandvoort et al. 2002, 2003	Co	Methanol degradation	Achieved conversion efficiency of 100%	Deprivation of micronutrients lead to rapid deterioration of reactor efficiency
Jarvis et al. 1997	Co	Grass silage	Increased biogas production	Adding iron, nickel, and molybdenum showed no effect
Fish 1999	Co	MSW	Increased stability	Effect was most noticeable when the methanogenic population was stressed
Paolo et al. 2003	Co	Methanol	Lack of Co destabilizers Acetotrophs	Thermophilic study
Gonzalez-Gil et al. (1999)	Co, Ni	Kinetic study	Additions should be continuous not pulsed for optimal bioavailability	Later work demonstrated that this might not be a limiting factor
Jansen et al. 2007	Ni, Co	Maize	Dissolution study	Suggested mode of addition may not be important

Pobeheim et al., 2010a	Ni	maize	Stimulatory effect on gas production	Batch study only
Pobeheim et al., 2010b	Ni, Co, Mo	Maize	Co and Ni have stimulatory effect	Eliminated Mo as an important additive
Fish, 1999	Fe, Ni, Co	MSW	Enhances start up time	Co addition might be either stimulatory when inoculum: substrate ratio was high or essential when it was low
Lin et al., 2006	Fe	Artificial media	Fe stimulates methanogenesis by enhancing cytochrome formation	Focus was on discovering how Fe enhances AD, not optimizing the process
Agridiotis et al., 2007	Al, Fe	WAS	maintenance of granular sludge	Effects attributed to surface salts
Jarvis et al. (1997)	Co, Fe, Ni, Mo	Grass clover silage	Co supplementation was critical for acetogenesis, not Fe, Ni and Mo	Recirculation may have a beneficial effect on micronutrient availability
Kumar et al., 2006	Ni, Zn and Cd	potato processing residue and animal excreta	Maximised biogas production	Only study to use Cd, results different from other studies
Plugge et al. 2009	W, Mo	Artificial media	Tungsten plays a significant role in propionate degradation	Only study giving significant importance to tungsten in AD, results are different from other studies
Zhang et al., 2003	Ni, Co, Mo, Fe, Zn	Artificial media	Fe and Ni are critical for certain methanogens	Purely microbial study

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From this review, cobalt appears to be the most important micronutrient as it was required for optimizing VFA uptake, especially that of propionate, enhancing methane production as well as stabilizing the process. Although many studies have contradictory results, the consensus on the importance of nickel and iron was very high. Hence, it will be very interesting to study the impact of addition of these elements as micronutrients to an AD process.

2.3 Anaerobic digestion of food waste

Although AD had been used for biological wastewater treatment processes for various organic feedstocks such as industrial wastewaters, MSW, various types of domestic wastewaters and sludges since early in the 20th century (Mata-Alvarez, 2003), the anaerobic digestion of pure food waste had been less well studied until recently. This section outlines the recent work in this field. There have been a number of studies on the anaerobic digestion of food wastes (from kitchens and markets) (Mtz.-Vituria et al., 1995; Kim et al., 2006; Bouallagui et al., 2005; Parawira et al., 2004; Wang et al., 2005; Bouallagui et al., 2009, El-Mashad et al., 2010). In most of these studies, a two-stage mode of anaerobic digestion was used; this was to allow for control VFA loading to the methanogenic stage, due to the sensitivity of the methanogens to inhibition by high concentrations of volatile fatty acids. Single-stage digestion, however, was still the preferred mode for digestion of biowastes at a commercial scale, as over 87% of plants in Europe are single-stage plants (De Baere, 2006). At a laboratory scale, single-stage digestion had been used for biodegradable municipal waste (Gallert et al., 2003; Bolzonella et al., 2003), from food processing and slaughterhouses (Beccari et al., 1999; Carucci et al., 2005; Salminen and Rintala, 2002; Siegrist et al., 2005).

Two-phase digestion was one possible strategy to mitigate VFA inhibition effects. Mtz-Viturtia et al. (1995) compared two-phase with single-stage mesophilic digestion for food wastes and found lower yields in the two-phase system. They also found that as OLR was increased, the bulk of biogas production shifted from the second-stage methaniser to the first-stage hydrolyser. They concluded that a two-phase system did not have significant advantages over a one-phase system on wastes with a C:N ratio above 15, unless more sophisticated control mechanisms were used. Later work by co-authors of this paper, however, had recommended two-phase systems for fruit and vegetable wastes, due to the susceptibility of one-phase systems to upset by VFA overproduction (Mata-Alvarez et al., 2000). Bouallagui et al. (2004) carried out two-stage digestion of fruit and vegetable wastes from a market in Narbonne, France, reaching a maximum OLR of 10.1 g of chemical oxygen demand (COD) per litre per day to the acidification stage, the effluent of which delivered an OLR of 0.72-1.65 g COD l⁻¹d⁻¹ to the methanogenic stage. Overall hydraulic retention time in the system was 13 days, but the methanogenic phase was operated in a fill-mix-settle-withdraw cycle that retained solids in the system. Solids retention time in the methanogenic stage was approximately 50 days.

In 2003, Wang et al. developed a hybrid anaerobic solid-liquid (HASL) bioreactor was developed to enhance food waste conversion. This was two-phase AD system with a SW reactor and an upflow anaerobic sludge blanket (UASB) reactor treating wastewater. The use of UASB reactor in semi-continuous mode was shown to be able to increase the methane content in biogas production. This system was further enhanced by recycling of the leachate in the acidogenic reactor. This increased the anaerobic digestion of FW and increased the rate of methane production by 40% in comparison with anaerobic digestion of food waste without recirculation (Stabnokova et al., 2008). It was also

shown to be able to utilize FW with a high lipid content, as long as the lipid content was below 40% of total solids (Wang et al., 2005).

Co-digesting FW with other substrates had also been investigated by various researchers. Carucci et al. (2005) co-digested two types of food processing wastes from a frozen food factory in Italy: fresh vegetable waste (scraps from selection and washing of fresh vegetables, primarily spinach) and pre-cooked food waste (quality control rejects from the preparation of precooked products, containing high quantities of flour, grease and cheese). Co-digestion achieved better results than digestion of each of the types of waste as a single stream. For the single streams, they found inhibition of methanogenesis which they attributed to high content of potassium in the vegetable waste, and high content of lipids in the pre-cooked waste.

As part of an investigation into the potential for digesting fruit and vegetable wastes at wastewater treatment plants, Gomez et al. (2006) compared co-digestion of the fruit and vegetable fraction of MSW and sewage sludge with digestion of primary sludge alone, and found a better biogas yield and specific gas production with the co-digestion mixture. They found that low mixing conditions were more effective for digestion than static conditions or a high mixing regime.

Kaparaju and Rintala (2005) co-digested various potato wastes (peel, tuber and stillage from glue production) with pig manure to determine the technical feasibility of using on-farm digesters to treat industrial food wastes from potato processing. From this work, they concluded that potato wastes could be co-digested successfully with pig manure in small scale digesters.

A number of studies have been done in support of anaerobic digestion of biodegradable municipal waste at a commercial scale. Bolzonella et al. (2003) used high-solids digestion (20% total solids) at bench scale to simulate the process at a full-scale plant in Verona, Italy. They used a combination of mechanically sorted organic fraction of MSW (OFMSW) from a refuse sorting plant and source-separated OFMSW from supermarkets and vegetable markets. The study was focused on plant start-up. The desired organic loading rate (OLR) of 9 kg total volatile solids (TVS) $\text{m}^{-3}\text{d}^{-1}$ was reached in approximately 30 days, with a specific biogas production of 0.23 $\text{m}^3 \text{kg}_{\text{TVS}}^{-1}$.

Gallert et al. (2003) processed biowaste collected from households in Karlsruhe, a city in Germany, in a bench-scale reactor as a test for increasing loading to a full-scale commercial plant operating on the same feedstock. Liquid pulp from the initial processing stages of the commercial plant was fed to a bench-scale reactor operating in CSTR mode on a 5-day feed schedule, the same mode as the commercial plant. The researchers were able to increase OLR stepwise from 4.3 to 19 g COD $\text{l}^{-1}\text{d}^{-1}$ while maintaining stable digestion, concluding therefore that it should be possible to increase load to the full-scale plant up to the desired OLR of 15 kg COD $\text{m}^{-3}\text{d}^{-1}$. This was borne out by results at the full-scale plant over the following year.

Lissens et al. (2004) tested a novel pre-treatment process (thermal wet oxidation) for biowastes, to increase methane yields from various components of BMW (food waste, yard waste, and post-digestion biowaste). Wastes were pre-treated at temperatures of 185-220°C and oxygen pressures of 0-12 bar. The thermal wet oxidation process resulted in higher methane yields in subsequent biochemical methane potential (BMP) tests, attributed to increased bioavailability of cellulose and lignin components.

Investigators have researched the effect of specific potential inhibitors, such as ammonia and cations. Kayhanian (1994) tested the performance of a thermophilic high-solids anaerobic digester processing a mix of paper, food waste and yard waste, under various concentrations of total ammonia nitrogen (TAN). TAN was varied by increasing or decreasing the C:N ratio of the feedstock by using greater or lesser fractions of nitrogen containing components (food waste and yard waste). Optimal digestion was found within the range of 600-800 mg l⁻¹ TAN. At TAN concentrations beyond 1 g l⁻¹ inhibition was observed; acclimation allowed operation at TAN concentrations up to 2.3 g l⁻¹, but failure occurred at 2.5 g l⁻¹. This was lower than the 4-6 g l⁻¹ TAN that had been observed in stable digestion of livestock wastes (Angelidaki and Ahring, 1993)

Kim et al. (2000) studied the effect of cations and food waste particle size in a series of thermophilic batch activity tests. In the degradation of glucose at varying concentrations of sodium, they found that a sodium concentration of 5 g l⁻¹ decreased the rate of methane production, and a sodium concentration of 20 g l⁻¹ resulted in methane production of only 50% of the theoretical methane potential. In batch flask tests degrading food waste of various particle sizes, they found that substrate utilization rate was inversely proportional to particle size, i.e., the smallest particle size was the fastest to degrade.

Neves et al. (2004) carried out a series of activity tests with kitchen wastes from the canteen of the university of Minho, Portugal, comparing the performance of granular and suspended sludge at different waste to inoculum ratios from 0.5-2.3 gVS_{substrate} gVS_{inoculum}⁻¹. They found that the granular sludge exhibited a similar strong performance (both methane production rate and yield) regardless of waste:inoculum ratio, while the suspended sludge only performed as well as the granular sludge at the

lowest waste:inoculum ratio, with decreasing activity as the waste:inoculum ratio was increased.

The same authors carried out a series of batch assays with a simulated food waste using different ratios of lipid, protein, starch and carbohydrate. They found that a high lipid content resulted in a lag in the onset of methane production (Neves et al., 2008).

In a study of food wastes collected from restaurants in San Francisco, USA, biochemical methane potential (BMP) trials gave a total methane yield of 435 ml gVS⁻¹, 80% of which was achieved after the first 10 days (Zhang et al., 2007). This showed restaurant food waste to be a good potential feedstock for anaerobic digestion, however since continuous digestion trials were not carried out; there was no investigation of long term stability for this feedstock. It was therefore important to carry out continuous digestion trials to explore the potential of this feedstock for sustained stable anaerobic digestion. In the UK, food waste digestion had been carried out at pilot scale. Kitchen waste was collected weekly from the village of Burford in Shropshire, and processed in 1500 litre CSTR digesters. Two digesters were operated in parallel at mesophilic and thermophilic temperatures, to compare system stability and pathogen kill at the different temperatures. It was found that thermophilic digestion was less stable than mesophilic digestion, which had a greater buffering capacity and was more robust with respect to changes in operational conditions or accumulation of inhibitory compounds. Stable digestion was maintained at concentrations of VFA up to 27 g l⁻¹ in the mesophilic digester; in thermophilic digestion VFA reached 45 g l⁻¹, at which point OLR was reduced from 4 kgVS m⁻³ to 1 kgVS m⁻³ to maintain process stability (Banks et al., 2008).

2.4 Thermophilic AD

There had been a revival of interest in using thermophilic AD for biogas production lately, due observations that it potentially increases both the rate of biogas production and quality. The simplest use of a thermophilic step was using it as a pre-treatment step for increasing hydrolysis rate in sludge digestion (Chiu et al., 1997; Weemans et al., 1998). Various studies (Stuckely and McCarty, 1984; Muller, et al., 2000; Valo et al., 2004; Lu et al., 2008; Nielson et al., 2004) have used various methods, from low temperature thermophilic (70°C) to extreme high temperature degrading (270°C) from pre-treatment and all have managed to get an improvement for 16-20% in biogas generation in both mesophilic and thermophilic AD processes. Climent et al. (2007) found a 50% increase in production from low temperature thermal pre-treatment.

There have been a few studies that investigated using a thermal pre-treatment step (TPS) and thermophilic single phase systems for production of biogas artificial kitchen waste. Sasaki et al. (1999) conducted a thermophilic single phase AD experiment and suggested that the appropriate organic loading rate (OLR) was 20gCOD/l/d, for an influent total solids (TS) concentration of 10%. Li et al. (2003) developed a TPS that was composed of thermophilic acidogenic and mesophilic methanogenic reactors and suggested that the appropriate OLR was 20 gCODcr/(l ·d) for an influent TS concentration of 20%. Sakamoto et al. (2005) proposed a mesophilic TPS combined with an aerobic solubilization process before using the TPS, and stable operation was obtained at an OLR of 9 gCOD/l/d. Park et al. (2008) performed a lab-scale comparison between single stage and two-phase thermophilic AD systems feed with kitchen wastes and concluded that a two phase system was easier to maintain and more stable due having a richer microbial community.

Thermophilic AD had gradually been used for biogas production from energy crops and agro wastes. In central and north Europe, AD was widely applied in the agricultural sector, e.g., Denmark had more than 80 centralised and farm-scale biogas plants treating livestock manure. The centralised plant mostly codigest these wastes with other sources at thermophilic temperatures (Nielsen and Angelidaki, 2008). About 1.5 million ton/yr of manure and other wastes are treated, producing 39,000,000m³ CH₄/yr (Angelidaki and Ellegaard, 2003). However, in many Swedish and Danish decentralized plants, temperature fluctuations often give rise to yield reductions, thus underlining the inherent instability of thermophilic AD (Alvarez and Lidé, 2008; Lettinga, 2004; Massé et al., 2003). However, when optimal conditions are stringently maintained, great improvements in yields are seen: Cavinato et al. (2010) showed that thermophilic anaerobic co-digestion of cattle manure with agro-wastes and energy crops can lead to an improvement in biogas yield from 0.45 to 0.62 m³/kg VS and methane content from 52% to 61% due to the use of a 'reduced' thermophilic range (47 °C). Vindis et al. (2009) compared mesophilic and thermophilic AD of three maize varieties and found that that Thermophilic digestion was 4 times more intense and biogas quality produced in thermophilic temperature range was better than biogas quality produced in mesophilic temperature range.

A way of getting over the stability issues using temperature phased anaerobic digestion (TPAD) which consists of adding a thermophilic hydrolysis step before mesophilic methanogenic phase in a two-step anaerobic digestion process. This allows for different bacterial populations to be provided the best conditions in which to operate (Lev et al., 2010). Two- phase processes have been widely applied in a wide range of configurations and capacities (Bhattacharya et al., 1996, Oles et al., 1997; Roberts et al., 1999; Cheunbarn and Pagilla, 2000; Vandeburgh and Ellis, 2002; Song et al., 2004; Watts et

al., 2005; Gray et al., 2006; Santha et al., 2006; Demirer and Othman, 2008). Extreme thermophilic hydrolytic steps operating at range 60–80°C have been used in TPADs, followed by the thermophilic methanogenic phase (Wang et al., 1999; Gavala et al., 2003; Gray et al., 2006; Lu et al., 2008; Ferrer et al., 2009, 2010; Ge et al., 2011). Bolzonella et al (2012) long term trial for the mesophilic (35°C), thermophilic (55°C) and TPAD (65 °C and 55°C) of waste activated sludge. This study greatly emphasised the potential of thermophilic AD as the performance improved with increases in temperature. The COD removal increased from 35% in mesophilic conditions, to 45% in thermophilic conditions, and 55% in the two stage temperature phased system.

2.5 Optimizing Anaerobic Digestion by Pretreatment of Food Waste

Suboptimal substrate characteristics and kinetics of hydrolytic reactions are an important cause of inefficient performance in AD processes. Hence, hydrolysis, which was also the first step in any anaerobic digestion process, was considered the rate limiting step (Vavilin, et al., 2008). Hydrolytic activity was influenced by a number of environmental factors such as pH and temperature, however, the rate of degradation of any substrate was directly linked to its substrate characteristics such as its bioavailability and biodegradability (Jash and Ghosh, 1996). These deficiencies stem from the substrate being present in large particles, the presence of non-biodegradable compounds such as lignin or complex compounds such as starch and proteins as well as structures such as cell walls and cellulose fibres which make their constituents hard to degrade (Salminen et al., 2003; Lissens et al., 2004).

Hence, organic substrates are often subjected to pretreatments which can convert them into to more easily bio-utilizable forms. Pretreatments mostly act on the substrates in three ways:

- i. reducing particle size
- ii. solubilizing the substrate
- iii. improving biodegradability

Measuring particle size reduction was a commonly used way of analyzing the effect of a pretreatment method (Izumi, et al., 2010), however it can misrepresent the effect of the pretreatment on some of the more common non-biodegradable fibrous substrates. This was because such compounds tend to increase their surface area due to shear forces without really decreasing their size. In addition, it does not account of solubilization of smaller particles due to the pretreatment and may only detect the size distribution of the larger particles, thus leading to incorrect conclusions (Kianmehr, et al., 2010)

Another common way of evaluating the effect of a pretreatment was calculating substrate solubilization. This was generally done by characterizing the increase in soluble COD or volatile solids in the substrate after the pretreatment. Since CODs and VS are the most easily biodegradable parts of the substrate, this was a good measure of the effectivity of the pretreatment method (Elbeshbishy et al., 2011)

Improved biodegradability due to a pretreatment was measured by directly detecting the increased bio utilization of the substrate due to its enhanced solubility, disintegration or transformation into smaller, simpler compounds. This was generally done using batch methane potential (BMP) tests and expressed as methane produced per unit of VS or COD utilized (Bougrier et al., 2006 ; Zhang et al., 2007). This gives a direct measurement of the effectivity of the pretreatment method.

2.5.1 Types of pretreatments

The three most commonly and successfully used methods of pretreatments for AD of food waste were found to be mechanical, chemical and thermal pretreatment. In addition, enzymatic pretreatment was also found to be commonly used.

Mechanical pretreatment:

Mechanical pretreatment was done by using physical force to break up substrate particles and reduce particle size as well as releasing intracellular compounds. This also increases the surface area of the substrate particles, which makes them more bio utilizable and improves the COD, which leads to higher methane production (Carrere et al., 2010; Esposito et al., 2011). One of the most widely used mechanical pretreatment method was ultrasonication, which achieves these effects by the use of ultrasound waves that disrupt cell and particle structures. It also forms various radicals like OH^* and H^* , which cause the oxidize complex organic compounds and converts them into simpler molecules (Bourgrier et al., 2006). The main advantages of this method are easy implementation, upgradability and moderate energy consumption (Ariumbaater et al., 2014)

For treatment of organic wastes, low frequencies between 20–40 kHz were most effective (Carrere et al., 2008). Elbeshbishy and Nakhla (2011) showed that the use of ultrasonication in a two phase AD process showed improved hydrogen as well as methane yields. Ormaechea et al. (2017) used ultrasonication to improve the yield of a mesophilic AD process utilizing a mixture of food waste and glycerine and found that methane yields increased by around 80%. Quiroga et al. (2015) used ultrasonic treatment to enhance methane production from mixed organic wastes (including food

waste) and showed that it improve volumetric productivity by up to 31% and 67% for mesophilic and thermophilic digestion, respectively. Houtmeyer et al. (2014) showed that ultrasonic pretreatment can improve biogas production from food waste by upto 27% in mesophilic AD. However they found that the process was energy-negative at pilot scale. Jiang et al. (2013) showed a 1.6-4.4 fold increase in susbtrate degradation and VFA production in anaerobic hydrolysis of food waste.

Chemical Treatment:

Chemical treatment involves the use of strong acids, oxidants or alkalis are used to degrade complex organic compounds. The most commonly used method was alkali (either sodium potassium or calcium hydroxide) addition as the AD process generally requires pH adjustment to increase the overall alkalinity (Li et al., 2012). Alkali pretreatment causes solvation and saponification reactions to occur in the substrate, which cause the chemical degradation of solids and subsequent increase in surface area, thus improving bioavailability (Carlsson et al., 2009). Process productivity was further improved by the alkali itself acting as a nutrient for biomass growth (Hendricks et al., 2009)

Acidic pretreatments and oxidative methods such as ozonation are also used to enhance the biogas production and improve the hydrolysis rate (Lim and Wang, 2013). Acid pretreatment was used more commonly for substrates with high lignocellulololic content as it hydrolyses such non-biodegradable compounds into monosaccharides and creates better pH conditions for hydrolyzing microbes (Hendricks et al., 2009). However, there are many reports of acidic pretreatment being detrimental to AD processes as they can lead to the formation of inhibitory agents such as furfurals (Modenbach and Nokes,

2012), loss of fermentable substrates and additional costs related to the use of acids (Taherzadeh and Karimi, 2008).

Thermal pretreatment:

Thermal pretreatment was the most commonly used pretreatment methods for food wastes and had been shown to be applicable for industry scale processes (Cesaro et al., 2013). Thermal pretreatment mainly works by increasing the solubilization of organic compounds. As COD solubilization had a correlation with both treatment time and temperature, this can be brought about by either short, high temperature treatments or long, low temperature treatments. In addition to its substrate enhancement, thermal pretreatment can also lead to pathogen removal.

Despite its successful use in treatment of other organic wastes, there are mixed reports of its use for treating food waste. Chamchoi et al. (2011) found no improvement in biogas yield after low temperature (70 °C) thermal treatment of household waste, while Wang et al. (2006) reported a 2-12% increase in biogas production from food waste in the same conditions with longer treatment time. Schieder et al. (2000) reported a 70% increase in solubilization and 3% increase in biogas yield for the same substrate using high temperature (160–200 °C) treatment. Using a hyperthermophilic pretreatment step was shown to improve process time, biogas yield, COD and VS destruction in both thermophilic and mesophilic AD of food waste (Kim et al., 2000; Kim et al., 2006; Kim et al., 2004). However, thermal treatment was also reported to be detrimental in certain conditions as a high temperature pretreatment (175 °C, 60 min) was shown to lead to a decrease in methane production from mesophilic AD of food waste (Liu et al., 2012)

Enzymatic pretreatment:

Enzymatic pretreatment offers an energy unintensified alternative that has the added advantage of not creating unwanted or inhibitory non products. Enzymes such as cellulases, xylanases, cellobiases, proteases, amylases, etc., can be used on a variety of substrates to break up complex compounds and increase their bioavailability for anaerobic microorganisms. Enzymes can be used to increase the biogas yield in form of a hydrolysis pretreatment of the feedstock before AD, or can be added directly to a single stage reactor or the acidogenic phase of a two step AD process.

Studies investigating enzymatic treatment of biogas feedstocks have shown mixed results. It had been shown to provide minor (13%) or no improvement, or in some cases a decrease in biogas production when used for treating lignocellulosic biomass (Romano et al., 2009). Schimpf et al., (2013) showed that using pectinolytic enzymes had a very low impact (4% increase) when used in large scale biogas plants. Since most enzymes used in pretreatment for AD are derived from fungi, it had been suggested that directly using fungi or their extract will be more suitable for AD than enzymes in pure form (Rouches et al., 2016). Using fungal mash rich in hydrolytic enzymes had been shown to greatly improve methane production from food waste by in solid state fermentation (Kiran et al., 2015) and enhance methane production and volume reduction from co-digestion of FW and activated sludge (Yin et al., 2016). The limitations of this technique were that the gains in productivity were low compared to other methods and there was a risk of endogenous microorganisms consuming the sugars created by the action of the enzymes. Hence the cost-benefit of this method was unclear for long term AD process utilizing food waste.

This literature review established that pre-treatment effects on substrate properties are difficult to compare quantitatively due to a lack of a standard characterization. Despite

this the common theme of most pretreatment studies using food waste was that pretreatment regimes were not optimized for the substrate. While alkaline, thermal and ultrasonic pretreatments could be qualitatively identified as the most promising methods for pretreatment of FW, most studies used parameters that were optimized for different substrates such as MSW, WAS or sludge, mostly for mesophilic single stage AD. This would lead to suboptimal performance or unnecessary energy or reagent consumption. This was the most evident in thermal pretreatment studies as most studies on food waste could not even come to consensus on whether thermal treatment was beneficial for AD of food waste. Hence there was major gap in the literature concerning pretreatment of food waste for thermophilic AD. There was much scope for investigations aimed optimizing chemical, ultrasonic and thermal pretreatments for producing biogas from food waste using thermophilic AD.

2.6 Microbial community analysis by use of 16S rRNA gene based clone libraries

The AD process was driven by a diverse microbial community that carries out the various reactions. While we have a general idea of the dominant bacterial species and families in this process, there are many gaps that exist in this field especially with respect to AD.

16S rRNA was the most widely used identifier group in the field of microbial ecology. Fig. 2.1 outlines the methodology of this process. In short, it involves the extraction of DNA and RNA, amplification and cloning of the 16S rRNA genes, sequencing and identification of the isolated clone with logenetic software.

Fig. 2.1 Studying a microbial community by cloning (Sanz and Kochling, 2006)

The main advantage of this technique was that it allows for precise taxonomic studies and the creation of high resolution phylogenetic trees. Most microbial groups, including minority groups are detected by this method. It also facilitates identification of uncultured and unidentified microorganisms. However, it was impractical for high sample throughput given its processing time and labour requirements. Perhaps its biggest drawback was that it was not quantitative.

There have been several community analysis studies done for mesophilic AD (McMahon et al., 2001; McHugh et al., 2003; Bagge et al., 2005). However, thermophilic processes are not so well studied. Sasaki et al. (2010) studied methanogenic pathways and community dynamics in a thermophilic AD of organic solid waste in a CSTR system by performing 16S rRNA analyses. Liu et al. (2008) studied the community dynamics of thermophilic anaerobic co-digestion of MSW, screened swine and manure using an upflow anaerobic filter reactor and identified the various dominant species. Tang et al. (2008) studied the sensitivity of microbial community under to

temperatures in hyperthermophilic chemostat conditions using Denaturing Gradient Gel Electrophoresis (DGGE) and discovered at what temperatures the various dominant methane producers cease to function. However, this study was done using synthetic wastewater. Tang et al. (2004) studied the effects of micro-aeration on the diversity of microorganisms in a thermophilic anaerobic municipal solid-waste digester by of bacteria by DGGE as well as fluorescence in situ hybridization (FISH) and quantitative real-time polymerase chain reaction (PCR). They determined the various dominant families and also studied the activity of sulphate reducing bacteria. The study was done to compare between different micro aeration strategies, but they failed to discover any significant changes between aeration and non-aeration.

Dohrmann et al. (2011) analysed the structural diversity in biogas reactors run with either cattle or pig manures as substrates, operated at mesophilic and thermophilic conditions, using genetic profiling by single-strand conformation polymorphism (SSCP) and PCR based detection of 16S rRNA genes. The study suggested that both bacterial and clostridial communities evolved in parallel, and substrate and temperature both had a great effect on community structures. This study was done mostly to determine the growth of pathogenic species, and determined that thermophilic AD was mostly free of them.

Goberna et al. (2009) investigated prokaryotic diversity of a plug-flow reactor to study the effect of biowaste sludge maturation on the diversity of thermophilic bacteria and archae. They determined the dominant families were more or less stable and suggested that significant changes in pH and volatile fatty acids did not much of an effect on them.

Ritari et al. (2012) conducted molecular analysis of meso- and thermophilic microbiota in AD to study the composition of archaeal, bacterial and fungal communities in

response to increasing organic loading in mesophilic and thermophilic AD processes by applying 454 amplicon sequencing technology. They found that the diversity and number of bacterial taxa decreased with increasing organic load, while archaeal remained more constant, and that of fungi increased. This suggested that while bacterial groups were very responsive to changes in various conditions, archaea were mostly stable and fungi mostly existed to metabolise nutrients for the prokaryotic groups. Lerm et al. (2011) also looked at community dynamics during organic overload by genetic profiling based on PCR-amplified partial 16S rRNA genes and on the whole came to similar conclusions with regards to the bacterial community.

The common theme of all these studies was that most of them were basic studies, aimed only at determining the community structure, with very few actually looking at the dynamics during different stages of the reactor except for organic overload. Also, they were all done with single stage systems, and previous mesophilic studies have shown that the findings from these and two phase (or multiple phase) systems do not agree with each other. It can be safely said that the case will be the same for thermophilic systems.

2.6 Summary

Although some research had been done on thermophilic AD, there are a lot of gaps in the state of the current knowledge. The major ones identified by this survey are:

1. There was very little research done on thermophilic AD of food waste using two-phase systems.

2. Vast improvements can be made in sludge adaptation for thermophilic AD in terms of reducing the time as well as investigating the changes that happen in the microbial community.
3. There was little or no investigations of the efficacy of sludge recycle in two-phase thermophilic systems. Potentially, this can greatly increase the efficiency of the process by increasing the SRT of the system, while keeping the HRT constant or even reducing it.
4. There was major gap in the literature concerning the use of pretreatment for optimizing the anaerobic digestion of food waste. More research needs to be done for optimizing chemical, ultrasonic and thermal pretreatments of food waste for producing biogas using thermophilic AD.
5. There was great potential for investigating the effect of micronutrient addition to thermophilic AD as it had shown great to improve the mesophilic AD process significantly. Theoretically, trace metal limitations can have even greater effects in a thermophilic process, so an investigation into this, especially for elements like Ca, Mg, Co, Fe, Ni and Mo in thermophilic AD will be of great interest.

CHAPTER 3

PRELIMINARY STUDIES

3.1 Introduction

As discussed in section 2.4, a major part of this thesis is dedicated to evaluating the use of two phase thermophilic AD for treating food waste and the use of pretreatments to optimise two phase thermophilic AD. Preliminary studies were done to characterise food waste, compare the hydrolysis of FW under thermophilic condition and ascertain whether two phase thermophilic AD was a significant improvement over conventional single phase mesophilic AD.

This chapter details some basic studies that were done to serve this purpose. First, the hydrolysis of food waste under mesophilic and thermophilic conditions was studied. As described in section 2.3.3 the optimisation of pretreatments under thermophilic conditions was under-studied in literature, hence the effect of some pretreatment methods on food waste in thermophilic AD was investigated.

The conversion of mesophilic sludge to thermophilic sludge was also described in this chapter. Finally, a long term comparison of continuous two phase thermophilic AD and single stage mesophilic AD was made. The objectives of this chapter were to evaluate the potential of certain pretreatment methods for the treatment of food waste at thermophilic conditions, describing a method of converting mesophilic AD to thermophilic conditions and lastly, to compare long term stable thermophilic and mesophilic AD.

3.2 Materials and Methods

3.2.1 Experimental Design

Three bioreactor systems were used to perform the various AD experiments. They were:

1. An AMPTS system
2. The 10L and 20L bioreactors with automated pH, temperature and stirring control

The first system was used in batch mode, while the others are used in continuous SBR mode.

3.2.1.1 AMPTS

The Automatic Methane Potential Test System (AMPTS) can allow 15 batches of up to 500ml of volume to be run simultaneously. It allows monitoring of the methane emission and dynamic degradation of the substrate without need for constant monitoring by the user.

Fig 3.1 shows the working principle of the AMPTS system. In Step 1, the AD process takes place in the sample incubation unit, which consists of a 500 ml glass bottle fitted with an automated, programmable stirrer. The temperature was maintained using a water bath. In step 2, the biogas generated from the previous step was passed through a small container filled with 3M NaOH (which was continuously stirred using a magnetic stirrer), which absorbs the CO₂ and H₂S present in the biogas, leaving only CH₄. In step 3, the volume of this methane was measured using a flow cell array unit which works on the principle of liquid displacement and buoyancy. In step 4, web based software

running on embedded servers detects and records real time gas flow and volume normalisation.

Fig 3.1: Automated Methane Potential Testing System

3.2.1.2 The 10L and 20L bioreactor system

For continuous AD experiments, three Winpact bioreactors, one of 10L and two of 20L effective volume were used. Both had automated pH, DO, temperature and stirring control, with online logging of all these parameters in addition to ORP and 4 programmable pumps. The temperature of the 10L reactor was maintained by a hotplate while that of the 20L reactors was maintained by a heating jacket. These reactors were used to create a 20L single stage continuous stirred tank reactor (CSTR) AD system using one 20L reactor and a 30L two phase CSTR AD system using a 10L reactor as the first (acidogenic) phase and a 20L reactor as the second (methanogenic) phase. The

HRT of the reactors was maintained at 20 days and the flow rates were 1L/day for the single stage system and 1.25 L/day for the two phased system.

Fig 3.3: 10L and 20 L automated Bioreactors

3.2.3 Feedstock and Seed Sludge

3.2.3.1 Food Waste

All the food waste was collected from various canteens of NTU. Typically, it contained various components of cooked food such as rice, residual fruit parts (peels, cores), vegetables, eggshell, noodles, leftover meat such as chicken and beef and bones and was mostly cooked. Before feeding it to the reactor, it was shredded by a blender and suspended in de-ionized water in the ratio 105g FW to 1L of de-ionized water. For AMPTS experiments carried out in this chapter, 400 ml of 105g/L food waste was used as the substrate, with no addition of seed sludge.

Typical characteristics of FW (measured in triplicate) are:

Table 3.1: General food waste characteristics

pH	TS g/L	VS g/L	CODT(g/L)	CODS (g/L)
	(wet weight basis)	(wet weight basis)		
5.95	6.14 ±1.33	3.77 ±0.27	12.49 ±1.2	4.80 ±0.21

3.2.3.2 Seed Sludge

Anaerobic sludge from the Ulu Pandan Wastewater Reclamation Plant (Singapore) was used as seed sludge. This was acclimatized to thermophilic temperatures before use wherever required. The sludge was starved for at least one week in advance to remove any residual methanogenic potential. Its characteristics (measured in triplicate) are:

Table 3.2: General seed sludge characteristics

pH	TS g/L	VS g/L	CODT (g/L)	CODS(g/L)
	(wet weight basis)	(wet weight basis)		
7.01	313 ±10.3	239 ±16.1	631 ±21.9	343 ±4.5

3.2.4 Parameters and Analytical Methods

The parameters chosen for following the performance of the AD systems were: pH, COD, VS, TS, Gas production and composition, TOC, VFA production and metal ion concentration for the micronutrient study. pH value was measured using a compact titrator (Mettler Toledo) equipped with a pH probe (Mettler Toledo DGi 115-SC). Total (TS) and volatile (VS) solids were analysed according to the Standard Methods (APHA, 1992). Total (CODT) and soluble (CODS) chemical oxygen demand measurements are made using COD digestion vials (Hach Chemical) and a spectrophotometer (DR/2800, Hach). CODS measurements are 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 are filtered through Membrane Solutions 0.45 μ m cellulose acetate membrane filters. The biogas composition (methane, carbon dioxide and nitrogen contents) was analysed by gas chromatograph (Agilent Technologies 7890 A, USA) equipped with a thermal conductivity detector (TCD).

3.3 Results and discussion

3.3.2 Hydrolysis

This study was undertaken to investigate the kinetics of the hydrolysis and acidogenesis of the above two substrates.

In all the experiments, regardless batch temperature or pre-treatment, the CODT was found to remain steady at around 27g/L while the pH would fall from ~7.2 to 4.5 in the first 12 hours and show a steady decline to ~4.2 by the forth day. The VFA production was minimal. The only parameter to vary the most according to the treatment was found to be CODS, therefore,, only CODS will be discussed.

As shown in Fig 3.1, at 55°C, the CODS availability within the first 12 hours was greater than that at 37°C by as much as 30% and its consumption started 2 days before, thus showing that thermophilic AD greatly enhances the hydrolysis of the substrate. This action was mostly due to physical forces, as the higher thermophilic temperatures make the FW more susceptible to solubilisation. Further experiments with seed sludge (both mesophilic and thermophilic) were done to see how much the thermophilic microbes themselves affect this process.

Fig 3.1: Hydrolysis at 37°C and 55°C

3.3.3 Hydrolysis after Pre-treatment

Three strategies were investigated: thermal, alkaline and ultrasonic pre-treatment.

Thermal pre-treatment: Here, high temperatures were used to destabilize and break bonds leading to higher solubility. The FW was heated at 80°C for 150, 70 and 30 minutes. The control was simple maintenance at 55°C without any pre-treatment. As shown in Fig. 3.2, the CODS availability was approximately doubled by heating at 80°C for 70 and 150 minutes, and increased 70% by heating for 30 minutes at the outset. For the first day, the availability was higher than the control by 30% for all the heating times. This shows that heating at 80°C for 70 minutes might be the optimum time for hydrolysis at thermophilic temperatures, but further studies at higher temperatures and shorter durations may provide even better results. While these results somewhat agree with some studies that sub-boiling temperatures can be optimal for solubilisation, e.g. Wang et al. (2006), other studies such as Tsukahara et al. (1999) and Scheider et al. (1999) maintain that pre-treatment at temperatures such 135°C was necessary for effective solubilisation for FW. Hence, a more in depth study will be done using more temperature set points that will also take into account methane production using thermophilic sludge in addition to production of CODS.

Fig 3.2 Thermal pre-treatment at 80°C on hydrolysis of FW (CODS changes)

Alkaline pre-treatment: Here, high pH was used to stabilize polar parts of the substrate to increase the solubility. As shown in in Fig. 3.3 the pH of the FW was set at 12, 11, 10, 9 and 8 using 3M NaOH. While almost all sets show increased CODS concentration from the start point itself, adjusting the pH to 9 appeared to be the best alternative here as it increases the CODS availability by almost 25% over the control (not shown here) and 5 to 13% higher than the other pH points. There was no comparable data in literature with regards to simple FW; and studies on AD processes utilizing other substrates such as waste activated sludge disagree with this finding as they maintain that higher levels of pH, such as 11 (Kim et al., 2003) or pH 13 are optimal. Therefore further research was required in this case so determine if (and why) food waste gave such a different result.

Fig 3.3 Effect of CODSalkaline pre-treatment at pH 8, 9, 10,11, and 12 on hydrolysis of FW

Ultrasonication: Ultrasonic waves are used to create micro bubbles in the substrate with break it into smaller pieces, thus enhancing solubility. Fig. 3.4 details the results from the study. The FW was exposed to ultrasonic waves of 40Hz frequency and 700W power output for 10, 20 and 30 minutes. It was observed that initially 30 min gives the best results, but 20 min at 40Hz more or less equals it after the second day. There are no comparable studies in literature utilizing food waste for thermophilic AD.

Fig 3.4 Effect of ultra-sonication pre-treatment at 40 Hz and 700W for 10, 20 and 30 min on hydrolysis of FW (CODs changes).

3.3.4 Sludge acclimatization

The objective in this subsection was to develop standardized strategies for acclimatization for a single phase thermophilic AD system and performing OLR studies.

This was tackled in three parts:

After 3 rounds of trial and error, a protocol for quick and efficient conversion of mesophilic seed sludge to thermophilic sludge was finalized. It was as follows:

- Stage 1: 2 days. Sludge allowed to stand at 55°C

This allowed for the full utilization of residual substrate in the sludge while also partly enriching the thermophilic population by eliminating obligate mesophiles.

- Stage 2: 1 week. Addition of 0.5g of glucose/L/day

The addition of a small amount of substrate gave a competitive advantage to thermophilic species, thus enriching them. Glucose was added as it is a simple substrate (it was found in previous rounds that the fledgling thermophilic community could not tolerate the addition of complex substrates like FW).

- Stage 3: 4 days. Addition of 1g of glucose/L/day + addition of Na Acetate to maintain pH above 5

This intensified the competition, in theory hastening the enrichment as well as making the community tolerant to higher OLRs. The addition of Na acetate was found to stabilize the methanogenic population and make them more tolerant to the intensifying VFA production.

- Stage 4: Addition of FW (0.5 g VS/L/d)

Here the transition was made to FW as a substrate. The reactor was converted from fed-batch to semi continuous with an HRT of 40 days. There was no more pH adjustment. The temperature and the agitation was maintained throughout at 55°C and 60 rpm, respectively.

Fig 3.6 shows the COD and VFA changes in the effluent during this process. Both CODT and CODS build up during the first 5 weeks after which the CODT stabilizes while the CODS starts dropping. There was a sudden rise in effluent COD in phase IV after week 5 most probably due to the substrate being changed from simple glucose to a more complex FW. Apart from the temperature, the community also needed to

acclimatize to hydrolyzing the carbohydrate and lipids present in the FW. The observed spike in the effluent COD can be explained by lag time required by acidogenic groups for switching to complex organic compounds for VFA production, leading underutilization of the substrate. The reduction in effluent VFA in the same period was also a reflection of this change.

Acetic acid built up till the second week, but after the substrate was switched to FW, it was rapidly consumed and by the end of the 8th week (no shown) the concentration of VFAs in the effluent was not significant. Stable biogas production began 5 weeks days after Stage 1 (500ml/gVS/L/d).

Fig. 3.6: Acclimatization of mesophilic sludge

3.3.5 Maintaining Single stage CSTR

Fig 3.7 shows the performance of the single phase CSTR. The initial OLR was 2gVS/L/day and the HRT was 40 days. This was maintained for 14 days. The TS and VS removal both were stable at around 80% while the CODs in the effluent was dropping rapidly, showing that the AD process was utilizing the substrate effectively.

After this the HRT was decreased to 20 days. There was a brief spike in VS removal and methane production (not shown there) but after that there was a precipitous decrease in solids removal, while the COD in the effluent (both total and soluble) increase rapidly. This showed that there was a large wash out event where a large amount of thermophilic biomass was lost. The pH (which had stabilised to 6.3) registered a slow, gradual fall which could mean that there was a large decrease in the buffering action of sludge, supporting the hypothesis of washout of micro-organisms. The ORP also increased rapidly above -350mV, signifying that the Methanogen activity was severely disrupted. This was reflected in the biogas production, which fell from 700ml/gVS/day to less than 400ml/gVS/day. This HRT was maintained for 4 weeks, but as there were no signs of improvement, the HRT was reverted to 40 days. Once this was done, the solids removal went back to its original state while there was a slight decrease in the effluent COD. The pH remained under 6 but had stabilized since.

Fig 3.7: Performance of single phase CSTR a: Solids removal, b: Effluent COD, c: ORP
d: pH.

Figure 3.8 shows the biogas yield of the two systems. The biogas yield was yet to recover however as it was still at approximately 500ml/gVS/day. Although the biogas yield was comparable to most other similar studies found in literature, this behaviour in response to the HRT changes had not been seen anywhere. Moreover, an AD process working at such low pH levels without the accumulation of VFA was not commonly seen (most processes maintain a neutral pH). Therefore, it was of interest to monitor the alkalinity of this process as well as analyse the microbial community to check for any significant deviations from the normal.

Fig 3.8 Biogas production of two phase thermophilic and single stage mesophilic AD

3.4 Conclusions

All the pretreatment methods studied here were found to have significant potential for improving the hydrolysis of food waste. Hence they will be studied in detail in subsequent chapters. The sludge acclimitization method was found to be very effective. Finally, two phase themophilic AD was found to be a substantial improvement over single phase mesophilic AD, hence further study and optimization of it was warranted.

CHAPTER 4

EFFECT OF PRETREATMENT TECHNIQUES ON FOOD WASTE SOLUBILIZATION AND BIOGAS PRODUCTION DURING THERMOPHILIC BATCH ANAEROBIC DIGESTION

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4.1 Introduction

As mentioned in section 2.2.1, several bio-chemical reactions usually take place in AD process, all of which have different optimum conditions. For instance, hydrolysis and solubilization phase was the rate limiting step in AD and operates more efficiently at lower pH and hydraulic retention time (HRT) than the methanogenic phase (Palmowski and Miller, 2000). The rate and efficiency of solubilization and volatile fatty acids (VFAs) production have a direct impact on the total biogas production (Mata-Alvarez et al., 2000). Furthermore, this phase can be used to produce hydrogen enhancing the quality of the biogas produced from the overall process (Cavinato et al., 2012). Therefore, it was of great interest to specifically improve this phase in order to improve the AD process as a whole. The improvements can be in the form of

increasing solubilization of the substrate, reducing process time, enhancing VFA production or increasing hydrogen production.

Food waste (FW) was the largest component of municipal solid waste (MSW) after the recyclables are separated. Due to its high organic and water content, its disposal by conventional means was improper but these very same reasons make it ideal for anaerobic digestion (Bai and Sutanto, 2002). It had been proven that pretreatment of FW could enhance the AD process (Lagerkvist et al., 2012). However, little had been done to specifically optimise pretreatment conditions for treating food waste for thermophilic AD. In this study, three different methods were studied and optimised: alkaline pretreatment, ultrasonication and thermal pretreatment.

Response surface methodology (RSM) is a collection of mathematical and statistical techniques useful for analysing the effects of several independent variables on the response (Box and Draper, 1987). It can be applied in design of experiments (DOE), improving process design and also process optimization. In the last few years it had been used to study and optimize the overall effect of various factors in anaerobic digestion (Kim et al., 2004, Wang et al., 2012).

The purpose of this study was to optimize the solubilization of FW by the application of pretreatment techniques like alkaline, ultrasonication and thermal. VFA production efficiency and hydrogen generation in the thermophilic acidogenic phase were also monitored. Optimal conditions of alkaline, ultrasonic pre-treatments and their combination for maximizing thermal pretreatment were predicted by the application of RSM. In addition, the optimized conditions were applied in single stage thermophilic AD process where its effects on biogas (methane) production were also studied.

4.2 Materials and Methods

4.2.1 Feedstock preparation and inoculum sources

FW was collected from a canteen at Nanyang Technological University (Singapore). It was leftover food consisting mainly of rice, noodles, peeling of fruits or vegetables, cooked potatoes, garden peas, cooked meat, and bread. Bones, plastics, and other non-biodegradable materials were separated from the FW manually, and it was grinded to a paste using a blender. It was then diluted with water to the concentration of 3 gVS/L. Its main characteristics are presented in Table 1.

Table 4.1 Characteristics of raw food waste used in the study

Parameter	Value	unit	Parameter	Value	unit	Parameter	Value	unit
pH	5.1±0.4	–	CODS	8.95±1.24	g/L	Fe	51.79±5.88	µg/L
C	47.2	%	NH ₄ ⁺ N–N	16±0.5	mg/L	Mg	15.80±2.66	µg/L
N	2.7	%	Carbohydrates	24.08±1.33	g/L	Mn	44.66±4.47	µg/L
H	7.4	%	Ag	1.13±0.13	µg/L	Mo	6.80±1.44	µg/L
C/N	17.5	–	Al	13.52±1.62	µg/L	Ni	14.18±0.92	µg/L
TS	5.88±0.84	%	As	48.19±1.83	µg/L	Pb	5.28±0.77	µg/L
VS/TS	93.51±1.76	%	Ba	9.33±0.91	µg/L	Sb	7.43±1.16	µg/L
Acetate	1.22±0.14	mM	Be	0.15±0.01	µg/L	Se	71.65±2.94	µg/L
Propionate	0.15±0.02	mM	Ca	12.09±0.04	mg/L	Sn	106.1±24.1	µg/L
Butyrate	0.05±0.01	mM	Co	2.27±0.31	µg/L	Sr	79.14±3.57	µg/L
TVFAs	1.98±0.31	mM	Cr	1.99±0.75	µg/L	V	9.94±1.62	µg/L
CODT	33.55±3.21	g/L	Cu	96.96±7.96	µg/L	Zn	5.29±0.59	µg/L

The original seed inoculum was mesophilic AD sludge obtained from Ulu Pandan Water Reclamation Plant, Singapore. Its conversion to thermophilic sludge was done using a method described by de la Rubia et al. (2005) with some changes. The reactor was initially operated with an organic loading rate (OLR) of 0.5 g VS/L/day with food waste as substrate and a hydraulic retention time (HRT) of 30 days for a period of 2 weeks at mesophilic conditions in a 20 L single stage semi-continuous stirred tank reactor setup. The temperature was then directly raised to thermophilic conditions (55 °C) and after a period of starvation for 2 days, the reactor was fed with 0.5 g glucose/L/day for 2 weeks while the other conditions remained unchanged. The substrate was then changed to food waste with an OLR of 0.5 g VS/L/day

4.2.2 Experimental set-up

Batch experiments for determining the anaerobic biodegradability, solubilization, and hydrogen and methane production of FW were carried out in Automatic Methane Potential Test System (AMPTS) (Bioprocess Control, Sweden). AMPTS consisted of 15 parallel reactors of 500 mL capacity (working volume of 400 mL) and the same number of gas flow meters connected to the acquisition system. An inoculum to substrate ratio (ISR) of 3:1 was used (Brown and Murphy, 2013). The reactors were closed with rubber stoppers and then connected to mechanical agitator, while mixing were set to be 1 min on and 1 min off alternately at 80 rpm during the entire experiment. The reactors were incubated at 55 °C and sparged with nitrogen gas before sealed to create anaerobic conditions. The gas produced was monitored online periodically using automated data logging system.

Two different sets of experiments were conducted. In the first set, acidogenic sludge from the hydrolytic phase of a two phase thermophilic CSTR was used to study the pretreatment techniques in hydrolysis, VFA production and biogas (mainly comprising of hydrogen) for 5 days. In the second set, methanogenic sludge from a single phase thermophilic CSTR was used as inoculum to study biogas production (mainly comprising of methane) for 25 days. Both sets were carried out in anaerobic thermophilic batch conditions (55 °C).

Alkaline pretreatment was done by adding 3 M NaOH to the FW. Batches were set at pH 8, 9, 10, 11 and 12. The pretreated FW was allowed to stand for 10 minutes before inoculum was added. Ultrasonic pretreatment was carried out by using a vessel ultrasonicator (Sonics, VCX 500-750W). Samples were placed in the vessel and exposed to ultrasonic waves of 20 kHz at 100 W for 15, 30 and 45 min. For thermal pretreatment an autoclave (Hirayama) with variable temperature controls was used to carry out the thermal pretreatment. Substrate was heated at 80, 105 and 130 °C for 20, 40 and 60 min each.

4.2.3 Response Surface Methodology (RSM)

RSM was used in this study to optimize time and temperature for thermal pretreatment. A central composite face-centered design (CCFD) involving two different factors was used to create the experimental conditions (Box and Draper, 1987). The two operating variables were considered at three levels namely, low (−1), central (0) and high (+1). 13 experiments were conducted with 9 experiments organized in a factorial design (including 4 factorial points, 4 axial points and 1 center point) and the remaining 4 involving the replication of the central point to get good estimate of experimental error.

The statistical software analysis program MINITAB (SBTI, San Marcos Texas) was used for this purpose.

The p-level chosen in the thesis was 0.05. Models considered applicable if the interaction between the model and the response variable was significant (p-value <0.05) and there was goodness of fit (R-squared values >90%).

4.2.4 Analytical procedures

pH was measured using a compact titrator (Mettler Toledo) equipped with a pH probe (Mettler Toledo DGi 115-SC). Total (COD_t) and soluble (COD_s) chemical oxygen demand measurements were made using COD digestion vials (Hach) and a spectrophotometer (DR/2800, Hach) as per standard methods. 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 µm) and the samples were filtered through Membrane Solutions 0.45µm cellulose acetate membrane filters. The methane, hydrogen and carbon dioxide content of the biogas was analyzed by gas chromatograph (Agilent Technologies 7890 A, USA) equipped with a thermal conductivity detector (TCD). Carbohydrate concentrations were determined by adapting the colorimetric method of Dubois et al. (1956) with UV visible wavelength at 490 nm using glucose as standard (Agilent Cary 50, USA). The C/N ratio was determined by measuring the total carbon and nitrogen using an elemental analyzer (Vario EL cube CHNOS, Germany). Trace metals concentration was measured by an Inductively Coupled Plasma – Optical

Emission Spectrometer (Perkin Elmer Optima 8300, USA). COD and NH_4^+ concentrations were measured colorimetrically using a DR 2800 spectrophotometer (Hach, USA).

4.3 Results and discussion

4.3.1 Alkaline pretreatment

Fig. 4.1 shows the effect of alkaline pretreatment on total biogas, VFA and CODs production. Apart from control and the batch pretreated at pH 12, all other experiments showed enhanced production of biogas comprising mostly H_2 (52%) and CO_2 (47%) in a short period of time. In batches pretreated at pH 9, 10 and 11 most of the hydrogen was generated within 80 hours, while at pH 12 it wasn't produced till 80 hrs. Among batches, pH 9 had overall biogas production almost 4 times over the control. In terms of COD solubilization or VFA formation, there was no significant improvement. As a result there was little correlation seen between final concentrations of VFAs and CODs and gas production, e.g., although the batches at pH10 had high VFA and CODs levels at day 5, they produced less gas than those at pH 8 and pH 9, both of which had lower final concentrations of VFA and CODs. That was possibly due to the utilization of CODs and VFA in the metabolic pathways to produce H_2 and CO_2 . Additional alkaline adjustment in the food waste changed the pH of the overall AD process drastically. In the control batch, the initial pH of the reaction mixture was 4.75, while batches set at pH 8, 9, 10, 11 and 12, the initial pHs were 5.1, 5.4, 6.1, 6.6 and 7.2, respectively. According to Fang and Liu (2002) the optimal pH for hydrogen production was 5.5, which was consistent with biogas produced in batch set at pH 9. This indicates that the batches fed with substrate at pH 9 performed better as their pH conditions were most

viable for hydrogen producers. At higher pH adjustment, hydrogen production was inhibited till the pH dropped to likely levels for the hydrogen producers. The final pH in all the batches was between 4.7-4.9.

(a)

(b)

Fig. 4.1 Performance of acidogenic batches fed with food waste alkaline pretreatment at pH 8, 9, 10, 11 and 12. (a) Total biogas accumulation; (b) Initial and final VFA and CODs

4.3.2 Ultrasonication

Fig. 4.2 shows the effect of ultrasonication pretreatment on total biogas, VFA and CODs. This pretreatment showed no significant improvement in either CODs or VFA production, only increase in the total biogas production, and reduction of reaction time as seen by the trends in biogas (consisting of 52% H₂ and 47% CO₂) production. Most of the biogas was generated in less than 38 hours, which was roughly half of the time taken in the control batch. All the pretreated batches had similar performance, with the batch fed with FW pretreated for the longest time (45 minutes) showing slightly higher gas production than the others. The control batch needed more than 2 days to utilize all the FW for gas production whereas the total production was also substantially lower. There was some correlation seen between the VFA and CODs production and gas production- all three ultrasonicated batches show similar levels of final VFA and CODs concentrations and similar gas production profiles, while the control batch had lower final VFA and CODs levels and correspondingly lower gas production, both in terms of total volume and rate.

The microbubbles produced during ultrasonication reduced the particulate size of the substrate, thus making it easy to metabolize (Suslick, 1998). This caused a physical change in the substrate and allowed it for easier initial utilization by making the hydrolysis step much faster. This was in agreement with previous studies in terms of hydrogen production dynamics, however, the increase in CODs was not as high as the values reported (Kim et al., 2013).

(a)

(b)

Fig. 4.2 Performance of acidogenic batches fed with food waste ultrasonic pretreatment for 15, 30 and 45 minutes. (a) Total biogas accumulation; (b) Initial and final VFA and CODs

4.3.3 Thermal Pretreatment

Batches treated thermally at 80, 105 and 130 °C for 20, 40 and 60 minutes (shown in Table 4.2) each showed no biogas production, which could be attributed to certain inhibitory species being produced that blocked hydrogen producers (Xiao and Liu, 2009). However, it greatly increased substrate solubilization. FW heated at 130 °C for 40 minutes showed 43 % increase in CODs, which was more than any other pretreatment method. Therefore, CODs production was selected as the response for optimizing the parameters.

Table 4.2: Variables for response surface analysis of thermal pretreatment

Coded variable	Temperature (°C)	Time (min)
+1	130	60
0	105	40
-1	80	20

Since there were two variables involved in thermal pretreatment (time and temperature), it was important to investigate their relation and identify the most efficient combination. For this purpose, RSM was used to predict the time and the temperature in order to maximize CODs production. The analysis of variance (ANOVA) was conducted to study the significance of fit from second-order polynomial equation for the experimental data. The ANOVA showed that quadratics models were the most significant, illustrated by the F-test value of 9.1 at a confidence level of > 0.05 . The regression equation used in the model was:

$$\begin{aligned} \text{CODs} = & -0.66 + 0.1147 \text{ time} - 0.0035 \text{ temperature} - 0.001995 \text{ time}^2 \\ & + 0.000697 \text{ time}^2 \text{ temperature} \end{aligned}$$

Fig. 4.3 (a) shows the results of RSM analysis of batches with thermal pretreatment. It was found that best possible temperature lie beyond conventional means and more sophisticated methods such as thermal processing would be required to treat the FW at such high temperatures. Fig. 3 (b) shows the optimization plot for the analysis. In this study, the optimal conditions were predicted to be 130°C and 50 minutes with an acceptable composite desirability of 0.75 with a p value of >0.05 , which showed that the model was significant.

(a)

(b)

Fig. 4.3 Response surface analysis of thermal pretreatment (contour plot of temperature and time with CODs production as the response)

Table 4.3: ANOVA of model used in Response Surface analysis

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	4	6.68364	1.67091	18.67	0
Linear	2	4.14153	2.07076	23.14	0
time	1	1.91959	1.91959	21.45	0.002
temperature	1	2.22194	2.22194	24.83	0.001
Square	1	2.05648	2.05648	22.98	0.001
time*time	1	2.05648	2.05648	22.98	0.001
2-Way Interaction	1	0.48563	0.48563	5.43	0.048
time*temperature	1	0.48563	0.48563	5.43	0.048
Error	8	0.71584	0.08948		
Lack-of-Fit	4	0.70709	0.17677	80.81	0
Pure error	4	0.00875	0.00219		
Total	12	7.39948			

Model Summary	
R-sq	R-sq(adj)
90.33%	85.49%

Table 4.2 shows the analysis of variance of the various terms in the RSM model. Based on the significantly strong relationship between the model and the response variable (p-value <0.0005) and high R-squared and R-squared (predicted) values of 90.33% and 85.49%, signifying acceptable goodness-of-fit, the model can reliably be used to predict the effect of time and temperature on CODs production. In the regression model, p-values of the both time and temperature linear coefficients are highly significant (<0.005), signifying that both have a large effect on methane production. While the squared time*time square factor was also highly significant, however, the temperature*temperature value was found to be highly insignificant (p>0.5) and hence was not included in this model. This indicates that time was the most crucial factor in

thermal pre-treatment. The interaction value time*temperature was significant (p-value < 0.05), suggesting that both factors have a strong synergistic effect on CODs production and hence the application of both was important for efficient pretreatment. Thermal treatment functions by reducing the particle size of the substrate and releasing carbohydrates (Carlsson et al., 2012) and at lower temperatures, this was more a function of time rather than temperature (Rani et al., 2011). Hence, the results of this study are consistent with available knowledge.

Thermal pretreatment was more efficient in high temperatures in order to cause physiological changes in the substrate to break long chained or otherwise complex compound into smaller chained molecules for easier digestion. The results are in agreement with previous studies in regards to solubilization, however, the hydrogen production here was lower (Elbeshbishy et al., 2011). These conditions were used in all subsequent experiments.

4.3.4 Combination of thermal, alkaline and ultrasonication pretreatment

The main advantage of ultrasonication was the reduction of processing time by physical breaking down of the substrate particles. In these experiments, it was examined the synergistic relation with alkaline and thermal pretreatment, both of which cause complex physiological changes in the substrate. Combination of the optimum efficiencies found in previous experiments (alkaline pretreatment at pH 9 and thermal pretreatment at 130°C for 50 minutes) and ultrasonication at 20 kHz for 15, 30 and 45 minutes were hence studied.

Fig. 4.4 presents the effect of the combined pretreatments on the total biogas, VFA and CODs production. The combination of ultrasonication and thermal pretreatment had no

significant effect. Although these batches showed hydrogen production as opposed to those treated only thermally, there was no increase in gas production or CODs solubilization and no shortening of processing time. The batch fed with FW ultrasonicated for 30 minutes after thermal pretreatment was an exception as it showed a slight reduction in processing time by 12 hours. The combination of ultrasonication and alkaline pretreatment, however, showed positive effect. The gas production (55% H_2) almost doubled, while there was a reduction in the reaction time in all batches. The batch fed with FW pretreated with the combination of adding NaOH to raise the pH of food waste to 9 and then ultrasonicated at 20 kHz for 30 minutes showed the best performance in term of hydrogen generation, with slightly higher total gas production in comparison to the other batches while having a slightly longer processing time. The better performance of the combination of ultrasonication and alkaline pretreatment can be ascribed to both methods improving the substrate utilization in different, non-overlapping ways, i.e., by reducing particulate size and enhancing the conditions for hydrogen producers, respectively. Interestingly, these results do not agree with other similar studies, such as Elbeshbishy et al., who found ultrasonication and alkaline pretreatment had lower efficiency than ultrasonication and thermal pretreatment. This could be ascribed to the different active microbial population in the two systems as that research dealt with AD process focused on mesophilic biohydrogen production, while the current study was dealing with the separated hydrolysis and acidogenic phase of a 2-phase AD process for biogas production.

(a)

(b)

Fig. 4.4 Effect of combination of thermal (T), alkaline (pH) and ultrasonic (U) pretreatments on acidogenic batches. (a) Total biogas accumulation; (b) Initial and final VFA and CODs

4.3.5 Effect of pretreatments on biogas production in single stage AD

For performance evaluation, the pretreatments that showed the best efficiencies (combination of alkaline pretreatment at pH 9 and ultrasonication for 30 minutes and thermal pretreatment at 130°C for 50 minutes) were applied on methanogenic sludge to investigate the effect on methane production, processing time and CODs and VFA utilization (Fig. 4.5). It was found that both pretreatments could increase the overall efficiency of the process. Thermal pretreatment gave the best results as it duplicated the total biogas production (comprising of 58% CH₄, 40% CO₂) over the period of 25 days, while there was complete utilization of available CODs. The combination of alkaline and ultrasonication also duplicated the biogas production, however the production rate was much slower and CODs was not fully utilized. The better performance of thermal pretreatment could be attributed due to inhibition in hydrogen production at the onset, which means all the solubilized CODs was available for methanogenic microbes. In the pH+U combination, however, there was a lot of hydrogen production at the onset, leading to most of the initially available CODs taken up by those metabolic pathways. Further CODs and VFAs should be generated for the methanogens to start producing methane. This process bottleneck could be described as the long plateau like region.

(a)

(b)

Fig. 4.5 Combination of alkaline pretreatment and ultrasonication (pH+U) and thermal pretreatment (Thermal) in batches with methanogenic (Meth) and acidogenic (Acid) inoculum. (a) Total biogas accumulation; (b) Initial and final VFA and CODs

The concentrations of the major VFAs (acetate, propionate and butyrate) at the end of 25 days are shown in Fig. 4.6. In the batch with thermal pretreatment, propionate and butyrate were completely utilized by the methanogens while a small portion of acetate was not fully utilized. On the other hand, substantial amounts of acetate and propionate remained in the pH+U combination. The fact that biogas was continued to produced indicates that methanogenesis was still in progress. The implication could be that both pretreatment methods had equal biogas generation potential, although thermal pretreatment could lead to more efficient utilization of the available substrates reducing the processing time in AD reactors.

Fig. 4.6 Combination of alkaline pretreatment and ultrasonication (pH+U) and only thermal pretreatment (Thermal) in batches with methanogenic (Meth) and acidogenic (Acid) inoculum. Residual concentrations of major VFAs at day 20

4.4 Conclusions

4.4.1 Summary

- The highest increase in hydrogen production in acidogenic phase was achieved by alkaline pretreatment at pH 9, which was found to increase hydrogen production almost 4 times.
- Ultrasonication showed the highest decrease in reaction time in acidogenic phase, with FW pretreated at 20kHz for 45 minutes utilizing all the available substrate for gas production in 38 hours compared to 75 hours in the control.
- Thermal pretreatment at 130°C for 50 minutes showed 43% increase in CODs production. Using response surface methodology, thermal pretreatment at 130°C for 50 minutes was predicted to be the best parameters was maximizing solubilization. However, thermal pretreatment showed a complete inhibition of hydrogen production.
- A combination of ultrasonication for 30 minutes and alkaline pretreatment at pH 9 showed a synergistic effect since hydrogen production was duplicated in half processing time compared to control experiment.
- In single stage AD for biogas (methane) production, a combination of thermal pretreatment (130 °C for 50 minutes) and ultrasonication for 30 minutes showed the best efficiency. It was found a 100 % increase in total biogas production with total CODs utilization in 25 days, without any significant lag phase.

4.4.2 Significance

Although there are many studies on pretreatment methods for anaerobic digestion of food waste, optimization of these methods had been neglected. This study hence plays an important role in filling this gap in available literature. It revealed the effects of

alkaline, ultrasonication, and thermal pretreatment and their combination on thermophilic anaerobic digestion of food waste, in both acidogenic as well as methanogenic conditions. It was established that different methods could enhance different parameters in the process, and new information was provided for choosing a pretreatment method based on the needs of the process, such as increasing hydrogen production, reducing processing time or increasing solubilization.

CHAPTER 5

LONG TERM MONITORING OF TWO-PHASE AND SINGLE STAGE THERMOPHILIC AD SYSTEMS AND EVALUATION OF BACTERIAL COMMUNITY DYNAMICS

5.1 Introduction

The previous chapter described the optimization of two pretreatment methods for food waste to increase the productivity of thermophilic AD process - Thermal pretreatment at 130°C for 60 minutes and a combination of ultrasonication for 30 minutes and alkaline (pH+U) pretreatment at pH 9. These methods were able to substantially increase solubilization of substrate and increase hydrogen and methane production. In this chapter, the optimized pretreatment methods are tested on long term two phase and single stage thermophilic AD systems.

While the microbial communities of various AD systems have been extensively investigated (Li et al., 2014), there was large gap in current research when it comes to microbial dynamic changes in thermophilic AD. In this study, the relatively new and more accurate method of next generation sequencing (NGS) was utilized with the service of Illumina's MiSeq platform (Luo et al., 2015) to analyze the bacterial community in the acidogenic reactor of a two phase thermophilic AD and the single stage thermophilic AD systems, and to study its dynamics when fed with pre-treated food waste as well the effect of these changes on the operation of the reactors.

The purpose of this study was to identify the most suitable pretreatment method to improve the productivity of continuous two-phase and single stage thermophilic AD

systems using food waste as well as to characterise the bacterial communities and study their dynamics over a long term.

5.2 Materials and Methods

5.2.1 Feedstock preparation and inoculum sources

The FW and inoculums were the same as the ones used in the previous chapter (Section 4.2.1)

Thermal pretreatment: An autoclave (Hirayama) with variable temperature controls was used to carry out the thermal pretreatment. Substrate was heated at 130 °C for 50 min.

Alkaline + ultrasonic pretreatment: This was done by adding 3 M NaOH to the FW till the pH was 9 and then using a vessel ultrasonicator (Sonics, VCX 500-750W) to expose the FW to ultrasonic waves at 20 kHz for 30 min.

5.2.2 Experimental setup

The effect of pre-treated FW on biogas production was studied in continuous two-phase thermophilic AD system consisting of a 10 L reactor for hydrolysis and acidogenesis, and a 20 L reactor for methanogenesis (Winpact, USA), and 20 L reactor for single stage thermophilic AD system. The reactors were monitored for pH, ORP and biogas production. In the two phase system, both reactors were maintained at 55 °C, while the HRT was 5 and 15 days for acidogenic and methanogenic phase, respectively. In the single stage system, the HRT was 20 days and the temperature maintained at 55 °C as well. The feedstock for the acidogenic phase and the single stage was FW with OLR 3 gVS/L/day and pH 4.5±0.3. The effluent from the acidogenic phase was the feedstock in the methanogenic phase. The AD systems were operated for 90 days with untreated FW and then for 60 days each with the two pretreatment methods mentioned above.

5.2.3 Bacterial community analysis

Sludge samples were collected from all three reactors at days 89, 149 and 209 of operation. Then, genomic DNA was extracted using chemical lysis and phenol-chloroform-isoamyl alcohol (25:24:1, v:v:v) purification protocol (Amann et al., 1995). The samples were sent to the Research and Testing Laboratory at Lubbock, Texas (USA) for processing on the MiSeq platform. The 2x250 read kits were used with 357wF-785R primer kit assay. The average number of reads per assay was 10,000. The fasta files obtained from the MiSeq analysed using Illumina's QIIME Basespace software suite's 16s genomic analysis program and visualized using Krona (Ondov et al., 2011).

5.2.4 Analytical techniques

The analytical methods were described in section 4.2.4.

5.3 Results and Discussion

5.3.1 Effect of pretreatment techniques on long term thermophilic AD systems

After optimization of the two pretreatment methods - a combination of alkaline and ultrasonic pretreatment (pH+U) and thermal pretreatment (T) - their efficiency was tested on two phase and single thermophilic AD. The performance profiles two different AD system were studied over a period of 210 days: A two-phase thermophilic AD system comprising of a 10L acidogenic phase reactor (R_A) and 20L methanogenic phase reactor (R_M) reactor and single stage thermophilic AD comprising of a single 10L reactor (R_S).

5.3.1.1 Gas productivity of thermophilic AD systems

Fig. 5.1 illustrates the methane yield of R_S and R_M and the hydrogen yield of R_A . Initially, both systems were carried out without any pretreatment of FW. The methane yield in two-phase reactor averaged a steady 0.33-0.38 L $\text{CH}_4/\text{gVS}_{\text{added}}/\text{day}$ through the entire period of 90 days. The methane yield for R_S was initially high at 0.4-0.46 L $\text{CH}_4/\text{gVS}_{\text{added}}/\text{day}$, however it lowered substantially after day 30 and by the day 90 it had stabilised to around 0.25-0.3 L $\text{CH}_4/\text{gVS}_{\text{added}}/\text{day}$. R_A had a sensible hydrogen production, which made up about 35-40% of the total gas produced in the reactor. It showed a steady production of 0.16-0.18 L $\text{H}_2/\text{gVS}_{\text{added}}/\text{day}$ till day 90. The productivity of the AD systems was comparable to other studies (Park et al., 2008, Nathao et al., 2013) and confirms the observations that single phase thermophilic AD was less stable and harder to control.

Fig. 5.1 Gas production profiles of RS, RA and RM.

On day 91, R_A and R_S were fed with FW subjected to thermal pretreatment as described in Section 5.2. The effluent from R_A was used as substrate in R_M . All three reactors

showed a significant drop in biogas production. The productivity of R_M dropped off sharply from day 116 onwards and by 128 it reduced only 0.21 L CH_4 /gVS_{added}/day, which was less than half the productivity seen before day 90. The reduction in gas production was even more drastic for R_S , the productivity fell immediately and very sharply, reducing to 0.16 L CH_4 /gVS_{added}/day by day 112, a 30% decrease compared to the production at day 90 and 50% decrease compared to day 0-60. R_A also encountered similar fall in productivity, with the hydrogen production stabilizing at 0.06-0.09 L H_2 /gVS_{added}/day. The decreases in productivity could be due to the creation of inhibitory compounds which adversely affect the AD process by lowering the pH as seen here (Fang and Liu, 2002). However, this observation was very different from what was observed in the previous study, where batches fed with thermal pretreated FW showed the most methane production. An explanation for this could be the inhibitory factors were retained in the reactors in continuous AD, increasing their effect on biogas production.

On day 150, the regime changed and the reactors R_A and R_S were fed with FW pretreated with a combination of alkaline and ultrasonic. The productivity of R_M recovered almost immediately and by day 164 it had reached its pre day 90 levels of 0.33 L CH_4 /gVS_{added}/day. There was a minor increase in the production (5-10%) which stabilised at 0.3-0.35 L CH_4 /gVS_{added}/day. R_S showed some recovery by day 172, with the production rising to 0.2 L CH_4 /gVS_{added}/day, however, there was no subsequent increase. Although the productivity didn't reach pre day 90 levels, it was able to recover a failed system. R_A on the other hand showed tremendous increase in the productivity when fed with FW under pH+U pretreatment regime. After a small lag phase, the production increased to 0.23 L H_2 /gVS_{added}/day, which was higher than pre day 90 levels. It kept increasing and by the final day of the study, it had stabilised at 0.31-0.33

$L H_2/gVS_{added}/day$, which was an almost 100% improvement over the pre-day 90 levels. Hence, the pH+U method showed a massive improvement of hydrogen production in the acidogenic phase of a two-phase thermophilic AD process. The high hydrogen production was most likely due to suitable pH conditions created for hydrogen producers and shows productivity similar to that in systems designed for high hydrogen production (Cavinato et al., 2012).

5.3.1.2 pH and ORP monitoring profiles

The pH and ORP variations of the three reactors were monitored (Fig. 5.2). From day 0 to 90 (no FW pre-treatment), the R_M reactor had a more or less stable pH and ORP profiles at optimal levels reported for methane production (7.2-7.6 for and -515 to -550 mV for ORP, respectively). This reflected the stable methane production seen in this reactor during the that period. There was a pH reduction between days 130 and 150 (thermal pre-treatment), which may indicate possible acidification or overloading. The R_S reactor started with very stable ORP levels for the first 30 days, however, later the ORP stayed at slightly suboptimal levels of -475 to -500 mV till day 90. This was consistent with the high methane production seen during the early period and subsequent decrease in productivity during day 30 to day 90, signifying some instability in the system. The pH remained constant at 6.5-7. The R_A reactor had stable profiles 3.8-4 (pH) and -350 to -400 mV till day 150 which are similar to the reported values in acidogenic phase. At day 150, after commencement of the pH+U pretreatment regime, the pH almost instantaneously increases to 5 and stays stable for most of the remaining duration of the study at 5.3-5.5. This was the optimal level for hydrogen production (Lim et al., 2013) and can interpret the high hydrogen production seen during this period. Similarly the ORP also remained very stable at -400 to -415 mV. This was lower than what was suggested in comparable studies (Kargi et al., 2012, Xu et al.,

2013), however, the hydrogen production in these studies was also lower than what was seen here. This may be an indication that the acidification phase in the AD process was operating at artificially created optimal ORP conditions for hydrogen producing bacteria. This suggests that ORP control was need to create optimal redox conditions for hydrogen production from the acidogenic phase.

Fig. 5.2 pH and ORP profiles of RM, RA and RS.

5.3.1.3 Effect of pretreatments on TVFA, CODs and Solids destruction

Fig 5.3a shows the effluent VFA profiles for all three reactors. The TVFAs from R_A are shown on the left Y axis while those from R_M and R_S are shown on the right Y (secondary) Y axis. The effluent from R_A also acted as influent for R_M . The effluent profiles of two-phase reactors were stable when FW was pre-treated; with the total concentration being maintained at 3.8-4 mM for R_m . There was an increase over the levels seen before day 90, implying that FW pre-treatment contributed to the acidification of the methanogenic reactor, which lead to the observed decrease in methane production. R_A presented minor variation in VFA production throughout the study, with the levels maintained at 60-65 mM throughout. Similarly, R_S was also very stable at 2.5-2.8 mM throughout the process. From this was was concluded that neither pretreatment method had much effect on VFA. This was similar to findings in the previous chapter. In the earlier study, it was found that the use of thermal as well as pH+U pretreated food waste could lead the VFA accumulation in the methanogenic reactor. There was no significant increase in the influent VFA levels, which could be due to other conditions created by the pretreatment methods such as an increase in CODs concentrations or changes in the bacterial community that lead to inhibition of utilization of VFAs. Interestingly, comparable studies disagree with these results and report an increase in VFA production from the acidogenic phase using similar pretreatment methods (Kim et al., 2013; Elbeshbishy et al., 2011). The differences can be attributed to observed instability induced into the system due using thermal pretreatment and the diversion of the substrate to hydrogen producing pathways in the pH+U pretreatment regime.

Fig. 5.3 Effluent VFA, CODs profiles of RS, RA and RM

Fig 5.3b presents the profiles of CODs in the effluent. There was no change in CODs in R_s throughout the study and the levels were stable at 5 g/L, showing that neither pretreatment could affect the solubilization of substrate in single state thermophilic AD. R_A on the contrary showed a significant change in CODs production, with the CODs in effluent being approximately 25-30% higher than those before pretreatment (12 g/L). This means that both pretreatment methods were effective at increasing production CODs, the large increase in hydrogen production should be taken into consideration for the pH+U method. The increase in CODs production could also be responsible for the observed acidification in R_M . Although there are no comparable studies, this finding broadly agrees other studies using a combination of pH adjustment and ultrasonication to enhance acidogenesis by increasing CODs production using similar substrates (Kang et al., 2011).

Fig 5.3c shows the volatile solids destruction in single stage and the overall two-phase AD. There was a significant reduction in the solids destruction in two-phase AD after the addition of the thermal pretreatment. This corresponds in the increased CODs production as well the observed fall in pH and methane production, which suggests that thermal pretreatment was detrimental to the productivity and stability of two-phase AD. However, the volatile solids destruction returns to the stable pre day 90 levels of 75-78%, signifying that the use of the pH+U method not only increases the hydrogen production from the system but also maintains the stability and high solids destruction seen in thermophilic AD.

5.3.1.4 Mass Balance

The mass balance of the AD process described in the chapter was carried on a kg TS basis. The total solids content of food waste and alkali addition per kg FW added were included as inputs. Methane, carbon dioxide and hydrogen volumes were corrected to STP. The TS of the effluent was also added to the output. Water vapour losses and moisture present in the effluent biogas could not be taken into account.

Table 5.1: Mass balance of two phase thermophilic AD

	No Pretreatment	Thermal Pretreatment	pH+U Pretreatment
	Mass (kg)	Mass (kg)	Mass (kg)
Food waste input TS	392.06	261.37	256.53
NaOH			23.50
Total input	392.06	261.37	280.03
Methane	5.84	3.41	6.33
Carbon dioxide	15.56	10.27	18.04
Hydrogen	0.12	0.07	0.26
Effluent TS	146.37	73.18	33.35
Total output	167.89	86.93	57.98
Balance	441.98	174.45	222.05
Balance %	72.47	66.74	79.29

Table 5.1 shows the mass balance of the two-phase AD during the phases of no pretreatment, thermal pretreatment and pH+U pretreatment.

The TS mass balance for AD of untreated FW was 72% while that of thermally treated FW was 66% and pH+U treatment was 79%. The difference in the balance can mostly be explained by water content of the effluent and water vapor losses which are not accounted for in this calculation. These findings confirm the results found in the previous sections- pH+U treatment leads to the most efficient process by increasing methane and hydrogen productivity as well as making small improvements in TS destruction. It was however important to note that this mass balance will have some

inherent inaccuracies due to the difficulty in estimating the TS on a daily basis. There will be some variability of the values of both FW and effluent TS which may not be reflected in the final data.

5.3.4 Bacterial community analysis

To study the microbial community structure and dynamics brought by the different pretreatment regimes, samples from R_A and R_S were retrieved at days 89, 149 and 209 to obtain DNA for the amplification and subsequent sequencing of a region of bacterial 16S rRNA genes. The bacterial community structures were analysed using the Illumina MiSeq platform for next-generation sequencing. High-quality sequences were obtained for different samples, ranging from 50,018 to 113,204; all sequences were aligned and clustered to calculate operational taxonomic units (OTUs) using 97% sequence identity as a cutoff, resulting in 459-740 OTUs per sample.

5.3.4.1 R_A Day 89 Bacterial analysis

The bacterial community shown in Fig 5.4 represents the dominant groups of bacterial active in R_A while the substrate was untreated FW. There were a total of 50,018 unique sequences found in this sample. Three main groups of bacteria were found to be active: Firmicutes which accounted for 60% of the total species; Proteobacteria, which accounted for 37% and Thermotogae, which accounted for the remaining 3%.

of just one species- *Thermoanaerobacterium thermosaccharolyticum*. This is an anaerobic thermophilic species that had been identified to be a promising route of biological hydrogen production using carbohydrate rich substrates (Nath and Das, 2004).

Fig. 5.5 Firmicutes and Proteobacteria break up of R_A at Day 89

Fig 5.5b shows the composition of the Proteobacteria group in R_A at Day 89. This group was found to be the most diverse in the acidogenic reactor. It mostly consisted of *Acetobacter peroxydans* (65% of Proteobacteria, 24% of total bacteria), and acetate producing bacteria. This was highly unexpected as it had been reported to be a mesophilic aerobe, which might indicate that the reactor may be operated by microaerobic yet highly reducing conditions as evidenced by the very low ORP. These conditions might be created during the process of addition of FW, which may contain significant amounts of dissolved oxygen and would momentarily raise the ORP of the reactor to less reducing levels of -50 to -80 mV. The dominance of *Lactobacillus* sp., an aerotolerant species fits in well with this explanation.

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. It had been shown that lactate can be oxidized to pyruvate followed by slow oxidation of pyruvate to acetate (De Ley and Schel, 1959). Therefore, the co-existence of *Lactobacillus* sp. and *Acetobacter peroxydans* possibly resulted in the high levels of solubilization and acidification observed in R_A.

The second major group in Proteobacteria was Gammaproteobacteria, which consist of 13% of all the bacteria in this sample. This group was highly diverse consisting 61% of Psuedomonales and 39% Enterobacteriaceae. The former in turn consisted of highly important species such as *Acinetobacter* sp., *Psuedomonas vernoii* and *Psuedomonas fragi*. These are all commonly present in anaerobic digestors (Guo et al., 2015). All these species are important for hydrolysis of various substrates as well as uptake of phosphate and denitrification. Their presence reflects the ability of the reactor to process a variety of substrates. Moreover, many of these groups, such as *Routella* species can

grow not only at thermophilic but also mesophilic and even psychrophilic temperatures, signifying that the reactor was not limited to a small window of thermophilic temperatures for its operation.

The third and smallest group, which accounts for only 4% of the total presence, was Thermotogae, which mostly consists of unidentified species belonging to the family Thermotogales. These are thermophilic bacteria that play a role in the production of various VFAs and hydrogenotropic methane production (Wang et al., 2011). Their presence reflects the diversity of the reactor and the availability of different groups that can produce substrates for methanogens, thus adding to the robustness of the system.

5.3.4.2 *R_A* Day 149 Bacterial analysis

Figure 5.5 shows the bacterial community found in *R_A* at day 149, representing the evolution of the community after 60 days of being fed with FW subjected to thermal pretreatment. There were large shifts in the make up of the community. The single most dominant species here was *Thermoanaerobacterium thermosaccharolyticum* which now consists of 34% of all bacteria. Meanwhile, the previous most dominant species, *L. delbruekii* had nearly disappeared and other lactobacillus species cumulatively consisted of only 37% of the community. The second most abundant species, *A. peroxydans* had also reduced to 10%, thus halving its presence, just like the various *Lactobacillus* sp. This signals a major change in the metabolic activity of the reactor. Earlier, the dominant species were those involved in the hydrolysis of the substrates but now since the substrate was hydrolysed by physical methods, the species that can take it up and produce hydrogen and acids are the one that become dominant. This was seen in the changes in the other species involved in the production of other products of AD rather

than hydrolysis as well. Thermotogae, the thermophilic group that produces acids and hydrogen now accounted for 10% of the OTUs, a threefold increase.

Fig. 5.5 Bacterial community break up of RA at Day 149

Furthermore, the emergence of the new dominant species of the Lactobacillales group explains the suboptimal operation of the reactor when fed with thermal pre-treated FW. As seen in section 5.2, this pretreatment did not show any increase in VFA production, only a modest increase in CODs production and a 50% decrease in hydrogen production. This can be attributed to the *L. casei*, which now comprises of 10% of all the bacteria present in the reactor and particularly to *L. plantarum*, which comprises of 11%. *L. plantarum* was a facultative anaerobe which can consume oxygen to produce hydrogen

peroxide. However, it had no cytochromes and superoxide dismutase like other aerobes and instead converts the peroxide to other forms which are used weapons to inhibit other non-related competing bacteria from using the substrate (Sankaran et al., 2014). It also produces other bacteriocins, which are toxins that inhibit the growth and metabolism of other bacteria (Jiménez-Díaz et al., 1994). Similarly *L. casei*, can also inhibit the growth of other bacteria while promoting the growth of other lactobacilli, so much so that it was used as a commercial probiotic (Klaenhammer et al., 2012). This can explain why there was so little productivity in the reactor despite the proper bacteria such as *Thermoanaerobacterium thermosaccharolyticum* being present in large amounts. The beneficiary of the probiotic nature of the other lactobacilli can be assumed to be *L. brevis*, which accounts for 11% of all bacteria. This was a VFA producer and efficient hydrolyser (Lee et al., 2008) and should, in addition to the action of the other lactic acid bacteria, account of the production of VFAs and CODs in the reactor.

5.3.4.3 *R_A* Day 209 Bacterial analysis

Figure 5.6 shows the bacterial community presented in *R_A* at day 149, which represents the evolution of the community after 60 days of being fed with FW subjected to pH+U pretreatment method. Here, the *Thermoanaerobacterium thermosaccharolyticum* was now the single most dominant species; effectively this pretreatment had changed the bacterial community such that it was the only major metabolic pathway going on in the reactor was hydrogen production. *T. thermosaccharolyticum* is a prodigious hydrogen producer and is the focus of other studies that isolate it and culture it for the production of biohydrogen from nutrient rich substrates (Cao et al., 2014; O-Thong et al., 2007). Here, it had been naturally enriched and other competing bacteria have been eliminated (there are effectively no Thermotogae or Lactobacillales present), meaning the vast majority of the substrate was diverted towards hydrogen production and other hydrolysis

and acid producing activities. This change in community structure could be attributed to the drastic change in pH and ORP brought in by the pH+U pretreatment. The higher pH eliminated the competing advantage of the lactic acid bacteria, which could function in acidic conditions while the substantially lower ORP levels created highly reducing and strong anaerobic conditions which are detrimental to those groups as they need small amounts of oxygen for survival.

However, the downside is other species like *Aeromonas caviae*, *Pantoea agglomerans* and *Klebsiella pneumonia* were taken up. While these bacteria carry out some functions in the AD process such as hydrolysis, nitrogen fixation, VFA production and hydrogen production, (Yang et al., 2008; Lee et al., 2009, Kim et al., 2011), they are all human pathogens that can cause mild to severe illnesses such as diarrhoea, pneumonia, urinary-tract infections, and even necrotizing fasciitis (Kumar et al., 2012). Thus, while pH+U pretreated FW can turn the acidogenic reactor into a high performing hydrogen producer, its effluent can be highly pathogenic in nature. Hence, the effluent may need disinfection before disposal

Fig. 5.6 Bacterial community break up of RA at Day 209

5.3.4.4 R_s Day 89 Bacterial analysis

Figure 5.7 shows the bacterial community presented in R_s at day 89, which represents the community after 90 days of being fed with untreated FW. Here the most dominant group was Thermotogae, which accounts for an overwhelming 85% of all bacteria present. The bacteria required for the early stages of AD such as hydrolysis and acidification, Firmicutes and Proteobacteria were present in very small numbers, accounting for only 6% and 7% each. This could explain the low productivity and VS destruction - the substrate utilization was not optimal due to the community being

dominated by methanogens with very few groups that are required to produce the products required for efficient methanogenesis. The three most prominent species apart from unidentified thermotogales species were *Fervibacterium*, which are a highly thermophilic methanogenic species (Salminen et al., 2003). The most dominant Firmicute was a *Coprothermobacter* sp which are a group of thermophilic hydrogen producing and hydrogenotropic methanogens (Tandishabo et al., 2010) while the most dominant Proteobacteria was *Psuedomonas caeni* which was a denitrifying bacteria playing a major role in acid production from carbohydrates. The available literature also suggests that these groups are more active at higher temperatures (55-65°C) and higher ORP levels (-350 to -400 mV), which might also explain why the reactor was not as productive as expected.

Fig. 5.7 Bacterial community break up of RS at Day 89

5.3.2.5 R_S Day 149 Bacterial analysis

Figure 5.8 shows the bacterial community presented in R_S at day 149, which represents the evolution of the community after 60 days of being fed with FW subjected to thermal. The community here was an extreme case of the one described in section 5.3.4. Thermotogae was by far the most dominant community in the reactor during this period, accounting for 89% of all the bacterial groups present. Proteobacter and Firmicutes combined accounted for less than 1%. As seen in the previous section, this change could be because of the easy availability of simple substrates causing the enrichment of Thermotogales. The limited diversity in this community along with the suboptimal pH

conditions could be the reason behind the observed instability and low methane production with thermally treated FW.

Fig. 5.8 Bacterial community break up of RS at Day 149

5.3.4.6 *R_s* Day 209 Bacterial analysis

Figure 5.9 shows the bacterial community presented in *R_s* at day 209, which represents the evolution of the community after 60 days of being fed with FW subjected to pH+U pretreatment. Here, the community started recovering compared to the previous section. Thermotogales were still the most dominant group, accounting for 62% of the total bacterial population, but Firmicutes now accounted for 8% and Proteobacteria 14%. This was reflected in the improved methane production and stability of the reactor. The increase in pH might have been the cause of both these changes. A new group of

5.4 Cost/Benefit Analysis

A cost benefit analysis was done to ensure that the improved gas production due to the pretreatment methods was commensurate with the added pretreatment costs. This was especially important here as the use of ultrasonication and addition on NaOH will incur continuous operational costs.

Table 6.7 shows the cost benefit analysis of the use of the alkaline and ultrasonic pretreatment for enhancing thermophilic AD. The analysis was done on a per year basis, hence all the results described in the chapter were projected over 365 days. NaOH addition costs were based on an addition rate of 2.4 g/L/day of substrate (FW mixture) added and the commercial wholesale price of the reagent as on 31st March, 2017.

The improvement in digester performance was estimated to be 6% for methane and 103% for hydrogen. The total increase in gas production was calculated based on project annual production using these figures and further energy estimations were done based on a similar cost/benefit study (Wolff et al., 2007). The energy density for methane and hydrogen was estimated to be 10kWh/L/m³ and 3kWh/m³ (Behlau, 2009). The monetary impact of this enhanced production on conversion to power was based on data used in the model study, conversion into energy at 40% CHP (combined heat and power) and 0.094 € net/kWh_{el}

Table 5.2: Cost/Benefit analysis of pretreatment regime on combined methane and hydrogen production

Cost	per year
Energy applied (kWh)	0.970
Cost of energy applied (SGD)	0.235
NaOH addition (SGD)	0.374
Total Annual Cost (SGD)	0.609

Benefit	L/year	kWh/year	kWh _{el} /year	SGD/year
Enhanced Methane production	289	2.89	11.56	0.976
Enhanced Hydrogen production	54.75	0.5475	2.19	0.185
Total Annual Benefit (SGD)				1.161

As seen from the calculations, the cost/benefit ratio of the use of this pretreatment regime was 0.069/1.1605 SGD/year, a clear indication of the net positive effect of the pretreatment regime. Furthermore, the electricity usage of the ultrasonicator was estimated to be 0.970kWh/year, while the power output from the increase gas production (methane and hydrogen combined) was estimated to be 2.89 kWh/yr, showing that the pretreatment regime created more energy than it used. It had to be noted that such a calculation does not account for the initial cost of the equipment and all the prevailing conditions and hidden costs involved in operating full scale waste treatment plants. Hence this result was only a rough estimate with an unpredictable error margin. However, such a large cost/benefit ratio points to a clear positive impact of the pretreatment process.

5.5 Conclusion

5.5.1 Summary

- Two-phase thermophilic AD was shown to substantially outperform single stage thermophilic AD. Furthermore, the separate acidogenic phase could be optimised for increased biohydrogen production. Hence, this study recommends its use for the conversion of food waste into methane and hydrogen.
- Thermal pre-treatment of food waste had negative effect on substrate solubilization, hydrogen or methane production in two-phase or single stage thermophilic AD process.

- The main reasons were less diverse bacterial community which lacked the presence of hydrolysing and acid producing bacteria as well the creation of unfavourable pH and ORP conditions.
- Alkaline + Ultrasonication (pH+U) pretreatment presented a small increase in CODs production in the acidogenic phase of two phase AD as well as led to a two fold increase in biohydrogen production, which converted the AD system from only methane producing to a methane and hydrogen producing set up.
- The microbial community was enriched with *Thermoanaerobacterium thermosaccharolyticum*, a bacterial species that shows prodigious biohydrogen production. Also, the removal of lactic acid bacteria due to the high pH and extremely reducing conditions stopped the production of inhibitory toxins in the system.
- However, this kind of pretreatment introduced large quantities of human pathogens into the sludge.
- The cost/benefit ratio for the use this pretreatment to enhance gas production (both hydrogen and methane) was found to be 0.069/1.1605 SGD/year, thus signifying that the addition of the pretreatment greatly improved the financial value of the AD process.

5.4.2 Significance

This study was the only one of its kind to use next gen sequencing to study the bacterial community in two phase and single stage thermophilic AD systems utilizing food waste and to follow the dynamics in the bacterial community due to the use of different pretreatment methods.

It also suggested a new method for enrichment high hydrogen producing species which converts the acidogenic phase into a standalone biohydrogen producing stage (hydrogen fermentation) which can also be used with a methanogenic reactor to produce methane, thus greatly increasing the productivity and value of the AD process.

CHAPTER 6

OPTIMIZATION OF MICRONUTRIENT SUPPLEMENT FOR ENHANCING BIOGAS PRODUCTION FROM FOOD WASTE IN TWO-PHASE THERMOPHILIC ANAEROBIC DIGESTION

6.1 Introduction

Anaerobic digestion (AD) is a promising approach for the bioconversion of FW into biogas with relatively low energy consumption, space requirement and cost (Speece, 1983). The two main groups of microorganisms (acidogens and methanogens) involved in AD differ widely in their nutritional needs, growth kinetics and environmental requirements. Any imbalance among them could cause process failure (Demirel and Yenigün, 2002). In particular, AD suffers with easily biodegradable substrate like FW due to enhancement of acidification stage (Ward et al., 2008). The scarcity of essential micronutrients, the presence of inhibitory agents such as long chain fatty acids (LCFA) and the excess of ammonia are some other factors that could further limit AD process (Chen et al., 2008).

Phase separation can improve the AD process through optimization of the acidogenic and/or the methanogenic stage (Jiang et al., 2012). The two-phase AD process mitigates imbalance and overloading while higher organic loading rates (OLRs) or biogas productivity could be achieved (Shen et al., 2013). Thermophilic AD (carried out at 50-60 °C) was another operation mode to increase biogas productivity. The higher

temperatures improve hydrolysis and physical degradation of the substrate, increase destruction of organic matter and enhance methane yields (Kim et al. 2002).

Recently, there had been a spotlight on the role of certain trace metals or micronutrients in AD process. The micronutrients play significant role in several metabolic pathways in AD, however, their concentration was usually inadequate to affect them (Schattauer, et al., 2011; Zhang et al., 2007). Micronutrients of particular interest are cobalt (Madigan et al., 2003), nickel, iron (Takashima and Speece, 1990), magnesium (Schmidt and Ahring, 1993) and calcium (Kleybocker et al., 2012). These micronutrients are specifically useful in thermophilic AD to improve VFA utilization, methane generation and cell lysis. While some research had been conducted to formulate a micronutrient supplement for use in AD, there was little work done towards tailoring a micronutrient supplement for enhancing thermophilic AD of food waste.

Usually, the micronutrient supplement is externally added in AD system (Facchin et al., 2013). However, it was imperative to determine the optimal levels as over dose can lead to excess cost and eco-toxicity. Response surface methodology (RSM), a collection of mathematical and statistical techniques can be used for analysing the effects of such independent variables and optimise their effect on a single response (Box and Draper, 1987). RSM had been used to optimize various factors in anaerobic digestion such as substrate mixing ratio for biohydrogen production (Kim, 2004), feeding rate, C/N ratio and substrate pretreatment for biogas production (Wang et al., 2012; Menon et al., 2015).

In this study, a micronutrient supplement containing Ca, Mg, Co, and Ni was formulated to improve methane generation in two-phase thermophilic AD system using food waste as substrate. RSM was initially applied to optimise the concentration of Ca, Mg, Co,

and Ni in batch experiments. The efficiency of the formulated supplement was tested in continuous mode thermophilic AD in terms of process stability and biogas yield.

6.2 Materials and methods

6.2.1 Substrate and inoculum

The primary substrate was FW collected from a canteen in Nanyang Technological University, Singapore. It mostly consisted of leftovers containing rice, noodles, fruit peels, boiled vegetables, cooked meat and oil. Non-biodegradable materials such as bones or plastics were manually separated. Afterwards, the FW was grinded to a homogeneous mixture and diluted with water to the concentration of 3 g VS/L. This was used as feedstock in the acidogenic phase of thermophilic AD process. The effluent from the acidogenic phase was used as substrate in the methanogenic phase of AD without any pre-treatment or dilution. Table 6.1 presents the main characteristics of FW and acidogenic effluent.

The original seed inoculum was mesophilic AD sludge obtained from Ulu Pandan Water Reclamation Plant, Singapore. Its conversion to thermophilic sludge was done using a method adopted from the one described by de la Rubia et al. (2005) with some changes. The reactor was initially operated with an organic loading rate (OLR) of 0.5 g/L/day with food waste substrate and a hydraulic retention time (HRT) of 30 days for a period of 2 weeks at mesophilic conditions in a 20L single stage semi-continuous stirred tank reactor setup. The temperature was then directly raised to thermophilic conditions (55°C) and after a period of starvation for 2 days, the reactor was fed with 0.5g glucose/L/day for 2 weeks while the other conditions remained unchanged. The substrate was then changed to food waste with an OLR of 0.5gVS/L/day. Once biogas

production and solids destruction was stabilised, the sludge was divided into two phases which were operated under the conditions detailed in section 2.3. The effluent from the methanogenic phase was collected and used as inoculum in the batch experiments.

Table 6.1. Characteristics of food waste original feedstock and acidogenic AD effluent.

Parameter	Food Waste(3 gVS/L)	Acidogenic effluent
pH	5.1	4.2
ORP (mV)	-59	-388
TS (%)	5.88	2.1
VS/TS (%)	93.51	74.54
Acetate (mM)	1.22	40.17
Propionate (mM)	0.15	1.86
Butyrate (mM)	0.05	15.52
TVFAs (mM)	1.98	59.69
CODT (g/L)	33.55	28.26
CODS (g/L)	8.95	16.57
Ca (mg/L)	13.3	36.2
Co (µg/L)	2.27	0.84
Fe (µg/L)	51.79	16.25
Mg (µg/L)	15.8	5.18
Ni (µg/L)	14.18	n.d

n.d. Not detected

6.2.2 Micronutrient supplement

The concentration of micronutrients (Ca, Mg, Co, and Ni) was measured by the quantity of individual component typically found in food waste and the actual requirement in AD process. Three levels were used to determine the optimal concentration for the trace metals: 0, 250 and 500 mg/L for Ca^{2+} ; 0, 500 and 1000 mg/L for Mg^{2+} ; 0, 10 and 20 mg/L for Co^{2+} , and 0, 5 and 10 mg/L for Ni^{2+} . All trace metals were added as dissolved salts ($\text{CaCl}_2 \cdot 4\text{H}_2\text{O}$, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$) in the feed for the

methanogenic phase. In addition, Visual Minteq, a chemical equilibrium model was used to calculate the metal speciation and solubility equilibria.

6.2.3 Experimental setups

The optimal concentration of micronutrient supplement was determined using biochemical methane potential (BMP) tests. The substrate to inoculum ratio (SIR) was maintained at 0.3 (based on VS) (Angelidaki et al., 2009). The BMP tests were conducted in the Automated Methane Potential Testing System (AMPTS II) (Bioprocess Control, Sweden). AMPTS consisted of 15 parallel reactors of 500 mL capacity (working volume of 400 mL), and the same number of gas flow meters was connected to the acquisition system. The reactors were sealed with rubber stoppers and then connected to mechanical agitator which stirred the sludge for 1 minute on and off at 80 rpm. The reactors were sparged with nitrogen gas before sealed to create anaerobic conditions and incubated at 55 °C. The gas produced was monitored using automated data logging system. Three separate runs were carried out - Run 1: Ca and Mg mixture (high concentration metals); Run 2: Co and Ni mixture (low concentration metals), and Run 3: optimized micronutrient supplement. Each run was conducted in duplicate and the experiments lasted for 20 days.

6.2.4 Response Surface Methodology (RSM)

A central composite design with two factors was used to design the experiments in this study. Methane production was chosen as the response variable and the concentrations of Ca, Mg, Co and Ni were chosen as the independent variables. The micronutrients were divided into two groups for evaluation on the basis of their similarity and

synergistic effect: Ca and Mg (similar chemical characteristics, required in larger concentrations), and Ni and Co (both involved in methanogenesis and substrate utilization, required in lower concentrations). The statistical software analysis program MINITAB 17 (SBTI, San Marcos Texas) was used for this purpose.

6.2.5 Analytical techniques

The pH-readings in the batch experiments were conducted with a compact titrator (Mettler Toledo) equipped with a pH probe (Mettler Toledo DGi 115-SC). Total (COD_T) and soluble (COD_S) chemical oxygen demand measurements were made using COD digestion vials (Hach) and a spectrophotometer DR2800 (Hach, USA) as per standard methods (APHA, 1995). Ammonia was measured colorimetrically using a DR2800 spectrophotometer (Hach, USA). The micronutrient content in the AD effluent was analysed using Inductively Coupled Plasma-Mass Spectrometer (ICP-MS NexION 300D, Perkin Elmer). Volatile fatty acids (VFAs) content was measured using a gas chromatograph (Agilent Technologies 7890A, USA), equipped with a flame ionization detector (FID) (Agilent Technologies, USA). A selective capillary column (DB-FFAP) was applied for good separation of nine VFA compounds including acetic, propionic, iso-butyric, n-butyric, iso-valeric, n-valeric, iso-caproic, n-caproic and heptanoic acids. A gas chromatograph (Agilent Technologies 7890 A, USA) equipped with a thermal conductivity detector (TCD) was used to measure the methane, hydrogen and carbon dioxide content in biogas. The C/N ratio was determined by measuring the total carbon and nitrogen using an elemental analyzer (Vario EL cube CHNOS, Germany).

6.3 Results and discussion

6.3.1 Micronutrient availability

The bioavailability of micronutrient elements was determined using the Visual Minteq software. Table 6.2 shows species concentrations used for modelling the metal speciation analysis. Normal methanogenic conditions of pH = 7, ORP = -500mV and ammonia, carbonate and phosphate species were used. For the determination of bioavailability the maximum concentration of trace metals was used (Ca: 500 mg/L, Mg: 1000 mg/L, Co: 20 mg/L, Ni: 10 mg/L).

Table 6.2 Dissolved species in methanogenic phase

Species	Concentration (mg/l)
H ⁺¹	0
Co ⁺²	20
CO ₃ ⁻²	6
Fe ⁺³	200
Ca ⁺²	500
Mg ⁺²	1000
PO ₄ ⁻³	125
NH ₄ ⁺¹	6
Ni ⁺²	10
Cl ⁻¹	1730

Table 6.3 shows the details of the speciation analysis for trace metal ions. 94% and 91% of added calcium and magnesium were predicted to remain in solution as Ca²⁺ and Mg²⁺ ions respectively while 92% of Cobalt was predicted to remain in the form of bioutilizable Co²⁺ ions. 83% of the trace metal that was to be added in the smallest

amounts, nickel, was predicted to be available in the form of Ni^{2+} ions while a further 15% was predicted to remain in the form of soluble ammonium and phosphate complexes. Therefore, all the trace metals to be added would mostly be bio available and the anaerobic system would not suffer with precipitation accumulation (insoluble crystals) that could inhibit biogas productivity and cause toxic effects.

Table 6.3 Metal speciation in methanogenic phase

Component	% of total concentration	Species name	Component	% of total concentration	Species name	Component	% of total concentration	Species name
Cl-1	93.645	Cl-1	Ca+2	94.04	Ca+2	NH4+1	91.201	NH4+1
	1.042	CaCl+		0.013	CaCO3 (aq)		8.205	NH3 (aq)
	5.307	MgCl+		4.077	CaCl+		0.365	NiNH3+2
Co+2	91.993	Co+2		1.472	CaHPO4 (aq)		0.027	Ni(NH3)2+2
	0.252	CoOH+		0.304	CaPO4-		0.177	Co(NH3)+2
	0.659	CoCl+		0.044	CaH2PO4+		0.023	CaNH3+2
	2.772	Co(NH3)+2		0.037	CaHCO3+	Ni+2	83.303	Ni+2
		CoHPO4 (aq)	Mg+2	91.633	Mg+2		0.973	NiOH+
	3.993			0.053	MgOH+		0.496	NiCl+
	0.068	CoCO3 (aq)		6.296	MgCl+		11.418	NiNH3+2
	0.257	CoHCO3+		1.98	MgHPO4 (aq)		0.421	Ni(NH3)2+2
				0.028	MgHCO3+		0.018	NiH2PO4+
CO3-2	0.379	CO3-2	PO4-3	16.21	HPO4-2		2.872	NiHPO4 (aq)
	1.676	CaCO3 (aq)		3.123	H2PO4-		0.121	NiCO3 (aq)
	0.222	Mg2CO3+2		0.372	NiHPO4 (aq)		0.369	NiHCO3+
	74.224	HCO3-		1.03	CoHPO4 (aq)	Fe+3	0.013	FeOH+2
	3.329	H2CO3* (aq)		0.145	MgPO4-		52.775	Fe(OH)2+
	0.206	NiCO3 (aq)		61.862	MgHPO4 (aq)		2.754	Fe(OH)3 (aq)
	0.628	NiHCO3+		13.949	CaHPO4 (aq)		44.456	Fe(OH)4-
	0.232	CoCO3 (aq)		2.884	CaPO4-			
	0.872	CoHCO3+		0.416	CaH2PO4+			
	1.941	MgCO3 (aq)						
	11.652	MgHCO3+						
	4.638	CaHCO3+						

6.3.2 Optimization of micronutrient mixtures

6.3.2.1 Run 1: Ca and Mg mixture

Figure 6.1 presents the effect of Ca and Mg mixture on methane productivity. It seems that the addition of micronutrients had a positive effect in biogas production. More specifically, the addition of micronutrients not only increases the productivity, but it accelerated the production rate as well. This suggests that the trace elements Ca and Mg were at suboptimal concentrations in the AD system and their addition was necessary for better process performance. The production of methane was increased by 30-60% at various dosages of the micronutrients, with the combination of 250 mg/L Ca^{2+} and 500 mg/L Mg^{2+} showing the highest yield.

Fig. 6.1 Effect of Ca and Mg on methane productivity

The RSM analysis of Ca and Mg concentrations for optimized methane production was shown in Fig. 6.2 in the form of the three dimensional response surface of the predicted methane productions for various combinations of Ca and Mg concentrations. The regression equation used for the model was:

$$\text{CH}_4 = 1170 + 4.178 \text{ Ca} + 1.381 \text{ Mg} - 0.00583 \text{ Ca}^2 - 0.000720 \text{ Mg}^2 - 0.000861 \text{ Ca}^* \text{Mg}$$

(1)

Fig 6.2 Three-dimensional response surface of the effects of Ca and Mg supplements on methane production.

Fig. 6.3 shows the 2D surface graphs for optimising Ca and Mg concentrations for maximum methane production. The predicted optimal concentrations for Ca and Mg were 303 and 777 mg/L, respectively, which could theoretically produce 2.3 L of methane, leading to a 75% increase in productivity.

Fig. 6.3 2D surface graph for optimizing Ca and Mg concentrations

Table 6.4 shows the ANOVA results for the response surface analysis of co-addition of Ca and Mg for maximising methane production. Based on the significantly strong relationship between the model and the response variable (p-value <0.005) and high R-squared and R-squared (predicted) values of 93.31% and 88.53%, signifying acceptable goodness-of-fit, the model can reliably be used to predict the effect of co-addition of Ca and Mg on methane production. In the regression model, p-values of the both Ca and Mg linear coefficients are highly significant (<0.005), signifying that both have a large effect methane production. The squared value Ca*Ca was highly significant (p-value <

0.005) while that of Mg*Mg was significant (<0.05), which suggests that while both are important for methane production, calcium was the more important element of the two. On the other hand, the Ca-Mg had a high p value (>0.05), which suggests that the synergistic effect of calcium and magnesium on methane production not significant. This was to be expected as both these micronutrients target different biological processes and their effects do not overlap. Ca and Mg are similarly important as activators of various enzymes which are important for the growth and functioning of bacterial communities, the primary role of Calcium was to inhibit the toxicity of long chain fatty acids (Keyblocker, 2012). Long chain fatty acids (LCFAs) are a major inhibitor of biogas production (Kuang et al., 2006; Luostarinen et al., 2009). These are a cause of major concern when biogas plants are and can cause reactor failure by overacidification. The role of Magnesium ions to stimulate the production of single cells (Harris, 1987; Xun et al., 1988; Schmidt and Ahring, 1993). The high sensitivity of single cells (which are necessary for catabolizing acetic acid) to the high sensitivity of single cells to lysis was an important factor in the loss of acetoclastic activity in anaerobic reactors. This problem was heightened in thermophilic reactors as cell lysis was accelerated here.

Table 6.4: ANOVA results for response surface analysis of co-addition of Ca and Mg

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	5	1299958	259992	19.53	0.001
Linear	2	558910	279455	20.99	0.001
Ca	1	260625	260625	19.57	0.003
Mg	1	298285	298285	22.4	0.002
Square	2	694758	347379	26.09	0.001
Ca*Ca	1	366375	366375	27.52	0.001
Mg*Mg	1	89551	89551	6.73	0.036
2-Way	1	46290	46290	3.48	0.105

Interaction					
Ca*Mg	1	46290	46290	3.48	0.105
Error	7	93201	13314		
Lack-of-Fit	3	87812	29271	21.73	0.006
Pure Error	4	5389	1347		
Total	12	1393159			

R-sq	R-sq(adj)	R-sq(pred)
93.31%	88.53%	37.66%

It was difficult to compare this result with available studies as there are very few studies done to determine optimal concentrations of these micronutrients for thermophilic AD of food waste. The result agreed with some similar studies (Takashima and Speece, 1990) as the optimal concentrations determined here fell squarely within the suggested concentrations for Ca and Mg.

6.3.2.2 Run 2: Co and Ni mixture

Fig. 6.4 shows the methane productivity after adding mixture of Co^{2+} (0, 5 and 10 mg/L) and Ni^{2+} (0, 2.5 and 5 mg/L). Similar to Run 1, the addition of micronutrients greatly increased methane production of thermophilic AD process. The enhancement was between 65-80% over control.

Fig.6.4 Effect of Co and Ni on methane productivity

Figure 6.5 shows the RSM based on optimization of Co and Ni concentrations for methane production. The quadratic equation used for this model was:

$$\text{CH}_4 = 1131.0 + 52.2 \text{ Co} + 158.2 \text{ Ni} - 2.13 \text{ Co} \cdot \text{Co} - 17.23 \text{ Ni} \cdot \text{Ni} - 6.59 \text{ Co} \cdot \text{Ni}$$

(2)

Fig. 6.5 Three-dimensional response surface of the effects of Co and Ni supplements on methane production.

Fig. 6.6 shows the 2D plots of Co and Ni concentrations optimised for maximizing methane production. The best combination was 7 mg/L Co^{2+} and 3 mg/L Ni^{2+} , which would theoretically increase biogas yield by 45%.

Fig. 6.6 2D surface graph for optimizing Co and Ni concentrations.

Table 6.6 shows the detailed analysis of variance results for the co-addition of Co and Ni for increasing methane yield. The relationship between the model and the response variable (methane generation) was highly significant (p-value <0.005) and the high R-squared value (>93%) suggests that the model had considerable goodness-of-fit, hence this model can be said be safely used to predict the relationship between the concentrations of Co and Ni and methane production. The effect of addition of both micronutrients was shown to be highly significant for methane generation (p-value < 0.005). The square term of Ni was highly significant (p-value < 0.005) while that of Co was only significant at low p-value (p<0.1), implying that Nickel addition was extremely important for increasing biogas production while Cobalt was still important but not as critical. Moreover, co-addition of both was significant (p <0.05), suggesting that the two micronutrients have an important synergistic effect. This might be due to

the fact that both elements are directly involved in metabolic processes involved in methanogenesis. Cobalt was a key part of vitamin B12, which was involved in the activity of methyl transferase, required for the transfer of methyl groups from compounds including methanol, in methanogenesis (Madigan et al., 2003). Cobalt was also found in carbon monoxide dehydrogenase (CODH), which plays an essential role in methanogenesis from acetate (Madigan et al., 2003, Zandvoort et al., 2006). CODH also requires nickel, which was also found in the cofactor coenzyme F430 (Takashima and Speece, 1990). Coenzyme F430 was a porphyrinoid (Shima et al., 2002) that plays a role in autotrophic methanogenesis, making nickel especially important when H₂ and CO₂ are the sole sources of energy. Nickel was at the catalytic centre of most hydrogenases (Shima et al., 2002) and may be involved in membrane stability (Jarrell and Sprott, 1982).

Table 6.5 ANOVA results for response surface analysis of co-addition of Co and Ni

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	5	176639	35327.8	19.16	0.001
Linear	2	88849	44424.7	24.1	0.001
Co	1	31284	31284.3	16.97	0.004
Ni	1	57565	57565.2	31.23	0.001
Square	2	60688	30344	16.46	0.002
Co*Co	1	7814	7814.1	4.24	0.079
Ni*Ni	1	32016	32015.6	17.37	0.004
2-Way Interaction	1	27101	27101.4	14.7	0.006
Co*Ni	1	27101	27101.4	14.7	0.006
Error	7	12904	1843.5		
Lack-of-Fit	3	12644	4214.5	64.66	0.001
Pure Error	4	261	65.2		
Total	12	189543			

R-sq	R-sq(adj)	R-sq(pred)
93.19%	88.33%	32.60%

As before, while it was difficult to find exactly similar studies, these findings somewhat agree with comparable studies (Pobeheim et al., 2010). The suggested concentration of Co agrees with the range mentioned in the study but that of Ni was higher by a degree of magnitude. However, since such studies use mesophilic conditions and different substrates, it was understandable for them to have much different results than this one.

6.3.3 Formulation of micronutrient supplement

Based on previous findings, the micronutrient supplement was formulated with Ca, Mg, Co and Ni containing 303, 777, 7, and 3 mg/L, respectively. Experiments testing the effects of formulated micronutrient supplement, optimized Ca and Mg mixture, and optimized Co and Ni mixture in methane productivity are presented in Fig. 6.7. The experiments were run for 20 days in triplicate. It was demonstrated that the synergistic effect of micronutrient supplement greatly enhanced the thermophilic AD process compared to individual mixtures. The addition of the full micronutrient supplement was shown to highly significant ($p < 0.005$) in improving biogas production. The total biogas production was 11.4 L CH₄/gVS_{added}, an increase of 2.7 times over control. There was also significant improvement ($p < 0.05$) in the productivity in comparison to the addition of individual mixtures (Ca+Mg and Co+Ni respectively) with at least 20% increase in total biogas production. The batches with the individual mixtures (Ca+Mg and Co+Ni) had more or less the same total productivity implying that the benefits of adding both these groups of elements were the same. However, the gas production profiles of both were different: the batches fed with the Ca+Mg mixture had a very short lag phase, likely resulting from the improvements in cellular stability and inhibition as

described in section 3.2. The batches fed with Co+Ni had a longer lag phase but their productivity caught up with the former quickly, which could be attributed to the metabolic enhancements in methane generation pathways offered by those micronutrients. The combined benefits of these two groups were seen the biogas production profile of the batches supplement with all four micronutrients, resulting in not just higher productivity by also a major reduction in the processing time. It took only 5 days for 85% of the total biogas production.

Fig.6.7 Methane productivity using the formulated micronutrient supplement, and mixtures of Ca and Mg, and Co and Ni.

Table 6 presents the effluent characteristics (VFAs, TS removal, total and soluble COD, and pH) at the end of the experiments. The experiment with formulated micronutrient

supplement showed a significant reduction in effluent VFAs, TS and COD compared to experiments with individual mixtures and control. The residual VFAs in the batches with full micronutrient supplementation show 28-30% and 45% reduction compared to addition of Ca-Mg and Co-Ni mixtures and control respectively. This points to the micronutrient supplement greatly enhancing VFA utilization, which contributes to both improved methane production and decreased acidification. The latter was apparent in the noticeably higher pH of the batches with micronutrient supplementation (7.8 compared to 7.1 in control). There was an almost 3-fold reduction in CODs on addition of the micronutrients, again pointing to the micronutrients improving substrate utilization. While there was a significant increase in TS destruction in TS destruction (76.8% in micronutrient supplemented batches against 72.5 in control), it was more or less the same as that in the batches Co and Ni addition, suggesting that Ca and Mg do not have a major role in solids destruction. Regarding trace metals residual concentration, it seems that more than 95% of elements in micronutrient supplement were utilized by the microbial activity. The fact that that no accumulation of precipitation was noticed was important because not only would it could be toxic in long run, it would also affect the economic viability and sustainability of micronutrients addition.

Table 6.6 Effluent characteristics after experiments with micronutrient addition.

	VFA (mM)	TS reduction (%)	CODs (g/L)	CODt (g/L)	pH
Micronutrient supplement	1.8	76.8 ± 0.03	3.03 ± 0.04	18.14 ± 0.14	7.8
Ca+Mg mixture	2.3	74.7 ± 0.12	7.80 ± 0.06	22.58 ± 0.16	7.4
Co+Ni mixture	2.5	76.8 ± 0.04	6.97 ± 0.02	24.36 ± 0.04	7.5
Control	3.1	72.5 ± 0.14	8.36 ± 0.09	26.56 ± 0.15	7.1

6.4 Conclusions

6.4.1 Summary

The influence of adding four trace metals (calcium, magnesium, cobalt, and nickel) in the form of a micronutrient supplement in the methanogenic phase of the thermophilic system was investigated in this study. Response Surface Methodology (RSM) was used applied to determine the optimal concentrations of micronutrients in the supplement in batch experiments. The results showed that optimal concentrations of were found to be 303, 777, 7 and 3 mg/L of Ca, Mg, Co and Ni , respectively, and the supplement increased the biogas productivity by 2.7 times and reduced VFA accumulation by 45% compared to control, in addition to reducing the processing time.

6.4.2 Significance

This study was the first of its kind to create an optimised micronutrient supplement containing calcium, magnesium, cobalt and nickel for enhancing methanogenesis in a two phase thermophilic AD system utilizing food waste. As such a supplement had commercial value, the results of this study are the subject of a provisional patent application (Wang, Menon and Giannis; Provisional Patent Application No. 10201600685W).

CHAPTER 7

INFLUENCE OF MICRONUTRIENT SUPPLEMENTATION ON LONG TERM CONTINUOUS TWO-PHASE AD SYSTEM AND EFFECT ON THE BACTERIAL COMMUNITY DYNAMICS

7.1 Introduction

The previous chapter described the formulation of a micronutrient supplement to improve the productivity of a two-phase thermophilic AD system. Batch experiments indicated that adding a micronutrient ‘cocktail’, which consisted of Calcium, Magnesium, Cobalt and Nickel in concentrations of 303, 777, 7, and 3 mg/L, greatly improved methane production in the methanogenic phase. In this chapter, the influence of this supplement was tested in long term continuous two-phase AD system.

The effect of Ca, Mg, Co and Ni addition on substrate utilization and methanogenic pathways could only be inferred based on previous studies. This study investigated on the effects of micronutrient addition on the composition of the microbial community in the methanogenic phase. Methane production in AD was attributed to two archaeal orders, *Methanosarcinales* and *Methanomicrobiales*, which produce methane through acetoclastic and hydrogenotropic routes, respectively (Sekiguchi and Kamagata, 2004). The dynamics between the two groups and the two major *Methanosarcinales* genus, *Methanosaeta* and *Methanosarcina* are now well understood (Raskin et al., 1994; Vrieze et al., 2012; Williams et al., 2013) and predictably vary according to the OLR and substrate (acetate and hydrogen) availability. However, there was no indication on the bacterial dynamics in the methanogenic reactors (Merlino et al, 2013). Furthermore,

because of the bacterial populations represent more diverse pathways including acetate and hydrogen production, denitrification and methane production (Qiu et al, 2015), a next gen sequencing based study with a focus on bacterial community dynamics was carried out to investigate the effects of micronutrient supplementation.

The objectives of this study were to test the micronutrient supplement on the methanogenic phase of long term continuous two-phase thermophilic AD using food waste and investigate its effects on process productivity and stability as well as characterise the bacterial community and study its dynamics before and after micronutrient addition.

7.2 Material and Methods

7.2.1 Feedstock preparation and inoculum sources

The FW and inoculums were the same as the ones described in section 4.2.1.

7.2.2 Experimental setup

The reactor setup for two-phase thermophilic AD was described in section 5.2.2 and the formulation of the micronutrient supplement was described in section 6.2.3. The two-phase AD was operated without any micronutrient addition for 150 days, after which the micronutrient supplement was added in 3 doses with 20 days intervals (i.e. on day 151, day 171 and day 191).

7.2.3 Bacterial community analysis

Sludge samples were collected on days 140, 170 and 210. The bacterial community was analysed using the method described in section 5.2.3.

7.2.4 Analytical techniques

The analytical methods were described in section 4.2.4.

7.3 Results and Discussion

7.3.1 Effect of micronutrient supplement on long term thermophilic AD

The micronutrient supplement was formulated in batch tests, and subsequently its efficiency to improve methane productivity was tested in the methanogenic phase of two-phase thermophilic AD system.

Figure 7.1a illustrates the methane yield in the methanogenic reactor. Initially, the two-phase thermophilic AD process was carried out without any micronutrient supplement or treatment of any kind. The methane yield averaged 0.3-0.35 L CH₄/ g VS_{added}/day till around day 80, where there was some sludge washout. This caused temporary reduction in methane productivity and afterwards it fluctuated due to unstable conditions. At day 150, the methane yield reached the lowest value 0.2 L CH₄/g VS_{added}/day. At this point, the micronutrient supplement was added to the reactor. This was done by preparing a 20X stock solution in distilled water and adding it to the feed (effluent from the acidogenic phase). The final concentrations of the various trace metals in the methanogenic phase were as specified in section 3.3. The methane production recovered rapidly and for a few weeks, the productivity continued to increase until trace elements

were depleted. The micronutrient supplement was added again on day 170 and 190 to maintain the optimal concentration of the micronutrients in the system. The methane production reached the ultimate yield 0.461 L CH₄/g VS_{added}/day, which was nearly twice the production before the addition of the supplement. Methane production stabilised at around 0.43 L CH₄/g VS_{added}/day, which was 40% increase over the stable methane production in the early days of the AD. This indicates that the micronutrient supplement not only increased the methane productivity, but also stabilized the failing thermophilic AD process.

The methanogenic reactor was also monitored for pH and ORP variations (Fig. 7.2b). Similar to methane yield, pH and ORP showed fairly stable profiles till day 80, after which some instability appeared. The pH dropped below 6 after day 110, which was at suboptimal level for methanogenesis. The pH decrease signified possible VFA accumulation. ORP presented similar trend (at suboptimal level), with some instability beyond day 110. However, after the addition of the micronutrient supplement on day 150, both pH and ORP quickly recovered. They reached optimal and stable levels again within two weeks and remained at those levels for the duration of the experiment.

Fig. 7.1 Monitoring the methanogenic phase of continuous thermophilic AD process, (a) methane production; (b) pH and ORP variations.

The results showing improvement of methane production could be compared to other studies that deal with similar substrates. Climenhaga and Banks (2008) used a micronutrient recipe described by Gonzales-Gil et al. (2000), which consisted of 2000 mg/L of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 142 mg/L of $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ amongst other trace elements such as Fe, Al, Zn, Cu, and Mn. to stabilize methane production from single stage mesophilic AD of catering wastes in semi-CSTR. The AD process was shown to fail and stop

producing biogas after 60 days of operation at 25 day HRT, while the micronutrient supplementation allowed for stable methane production at 0.4-0.5 L/g VSadded/day. In our study, methane production was stabilized at similar levels while using lesser micronutrients (4 opposed to 10), at lower concentrations. This emphasizes the importance of determining the optimal concentrations of the micronutrients to minimise costs and potential toxicity. Zhang and Jahng (2012) used trace metal supplementation to stabilize methane production from AD of food waste in CSTR. A mixture of Co, Fe, Mo and Ni (2, 100, 10 and 5 mg/L each) was shown to stabilize methane production at 0.3-4.3 L/g VSadded/day, agreeing with the results found in this study. Although the studies differ in the kind of substrates used and reactor setups, the common theme was improvement in substrate utilization and methane production on the addition of trace metals. This can be attributed to the biological roles of these elements.

Fig. 7.2a shows the influent and effluent profiles for the methanogenic reactor. Although the concentration of VFAs in the influent was nearly constant, there was a major build-up of VFAs in the reactor. By the day 150, the amount of VFAs almost doubled compared to initial stages of the AD. This caused pH drop (as seen in Fig. 7.1) and hence reduced methane productivity. After micronutrient supplement addition on day 150, there was a drastic increase in the utilization of VFAs, indication the direct impact of micronutrient availability on substrate utilization. This was also apparent in the rapid increase of methane yield. The VFA levels reduced further after the second addition of the supplement and stabilised by the time of the third addition. The stable VFA levels showed more than 50% reduction compared to pre micronutrient supplementation levels. These results could be attributed to the addition of Ca and Co in particular. Calcium was well known to play an important part in cell permeability (Chen, et al., 2007) as well as enhancing the actions of other metals (Oleszkiewicz and Sharma, 1990). Cobalt addition

had been shown to be critically important in improving VFA utilization (Zandvoort, et al., 2006; Gallert and Winter; 2006) and for acting synergistically with Ni for VFA uptake and enhancing methanogenic activity (Murray and van den Berg, 1981). Because of this specific utility in improving VFA metabolism, micronutrient formulations have been shown useful in combating VFA accumulation in thermophilic AD (Yirong et al., 2015), although not as successfully as in this study.

Figure 7.2b shows the influent and effluent CODs variations of the methanogenic reactor. While the CODs was fairly constant till day 150, varying between 6-8 g/L, a drastic reduction was seen after the addition of the micronutrient supplement. After the first addition, the CODs levels dropped rapidly to 3g/L and stayed in the 2.5-1.5g/L region after the second and third additions. This compared favourably with effluent CODs seen levels in similar studies (Zhang et al., 2011; Zhang and Jahng, 2012). Such drastic reductions in CODs could explain the significant improvement in methane production, as more CODs utilization raised methane productivity on addition of the supplement. Micronutrient availability was critical for such improvements: Co and Ni affect the metabolic pathways in the methanogenesis stage, they are essential for the synthesis and activity of biomolecules like corrinoids, CODH, Coenzyme A, methylreductase, methyltransferase and other hydrogenases, (Oleszkiewicz and Sharma, 1990), all of which are crucial for metabolic pathways involved in conversion of hydrolysis products to methane. Furthermore, as stated before, Ca and Mg are important for substrate uptake and the longevity and function of cells required for converting available substrates to methane.

The VS and TS destruction profiles are presented in Fig. 9.2c. VS destruction was around 70% before the process became unstable. After day 70, the VS destruction was

reduced to 35% suggesting low substrate utilization efficiency and increased washout of inoculum. The washout increased drastically after day 70 and this was reflected in the rapidly lowered VS destruction. Although there was a slow recovery after day 120, micronutrient supplementation on 150 lead to an immediate improvement in VS destruction. By day 160, it had recovered to its pre supplementation stable condition and within a week it had increased by 10-15%. Similarly, TS destruction also showed a small increased on micronutrient supplementation. This improvement can be attributed to a healthier biomass with enhanced substrates utilization rates for the same reasons stated above. Similar studies shows an improvement in VS removal (Yirong et al., 2015), although not to this extent. This can be attributed to a healthier, more effective biomass. While cobalt had been specifically identified to be responsible to improving the health of the biomass (Agler et al., 2008), the inclusion of Mg in the supplement was also of vital importance, as stated before, it had been known to spur the growth of cells directly involved in substrate utilization and prevention of biomass washout. This was especially significant as the role magnesium was mostly neglected in most studies involving improving AD by micronutrient addition.

Fig. 7.2 Methanogenic reactor monitoring, (a) influent/effluent VFAs; (b) influent/effluent soluble COD; (c) solids destruction.

7.3.1.1 Mass Balance

The mass balance of the AD process described in the previous section was also carried on a kg TS basis. The mass inputs were the total solids content of the FW and the total micronutrient supplementation. The outputs were the weights of methane and carbon dioxide (corrected to STP) and the TS of the effluent.

Table 7.1: Mass balance of two phase thermophilic AD before and after micronutrient supplementation

	No supplemetation	Micronutrient supplementation
	Mass (kg)	Mass (kg)
Food waste TS	609.87	261.37
Micronutrients		3.27
Total input	609.87	264.64
Methane	8.58	5.08
Carbon dioxide	29.76	14.77
Hydrogen	0.00	0.00
Effluent TS	323.23	54.89
Total output	361.57	74.74
Balance	248.30	189.90
Balance %	40.71	71.76

The mass balance of the micronutrient supplemented AD process was shown in table 7.1. The TS mass balance of unsupplemented AD was 41% while that of the supplemented process was 72%. . The difference in the balance will mostly be explained by the water content of the effluent and water vapor losses which are not accounted for in this calculation. The low efficiency of the former was largely due to the washout and failure event due to acidification which led to a large amount of TS present in the effluent. The low balance indicates a high water content, possibly due to most of the biomass being lost due to cell death owing to the unsuitable conditions for cell growth and activity. The greater balance on micronutrient addition agrees with the earlier results

which indicate that the supplement makes the process more efficient by greatly improving methane yield and promoting a stable, healthy microbial community.

7.3.2 Bacterial community analysis

To study the effect of micronutrient addition on the bacterial community structure, samples were retrieved from the methanogenic reactor at days 145, 170 and 210 and the sequencing of bacterial 16s rRNA genes was carried out after DNA isolation and amplification. As in Chapter 5, the bacterial communities was analysed using the IlluminaMiSeq platform for next generation sequencing.

7.3.2.1 Bacterial analysis of Methanogenic reactor at day 140

The bacterial community shown in Fig. 7.3 shows the dominant groups of bacteria active in the methanogenic reactor at day 145, i.e. before any micronutrient addition.

Fig. 7.3 Bacterial community break up of methanogenic reactor at day 145

About 240 different bacterial species were found in the community from 70,219 reads PF. The Shannon Species Diversity was 1.112. The most dominant group here was phylum *Thermotogae*, which accounted for 89% of the all the bacteria in the reactor before micronutrient addition. Most of this group (93%) was an unidentified species belonging to the order *Thermotogales* and the rest were *Fervidobacterium* species. The *Thermotogales* species was unidentified; however, it was most similar to *Thermosipho ferriphilus*. The *Thermosipho* genus consists mostly of species that utilise simple and complex carbohydrates to mostly produce hydrogen and some acetate (Serve et al, 2009), thus it can be concluded that the methanogens present here mostly methane through both the acetoclytic route using the influent VFAs from the first phase as well

as the hydrogenotropic route using the hydrogen produced by the bacteria in the second phase. Interestingly, *Thermotogales* are a hyperthermophilic species mostly found in hydrothermal vents. Their optimal temperature is 70-75°C, and are known to be extremely efficient hydrogen producers (Olliver et al, 2000) which suggests that this reactor can be turned into a purely hydrogen producing system by operating it at hyperthermophilic temperatures and using this enriched *Thermotogales* community to its full potential. The *Fervidobacterium* species were found to be *Fervidobacteriumgondwanense* and *Fervidobacteriumpennivorans* which were present in a 5:1 ratio. These species are functionally similar to *Thermosipho*, however they are known to produce a wider variety of acids in addition to hydrogen from both simple and complex carbohydrates (Bonch-Osmolovskaya, 2008).

Firmicutes were found to have a minor presence in the reactor, accounting for 6% of the community. *Clostridium* species formed 74% of this group, dominated by *Clostridium caenicola*. As with the *Thermotogales* species, these are chemo-organotrophs which utilise carbohydrates and produce hydrogen, carbon dioxide and acetate (Shiratori et al, 2009). Other minor groups were *Proteobacteria* which accounted for 2%, with more or less equal numbers of *Comamonas* and *Pseudomonas* species. The complete dominance of hydrogen producing groups in the methanogenic phase could mostly be due to the overabundance of acetate from the acidogenic phase, leading to inhibition of the acidogens.

The bacterial community seen here was very similar to the one seen in section 5.3.4.4, the single stage thermophilic reactor fed with untreated FW. This shows that the methanogenic phase in a two-phase AD system was functionally identical to a single phase system. This revealed an important aspect of the two-phase system: that the

acidogenic phase existed purely as a substrate-processing stage and its community did not have a major impact in the methanogenic phase and that the methanogenic phase can, if needed perform independently of the acidogenic phase as it had all the bacterial communities required for the conversion of food waste into methane, although at much lower rates than a two-phase system. Such built in redundancy was important as it means that the system had the robustness to survive the failure of one of the phases due to reasons such as overloading or accidental washout.

7.3.2.2 Bacterial analysis of Methanogenic reactor at day 170

The bacterial community shown in Fig. 7.4 shows the dominant groups of bacteria active in the methanogenic reactor at day 170, i.e. after 20 days of micronutrient supplementation.

Fig. 7.4 Bacterial community break up of methanogenic reactor at day 170

About 328 different species were found in this community from 69,351 reads PF. The Shannon Species Diversity had improved to 1.683. As theorised in the earlier section, the addition of micronutrients had produced a richer, more diverse community within 20 days of addition. *Thermotogales* were still the most dominant group, however their presence had fallen to 60%, suggesting more redundancy in the hydrogen production pathways in the methanogenic stage.

Proteobacteria increased their presence from 2% to 11%, with *Psuedomonascaeni* accounting for most of the phylum. This was a species involved in denitrification and removal of inhibitory xenobiotic organic compounds and hence was important in improving the performance of biogas producing reactors (Zhang et al., 2016)

Firmicutes now accounted for 18% of all the bacterial groups. Fig 7.5 shows the breakdown of Firmicutes in the methanogenic phase at day 170.

Fig 7.5 Composition of phylum Firmicutes in methanogenic reactor at day 170

The most dominant genus here was *Coprothermobacter* (*Coprothermobacter proteolyticus* being the most represented species), which accounted for 64% of Firmicutes and 12% of all bacteria. This was a significant development as *C. proteolyticus* is a known proteolytic anaerobe, which degrades proteins and lipids to form hydrogen and acetate. Its presence had been well studied to be beneficial to methanogens due to syntrophic interactions and its presence was postulated to improve methane production (Sasaki et al, 2011). Furthermore, it had been suggested that *Coprothermobacter* sp. are able to degrade LCFAs, which are inhibitors for methanogens as well as recovering nutrients from dead cells, thus improving productivity (Palatsi et al, 2011). A study Tandishabo et al. (2012) suggested that *Coprothermobacter* sp. may be enriched with the use of a separate acidogenic stage, and indeed a small

Coprothermobacter population was seen in the acid reactors in Chapter 5. It was possible that the action of the micronutrient supplement, specifically Co and Mg helped it survive in the methanogenic phase and in turn improve the productivity

7.3.2.3 Bacterial analysis of Methanogenic reactor at day 210

The bacterial community shown in Fig. 7.6 shows the dominant groups of bacteria active in the methanogenic reactor at day 210, i.e. after 60 days of micronutrient supplementation.

Fig. 7.6 Bacterial community break up of methanogenic reactor at day 210

About 347 different species were found in this community from 74,177 reads PF. The Shannon Species Diversity was also higher at 2.046. Thus, long term micronutrient

supplementation was shown to improve the diversity of the bacterial community in the methanogenic phase of the two-phase thermophilic AD system.

Thermotogae were reduced to 38% after 60 days of supplementation of micronutrients and hence this route of hydrogen production for syntropic methane production had ceased being the dominant one. *Firmicutes* were also reduced to only 5% and *Coprothermobacter* sp. accounted only for 2% of the community.

The largest increase was seen in phylum *Bacteroidetes*, whose genus *Bacteroides* (comprising mostly of *B. graminisolvans* and *Parabacteroides goldsteinii*) accounted for 15% of the total bacterial species in the community. This was a major change from the earlier communities as the *Bacteriodes* spp. are known to be engaged in utilizing starch and converting it primarily to acetate and other acids (Meslé et al., 2015; Nishiyama et al., 2009; Sakamoto et al., 2006). Thus, the emergence of this group signifies the production of acetate in the reactor in addition to hydrogen thanks to the action of the micronutrient addition. This enrichment of acetogens can be attributed to the availability of micronutrients such as Co and Ni allowing such bacteria to overcome the product inhibition caused by the already high levels of acetate present due to the acidogenic phase. The diversion of the available substrate to acetate production over hydrogen production was important for improving methane production as the acetoclastic methanogens are known have higher productivity than hydrogenotrops(Vrieze et al, 2012).

Proteobacteria was seen to the co-dominant phylum in the methanogenic reactor after long term supplementation of micronutrients, accounting for 40% of the total bacterial species. Figure 7.7 shows the internal composition of *Proteobacteria* group.

Fig. 7.7 Breakup of Proteobacterial community in methanogenic reactor at day 210.

Proteobacteria mainly consisted of two groups: *Gammaproteobacteria* and *Epsilonproteobacteria*. The main species present in *Gammaproteobacteria* was *Pseudomonas caeni*, which as discussed above was involved in improving methane production by breaking down organic inhibitors and complex carbohydrates. It maintained its presence at 10% from day 170, indicating that it had reached stability in the reactor and was playing an important role in the bioprocess. The other groups with minor presence were *Buttiiaxella*, *Klebsiella* and *Aeromonas* sp., which as discussed in Chapter 5, are bacteria found in food and human digestive systems and can act as pathogens. Their presence in both phases optimised by different methods suggests that a highly productive process and a healthy, diverse bacterial community comes at the cost of the presence of some pathogens.

The major *Proteobacteria* group however was the genus *Arcobacter*, which accounted for 20% of all the bacteria in the community. These species are involved in denitrification (Heylen et al, 2006) and acetate oxidation (Vandieken and Thamdrup, 2012). This was another important addition to the community as denitrification had been linked to removal of sulphides from anaerobic reactors (Liu et al, 2015) and acetate oxidation had been shown to be an alternative route to methane production in the event of the collapse of the *Methanosaetaceae* population due to temperature shocks or other inhibitory conditions. Thus, the enrichment of *Arcobacter* by micronutrient addition increases the robustness of the AD process and adds another redundancy to the hydrogen producing and denitrifying pathways in the system.

In overall, it was demonstrated that the use of the micronutrient supplement greatly increased the microbial diversity both in terms of species richness and relative abundance. Such enrichment of the community was known to have a vital role in the process productivity (Venkiteshwaran et al., 2016). The addition of functional redundancy as seen above had a direct impact on functional stability as it adds a layer of biological insurance to the system. A richer ecosystem can survive the loss of one or more functional species due to process inhibition or system perturbation as other species from a different biological but the same functional group can quickly take their place, making the process more robust and resistant to failure (Werner et al, 2011; Vanwonterghem et al, 2014). Thus, the rapid improvement and high stability in productivity seen after the addition of the micronutrient cocktail can be attributed not only to removal of nutrient limitations but also the enrichment of the microbial community.

7.3.3 Cost/Benefit analysis of micronutrient addition.

As the addition of metal species to the AD process will lead to an increase in the operational costs, a cost/benefit analysis was carried out to justify the use of the micronutrient supplement described in this chapter. The cost to benefit analysis was described in Table 7.1. As the commercial price of the power generated by methane was used for the calculations, the costs of the micronutrient addition were calculated based on the bulk commercial prices of the metal salts as on 31st March, 2017. All calculations were carried out on a basis of kg of supplement added kg/L_{substrate added}/per year. Since the supplement was added once every HRT (20 days), it was projected that it would need to be added 18.5 times every year. No further operational costs were included as the supplement as used by simple addition into the feed, therefore no special equipment would be needed.

The benefits of improved methane production were calculated on the basis of the 40% (per litre of substrate added for 365 days) increase seen in process productivity seen in the last 40 days of supplement addition. Further calculations for energy and monetary gain from the extra methane produced done as shown in section 5.4: the energy density being 10lWh/m³ (Behlau, 2009), conversion into energy at 40% CHP and price of the extracted extra energy being 0.14 SGD net/kWh_{el}

Table 7.2: Cost/Benefit analysis of micronutrient supplement addition to enhance thermophilic AD

Supplemented metal	Total addition	Total cost
	kg/yr	SGD/yr
Calcium	14.3745	0.06641
Magnesium	5.6055	0.049328
Cobalt	0.1295	0.042735
Nickel	0.0555	0.006227
Total cost		0.1647

Benefit	L/year	kWh/year	kWh _{el} /year	SGD/year
Enhanced Methane production	1365.1	13.651	54.604	4.608578

As seen from table 7.1, the cost/benefit ratio was found to be 0.165/4.609 SGD/year. This indicated a clear positive financial impact of micronutrient addition to thermophilic AD. Furthermore, as described in this chapter, there were additional advantages to using the supplement beyond improvement in methane production- it had been shown to stabilise the AD process and aide in its recovery for near-failure conditions. While these benefits cannot be quantified easily in a financial analysis, they still add to the value the overall value of the enhanced thermophilic AD process.

7.3.4 Fate of metal ions and effluent from micronutrient supplemented AD process

The various enhancement methods detailed in both the current as well as the previous chapter entail the introduction of a large amounts of cations into the AD process, namely, Na⁺, Ca²⁺, Mg²⁺, Co²⁺ and Ni²⁺. All these metal species are important for the metabolic activities of microbial cells and are vital parts of various cell machinery such as cell membranes, organelles and enzyme structures. As the addition of all these ions were optimised in this study, they were not present in superfluous quantities and hence, as mentioned in Chapter 4, there was no external precipitation or presence in aqueous phase observed. This suggests that the metal ions added to the sludge were absorbed by the microbial communities and made a part of the cells themselves. The functions of

these cations are detailed in Section 2.2.2 and it was postulated that the cations will be present in the AD biomass as a part of the structures mentioned there. Sodium will be present in the various cell membrane pumps that regulate active transport of molecules in and out of the cells. Calcium and magnesium will be a part of cell walls and regulatory mechanisms similar to the sodium-potassium pumps. Cobalt will be a part of various enzyme complexes such as methyltransferase, transcarboxylase, carbon monoxide dehydrogenase. Similarly, nickel will be a part of other enzymes such as coenzyme F430 and most hydrogenases.

All the cations added to the AD process will be present in the effluent as a part of the biomass or the aqueous fraction. Although the cations added as a part of the supplement will form less than 1% of the total solids, their presence in the AD sludge will have a major impact on the disposal or downstream use of the effluent. As all these cations are present in far below toxic concentrations as reported in literature (Chen et al., 2008), the effluent can be used as a substrate in biological processes that use waste sludge for energy recovery, such as anaerobic digestion or biodiesel production by microalgae. However, it will have to be carefully characterised before use as the presence of the cations will drastically affect its nutritional qualities.

While cobalt concentrations are within acceptable limits, nickel concentrations will be high enough make the effluent unsuitable for soils used for agriculture or grazing, according to EU and USEPA guidelines (Inglezakis et al., 2012; McFarland et al., 2012). It will not be suitable candidate for any process that would lead to accumulation of these metal ions such as landfilling or conversion to fertiliser or soil conditioner as over time their concentrations will build up to toxic proportions. The effluent from micronutrient supplemented AD would be classified as “Pollutant Class B sludge” under

EU regulations, which means will have to undergo some simple post-treatment such as liming to reduce the Ni to $<0.75\text{mg/L}$ before being disposed on non-agricultural land (LeBlanc et al., 2009). Care had to take to ensure that all metals are sufficiently immobilised as accumulation can lead to contaminated land and soils which are difficult to remediate (Bolan et al., 2014). In addition, them being leached or washed into water bodies such as lakes or rivers will can lead to eutrophication, which was a major source of water pollution (Larson et al., 2015). In localities that have even stronger regulations for disposal or use, the effluent will require specialised treatment methods such as biochar and incineration (Inyang et al., 2016). This will lead to increased costs which will have to be taken into account while carrying out the cost/benefit analysis of the use of this supplement on industry scale projects..

7.4 Conclusions

7.4.1 Summary

- The deficiency of trace metals in food waste was exacerbated in two-phase thermophilic AD and lead to limited productivity and reactor instability in the Methanogenic phase.
- The addition of the micronutrient supplement to the methanogenic phase was found to help process recovery and increase the methane production by up to 40% and increase utilization of soluble COD by 120%.
- A study of the bacterial dynamics before and after micronutrient addition showed that the supplement greatly increased the bacterial diversity of the community.

- The functional redundancy of hydrogen production, denitrification and substrate utilization was greatly increased.
- In addition, the community species involved in acetate oxidation, acid production and removal of organic inhibitors were added to the system, making it more functional.
- Thus, the enhancement in methane production was achieved by addressing nutrient limitation and increasing the resistivity and robustness of the microbial community.
- A cost/benefit ratio of the micronutrient supplement addition to was found to be 0.165/4.609 SGD/year, thus indicating at the supplement had a positive effect on enhancing the financial value of this two phase thermophilic AD process.

7.4.2 Significance

This study tested the micronutrient supplement formulated in the previous chapter and determined that it recovers the two-phase AD system from a near failure and further improves the stability and productivity of the system.

This study was also the first of its kind to use next generation sequencing to investigate the changes in the bacterial community of a two-phase thermophilic AD system utilizing food waste to produce methane.

It suggested that methane productivity could be greatly increased by increasing the biological diversity of the system and adding functional redundancy to the biological processes.

CHAPTER 8

CONCLUSIONS AND RECOMMENDATIONS

8.1 Major findings

This study aimed to enhance the productivity of the two-phase thermophilic AD utilizing food waste by optimizing the acidogenic and methanogenic phases separately using different methods.

8.1.1 Evaluation of pretreatment methods for optimizing food waste utilization by thermophilic AD

- Five different pretreatment methods were evaluated for optimizing the conversion of food waste into CODs, VFAs and hydrogen - alkaline pretreatment, thermal pretreatment, ultrasonication and the combination of ultrasonication and thermal and alkaline pretreatment, respectively.
- In batch tests, it was found that different methods could be optimised to give different beneficial effects - hydrogen productivity was enhanced by up to 4 times by a combination of alkaline pretreatment and ultrasonication, while thermal pretreatment at maximised substrate solubilization and biogas production.

- However, when these methods were tested in single stage and two phase thermophilic AD, thermal pre-treatment was found to have negative effect on substrate solubilization, hydrogen and methane production.
- It was found that the changes in the bacterial community of the acidogenic reactor caused by thermal pretreatment lead to a large increase in lactic acid bacteria which produced toxins that inhibited the growth and function of other bacteria that were required for hydrogen and VFA production.
- Alkaline + Ultrasonication (pH+U) caused a small increase in substrate solubilization, and a two fold increase in hydrogen production in the acidogenic phase of the two-phase system, effectively turning the system from a purely methane producing one to a combine biohydrogen and biogas producing set up.

8.1.2 Using micronutrient supplementation to enhance the productivity of the methanogenic phase

- The methanogenic phase was found to be prone to acidification and instability in substrate utilization and methane production in the long term. This was attributed to the deficiency of trace metals or micronutrients in the sludge.
- Four key micronutrients were identified- calcium, magnesium, cobalt and nickel- to address this matter and a novel micronutrient supplement containing the optimal concentration of these elements was prepared using Response Surface Methodology.
- The use of formulated supplement in the methanogenic stage of the two-phase AD system was found to help the process recover from instability and increase the methane production by up to 40% and increase utilization of soluble COD by 120%.

- The micronutrient supplement greatly increased the bacterial diversity of the community both in richness and abundance of different species.
- Before supplementation, the process only had two major functional groups- a dominant Thermotogae group which used the substrate to produce hydrogen for hydrogenotropic methanogenesis and a small Firmicutes population which solubilised the substrate and produced VFAs
- After supplementation, the process had higher diversity which did not only create multiple functional redundancies with respect to solubilization and hydrogen and VFA production, it was also seen to have additional functional groups involved in denitrification, acetate oxidation and removal of inhibitors.
- It increased process robustness and resistivity, along with the removal of nutrient limitation was the cause behind the drastic improvement in the performance and stability of the methanogenic phase.

8.2 Conclusions and implications

This research developed two different methods to greatly increase the productivity of two-phase thermophilic AD. Knowledge gained from the studies can be used to optimise the existing AD processes by creating an enriched hydrogen producing acidogenic stage and a stable methanogenic with a large improvement in methane production. The main insights gained from this research were that the bacterial communities were key to the functioning of the AD process and that they can be engineered to have extra functionalities and diversity which can help the process with both the productivity and the robustness of the process. This was also the first study that characterised the changes in bacterial communities of a two phase

thermophilic AD utilizing food waste and hence added the existing knowledge on this topic.

8.3 Recommendations

While the pretreatment methods and micronutrient supplement produced by this study had shown great improvements in the thermophilic AD process, the process can be further improved by basic studies that were beyond the scope of this research.

The results suggested that the improvements weren't only in the productivity of the process but also the rate of substrate utilization. Hence, a long term study that aims at optimising the OLR and HRT of both phases deserves to be carried out. Only standard OLR and HRT were used in this study as it was more of demonstration of concept, a substantial increase in OLR and/or decrease in HRT was possible, making the process truly commercially viable.

The micronutrient supplement produced in this study had obvious commercial applications and hence it was under provisional patent application. However to make it more commercially viable, it requires some follow up research and development. Firstly, its efficacy over a range of substrates needs to be investigated. Since it was tailored for the methanogenic phase which was not fed directly with a substrate, it should, in theory have a wide applicability. Secondly, it was added to the AD process by the simple process of dissolving in the feed, while this works at the lab scale, it may not be viable for full scale plants. Hence, an efficient mode of dispersal needs to be developed for the supplement. Most importantly, the economic viability of this method needs to be studied.

While the microbial study gave substantial insights on the impact of the micronutrient supplementation on the biological process of the system, it was only half the story. More research was required to identify and confirm the direct effects of these micronutrients. This can be achieved through investigations on their effect on gene products and protein synthesis which could pinpoint the changes caused by the addition micronutrients to the actual metabolic activity of the methanogenic community. While community dynamics studies could shed some light on the effect of such additions, such metagenomic studies have their limitations, considering many species present in AD process are unculturable and their functionality was not well understood. Of particular interest would be metatranscriptomics based studies on the changes in gene activity diversity and different gene expressions could further pinpoint the effects of micronutrients on metabolic pathways and enzyme production, thus giving us a better understanding of enhancing the anaerobic digestion process through external nutrient addition and community engineering.

CHAPTER 9

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