The University Of Sheffield.

Anaerobic Digestion of Fatty Slaughterhouse Waste

Optimising the Digestion Process

by

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Abstract

The continued growth of world population and food production leads to a continued increase in organic waste, responsible for many environmental and health problems when disposed in the wrong way.

In Makkah city, huge amount of raw slaughterhouse waste from the Hajj sacrifice is generated and disposed in landfill without any treatment each year during Hajj. The huge number of pilgrims and a slaughterhouse waste generated from sacrificed animal (2.5 million animals in 2014) results in an environmental and health problems from the landfill area. One of the most applicable and effective methods to treat organic waste materials is anaerobic digestion (AD). In AD, bioactivity of four different groups of microorganisms in sequential metabolic steps under oxygen free conditions is responsible for breaking down complex organic waste into the simplest compounds and producing energy in form of biogas (a mixture of methane and carbon dioxide).

However, the process can collapse when the organic waste contains high fatty materials (such as slaughterhouse waste) as long chain fatty acids (LCFAs) produce a strong inhibitory effect on some AD microorganisms.

In a batch system, adding a small amount of fat to a large volume of anaerobic seeding sludge in order to provide sufficient amount of anaerobic inoculum could work effectively. However, this will lead to the disadvantage of wasting most of the reactor volume and therefore reducing the capacity to receive fresh organic waste.

This research looks into the possibility of optimising the digestion process in order to achieve a good anaerobic digestion process for high fat concentration substrate with a minimum amount of seeding sludge in the reactor.

Different strategies were studied to achieve this goal. These included the use of different fat concentrations in co-digestion with vegetable waste, different I/S (inoculum to substrate) ratios vs/vs, different operating conditions of single stage reactors (mesophilic CSTR, thermophilic CSTR, and mesophilic up-flow), and study of different multi stage reactor sets (two, three, and four stages) in order to optimise the digestion process.

In term of fat concentration, at I/S ratio of 1, more fat in the feed results in more methane production, however, late start-up of the digestion process was associated with higher fat concentrations.

In case of the I/S ratio, different ratios were studied including I/S of 1, 0.5, 0.25, and 0.1 vs/vs. The higher ratio result in faster and much more stable start-up. However, obtaining an I/S ratio of 1 is difficult without using a large volume of seeding sludge. Pre-concentrated seeding sludge occupied ≥80% of the reactor volume to achieve an I/S ratio of 1. Lower I/S ratios result in slower and instable start-ups. However, higher hydrogen production was observed at the start-up of low I/S experiments.

Single stage CSTR reactors showed very low methane production and could not effectively start-up the digestion process.

Two stage reactors (with pH controlling and reseeding steps), and a three time re-seeding strategy showed the best ability in treating high fat concentrations of 20g L^{-1} VS and minimising the required seeding sludge for the process at the same time. Use of a two stage reactor with two seeding steps of 0.1 I/S ratio was found to successfully treat the 20g L^{-1} VS fat substrate with 80% less seeding sludge volume comparing to the I/S ratio of 1. A reactor with four seeding steps of 0.1 I/S ratio each, was able to successfully treat the same amount of fat substrate with 60% less seeding sludge volume compared to the I/S ratio of 1. Due to the complexity and overlapping of the stages of the digestion process, it was found to be very difficult to physically separate each stage in a separate reactor through optimising the pH conditions.

From the results of this study, in the case of Makkah city where many tones of slaughterhouse waste are generated in the four days of Hajj, anaerobic digestion treatment is recommended to be conducted through a combination of two stage digesters and high I/S ratios in the second stage. The first stage should be a large storage tank to store the raw slaughterhouse waste after grinding pre-treatment and allow natural hydrolysation during storage. The second stage could be a medium tank, or several tanks, that can be regularly fed with a small amount from the first stage in a semi- continuous system over the year.

Dedication

I would like to dedicate this work to the loving memory of my father Abdulhadi. Also, I would like to extend my deepest gratitude and dedicate this work to my wonderful mother Jawaher, my wife Bashaer, and my daughter Almas.

Ahmad Alsaigh

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Abbreviations

AD : Anaerobic digestion

BFP : Bovine fat powder

BOD : Biochemical oxygen demand

C/N ratio : Carbon-nitrogen ratio

COD : Chemical oxygen demand

CSTR : Completely stirred tank reactor

DO : Dissolved Oxygen

LCFA : Long chain fatty acid

OLR : Organic loading rate

SD : Standard deviation of samples.

TCD : Thermal conductivity detector

TS : Total solids

UASB : Up-flow anaerobic sludge blanket

V/V : Volume per volume.

VFA : Volatile fatty acids

VS : Volatile solids

W/W : Wieght by wieght.

Chapter 1 Introduction

1.1 Introduction:

1.1.1 Population and Organic Waste Generation in the World:

Mankind produces a huge amount of organic waste, which originally was left for the processes of natural decay to deal with.

In the past, there was no real problem with all these complex organic wastes, due to the low population and smaller amount of waste generation compared with today's world. Natural decomposition activities of both macro (scavenging animals etc.) and microorganisms (bacteria and fungi) will break down this material and turn it into simple substances that can be recycled in the environment.

However, problems of organic waste become more pressing with increasing world population, high density of human activity and improvements in living quality. The increase in both food production and industrial activities, together with human waste, leads to a continuous rise in municipal waste. As stated in the world population prospects published by the United Nations (UN, 2017) the population of the world, based on to the medium variant as can be seen in (Figure 1-1), is likely to continue increasing from 7.6 billion in 2017 to around 9.8 billion in 2050 and even expected to touch 11.2 billion in 2100. This will result in ever increasing amounts of wastes produced by people, agriculture activities, farm animals, and food factories.

Historically, primitive methods such as burning or dumping were used to deal with accumulated solid wastes, whereas liquid wastes and some other solids were poured directly into seas, rivers or lakes. The direct disposal of waste by these methods caused environmental and health problems. Ground and surface water pollution by leachate of waste materials can cause serious problems such as breeding pathogens, odour and spreading diseases, in addition to destruction of aquatic life. Air pollution leads to the spread of respiratory diseases, pollution of surface water as well as increasing the negative impact of global warming. Dumping and landfill causes soil and ground water pollution by

the leachate. Waste management has had to continue to develop to alleviate these problems.

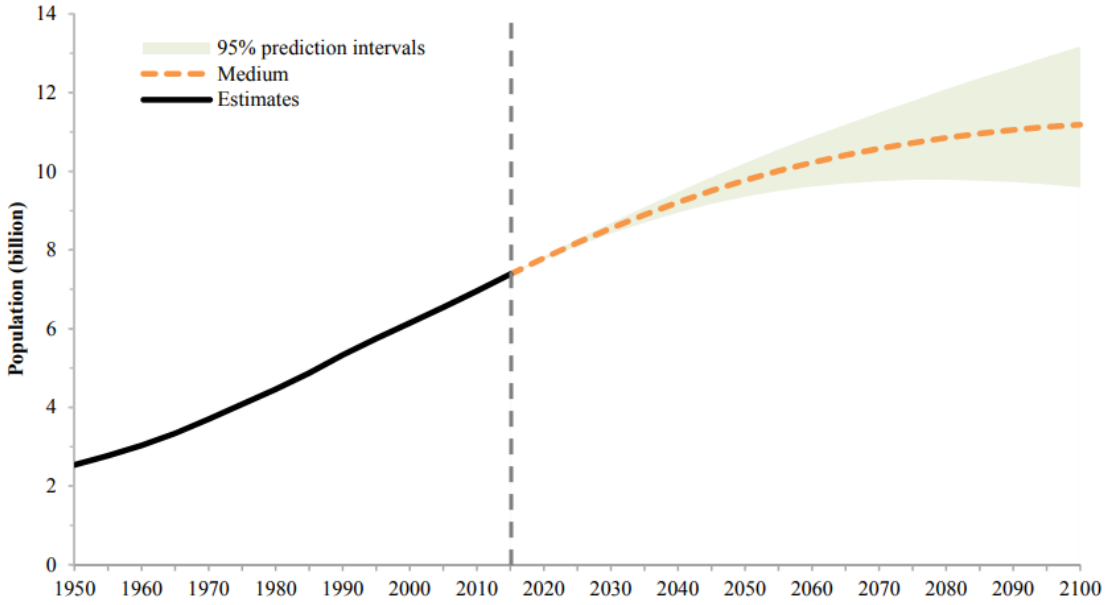

Figure 1-1: Increase in world population between the years 1950 – 2017 and projected growth till 2100 (UN, 2017).

1.1.2 The City of Mecca (Makkah), it's Waste Problems and Anaerobic Digestion as a Promising Solution.

1.1.2.1 *History of Makkah and Hajj and sacrificing origin:*

Makkah is recognised as the holiest city among the three holy cities in Islam. In Islamic tradition, the beginning of Makkah's history from a valley barren from any cultivation, includes the appearance of the Holy water well (Zamzam), the building of the Sacred House, the Hajj (pilgrimage) as well as animal sacrifice after Hajj, is directly related to prophet Abraham, his son Ishmael, and the mother of Ishmael, Hagar.

The story of Abraham when he left his son Ishmael and Hager is narrated in the Holy Bible and the Holy Quran with high similarity and some differences between them. The dessert place where Abraham left Ishmael and Hagar was Makkah as narrated in the Quran. The holy Zamzam well is mentioned to be the water well opened by the angel Gabriel when God heard the thirsty boy Ishmael crying in the desert. The Sacred House was stated to be rebuilt later in its place by Abraham and his adult son Ishmael, who performed the first Hajj after finishing the building. Even the animal sacrifice in Hajj is related to the sacrifice of ram in the story of Abraham instead of sacrificing his son.

Hajj is one of the five pillars of Islam, that every physically and financially capable adult should perform at least once in their lifetime. Nowadays, millions of Muslims come to perform Hajj in Makkah every year.

1.1.2.2 *Number of pilgrims, vehicles, and sacrificed animals.*

The number of pilgrims has shown a continuous increase between 2009 and 2012 as stated by the general authority for statistics in the Kingdom of Saudi Arabia (KSA). The highest recorded number of pilgrims was over 3 million during 2012 (Figure 1-2). From 2013 government reduced the number of pilgrims accepted due to huge reconstruction and development projects in Makkah, which were established in order to increase Makkah's capacity and service level for pilgrims in the future. The annual number of pilgrims was kept between 1.86 and 2.35 million since then until 2017 (GAFS, 2017). The projected number for Hajj pilgrims will be around 4.5 million by 2050 (Shahzad

et al., 2017). As general authority for statistics in KSA stated, the recorded number of vehicles used in transporting pilgrims into Makkah was over 99 thousand vehicles in 2012. This number has dropped into a range between 29,169 and 46,108 vehicles from 2013 to 2017 (Figure 1-3). The harmful exhaust from this high number of vehicles in a small and crowded area is one of the challenging problems and can cause respiratory problems for both pilgrims and people working in pilgrim services during Hajj.

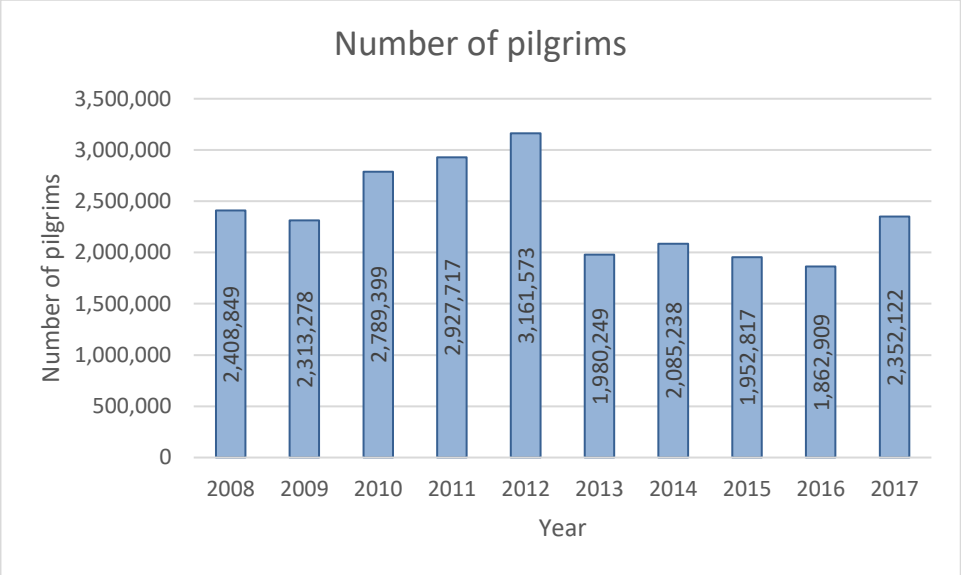

Figure 1-2: Number of pilgrims to Makkah between 2008 and 2017 (GAFS, 2017).

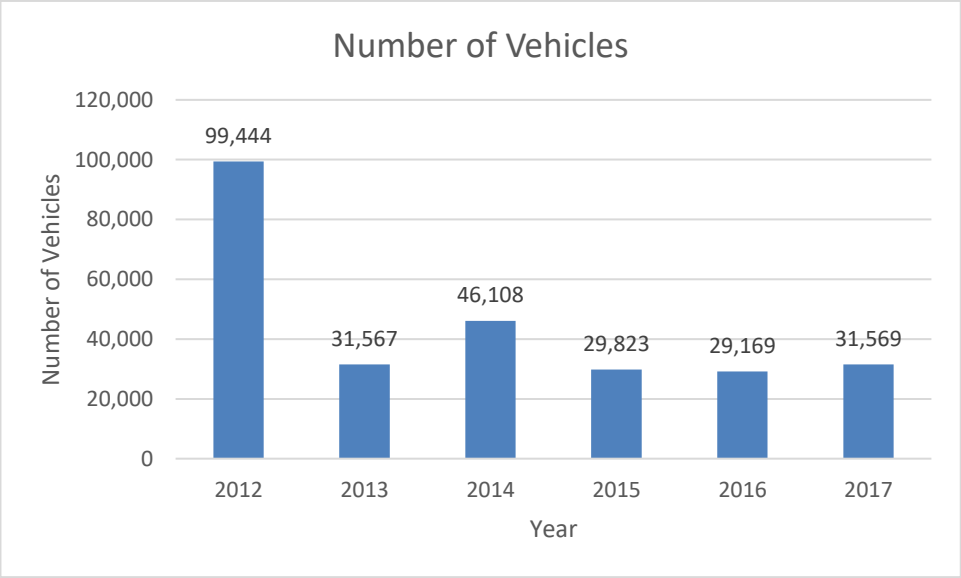

Figure 1-3: Number of vehicles Used in Hajj transportation in Makkah during Hajj between 2012 and 2017 (GAFS, 2017).

During Hajj, performing of animal sacrifice can be done only during four days from day 10 and until day 13 of the Hajj month. Millions of animals are sacrificed every year in these four days. The meat is distributed to poor people inside and outside the country. Nowadays, hundreds of thousands of flash frozen whole lamb from Hajj sacrifice are exported to more than 24 countries to be distributed to the poor people (Figure 1-4). As stated by Amtul (2014), over 2.5 million animals were sold in the 2014 Hajj season for sacrificing in Makkah. Slaughtering of such a high number of animals in one place and during just four days, results in a huge amount of animal waste including blood, inedible parts, rumen, bowels and other by-products. This organic waste was disposed of in Makkah landfill with no treatment until 2015. In 2015, an incineration plant, costing around £40 million and covering an area of 4,000 m^2, was established to treat part of the generated waste (SPA, 2015). However, the largest part is still disposed of in landfill.

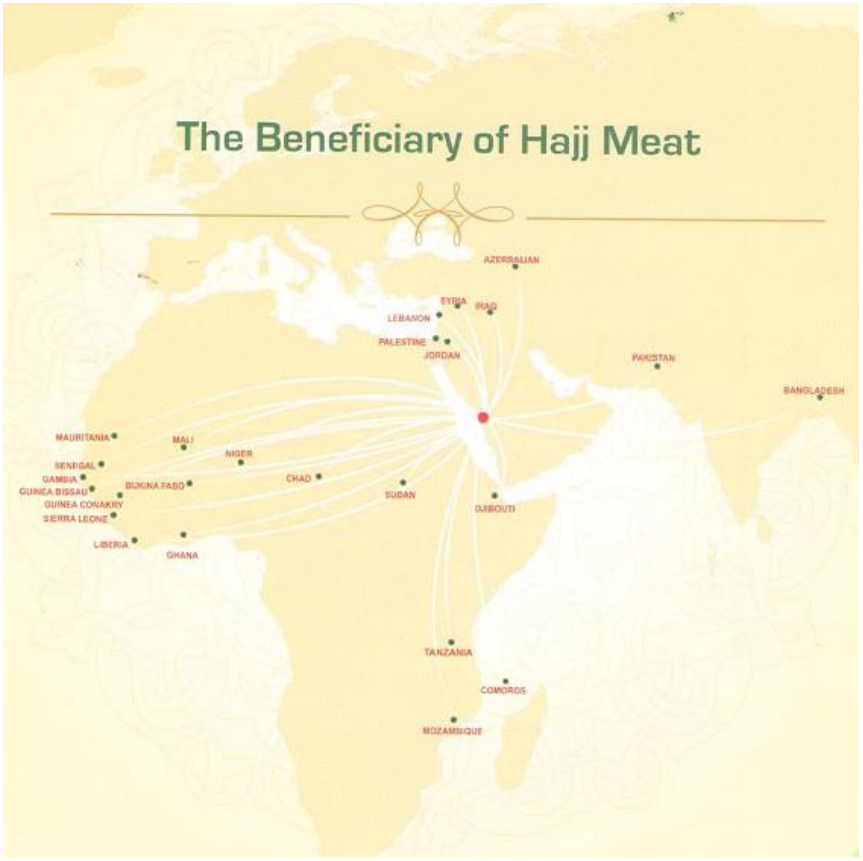

Figure 1-4: The beneficiary 24 countries of free Hajj meat (Adahi, 2016).

1.1.2.3 *Environmental and health problems:*

Dumping this huge amount of slaughterhouse waste in landfill causes soil and ground water pollution by the leachate, as well as breeding pathogens, causing odour, air pollution and increasing the negative impact of global warming by the greenhouse gas emissions.

Due to the significant environmental and health problems associated with the high production and poor disposal of liquid and solid slaughterhouse waste, waste management has to continue to develop to alleviate these problems.

1.1.2.4 *Anaerobic digestion as a promising solution:*

Apart from controlling and minimising the production of effluents, waste management must look to increase the recovery of beneficial materials from the waste. This is equally important for organic waste as for other (non-organic) wastes. For instance, the residues of the slaughtering process such as lungs, kidneys, livers etc. are more valuable if used in pet food production rather than disposed of.

However, even with high by-product recoveries, the amount of organic waste generation is still high and there is a need for suitable treatment methods. One of the most efficient methods to deal with high organic-content waste is anaerobic digestion (AD). AD is a microbial conversion method using several groups of bacteria to break down complex organic material into simpler compounds in an anaerobic environment. AD does not only treat the problem of organic waste accumulation, but also produces valuable by-products such as biogas (methane and carbon dioxide) which is a renewable alternative energy source to fossil natural gas. Biogas can fuel vehicles for Hajj transportation, provide cooking gas and generate electricity. Biogas vehicles will reduce the harmful exhaust from the current petroleum diesel buses in Makkah. In addition, the digestate can be used in agriculture applications as a high nutrient fertiliser. It can be used to improve the soil fertility inside and around Makkah in order to increase urban green areas for better air quality and to improve the visual appearance of the area. The digestion process can be very efficient with biomass sources that contain high water content without any pre-treatment (Ward *et al.*, 2008).

In contrast, most of the other conversion technologies require waste pre-treatment when they deal with high water content organic waste. Combustion, for instance, can only give positive net energy balances when the content of water in the biomass is less than 60% w/w, however, even then most of the stored energy in that biomass is used to evaporate the biomass moisture (Appels *et al.*, 2011). Similarly, high water content in biomass causes a considerable decrease in the energy productivity of both gasification and pyrolysis in addition to the unwanted water contaminated in the produced bio-oil (Van de Velden *et al.*, 2010). Using any of these technologies would consume energy in a required pre-drying step of wet biomass. Biogas, produced from anaerobic digestion contains methane (CH_4) of 65% of the biogas volume, and carbon dioxide at 35%, as well as small traces of some other gases such as hydrogen, nitrogen and hydrogen sulphide is energetically efficient and has low hazardous emissions into the environment on combustion (Appels *et al.*, 2011). It is relatively easy to produce and store, and the carbon dioxide and other trace gasses can be easily removed to give a gas that is similar to, and thus substitutable for, natural gas.

1.2 Aim and Objectives:

The aim of this thesis is to identify ways to optimise the anaerobic digestion process in order to achieve a good anaerobic digestion treatment of high fat substrates with a minimum amount of seeding sludge in the reactor.

In order to reach this aim, this study includes several objectives as follows:

- To investigate the effect of varying the concentrations of fat contributing to 20 g L^{-1} total volatile solid (VS)in co-digestion with vegetable waste.

- To examine the effect of using low inoculum to substrate ratio I/S (below 1) at high fat concentrations (75% and 100% w/w) in the 20g L^{-1} VS feed condition.

- To examine the effect of seeding sludge sources (acclimatised and not acclimatised to fat) on the digestion process at different concentrations of fat and at low inoculum to substrate (I/S) ratios from 0.1 to 0.4 (vs/vs).

- To study reseeding as a recovery or enhancing step to the digestion process.

- To investigate the overall changes in the pH behaviour under different feed and seeding conditions. In addition to test the ability of pH electrodes to work consistently under long exposures to the reactor contents at high fat levels during the pH monitoring.

- To investigate the possibility of improving the digestion process and minimising the inhibitory effect of LCFAs through physically separating the AD process into two phases.

- To investigate the possibility of improving the digestion process and minimising the inhibitory effect of LCFAs through physically separating the AD process into three and four phases.

1.3 Thesis Outline:

The thesis format is as follows:

- **In chapter 2** existing literature related to anaerobic digestion and slaughterhouse waste is covered.
- **In chapter 3,** the material feeds, the experimental set-up as well as the methodologies used for analysis are discussed.
- **In chapter 4,** initial experiments were conducted to test experimental design and equipment, as well as to observe any problems that might appear during reactor operation.
- **In chapter 5,** the effect of varying the fat concentration and seeding sludge to substrate ratio (in term of VS content) upon gas production was investigated.
- **In chapter 6,** co-digestion of fat and vegetable waste. The effect of fat concentration and I/S ratio on the digestion process was examined.
- **In chapter 7,** a comparison was made between a single stage reactor and a 2 stage reactor to see how this affects the fat digestion rate.
- **In chapter 8,** a comparison was made between a 4 stage reactor and a 3-stage reactor.
- **In chapter 9,** conclusions of the present work are drawn with recommendations for future work.

Chapter 2 Literature Review

2.1 Anaerobic Digestion:

The term anaerobic digestion refers to the process of breaking down complex biodegradable organic matter into simpler chemical materials through the activities of several groups of bacteria under oxygen-free conditions (Monnet, 2003, Ek *et al.*, 2011, Nayono, 2009). These activities run in a sequence of metabolic steps that end with a nearly full conversion of biodegradable organic substances into bacterial biomass, biogas (mostly methane and carbon dioxide) and indigestible materials (Holm-Nielsen *et al.*, 2009). The sequence is often defined as four processes: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Figure 2-1).

During hydrolysis, different types of insoluble complex carbon-based polymers, such as proteins, lipids and polysaccharides, are hydrolysed and transformed into soluble molecules (proteins to amino acids and peptides, polysaccharides into soluble sugars, and lipids to some higher fatty acids (LCFAs) in addition to glycerol). The hydrolysis process is achieved by a wide range of extracellular enzymes produced by different types of hydrolytic bacteria (Nayono, 2009).

Acidogenesis (fermentation) is the next step where soluble organic materials produced during hydrolysis are further degraded through several fermentative pathways. The results are carbon dioxide (CO_2), hydrogen (H_2), organic acids, alcohols, and some other sulphur and nitrogen compounds. In case of lipid degradation, glycerol, produced during the hydrolysis step, is fermented into alcohols and some short chain fatty acids. One of the organic acids that are produced during the fermentation step is acetate, which considered as a main substrate to be used by methanogenic microorganisms directly in methane production (Nayono, 2009).

Acetogenesis is the third step where acetate also can be produced through the conversion of volatile fatty acids with low molecular weight into acetate, carbon dioxide and hydrogen. LCFAs produced during the hydrolysis step can also be degraded into acetate and hydrogen in this stage through β-oxidation cycle (Equation 2-1, $n \geq 2$). This yields acetate, hydrogen and a shorter LCFA molecule by two carbon atoms (Weng and Jeris, 1976). The cycle can be repeated until

the LCFA molecules are completely converted to acetate and hydrogen (Templer *et al.*, 2006).

$$CH_3(CH_2)n\,COOH + 2\,H_2O \rightarrow CH_3(CH_2)n\text{-}2\,COOH + CH_3COOH + 4\,e^- + 4\,H^+$$

(Equation 2-1)

During the fourth stage (methanogenesis), methane gas (CH_4) is produced through the bioactivities of methanogenic microorganisms. During this stage, methane can be produced through two pathways. The first is via acetoclastic methanogenic microorganisms activities where two acetic acid molecules are cleaved and generate methane in addition to carbon dioxide in an acetate decarboxylation process (Monnet, 2003). In the second pathway methane gas is formed through the reduction of CO_2 by H_2 molecules via methanogenic hydrogen utilizing bacteria (Padilla-Gasca *et al.*, 2011, Nayono, 2009).

The main pathway for methane production is the first one (acetate reaction) due to limiting hydrogen availability in this stage (Monnet, 2003). As stated by Nayono (2009), around 66% of methane production during methanogenesis comes from acetate decarboxylation, and only around 34% comes from the carbon dioxide and hydrogen reaction.

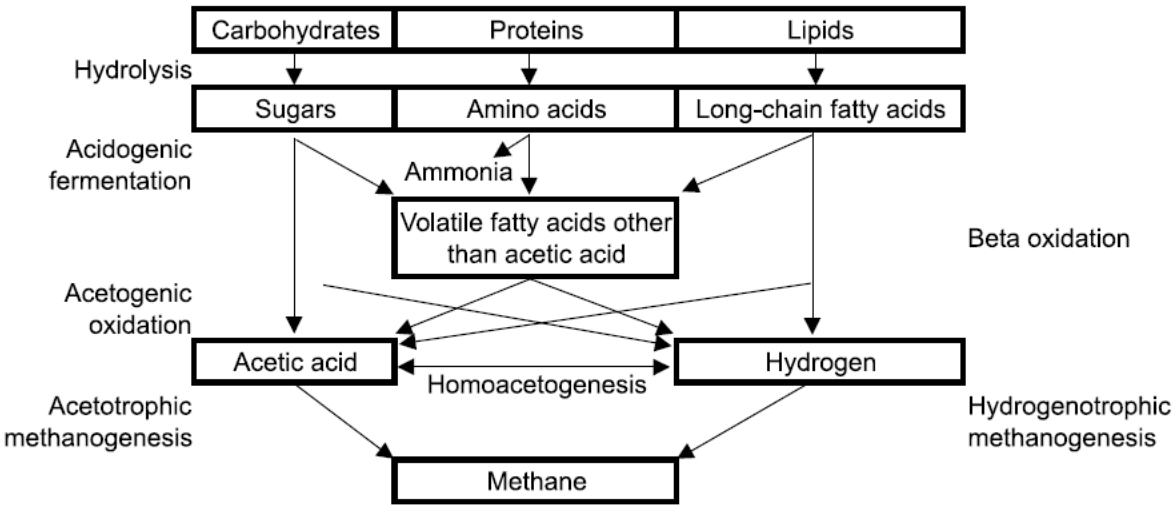

Figure 2-1: Anaerobic degradation pathways for organic materials (Salminen and Rintala, 2002).

2.1.1 Organic Substrate (Reactor Feed) and the Digester by-products

Most agricultural waste and residues, organic municipal solid waste, industrial food waste, and sewage sludge are ideal materials for AD as they are rich in easily biodegradable substances.

The main by-products of the AD process are biogas and digestate. Biogas is a renewable and high value energy source that can be used in the same applications as natural gas. As stated above, the main components of biogas are methane (CH_4) and carbon dioxide (CO_2), in addition to traces of hydrogen sulphide (H_2S), hydrogen (H_2) oxygen (O_2) and nitrogen (N_2). Biogas can be upgraded by removing water vapour and unwanted gases (particularly the CO_2) to maximise the methane content (to around 98%) and thus to increase the biogas quality. Biogas yield and composition are highly related to the composition of the digester feedstock (Monnet, 2003).

Digestate is the remaining solids and liquid material in the digester after the digestion processes. It is considered to be a high nutrient material to fertilise agricultural land and provide soil with the necessary nutrients as well as protect it against erosion (a “soil enhancer”). Digestate must undergo analyses to confirm that it is safe and of good quality before being applied to agricultural land.

2.1.2 Widespread Simple of Anaerobic Digestion Reactor Technologies

Different designs and mixing technologies of anaerobic digester can be found around the world. Digester design and mixing technologies vary according to factors such as the composition and characteristics of the organic waste substrate as well as digester water content. The geographic location of the digester plant can define the temperature conditions for the digester system.

2.1.2.1 *Completely mixed reactor:*

A completely mixed digester is a tank reactor with continuous mixing system that runs on one or more of either mechanical mixing, gas or sludge recirculation (mixing) system (Karim *et al.*, 2005). A completely mixed reactor can provide better contact between the bacteria and the organic waste in addition to homogenising the reactor temperature and minimise the settling and

accumulation of solid materials on the ground of the digester tank (Polprasert, 2007).

2.1.2.2 *UASB Reactor:*

This reactor contains two parts, a cylinder-shaped column and a gas-liquid-solids separator as shown in Figure 2-2. Liquid feedstock is introduced near to the reactor bottom which causes a washout of light particles, while heavier material will return and remain, enhancing the formation of bacterial aggregates that allows the reactor to treat higher organic loading rates comparing to non-granule digesters (Pol *et al.*, 2004). Continuously running a UASB for 2-8 months (depending on the waste water characteristics, initial seeding sludge, and the operational conditions) should result in development of two layers, a very dense layer, the sludge bed, located at the reactor bottom and a lower density sludge blanket located directly on top of it (Aiyuk *et al.*, 2006). In UASB reactors, the AD process happens mainly through the sludge bed and sludge blanket, which are highly bio-active and rich in AD microbial communities (Chong *et al.*, 2012).

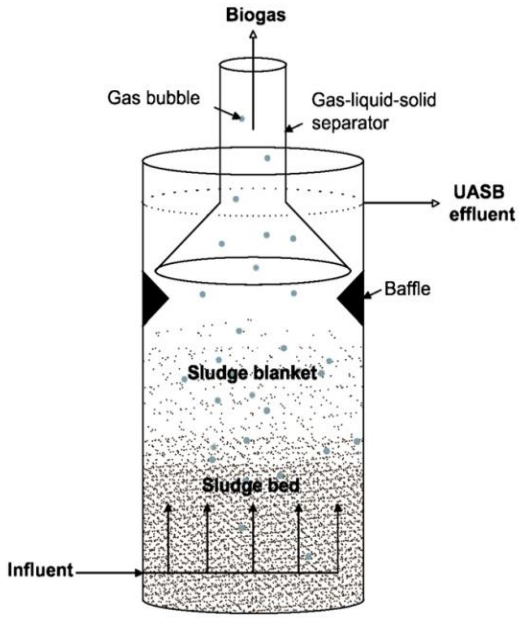

Figure 2-2: Schematic diagram of Up-flow anaerobic sludge blanket reactor (UASB) (Chong *et al.*, 2012).

2.1.2.3 *Single Stage and Two Stages Technologies:*

A single stage reactor is a basic simple anaerobic digester where all the anaerobic process (hydrolysis, acidogenesis, acetogenesis and methanogenesis) runes in the same reactor. In a two stage system, AD phases are physically separated with the first one running the hydrolysis and acidification phases and the second reactor running the methanogenesis phase (Cysneiros *et al.*, 2012). Many studies report that two-stage anaerobic digestion technology provides higher degradation ability, methane gas production and higher stability in AD processes compared to a single stage reactor. In a two phases experiment composed of an acidogenic phase followed by a methanogenic phase run in an up-flow bed reactor to treat high lipid content waste water from a food factory (milk & ice-cream factory), Kim and Shin (2010) found around 20% higher COD removal, 90% higher lipids removal and 40% higher methane gas production when compared to a single stage system. These positive results from using two-stage technology resulted from separating and optimising the environmental requirements of both acidification and methanogenesis stages. The stages can usually be differentiated according to temperature (higher in the first stage) or pH (acidic in the first stage).

2.1.3 **Type of Anaerobic Digesters According to its Operational Methods.**

2.1.3.1 ***Batch:***

In batch mode, the digester is filled completely with organic substrate and seeded with an inoculum (a seeding sludge from another working digester that provides the required microbial cultures for the digestion process). The microbial digestion starts and is left until the production of biogas decreases to a very low rate (nearly stopped). Around 80 to 90% of the reactor content (volume) is then removed, and the remaining 10-20% (volume) kept inside as a seeding inoculum. The digester is refilled with new organic substrate and the operation continued (Polprasert, 2007).

Biogas production from a batch digester is expected to be unsteady, and the production rates vary from a low rate at the digester start-up to high at the middle stages and then low again in the later stages. Digester failure is a common problem particularly when the digester is overloaded with organic

materials due to the shock effect. Nonetheless, this operational mode is suitable to deal with large amounts of organic material being provided at long intermittently (Polprasert, 2007).

2.1.3.2 *Semi-continuous:*

In semi-continuous operation, the digester feeding process is run on a more regular basis (one or twice a day). Digested organic materials are removed from the digester simultaneously with introducing new feed to the reactor. This kind of operation is recommended when a steady flow of organic substrate is available. Most commercial biogas plants follow this system of operation (Polprasert, 2007).

2.1.3.3 *Continuous:*

In the continuous mode of operation, feeding and removing of organic matter are continually running. The material that needs to be digested is kept constant in the digester content through overflow or pumping. Continuous operation requires high external energy inputs for the mixing and pumping process, and leads to limited application of this mode. At start up, seeding inoculum is added to the reactor and kept till the microbial community achieves a high population and both biogas production and methane concentration stabilise before the continuous operational mode is started (Polprasert, 2007).

2.1.4 Significant Parameters and Environmental Requirements for Anaerobic Digestion Operation:

2.1.4.1 *Temperature:*

Temperature conditions are considered to be one of the most critical factors for the success of the whole AD process. Temperature has an important effect not only on the growth and metabolic activity of the AD microorganisms, but also on other physical factors inside the digester such as substrate density, viscosity and gas transfer rate (Nayono, 2009). In a study of temperature and temperature shock on a mesophilic digestion system, (Chae *et al.*, 2008) stated that even small change in the digester temperature from 35 ˚C to 30 ˚C and then from 30 ˚C to 32 ˚C would cause a temperature shock for the

microorganisms, and reduce the production of biogas. However, the digestion system can rapidly return to the average biogas production rate (Chae *et al.*, 2008).

Interestingly, the temperature range of anaerobic digestion processes in nature varies from 4 ˚C in some cold lake sediments to a 60 ˚C where thermophilic digestion is taking place (Parawira *et al.*, 2004). However, the temperature range of anaerobic digestion processes used in industrial practice is more controlled. In general, as can be seen in Figure 2-3, the optimum temperature ranges for organic solid waste anaerobic digestion are around 35˚C for mesophilic and 55˚C for thermophilic systems (Mata-Alvarez, 2003).

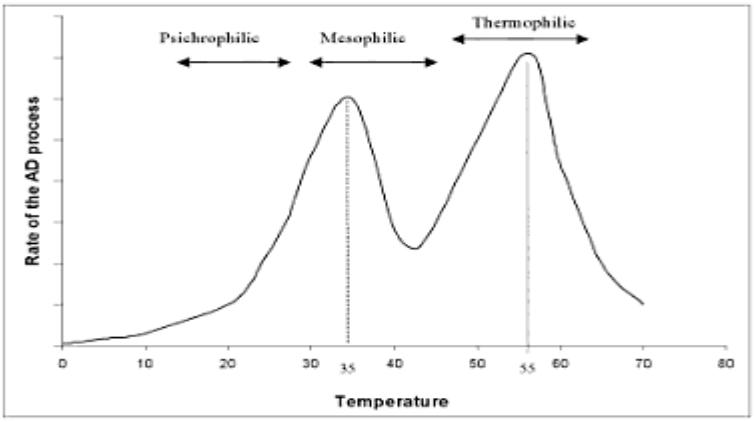

Figure 2-3: Temperature effects on the rate of AD process.
The ranges of optimum temperature for AD are around 30 – 35˚C for mesophilic conditions and 55 – 60 ˚C for thermophilic conditions. Source: (Mata-Alvarez, 2003)

Mesophilic anaerobic microorganisms are able to live and work effectively under changes of digester parameters more than thermophilic bacteria. The advantages of the mesophilic process is stability which makes it popular in many facilities, nonetheless, it requires much longer retention time than anaerobic digestion under thermophilic conditions (Zaher *et al.*, 2007). Although thermophilic digestion offers faster retention time, higher loading rate, maximum methane production as well as significant pathogen removal, it still more sensitive to operation parameters changes comparing with mesophilic process (Mata-Alvarez, 2003, Zaher *et al.*, 2007).

2.1.4.2 ***pH:***

pH is considered to be one of the most important factors and a good indicator for the performance, stability and success of the whole AD process. Many changes in pH occur as a result of different biological activities during the AD process. A fall in pH indicates acid accumulation from the acidogenesis process and therefore, if not transitory, it may cause process inhibition and instability in the digester performance (Zaher *et al.*, 2007). AD reactor failures have been reported in many studies as result of pH drop caused by high volatile fatty acids accumulation (Poh and Chong, 2009). Accumulation of organic acids occurs when the loading of volatile solids increases sharply in the digester as a result of feeding the digester a large amount of organic waste (Zaher *et al.*, 2007). Studies suggest that the pH required for good activity and stability of an anaerobic digestion process is between 6.5 and 7.5 , however, stable AD processes have been achieved outside this range (Zaher *et al.*, 2007).

Controlling pH for AD digester contents can be achieved through introducing chemical buffers such as sodium bicarbonate ($NaHCO_3$), potassium bicarbonate ($KHCO_3$), sodium carbonate (soda ash – Na_2CO_3), calcium carbonate (lime – $CaCO_3$), potassium carbonate (K_2CO_3), calcium hydroxide (quick lime – $Ca(OH)_2$), anhydrous ammonia (NH_3 – gas), as well as sodium nitrate ($NaNO_3$). Calcium carbonate (lime) would be useful to increase the pH value to 6.4 , then other carbonate or bicarbonate salts of either sodium or potassium are applied to raise the pH to the required optimum values (Mendez *et al.*, 1989). Chemical buffers such as sodium bicarbonate ($NaHCO_3$) and potassium bicarbonate ($KHCO_3$) are preferred as they directly release bicarbonate alkalinity which is required by methanogenic microorganisms, in addition to its solubility, ease of handling, and low negative impact on the microorganism and process performance (Mendez *et al.*, 1989). Introducing chemical buffers into the digester should be done carefully and slowly to minimise any negative impact on the digester microorganisms (Nayono, 2009). Decreasing the pH can be achieved through adding more fresh organic material to the digester, which will stimulate the acidogenesis process (Zaher *et al.*, 2007).

2.1.4.3 *Substrate Characteristics:*

Potential biogas production and biodegradation efficiency depend highly on the main organic components of the waste such as lipids, carbohydrates, proteins, cellulose, hemicellulose and lignin (Hartmann and Ahring, 2006). Anaerobic digester production of methane may differ depending on the different percentages of the materials in the digester feedstock. Appels *et al.* (2011) have reported that using high lipid materials as a feedstock will increase the digester methane yield. Compared with carbohydrates and proteins, lipids seem to be the most attractive and highest methane yield materials (Cirne *et al.*, 2007).

However, high content of lipid and protein materials can cause problems in anaerobic digestion (Banks and Wang, 1999).

The C:N ratio (the ration of carbon to nitrogen present in the digester feedstock), is an important parameter for digestion performance. Feedstock materials with high C:N ratio show low biodegradability and gas production since high C:N ratios cause nitrogen deficiency which affects bacterial growth. In contrast, when the C:N ratio is too low, ammonia accumulation can occur. Accumulation of ammonia leads to an increase in pH and inhibition of the digester microorganisms (Hartmann and Ahring, 2006).

The optimum range of C:N ratio for anaerobic digestion and methane production is between 25 and 30 (Ward *et al.*, 2008, Wang *et al.*, 2012). With high C:N ratio materials such as lignocellulose and waste paper, this optimum range of C:N ratio can be achieved through mixing these high C:N ratio materials with other low C:N ratio materials such as municipal sewage sludge or animal manure (Hartmann and Ahring, 2006, Monnet, 2003).

Particle size of the feedstock plays a significant role in anaerobic digestion, mainly during the hydrolysis process, where smaller particle sizes offer greater contact surface area with hydrolysing enzymes (Hartmann and Ahring, 2006). Mshandete *et al.* (2006) reported that an increase of 23% in methane yield can be achieved with a pre-treatment reduction of particle size from 100 mm to 2 mm for a sisal fibres feedstock. Moreover, total mass degradation also showed an increase from 31% to 70% after particle size reduction.

2.1.4.4 *Retention Time:*

hydraulic retention time (HRT) refers to the time required for the AD digester to complete the whole digestion process of the organic substrate (Monnet, 2003). Determination of HRT can be done through measuring Chemical Oxygen Demand (COD) and Biological Oxygen Demand (BOD) of the remaining substrate to assure it fully digested. The optimal required retention time varies depending on other parameters such as temperature, organic loading rate and solid waste characteristics. Minimising the retention time for the digestion process allows the use of smaller digesters and therefore reduced capital costs (Zaher *et al.*, 2007).

2.1.4.5 *Organic Loading Rate (OLR):*

Organic loading rate refers to the amount of organic material that an anaerobic digester is required to deal with in a specific period of time. OLR is usually given in kg of volatile solids (VS) or the chemical oxygen demand concentration (COD) of the digester feeding substrate per unit volume of the AD reactor per unit time (Zaher *et al.*, 2007, Monnet, 2003). Production of biogas theoretically increases continually with increasing OLR until a point where methanogenic microorganisms can no longer convert acetic acid into methane at the same rate as acetic acid production. Therefore, OLR must be optimised to obtain a sustainable AD processes and high methane production. Optimisation of OLR can be achieved through controlling both HRT and substrate concentration and applying a good balance between them. Contact time between substrate and bacterial cells can be reduced by offering shorter HRT (Poh and Chong, 2009).

2.1.4.6 *Mixing Conditions:*

Adequate mixing can improve the surface contact between the bacteria and substrate which provides a good environment for the bacteria to obtain the required nutrients. Mixing is also an important factor to obtain better temperature control as it improves heat transfer throughout the digester. In addition, it helps in reducing organic particle size, avoiding substrate stratification and surface crust layer formation, as well as enhancing gas transfer throughout the reactor (Karim *et al.*, 2005, Sulaiman *et al.*, 2009, Poh and Chong, 2009). Mixing can be achieved through various methods such as mechanical mixers, digester slurry recirculation or biogas recirculation (Karim *et al.*, 2005).

2.2 Anaerobic Digestion of Slaughterhouse Waste

2.2.1 Introduction:

In many regions around the world, thousands tons of protein and lipid rich substrates are produced from daily slaughterhouse activities. This enormous amount of biological waste can cause a significant risk to the environment and people's health if it is disposed in an incorrect way. Recently, possible solutions to dispose of high lipid and protein waste safely are either aerobic or anaerobic process. During the last two decades, the use of anaerobic digestion reactors to treat complex biological waste shows a significant increase (Cammarota and Freire, 2006) (Salminen and Rintala, 2002). As stated by Appels *et al.* (2011), studies have reported that using high lipid materials as a feed stock for anaerobic digestion will increase the digester methane yield. The remaining solids of digested materials are considered to be very good fertilizer for agricultural lands. However, there are still many obstacles facing the anaerobic digestion process when slaughterhouse waste is used to feed the digester.

2.3 Slaughterhouse Waste Characteristics:

Characteristics and composition of slaughterhouse waste effluents are related to the kind of animals, facilities and the technical methods used in the slaughtering process. For instance, at some stages of slaughtering process, washing of carcass, slaughtering area and floor take place. Different facilities use different amounts of water for washing and cleaning according to their operational system; this cleaning process generally leads to an increase in effluent volume and contamination. As stated by Polprasert (2007) the BOD and total solids concentration in the plant effluent are highly related to the plant water use, waste separation, by-product recovery and plant management.

In general, slaughterhouse effluents are heavily contaminated with bio-solids, blood, fat and manure. This requires an effective and suitable treatment method before they are released to the environment. The anaerobic biodegradability, composition and digesting properties of animal fat and proteins in waste materials from slaughtering processes are presented in Table 2-1 and Table 2-2.

Table 2-1 Anaerobic biodegradability of fat and proteins materials (Steffen *et al.*, 1998).

Compounds	Sources	Anaerobic biodegradability	Disturbing effects	Inhibitory effects
Fat	Animals & animal product	Excellent	Poor water solubility, Scum layers	VFAs increase, pH decrease
Proteins	Animals & animal product	Excellent	Foaming	Ammonia increase

Table 2-2 characteristic and digesting property of some AD feed stocks from slaughterhouse waste as investigated in laboratory and pilot scale at the Institution for Agro-biotechnology, Tulln, Austria (Steffen *et al.*, 1998).

Feedstock	Total Solids %	Volatile Solids (% of TS)	Biogas Yield (m^3 /Kg VS)
Animal blood	9.7	95	0.65
Homogenised animal carcasses	33.6-38.8	90-93	1.14
Rumen contents	14.3	88.5	0.35
Gut and stomach contents	16.5	82.5	0.68
Animal fat (rendering plant)	89.90	90-93	1.00

2.4 Challenges, Problems and Limitations in treating Slaughterhouse Waste:

A high content of protein and lipid causes problems in the anaerobic digestion of slaughterhouse waste (Banks and Wang, 1999). The degradation of protein materials releases ammonia, which in high concentration is an inhibitor of anaerobic microorganisms (Angelidaki and Ahring, 1992). In addition, high lipid concentration is also considered to be a problem in anaerobic digestion as it promotes floating scum as well as causing the accumulation of long-chain-fatty-acids (LCFAs) which are strong inhibitors of acetogenic and methanogenic microorganisms (Long *et al.*, 2012). The inhibitory effect of LCFAs occur even in a low concentration (Hejnfelt and Angelidaki, 2009). Nevertheless, materials with high lipid content can also cause a transport limitation of digestion substrate and products, as well as blockages of the digester's pipes and pumps. Digester foaming and clogging, and challenges in the handling and collection system are all problems of using high fat material in AD (Long *et al.*, 2012). For these reasons, solutions must be founded to these obstacles to increase the efficiency of treating animal by-products through anaerobic digestion.

2.4.1 Main Inhibitors for Slaughterhouse Waste Anaerobic Digestion:

2.4.1.1 *LCFAs*

One of the most critical problems facing the anaerobic degradation of high fat materials is long chain fatty acids (LCFAs) produced during hydrolysis of lipid rich substrates. Hydrolysis is started by some extracellular lipases secreted by acidogenic bacteria to degrade fat into primarily glycerol and LCFAs (Cirne *et al.*, 2007). Glycerol is reported to be a good substrate in a co-digestion with rapeseed meal (Kolesarova *et al.*, 2013). However, saturated LCFAs with 12-14 carbon atoms as well as unsaturated LCFAs with 18 carbon atoms were found to be responsible for inhibition of both acetogenic and methanogenic microorganisms (Cammarota and Freire, 2006).

Results by Hanaki *et al.* (1981) who examined the inhibitory effect of LCFAs on the AD process in batch experiments, showed that LCFAs negatively affect the acetogenic bacteria's hydrogen production. Lower hydrogen production indicates an inhibition effect of LCFAs to the acetogenic bacteria, the

responsible group for the β-oxidation process of LCFAs. Toerien and Hattingh (1969) report that LCFAs have an inhibitory effect, even at low concentration, for Gram-positive microorganisms but not for Gram-negative bacteria. As Roy *et al.* (1985) has stated, the sensitivity of bacteria to the LCFAs inhibitory effect is highly related to the structure of the bacteria cell wall, as methanogens and other Gram-positive species are more likely to be inhibited by LCFAs than the Gram-negative bacteria.

The mechanism of LCFAs inhibition is suggested to result from the adsorption of LCFAs on the microorganism's cell membrane, which affects the metabolic processes of transportation and causes limitations in the nutrient transfer into the cell (Pozdniakova *et al.*, 2012). Also accumulation and sorption of a thin LCFA layer on bacteria cells and biomass can lead to biomass flotation and washout (Cysneiros *et al.*, 2012).

The severity of LCFA toxic effects on methanogenic microorganisms is believed to increase with an increase in both the double bonds in the LCFAs structure and the total LCFA concentration in the digester medium (Schnurer and Nordberg, 2008). The relation between increasing the toxicity of LCFAs with increasing its double bond number could result from increasing the LCFAs surface area with more double bonds in the fatty acid chain (Long *et al.*, 2012). Increasing the LCFAs surface area would result in less LCFA molecules required to cover the methanogenic microorganisms cells, in other words, a larger LCFAs surface area means that each LCFAs molecule can cover larger cell surface area of the methanogenic microorganisms (Long *et al.*, 2012).

Operation conditions such as reactor temperature play a role in the bacterial sensitivity to the inhibitory nature of LCFAs. Hwu and Lettinga (1997) reported that the sensitivity of methanogenic microorganisms to LCFAs under thermophilic conditions (55˚C) is higher than under mesophilic conditions (40 and 30˚C).

2.4.1.2 *Ammonia*

Ammonia is one of the common inhibitors in AD digestion of proteins and urea rich materials (Zaher *et al.*, 2007). Free ammonia (NH_3) and ammonium ions (NH_4^+) are the principal reduced nitrogen forms of inorganic nitrogen in aqueous

solutions (Chen *et al.*, 2008). The presence of ammonium ions inside a digester would positively affect the process of anaerobic digestion, since it can be used by the digester bacteria as a nitrogen source, whereas, free ammonia is toxic especially to methane producing bacteria (Mendez *et al.*, 1989). In a study of an anaerobic digestion under extreme ammonia levels, Koster and Lettinga (1988) have reported that after ammonia levels were increased in a range of 4051-5734 mg NH_3-N L^{-1}, acidogenic bacteria and volatile acid production were barely affected while the methanogenic microorganisms activity dropped by about 56.5%. The negative effect of ammonia on the bacterial cells comes from the ability of ammonia molecule to freely pass through the cell membrane and diffuse into the cell disrupting the intracellular pH and ion concentration (Ganesh *et al.*, 2013).

Ammonia and ammonium ions can be transferred into the digester environment through the feeding process; nevertheless, ammonia and ammonium ions can also arise via the biodegradation of organic nitrogenous materials such as proteins, amino acids and urea inside the digester (Mendez *et al.*, 1989, Zaher *et al.*, 2007).

In anaerobic digesters, the amount of ammonium ions and free ammonia is determined by the digester pH. Lowering the pH will cause an increase in the amount of ammonium ions, whereas increasing the pH increases the amount of free ammonia. At pH 7, the amount of free ammonia would be around 0.5% of the full amount of reduced nitrogen in the digester environment. The amount of both free ammonia and ammonium ions are equal at pH 9.3 (Equation 2-2) (Mendez *et al.*, 1989).

$$NH_4^+ \leftrightarrow NH_3 + H^+ \qquad \textit{(Equation 2-2)}$$

Methane forming bacteria are very sensitive to the toxicity of free ammonia. However, even though non-acclimated methanogens can be inhibited at concentrations of free ammonia higher than 50 mg L^{-1} (Mendez *et al.*, 1989), given a suitably long acclimation time they can tolerate a high concentration of ammonia-nitrogen (around 7000 mg L^{-1}) without a significant drop in methane gas production (Parkin and Owen, 1986) and can adapt to free ammonia in high concentrations (1500-3000 mg L^{-1}) (Zaher *et al.*, 2007).

An explanation of this adaptation is provided by Schnurer and Nordberg (2008), who report that the mechanism of methane gas production from acetate shifts from acetetotrophic methanogens pathway to the syntrophic-acetate-oxidation pathway as free ammonia concentration increases in the range 128-330 mg L^{-1}. The reason for the long acclimation period seems to be the variation of the doubling times between the acetoclastic methanogens of around 2–12 days (Caselles-Osorio *et al.*, 2007), and mesophilic syntrophic acetate oxidation microorganisms' doubling time of around 28 days (Peyong *et al.*, 2012).

2.4.1.3 ***Sulphide***

Sulphides can be produced inside the digester environment through two pathways, degradation of the feedstock protein content, and through microbial reduction of sulphates in the digester feed.

Sulphur is an important nutrient for bacterial cell growth, as a result, bacteria cells consume soluble sulphide (HS^-) to satisfy the need. However, high levels of sulphide or dissolved hydrogen sulphide (H_2S) cause a strong toxicity effect inside the digester (Mendez *et al.*, 1989). Sulphide in complex with heavy metals such as iron, copper or zinc is insoluble and nontoxic, whereas unionized hydrogen sulphide in soluble form acts as an inhibitor. Microorganisms can tolerate soluble sulphide in concentrations between 100-200 mg L^{-1} after some acclimatisation. However, concentrations over 200 mg L^{-1} are toxic (Zaher *et al.*, 2007). Hydrogen sulphide gas is relatively insoluble and it is partially stripped and removed from the digester sludge during the normal production of biogas (Mendez *et al.*, 1989). Among the anaerobic digestion bacteria, methanogenic microorganisms are much more sensitive to hydrogen sulphide toxicity than acid-forming bacteria. Hydrogen utilizing methanogenic microorganisms are more sensitive to hydrogen sulphide toxicity than acetoclastic methanogenic microorganisms (Mendez *et al.*, 1989).

2.4.2 Recent Methods to Deal with AD Problems of Fat and Protein Rich Substrates:

Ammonia and sulphide formation are the main problems associated with the degradation of protein rich materials in the digester environment. At high levels, ammonia can cause an inhibition of anaerobic microorganism activity both by increasing the digester pH and by ammonia molecules passing through the anaerobic microorganisms cell membrane, causing a disruption in intracellular ions and pH (Ganesh *et al.*, 2013).

Different methods have been studied and used to improve the overall anaerobic digestion of protein rich materials. One of the most applicable solutions is co-digestion of nitrogen rich materials with poor nitrogen materials. Co-digestion of these materials results in two advantages, minimising the inhibition effect of ammonia production and enhancing the anaerobic degradation of high C:N ratio materials such as lignocellulosic materials. Wang *et al.* (2000) examined five different mixtures with different concentration of bovine serum albumin and starch. The results showed a positive effect of co-digesting high protein materials with carbohydrates. The highest methane production was achieved under co-digestion of 20% bovine serum albumin with 80% w/w starch. A 15 year experience of a full scale slaughterhouse waste co-digestion plant in Linköping, Sweden showed that co-digestion of protein rich materials with other substances is a good way to avoid over loading of nitrogen/ammonia (Ek *et al.*, 2011).

Controlling the pH during the digestion process of protein rich materials will minimise the inhibition effect of free ammonia as lowering the reactor pH will shift the reversible reaction from free ammonia to ammonium ions (Mendez *et al.*, 1989).

Another inhibitor associated with protein rich material is hydrogen sulphide gas, which is a known inhibitor at concentrations higher than 200 mg L^{-1}. This gas is relatively insoluble and it is partially stripped and removed from the digester sludge during the normal production of biogas. Nonetheless, to prevent the toxicity effect of soluble sulphide, a common practice is to add iron to the digester environment. Soluble sulphide will react with iron and provide iron

sulphide (Fe_2S_3) a nontoxic, insoluble, component that gives the digestate a black colour (Mendez *et al.*, 1989).

However, in the case of fat rich materials, co-digestion seems to be a good solution to minimise the operational problems that might appear. As stated by Appels *et al.* (2011) co-digestion of lipid material with other bio waste can increase methane production. However, co-digestion slaughterhouse waste (high in fat concentration) only gives positive results under relatively low concentrations of slaughterhouse waste co-digested with high concentrations of other feed substrate such as agricultural waste (Pitk *et al.*, 2014).

As Roy *et al.* (1985) has stated, the addition of calcium to an anaerobic digester dealing with high lipid materials will minimise the inhibition effect of LCFAs, mainly because of insoluble salts formation. However, calcium addition is still unable to solve the sludge floatation and biomass washout problems (Chen *et al.*, 2008). Acclimatising inocula to high concentrations of lipid and protein may result in a good performance in the digestion process as well as reducing the required start-up time for biogas production, as reported by Goncalves *et al.* (2011), who obtained an increase in the biodegradation rate as well a reduction in the start-up required time for olive mill waste water by using an inoculum that was previously acclimatised to oleate.

2.5 Conclusion:

As reported in the literature, the present methods for anaerobic treatment of lipid rich materials cannot deal effectively with high concentrations of LCFAs, the main products of lipid hydrolysis. The reason is the strong inhibiting effect of the LCFAs on the acetogenic and methanogenic groups, which are responsible for converting the LCFAs into biogas. Inhibition occurs at low concentrations of LCFAs around 1.0 g L^{-1} (Palatsi *et al.*, 2009). Anaerobic degradation of LCFAs is possible through the β-oxidation cycle reaction in equation 2-3, which yields acetate, hydrogen and a shorter LCFA molecule by two carbon atoms (Weng and Jeris, 1976). As reported by Templer *et al.* (2006) this cycle can be repeated until all the molecule of LCFA is completely converted into acetate and hydrogen.

$$CH_3(CH_2)_n COOH + 2\,H_2O \rightarrow CH_3(CH_2)_{n-2} COOH + CH_3COOH + 4\,e^- + 4\,H^+ \qquad (n \geq 2)$$

(Equation 2-3)

Therefore, different strategies will be studied during this project in order to achieve a good digestion process for high fat concentration with a minimum amount of seeding sludge in the reactor.

This includes:

- A study of using different concentrations of fat in co-digestion with vegetable waste.
- A study of different Inoculum to substrate (I/S) ratios.
- Study the effect of using acclimatised seeding sludge with re-inoculating processes.
- Study of different operating conditions of single stage reactors (mesophilic CSTR, thermophilic CSTR, and mesophilic up-flow) under low I/S ratios.
- Study of multi-stage reactors (two, three, and four) to optimise each phase in the digestion process.

The idea of multi stage reactors is to investigate different factors. First, in anaerobic digestion, there are four different groups of microorganisms that live close to each other in a mixed culture to gain the benefit from their syntrophic relationship. Each group has different environmental requirements (Table 2-3) as well as different sensitivity to some inhibitors, such as LCFAs (for example

both acetogenic and methanogenic microorganisms are much more sensitive to LCFAs inhibitory than the other hydraulic and acidogenic bacteria).

Separating the process of anaerobic digestion into 2, 3 and 4 stages according to the optimum pH conditions and the sensitivity to LCFAs; in order to enhance the degradation performance of high lipid substrate, is one of the strategies in this project.

Thermosyntropha lipolytica is known as one the acetogenic bacteria capable of degrading LCFAs (Long *et al.*, 2012). It can be used in the second stage of a three or four stage experiment in order to help break down LCFAs and prevent their inhibitory effects in the next stage. This bacterium was thus selected for a pure culture stage as it requires completely different pH and temperature conditions (Table 2-4) compared to the optimum conditions for the early stages of a normal mixed anaerobic culture (Table 2-3). The scenario is that sudden increase in the pH and temperature in a second stage to pH 8.5 and 60˚C would inhibit the mixed AD bacterial culture and provide optimum conditions for the growth of *T. lipolytica* without the need for a sterilisation step.

Table 2-3 The optimum pH values for anaerobic digestion bacteria.

Anaerobic Bacteria type	Optimum pH value	Calculated average	Reference
Hydrolytic & Acidogenic bacteria	5.5 – 6.5	6	(Yu and Fang, 2002)
Acidogenic bacteria	5.2 – 6.5	5.85	(Solera *et al.*, 2002)
Acetogenic bacteria	6.6 – 7.6	7	(Speece, 1996)
Methanogenic archaea	7.5 - 8.5	8	(Hobson and Wheatley, 1993)

Table 2-4 Acetogenic bacteria that can degrade LCFAs longer than 12 carbons, data from (DSMZ, 2013).

Bacteria	*Thermosyntropha lipolytica*	*Syntrophomonas zehnderi*	*Syntrophomonas sapovorans*	*Syntrophomonas curvata*
Optimum Temperature	60°C	37°C	35°C	37°C
Optimum pH	pH 8.5	pH 7.2	pH 7.2	pH 7.2

Chapter 3 Materials and Methods

3.1 Introduction:

Anaerobic digestion of fat has been investigated through several steps. This chapter presents the materials and methodologies for different experimental setups and operational conditions.

3.2 Materials and methods:

3.2.1 Experimental Reactors:

3.2.1.1 *Single two litre stirred tank batch reactors.*

The stirred tank batch reactor consists of a 2L working volume quick-fit glass vessel, surrounded by a water jacket for temperature control, and placed on a magnetic stirrer for mixing. Each reactor has a pH probe connected to a continuous monitoring pH meter, a flexible sampling tube connected to a syringe for sampling purposes and ports for temperature measurement, and adding or removing contents. The reactor is connected to a gas collector (water displacement system) through a pipe for the daily measurement of biogas production as well as obtaining gas samples for gas composition analysis (Figure 3-1). All openings have gas tight seals.

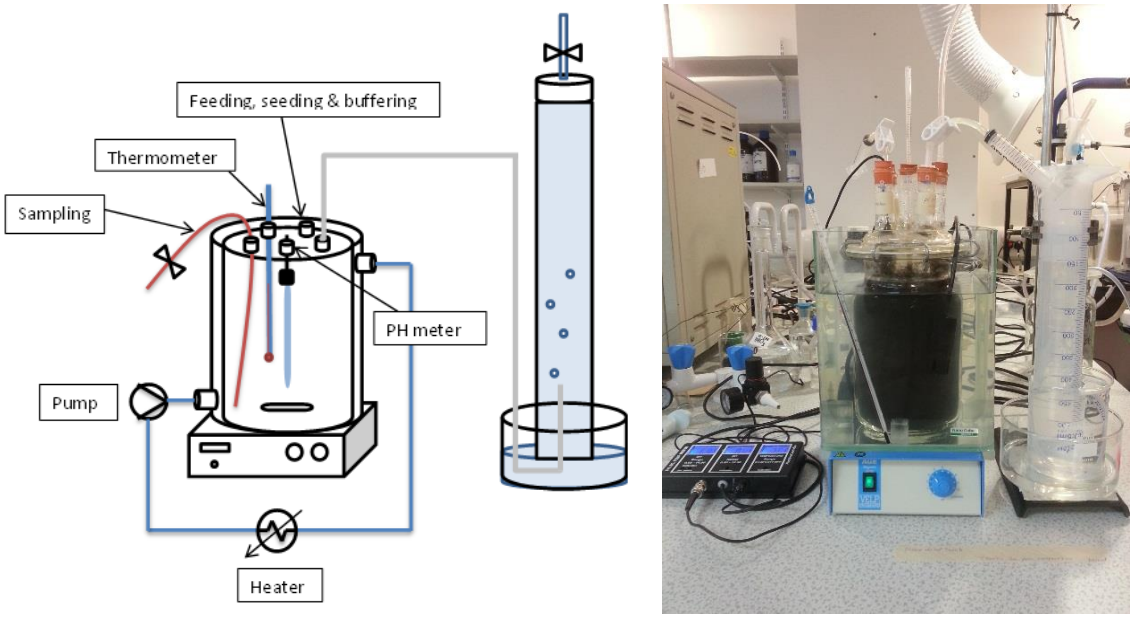

Figure 3-1: Single Stirred tank batch reactor.

3.2.1.2 *Serum bottles for batch digestion experiments:*

Serum bottles, each with a total volume of 120 mL, were used as mini digesters in studies to compare co-digestion of different fat concentrations with vegetable waste. Each experiment consisted of 18 serum bottles located in a clear plastic water bath. The water bath was equipped with a submersible heater to control the temperature to 35 °C and a water pump to circulate the water and thus homogenise the temperature in the water bath (Figure 3-2 and Figure 3-3).

Figure 3-2: Flushing the head spaces with nitrogen gas to provide anaerobic conditions.

Figure 3-3: Serum bottle batch experiment set-up.

3.2.1.3 *Stirred 500mL flask batch reactors*

The flask reactors were designed and built to study: firstly, the effect of different seeding sludge sources (adapted and not adapted) on different fat concentrations in the feed substrate; secondly, to study the effect of different concentrations of fat (from 0 to 20 g L^{-1} (VS)) in co-digestion with vegetable waste; thirdly to study pH behaviour and influence of fat concentration on the accuracy of the pH sensors.

Each experiment consisted of 36 reactors each of 500 mL working volume and each with its own water-displacement gas collector, 3 12 place multi-place magnetic stirrers, 3 temperature controlled water baths (each with 12 reactors), and two continuous pH reading units connected to 36 pH sensors (one in each reactor) (Figure 3-4).

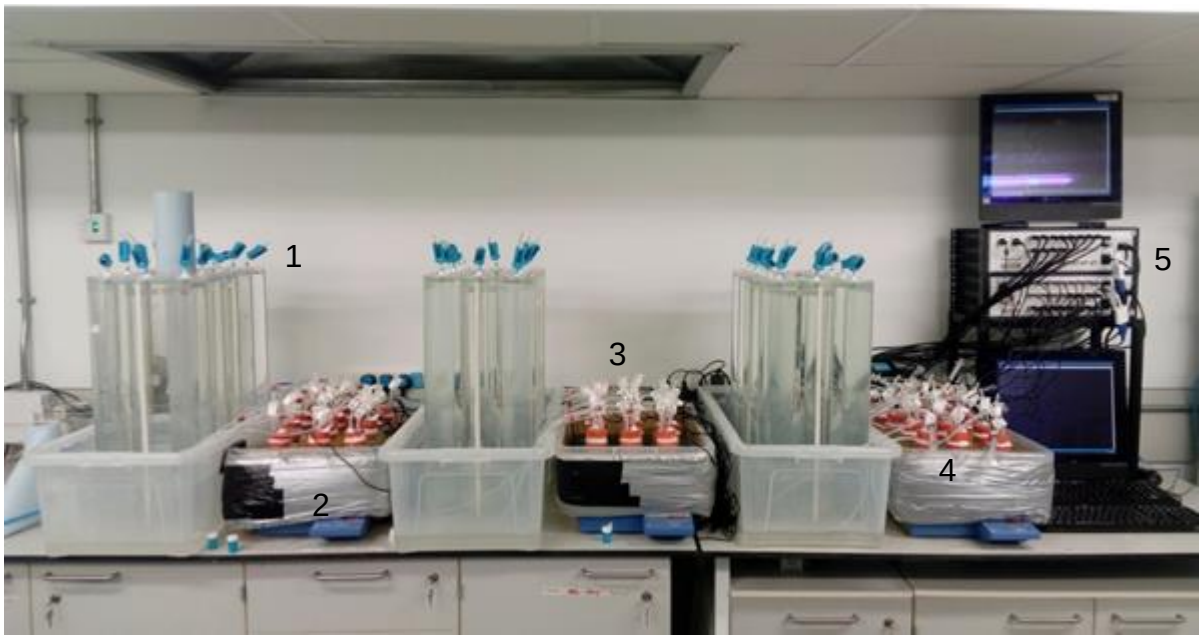

Figure 3-4: 36 flask batch reactors

(1-Biogas collectors, 2-Multi-position magnetic stirrer, 3- 500 mL flask batch reactor set, 4-Insulated water bath 35˚C, 5- Continuous pH reader units).

3.2.1.3.1 The 500 mL flask reactor

Each reactor consisted of a 500 mL volume glass wide neck flask equipped with a size 43 modified rubber bung through which passes a glass-sampling tube for liquid samples, a short glass tube for buffer addition, a gas sampling tube and a pH sensor probe (Figure 3-5). A magnetic stirrer bar was introduced into each reactor to provide the required mixing.

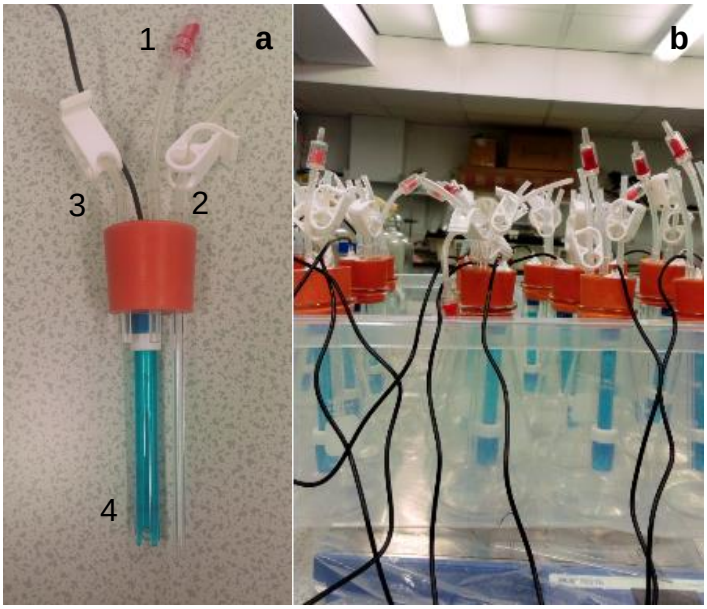

Figure 3-5: Reactors preparation.

a: rubber bung fitted with (1- gas outlet tube, 2- liquid sampling tube, 3- buffer and feeding tube and 4- pH probe).
b: flask reactors set up in the water bath.

3.2.1.4 *Multi-stages reactors: Two, Three and Four stages.*

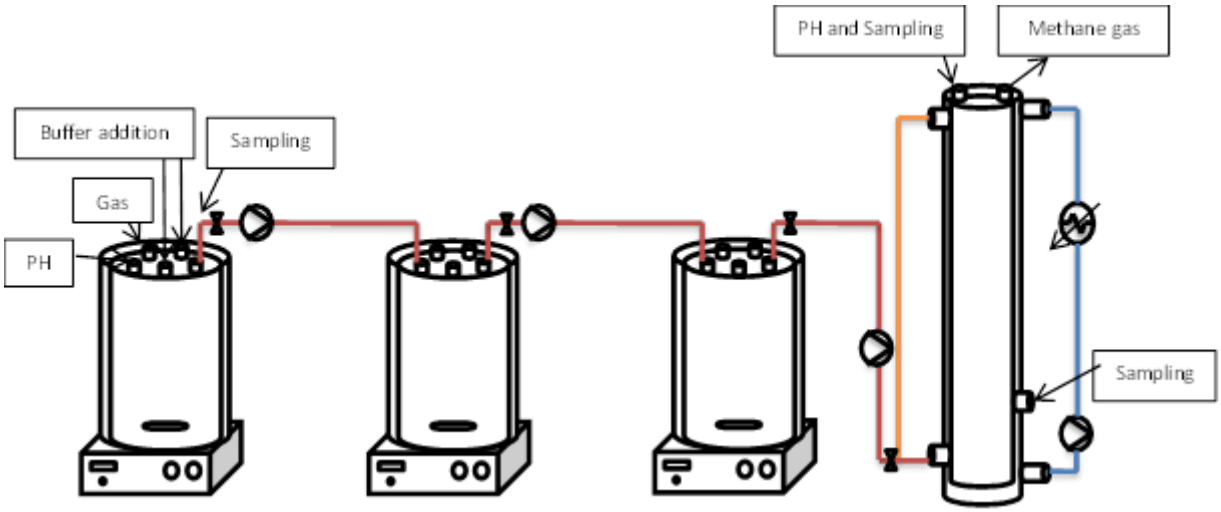

Figure 3-6: Four stage anaerobic digester.

Different multi stage reactors (in terms of the number of stages) were used during this study. Two, three, and four stage reactors were built and modified in order to test the separation of the breakdown process into different controlled stages, and how these influenced the overall digestion process. Figure 3-6 shows an example of the four stage reactor with two types of the reactor vessels.

Stirred tank reactors (CSTR) were used for the first stages of the process (hydrolysis, acidogenesis and acetogenesis) whereas an up-flow column reactor was used for the last (methanogenesis) stage. Stirred tank reactors (using magnetic stirrers) were chosen for the first three stages as it has been reported that strong mixing results in a higher percentage of lipid degradation. As described by Elefsiniotis and Oldham (1994), stronger mixing conditions are the reason behind the high percentage of lipid degradation provided by a completely stirred tank reactor (CSTR) anaerobic digester (63.4–83.2% digestion of lipids) compared to a UASB reactor (47.5–67.3% digestion of lipids). Strong mixing in case of high percentage lipid substrate leads to better lipid distribution and increased contact between enzyme (bacteria) and substrate.

An anaerobic up-flow reactor was chosen for the methanogenic step to provide higher methanogenic enrichment through the formation of a blanket of sludge that is suspended in the reactor medium giving higher bacterial density than CSTR under continuous operational condition.

The stirred tank batch reactor consists of a 2L volume quick-fit glass vessel, surrounded by a water jacket for temperature control, and on a magnetic stirrer for mixing, automatic pH controller with acid and base dosing pumps, and a flexible sampling tube connected to a syringe for sampling purposes. The reactor is connected to a gas collector (water displacement system) through a pipe to measure biogas production as well as to obtain gas samples for gas composition analysis.

The Up-flow reactor consists of a 2L volume acrylic vertical cylinder (8 cm internal diameter and 55 cm high), surrounded by a water jacket for temperature control, centrifugal pump for mixing, automatic pH controller with acid and base dosing pumps, and a flexible sampling tube connected to a syringe for sampling. The reactor is connected to a gas collector (water displacement system) through a pipe to measure biogas production as well as obtaining gas samples for gas composition analysis.

3.2.2 **Continuous multi pH readers:**

A continuous pH reader unit with 36 pH sensors was designed and fabricated by the electronics workshop at Sheffield University. The units were built using scientific grade reading pH circuits obtained from Atlas Scientific LLC, (New York, USA). The pH readers have a measuring range of pH 0-14 with ± 0.01pH accuracy at 25 °C. The units were equipped with 36 single junction standard KCL pH electrodes. Each pH reader was calibrated and tested at pH 7 and pH4 according to the pH circuits manual using calibration buffer solutions (pH 7 and 4) from (Fisher Scientific UK Ltd, UK) prior to experimental use.

3.2.3 **Automatic pH controller:**

Automatic pH controller units, obtained from Cyber Plant LLC, (Russia) were used to automatically control the pH according to the experimental conditions.

Each unit consists of an automatic pH controller connected to two peristaltic pumps for acid and base, and equipped with a combination glass pH electrode and temperature sensor (Figure 3-7).

The pH unit has a measuring range of pH 0-14 with ± 0.01pH accuracy at 25 °C. The pH controller units were calibrated at pH 7 and pH4 using calibration buffer solutions from (Fisher Scientific UK Ltd, UK) before use in any experiment.

The peristaltic pumps were connected to acid and base buffers containers through PVC pipes. Hydrochloric acid (HCl) was used at a concentration of 1 M to lower the pH and a saturated solution of sodium bicarbonate ($NaHCO_3$) was used to raise the pH value through the controller. The dosing rate was set at 0.1 mL min^{-1} for both acid and base, and waiting time between doses was set to 5 minutes.

Sodium bicarbonate ($NaHCO_3$) was preferred as it directly release bicarbonate alkalinity, in addition to its solubility, ease of handling, and low negative impact on the microorganism and process performance (Mendez *et al.*, 1989). However, it is important to mention that the CO_2 added to the system by bicarbonate buffer when react with acids (organic acids or HCl inside the reactor during the pH control process), may contribute to the carbon balance of the AD system.

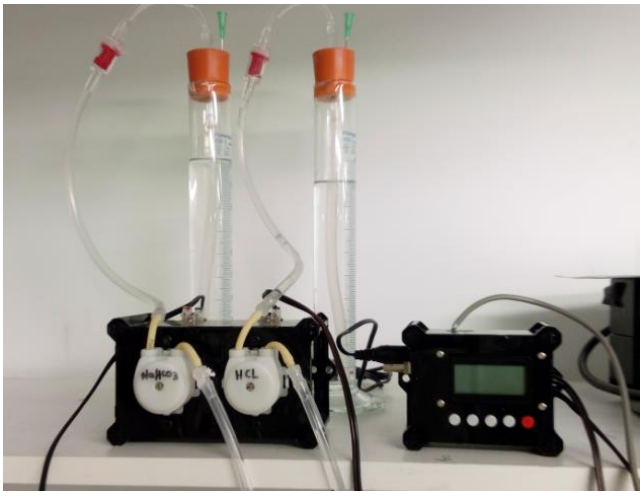

Figure 3-7: pH controller with acid and alkaline dosing pumps.

3.2.4 **Liquid displacement gas collectors:**

Biogas from the experimental lab scale digesters was captured and measured using a volumetric water displacement system.

Each water displacement gas collector consists of a vertical graduated clear acrylic cylinder, fitted with a sampling port in the top and standing inside a water container. The outlet gas pipes from the anaerobic digesters were connected to the lower part of the gas collector that was submerged under the water level.

Solubility of biogas components in the barrier liquid used in the collector system were taken into account during the system design. In the case of methane and hydrogen gases, both have a poor solubility in the water, whereas carbon dioxide shows a higher solubility.

Accurate monitoring of CO_2 is important during the anaerobic digestion process. The increase in CO_2 content and the following decrease (in case of batch digester systems) / or stabilisation (in case of continuous and semi-continuous digester systems) represent the progress of digester start up process.

To avoid losing of CO_2 in the water, the tap water which was used in the gas collection system was acidified to pH ~2 using Hydrochloric acid. Acidification of water in the volumetric displacement gas collector results in lower solubility of CO_2.

3.2.5 **Sampling technique:**

3.2.5.1 ***Liquid sampling:***

Liquid samples were withdrawn through flexible double walled silicon-PVC sampling tubes (Figure 3-8). This tube was selected in order to combine the advantages of flexibility, reliability and ease of control from the internal silicon wall, and the relatively low gas permeability of the external PVC wall.

Samples from the reactors were taken using disposable plastic syringes.

After connecting the syringe to the sampling tube, the clip valve is opened and the syringe plunger pulled to withdraw the nitrogen gas filling the internal part of the sampling tube and the liquid sample. To ensure that every sample was fresh and derived directly from inside the reactor, liquids in the internal part of the sampling tube was pushed back and displaced with the nitrogen gas from the same syringe. This can be achieved by turning the syringe tip upwards and pushing the gas level back up the tube (Figure 3-9). The clip valve now can be closed before disconnecting the syringe.

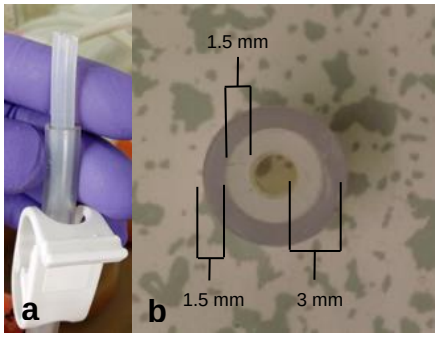

Figure 3-8: flexible double walled costumed silicon-PVC sampling tube.

a: double walled costumed silicon-PVC sampling tube to minimise oxygen permeability in AD experiments.
b: cross-section in the double walled tube showing tubes thickness and diameters.

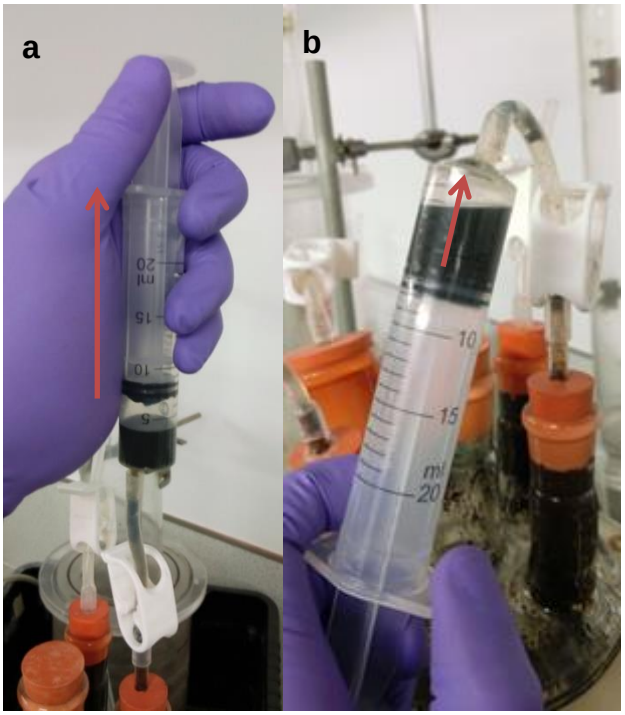

Figure 3-9: Liquid sampling technique.

a: withdrawing the nitrogen gas from the sampling tube followed by fresh samples from inside the digester.

b: ejecting the nitrogen gas to the sampling tube to ensure the sampling tube is empty from liquid sample residues so that next sampling will be fresh from inside the digester.

3.2.5.2 *Gas sampling*

3.2.5.2.1 From Lab scale digesters:

Samples of biogas from the lab scale digesters were obtained from the water displacement gas collectors. The samples were taken through a plastic syringe connected to the flexible sampling tube in top of the water displacement collectors. The first two samples were discarded each time to lower the possibility of contamination with air. After the third sample was taken, a gas tight cap was placed on the syringe to immediately close the plastic syringe tip.

3.2.5.2.2 From serum bottles mini digesters:

Plastic syringes fitted with stainless steel needles were used to measure the gas production and to obtain gas samples from the 100 mL serum bottles digesters. Measuring gas production and obtaining samples was carried out under water level. Syringes were flushed several times under the water level to remove any air bubbles before being inserted into the serum bottle through the rubber septum. All the syringes were previously lubricated from inside with silicon grease to ease the movement of the pistons.

Lubricated syringes were tested as a method to measure gas from a positive pressure area. These were tested against a known volume of air and showed accurate results during the preparation and testing for the serum bottles experiment (Figure 3-10).

When higher volume of gas was expected from any serum bottle digester, then a water displacement system connected to a stainless steel needle through a PVC tube was used to collect and measure the produced gas volume.

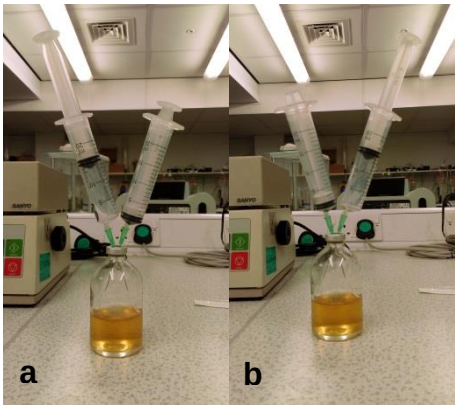

Figure 3-10: Testing of the lubricated plastic syringes against a known volume of gas (air).

a: 15 mL air injected into sealed serum bottle.

b: positive pressure lifting lubricated syringe with approximately equal volume of the injected gas.

3.2.6 **Feed substrate:**

Two types of feed substrate were used in this research. The first was a vegetable mix to represent vegetable waste. The mix was obtained and kept frozen until use, and contained 40% carrots, 30% cauliflower, and 30% w/w garden peas and broccoli.

The frozen mixed vegetables were processed by defrosting then blending in an electric blender in order to form a smooth textured feed substrate. The blended vegetables were then repacked into 500 g containers and stored in a freezer at -18°C.

Prior used, the frozen blended vegetables were defrosted at room temperature. Total solids and volatile total solids were measured and the feed then diluted according to the required VS of each experimental condition.

The second type of feed substrate used in this research was animal fat. Powdered bovine fat (Grau GmbH Company, Germany) was chosen as an alternative option over fresh bovine fat for many reasons. Fat powder is stable and easier to handle and store in the lab with less biosafety issues, also the use of a powder reduces clogging of the reactor pipes as it forms a suspension when mixed with water, thus providing better control of feeding and sampling.

Total solids and volatile solids of fat power were measured to calculate the required amount of fat for each experimental condition.

3.2.7 **Anaerobic seeding sludge:**

The main inoculum used in this study is an anaerobic seeding sludge obtained from Hull wastewater treatment works (Hull, UK). The sludge was concentrated through gravity settling. The supernatant was then removed in order to increase the volatile solids content per unit volume. Prior to use, the anaerobic seeding sludge was incubated at 35 °C for two to four weeks until gas production ceases.

3.2.8 **pH**

pH values of the initial experiment in chapter four were measured using a RS-200A continuous monitor pH meter with resolution of 0.10 pH and ±0.1 accuracy. The pH meter was calibrated at two points of pH7.00 and pH4.01 before use.

A benchtop pH meter HI2211 from HANNA instruments Ltd (Bedfordshire, UK) was used for the rest of the manual pH value measurements during this research. The pH meter has a resolution of 0.01 and was equipped with a combination single junction glass pH electrode HI-1131B (HANNA instruments Ltd, Bedfordshire, UK). The instrument was calibrated at pH 7.00 and 4.01 using standard calibration buffer solutions from Fisher Scientific UK Ltd, prior the experiment.

Problems of contamination and accumulation of solid fat around the pH probes were experienced during some experiments (Figure 3-11). Accumulation of fat can limit the contact between the probe and the sample causing reading errors and affect probe's responding time and accuracy.

Therefore, to maintain accurate readings and fast probe response, the probe was cleaned and its calibration tested in the pH 7 and 4 standards before and after every sample. The cleaning step was done through soaking the pH probe in a detergent solution and rinsing it with deionised water. Re-calibration on pH 7 and 4 was done when necessary. Replacements of failed probes were made when required.

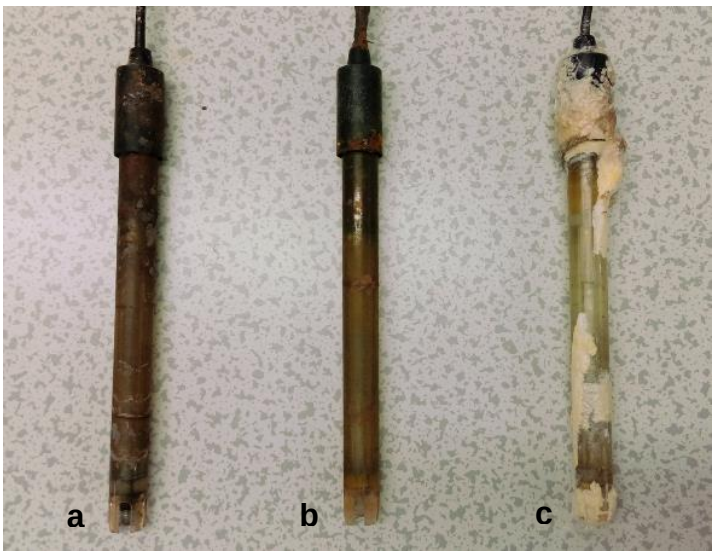

Figure 3-11: accumulation of fat around the pH electrode body.

a: pH probe from co-digestion reactor of fat powder and vegetable waste.
b: probe from only vegetable waste reactor, c: probe from only fat powder digester.

3.2.9 Developing an anaerobic pH probe case for laboratory use:

Good sealing is very important in anaerobic digestion experiments to prevent introducing oxygen from the surrounding environment into the anaerobic system. For this reason, pH electrodes were installed inside the reactor to monitor the pH (specifically for online, continuous pH monitoring) during the digestion process.

Long exposures of the pH electrode to the reactor contents can cause a drift from the actual pH value, giving inaccurate readings. This became a significant problem when fat was used to feed the anaerobic digester, where a longer time was required to finish the digestion process in addition to the accumulation of fat around the probe body.
To overcome this problem, manual pH testing was regularly done to correct any drift in the automatically measured pH values.

In some small scale experiments with limited sampling volumes, pH values were measured in an anaerobic atmosphere outside the reactor before the sample was returned back inside the digester. This was achieved using a small probe case designed and made for the purpose. A first plastic prototype was made in the lab using basic materials (Figure 3-12). Later, a glass version was made to replace the plastic one by the glass blowing workshop (Sheffield University, Sheffield, UK).

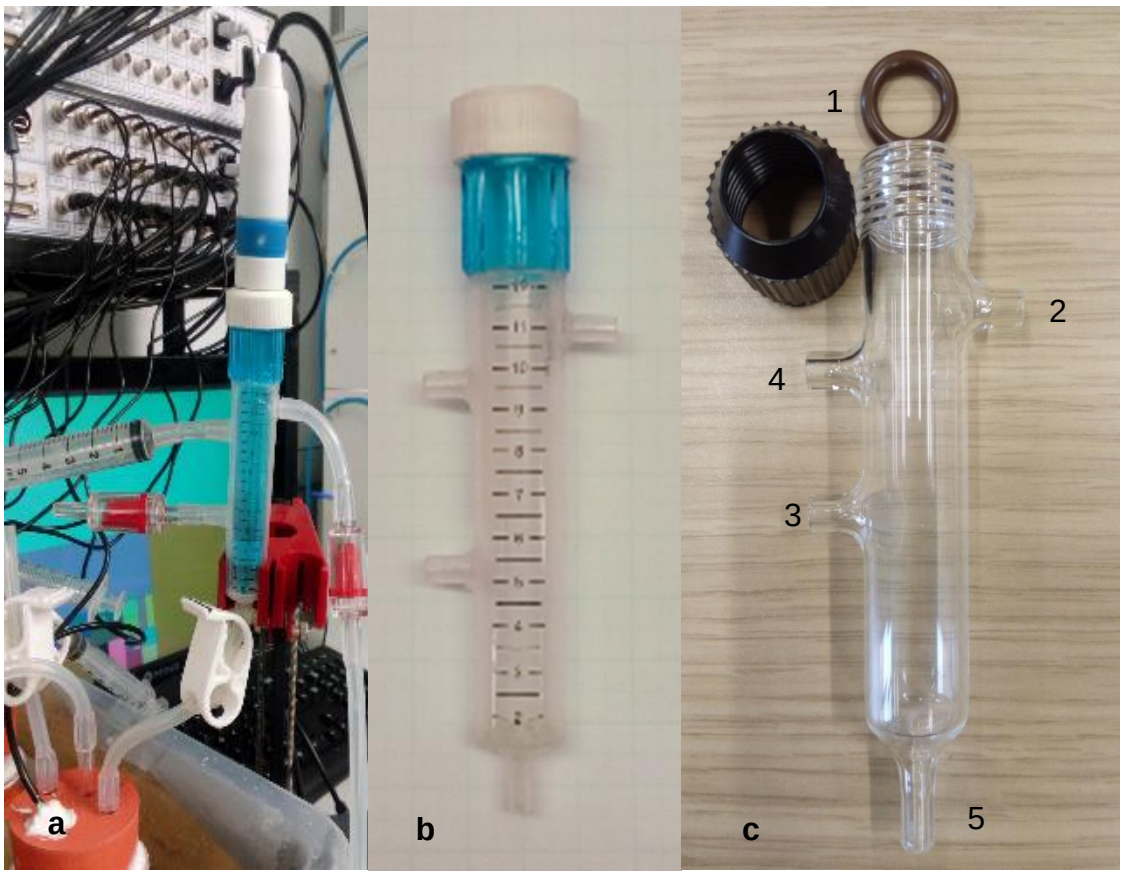

Figure 3-12: anaerobic pH probe case.

a: plastic anaerobic pH probe case connected to a small anaerobic digester for pH measurements.
b: first plastic prototype pH probe case.
c: glass anaerobic pH probe case, (1- O-ring rubber seal and cap, 2- nitrogen gas flush inlet, 3- nitrogen gas outlet, 4- syringe connection port to withdraw the anaerobic samples into the case, 5- connection port to sampling tube in the digester.

The probe case provides several advantages for limited volume anaerobic digestion experiments. It provides the ability of measuring the pH value without exposing the sample to air or loosing/disposing it. It gives more flexibility and eases the cleaning, re-calibration or replacing the probes compared to probes placed inside the reactor.

With some modifications, the probe can be a beneficial lab tool for single strains and strict sterilization experiments. A nitrogen flush can be replaced with an ethanol stream followed by autoclaved DIW to sterilize inside the case and the probe. A 0.22µm pore size inline filter can be used to maintain the sterile environment inside the case when an additional gas flush/injection is required.

The customised case can be used with automated sampling and measurements when fitted with appropriate analytical probe (such as pH, DO, conductivity, ammonia, etc.) and suitable software.

3.2.10 Total Solids and Volatile Solids

TS and VTS were analysed according to standard methods (Andrew, 2005). To determine the total solids content, a known weight of wet sample was dried in an oven at 103-105 ˚C for two hours. The total solids were then calculated by the expression:

$$TS\% = \frac{\text{weight of dry sample}}{\text{weight of wet sample}} \times 100\%.$$

To determine the volatile total solids, the dry samples were combusted in a muffle furnace at 550 ˚C for 30 minutes. The total volatile solids were then calculated:

$$TVS\ \% = \frac{(\text{dry sample weight} - \text{weight of the ash})}{\text{dry sample weight}} \times 100\%.$$

3.2.11 Chemical Oxygen Demand (COD)

Dissolved COD values of the supernatant were measured through HACH LANGE photometric tests with COD ranges of (200-1000 and 1000-10000 mg L^{-1}). The COD test gives a measurement of the amount of organic material in the sample by measuring the oxygen equivalent to this organic matter.

The principle of the COD cuvette test is that any oxidisable substances in the sample will react with potassium dichromate in a sulphuric acid solution. Silver sulphate is present as a catalyst to enhance the oxidation of the organic material. Mercury sulphate is present to mask chloride ions and prevent any interfering oxidation of chloride ions by the potassium dichromate. After the oxidation process was completed, the green coloration of Cr^{+3} was evaluated using the HACH DR2800 spectrophotometer.

The spectrophotometer was set to zero using a new COD cuvette test with deionised water as a blank. The cuvette was run into the same analytical procedure for the main experimental samples before it being used as a blank.

The COD values of the supernatant were measured after separating the solid material from the anaerobic sludge samples by centrifugation at 13000 rpm for 10 minutes followed by a filtration of the supernatant through 0.22µm pore size syringe filter units.

3.2.12 Volatile Fatty Acids (VFAs) by Gas Chromatography

3.2.12.1 *Preparation of sludge samples*

The preparation of samples for VFAs analysis was adapted from SCA (1979): Determination of Volatile Fatty Acids in Sewage Sludge (1979). The samples were prepared for GC analysis by centrifugation at 13000 rpm in 1.5 mL micro-centrifuge tubes for 10 minutes. The supernatant was withdrawn by using disposable 2.5 mL plastic syringes and filtered through a syringe filter with a pore size of 0.22 µm. 0.5 mL of the filtered samples were transferred by pipette into new 1.5 mL tubes with 0.5 mL of deionised water to form a total of 1 mL 50% diluted samples. 1 to 4 µL of formic acid was added to acidify all the samples to pH ~2. After the acidification step, 200 µL aliquots of the acidified samples were transferred into GC vials and loaded onto the auto-sampler for VFA analysis.

3.2.12.2 *GC method and calibration*

The VFAs were analysed using a Trace 1300 Gas Chromatograph (Thermo Scientific, UK), fitted with a flame ionisation detector and a Thermo TR-FFAP – 50m length, 0.32 mm diameter and 0.50µm film thickness. The carrier gas was Nitrogen at a flow rate of 1.5 mL min^{-1}. The temperature was programed for the GC oven to be increased from 100 to 180 °C in 15 minutes with an initial hold time at 100 °C for 1 minute and a final hold time at 180 °C for 6 minutes. The temperatures were set at 200 and 250 °C for the injector and the detector, respectively.

A standard volatile free acid mix containing acetic, propionic, isobutyric, butyric, isovaleric, valeric, isocaproic, hexanoic, and heptanoic acids in deionised water (Volatile Free Acid Mix, Sigma Aldrich, UK) was used at four different dilutions for calibration. The concentration of calibration standard was 1%, 10%, 50%, and 100% of the original volatile free acid mix standard.

The volume of injection samples into the GC was 1 µL. The auto-sampler was programed to prewash the GC syringe with deionised five times prior to the sample and three times with the sample before injecting 1 µL of it into the GC. After injecting the sample, the syringe was post washed five times with acetone.

3.2.12.3 *Validity and maintenance of the VFAs analysis*

Good peak separation and calibration curves were obtained through the VFA analysis method.

Standard checks were regularly made to maintain the validity and accuracy of the analytical results. In addition, three washes were made between samples by injecting 1 µL acidified DIW pH 2 (through formic acid addition) and 2 µL pure DIW (1 µL each time) were performed to reduce the possibility of contamination between samples.

However, liquid samples from anaerobic digestion are quite complex since they contain a wide range of different organic and inorganic components, and contaminations of the injection liner were regularly observed after number of samples.

Selected peaks such as acetic acid and propionic acid were reduced in size after several sample injections. Troubleshooting the software, various methods of restoring the peaks through washing injections using acidified DIW, pure DIW or acetone, all showed no significant recovery.

The most effective practice was found to be to change the inlet glass liner, when good peaks can be restored again. The glass liner was then changed every ~80 injections (every 20 samples and 60 washes in between samples), and calibration checks were performed after each liner change in order to maintain the accuracy in the results. Repeatability of samples analysis was tested, and showed good repeatable results under this protocol with a relatively low standard deviation based on the replicated sample (Table 3-1).

Table 3-1: Technical replicate of a VFAs sample from an experimental reactor at 100% w/w fat condition of the 20g L^{-1} total volatile solid feed condition.

VFA mg L^{-1}	Replicate No 1	Replicate No 2	Replicate No 3	Average	*SD*
Acetic	527.13	532.62	534.14	531.30	3.68
Propionic	24.99	24.87	25.24	25.03	0.18
Isobutyric	17.37	17.56	16.95	17.29	0.31
Butyric	382.97	385.67	386.21	384.95	1.73
Isovaleric	15.42	15.14	15.64	15.40	0.24
Valeric	10.87	10.39	10.43	10.56	0.26
Isocaproic	1.04	0.28	0	0.44	0.53
Caproaic	201.92	189.99	194.36	195.42	6.03
Heptanoic	2.81	1.01	1.764	1.86	0.90

3.2.13 **Biogas Production and Composition**

3.2.13.1 ***VARIAN TCD-GC***

During the initial experiments described in chapter four, biogas compositions were analysed using a Varian CP-3800 gas chromatograph fitted with a molecular sieve 5A 80/100 2 m, 2 mm internal diameter Varian column, thermal conductivity detector and argon as a carrier gas with a flow rate of 3.6 mL min^{-1}. The temperature was adjusted to 50 ˚C for the oven and 70 ˚C for the detector.

3.2.13.2 ***THERMO 1310 TCD-GC***

The composition of biogas produced in the main experiments during this study was analysed using a manufacturer customised TRACE 1310 Gas Chromatograph (Thermo Scientific, UK). The GC was fitted with a 250 µL gas-sample loop, in addition to two Restek Packed Columns, Molecular sieve 5A (60/80 mesh) - 2.0 m length x (1.0 mm ID 1/16" OD) and Hayesep Q (60/80 mesh) – 2m length x 1mm ID, 1/16" OD.

The detector was a thermal conductivity detector and argon was the carrier gas with a flow rate of 1.0 mL min^{-1}. The GC oven program was set to increase the temperature from 50 to 150 °C, over a 13 minute run time with an initial hold time at 50 °C for 2.5 minutes. The temperature is then increased again at a rate of 30 °C min^{-1} to 70 °C and held for 8.83 minutes. With a rate of 80 °C min^{-1}, the final temperature of 150 °C is reached at the end of the run. The temperature of the TCD detector is set at 150 °C.

The GC was calibrated using a standard gas mix containing 65% methane, 30% carbon dioxide, 2% hydrogen, 2% nitrogen, and 1% oxygen v/v (Air Products PLC, Worcester, UK). This calibration gas was used in different dilutions with argon to achieve three calibration points. Other lab-prepared gas standards were used as well to achieve higher calibration points for hydrogen, nitrogen and oxygen.

3.2.14 **Dissolved Oxygen**

A JENWAY Model 9150 dissolved oxygen meter with a DO/Temperature sensor that has a resolution of 0.01 mg L^{-1} and accuracy of ±2% within 10°C of calibration temperature was used. The dissolved oxygen meter was calibrated at room temperature and zero oxygen solution. The zero oxygen solution was prepared according to the instrument manual by mixing 2 g of sodium sulphite anhydrous in 100 mL of distilled water and then left for few minutes to stand before in being used.

3.2.15 **Carbon to Nitrogen ratio**

The carbon to nitrogen ratio was calculated after measuring organic carbon and nitrogen content in the mixed vegetable feed using a Flash 2000 gas chromatograph elemental analyser equipped with quartz reactor and 6x5mm I.D. 2m steel column and TCD detector. Helium was the carrier gas with a flow rate of 140 mL min^{-1} and oxygen as a sample oxidisation gas with flow rate of 300 mL min^{-1}. The temperatures were adjusted to 900 °C for the left furnace, 800 °C in the right furnace and 50 °C for the oven temperature.

3.2.16 **Elemental composition**

C, H, N, S content in the original samples (mixed vegetable, bovine fat powder, and seeding sludge) were analysed using the Elementar Vario MICRO Cube CHN/S Elemental Analyser. The analyser is equipped with a TCD detector and helium was the carrier gas with flow rate of 200 mL min^{-1}. Oxygen content in the previous samples was calculated through a subtraction of C, H, N, S and ash percentages from 100% w/w (Liwarska-Bizukojc and Ledakowicz, 2003).

Oxygen % = 100 - (C% + H% + N% + S% + Ash %)

3.2.17 **Theoretical Methane production**

Theoretical methane production was calculated through the equation of Buswell (Buswell and Mueller, 1952) using the elemental composition (CHNS-O) results for both fat and vegetable waste feed substrates (Equation 3-1).

$$C_cH_hO_o + \left(c - \frac{h}{4} - \frac{o}{2}\right)H_2O \rightarrow \left(\frac{c}{2} + \frac{h}{8} - \frac{o}{4}\right)CH_4 + \left(\frac{c}{2} - \frac{h}{8} + \frac{o}{4}\right)CO_2 \qquad \textit{(Equation 3-1)}$$

Direct calculations of the maximum theoretical methane production were made using the COD values of both fat and vegetables under different experimental feeding conditions.

From (Equation 3-2), two moles of oxygen are needed to fully oxidise one mole of methane. As CH_4 and O_2 have molecular mass of 16 and 32 respectively, the production of one gram of CH_4 links to the removal of four grams of COD from the reactor's substrate (Equation 3-2).

$CH_4 + 2O_2 = CO_2 + 2H_2O$ *(Equation 3-2)*

16 + 64 (molecular mass)

- The removal of 1 gram of COD will yield 0.25 gram of CH_4
- 0.25 gram of CH_4 / 16 (molecular mass) = 0.015625 moles of CH_4
- 0.015625 moles of CH_4 x 22.4 L (molar volume constant) = 0.35 STP litres of CH_4

Therefore, assuming that all the COD removed from the reactor is converted to CH_4, one gram of the COD removed will yield 350 mL of CH_4 at standard temperature and pressure.

3.2.18 Long Chain Fatty Acids (LCFAs)

3.2.18.1 *Preparation of the sludge samples*

Sludge samples in 2 mL Eppendorf tubes were first stored at -80 °C before been transferred to the freeze dryer for the drying process. The freeze-dried sludge samples were treated by adding 300 µL toluene, vortexing for 2 minutes and transferring into glass vials. 300 µL of sodium methoxide was added to each vial followed by incubation at 80 °C for 20 minutes. After the samples were cooled to room temperature, another 300 µL of 14% boron tri-fluoride in methanol was added followed by incubation at 80 °C for another 20 minutes. After cooling to room temperature, the contents of the glass vials were transferred to new 2 mL

Eppendorf tubes where 300 µL of ultrapure water and 600 µL of hexane were added. The tubes were vortexed for 1 minute and centrifuged at 8500 rpm for 10 minutes. 800 µL of the upper organic phase (hexane and toluene layer) was transferred to separate Eppendorf tubes followed by a drying step using an inert nitrogen gas flush. Samples were re-suspended in 80 µL of toluene and centrifuged for 3 minutes. 30 µL of the contents were transferred into GC vials and loaded onto the auto-sampler for the LCFA analysis. Pre dilution of the samples with DI water and/or post dilution after the transesterification processes with toluene were done when necessary.

3.2.18.2 *GC method*

The LCFAs were analysed using a Trace 1310 Gas Chromatograph (Thermo Scientific, Hertfordshire, UK), fitted with a flame ionisation detector and a Thermo TR-FAME – 25 m length, 0.32 mm internal diameter and 0.25 µm film thickness. The carrier gas was Helium at a flow rate of 1.5 mL min^{-1}. The temperature was programmed for the GC oven to be increased by 10˚C min^{-1} from 150 to 250 °C in 12 minutes total running time with an initial hold at 150 °C for 1 minute and a final hold at 250 °C for 1 minute. The temperatures were set at 250 °C for both the injector and the FID detector.

A standard FAME mix in deionised dichloromethane (Supelco 37-component FAME Mix, Sigma Aldrich, UK) was used to calibrate the GC.

The volume of samples injected into the GC was 1 µl. The auto-sampler was programed to prewash the GC syringe with toluene three times prior to taking the sample and washed three times with the sample before injecting 1 µl into the GC. After injecting the sample, the syringe is post washed four times with toluene.

3.2.19 **Culturing and preparation of *Thermosyntropha lypolitica*: Media and OD.**

An active single strain bacterial culture of *Thermosyntropha lypolitica* used in this research was obtained from DSMZ; (Braunschweig, Germany). The bacteria were subcultured in serum bottles according to the supplier's instructors. The process was performed under anaerobic conditions inside an automatic nitrogen flush anaerobic chamber.

3.2.19.1 ***Growth Medium:***

The medium was prepared according to the 731 Thermosyntropha Medium recipe provided by DSMZ (Table 3-2). The preparation was carried out anaerobically under 100% N_2 before it was autoclaved for sterilisation. All the medium stocks were sterilised in the autoclave except the vitamin solution, which is heat and light sensitive, it was filter sterilised in a direct injection to a sterile serum bottle covered with foil. Sterile stocks were stored in the fridge 4 °C until further use. Vitamins, sulphide and cysteine from sterile oxygen free stock solutions were added prior inoculation under 100% N_2 conditions.

The growth was visually evident by the turbidity of the cultured broth after 48 hours of incubation at 60 °C. Growth curve was obtained after measuring the optical density (OD) at the wavelength of 600 nm as well (Figure 3-13).

In the four stage anaerobic digestion experiment, a 3 days old active stationary phase *Thermosyntropha lypolitica* culture was used as an inoculation to seed the experimental reactor.

Table 3-2: 731 Thermosyntropha Medium recipe (DSMZ, 2015)

731. Thermosyntropha Medium	
K_2HPO_4	0.30 g
KCl	0.30 g
NaCl	0.50 g
NH_4Cl	1.00 g
$MgCl_2$ x 6 H_2O	0.30 g
$CaCl_2$ x 2 H_2O	0.05 g
Trace element solution	10.00 mL
Yeast extract	10.00 g
Na-resazurin solution (0.1% w/v)	0.50 mL
$NaHCO_3$	3.00 g
K_2HPO_4	0.30 g
Na_2CO_3	3.00 g
Vitamin solution	10.00 mL
L-Cysteine-HCl x H_2O	0.15 g
Na_2S x 9 H_2O	0.50 g
Distilled water	1000.00 mL

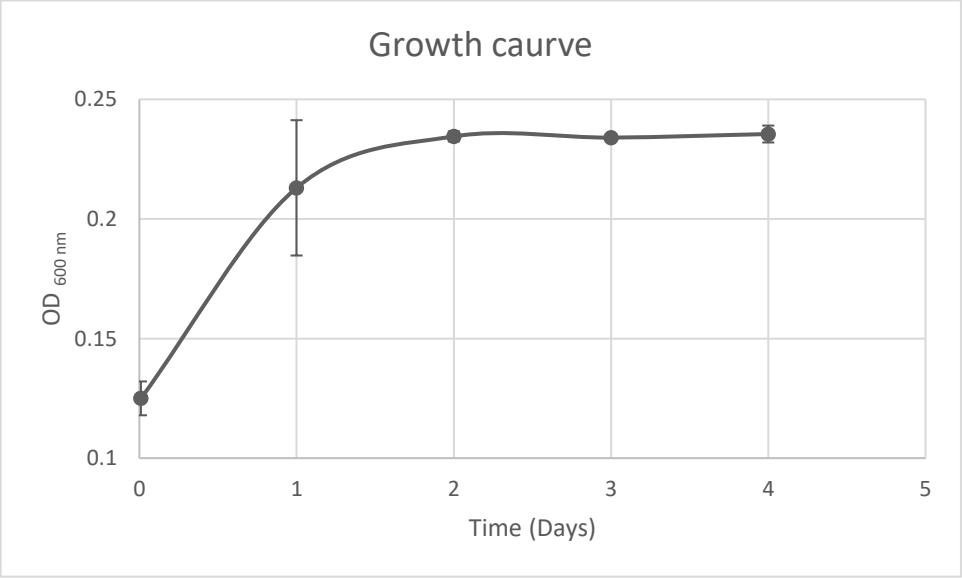

Figure 3-13: The growth curve of the *Thermosyntropha lypolitica.*

Chapter 4 Initial Anaerobic Digestion Experiments

4.1 Anaerobic digestion of mixed vegetable material in a stirred tank batch reactor.

The objective of this experiment was to identify the ideal organic concentration of vegetable feed that can work effectively for anaerobic digestion experimental purposes. This allowed the experimental design and equipment set-up to be verified to show its applicability. It also allowed any potential issues to be highlighted early on and rectified before conducting long term anaerobic digestion studies.

4.1.1 Experiment Design and Operation Conditions:

Experiment reactor: A stirred tank batch reactor consists of a 2L working volume quick-fit glass vessel, surrounded by a water jacket for temperature control, magnetic stirrer for mixing, continuous monitoring pH meter, and a flexible sampling tube connected to a syringe for sampling purposes. The reactor is connected to a gas collector (water displacement system) through a pipe to daily measure biogas production as well as obtaining gas samples for gas composition analysis (Figure 3-1). The experiment was run under conditions of 2L total working volume, 25g L^{-1} TS with >95% w/w VTS mixed vegetable feed and 35 ˚C mesophilic temperature condition.

4.1.2 Examination of the required time and flow rate for nitrogen flush process to provide anaerobe conditions for the AD experiment:

Aim: to measure the dissolved oxygen concentration after bubbling nitrogen gas through distilled water in a reactor for 5 min, 10L min^{-1} N_2 flow rate and 35 ˚C conditions.

Material and methods: 2L working volume quick-fit glass vessel, surrounded by water jacket to control the temperature at 35 ˚C, magnetic stirrer to provide a homogenisation of temperature and dissolved gasses content, gas flow meter and JENWAY Model 9150 dissolved oxygen meter. The dissolved oxygen meter was calibrated at room temperature and zero oxygen solution. The zero oxygen

solution was prepared according to the instrument manual by mixing 2 g of sodium sulphite anhydrous in 100 mL of distilled water and then left for few minutes to stand before being used.

Procedure: the glass reactor provided with a DO probe and one outlet one-way-valve was filled with 2L of distilled water and sealed. The reactor then left until the internal temperature increased to 35 ˚C. Then, nitrogen gas was bubbled into the reactor through the sampling pipe shown in Figure 3-1 with a flow rate of 10 L min^{-1} for 5 minute. After each minute, the nitrogen gas was turned off and the dissolved oxygen content were recorded after it had stabilised.

Results: as can be seen from Figure 4-1, bubbling nitrogen with flow rate of 10L min^{-1} for 5 minute decreases the dissolved oxygen content from 6.5 mg L^{-1} to 0.03 mg L^{-1} DO and thus provides suitable anaerobic conditions for AD bacteria.

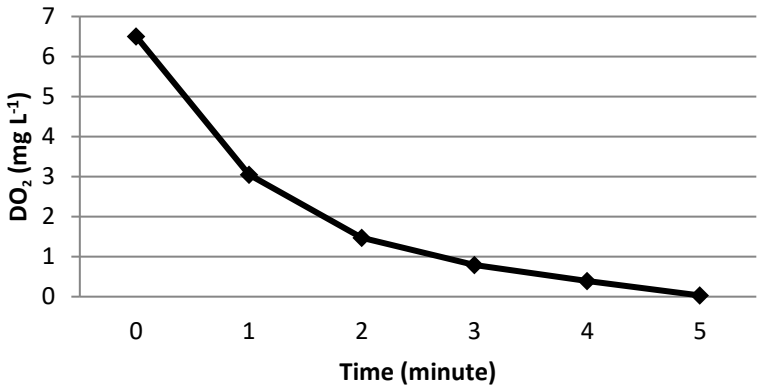

Figure 4-1: Dissolved oxygen concentration mg L^{-1} in distilled water during 5min of 10L min^{-1} flow rate nitrogen bubbled.

4.1.3 Testing the system against oxygen leaks:

The reactor was left running for two weeks and the dissolved oxygen concentrations were measured in a daily basis to test the system for any oxygen (air) leaks.

Results:

As can be seen from Figure 4-2, dissolved oxygen shows a slight decrease from 0.03 to -0.22 mg L^{-1} DO on day 1, this decrease being within the accuracy range of the DO meter.

The steady state of dissolved oxygen concentration inside the reactor (between days 2 to 14) indicates that there is no significant leak of air into the reactor vessel. Thus anaerobic conditions can be maintained successfully inside the reactor.

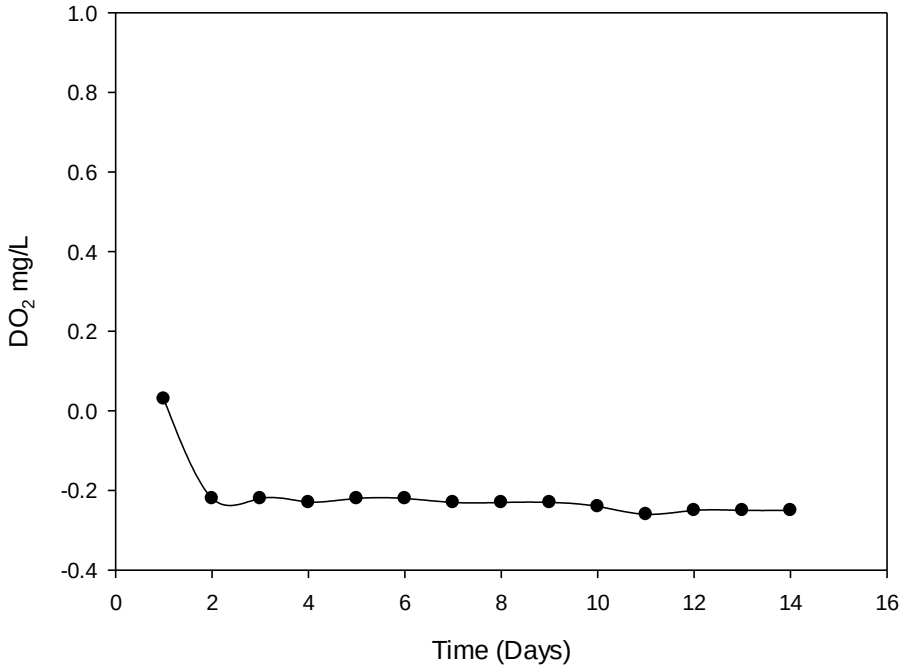

Figure 4-2: Dissolved oxygen concentrations in distilled water during 14 days oxygen leak experiment.

4.2 Start-up of anaerobic batch experiments with mixed vegetable feed:

In this experiment, blended vegetables (frozen mixed vegetables containing 40% carrots, 30% cauliflower, 30% w/w garden peas and broccoli) were used to start up a batch reactor with 25g L^{-1} TS vegetable mixture that contains >95% VS of TS weight. After the reactor vessel was filled with 25g L^{-1} TS vegetable mixture, the reactor pH was adjusted to 6.8 using 1M sodium hydroxide (NaOH). Nitrogen gas was bubbled through the reactor for 5 minutes at 10L min^{-1} through the flexible sampling tube to provide an oxygen free environment and to flush the reactor head space. After that, the reactor was seeded with 2.5% v/v of an anaerobic mixed culture inoculum (anaerobic sludge) obtained from a local anaerobic digestion plant with a VS content of 15 g L^{-1}. This experiment was run for 84 days and the reactor was re-fed three times on days 34, 36 and 67 with 20 mL, 20 mL and 200 mL of 20g L^{-1} TS vegetable feed, respectively. The selection of these feeding points was determined from the biogas production, composition and COD behaviour. These refeeding steps were performed after the decrease and low or no production of biogas, concentration of methane gas and the slow change in the COD.

4.2.1 **Results and Discussion:**

4.2.1.1 ***Carbon/Nitrogen Ratio and the Availability of Volatile Total Solids in the Feed.***

Organic elemental analysis of the prepared mixed vegetables, (the experiment feed substrate discussed earlier in chapter 3), shows a carbon/nitrogen (C/N) ratio in average of 15.62 which is below the optimum C/N ratios average (25 to 30) that has been reported by (Ward *et al.*, 2008). This was due to the higher nitrogen content in the prepared mixed vegetables substrate. On the other hand, results of volatile total solids analysis for the same mixed vegetable feed were always around ≥95% w/w, which support the findings of (Jiang *et al.*, 2012) of high availability of digestible organic material fruit and vegetable waste (volatile solids >95% of total solids).

4.2.1.2 ***Biogas Production, Composition, Reactor pH and COD Concentrations.***

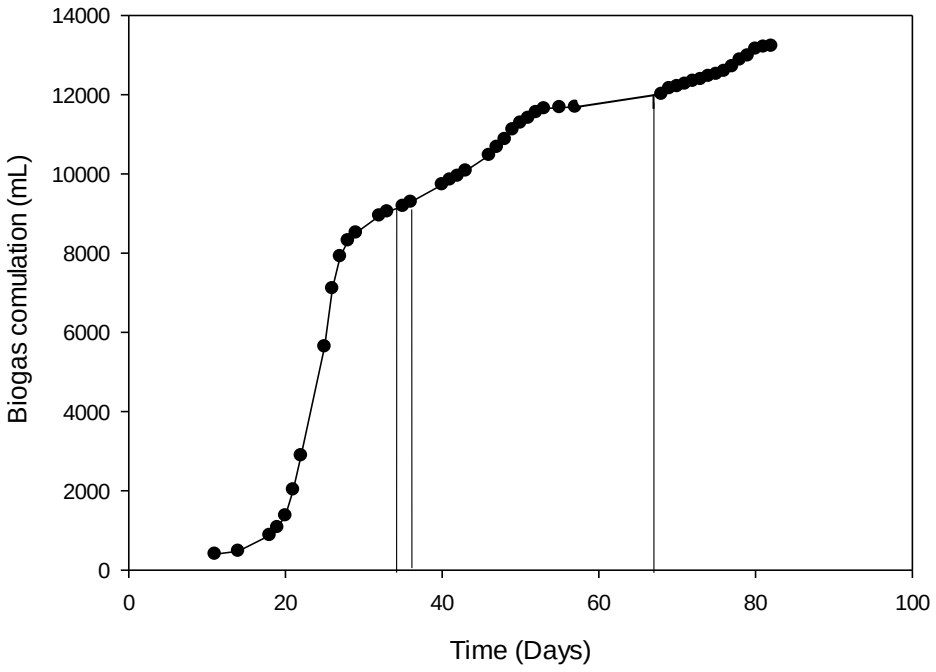

Figure 4-3: Cumulative Biogas produced from 2L stirred tank batch anaerobic reactor during 84 days

X axis represents the time duration in days; Y axis represents the biogas in millilitres. The three lines on days 34, 36 and 67 represent the re-feeding process with 20 mL on day 34, 20 mL on day 36 and 200 mL on day 67.

The production of biogas might have started earlier than the data show (Figure 4-3) as the amount of gas produced from day 1 to day 4 was unmeasured. On day 4 the 25 mL gas collecting cylinder was found to be completely empty of water and full of non-flammable gas, which meant that there was an unknown volume of gas (presumably CO_2) released to the atmosphere. As a result, the small collector cylinder was replaced with a 500 mL cylinder in order to avoid losing any gas and to obtain more accurate measurements. After only 24 hours (day 5) the new 500 mL collector was also found to be completely empty of water and this time full of highly flammable gas producing a noise when it burned, (later found to be hydrogen gas with concentration of ~16% v/v (Figure 4-4 and Figure 4-7). the 500 mL collector was found completely filled with gas again after only 5 hours on the same day. In addition, high gas production (tiny gas bubbles produced continually) was observed inside the reactor (Figure 4-5). On the following days of the experiment (day 6 and 7) the production of gas bubbles clearly decreased. However, on day 8, 200 mL of water (from water displacement cylinder) was found to have transferred into the reactor.

The reason of this found to be possibly results of using NaOH as a pH buffering solution, which leads to a chemical reaction between NaOH and CO_2 gas inside the digester environment to create sodium carbonate Na_2CO_3 (Equation 4-1). This causes a vacuum effect inside the reactor (low pressure) as a result of CO_2 absorption which leads to water suction. In order to return to the original reactor volume and the reactor pressure balance without introducing oxygen or air to the experiment, 230 mL of the reactor content was removed under a nitrogen flush and 30 mL of fresh feed substrate (25g L^{-1} TS) was introduced into the reactor. The reactor pH was readjusted to 7 with 1M NaOH.

$$2NaOH\ (aq) + CO_2\ (g) = Na_2CO_3\ (aq) + H_2O\ (l) \qquad \textit{(Equation 4-1)}$$

Figure 4-4: Biogas flammability test on day 5 of the experiment.

Figure 4-5: Tiny biogas bubbles continually produced inside the glass reactor on day 5 of the experiment.

The batch system recovered after three days (day 11) as 400 mL of non-flammable gas (N_2, CO_2 and O_2 (Figure 4-7)) accumulated in the gas collector over 3 days. Biogas then started to be produced (~20-30 mL day^{-1}) till day 15 where the reactor content colour changed from brown to nearly black and was found to be generating 400 mL of biogas with 29.2% v/v methane on day 18. Both methane gas percentage and biogas production started to increase from day 18 Figure 4-3 and Figure 4-7). A flammability test on day 19 gas sample showed a positive result with a quiet flame of >50% methane v/v (Figure 4-6 and Figure 4-7).

Figure 4-6: Biogas flammability test on Day 19, biogas with >50% v/v methane.

The rapid increase in biogas production between days 18 and 28 (Figure 4-3), the continuous increase of methane concentrations in the biogas (Figure 4-7), the rapid increase methane gas yield (Figure 4-8), a sharp decrease in dissolved COD (Figure 4-9), and an increase in pH (Figure 4-10) all indicate a rapid consumption of nutrients by the anaerobic microorganisms and an increase in its activity. The pH increased during this period presumably as a result of methanogenic microorganisms' activities consuming VFAs and lowering the reactor acid content.

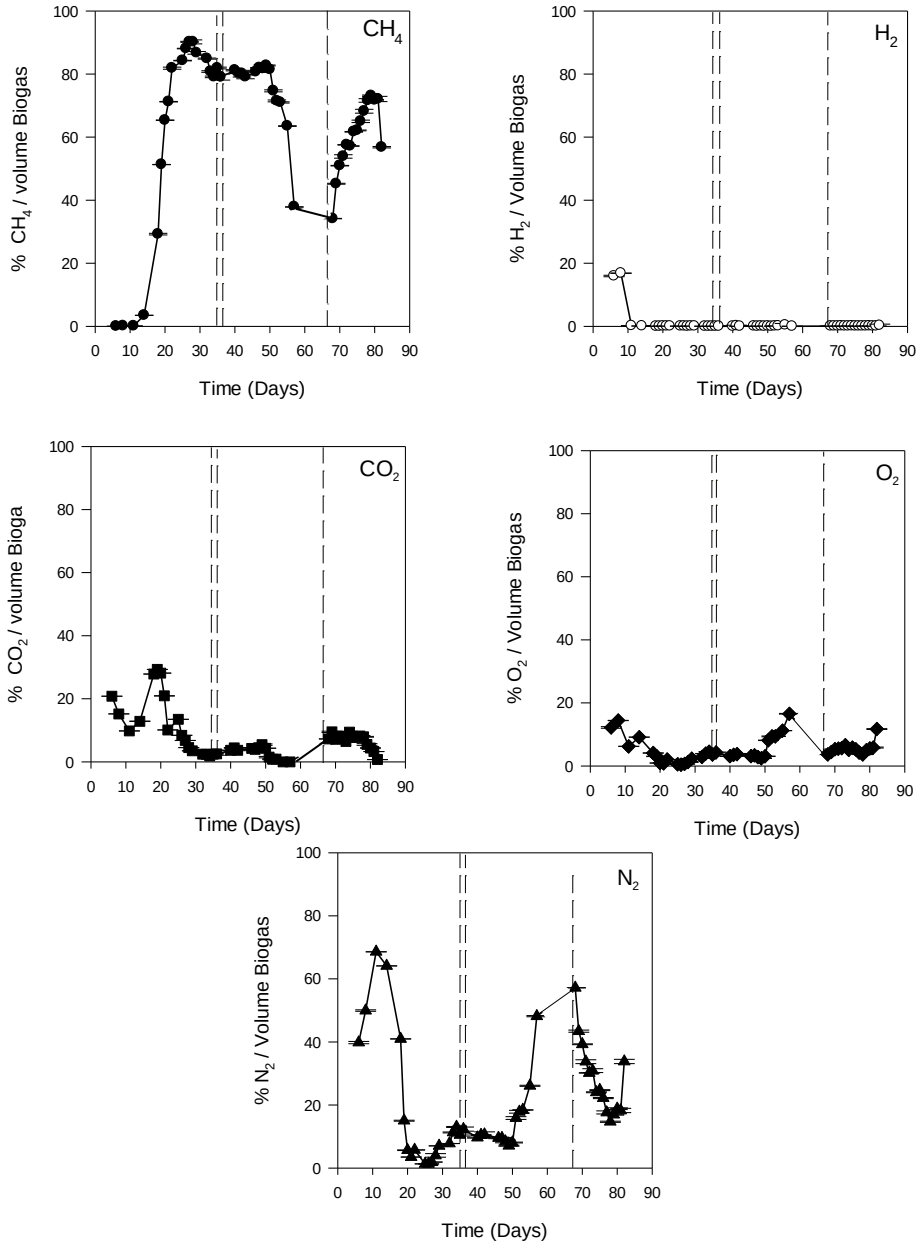

Figure 4-7: Biogas compositions from 2L stirred tank batch anaerobic reactor during 84 days

X axis represents the time duration in days; Y axis represents the biogas composition. The three dashed lines point to days 34, 36 and 67 and represent the re-feeding process with 20 mL on day 34, 20 mL on day 36 and 200 mL on day 67. (Error bars represent standard deviation for three separate measurements taken from the gas sample.)

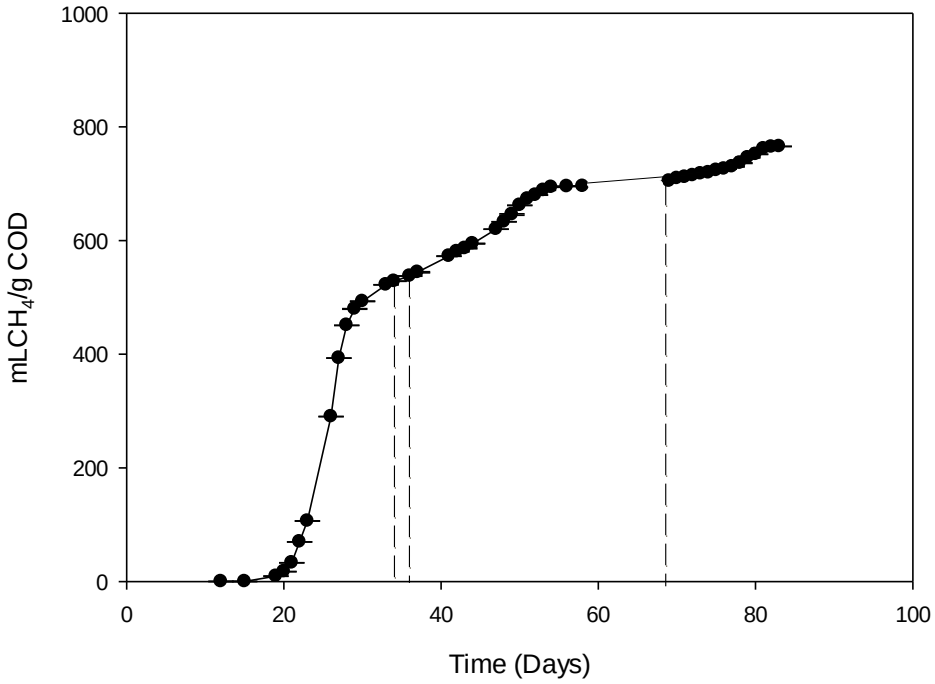

Figure 4-8: Methane gas yield during 84 days anaerobic batch reactor experiment on of vegetable mixture.

X axis represents the time duration in days; Y axis represents the methane gas yield in millilitres. The three dashed lines point days 34, 36 and 67 and represent the re-feeding process with 20 mL on day 34, 20 mL on day 36 and 200 mL on day 67. (Error bars represent standard deviation for three separate measurements taken from the gas sample.)

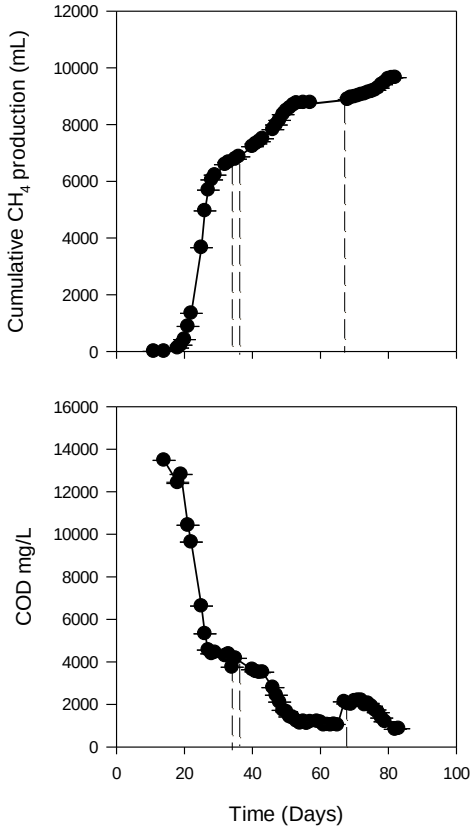

Figure 4-9: D-COD reduction during AD of vegetables mixture in a stirred tank batch reactor experiment.

X axis represents the time duration in days; Y axis represents the D-COD production in milligram per litre. The three dashed lines point days 36, 34 and 67 indicating the re-feeding process with 20 mL on day 31, 20 mL on day 34 and 200 mL on day 67. (Error bars represent standard deviation for three separate measurements taken from the gas sample.)

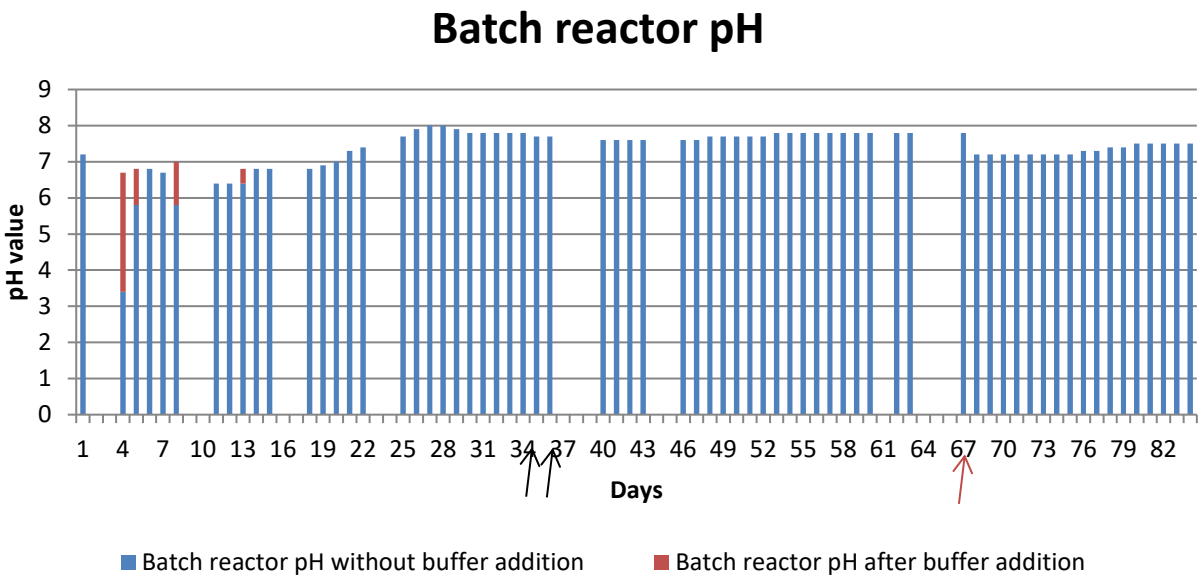

Figure 4-10: pH variations during 84 days of anaerobic batch reactor experiment on vegetable mixture.

Figure 4-10 shows the changes in pH values during the experiment time. X axis represents the time duration in days; Y axis represents the pH value. The blue colour in this graph represents the pH value either without or before buffering process, whereas the red colour represents the pH value after buffering addition. The three arrows point the days 34, 36 and 67 indicating the re-feeding process with 20 mL on day 34, 20 mL on day 36 and 200 mL on day 67.

An interesting point is that the methane percentages in the biogas remain high even when the biogas production decreases to a very low level. This indicates a high population of active methanogenic microorganisms in the culture. However, between day 50 to 57, after the methane gas reaches its highest concentration and starts to decrease slowly, the nitrogen percentage starts increase as shown in Figure 4-7). Decreasing methane concentration with a concomitant nitrogen increase is a possible sign of the ammonia accumulation and a presumable activity of the anaerobic Anammox bacteria that convert nitrate and ammonium from protein degradation into nitrogen gas and water. This can explain the relation between pH and nitrogen gas production from day 46 to 57, where both pH and nitrogen gas increased (pH from 7.6 to 7.8) (Figure 4-7 and Figure 4-10). Another possible reason for the increase in nitrogen concentration might be a hidden leak of atmospheric air into the reactor head space.

4.2.1.3 *Re-feeding Effects on Batch Reactor Experiment, Before and After the Reactor Becomes Exhausted.*

Data shown in Figure 4-3, Figure 4-8, Figure 4-9, and Figure 4-10 give information about the effect of re-feeding before and after the digester becomes exhausted. These graphs show variations in biogas production, composition, COD concentration and the pH values before and after the three black dashed lines that mark re-feeding with 20 mL on day 34, 20 mL on day 36 and 200 mL on day 67.

4.2.1.3.1 Before The Reactor is Exhausted:

Re-feeding the reactor on days 34 and 36 with a small amount of vegetable substrate (20 mL of 20g L^{-1} TS) each day, results in small increase in biogas production during the following 15 days. However, biogas production started to decrease again after day 49 and completely stopped after day 57, which is considered a sign of digester exhaustion. Re-feeding clearly delays any decrease in methane percentage. Methane concentration in the biogas had started to decrease after day 28 (before re-feeding), however, after re-feeding on days 34 and 36, methane concentrations fluctuated between (79-82% v/v) for the following 15 days before decreasing again after day 49.

The effect of re-feeding on reactor COD concentration was small and only a minor increase was observed before the COD continued decreasing. A slight decrease occurred in the pH value from 7.8 to 7.6 after the re-feeding step, expected as feeding the reactor with fresh organic feed would stimulate the acidogenesis process to produce VFAs and lower the pH (Zaher *et al.*, 2007).

4.2.1.3.2 After The Reactor Becomes Exhausted:

On day 57, gas production had completely stopped and the reactor remained producing no gas for 10 days (Figure 4-3) before a re-feeding step with 200 mL of 20 g L^{-1} TS was applied. COD concentrations showed no obvious change during those 10 days and the pH values were 7.8.

After re-feeding on day 67, biogas production started from the following day with a volume of 326 mL. Analysis showed a high (57% v/v) percentage of nitrogen

with 34% v/v methane. Biogas production then fluctuated till it stopped again on day 83. However, the percentage of methane gas continuously increased and nitrogen gas continuously decreased over this time (Figure 4-7). After day 79, the methane gas concentration started decreasing again and nitrogen gas started increasing till the production of biogas stopped at day 83.

COD concentration increased directly after this re-feeding step from 1025 to 2122 mg L^{-1}. However, COD concentrations showed a slight fluctuation before it declined again to 860 mg L^{-1} at day 84. The pH values dropped from 7.8 to 7.2 after raw feed addition and remained without any changes for seven days. At day 76, the pH value started to increase slightly, reaching 7.5 before the end of the experiment.

4.2.2 **Conclusion of the initial trails:**

Most of the initial experimental objectives were achieved and a good experience in lab scale AD reactor set-up and operation gained.

The highest methane yield for the batch experiment occurred between days 22-27. The highest concentration of methane in the biogas was 90% v/v at day 28 in the experiment.

During the operation and monitoring a difficulty in manually controlling the pH to a steady state was observed, especially during the first few days of the experiments. Daily monitoring and controlling of the pH did not provide a fixed pH value. Therefore, it was decided to use an automatic pH control for future pH controlled experiments.

Chapter 5 Co-Digestion of bovine fat and vegetables waste.

The effect of bovine fat concentration and I/S ratio on the digestion process

5.1 Objectives:

The experiments conducted in this chapter had two objectives;

- Firstly, to investigate the effect of varying the concentrations of fat contributing to 20 g L^{-1} total volatile solid (VS) in co-digestion with vegetable waste, further elaborating on the initial findings from the previous chapter of vegetable waste solely to see the effect of fat being added to the feed.
- Secondly, to examine the effect of using a low inoculum to substrate ratios (I/S below 1) at high fat concentrations (75% and 100% w/w) in the 20g L^{-1} VS feed condition on the overall digestion process and biogas production.

5.2 Experiment design and operation conditions:

Serum bottles each with a total volume of 120 mL were used as mini digesters in a comparison co-digestion study for different fat concentrations in vegetable waste. Figure 5-1 shows the approach taken for the experimental work, which was separated into two pathways. The first focused on the effect of different fat concentrations at the optimum I/S ratio of 1, while the second was to study the effect of lower I/S ratios (below 1), at high fat conditions (75% and 100% w/w) at the 20g L^{-1} VS feed condition.

Each pathway consisted of a set of 18 serum bottles. These were placed in a clear plastic water bath equipped with a submersible heater and a water pump to circulate the water and create a uniform temperature throughout the water bath. Gas production was measured and sampled using plastic syringes, which were lightly lubricated with silicon grease and tested as described in the materials and methods chapter.

The temperature condition for both pathways was mesophilic. The temperature was set to 35±1°C. The feed condition was set at 20g L^{-1} total VS for all the batch digesters. Bovine fat powder (BFP) and vegetable waste were used as a

feed substrate, with different percentage of BFP making up part of the 20g L^{-1} total VS. The percentages of fat in the first pathway were 0%, 10%, 25%, 50%, 75%, and 100% w/w with the remainder made up of vegetable waste. The seeding sludge was incubated at 35°C, until degassed before being used to inoculate the first pathway of the experiment with an I/S ratio of 1 in terms of the VS content. The fat conditions used in the second pathway were 75% and 100% w/w of the 20 g L^{-1} total VS feed condition. The I/S ratio conditions in the second pathway were 0.5, 0.25, and 0.1 in terms of the total VS (Figure 5-1).

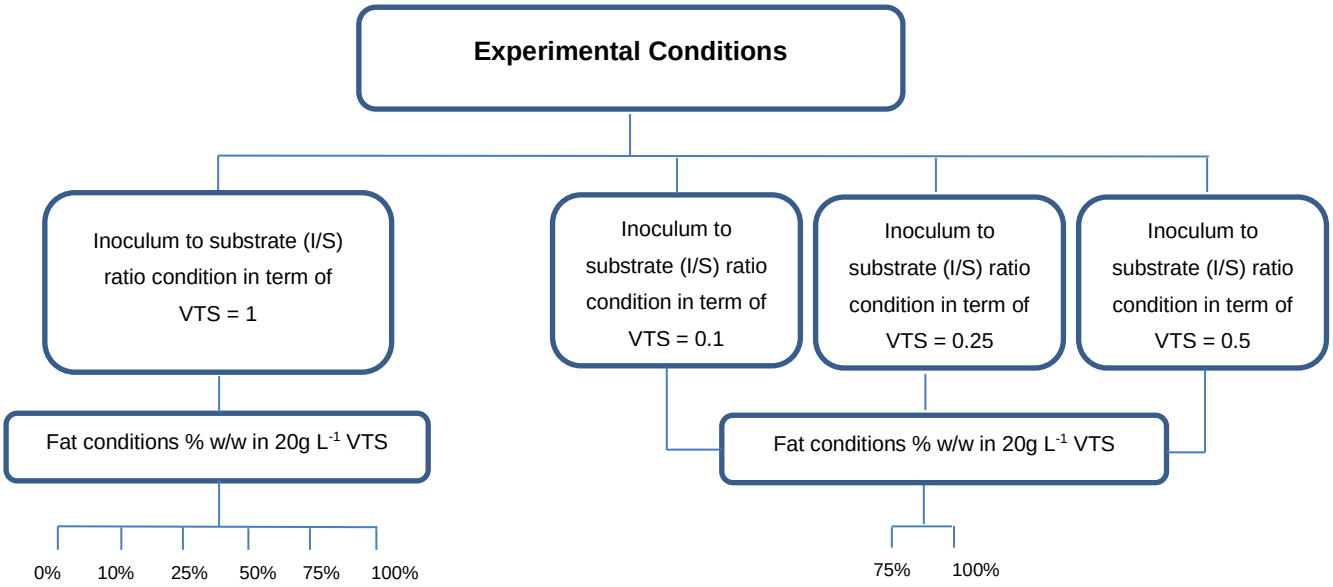

Figure 5-1: Experimental conditions for the 120 mL total volume serum bottle digesters to study both the effect of fat concentrations in the digester feed and the effect of low I/S ratios at the digester performance.

The total solids and volatile solids content of all of the components (degassed seeding sludge, fat and pre-prepared vegetable waste) were mesured to calculate the required amount of each individual substrate in the experimental feed. The required amount of each substrate was transferred into labled serum bottle digesters to obtain 20 g L^{-1} TVS with 0%, 10%, 25%, 50%, 75%, and 100% w/w bovine fat. The volume of seeding sludge required to obtain the desired I/S ratio (for example: I/S ratio of 1 in photo (b) Figure 5-2) was transfered under nitrogen flush and the reactors were immediately sealed. The head spaces were flushed with nitrogen gas for 30 minutes with manual shaking every ~5 minutes to insure the required anaerobic conditions (Figure 5-2). The digesters were placed in the 35°C water bath and mixing was achieved by shaking the bottles once per day.

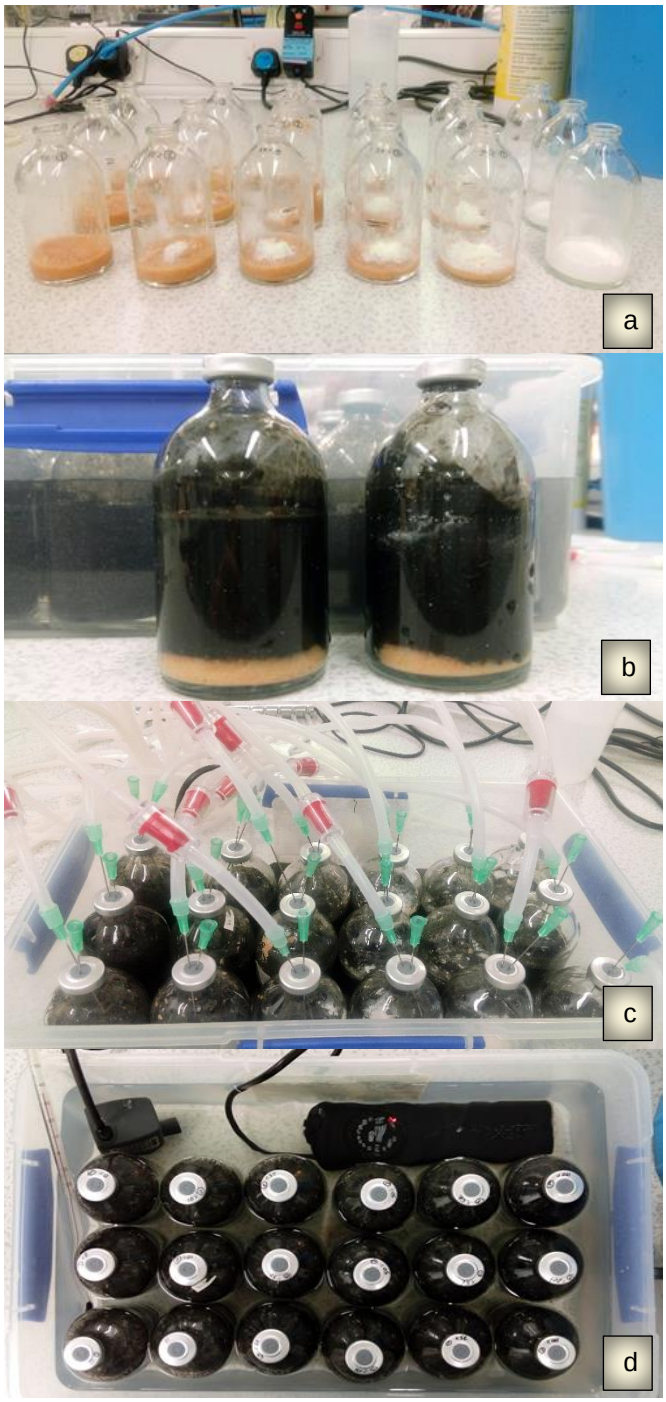

Figure 5-2: Setting up the first set of the 18 serum bottle digesters experiment.

a: Filling the serum bottle reactors with the required feed substrate (20g L^{-1} total VS with 0%, 10%, 25%, 50%, 75%, and 100% w/w of fat in co-digestion with vegetable waste).
b: Transferring the required volume of seeding sludge to obtain the desired I/S ratio (I/S ratio of 1 in this photo). This was done under nitrogen flush and followed by sealing the reactors.
c: Flushing the reactors' head space with nitrogen gas for 30 minutes with manual shaking every 5 minutes to ensure the required anaerobic condition for this experiment.
d: Placing the digesters in the 35°C water bath. Mixing was by shaking the bottles once every day.

5.3 Results and discussion:

5.3.1 Carbon to nitrogen ratio:

Table 5-1 illustrates the results of the amount and ratios of carbon and nitrogen for bovine fat, vegetable waste and anaerobic seeding sludge. The calculated C/N ratio for the bovine fat was the highest at 741.84 compared to 15.62 and 6.35 for vegetable waste and seeding sludge respectively.

In general, most of the literature considers the seeding sludge as (only) an inoculum source, to provide the required anaerobic microorganisms for the digestion process. Therefore, carbon and nitrogen in the seeding sludge are often not involved in their calculation of C/N ratio. Nurliyana *et al.* (2015), as an example, focused on the effect of C/N ratio on the biodegradability and methane productivity in a co-digestion study of empty fruit bunch and palm oil mill effluent. However, the carbon and nitrogen in the seeding sludge were not measured nor mentioned or included in any calculation of C/N ratio. Another example is that of Wang *et al.* (2012), the carbon and nitrogen content of the seeding sludge was not measured or included in any C/N ratio calculation during the study of optimising the C/N ratio, even though a high I/S ratio of 2 (two time higher than the highest I/S ratio used in this research) was used and one third of the experimental reactors volume was occupied with inoculum seeding sludge.

In fact, the anaerobic seeding sludge not only contains a mixed inoculum culture, but also residues of organic and inorganic materials that might be involved directly or indirectly in the digestion process. Since the anaerobic seeding sludge is naturally low in volatile solid content, controlling the I/S ratio to the recommended optimum value of 1 is very difficult without adding a large volume of seeding sludge to a small volume of feed substrate (normally high in VS) to balance the VS contents between them, a and b in Figure 5-2 as an example. Therefore, if the seeding sludge was high in ammonia nitrogen for example, then larger volume of seeding sludge will lead to relatively higher nitrogen content among the reactor's substrates.

The effect of taking the seeding sludge's carbon and nitrogen contents into account on the C/N ratio of the reactor mixture is further discussed in the following subsections.

Table 5-1 Carbon and nitrogen content and C/N ratios for the main feeding substrates (fat and vegetable waste) and the seeding sludge.

Material	C% w	N% w	C/N	*SD*
Fat powder	69.43	0.093	741.84	0.93
Vegetables waste	41.10	2.63	15.62	0.90
Anaerobic seeding sludge	28.14	4.42	6.35	0.14

5.3.1.1 *C/N ratios of the first experimental pathway conditions:*

In these experiments the amount of added seeding sludge for an I/S ratio of 1 is the same for all the reactors. However, the difference in these experiments was in the proportions of fat and vegetable waste making up the total 20g L^{-1} VS feed condition.

As can be seen from Table 5-2, the values of C/N ratio decrease when the carbon and nitrogen content of the seeding sludge is included in the calculations, this is due to the higher nitrogen content in the seeding sludge compared to the fat and the vegetable waste.

The effect of the decreasing value of C/N ratio becomes clearer at higher proportions of fat, as the fat is very low in nitrogen while the seeding sludge is relatively high in nitrogen. For example, with no fat content the C/N ratio is 37.14% w/w lower when including the seeding sludge than the C/N ration of the vegetable waste substrate. At higher fat concentrations, the decrease in C/N ratio when including the seeding sludge in the calculation is 42.76%, 51.08%, 65.36%, and 81.19%, for 10%, 25%, 50% and 75% fat respectively. The greatest effect was at a fat concentration of 100%, with a decrease of 97.02% in the C/N ratio, when the seeding sludge is included in the calculation.

Table 5-2 Comparison between the C/N ratios of the first experimental pathway reactor's mixture, with and without taking the carbon and nitrogen content of the anaerobic seeding sludge into account during the calculation.

1st Pathway serum bottles experiment	Excluding sludge	Including sludge
Fat Condition (%) w/w of 20g L^{-1} VS	C/N	C/N
0%	15.59	9.80
10%	18.52	10.60
25%	24.37	11.92
50%	41.92	14.52
75%	94.59	17.79
100%	741.84	22.04

5.3.1.2 *C/N ratios of the second experimental pathway conditions:*

In this experiment, the amount of added seeding sludge was variable, in order to get the required experimental condition of I/S ratio of 0.5, 0.25 and 0.1. The fat was either 75% or 100% w/w of the total 20g L^{-1} VS feed condition.

As can be seen from (Table 5-3), a similar effect of decreasing the C/N ratio occurred when the carbon and nitrogen contents of the seeding sludge were taken into account during the calculations. This is due to the lower amount of seeding sludge introduced to the reactor, and therefore, lower nitrogen content.

The reduction to the original C/N ratio (i.e. the ratio without taking seeding sludge into account) after including the seeding sludge in the calculation is still considerable, even at a low I/S ratio (i.e. lower amount of seeding sludge to feed content). The greatest reductions occur at 100% w/w fat with 94.91%, 90.68%, and 76.71% reduction in the C/N ratio for the feed alone at I/S ratios of 0.5, 0.25, and 0.1 respectively when the seeding sludge is included. For 75% fat w/w, the reduction in the C/N ratio from the original values were 71.87%, 58.46%, and 37.47% for I/S ratios of 0.5, 0.25, and 0.1 respectively.

Table 5-3 Comparison between the C/N ratios of the second experimental pathway reactor's mixture, with and without taking the carbon and nitrogen content of the anaerobic seeding sludge into account during the calculation.

2nd Pathway serum bottles experiment	condition	Excluding sludge	Including sludge
Fat Condition (%) w/w of 20g L^{-1} VS	I/S ratio	C/N	C/N
75%	0.5	94.59	26.60
	0.25		39.29
	0.1		59.14
100%	0.5	741.84	37.73
	0.25		69.10
	0.1		172.75

5.3.2 Theoretical methane production

5.3.2.1 *Theoretical estimation of the methane in the biogas:*

The Buswell equation was formulated in 1952 based on an assumption of complete conversion of the organic substrate into two simple gases, methane and carbon dioxide (Buswell and Mueller, 1952). The equation was developed to calculate the theoretical methane composition based on the organic elemental composition of the reactor's feed or substrate (Equation *5-1*).

$$C_cH_hO_o + \left(c - \frac{h}{4} - \frac{o}{2}\right)H_2O \rightarrow \left(\frac{c}{2} + \frac{h}{8} - \frac{o}{4}\right)CH_4 + \left(\frac{c}{2} - \frac{h}{8} + \frac{o}{4}\right)CO_2 \qquad \textit{(Equation 5-1)}$$

A theoretical estimation of the biogas composition was calculated using Equation 5-1 for the feed substrates used in these experiments. The calculation was carried out using the results of the organic elemental analyser for the experimental feed substrates (i.e. the bovine fat powder and the vegetable waste).

As can be seen from Figure 5-3, the calculated methane generated from full conversion of fat and vegetable waste to biogas show higher amounts of methane from fat. The biogas generated from fat will contain 68% CH_4 and 32% CO_2. Whereas in the case of vegetable waste, the theoretical biogas composition was 49% CH_4 and 51% CO_2 v/v.

These theoretical results agree with reports in the literature (Cirne *et al.*, 2007, Appels *et al.*, 2011, O-Thong *et al.*, 2012) that lipids and fat materials can generate more methane than other waste substrates.

Nonetheless, the Buswell equation does not take into account any possible subsequent methane production and carbon dioxide consumption through the hydrogenotrophic methanogenesis pathway, where methane can be produced from CO_2 reduction by H_2 (Equation *5-2*).

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \qquad \textit{(Equation 5-2)}$$

Experimental results can normally show higher methane percentages in the biogas composition than the maximum theoretical estimation by the Buswell equation. The reason behind this is that carbon dioxide can be re-dissolved into the liquid phase and become involved in other reactions inside the reactor. For example, it can be reduced by hydrogenotrophic methanogenesis to produce more methane, as well as being involved in several possible side chemical reactions in the liquid phase.

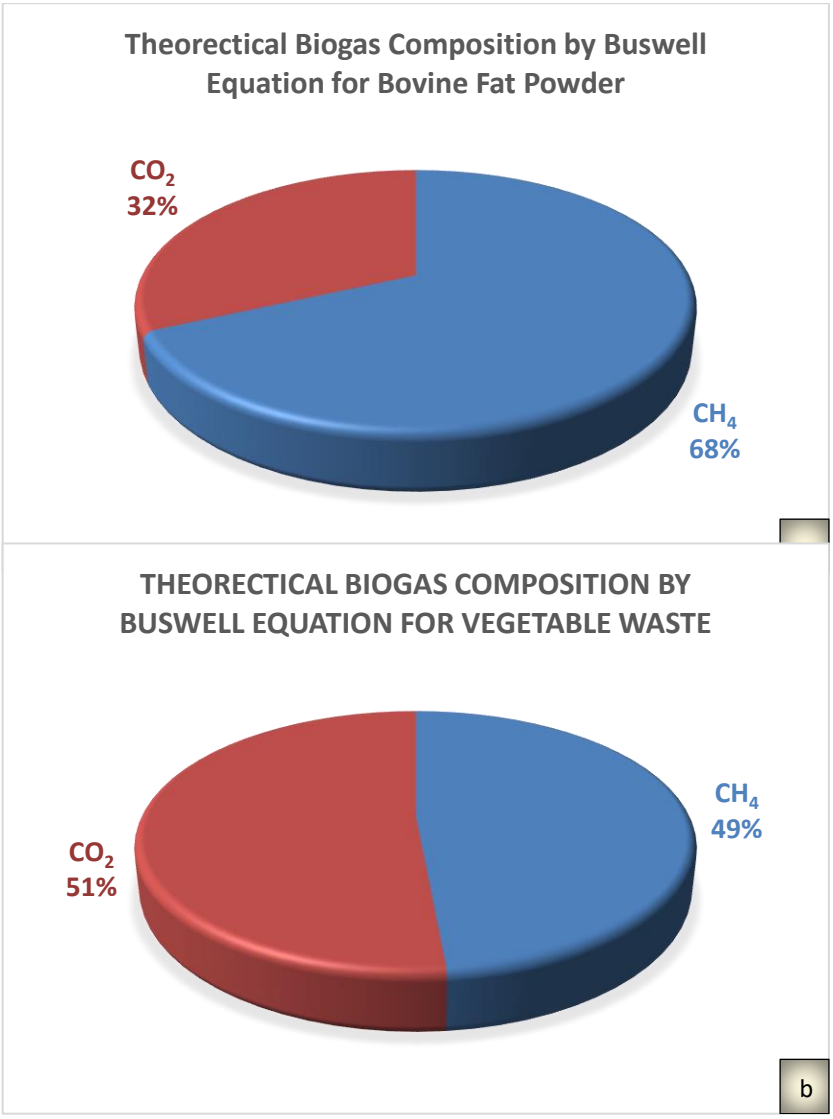

Figure 5-3: Theoretical Biogas composition using the Buswell equation, according to the experimental results of the organic elemental analyser of carbon, hydrogen, oxygen contents in the feed substrate.

a: is the estimated CH4 and CO_2 generated from fat feed substrate.
B: is the estimated CH4 and CO_2 generated from vegetable waste feed substrate.

5.3.2.2 *Maximum theoretical methane productions through the total D-COD consumption:*

5.3.2.2.1 First pathway:

Theoretical methane production was calculated using the value of removed D-COD from the 18 serum bottle reactors after 41 days' digestion. As can be seen from Figure 5-4, theoretical methane production shows a consistent increase related to increasing the fat concentration from 10% to 100% w/w.

The triplicated reactors running at 0% fat showed slightly higher theoretical methane production than the 10% fat ones. This might be as a result of the higher initial D-COD measured for the 0% fat (100% vegetable w/w) than the 10% fat ones. Another possible reason could be the faster biodegradability of vegetable waste, (and therefore higher consumption rate), compared to the 10% fat reactors.

The results presented in Figure 5-4, are only possible when 100% of the removed COD is converted to CH_4. Therefore, as these calculated results have ignored other possible conversions to materials such as CO_2 and biomass, it is normal to get higher estimated values than those that can be obtained experimentally.

The highest estimated methane production was for 100% w/w fat with just over 18 litre of methane. This might be due to the highest initial D-COD and the highest reduction value under this condition. Initial D-COD removal efficiency will be discussed further in the COD section of this chapter.

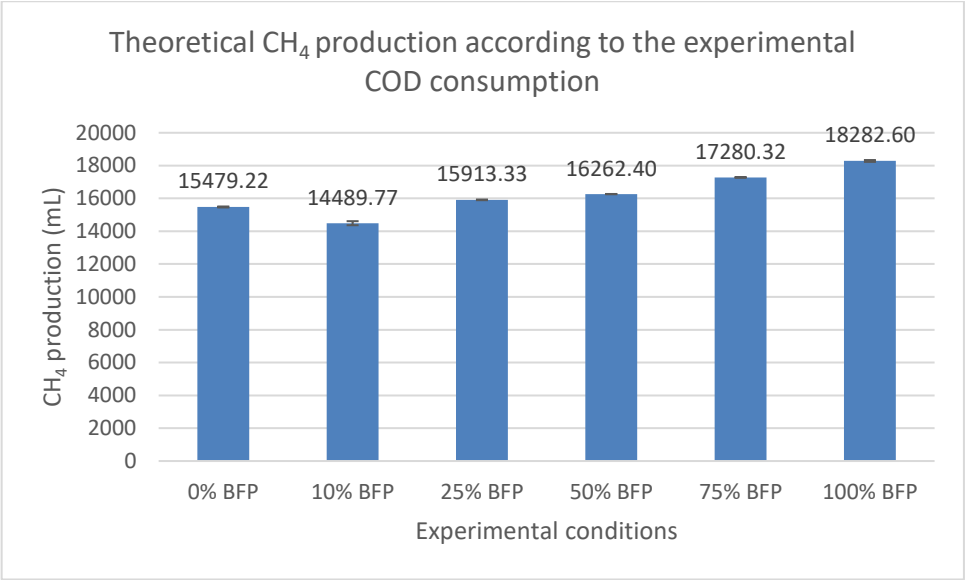

Figure 5-4: Theoretical methane production from total D-COD consumption under different fat concentrations (0%, 10%, 25%, 50%, 75%, and 100% w/w) in the 20g L^{-1} TVS feed. (Error bars represent standard deviation of samples from three replicate reactors.)

5.3.2.2.2 Second pathway:

Inoculum to substrate (I/S) ratio shows a high impact on the total reduction of D-COD which is reflected in the theoretical estimation of the CH_4 from D-COD consumption.

Overall, a higher percentage of fat in the reactor's feed leads to higher theoretical CH_4 production. As can be seen from Figure 5-5, reactors with 100% fat show higher estimated theoretical CH_4 production than for 75% fat. The reason seems to be due to higher initial D-COD values and a higher amount of removed D-COD for 100% w/w fat when compared to 75% w/w fat.

The effect of the I/S ratio can be clearly seen from Figure 5-5. A lower I/S ratio means less bacterial culture provided to the reactors as an inoculum, which ends up with poor degradation and lower reduction in the D-COD values. This is reflected in the estimated CH_4 production calculated from the removed D-COD value.

The results of the first and second pathways (Figure 5-4 and Figure 5-5) show that, under conditions of 75% and 100% fat, both I/S ratio conditions of 1 and 0.5 give similar results with slightly higher values for the 0.5 I/S ratio.

The I/S ratio of 0.5 gives slightly higher theoretical methane production compared to the I/S ratio of 1 as the reduction in D-COD was higher, possibly this was due to the longer experimental time of 250 days for the lower I/S ratio experiment (second experimental pathway) with I/S ratio of 0.5, 0.25, and 0.1 compared to only 41 days for the I/S ratio of 1 (first experimental pathway). The longer experimental run allows bacteria more time to break down the organic materials and therefore end up with lower D-COD values at the minimum sufficient I/S ratio of 0.5.

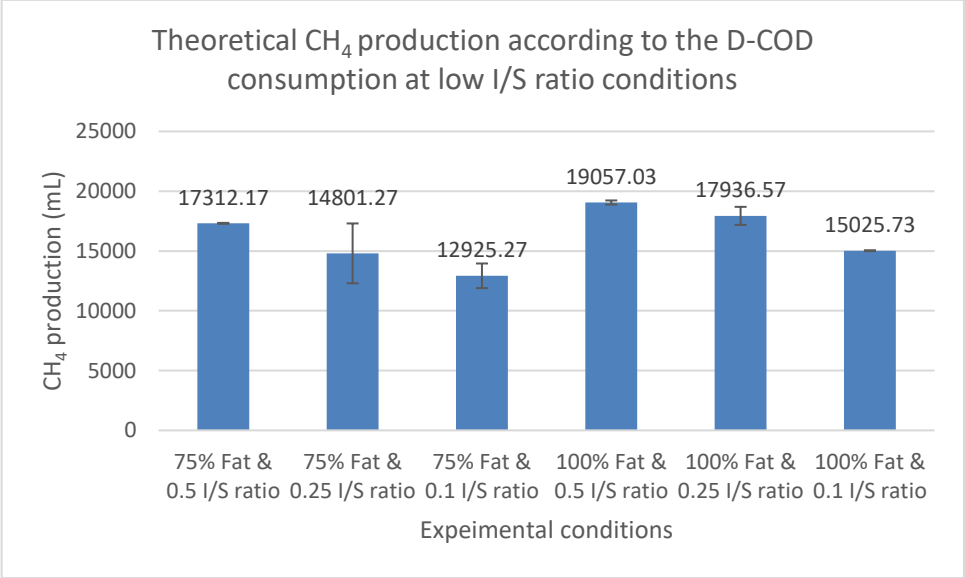

Figure 5-5: Theoretical methane production calculated from the consumption of total D-COD after 250 days experimental run. The results are for high concentrations of fat (75% and 100% w/w) among the 20g L^{-1} TVS at lower I/S ratio of 0.5, 0.25 and 0.1. (Error bars represent standard deviation of samples from three replicate reactors.)

5.3.3 **Biogas production:**

At 20g L^{-1} total volatile solids, the experimental results from both experimental pathways show a direct influence of fat on the biogas production times, compositions and total methane gas produced.

Overall, under I/S ratios ≥ 0.5, higher concentrations of fat lead to higher total methane production but a later start of active methane production.

5.3.3.1 ***Biogas from the first experimental pathway:***

In the first pathway, where different concentrations of fat were studied under a fixed I/S ratio of 1, there is a clear variation in the methane production during the first five days as illustrated in Figure 5-6. Reactors with fat concentrations of 0%, 10% and 25% w/w show higher initial methane production (35.5 mL, 35.12 mL and 36.5 mL) respectively compared to the reactors with higher fat concentrations of 50%, 75% and 100% w/w (19.6 mL, 14.8 mL, and 8.28 mL) respectively. The possible reasons for this variation at the beginning of the experiment could be the availability of simple and fast degradable materials in the vegetable waste substrate compared to the fat. More vegetable waste in the co-digestion mixture means a higher proportion of simple consumable materials that can be easily used by the anaerobic bacteria to generate methane in the early days of the experiment. This can be combined with the inhibitory effect of fat on the anaerobic bacteria, as well as the expected faster adaptability of the bacteria to vegetable waste than to the fat.

The decrease in methane production during the first five days may be due to a combination of the following. First, is the lower availability of simple organic materials which have been consumed by earlier bacterial activity. Second, the fast growth and activity of hydrolytic and acidogenic bacteria (acid producers) which allow them to take over control during the early days of the digestion and drop the pH value inside the reactors causing a shock effect thus lowering methanogenic microorganisms growth. Third, the production of LCFAs during the hydrolysis process of fat could cause an inhibition, slowing down some bacterial activity and growth and therefore lowering methane production.

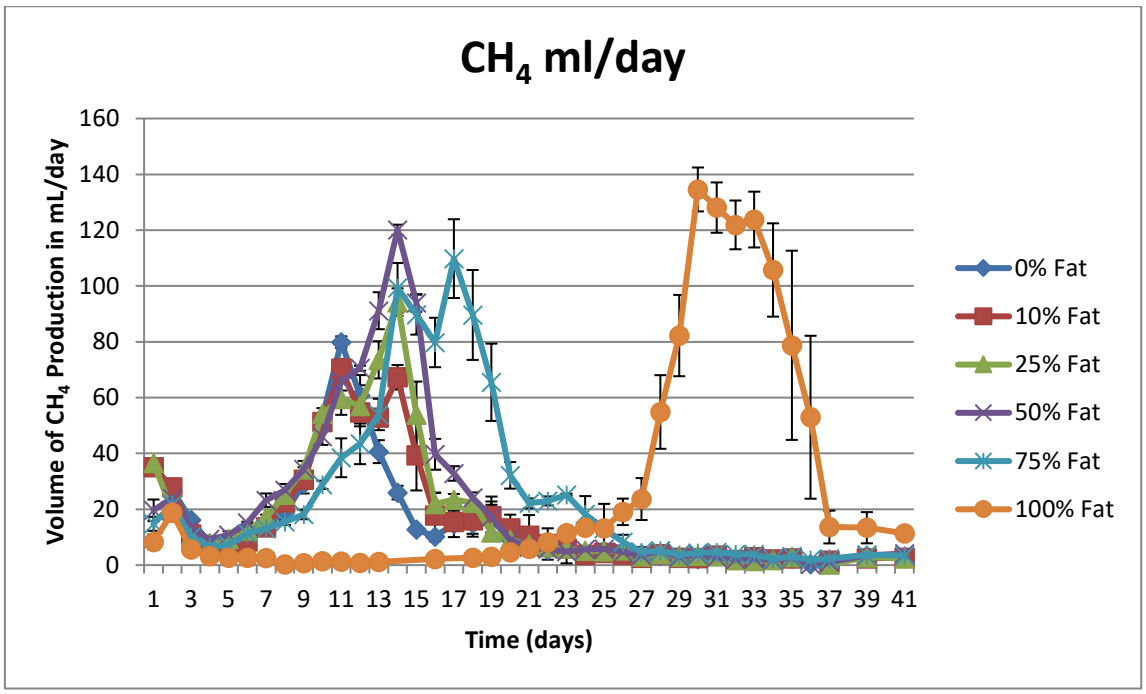

Figure 5-6: Daily methane production from 60 mL working volume serum bottles digesters at different fat concentrations (0%, 10%, 25%, 50%, 75%, and 100% w/w) of 20g L^{-1} total VS in a co-digestion with vegetable waste. (Error bars represent standard deviation of samples from three replicate reactors.)

As can be seen in Figure 5-6, the production of methane starts after day 5 in most of the reactors except for 100% w/w fat. The availability of simple organic materials produced through the previous activity of hydrolytic and acidogenic bacteria might lead to an enhancement for both acetogenic and methanogenic microorganisms, which is shown by the increase of methane production after day 5.

The influence of fat concentration in the digester feed can be clearly seen on both the lag phase length and the period of time where highest peaks of the methane production appear.

From Figure 5-6 and Figure 5-7, it can be seen that the lag phase is extended when higher concentrations of fat are used, especially at 100% w/w fat. Similar results of extended lag phase are reported by Hanaki *et al.* (1981) in a study of the inhibitory mechanism of LCFAs during the anaerobic digestion process.

Biogas production peaks are also delayed with higher concentrations of fat in the reactor's feed. The highest peaks of bio-methane production were detected on day 11 for 0% and 10% fat, and on day 14 for 25% and 50%fat. For 75% and

100% fat the highest productions of biogas were detected on days 17 and 30 respectively.

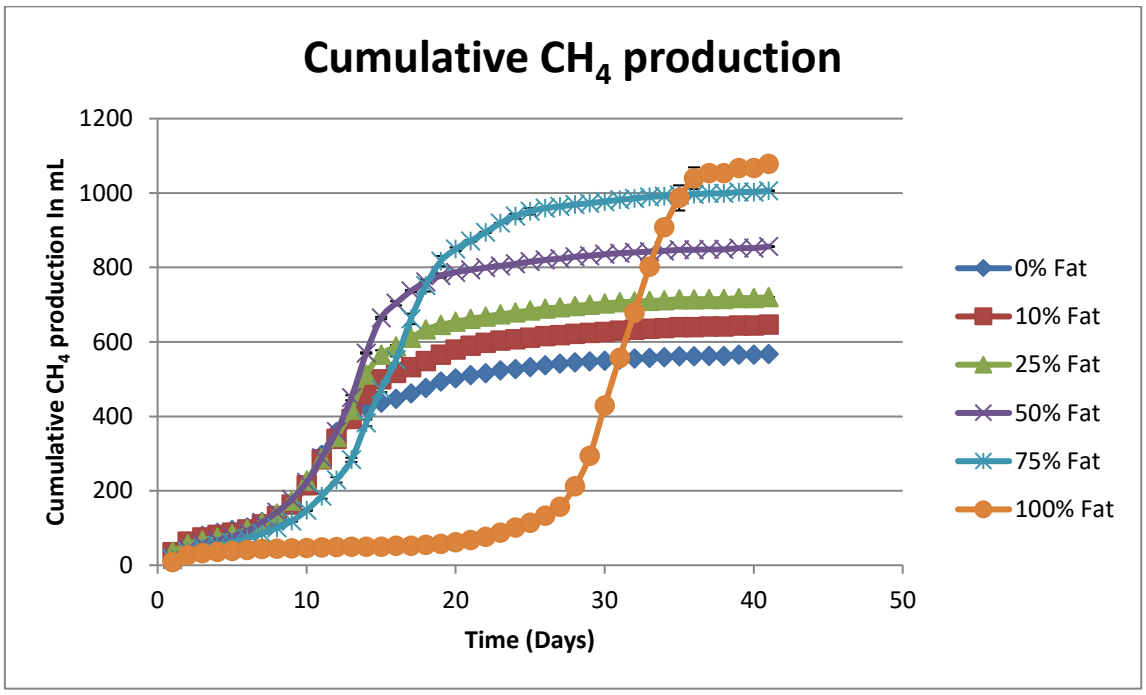

Figure 5-7: Cumulative methane production from 60 mL working volume serum bottles digesters at different fat concentrations (0%, 10%, 25%, 50%, 75%, and 100% w/w) in 20g L^{-1} total VS in a co-digestion with vegetable waste and an experimental time of 41 days. (Error bars represent standard deviation of samples from three replicate reactors.)

The cumulative methane production results illustrated in Figure 5-7 provide an indication about the effect of different concentrations of fat on methane production under the same levels of VS. It shows that higher fat concentrations lead to slower anaerobic digestion processes, possibly as a result of the limited degradation rate of lipid and the inhibitory effect of the cumulated LCFAs inside the reactor.

However, as can be seen in Figure 5-8 and Figure 5-9, the total methane gas production was directly proportional to the fat percentage. In other words, the higher the proportion of fat powder contributing to the 20g L^{-1} VS feed the higher the total methane production. An interesting example from Figure 5-8 is that under the same volatile solids feed condition of 20g L^{-1} VS, 100% fat has produced 47.35% more methane than 20g L^{-1} VS with 100% vegetable waste. The theoretical calculations by Alves *et al.* (2009) of methane production from fat, protein, and carbohydrate substrates are illustrated in (

Table 5-4).

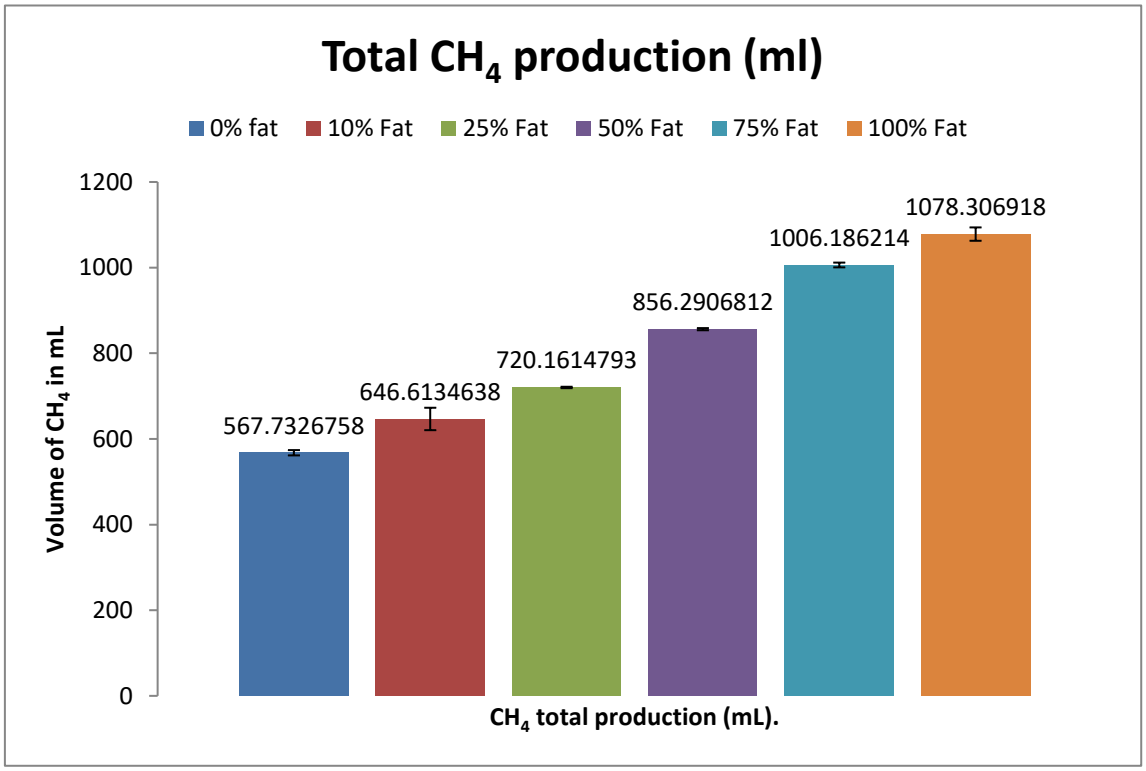

Figure 5-8: Total methane production from 60 mL working volume serum bottle digesters at different fat concentrations (0%, 10%, 25%, 50%, 75%, and 100% w/w) in 20g L^{-1} total VS in a co-digestion with vegetable waste. (Error bars represent standard deviation of samples from three replicate reactors.)

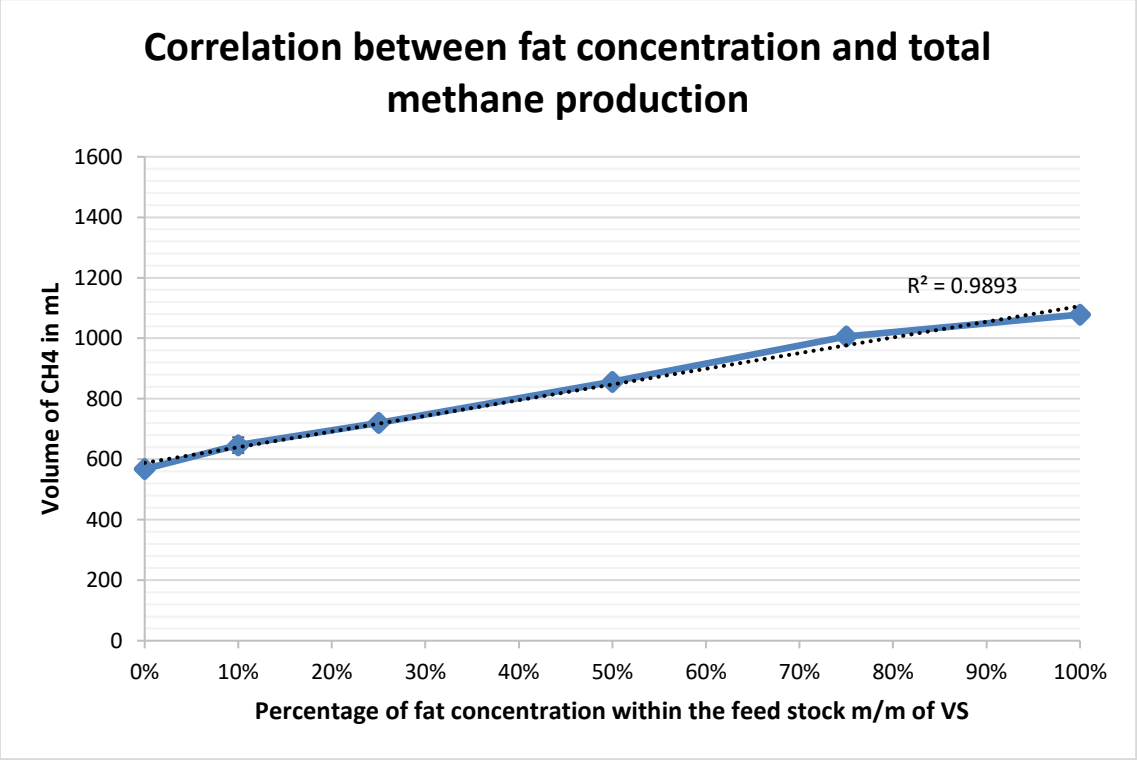

Figure 5-9: Correlation between the total methane production and the fat concentration within the feed stock (0%, 10%, 25%, 50%, 75%, and 100% w/w) in 20g L^{-1} total VS in a co-digestion with vegetable waste.

Table 5-4 Theoretical Biogas and methane from lipids, proteins, and carbohydrates, as reported by Alves et al. (2009).

Component	Chemical formula	Biogas (L g^{-1} of VS)	CH_4 (% v/v)
Lipids	$C_{50}H_{90}O_6$	1.425	69.5
Proteins	$C_{16}H_{24}N_4$	0.921	68.8
Carbohydrates	$C_6H_{24}O_5$	0.830	50.0

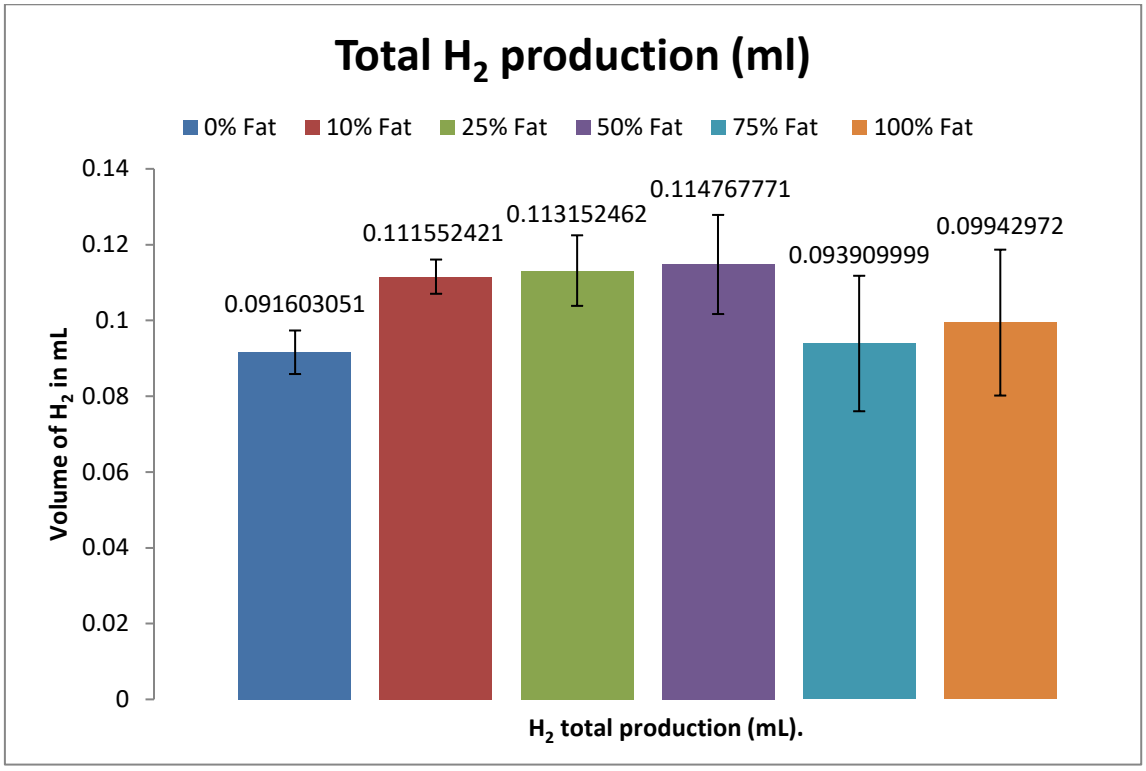

Figure 5-10: Total hydrogen production from 60 mL working volume serum bottles digesters at different bovine fat powder concentrations (0%, 10%, 25%, 50%, 75%, and 100% w/w) in 20g L^{-1} total VS in a co-digestion with vegetable waste. (Error bars represent standard deviation of samples from three replicate reactors.)

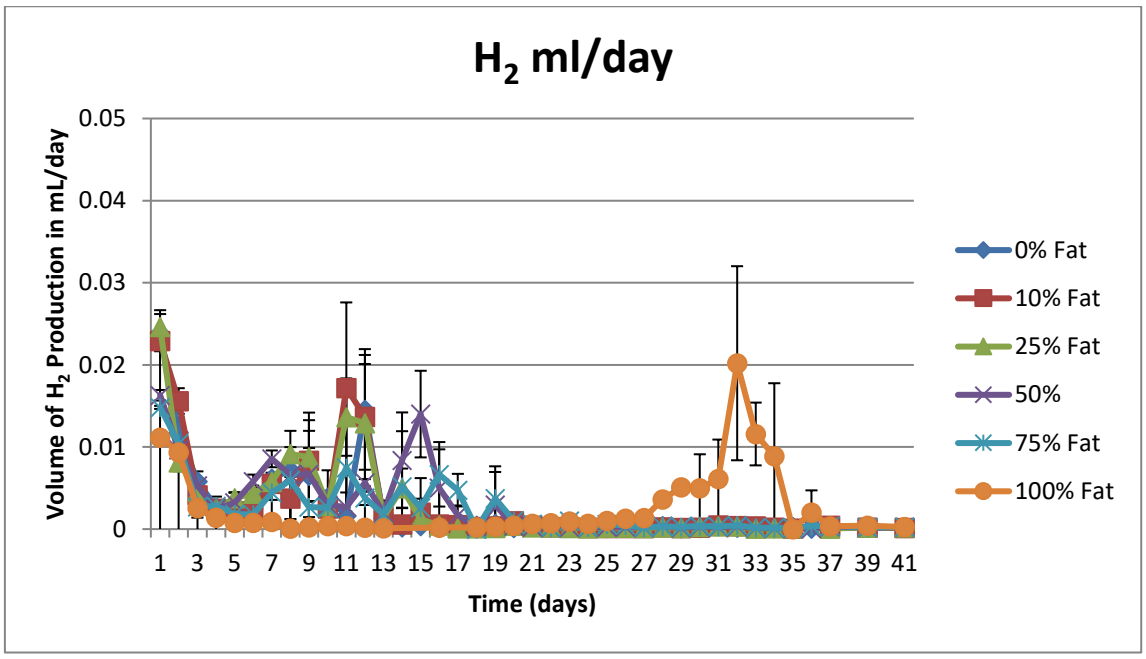

Figure 5-11: Daily hydrogen production from 60 mL working volume serum bottles digesters at different fat concentrations (0%, 10%, 25%, 50%, 75%, and 100% w/w) in 20g L^{-1} total VS in a co-digestion with vegetable waste. (Error bars represent standard deviation of samples from three replicate reactors.)

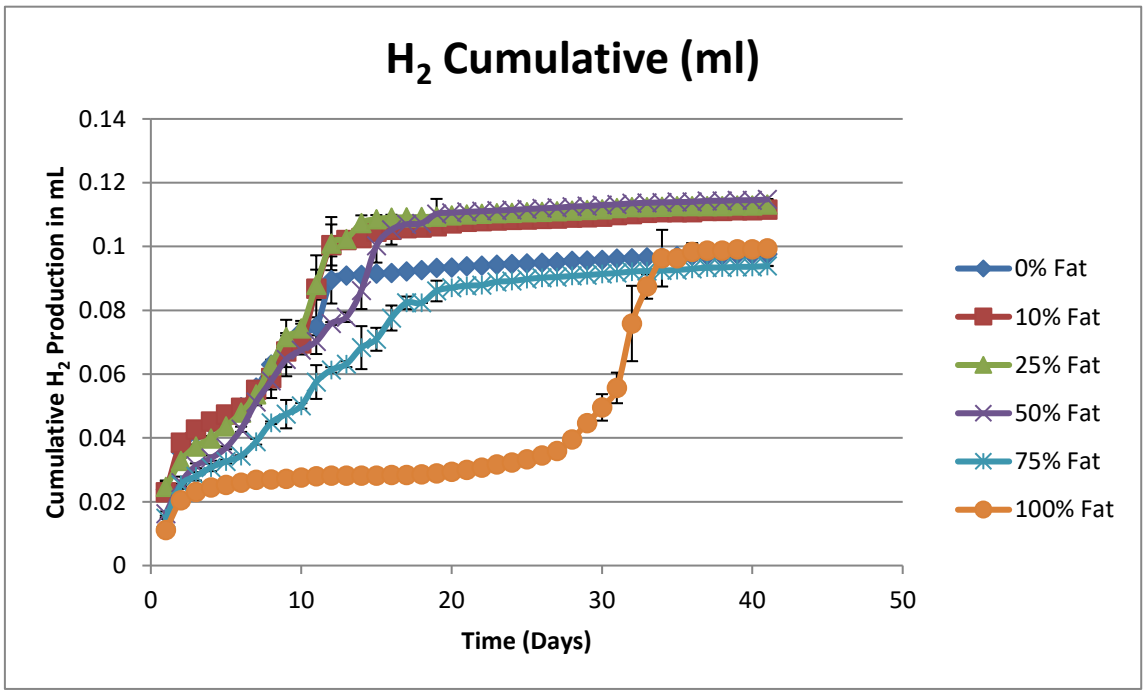

Figure 5-12: Cumulative hydrogen production from 60 mL working volume serum bottles digesters at different fat concentrations (0%, 10%, 25%, 50%, 75%, and 100% w/w) in 20g L^{-1} total VS in a co-digestion with vegetable waste and a running experimental time of 41 days. (Error bars represent standard deviation of samples from three replicate reactors.)

As can be seen in Figure 5-10, the total production of hydrogen gas during the experiment was very low (<0.12 mL) for all fat concentrations conditions at an I/S ratio of 1.

However, by looking to the overall behaviour of both CH_4 and H_2 daily production in Figure 5-6 & Figure 5-11, as well as the cumulative production of both gases shown in Figure 5-7 & Figure 5-12, it can be clearly seen that increasing CH_4 production is associated with a similar increase in H_2 production. This indicates that the inhibitory effect of higher fat concentration affects both the acetogenic (hydrogen forming) and methanogenic (methane forming) microorganisms before active production starts. This is similar to what Hanaki *et al.* (1981), reported about the inhibition of both hydrogen forming acetogenic and methanogenic microorganisms.

5.3.3.2 *Biogas from the second experimental pathway:*

In the second experimental pathway, high concentrations of fat (75% and 100% w/w of the 20g L^{-1} VS feed condition) were studied under lower I/S ratios of 0.5, 0.25, and 0.1.

When compared at the same I/S ratios, both concentrations of fat (75% and 100% w/w) in the feed showed similar overall behaviour and methane production. There is a slightly longer lag phase and slightly higher methane production for the 100% fat condition. As can be seen from (Figure 5-13) high methane production was found for the 0.5 I/S ratio for all of the replicate reactors at both fat concentrations (charts a and b). However, some interesting observations can be made from the reactors run at lower I/S ratios (<0.5).

At an I/S ratio of 0.25, large variations were observed between the results from the three replicates at both fat concentrations as can be seen in Figure 5-13 (charts c and d), Figure 5-14 (chart b), and Figure 5-15 (chart b). The expected production of methane was only achieved from one reactor among the three reactors of each fat concentration.

At 75% fat and 0.25 I/S ratio, only the replicate reactor No.3 was producing methane, after a long lag phase of 111 days compared to 31 days at an I/S ratio of 0.5 and only 8 days at an I/S ratio of 1. Replicate reactor No.1, showed almost no methane gas production during the whole 250 days of the experiment. In replicate reactor No. 2, low methane production was detected after 111 days, but this was 80.75% lower total production than the active replicate reactor (No. 3). Similar results were observed from the 100% fat concentration triplicated reactors at the same 0.25 I/S ratio. Gas production was seen in reactor No. 2 with a total methane production of 1012 mL after a lag phase of 160 days. The other two replicate reactors showed 88.78% and 67.57% lower methane production with total volumes of 113.5 mL from replicate reactor No.3 and 328.15 mL from replicate reactor No. 1.

However, at 0.1 I/S ratio (the lowest I/S ratio used in this experiment), there was almost no methane production. The total methane production was between 2.52 and 2.65 mL at 100% fat, and between 0.4 and 0.9 mL at 75% fat during the 250 days running time.

The overall results suggest that the optimum I/S ratio for an affective anaerobic digestion process is an I/S ratio of 1.

An I/S ratio of 0.5 extended the methane production lag phase between 31 days and 46 days for 75% and 100% fat conditions respectively.

An I/S ratio of 0.1 was insufficient to actively treat the fat anaerobically. An I/S ratio of 0.25, appears to be on the borderline between sufficient and insufficient as the anaerobic process may work or may not work at this ratio.

At I/S ratios ≥0.5, higher concentrations of fat will not only lead to longer lag phases, but also to higher total methane production.

One of the drawbacks of using an I/S of 1 in batch reactors is that a large amount of anaerobic seeding sludge (which is naturally low in VS content) will be required in order to achieve the I/S ratio and thus get sufficient anaerobic bacteria for a good anaerobic degradation process.

Even though in the current study the seeding sludge was pre-concentrated by gravity to increase the VS solids content and therefore minimise the required volume of seeding sludge added to the reactors, ≥80% of the reactors' working volumes were occupied by the seeding sludge when I/S ratio of 1 was applied.

An interesting point is that there is a direct correlation between lower I/S ratios and hydrogen gas production during the early days of the experiments. Results of total hydrogen production illustrated in Figure 5-16 show that lowering the I/S ratio below ≤0.5 leads to higher production of hydrogen. The 100% fat condition produces more hydrogen gas than the 75% one for all I/S ratios (Figure 5-16). The maximum hydrogen production was obtained for 100% fat and 0.1 I/S ratio (a total hydrogen volume of 24.39 mL). The second highest hydrogen production was obtained for the same I/S ratio condition of 0.1 at 75% fat condition (a total hydrogen volume of 13.96 mL). As can be seen in Figure 5-17 (charts a and b), there were no (significant) further increases in hydrogen production after day 3 under all I/S ≤0.5 during the experiment. The increase in hydrogen production when lowering the I/S ratio could be the result of fast initial growth rates of hydrolytic and acidogenic bacteria. The hydrolytic and acidogenic bacteria are thus dominant during the early days of the reaction, which results in a drop in the pH and hydrogen production. At higher I/S ratios, more methanogenic

microorganisms are present from the beginning of the experiment and take over control of the system earlier as is evident by the earlier production of methane and shorter duration of low pH before it naturally increases as a result of methanogenic activity.

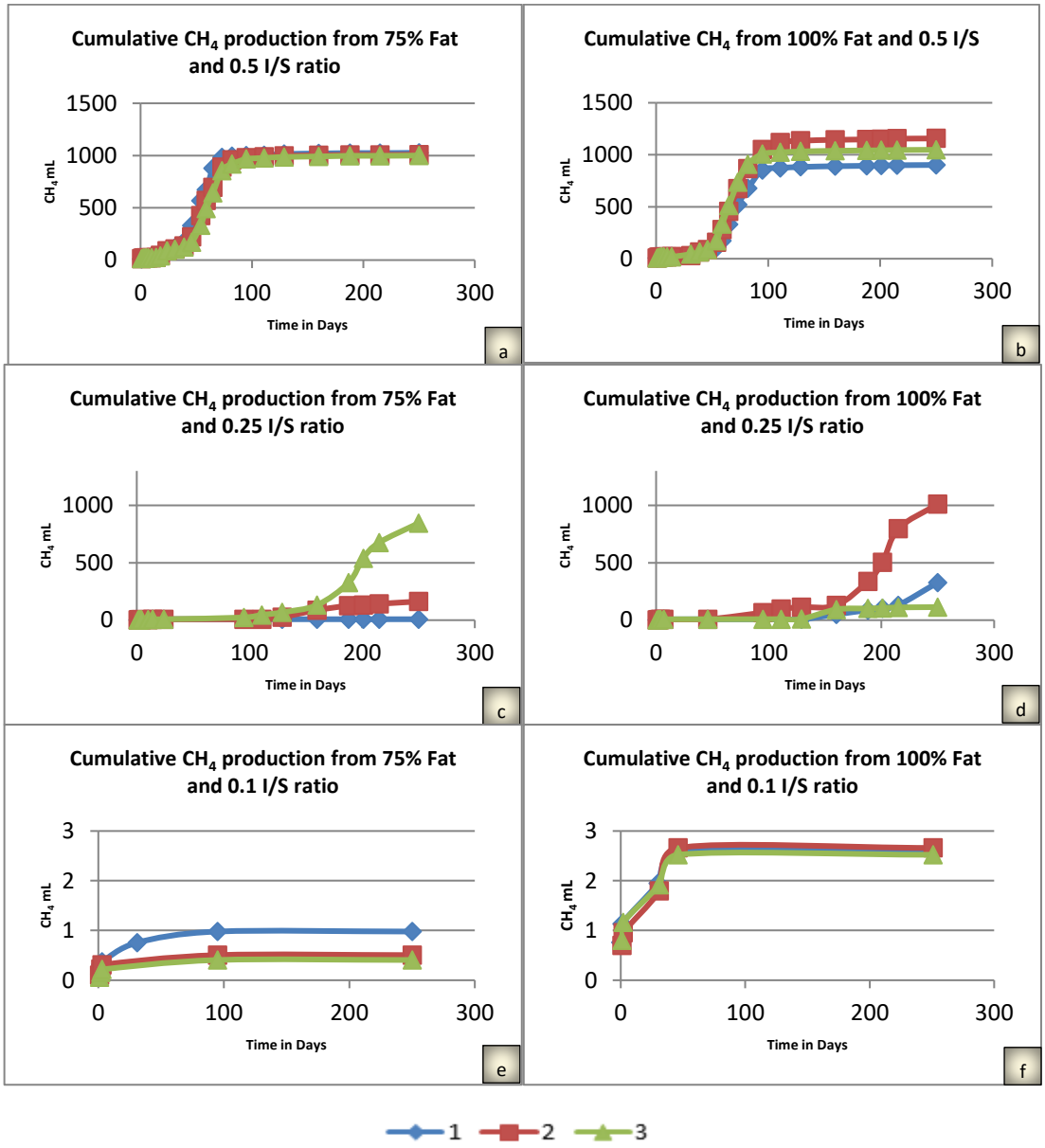

Figure 5-13: Cumulative methane production from 60 mL working volume serum bottles digesters at fat concentrations of (75%, and 100% w/w) in 20g L^{-1} total VS in a co-digestion with vegetable waste and at different I/S ratio conditions of (0.5, 0.25 and 0.1, and running experimental time of 250 days.

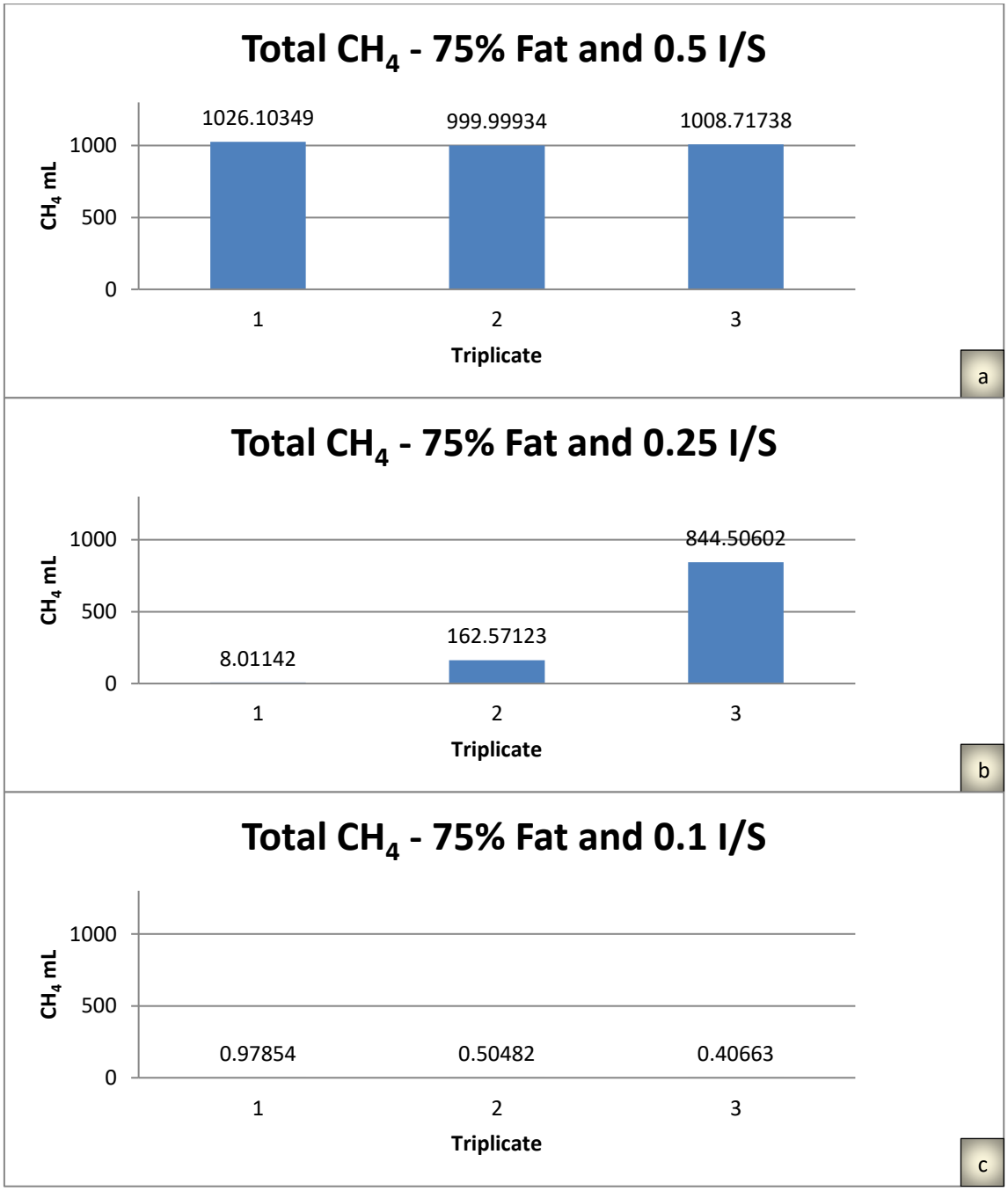

Figure 5-14: Total methane production from 60 mL working volume serum bottles digesters at a fat concentration of 75% w/w, of 20g L^{-1} total VS in co-digestion with vegetable waste and at different inoculum to substrate ratios of 0.5, 0.25, and 0.1 in term of VS.

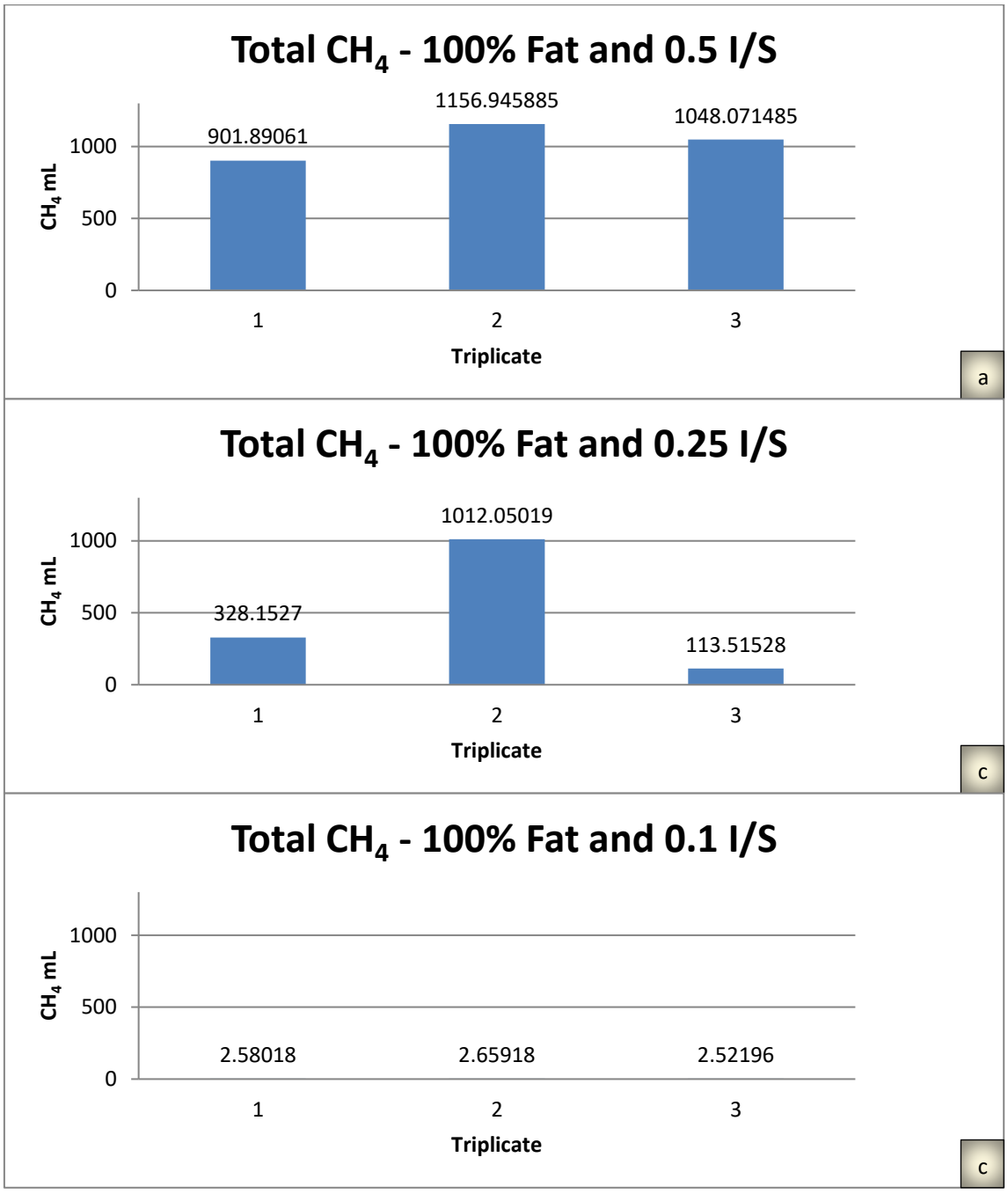

Figure 5-15: Total methane production from 60 mL working volume serum bottles digesters at a fat concentration of 100% w/w in 20g L^{-1} total VS feed substrate and at different inoculum to substrate ratios of 0.5, 0.25, and 0.1 in term of VS.

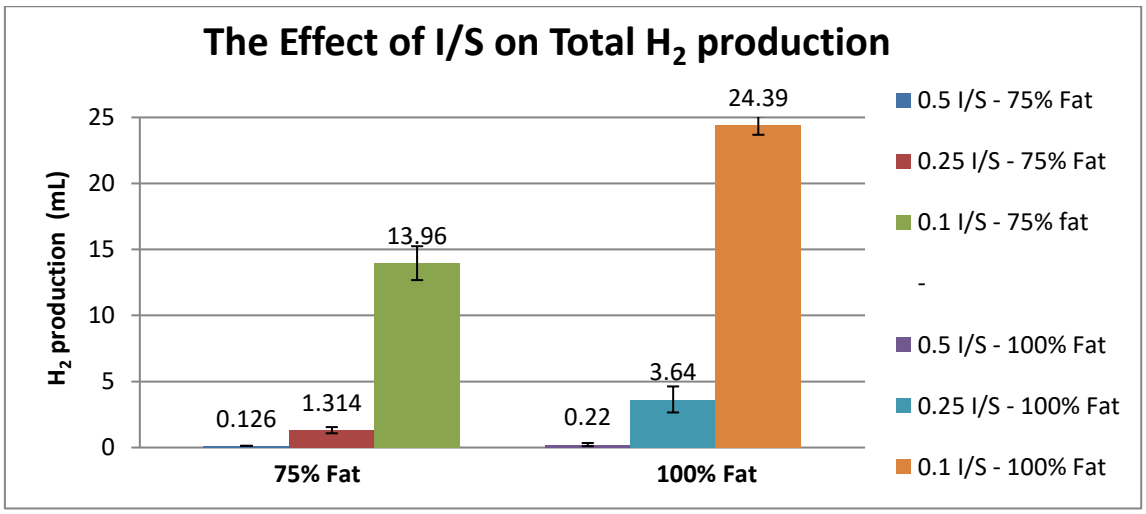

Figure 5-16: Total hydrogen production from 60 mL working volume serum bottles digesters at fat concentrations of (75%, and 100% w/w) in 20g L^{-1} total VS in co-digestion with vegetable waste and at different inoculum to substrate ratios of 0.5, 0.25, and 0.1 in terms of VS. (Error bars represent standard deviation of samples from three replicate reactors.)

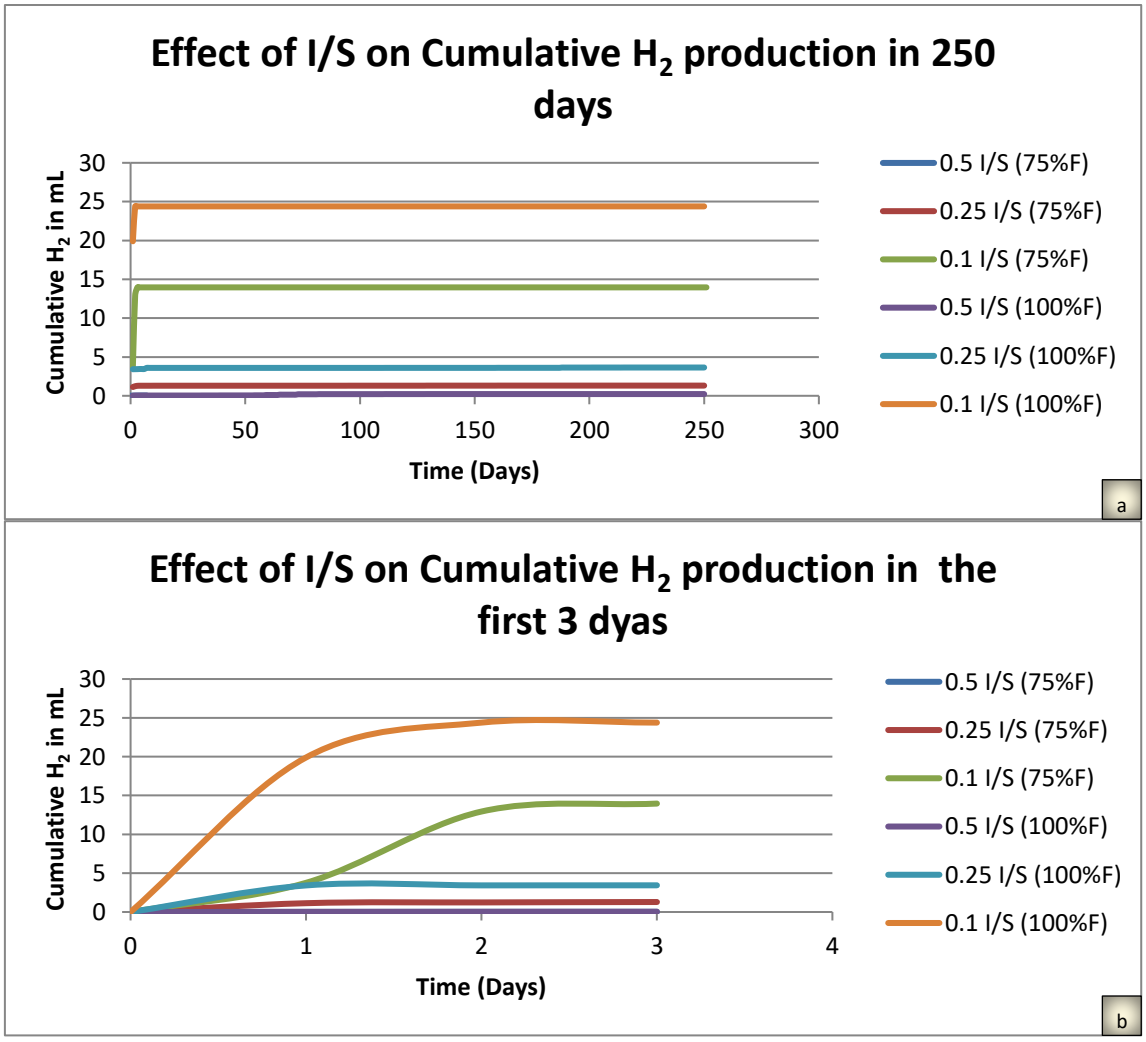

Figure 5-17: Cumulative hydrogen production from 60 mL working volume serum bottles digesters at fat concentrations of (75%, and 100% w/w) in 20g L total VS in co-digestion with vegetable waste and at different inoculum to substrate ratios of 0.5, 0.25, and 0.1 in terms of VS.

5.3.4 **Long Chain Fatty Acids**

5.3.4.1 ***LCFAs from the first experimental pathway***

The initial total LCFA concentrations at the beginning of the experiment show a direct relationship with the amount of fat in the feed substrate. At 0% fat (or in other words 100% w/w vegetable waste), the lowest initial total LCFA value was 244.78 mg L^{-1}. Increasing the proportion of fat making up the 20 g L^{-1} TVS feed from 0% to 100% results in a continuing increase in LCFA content as shown in Figure 5-18. The highest value of total LCFAs (5001.58 mg L^{-1}) was at 100% fat and is five times higher than the inhibitory level stated by Palatsi *et al.* (2009) who state that concentrations of 1.0 g L^{-1} LCFAs can be inhibitory. By this measure, four of the current experimental conditions (25%, 50%, 75%, and 100% fat w/w), are above the inhibitory LCFAs concentrations.

Final total LCFAs values are illustrated in Figure 5-18, and show a reduction from the initial levels for all fat conditions after 41 days. Combining these results (Figure 5-18) with methane production (Figure 5-7 and Figure 5-8), it is shown that an effective anaerobic digestion process was obtained with good conversion rates of LCFAs to methane gas under the current operational conditions.

The results in Figure 5-18 show some LCFA residues on the last day of the experiment (Day 41). These residues are greater in the higher fat content conditions. This might be due to the slower starting point (delay) of active anaerobic degradation, which can be demonstrated by the delay in production of methane gas (Figure 5-6 and Figure 5-7). However, the highest residue value among all reactors was 159.73 mg L^{-1} at 100% fat condition, which is lower than the reported inhibitory concentration of 1 g L^{-1} (Palatsi *et al.*, 2009), and thus indicates an effective anaerobic digestion of fat with high initial total LCFAs of ~ 5 g L^{-1} under an I/S ratio of 1.

The efficiency of LCFA reduction from 10% to 100% fat is between 91.23% and 97.38% degradation of the initial total LCFAs as illustrated in (Table 5-5).

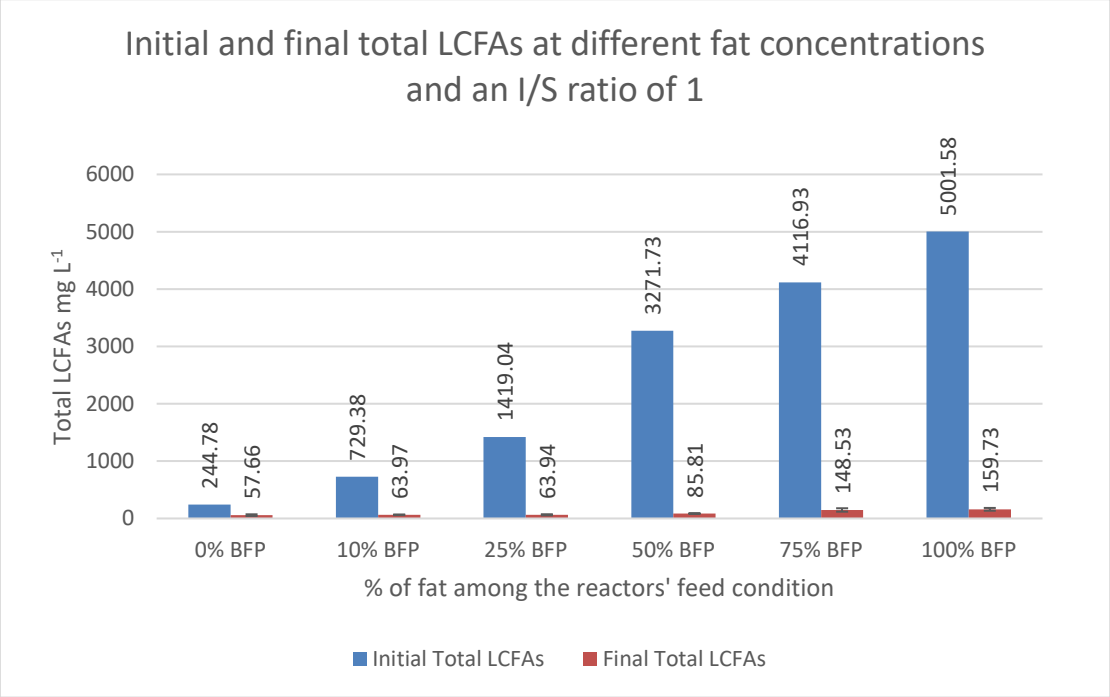

Figure 5-18: Initial and final remaining concentrations of total LCFAs in the serum bottle reactors with fat concentrations of 0%, 10%, 25%, 50%, 75%, and 100% w/w and an I/S ratio of 1 at the end of 41 days of the experimental run.

Table 5-5 Average total LCFA reductions in percent, after 41 days experimental time under I/S ratio of 1 and at different fat concentrations.

fat concentrations the in 20g L^{-1} VS	% of total LCFAs removed	*SD*
0%	76.44	6.07
10%	91.23	0.80
25%	95.49	0.67
50%	97.38	0.10
75%	96.39	0.72
100%	96.81	0.48

5.3.4.2 *LCFAs from the second experimental pathway*

Total LCFAs remaining after 250 days are shown in Figure 5-19 and Figure 5-20 for I/S ratios of 0.5, 0.25, and 0.1 and at fat concentrations of 75% and 100% of the total 20g L^{-1} volatile solid feed. The overall LCFA profiles align with the methane production results from the same pathway. For both fat conditions, reactors at the I/S ratio of 0.5 showed an effective reduction of the initial total LCFAs associated with a good methane production from all replicates.

At an I/S ratio of 0.25, replicate reactors under both fat concentrations (75% and 100% w/w) show inconsistent results. The triplicate reactors show a high variation in LCFA content on the last day of the experiment, similar to the variation in gas production and the VFAs content under the same I/S ratio and, probably, for the same reason of the I/S ratio being on a borderline between sufficient and not sufficient inoculum for an adequate anaerobic process. At an I/S ratio of 0.25 and 75% fat the replicate reactor No. 3 showed a relatively high reduction of LCFAs, while the other replicates appear to be accumulating LCFAs rather than reducing them. The higher LCFAs values might be due to the effect of non-digested aggregates of fat particles transferred with the liquid samples during the sampling process. Similar behaviour was found in the results of total LCFAs at 100% fat and the same 0.25 I/S ratio (Figure 5-20). In this case, replicate reactor No. 2 performed well in terms of reducing the LCFAs while replicates No. 3 and 1, show the same problem of accumulating LCFAs.

At the I/S ratio of 0.1, none of the reactors actively treated the fat and LCFAs were accumulated in all of the replicates under both fat conditions of 75% and 100%.

The LCFA show a high similarity, in terms of overall behaviour, to the methane production during the experiment. A direct relationship can be drawn between LCFA reduction and methane production under all experimental conditions as illustrated in Figure 5-8, Figure 5-14, Figure 5-15, Figure 5-18, Figure 5-19, and Figure 5-20).

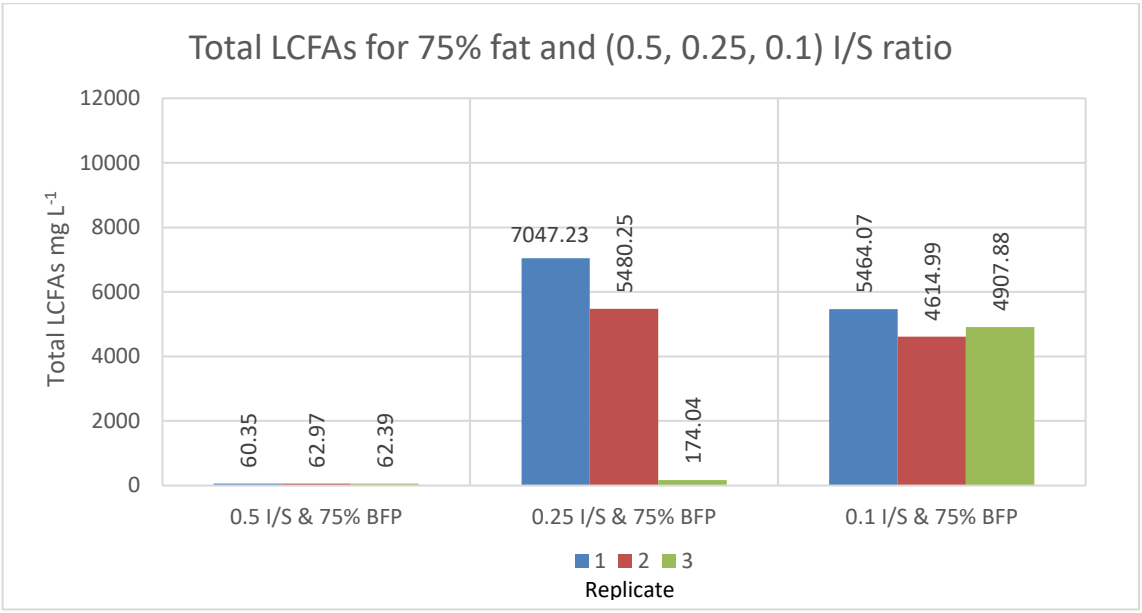

Figure 5-19: Total LCFAs remaining in the serum bottle reactors with fat concentrations of 75% w/w and I/S ratio of (0.5, 0.25, and 0.1), after 250 days experiment.

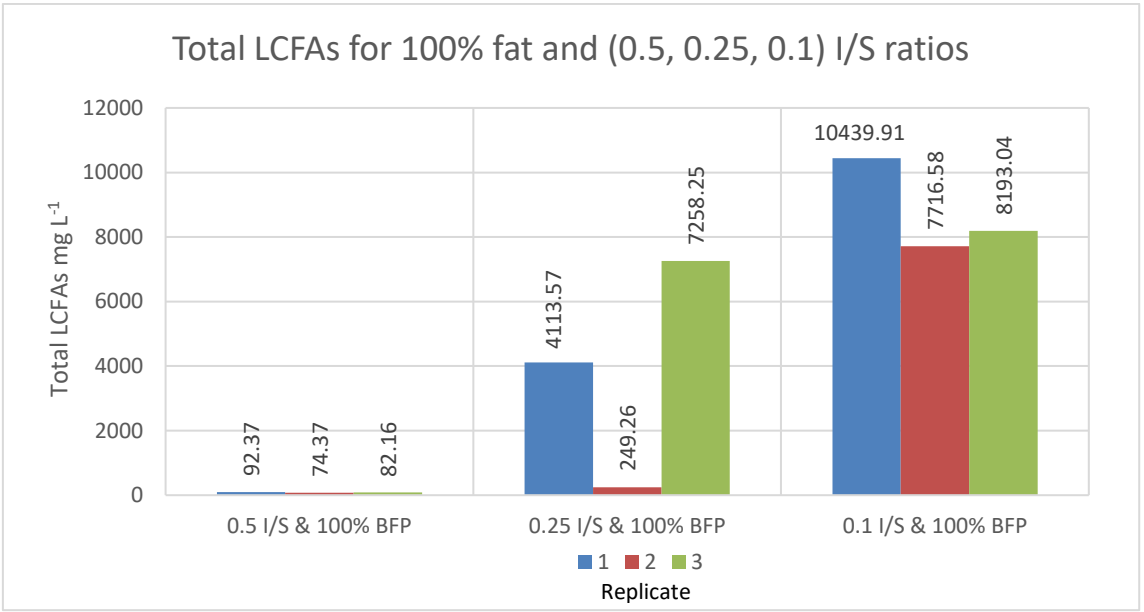

Figure 5-20: Total LCFAs remaining in the serum bottle reactors with fat concentrations of 100% w/w and I/S ratio of (0.5, 0.25, and 0.1), after 250 days experiment.

5.3.5 **Volatile Fatty Acids**

5.3.5.1 ***VFAs from the first experimental pathway***

As illustrated in Figure 5-21, the VFA results from the first experimental pathway show some small concentrations of volatile fatty acids remaining even after gas production had almost ceased at the end of the experiment. Under fat conditions of 0%, 10%, 25%, 50%, and 75% w/w within the feed stock, the main VFAs were acetic acid, butyric acid and isovaleric acid, in addition to small fractions of other VFAs.

However, at 100% w/w fat within the feedstock, the replicated digesters show an accumulation, mainly of propionic acid with a concentration of 1084.4 mg L^{-1} isobutyric and isovaleric acids were also found at higher concentrations in these reactors with concentrations of 45.8 mg L^{-1} and 27.4 mg L^{-1} respectively. The relatively high concentration of propionic acid may indicate an incomplete digestion which might produce further biogas if it were left longer.

The small concentrations of VFAs that remained in the reactors can be an indication of slower bacterial activity due to lack of nutrients by the end of the digestion process. Lower activity can also be affected by an accumulation of toxic material such as ammonia by the end of the experiment.

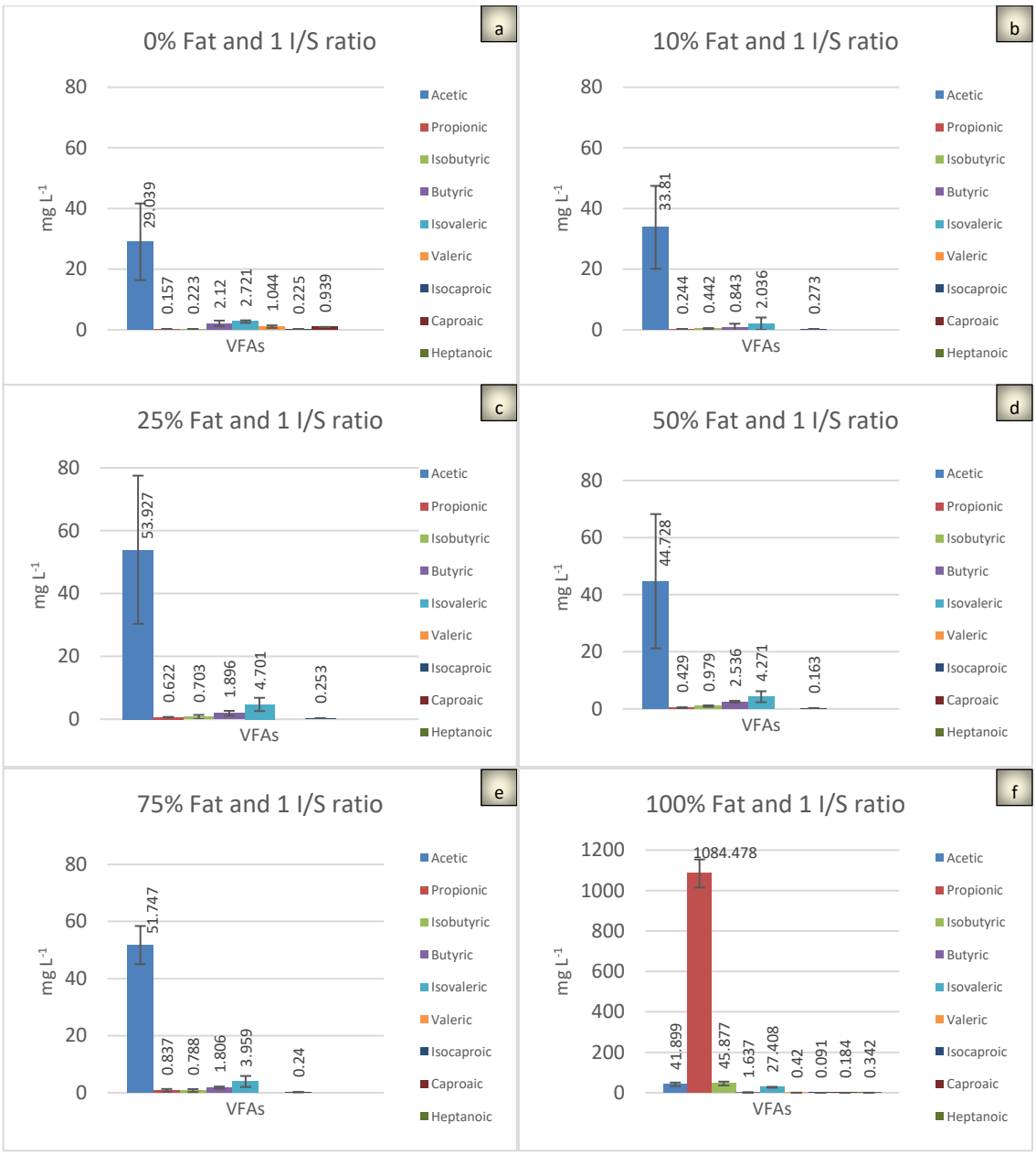

Figure 5-21: Volatile fatty acids remaining in the serum bottles reactors which have been run for 41 days at different fat concentrations of (0%, 10%, 25%, 50%, 75%, and 100% w/w) in 20g L^{-1} total VS in a co-digestion with vegetable waste. (Error bars represent standard deviation of samples from three replicate reactors.)

5.3.5.2 *VFAs in the second experimental pathway*

At low I/S ratios of 0.5, 0.25, and 0.1, reactors running on both fat concentrations (75% and 100% w/w) show different abilities to treat VFAs.

At an I/S ratio of 0.5, rectors show a good performance, evident by both VFAs consumption (Figure 5-22, Figure 5-23) and biogas production (Figure 5-14, Figure 5-15). However, inconsistent results were found from the triplicated reactors of 0.25 I/S ratio at both fat concentrations. There is a high variation in VFAs content at the end of the experiment, similar to the variation in gas production and LCFA content under the same I/S ratios and for the same reason of being on the borderline in terms of providing enough inoculum for the anaerobic process to complete. Replicate reactor No. 2 at an I/S ratio of 0.25 and 100% fat showed a good total methane production (Figure 5-15), indicating an effective anaerobic conversion. In chart B of Figure 5-23, propionic acid is in relatively high concentration in this reactor, with a similar observation from chart F in Figure 5-21. As both reactors show a high production of methane, this remaining high concentration of propionic acid may indicate an incomplete digestion which would produce more biogas if it were left longer.

Results shown in chart A in Figure 5-22 and Figure 5-23 indicate the inhibition of methanogenesis at low I/S ratios. Under both fat conditions the VFAs accumulated instead of being converted to methane. Comparing these results and the total LCFA results in Figure 5-19 and Figure 5-20 is further indication of an inhibition situation.

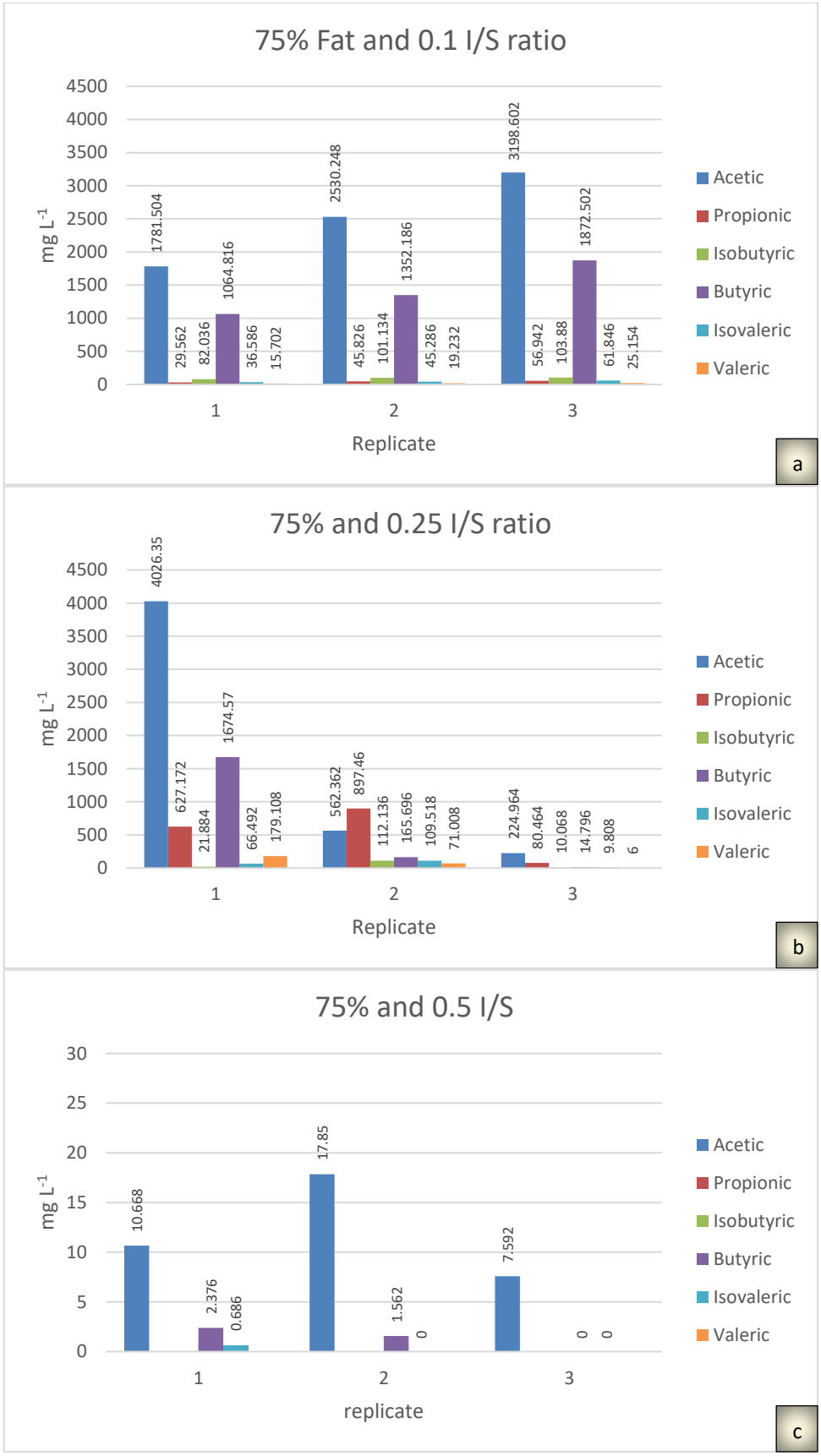

Figure 5-22: Total volatile fatty acids concentrations remain after 250 days' experimental time, for a fat concentration of 75% w/w of 20g L^{-1} total VS in co-digestion with vegetable waste and at different inoculum to substrate ratios of 0.5, 0.25, and 0.1 in terms of VS.

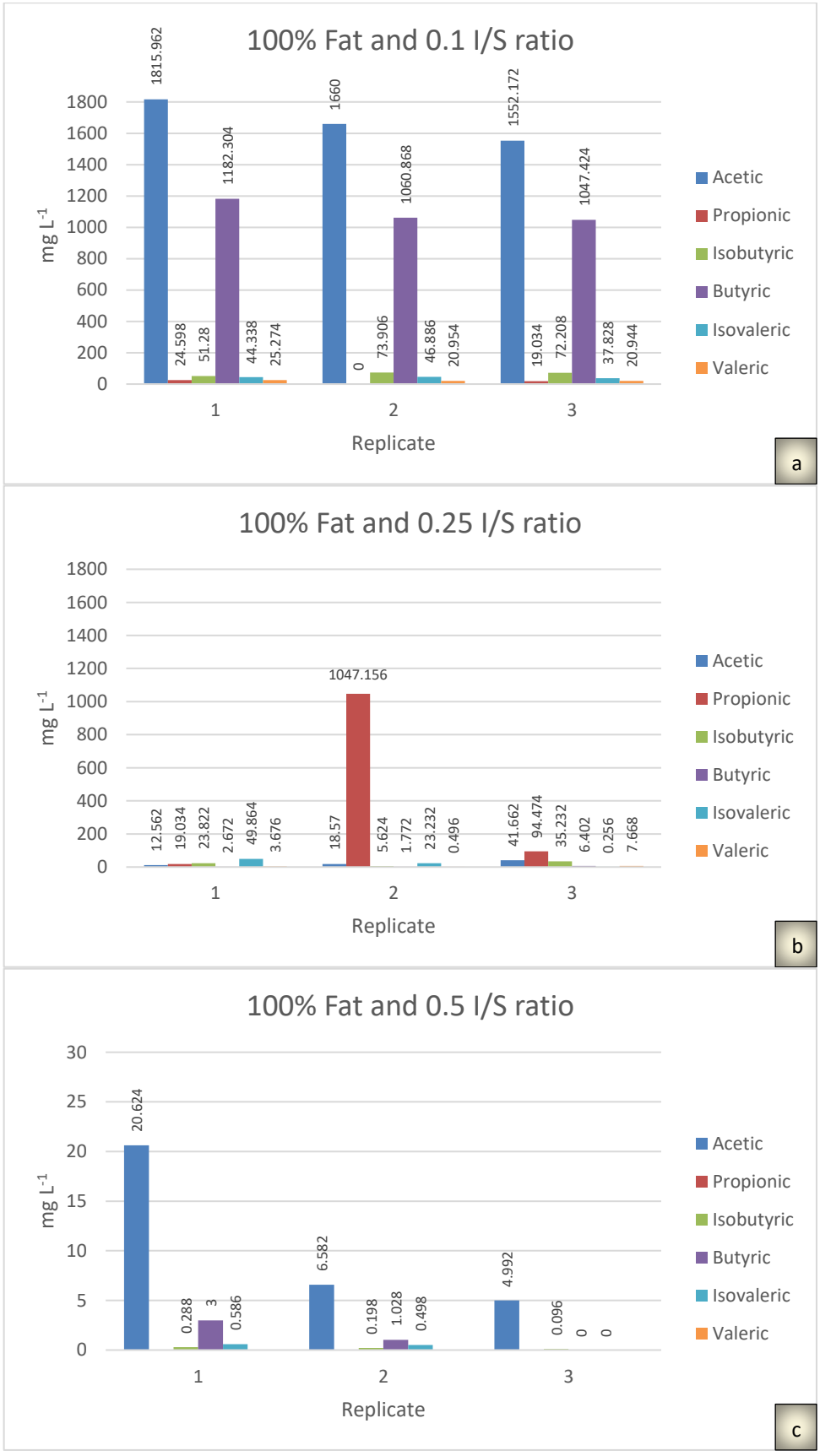

Figure 5-23: Total volatile fatty acids concentrations remain after 250 days' experimental time, for fat concentrations of 100% w/w of 20g L^{-1} total VS and at different inoculum to substrate ratios of 0.5, 0.25, and 0.1 in terms of VS.

5.3.6 **Chemical Oxygen Demand**

5.3.6.1 ***COD for the first experimental pathway***

Figure 5-24 shows that higher percentages of fat in the feed will mostly lead to higher initial D-COD values. The reason behind this is that the fat will naturally have higher carbon content compared to the vegetable waste as shown by the elemental analysis earlier in this study (Chapter 5).

As shown in Figure 5-24, reactors with 0% fat (100% of the 20g L^{-1} TVS is vegetable waste) show higher D-COD values than for a fat concentration of 10%. This result was expected as these COD values are dissolved COD and not total COD values. In dissolved COD measurement, samples undergo centrifugation and then filtration through 0.22µl pore size filter units, to remove any solids before the analyses (the reason of measuring dissolved and not total COD is discussed in the materials and methods chapter). The second possible reason is that the organic components from blended vegetable waste are more quickly and easily dissolved than the fat. The fat is hydrophobic and will not dissolve directly into the water. Therefore, some of the solid fat particles will be removed through the filtration process and might be eliminated from dissolved COD analysis. The increase in initial D-COD values associated with increasing percentage of fat from 10% to 100%, as illustrated in Figure 5-24, is evidence to support the higher D-COD at higher fat conditions.

The efficiency of D-COD reduction shows a very good association with methane production after 41 days' experimental time (Figure 5-8 and Figure 5-24).

As illustrated in Table 5-6, by the end of the experiment, the D-COD values were reduced by an average of 93.73% to 96.97% in all of the experimental reactors.

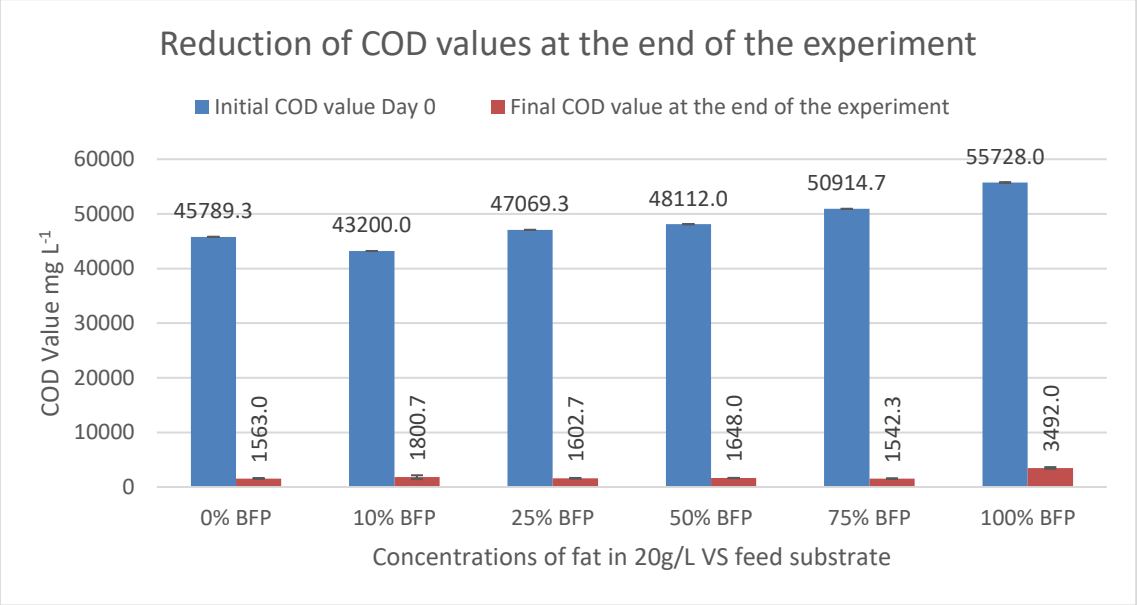

Figure 5-24: Initial and final dissolved-COD values for serum bottle reactors content which been run at I/S ratio of 1 and fat concentrations of 0%, 10%, 25%, 50%, 75% and 100%.

Table 5-6 The average total percent of dissolved-COD reductions after 41 days experimental time under I/S ratio of 1 and at different fat concentrations.

Fat concentration in 20g L^{-1} VS	% of D-COD removed	*SD*
0%	96.58	0.21
10%	95.83	0.83
25%	96.59	0.11
50%	96.57	0.04
75%	96.97	0.04
100%	93.73	0.27

5.3.6.2 *COD for the second experimental pathway*

Figure 5-25 shows that, at both 75% and 100% w/w fat, there is a direct link between the I/S ratio and the efficiency of removing the D-COD from the reactors. It can be clearly seen that at lower I/S ratios, the efficiency of removing D-COD is decreased.

The highest removal of D-COD in the second pathway was observed at the highest I/S ratio condition of 0.5, for both 75% and 100% fat concentrations with a removal efficiency of 97.1% and 97.7% respectively (Table 5-7).

The experiments at a lower I/S ratio of 0.25 show a lower efficiency in removing D-COD of 83% and 91.9% from 75% and 100% fat respectively (Table 5-7).

At an I/S ratio of 0.1, the lowest efficiency of D-COD removal was observed (72.5% and 77% for 75% and 100% respectively). Even though there was not much methane produced from the 0.1 I/S ratio reactors, a relatively high removal of D-COD was observed. An explanation for this could be acidogenic bacteria activity which is dominant at the beginning of the experiment and is responsible for releasing CO_2 and H_2 gases during the digestion process. Similar behaviour of reducing COD by the activity of acidogenic bacteria in an anaerobic digestion study was observed by Thanwised *et al.* (2012).

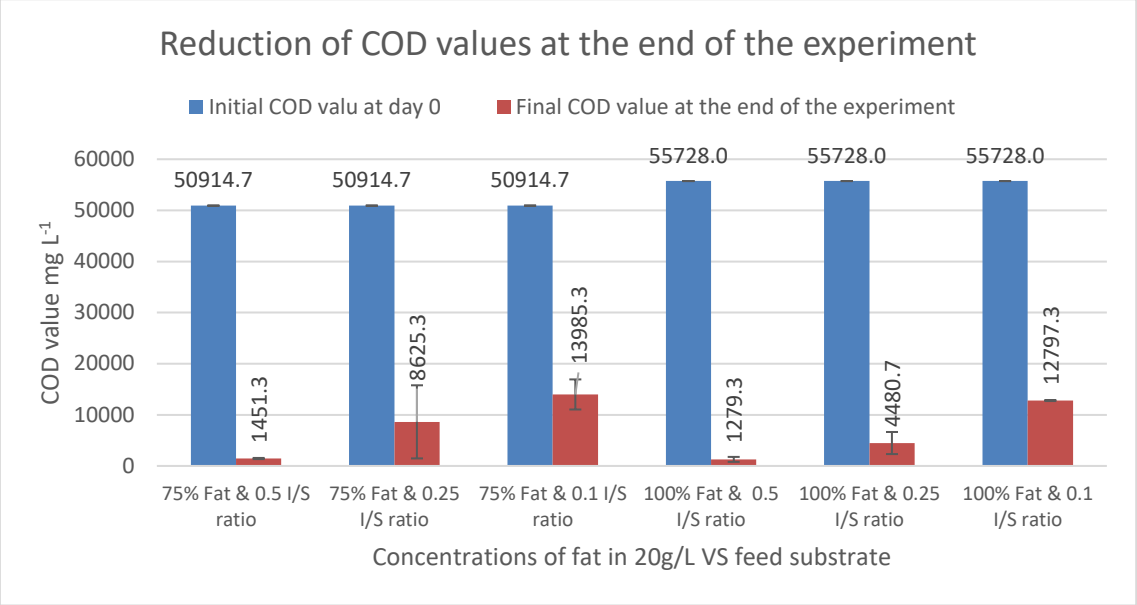

Figure 5-25: Initial and final dissolved-COD values for serum bottle reactors content which been run under I/S ratios of 0.5, 0.25, and 0.1 with fat concentrations of 75% and 100% w/w in 20g L^{-1} TVS feed condition.

Table 5-7 Averages of dissolved-COD removed after 250 days experimental time at I/S ratios of 0.5, 0.25, and 0.1, under FAT concentrations of 75% and 100% w/w of the total volatile solids feed condition.

Fat concentrations w/w in the 20g L^{-1} VS	I/S ratio condition	% of D-COD removed	*SD*
75%	0.5	97.14	0.22
	0.25	83.05	14.03
	0.1	72.53	5.78
100%	0.5	97.70	0.87
	0.25	91.95	3.87
	0.1	77.03	0.18

5.4 Overall discussion:

5.4.1 The effect of fat concentrations:

The effect of different fat concentrations in the feed substrate of the anaerobic reactors was examined in the first experimental pathway of this chapter.

From the overall results, at an inoculum to substrate ratio (I/S ratio vs/vs) of 1, more fat in the feed results in more methane production from the reactor (Figure 5-8). The calculated theoretical results and the experimental results shown in (Figure 5-3, Figure 5-4, and Figure 5-8) agree with reports in the literature (Cirne *et al.*, 2007, Appels *et al.*, 2011, O-Thong *et al.*, 2012) that lipids and fat materials can generate more methane than other waste substrates.

However, it was observed that there is a late start-up of active biogas production (long lag phase) associated with higher fat concentrations in the feed (Figure 5-6). Similar results of extended lag phase are reported by Hanaki *et al.* (1981) in a study of the inhibitory mechanism of LCFAs during the anaerobic digestion process.

Biogas production peaks are also delayed with higher concentrations of fat in the feed. The highest peaks of methane production were detected on day 11 for 0% and 10% fat, and on day 14 for 25% and 50% fat. For 75% and 100% fat the highest methane was on days 17 and 30 respectively (Figure 5-6). It is thus clear that higher fat concentrations lead to slower anaerobic digestion processes, possibly as a result of the limited degradation rate of lipid and the inhibitory effect of accumulated LCFAs in the reactor.

The total methane gas production was directly proportional to the fat content (Figure 5-9). In other words, the higher the proportion of fat contributing to the 20g L^{-1} VS feed the higher the total methane gas production. An interesting example from the experimental results is that under the same volatile solids feed condition of 20g L^{-1} VS, 100% fat produces 47.35 % volume more methane gas than the same 20g L^{-1} VS from 100% vegetable waste (Figure 5-8).

Looking to the overall behaviour of both CH_4 and H_2 daily production, as well as the cumulative production of both gases, the late increase of CH_4 production seem to be associated with a similar increase in H_2 production (Figure 5-6,

Figure 5-7, Figure 5-10, and Figure 5-11). This might indicate that the inhibitory effect of higher fat concentration on both the acetogenic (hydrogen forming) and methanogenic (methane forming) microorganisms before active production starts. This is similar to a report from Hanaki *et al.* (1981) about the inhibition of both hydrogen forming acetogenic and methanogenic microorganisms in similar circumstances.

Even though the reactors were able to deal with a 100% w/w fat feed at an inoculum to substrate (I/S) ratio of 1, controlling the I/S ratio to 1 is difficult and has the disadvantage of wasting a large proportion of the working volume of the reactor. As the anaerobic seeding sludge is naturally very low in volatile solid content, controlling the I/S ratio to a value of 1 will be very difficult without adding a large volume of seeding sludge to a small volume of feed substrate (normally high in VS) to balance the VS contents between them. However, on a commercial scale this is more likely to be a viable scenario as it is usual to add small amounts of feed to a large volume of active reactor contents (equivalent to the seeding sludge here). Even though in the current study the seeding sludge was pre-concentrated by gravity to increase the VS solids content and therefore minimise the required volume of seeding sludge added to the reactors, ≥80% of the reactors' working volumes were occupied by the seeding sludge when I/S ratio of 1 was applied. Again this indicates a suitable potential for an industrial scale plant where it would be usual to add 5-10% v/v "feed" to an active reactor in continuous or semi-continuous operation condition.

5.4.2 The effect of inoculum to substrate ratio (I/S):

Overall, higher I/S ratios (>0.5) result in faster and much stable start-up of the digester and higher methane production against the hydrogen gas production.

Lower I/S ratios (<0.5) result in instable and slower start up with higher hydrogen gas production during start-up.

Lower I/S ratios mean a smaller bacterial culture is being provided to the reactors as an inoculum, which end up with poor degradation and lower reduction in the D-COD values. This is reflected in the estimated CH_4 production from the removed D-COD value (Figure 5-5). High methane production was reached at the I/S ratio of 0.5 from all replicate reactors of both fat concentrations. However, some interesting observations can be made from the

reactors run at lower I/S ratios (<0.5). At an I/S ratio of 0.25, large variations were observed between the results from the three replicates of both fat concentrations. The expected production of bio methane was only achieved from one reactor among the triplicate reactors of each fat concentration (Figure 5-13). From the overall results, I/S ratio of 0.25 appears to be on the border line between the sufficient and insufficient as the anaerobic process may work or may not work at this ratio.

An I/S ratio of 0.1 was insufficient to actively treat the fatty substrate anaerobically. An interesting point is that there is a direct correlation between lower I/S ratios and hydrogen gas production during the early days of the experiments. Results of total hydrogen production show that lowering the I/S ratio below 0.5 leads to higher production of hydrogen. The maximum hydrogen production was obtained for 100% fat and 0.1 I/S ratio (a total hydrogen volume of 24.39 mL). There were no (significant) further increase in hydrogen production after day 3 under all I/S ≤0.5 during the experiment.

The increase in hydrogen production when lowering the I/S ratio could be the result of fast initial growth rates of hydrolytic and acidogenic bacteria (Figure 5-17). The hydrolytic and acidogenic bacteria are thus dominant during the early days of the reaction, which results in a drop in the pH and hydrogen production. At higher I/S ratios, more methanogenic microorganisms are present from the beginning of the experiment and take over control of the system earlier as is evident by the earlier production of methane and shorter duration of low pH before it naturally increases as a result of methanogenic activity. Starting up a mesophilic anaerobic reactor with a low seeding sludge concentration has been shown to require more time and more careful management compared to the use of a high concentration of seeding sludge (Hatzigeorgiou *et al.*, 2006). A similar effect of extended lag phase as a result of using a low I/S ratio (0.1) was also observed by Ma *et al.* (2015) in a study of LCFAs inhibition to the anaerobic digestion of algae biomass at different inoculum to substrate ratios. As mentioned by Maier *et al.* (2009), in some cases a very small initial population can be the reason for a long lag phase in microorganism growth and function.

Next chapter will look into the possibility of improving the digestion process at low I/S ratio (low volume of seeding sludge) to address the findings of this chapter.

Chapter 6 Co-Digestion of fat powder and Vegetables waste.

The effect of fat powder concentration, inoculum sources, and reseeding on the digestion process

6.1 Objectives:

The objectives of this chapter is as following:

- Firstly, is to study of the effect of seeding sludge sources (acclimatised and not acclimatised to fat) on the digestion process at different concentrations of fat and at low inoculum to substrate (I/S) ratios from 0.0675 to around 0.27 (vs/vs). This is to address the findings from Chapter 5, where it was found high inoculum to substrate ratios were required, but were not ideal due to the volume being occupied in the reactor.
- Secondly, is to study the potential of reseeding as a recovery or enhancing step to the digestion process.
- Thirdly, is to investigate the overall changes in the pH behaviour under different feed and seeding conditions. In addition, these experiments tested the ability of pH electrodes to work consistently under long exposures to the reactor contents, especially during high levels of fat concentrations.

6.2 Experimental design and operation condition:

The experiment follows two pathways, each consisting of 6 batches of 500 mL flasks in triplicate. In pathway one, the flasks were seeded from an AD lab reactor dealing with vegetable waste, whereas in the second pathway, the flasks were seeded with an inoculum from a lab reactor dealing with bovine fat. Both pathways were run under initial fat concentrations of 0, 10, 25, 50, 75, 100% w/w of the total volatile solids of 20g L^{-1}, in co-digestion with vegetable waste (Figure 6-1).

36 reactors with a working volume of 500 mL each were used. Each reactor consists of a wide neck 500 mL flask equipped with a magnetic bar for mixing purposes and a large rubber bung which been customised to hold a standard pH probe and three glass tubes for sampling, feeding, and gas collection (Figure 6-2 (a)). All reactors were placed in water baths equipped with submersible

heaters to control the temperature and water pumps to circulate the water thus maintaining a uniform temperature. The reactors were connected to 36 water displacement gas collectors for measuring and sampling the biogas. The pH probes were connected to two continuous monitoring pH units which had been built for this project. All pH probes were previously tested and calibrated to pH 7, and pH4 using standard calibration buffers. Multi-place magnetic stirrers were placed under the water baths ensured continuous mixing (Figure 6-2 (d)).

The temperature condition for both pathways was mesophilic and set to 35±1°C. Both seeding sludges (adapted and not adapted to fat), were incubated at 35°C, until degassed before being used to inoculate the experiment. As can be seen from Table 6-1, the original total volatile solids of the fat adapted seeding sludge was very low (1.35 mg L^{-1} TVS), therefore the inoculum to substrate (I/S) ratio, was 0.0675 in terms of the VS content of the seeding sludge and the substrate feed, for both experimental pathways. The non-acclimatised seeding sludge was diluted to the same I/S ratio inside the reactors by adjusting the volume with distilled water. The amount of fat adapted seeding sludge added at the beginning of the first experimental pathway was 50 mL for each flask reactor, equal to 10% of the total working volume of the reactor. The amount of the non-adapted seeding sludge added in the second experimental pathway was 14 mL per flask followed by 36 mL of distilled water to obtain the same I/S ratio conditions for both pathways.

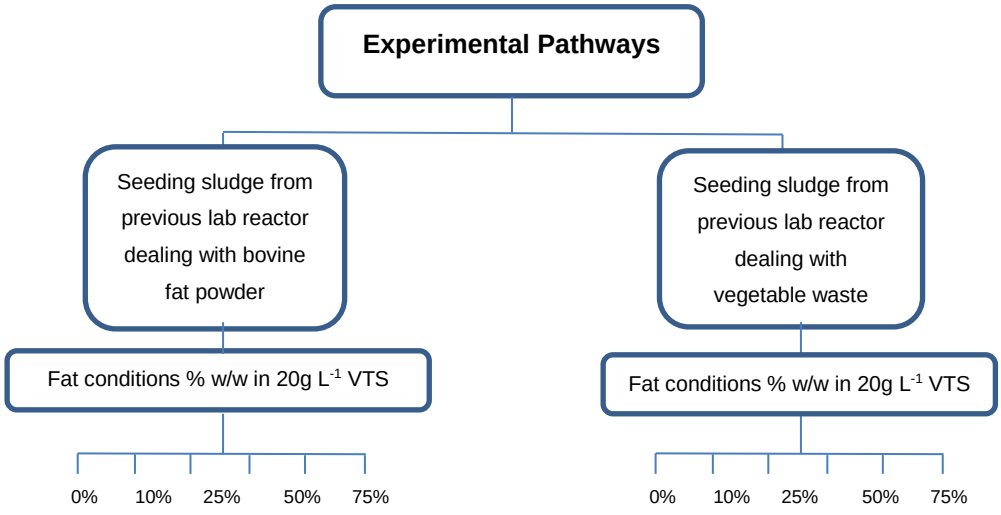

Figure 6-1: Experimental conditions for the continuous pH monitoring 500 mL total volume flask digesters.

Table 6-1 Original total volatile solids for both seeding sludge sources after degassing and before starting the experiment.

Seeding sludge	Seeding sludge source	Original total volatile solids (mg L^{-1})	*SD*
Adapted to fat	From a 1.5L laboratory CSTR dealing with fat.	1.35	0.046
Not adapted to fat	From a 5 L laboratory CSTR dealing with vegetable waste.	4.84	0.443

After the required amounts of fat powder and vegetable material were placed in the digesters (Figure 6-2 (b and c)), the required volume of distilled water was added. Drops of either sodium bicarbonate ($NaHCO_3$) or hydrochloric acid (HCl) were added to the reactors to adjust the pH to 7. The reactors were then tightly closed with the customised rubber bungs and sealed with silicone sealant. After placing the reactors in the temperature controlled water bath, the required amount of seeding sludge was transferred under nitrogen flush followed by further sparging with nitrogen gas for 15 min through the sampling tube to ensure the required anaerobic conditions. Re-seeding was repeated three times (on days 40, 75, and 110) during the experimental run.

Figure 6-2: Setting up the 36 flask reactor experiments.

a: building and preparation of the experimental reactors.
b: measuring the volatile solids contents of the seeding sludge to control and equalise the I/S ratio conditions for the experimental pathways.
c: the required amounts of fat powder and vegetable substrates were placed in the flask digesters
d: experimental setup was connected, flushed with nitrogen, inoculated, and started.

6.3 Results and discussion

6.3.1 Biogas production:

Under the same conditions of 20g L^{-1} total volatile solids, the results from both pathways show a direct influence of the proportion of fat powder making up the 20g L^{-1} VS feed, and the reseeding steps on both the time to production and total methane gas produced (Figure 6-3 and Figure 6-6).

Overall, the fat acclimatised anaerobic seeding sludge shows a better ability to deal with the substrates than non-acclimatised seeding sludge. The initial I/S ratio of 0.0675 was clearly insufficient to start up methane production from the reactors in both experimental pathways (Figure 6-3 and Figure 6-6).

Re-inoculating the reactors had a positive effect on methane production, especially at lower concentrations of fat (Figure 6-3).

6.3.1.1 *Methane gas production from the first experimental pathway:*

In the first experimental pathway, where seeding sludge acclimatised to fat was used, very long lag phases were observed before methane production star, even at a fat concentration of 0% (Figure 6-3). The reason seems to be the low I/S ratio which results in insufficient anaerobic microorganisms in the reactors. Starting up a mesophilic anaerobic reactor with a low seeding sludge concentration has been shown to require more time and more careful management compared to the use of a high concentration of seeding sludge (Hatzigeorgiou *et al.* (2006). A similar effect of extended lag phase as a result of using a low I/S ratio (0.1) was also observed by Ma *et al.* (2015) in a study of LCFAs inhibition of the anaerobic digestion of algal biomass at different inoculum to substrate ratios. As mentioned by Maier *et al.* (2009), in some cases a very small initial population can be the reason for a long lag phase in microorganism growth and function.

During the first 40 days of the experiment, there was no significant methane production from any of the experimental reactors. Even after the first re-inoculation, performed on day 40, there were no noticeable change in the gas production behaviour, and the lag phases remain until day 75. After the second re-inoculation on day 75, methane gas production started from the reactors with

0% fat condition, and from only one reactor out of the three replicates running with 10% fat (a and b in Figure 6-3). However, methane production was observed from most of the reactors after the third re-inoculating step on day 110, except one of the replicated reactors running at 50% fat and two of the replicated 100% fat reactors (Figure 6-3). These re-inoculations increase the bacterial biomass and are found to be one of the best strategies to recover a reactor from LCFAs inhibition (Palatsi *et al.*, 2009).

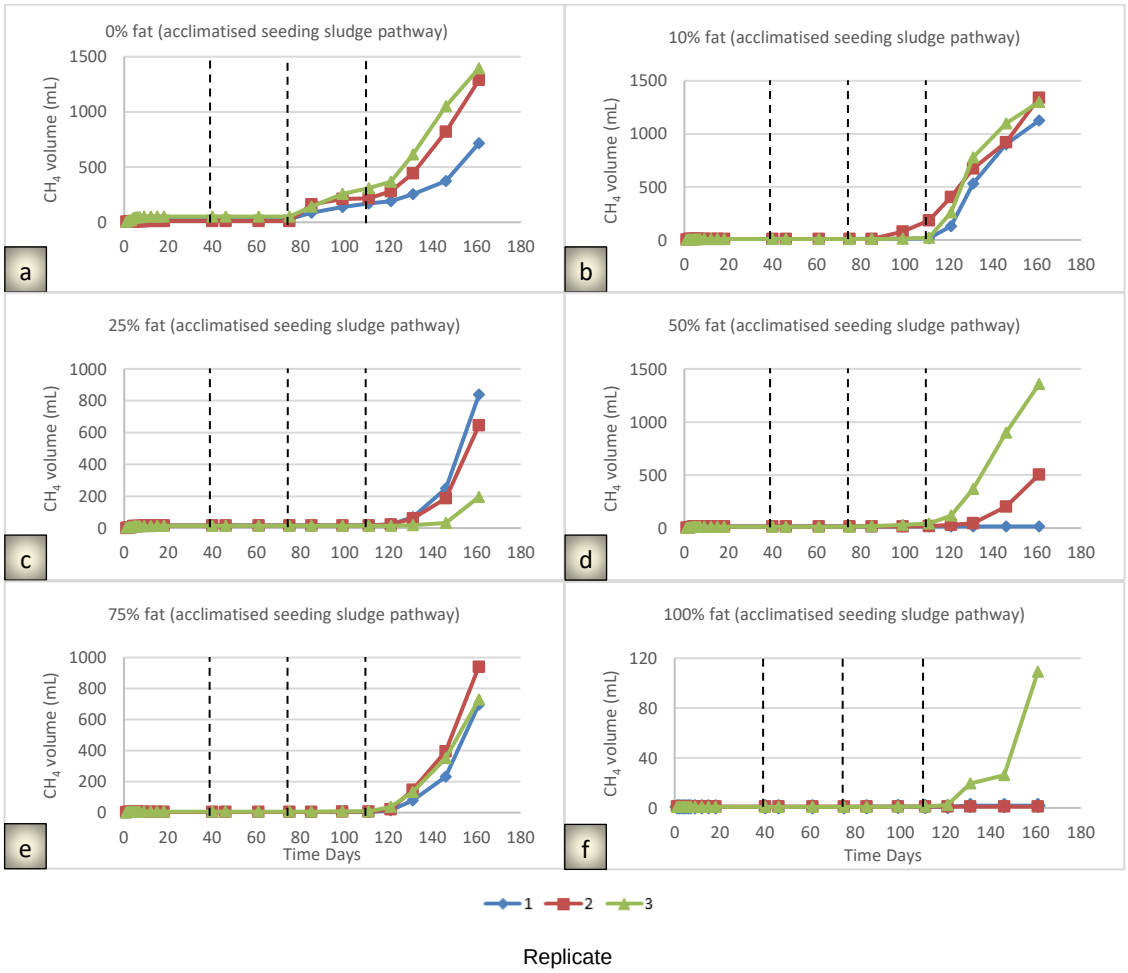

Figure 6-3: Cumulative methane production from the first experimental pathway.

The reactors were inoculated with a seeding sludge acclimitised to fat from a previous working lab reactor.
X axis represents the time duration in days; Y axis represents the methane accumulation in millilitres. The three dashed lines on days 40, 75 and 110 represent the re-inoculating process with seeding sludge. The three coloured lines (blue, red and green) represents the three replicated reactors under each condition.

According to the theoretical methane production, reports in the literature, and the experimental results discussed in Chapter 5, it is expected that higher fat conditions will lead to higher methane production. However, the current total methane production results in Figure 6-4 showed a different scenario, where higher fat concentrations can lead to lower methane production, possibly due to the lower inoculum provided to the reactors in the face of higher inhibitory level of fat concentrations. The highest methane production was obtained from the third replicate under the feed condition of 0% fat with 1395.29 mL of methane gas whereas the lowest methane production was observed to be from the second and first replicates of 100% fat feed with 1.04 mL and 1.78 mL of methane gas respectively (Figure 6-4).

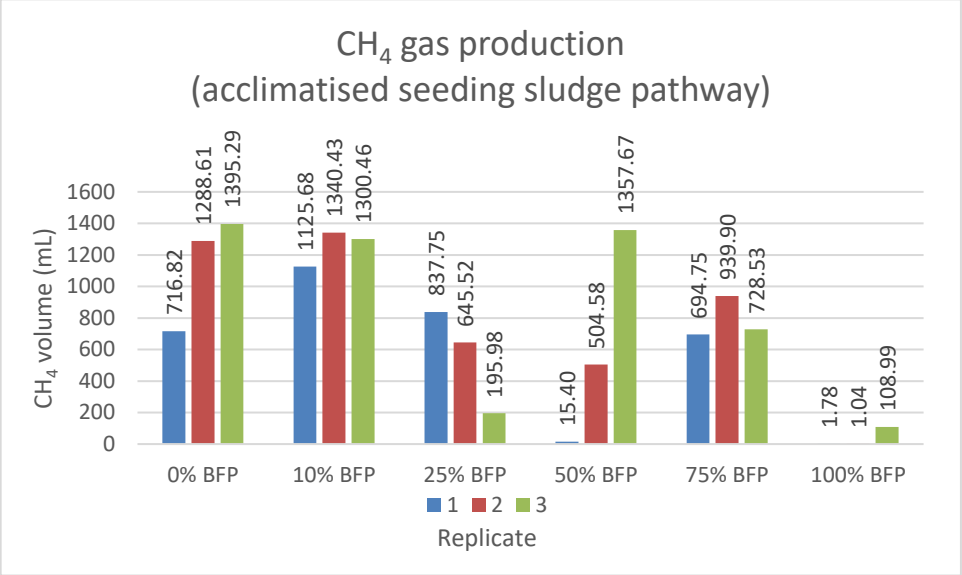

Figure 6-4: Total methane production from the first experimental pathway of 500 mL working volume flask digesters at different fat powder concentrations (0%, 10%, 25%, 50%, 75%, and 100% w/w) for 20g L^{-1} total VS in a co-digestion with vegetable waste.

X axis represents the experimental fat conditions; Y axis represents the total methane production in millilitres. The three colours (blue, red and green) of the columns represent the three replicated reactors under each condition.

Under each fat condition, the replicate reactors show some variations in cumulative methane gas production. These variations can be slight, as in (b) and (e) in Figure 6-3, or more marked, like the results in (d) and (f) in Figure 6-3. The total methane production, illustrated in Figure 6-4, provides a clearer indication of the variation between replicates for each fat percentage.

The possible reason for the variations in some of the experimental replicates can be related to the nature of the seeding sludge used. The seeding sludge naturally contains some solids and non-digested residue of organic materials such as fibres. The surfaces of these materials can be used by the bacteria to attach and grow forming a biofilm. The anaerobic microorganisms can also exist in clumps among the seeding sludge. In addition, rapid sedimentation of solid particles was observed in the acclimatised seeding sludge during the preparation for total solids and volatile solids analysis (Figure 6-2 (b)). Thus, even when every effort was made to provide the same conditions for each replicate by thorough mixing and fast transfer of seeding sludge, it is very difficult to control the microorganism content and concentration due to the nature of the material (Figure 6-5).
It is believed that the amount of solid material might be not the same for all the replicates, and this might be a possible reason for the variation in results between the replicates.

Comparing the variation between replicates in this chapter with the results from Chapter 5, the variation between replicates is higher with a less dense and lower volatile solids seeding sludge (and, therefore, low I/S ratio ≤0.25). There is lower variation with denser seeding sludge and higher I/S ratio of between 0.5 and 1. This is believed to be due to sufficient amounts (and possibly, types) of anaerobic microorganisms being provided to the reactors leading to more stable processes.

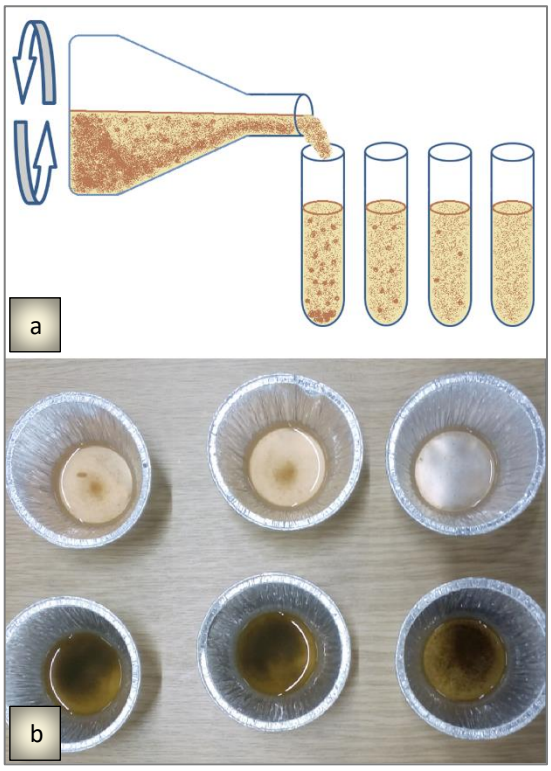

Figure 6-5:Demonstration of the difficulties in control of the solid content among the same equal volumes of seeding sludge due to low density and fast sedimentation.

a: an Illustration of the difficulty in obtaining equal solids content even with continuous mixing. b: an example of solids content after transferring equal volumes of seeding sludge under a mixing process.

6.3.1.2 *Methane gas production from the second experimental pathway:*

In the second experimental pathway, where a seeding sludge not acclimatised to fat was used, similar results of long lag phases before methane production were observed under all of the experimental feed conditions.

In this pathway, there was no significant methane production from any of the experimental reactors during the first 40 days of the experiment. This situation remained the same with no active production of methane gas even after several re-inoculating steps. The first re-inoculating step was at day 40, but no increase in methane production was observed. After the second re-inoculating step on day 75, only one replicate out of three running at 75% fat showed an increase of methane production after 30 days from the re-inoculation step as illustrated in Figure 6-6 (e). Most of the experimental reactors showed no improvement in methane production even after the third re-inoculating step made on day 110.

Only 7 reactors out of 18 showed average methane production, 6 of these started methane production after the third and final re-inoculating step.

This very long lag phase in some reactors and the failure of start-up in other reactors is believed to be due to an insufficient amount of anaerobic microorganisms added to the reactors for a rapid start of activity and a low I/S ratio. In a study by Ma *et al.* (2015), a similar delay in starting up of methane production and an extended lag phase were experienced at a low I/S ratio of 0.4, while at lower I/S ratio of 0.1 the experiment ended still in the lag phase, with an incomplete process and, therefore, ineffective production of methane.

Overall, the total methane production in this experimental pathway showed considerably lower methane production compared to the first pathway which used an acclimatised seeding sludge. In a comparison of the two pathways, the highest methane production of the replicates at 0% fat was 1395.29 mL for the acclimatised pathway compared to only 329.45 mL in the second pathway. Similarly, at 10% fat, the highest methane production was 1340.43 mL from replicate 2 in the first experimental pathway, compared to 125.15 mL for the highest replicate in the second experimental pathway. At 25% fat, the highest replicate in the first experimental pathway was 837.75 mL compared to only 15.56 mL in the second pathway.

For 50%, 75% and 100% w/w fat, the highest methane production among the replicates of the first experimental pathway were 1357.67 ml, 939.9 ml, and 108.99 mL compared to 118.28 mL, 398.53 mL, and 2.59 mL respectively.

The highest methane production among the replicates at 50% and 75% fat conditions in the second experimental pathway, (represented by red columns in Figure 6-7), are considered as out of range in this study, (due to the massive difference between replicate number two and the other replicates of the same 50% and 75% fat conditions). In this case, it can be stated that less methane was produced from the second experimental pathway with non-acclimatised seeding sludge, compared to the first experimental pathway with acclimatised seeding sludge. The methane production from the second pathway was lower than the first pathway by 76.75%, 90.7%, 98.14%, 99.5, 99.92%, and 97.63 at the fat conditions of 0%, 10%, 25%, 50%, 75%, and 100% w/w respectively.

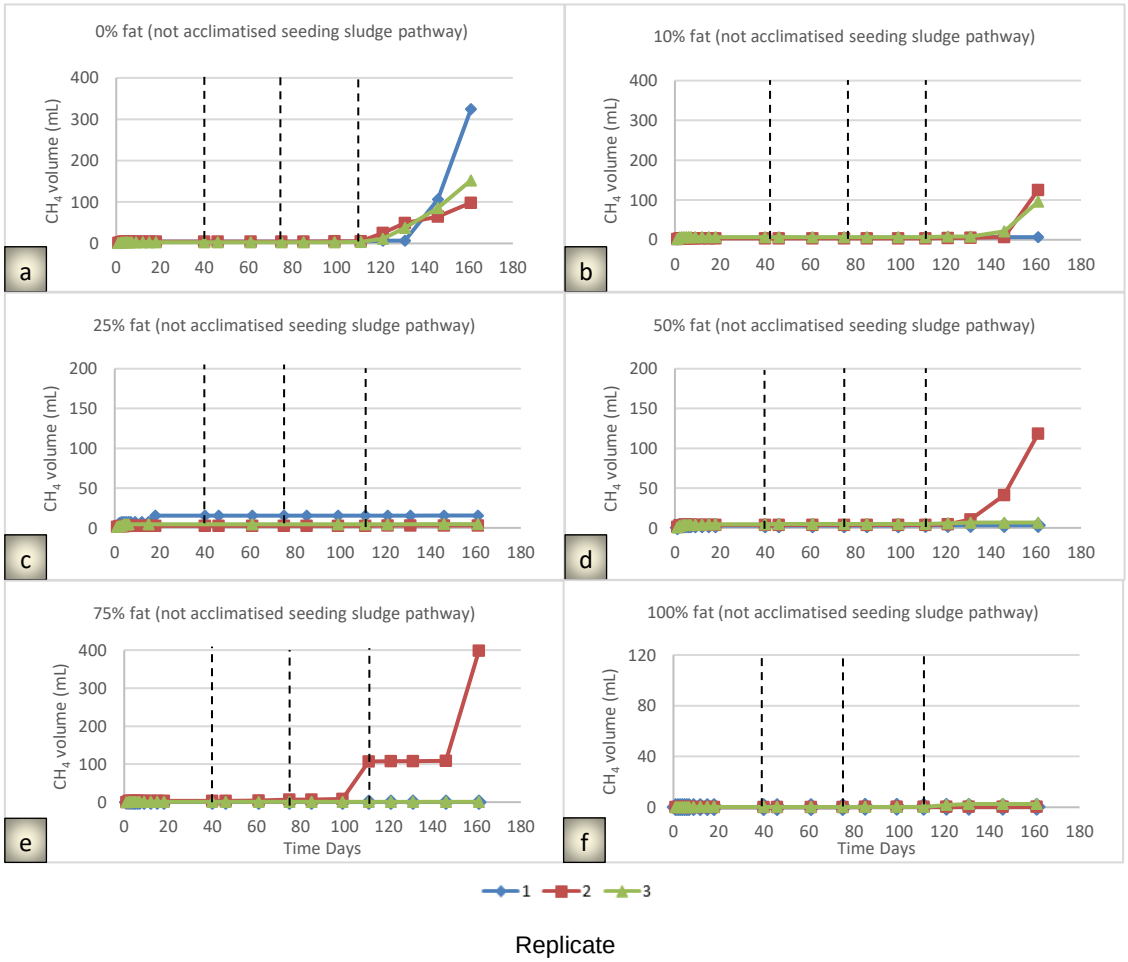

Figure 6-6: Cumulative methane production from the second experimental pathway.

The reactors were inoculated with seeding sludge not acclimitised to fat.
X axis represents the time duration in days; Y axis represents the accumulation of methane in millilitres. The three dashed lines on days 40, 75 and 110 represent the re-inoculating process with seeding sludge. The three coloured lines (blue, red and green) represents the three replicated reactors under each condition.

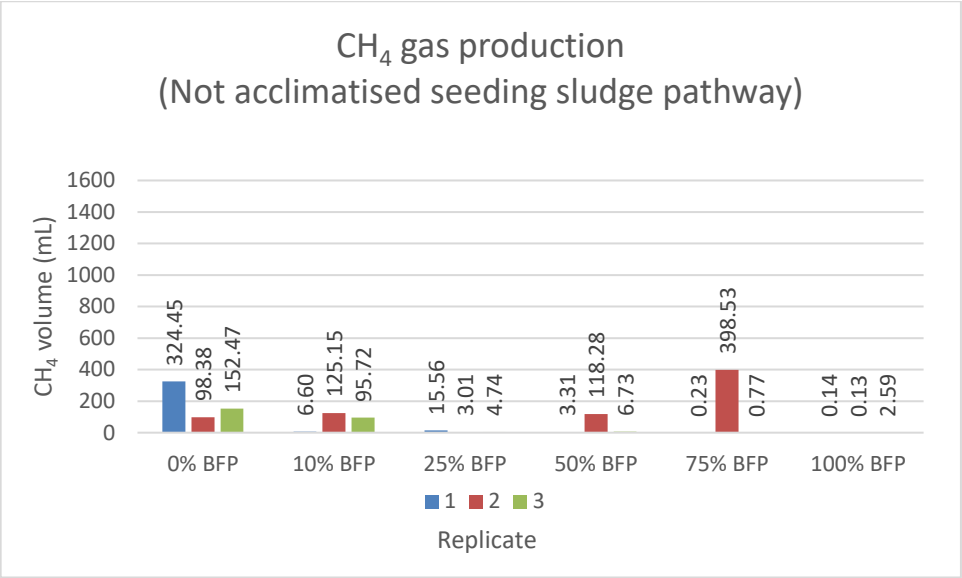

Figure 6-7: Total methane production from the second experimental pathway of 500 mL working volume flask digesters at different fat concentrations (0%, 10%, 25%, 50%, 75%, and 100% w/w) making up 20g L^{-1} total VS in a co-digestion with vegetable waste.

X axis represents the experimental fat conditions; Y axis represents the total methane production in millilitres. The three colours (blue, red and green) of the columns represents the three replicated reactors under each condition.

The lower methane production from the second experimental pathway, compared to the first experimental pathway, is believed to be due to both the low I/S ratio and a shock effect. In the second experimental pathway, non-acclimatised seeding sludge was transferred from a reactor dealing with vegetables to a new type of substrate with different levels of fat concentrations which caused a shock effect for the non-acclimatised microorganisms. However, in case of the 0% fat condition, the results of methane production were also lower compared to the ones from the first experimental pathway that had been seeded with seeding sludge acclimatised to fat. This is believed to be due to the low I/S ratio and insufficient active microorganisms to start-up the process. In the case of seeding sludge acclimatised to fat, the inoculum was transferred from the stressful environment of a lab reactor dealing with 100% fat to a less stressful condition of 0% fat, which, while there still could be a shock effect, the microorganisms should easily break down and generate more methane gas compared to the second experimental pathway.

6.3.1.3 *Hydrogen gas production from the first experimental pathway:*

Hydrogen production from the first experimental pathway occurred early in each experimental run (Figure 6-8). The highest levels were obtained from the reactors running at 0%, 10%, and 100% fat conditions (Figure 6-8 and Figure 6-9).

As can be seen from Figure 6-9, it is clear that hydrogen production is negatively affected by the co-digestion conditions of vegetable waste and fat. Introducing fat to the vegetable waste in co-digestion showed a continuous decreasing effect in hydrogen gas production with increasing fat in the mixture (Figure 6-9 b). Hydrogen production continuously decreased from an average of 114.70 mL at 10% fat to an average of 8.58 mL at 75% fat.

However, the highest hydrogen gas production was from the reactors running on 100% fat, with an average of 190.76 mL hydrogen. This is associated with a decrease in the pH in these reactors as can be seen in Figure 6-15 (graphs: p, q, and r), which is a clear indication of hydrolysis and acidogenesis microorganism activity.

Through a combination of the overall results of hydrogen production (Figure 6-9 b), pH (Figure 6-15) and visual observations of the experimental reactors during the first week of the experimental run (Figure 6-10), it is believed that the decrease in hydrogen production with the increasing proportions of fat could be due to the inhibitory effect of fat on the microorganisms, especially at this very low I/S ratio of 0.0675. Fast solidification and separation of fat from the water based medium due to the hydrophobic properties of fat, was only observed in the 100% w/w fat condition in the early stages of the experiment.

It is believed that, during the co-digestion conditions of fat and vegetable waste, for example in the experimental conditions of (10%, 25%, 50%, and 75% w/w fat), vegetable waste particles were disrupting the accumulation of fat by providing more alternative contacting surface area, and thus preventing the fat particles from forming larger solid parts as happened in the 100% fat condition (Figure 6-10). Therefore, it is believed that co digestion could help to minimise the ability of small fat particles to accumulate together causing fat solidification or separation from the reactor medium. The higher amount of hydrogen

produced from the 100% fat reactors is thus believed to be due to the lower fat surface area (due to fat separation) and thus, less direct contact between the microorganisms and the inhibitory material compared to the co-digestion mixtures.

Within a single experimental condition, the replicate reactors showed inconsistent hydrogen production, similar to the variation in the methane gas production that occurred at the I/S ratio of 0.25 in the previous chapter (Chapter 5). The reason is probably the same, that the I/S ratio is borderline between sufficient and non-sufficient inoculum for the anaerobic process to establish. But in the case of hydrogen production, the reason is believed to be the extremely low I/S ratio of 0.0675, resulting in insufficient levels of hydrolytic and acidogenic microorganisms to produce consistent hydrogen production in contrast to the results shown in chapter 5 under an I/S ratio of 0.1. Another factor is variation in the amount of solid particles in the fixed volumes of seeding sludge added to the reactors, even when every effort was made to provide the same conditions for each replicate by well mixing and fast transferring of seeding sludge, it is difficult to control the microorganisms content due to the nature of the seeding sludge (Figure 6-5).

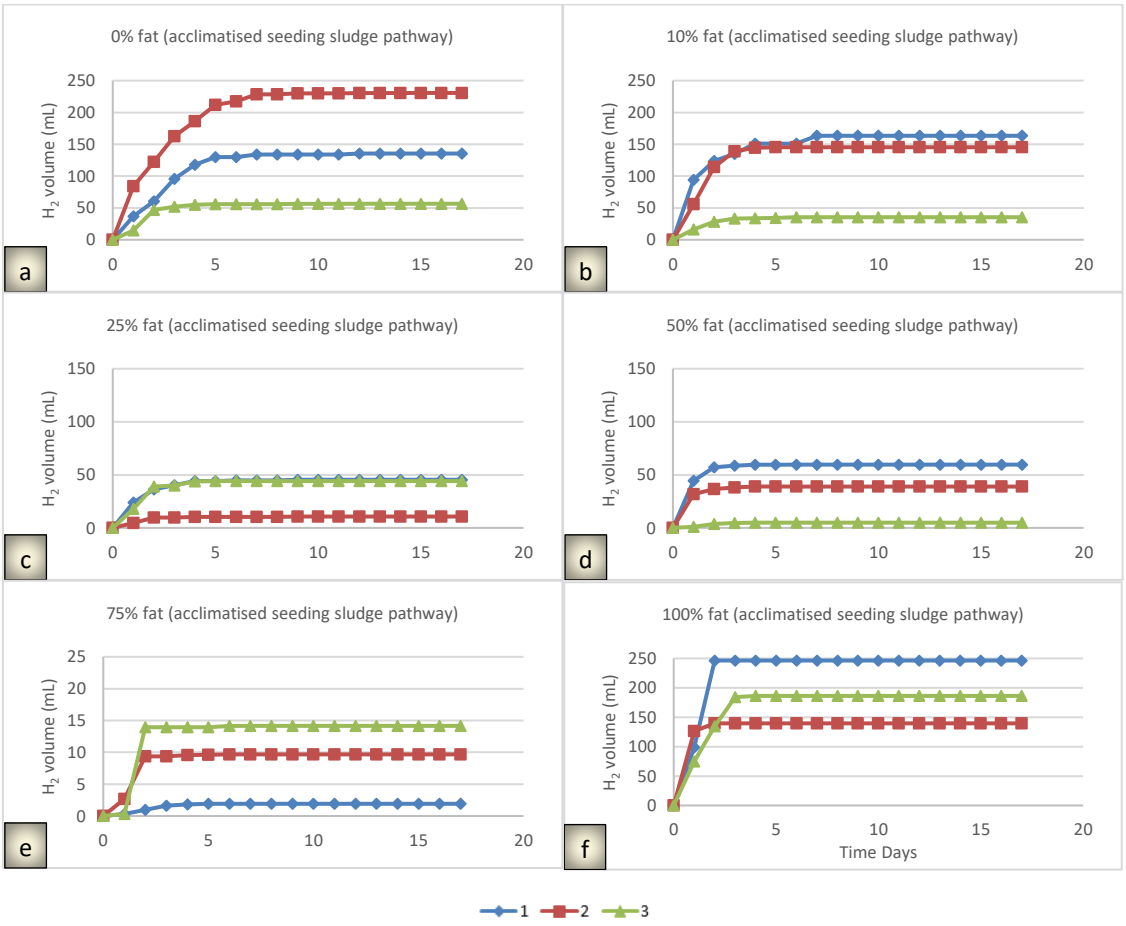

Figure 6-8: Cumulative hydrogen production from the first experimental pathway of 500 mL working volume flask digesters at different fat powder concentrations (0%, 10%, 25%, 50%, 75%, and 100% w/w) making up 20g L^{-1} total VS in co-digestion with vegetable waste.

The reactors were inoculated according to the first pathway conditions, with a seeding sludge acclimatised to fat from a previous working lab reactor.
X axis represents the time duration in days; Y axis represents the hydrogen cumulative in millilitres. The three coloured lines (blue, red and green) represents the three replicated reactors under each condition.

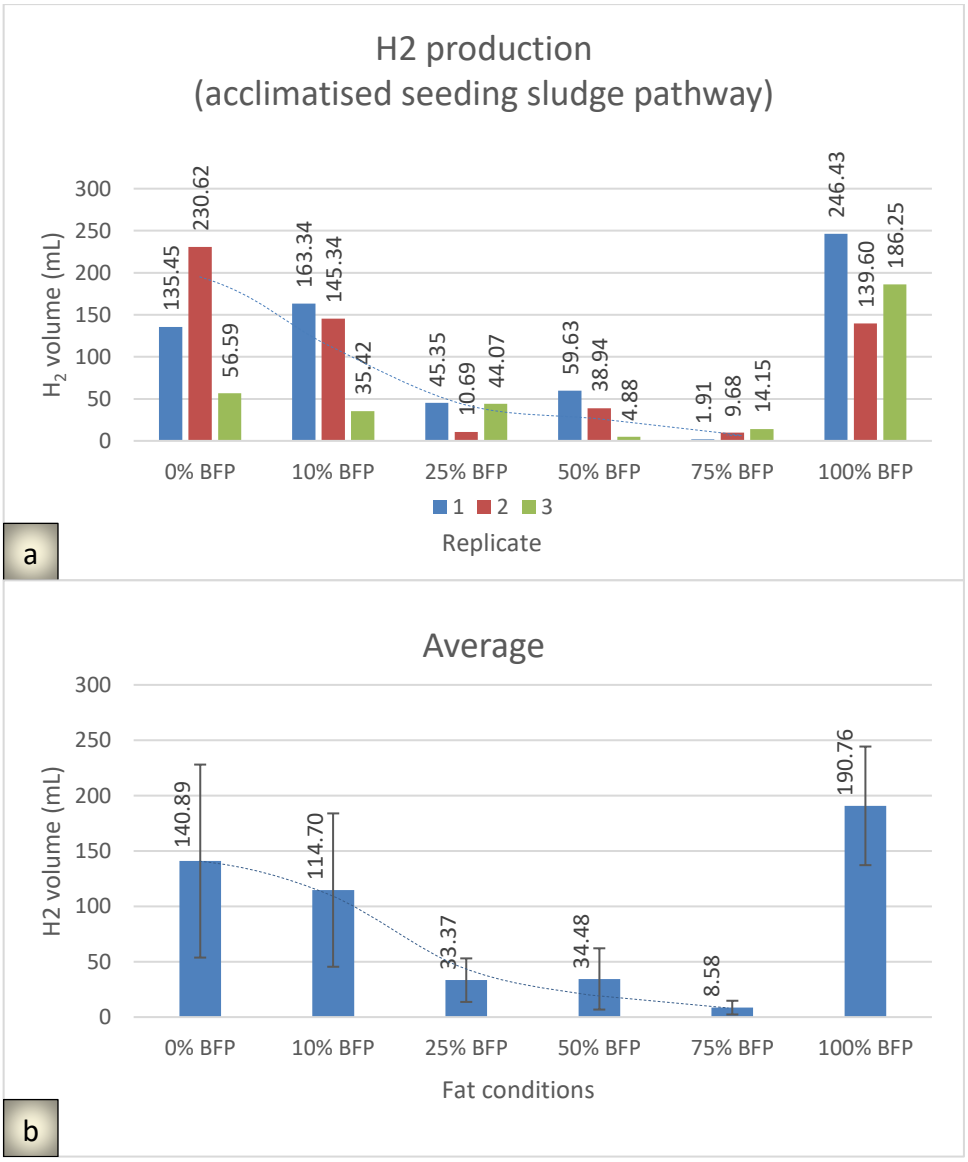

Figure 6-9: Total hydrogen production from the first experimental pathway. 500 mL working volume flask digesters at different fat powder concentrations (0%, 10%, 25%, 50%, 75%, and 100% w/w) making up 20g L^{-1} total VS in co-digestion with vegetable waste. (Error bars represent standard deviation of samples from three replicate reactors.)

a: Hydrogen production from replicates reactors under each fat concentration.

b: Average of hydrogen production under each fat concentration.

X axis represents the experimental fat conditions; Y axis represents the total hydrogen production in millilitres. The dashed line represents the hydrogen production behaviour for feed conditions between 0% to 75% fat. The three colours (blue, red and green) of the columns represents the three replicated reactors under each condition.

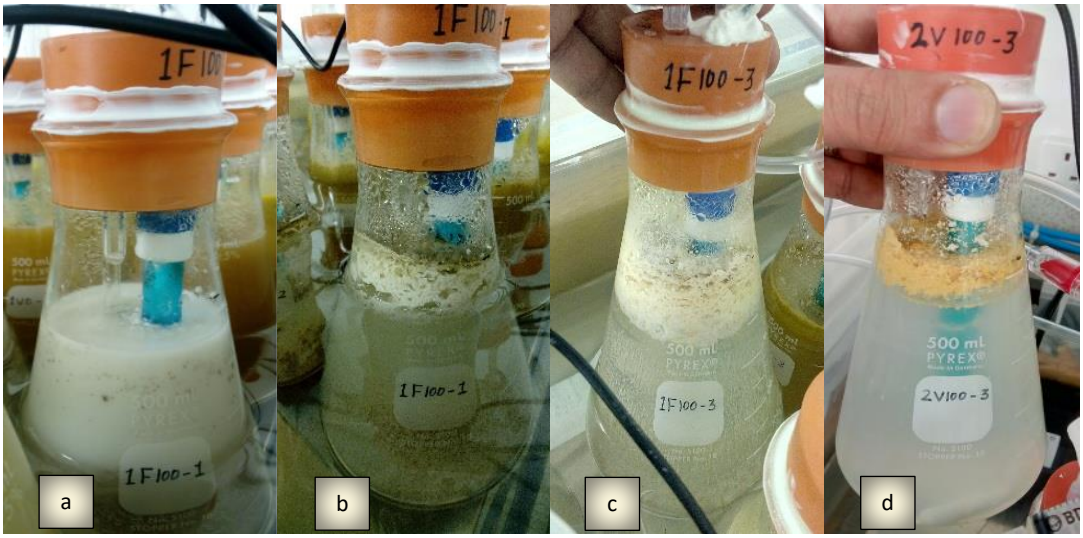

Figure 6-10: Hydrophobic separation of fat in the 100% fat anaerobic reactors early in the experiment (During the first five days).

a: Fat powder formed a suspension in the water based medium (day 0).

b: Fat starting to separate, forming a layer on top of the water based medium (day 1).

c: Fat continues separate to the top and the medium becomes semi-clear (day 2).

d: Fat starts to solidify on top of the media, the medium becomes clearer (day 5).

6.3.1.4 *Hydrogen gas production from the second experimental pathway:*

Similar overall hydrogen production behaviour was observed in this pathway as in the first pathway. Higher production was found from reactors running with 0%, 10%, and 100% fat. Hydrogen production shows sensitivity to the fat content of the co-substrate mixtures with a decrease in hydrogen production associated with an increase of the percentage fat in the co-digestion mixture (Figure 6-12). The hydrogen decreased from 68.45 mL at 10% fat to 3.07 mL at 75% fat. However, the highest hydrogen production was, again, from the reactors running at 100% fat with an average of 212.49 mL. This may be due to the same factors which were given for the first pathway section. The decrease in hydrogen production under co-digestion conditions seems to be due to the inhibitory effect of fat on the microorganisms especially at this very low I/S ratio of 0.0675. However, fast solidification and separation of fat particles from the water based medium in the 100% fat reactors in the early part of the experiments (shown in Figure 6-10) is believed to lower the exposure of the microorganisms to the inhibitory fatty materials at the beginning of the experiment, and, therefore, allowing the initial processes to start quickly and lead to more hydrogen production. This was associated with a decrease in the pH as illustrated in Figure 6-16 (graphs: p, q, and r), which is a clear indication of hydrolytic and acidogenic microorganism activity.

The replicated reactors again show inconsistent results for hydrogen production at each experimental condition (Figure 6-11). The reason is believed to be due to the extremely low I/S ratio of 0.0675, which results in lower and insufficient numbers of hydrolytic and acidogenic microorganisms to produce consistent levels of hydrogen. Another factor is the solid particle content of the inoculating seeding sludge as discussed earlier under the methane production section (Figure 6-5). Even when every effort was made to provide the same conditions for each replicate by good mixing and fast transfer of the seeding sludge, it is very difficult to control the microorganisms content and concentration due to the nature of the materials.

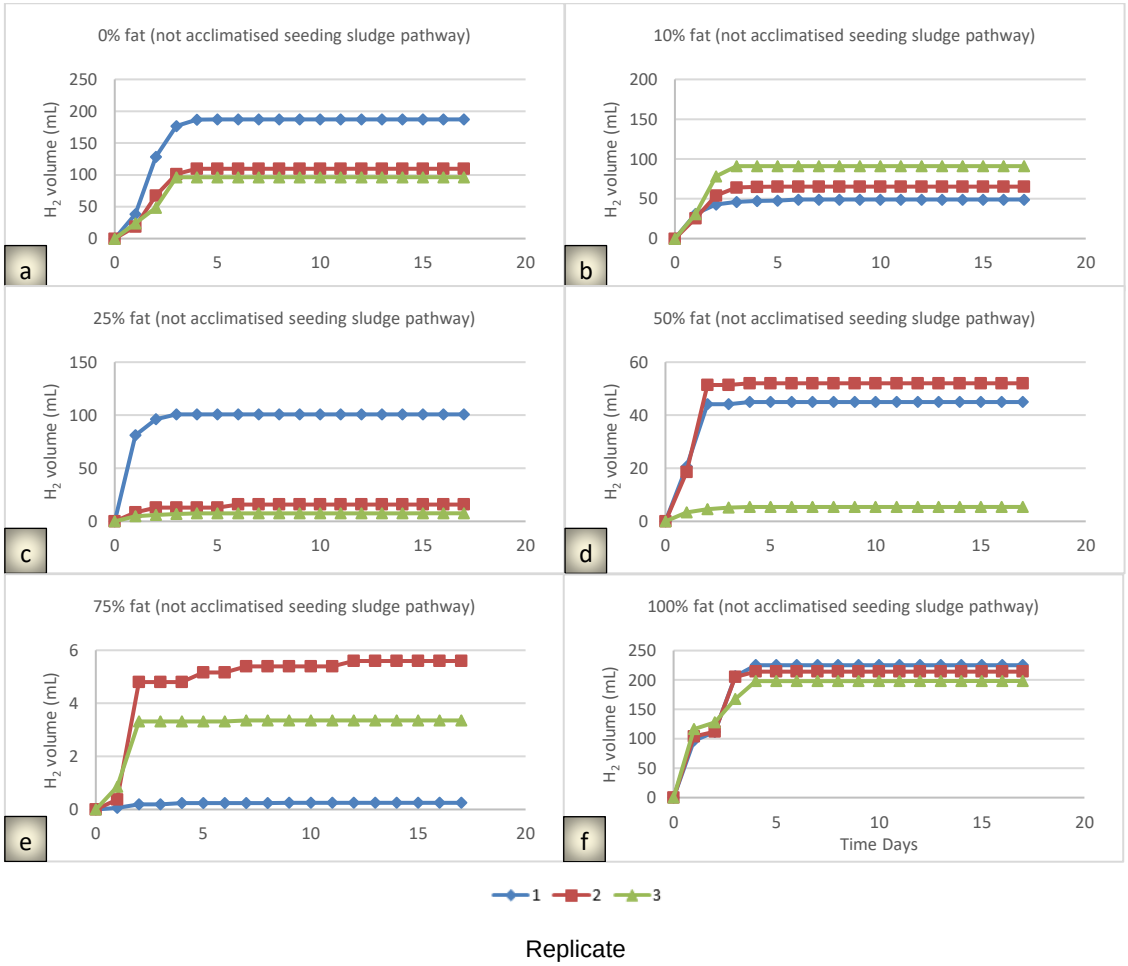

Figure 6-11: Cumulative hydrogen production from the Second experimental pathway of 500 mL flask digesters at different fat concentrations (0%, 10%, 25%, 50%, 75%, and 100% w/w) to the 20g L^{-1} total VS in co-digestion with vegetable waste.

The reactors were inoculated according to the second experimental pathway conditions, with seeding sludge that was not acclimatised to fat.
X axis represents the time duration in days; Y axis represents the hydrogen accumulation in millilitres. The three coloured lines (blue, red and green) represents the three replicated reactors under each condition.

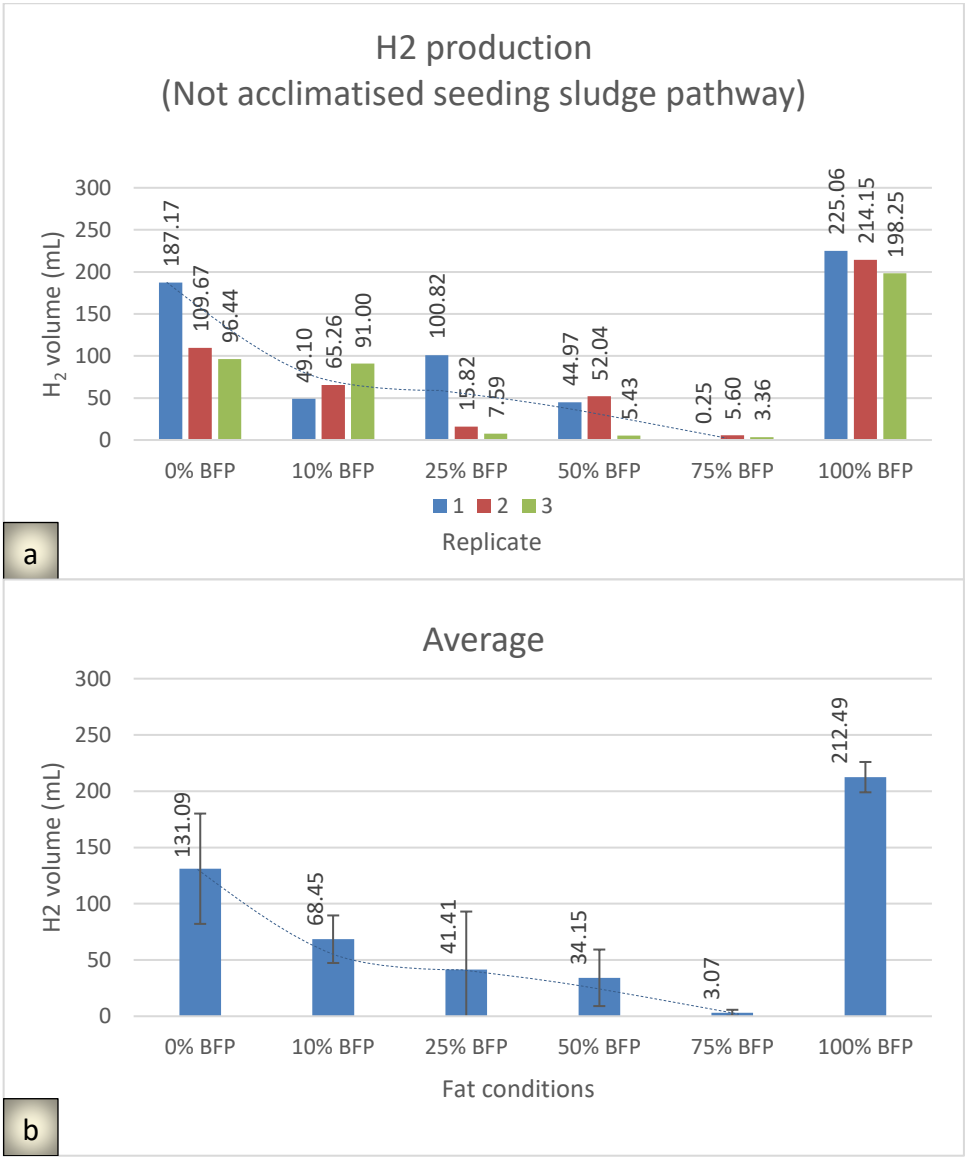

Figure 6-12: Total hydrogen production from the second experimental pathway of 500 mL flask digesters at different fat concentrations (0%, 10%, 25%, 50%, 75%, and 100% w/w) to the 20g L^{-1} total VS in co-digestion with vegetable waste. (Error bars represent standard deviation of samples from three replicate reactors.)

a: Hydrogen production from replicates reactors under each fat concentration.

b: Average of hydrogen production under each fat concentration.

X axis represents the experimental fat conditions; Y axis represents the total methane production in millilitres. The dashed line represents the hydrogen production behaviour for feed conditions between 0% to 75% fat. The three colours (blue, red and green) of the columns represents the three replicated reactors under each condition.

6.3.2 Automatic and manual pH readings:

The pH behaviour under different feed and seed conditions was designed to be monitored automatically through online pH units gathering readings from the 36 standard pH probes in the flasks. At the same time, manual pH readings were performed in parallel to check the results and to find any problems that might be associated with the long exposures of the pH probes to the reactor contents in the automatic monitoring condition, especially at high fat content. The manual pH readings were performed under anaerobic conditions through the use of a simple anaerobic pH probe case that was developed specifically for this purpose (Figure 6-13). The design and construction of the anaerobic probe case is discussed in the materials and methods chapter.

The results give a better understanding of the ability of the standard pH electrodes to consistently work under long exposures to the reactor substrate. It is clear that long exposure leads to more problems in the pH probes accuracy, response and overall performance. However, some of the probes experienced problems early in the experiment. This might be due to the nature of the experimental substrate clogging the small ceramic reference junction or coating the electrode's glass membrane. Manual reading probes have the advantage of being much more flexible to clean, check, and recalibrate when needed.

Discussion of the pH behaviour will refer only to the manual pH readings, as these provide much more reliable results.

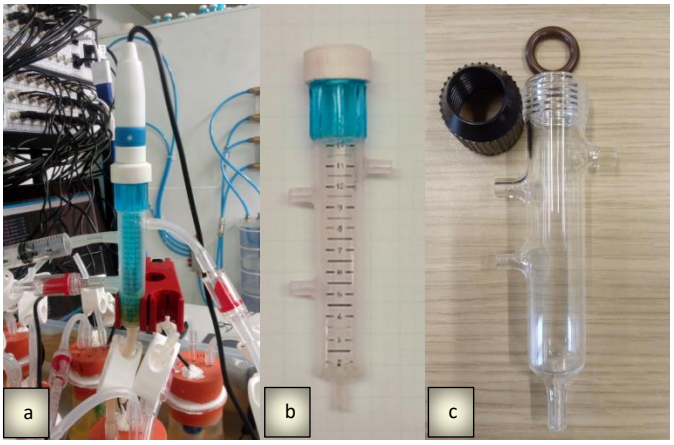

Figure 6-13: The anaerobic pH probe case.

(a: plastic anaerobic pH probe case connected to a small anaerobic digester for pH measurements. b: first plastic prototype pH probe case. c: final glass anaerobic pH probe case).

6.3.2.1 *Automatic and manual pH readings from the first experimental pathway:*

In the first experimental pathway, where an acclimatised seeding sludge was used, the changes in pH behaviour inside 18 anaerobic flask reactors were monitored. Each graph in Figure 6-15 represents both the automatic and manual pH results for an individual reactor.

As illustrated in Figure 6-15 graphs a, b, and c, the manual pH monitoring of 0% fat reactors, showed a decrease in the pH values at the early days of the experiment. This normally results from hydrolysis and acidogenesis processes. The increases in pH values after 75 days is associated with the production of methane (Figure 6-3 graph a). Thus, the increase in pH at this time indicates the removal of organic acids from the reactor through both acetogenic activity, which converts organic acids into acetate, CO_2, and H_2, and then methanogenic activity which converts these products into methane. Similar behaviour was observed for all the reactors under different fat conditions.

The decrease in pH values early in the experiment was associated with hydrogen production. The drop in pH in most of the reactors was 1 to 2 pH units. However, some reactors showed decreases of 3 to 4 units (Figure 6-15 graphs p, q, and r).

The slight fluctuations in the manual pH values inside some of the reactors is believed to be due to the interference between late organic acid production from the degradation of substrate materials and some solidified fat particles (Figure 6-14), which causes the pH to drop, and the acetogenic and methanogenic activity which removes these organic acids and converts them into methane and carbon dioxide, leading to the pH going up.

Combining the results of hydrogen production and pH reduction, it is clear that the amount of hydrogen produced, and the drop in pH both indicate the activity of hydrolytic and acidogenic microorganisms. For example, the reactors running at 100% fat, which show higher hydrogen production, as illustrated in Figure 6-9, also show lower pH values (Figure 6-15 graphs p, q, and r).

Figure 6-14: Examples of solidified fat particles inside some of the reactors.

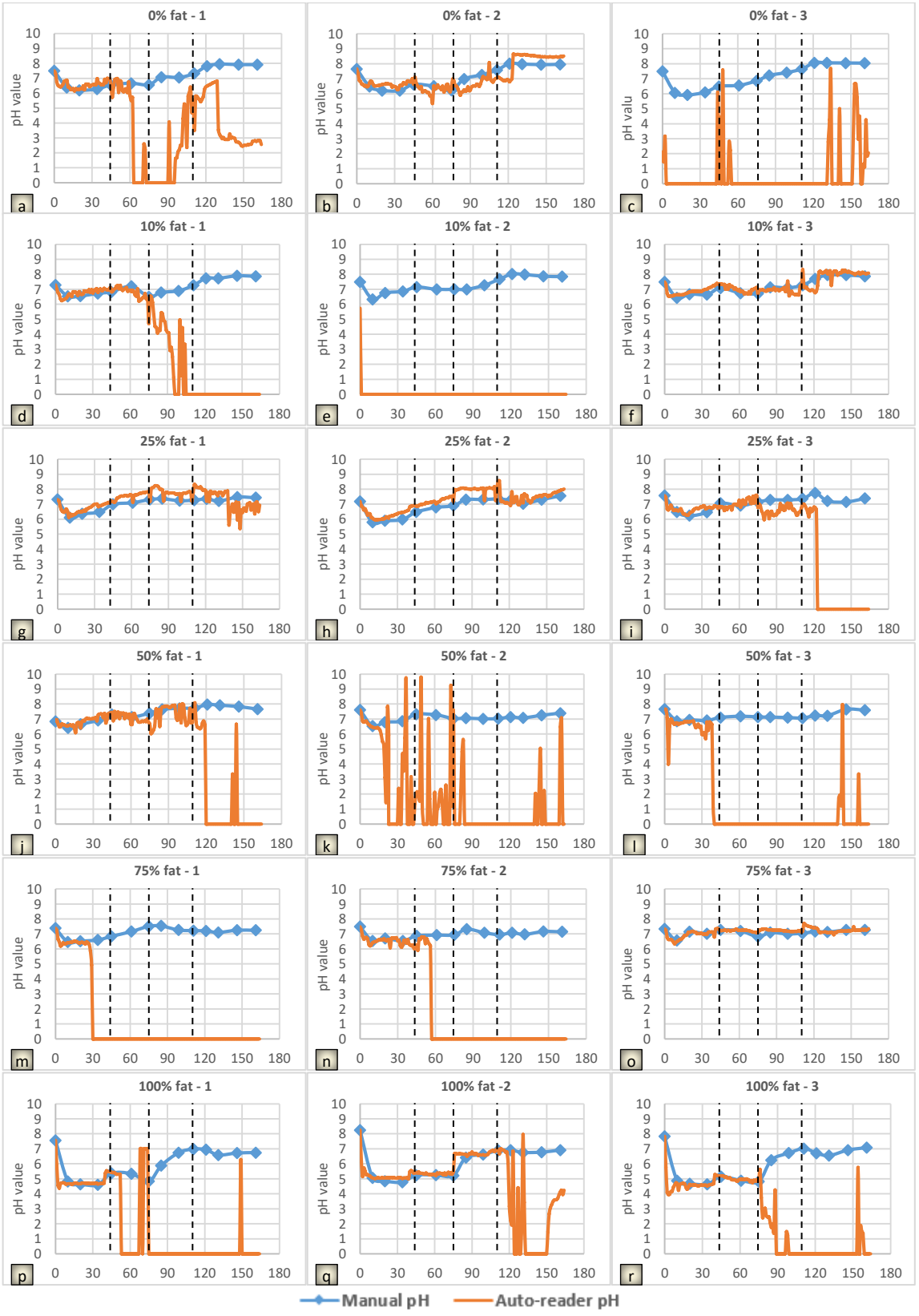

Figure 6-15: Automatic monitoring and manual pH readings from the first experimental pathway reactors.

X axis represents time in days; Y axis represents the pH value. The three dashed lines on days 40, 75, and 110 represents the re-inoculating step with anaerobic seeding sludge.

6.3.2.2 *Automatic and manual pH readings from the second experimental pathway:*

In this experimental pathway, a non-acclimatised seeding sludge was used to inoculate the reactors. The pH behaviour of 18 anaerobic flask reactors was monitored both automatically and manually. Most of the anaerobic reactors showed a decrease in pH value in the early days of the experiment associated with hydrogen production. This is normally associated with hydrolysis and acidogenesis activity, the first two stages in the anaerobic digestion process. The slight increases in pH after day 110 were associated with the production methane gas in some reactors (Figure 6-6 - Figure 6-16). This is due to the conversion of organic acids to methane.

In case of automatic pH monitoring, only four out of eighteen internal pH probes of this pathway were still working, but with some problems (noise, fluctuations, and drift), by the end of the experiment.

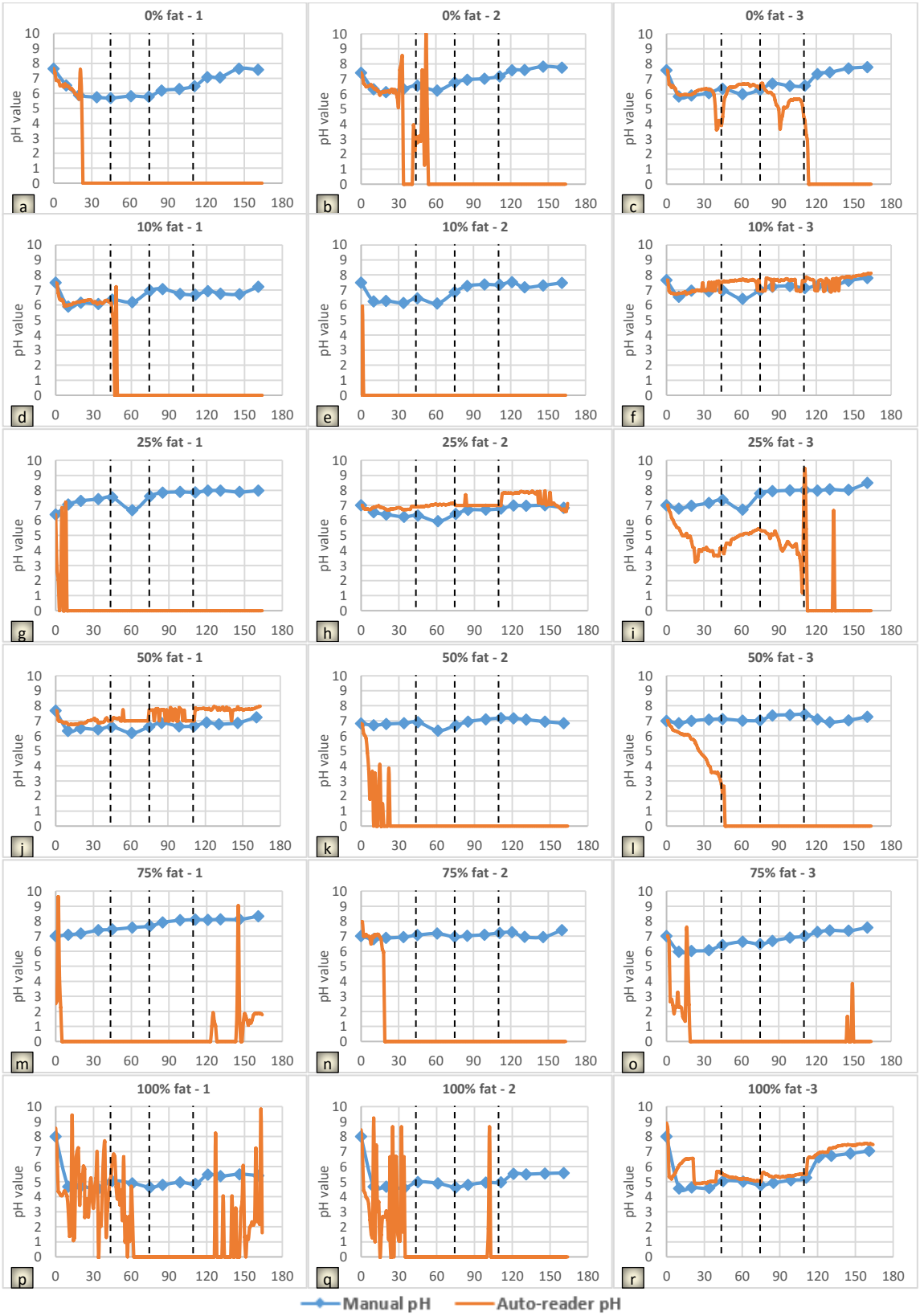

Figure 6-16: Automatic monitoring and manual pH readings from the second experimental pathway reactors.

X axis represents time in days; Y axis represents the pH value. The three dashed lines on days 40, 75, and 110 represents the re-inoculating step with anaerobic seeding sludge.

6.3.2.3 *Problems associated with the automatic pH monitoring:*

As Figure 6-17 illustrates, more than a quarter of the total internal pH probes connected to the automatic pH monitoring system showed functional problems and were faulty by the end of first week of the experiment. With longer exposure time inside the reactors, the number of faulty probes increased.

After 161 days, 75% of the probes were faulty (Figure 6-18). The rest were still working but with some functional issues such as high noise, fluctuations, and drifting. Overall, as can be seen from Figure 6-15 (graphs b, f, g, h, and o) and Figure 6-16 (graphs f, h, j, and r), 8 out of nine working probes were not giving accurate results. Only one pH probe was providing a constant and acceptable monitoring during the experimental run (Figure 6-15 graph o). The problems relate to the long exposure and are probably due to junction clogging by substrate materials, fat coating the electrode's glass membrane (Figure 6-19), and, possibly, electrolyte contamination.

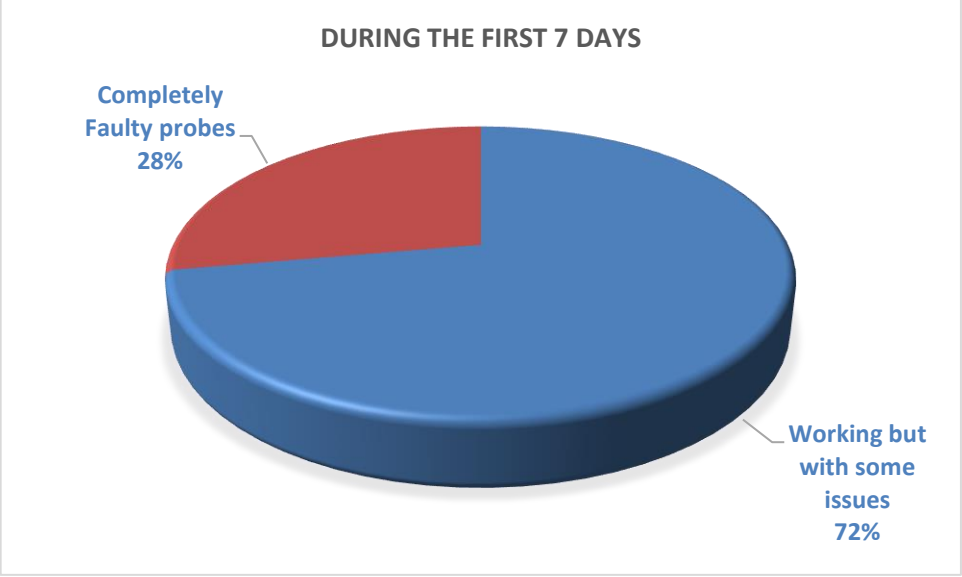

Figure 6-17: The status of the pH probes workability after one week of direct contact with the substrate inside the reactors.

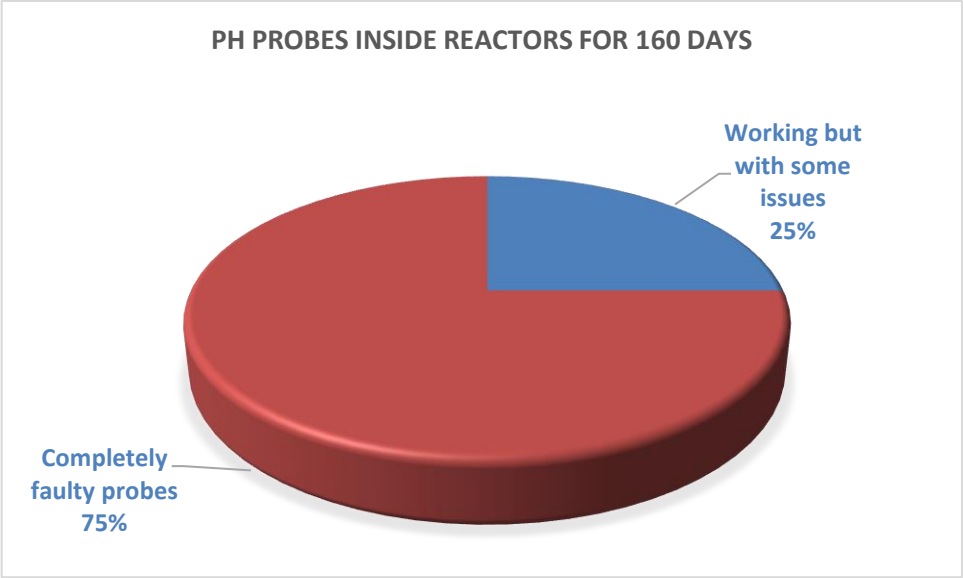

Figure 6-18: The status of the pH probes workability after being in continuous direct contact with the substrate inside the reactors for 161 days.

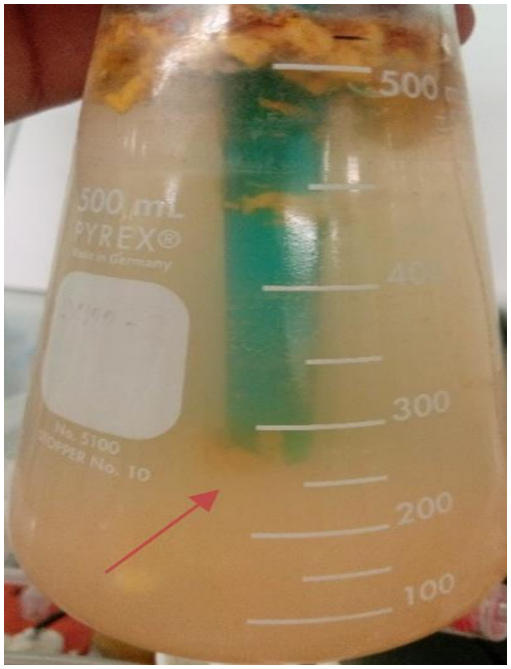

Figure 6-19: Fat accumulation on an electrode's glass membrane and junction area.

6.4 Overall discussion:

6.4.1 Effect of using pre-acclimatised seeding sludge under re-inoculation condition with low I/S ratio:

Overall, pre-acclimatised seeding sludge showed better ability to treat fat than non-acclimatised after several re-inoculating steps on both pathways.

Re-inoculating the seeding sludge led to a start of methane production in most of the experimental reactors. During the first 40 days of the experiment, there was no significant methane production from any of the experimental reactors. Even after the first re-inoculation, performed on day 40, there were no noticeable changes in the gas production behaviour, and the lag phases remain until day 75. The long lag phase is believed to be due to the low and insufficient starting-up inoculum. Similar lag phase was also observed by Ma *et al.* (2015) in a study of LCFAs inhibition to the anaerobic digestion of algae biomass at low inoculum to substrate ratios.

After the second re-inoculation process on day 75, methane gas production started from the reactors with 0% fat, and from only one reactor out of three replicates running with 10% fat. However, methane production was observed from most of the reactors after the third re-inoculating step on day 110, except one of the replicated reactors running at 50% fat and two of the replicated 100% fat reactors. These results are in agreement with a report by Palatsi *et al.* (2009), in a study of anaerobic reactors recovering from LCFA inhibition through diluting the reactor content by re-inoculating steps. These re-inoculations increase the bacterial biomass and are found to be one of the best strategies to recover a reactor from LCFA inhibition.

Less methane was produced from the second experimental pathway with non-acclimatised seeding sludge, compared to the first experimental pathway with acclimatised seeding sludge. The methane production from the second pathway was lower than the first pathway by 76.75%, 90.7%, 98.14%, 99.5, 99.92%, and 97.63% v/v at the fat conditions of 0%, 10%, 25%, 50%, 75%, and 100% w/w respectively.

The lower methane production from the second experimental pathway compared to the acclimatised seeding sludge in the first experimental pathway is

believed to be due to both the low I/S ratio and the shock effect. In the second experimental pathway, non-acclimatised seeding sludge was transferred from a reactor dealing with vegetable material to a new type of substrate with different fat concentrations which causes a shock effect on the non-acclimatised microorganisms. However, in case of 0% fat condition, the results of methane production were also lower compared to the ones from the first experimental pathway that had been seeded with sludge acclimatised to fat. This is believed to be due to the low I/S ratio and insufficient active microorganisms to start-up the process. In the case of seeding sludge acclimatised to fat, the inoculum was transferred from the stressful environment of a lab reactor dealing with 100% fat to a less stressful condition of 0% fat, which the microorganisms could easily break down and generate more methane gas more quickly. Overall, acclimatised seeding sludge does improve the reactor process during co-digestion comparing to non-acclimatised seeding sludge. However, this option required a very long time to start the methane production, and could not deal effectively with fat only substrate (Figure 6-3).

Thus, the next chapter will look into the possibility of improving the yield of gas production over a shorter period of time through physically separating the AD process into two phases in order to minimise the inhibitory effect of LCFAs on the AD process.

Chapter 7 A Comparative study between one and two stage batch reactors

(using pH separation and re-inoculating)

7.1 Objective:

The objective of this chapter is to investigate into the possibility of improving the digestion process and minimising the inhibitory effect of LCFAs through physically separating the AD process into two phases. As identified in Chapter 6, acclimatised seeding sludge does improve the reactor process during co-digestion, though the long duration required for the production of gas makes this option unrealistic for implementation. Thus, separating the phases may aid in improving the yield of gas production over a shorter period of time.

The separation strategy included pH control, re-inoculating to provide new inoculum for the second stage start up, and the use of an up-flow column reactor for the second methanogenic stage (Figure 7-1).

The experimental set consisted of six separate reactors. A CSTR was used for the single stage set of experiments while two reactors (CSTR and Up-flow) were used for the two stage set. Another three reactors (2 CSTR and 1 Up-flow) were used as controls to get better understanding of the factors in this study.

7.2 Experiment design and operation conditions:

Continuously stirred tank reactors were used for the both the single stage reactor and the first stage of the two stage reactors. In the single stage, all of the anaerobic digestion process will take place in one reactor without any buffer addition or re-inoculation steps. In the two stage design, the digestion processes are divided between two separate reactors. The initial processes of hydrolysis, acidogenesis and acetogenesis are expected to take place in the first stage CSTR, while the later methanogenesis step is expected to take place in the second stage, separate, up-flow column reactor (Figure 7-1).

CSTRs (using magnetic stirrers) were chosen for the first stage as it has been reported that strong mixing results in a higher percentage of lipid degradation.

Elefsiniotis and Oldham (1994) found that stronger mixing conditions in a CSTR gave a higher percentage of lipid degradation (63.4–83.2%) compared to a UASB reactor (47.5–67.3%). This is probably due to better enzyme-substrate contact.

An anaerobic up-flow reactor was chosen for the second stage in order to provide conditions whereby methanogenic microorganisms can form a blanket of sludge in the lower part of the reactor while any inhibitory fatty material would accumulate in the higher part of the column. This would allow the microorganisms to reach higher bacterial mass and to gradually break down the fatty materials at the same time.

The stirred tank batch reactors consist of 2L volume quick-fit glass vessels, surrounded by a water jacket for temperature control, magnetic stirrer for mixing, automatic pH controller with acid and base dosing pumps, and a flexible sampling tube connected to a syringe for sampling purposes. The reactor is connected to a gas collector (water displacement system) through a pipe to measure biogas production as well as obtaining gas samples for gas composition analysis.

The up-flow reactors consist of 2L volume acrylic vertical cylinders (8 cm ID and 55 cm L), surrounded by a water jacket for temperature control, centrifugal pump for circulating process (from top down), automatic pH controller with acid and base dosing pumps, and a flexible sampling tube connected to a syringe for sampling purposes. The reactor is connected to a gas collector (water displacement system) through a pipe to measure biogas production as well as obtaining gas samples for gas composition analysis.

Three separate reactors were set as controls for this experimental work. Control No. 1 was a single up-flow single stage column reactor to investigate the effect of the reactor design on the digestion process under the current experimental conditions. Control No. 2 was a single CSTR to study the effect of only the re-inoculating (re-seeding) step without controlling the pH in order to establish the second stage inside the same reactor. Control No. 3 was a single CSTR to study the effect of only pH control without the re-inoculating process (Figure 7-1).

The experimental feed condition was 20g L^{-1} VS derived from 100% bovine fat powder for all experiments. The experiments were run at 35 ˚C (mesophilic).

The initial working volume was 1L for all the reactors at start up. Inoculum to substrate ratio was 0.1 in terms of the volatile solids content for all the reactors. At re-inoculation, the same I/S ratio of 0.1 was used. For pH controlled reactors (the two stage sets, and control No.3) the pH was 5.5 for the first stage (with the controller being set at a lower pH of 5 and higher pH of 6), and 8.5 for the second stage (with the low and high values of the controller set at pH 8 and 9 respectively). The pH was controlled by two automatic pH controllers equipped with alkaline and acid dosing pumps and combination glass pH electrodes. Hydrochloric acid (HCl) was used at a concentration of 1 M to lower the pH and a saturated solution of sodium bicarbonate ($NaHCO_3$) was used to raise the pH. The dosing rate was set at 0.1 mL min^{-1} for both acid and base, and waiting time between doses was set to 5 minutes.

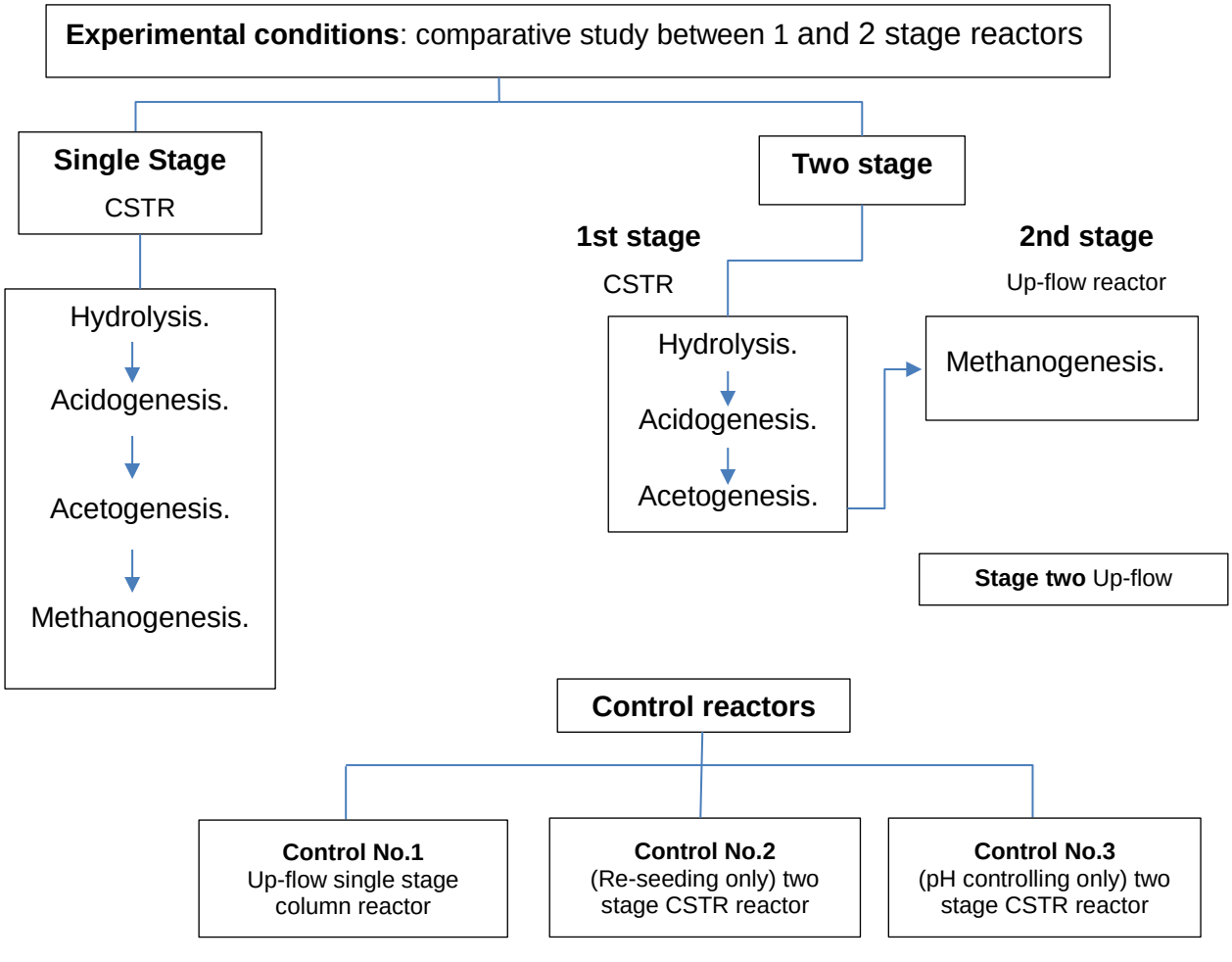

Figure 7-1: Experimental condition for the single and two stages anaerobic reactors

7.3 Results and discussion

7.3.1 Biogas production:

From the overall results, the two stage reactors showed higher methane and hydrogen gas production and higher ability to treat the fat substrate. Control No.3 (the pH controlled CSTR) showed an increase in methane production during the first month, however, the process inside the reactor seems to be interrupted as the reactor stopped producing any gas after 38 days. The rest of the experimental reactors did not generate much methane and were not able to effectively start-up.

7.3.1.1 *Methane production*

As can be seen from Figure 7-2 and Figure 7-3, the highest methane production was obtained from the two stage reactor with a total methane production of 927.82 mL. Reactor control No.3 with only pH control, showed nearly three times lower methane production of 304.67 mL. However, it was still much higher than the single stage, control No.1 (the single column reactor), and control No.2 (the re-seeding step control CSTR).

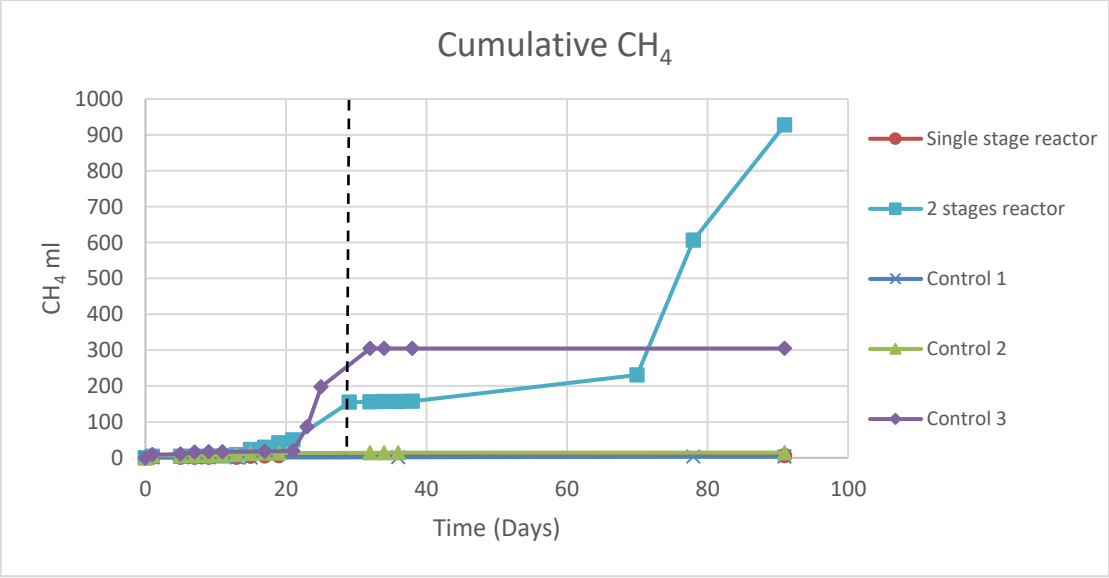

Figure 7-2: Cumulative methane production from the experimental reactors.

X axis represents time in days; Y axis represents cumulative methane in millilitres. The dashed line on day 29 represents the start of the second stage with pH control or reseeding in case of the two stage setups.

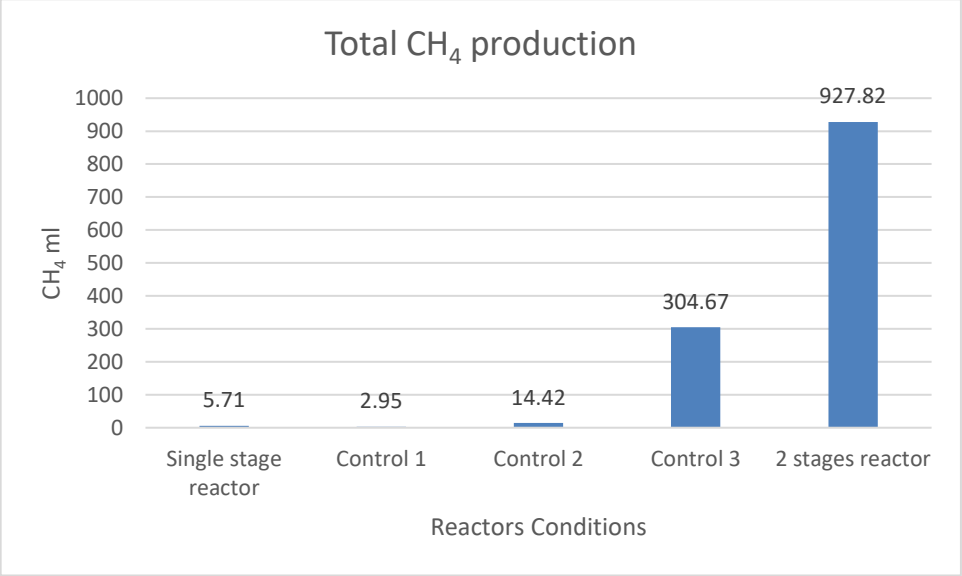

Figure 7-3: Total methane production from the experimental reactors.

X axis represents the reactors conditions; Y axis represents the total methane in millilitres.

As illustrated in Figure 7-3, Control No.2 (with only the re-seeding step) showed slightly better gas production of 14.42 mL than the CSTR single stage reactor with 5.71 mL total methane production and the Up-flow control No.1 reactor with a total methane production of 2.95 mL.

It can be seen from Figure 7-2, that methane production started before starting the second stage for both the two stage reactor and the No.3 control reactor. The only difference in the start-up of these two reactors and the rest of the experimental reactors was the pH at start-up. As can be seen from Figure 7-8 and Figure 7-9, the start-up pH for all of the single stage CSTRs, control No.1, and control No.2, was pH 8. This was due to the natural pH of both the fat powder substrate and the seeding sludge, no external interference was applied to change the pH at the starting up point in these reactors. In case of the first stage of the two stage reactor and the control No.3 reactor, the start-up pH value was lowered to pH 6 by the addition of 1 molar HCl through the pH controlling system before seeding the reactors with the inoculum. This was applied for these two reactors from day 0 in order to provide the proposed two stages separation through pH control.

It appears that lowering the pH at set-up is the reason for the fast start up of the reactor. The initial low pH value might play a role in enhancing both hydrolytic

and acidogenic microorganisms. It has been reported by Yu and Fang (2002), that the optimum pH for hydrolytic and acidogenic bacteria is between pH 5.5 and pH 6.5. Solera *et al.* (2002), gave a similar optimum pH range for acidogenic bacteria of between pH 5.2 and 6.5.

Enhancing the hydrolytic and acidogenic bacteria by providing initial optimum pH conditions can result in better fat hydrolysis by the activity of these microorganisms. The increase in hydrolysis and acidogenesis activity is indicated by the hydrogen production (Figure 7-4 and Figure 7-5), as well as by the increase in volatile fatty acid content inside the reactor. The highest production of hydrogen gas was from the two pH controlled reactors (control No.3 and the first stage of the two stages reactor) with 366.11 mL and 426.25 mL respectively, compared to between 219.89 to 239.97 mL from the other, non-pH controlled, reactors.

Better hydrolysis and acidogenesis processes will lead to higher availability of simpler organic materials (simpler volatile fatty acids including the sequence of splitting that results eventually in acetic acid). The availability of these simpler organic materials can enhance the activity of acetogenic and methanogenic microorganisms during the digestion process which is evident by the increase in methane production. Appropriate availability of volatile fatty acids will stimulate acetogenic bacteria, responsible for converting these into acetic acid, hydrogen, and carbon dioxide. The availability of acetate, hydrogen, and carbon dioxide would stimulate the activity of acetoclastic and hydrogenotrophic methanogens and thus result in an increase in methane gas production.

Towards the end of the first stage (day 29), acetogenesis activities were decreasing in both the first stage of the two stage reactors and the control No. 3 reactor (Figure 7-15 and Figure 7-18). The decrease in acetic acid shown in Figure 7-15 and Figure 7-18 is possibly related to the shortage of acetogenic activity and to the consumption of the available acetic acid by methaneogenic microorganisms (Figure 7-2). A limitation of acetogenesis activity as a result of inhibition by accumulated LCFAs released from the ongoing hydrolysis of fat is believed to be a limiting step in the progress of the reactors.

On day 29, the second stage was started in the two stage reactors in addition to control No. 2 (the reseeding only control), and control No. 3 (the pH-controlling only control). The pH values were set to 8.5 for these reactors. After changing the pH value to pH 8.5, the second stage of the two stages reactors and control No.2 (re-seeding only control) were re-inoculated then with seeding sludge at an I/S ratio of 0.1.

After the second stage was started, cumulative methane production increased to around 300 mL in control No.3, before it stopped producing methane from day 34 until the end of the experiments on day 91. However, in case of the second stage in the two stages reactor, no active methane production was observed from the setting up of the second stage until day 70, where a continuous increase in methane production began. The start of active methane production from day 70, was associated with an increase in acetic acid as can be seen in Figure 7-15. This is a clear indication of higher activity of both acetogenesis (acetic acid production), and methanogenesis (methane gas production) (Figure 7-2and Figure 7-15).

7.3.1.2 *Hydrogen production*

As can be seen from Figure 7-4 and Figure 7-5, the active production of hydrogen gas was only observed in the early days of the experiment. The highest production of hydrogen gas was from the two pH controlled reactors (control No.3 and the first stage of the two stage reactor) with 366.11 mL and 426.25 mL respectively, compared to between 219.89 to 239.97 mL from the other non-pH controlled reactors (Figure 7-5).

The higher hydrogen production could be related to the initial pH lowering, which was only used for these two reactors.

Providing an initial optimum pH condition would enhance the hydrolytic and acidogenic microorganism activity, and therefore better fat hydrolysis and degradation are expected. The increase in hydrolysis and acidogenesis is indicated by the hydrogen production from the experimental reactors (Figure 7-4 and Figure 7-5), as well as by the increase in volatile fatty acid content inside the reactor (Figure 7-15 and Figure 7-18).

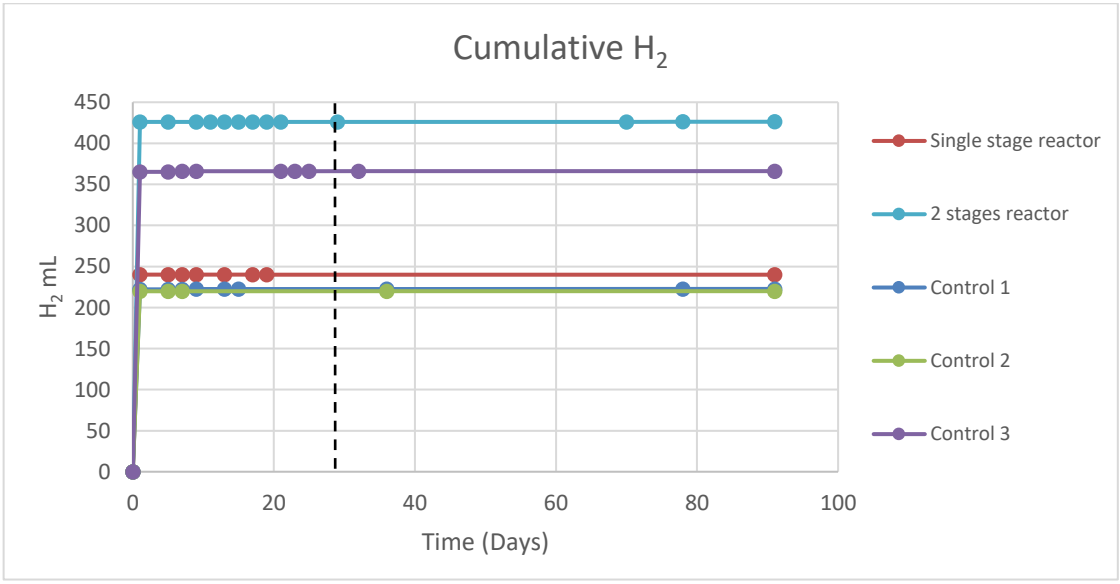

Figure 7-4: Cumulative hydrogen production from the experimental reactors.

X axis represents the time in days; Y axis represents the cumulative hydrogen in millilitres. The dashed line on day 29 represents the start of the second stage with pH control and reseeding steps in the case of the two stage setup.

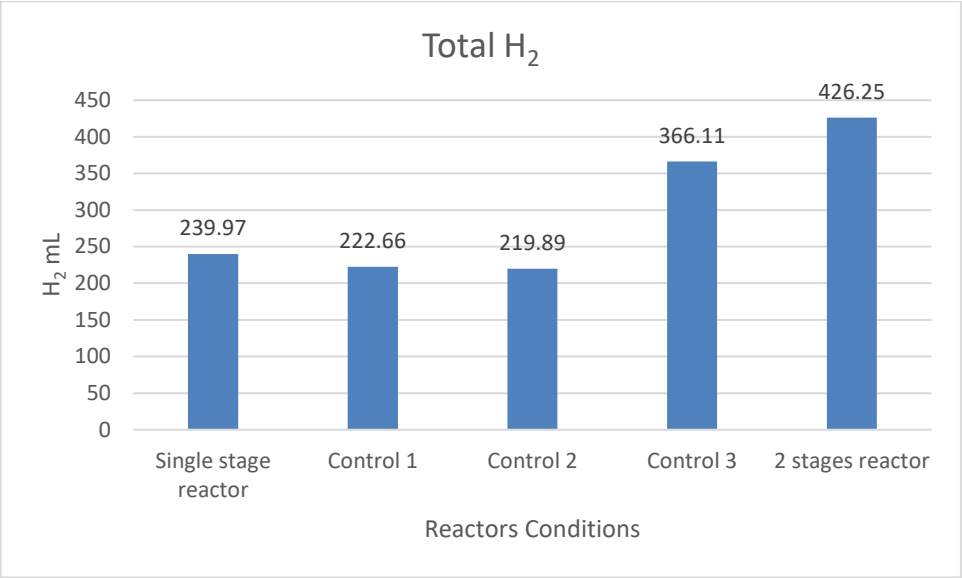

Figure 7-5: Total hydrogen production from the experimental reactors.

X axis represents the time duration in days; Y axis represents the total hydrogen production in millilitres.

7.3.1.3 *Carbon dioxide*

Carbon dioxide shows similar behaviour to the methane gas production. Increasing carbon dioxide, shown in Figure 7-6, can be an indication of acetoclastic methanogenesis activity, since the increase in carbon dioxide is associated with an increase in methane gas production (Figure 7-2). The situation would be different if the majority of the active methanogens were hydrogenotrophic microorganisms, as a decrease in carbon dioxide would be expected due to the conversion of carbon dioxide and hydrogen into methane.

As can be seen from Figure 7-7, the highest total carbon dioxide production was from the active biogas reactors (the two stages reactor, and the control No.3 (pH controlled) reactor).

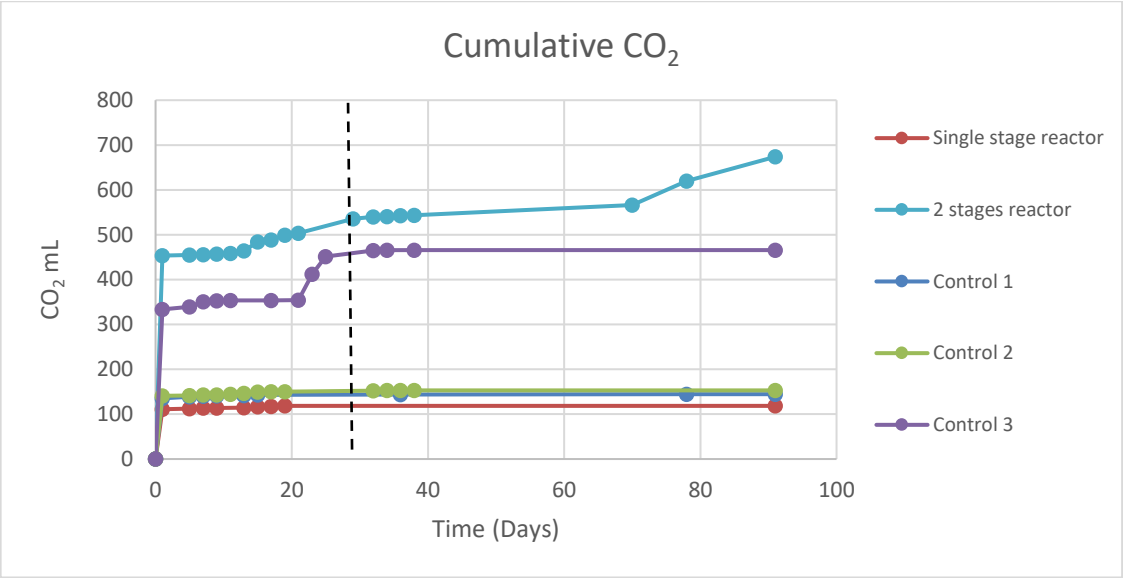

Figure 7-6: Cumulative carbon dioxide generation from the experimental reactors.

X axis represents time in days; Y axis represents the cumulative carbon dioxide in millilitres. The dashed line on day 29 represents the start of the second stage with pH control and reseeding steps in the case of the two stage setups.

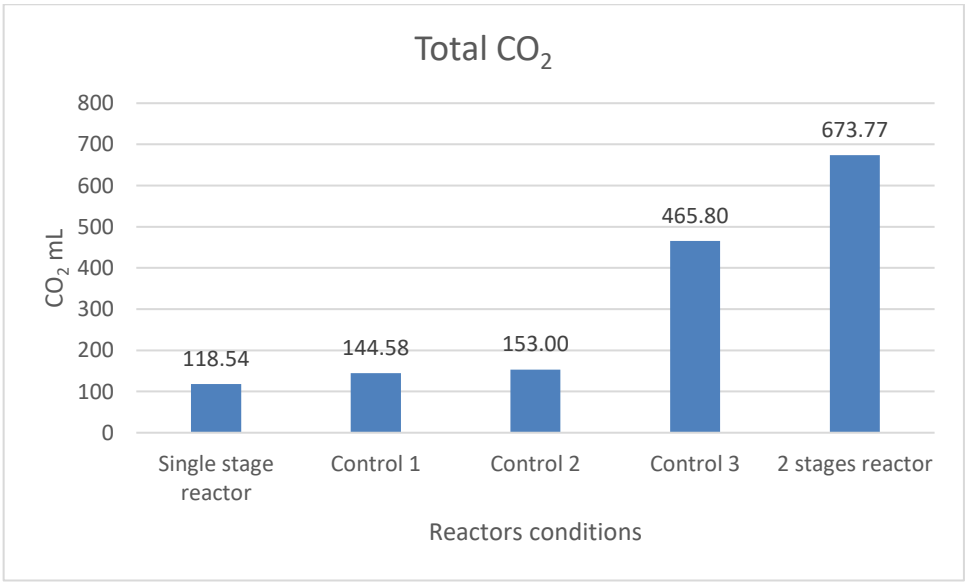

Figure 7-7: Total carbon dioxide generated from the experimental reactors.

X axis represents the time duration in days; Y axis represents the total carbon dioxide production in millilitres.

7.3.2 **pH behaviour:**

pH measurements were made both manually and with the automatic pH controllers. This was to overcome possible errors or drifts in the internal pH sensors, which might result from clogging of the ceramic reference junction or an accumulation of fat around the electrode's glass membrane, leading to incorrect automatic readings and control of the pH. Manual pH was used to correct any drift in the automatic controllers in order to maintain the required experimental pH conditions.

As can be seen from Figure 7-8, the start point of the pH was around pH 8 for the single stage CSTR, control No.1, and control No.2. This was due to the natural pH of both the fat powder substrate and the seeding sludge, no additions were made to change the pH at the start-up of these reactors.

The pH naturally dropped down in these reactors as a result of hydrolysis and acidogenesis activity during the first few days. The pH decreased from around pH 8 on day 0, to around pH 5 then remained around pH 5 without noticeable change in the single stage CSTR and the control No. 1 column reactor. In the reseeded control reactor No. 2, the pH increases to nearly pH 6 from pH 5 as a result of the seeding sludge addition on day 29, which had an original pH of 7.9. The pH of control No.2 then shows a slight and continuous decrease from pH 6 to pH 5.65 over a period of 70 days. This might be an indication of slow degradation by acidogenesis and/or acetogenesis activities.

However, in case of the first stage of the two stage reactor and the control No.3 reactor illustrated in Figure 7-9, the start-up pH value was lowered to pH 6 by the addition of 1 molar HCl before seeding the reactors with the inoculum. For these pH controlled reactors the pH conditions were set at 5.5 for the first stage (controlled between 5 and 6 in the controller settings), and 8.5 for the second stage (between 8 and 9 in the controller settings using $NaHCO_3$).

As can be seen from Figure 7-9 some fluctuation in pH occurred in the two pH controlled reactors after day 29. This fluctuation could result from a drift and re-correction of the measurements of the pH controlling units. Drift in pH measurement and slower response are common limitations with long exposure of the pH electrode to the reactor's substrate. Another reason for the fluctuation

could be natural pH changes due to the microorganisms' activities and correction of this pH by the controllers.

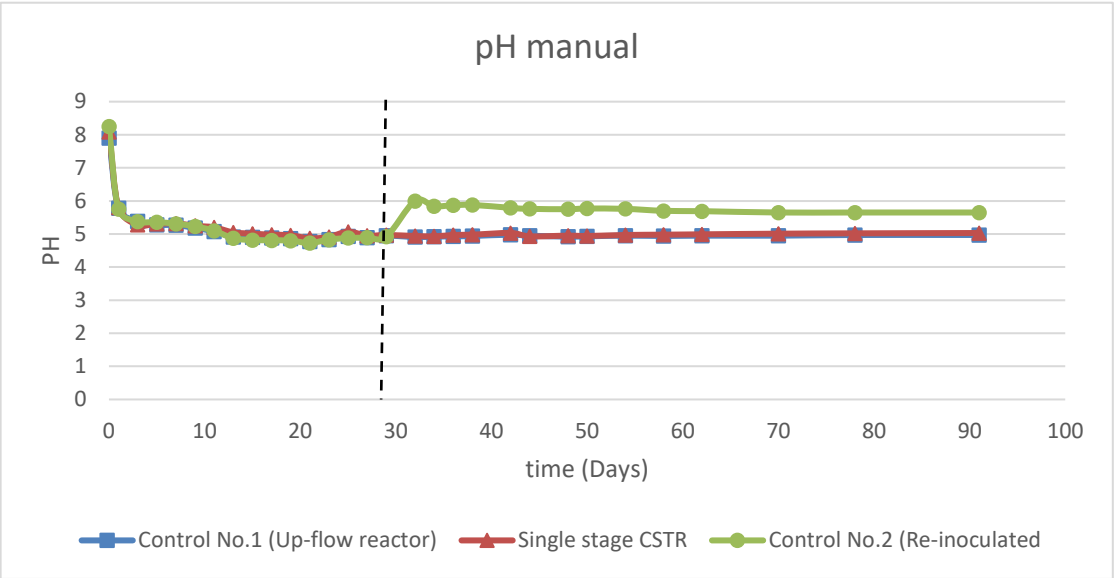

Figure 7-8: pH behaviour for the anaerobic reactors with no pH control.

X axis represents time in days; Y axis represents the pH. The dashed line on day 29 represents the start-up of the second stage (in Control no.2).

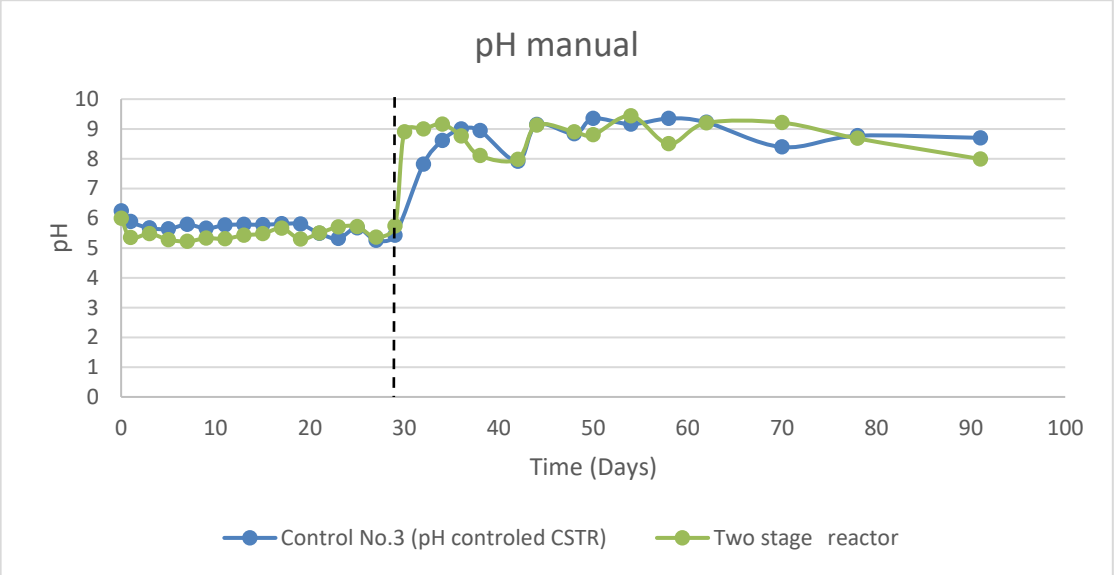

Figure 7-9: pH behaviour of the anaerobic reactors fitted under pH control.

X axis represents time in days; Y axis represents the pH. The dashed line on day 29 represents the start of the second stage with pH control and reseeding in the case of the two stage reactor, and with only pH control in the case of control reactor No.3.

7.3.3 **Long chain fatty acids:**

The total LCFA concentration showed variations according to the experimental condition of each reactor. There are some limitations to the LCFA analyses from the single stage reactors and the first stages of the two stage reactors due to physical limitations in the homogeneity of the samples. First, some semisolid fat particles were sticking to the pipe wall and the sampling syringe during sampling which may lead to a reduction in fat content of the sample. Second, physical separation of solidified fat was observed in the reactors (e.g. in the single stage reactor, Figure 7-10). This may be due to the hydrophobicity of the fat and the circular mixing action leading to spherical solidified fat particles. Solidified fat was also observed in the single stage up-flow column reactor (control No.1), which has the same substrate and I/S ratio conditions to the CSTR single stage reactor (Figure 7-11).

As whole, non-filtered samples were used to determine the LCFAs in the reactor environment, solidification and separation of fat will have a direct effect on the LCFAs results in the single and first stage reactors. In case of the second stage of two stages reactors, the addition of alkaline buffer leads to alkaline hydrolysis of the fat, giving more homogenised samples. The re-seeded reactor showed less formation and separation of solid fat particles after the start of the second stage. This may indicate better degradation due to more active anaerobic microorganisms provided by the re-seeding step.

In the single stage CSTR reactor, the total LCFAs results fluctuated. As can be seen from Figure 7-12, separation of fat and the formation of solidified fat particles are expected to cause a decrease in the LCFAs results, whereas breaking down of some of the solidified fat particles during the stirring process will lead to an increase in the detected LCFAs.

However, in case of the control No.1 (up-flow single stage column reactor), the mixing regime led to the floatation of fat particles at the top of the reactor. This limitation is believed to be the cause of the relatively low concentration of LCFAs as shown in Figure 7-12.

In the control No.2 (re-inoculated) CSTR, the results show some fluctuation during the first month, (Figure 7-12). However, after the reseeding step, more

consistent LCFA results were obtained. These show similar behaviour to its VFAs results (see next section), which indicate low active digestion.

In the case of the two stage reactors and control No.3 (pH controlled reactor), LCFA results show fluctuations similar to the other reactors in the same period, probably for the same reason. However, after the addition of the alkaline buffer to start the second stage on day 29, fat particles were homogenised among the reactor content. This results in more consistent LCFA analysis which showed a decrease in LCFAs in the second stage of the two stage reactor. This decrease in total LCFAs inside the second stage reactor illustrated in Figure 7-13, mirrors the increase in methane gas production (Figure 7-2) and carbon dioxide production (Figure 7-6), and is a good indication of a successful digestion process. In the case of control No.3 reactor (Figure 7-13), the unchanging LCFA concentration from day 48 till the end of the experiment is an indication of a poor digestion process due, possibly, to inhibition of the microorganisms by the high concentrations of LCFAs. This is reflected in poor methane production.

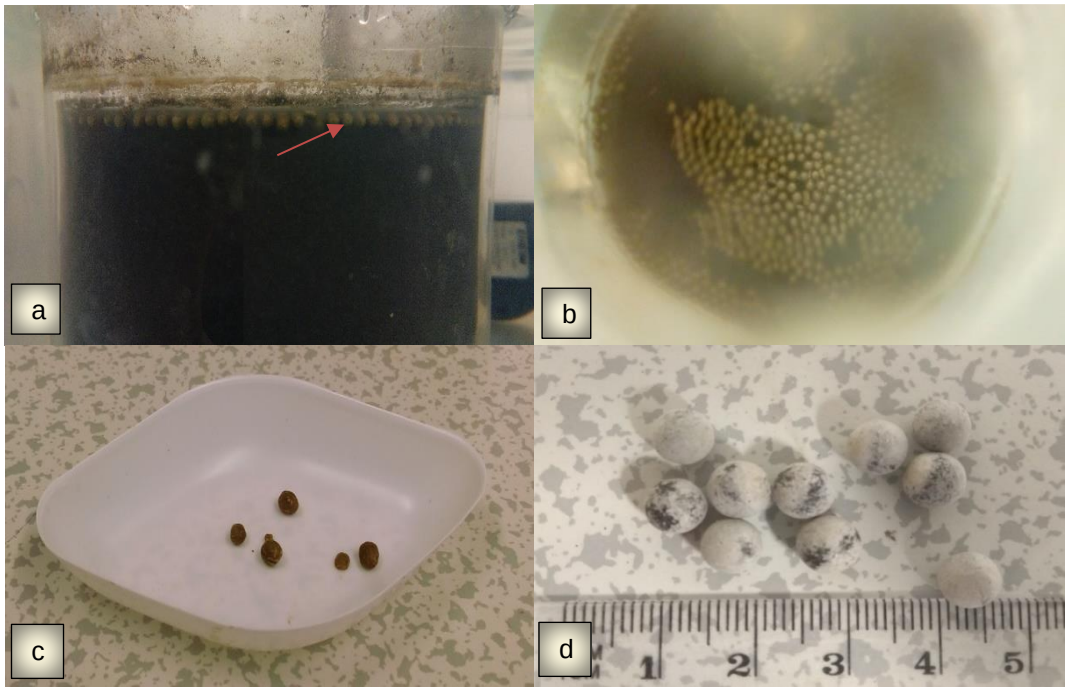

Figure 7-10: Spherical fat particles formed in the single stage reactor after 21 days.

a: side view of the reactor showing the solidified fat floating near to the surface.
b: overhead view of the solidified particles inside the reactor.
c: a sample of the solidified fat particles taken from the reactor.
d: a dry sample of the solidified fat particles taken from the reactor on the last experimental day.

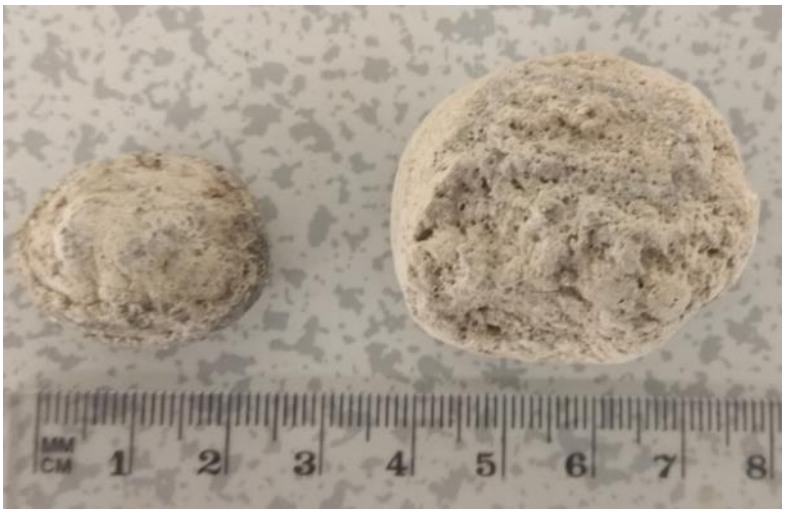

Figure 7-11 large solidified fat from the up-flow single stage column reactor (control No.1).

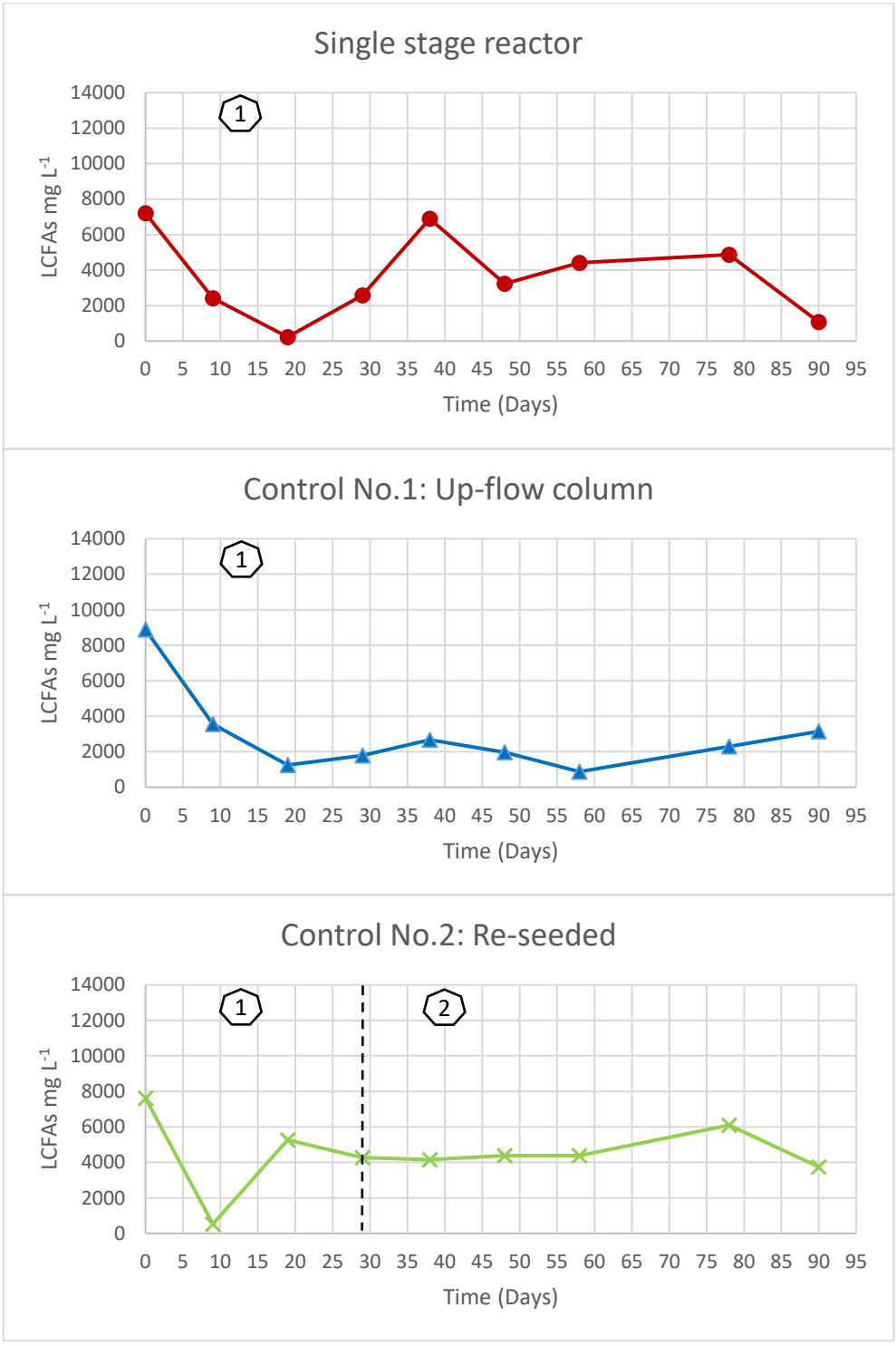

Figure 7-12: Total LCFA behaviour from the experimental reactors.

X axis represents time in days; Y axis represents total LCFAs in mg L^{-1}. The dashed line on day 29 represents the start of the second stage with only reseeding steps in control No.2.

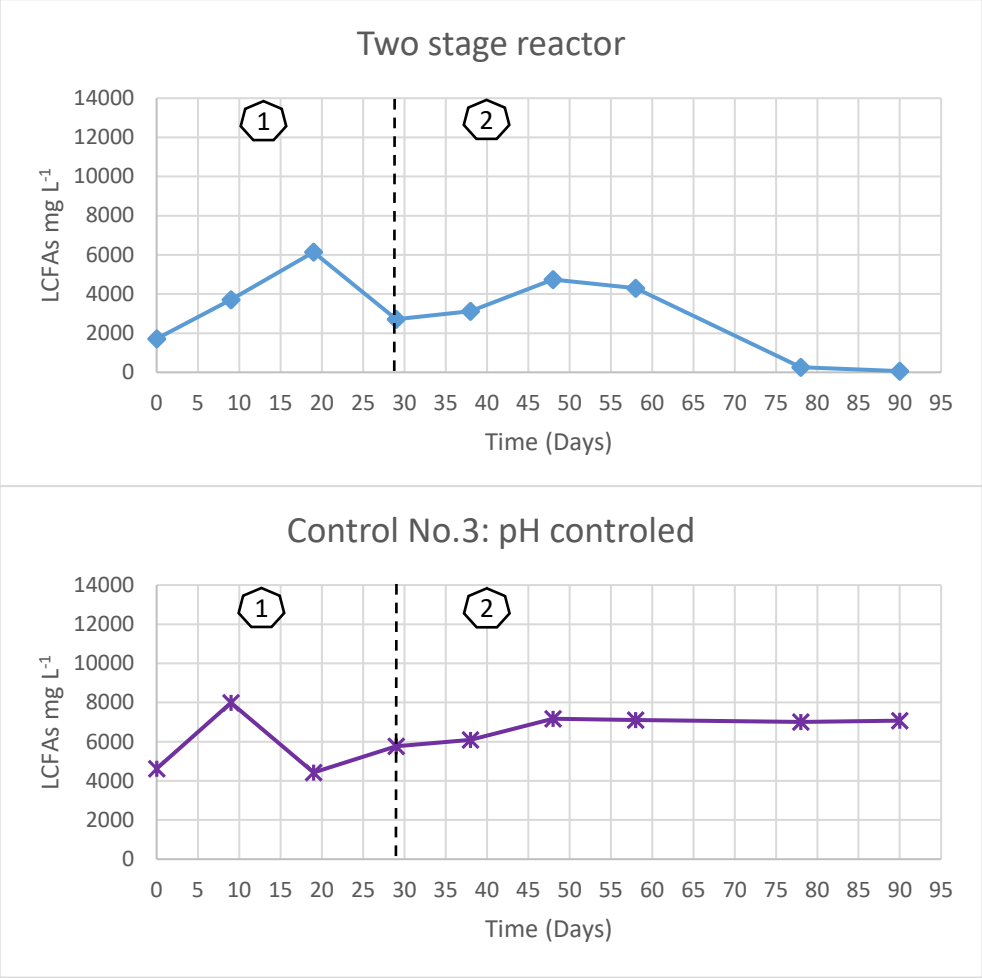

Figure 7-13: Total LCFA behaviour for the two stage reactor and the control No.3 (pH controlled) reactor.

X axis represents time in days; Y axis represents the total LCFAs in mg L^{-1}. The dashed line on day 29 represent the start of the second stage with pH control and reseeding steps in case of the two stage setup.

7.3.4 **Volatile fatty acids:**

All the reactors showed an increase in volatile fatty acids from the early days of the experiment. This increase can be directly related to the acidogenesis and acetogenesis activity of microorganisms. Acetic acid levels are one of the clearest indications of the digester progress, especially when combined with the methane and carbon dioxide production and the overall pH behaviour.

The single stage reactor showed an increase in VFAs at the beginning of the experiment. However, the VFAs then reached a stationary phase. The absence of change in the VFAs concentrations can be an indication of inhibition of both acid producers (acidogenesis) and acid consumers (acetogenesis and methanogenesis). The activity of acidogenesis can be identified through an increase in VFAs, whereas the activity of the acetogenesis can be identified by the decrease in the concentrations of VFAs other than acetic acid, associated with an increase of the acetic acid concentration. A clear example of this is the behaviour of the VFAs in the two stage reactor after day 70 (Figure 7-15).

The methanogenesis activities can be identified by the consumption (decrease, or at least slower increase) in the acetic acid levels, when both methanogenesis and acetogenesis occur at the same time.

Looking at the VFA behaviour for single stage reactor (Figure 7-14), along with methane, carbon dioxide and hydrogen gas production (Figure 7-2, Figure 7-4, Figure 7-6), and the pH behaviour (Figure 7-8), it is clear that the process of anaerobic digestion has been interrupted after day 14 in this reactor due to the observed lack of any active changes in these parameters.

The failure of this reactor could be due to several reasons. First, the I/S ratio was very low and may not provide enough anaerobic microorganisms to the reactor. Second, the accumulation of the initial by-products of fat degradation (VFAs and LCFAs) might have an inhibitory effect on the microorganisms. The drop in pH during the first few days of the experiment could also affect the growth and activity of the acetogenic and methanogenic microorganisms.

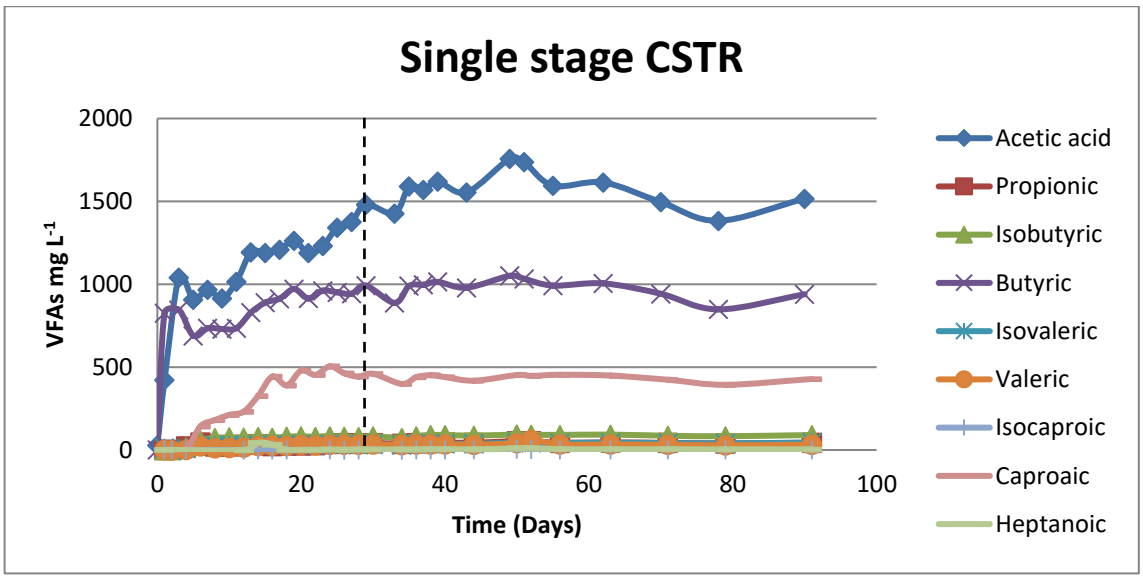

Figure 7-14: Volatile fatty acid behaviour from the single stage anaerobic reactor.

X axis represents time in days; Y axis represents the amount of VFAs in mg L^{-1}. The dashed line on day 29 represents the start of the second stage in the other (two stage) experiments

In case of the two stage reactor, the VFAs showed a continuous increase from day 0 until day 21. On day 21, the concentration of the acetic acid started to decrease and this was associated with an increase in methane production, indicating the activity of methanogenic microorganisms. The decrease in the acetic acid can also possibly indicate to a lower activity of the acetogenic microorganisms opposite to the consumption of acetic acid by the methanogenesis activities. The decrease in the acetic acid productivity could possibly result from an inhibition of the acetogenic microorganisms or a lack in the availability of the proper intermediate for a fast acetic acid conversion.

The experimental results showed that the removed amount of acetic acid from the reactor was during the 8 days (from day 21 to day 29) was 1.43 gram, equal to 0.0238 mole of the acetic acid. Whereas the produced amount of methane gas during the same time was 105.24 mL of CH4, equal to 0.004698 mole of CH_4.

Carbon balance calculation for both acetic acid consumption and methane gas production during the period of time from day 21 to day 29 was calculated using Buswell equation (Equation 5-1). The results showed that the estimated methane gas production from a full conversion of 0.0238 mole of acetic acid into methane is 534.1 mL of CH_4 (Table 7-1). However, this result shows an 80.29 % higher estimated methane in volume than the actual measured volume of methane gas in the experiment. It is normal that the maximum theoretical estimation of methane production has a higher methane estimation than the experimental results. This is due to the assumption of an ideal and full conversion of the acetic acid into biogas which is not applicable in the real experimental condition.

Table 7-1 Acetic acid consumption and theoretical and experimental methane production between days 21 and 29 in the two stage reactor.

Time	Acetic acid consumption	Theoretical CH_4 production	Experimental CH_4 production
(8 days) From day 21 to day 29	1.43 g = 0.0238 mole	0.0238 mole = 534.1 mL	0.004698 mole = 105.24 mL

The decrease in concentration of all volatile fatty acids on day 29, is associated with the start-up of the second stage. The reactor content was transferred into a column reactor, followed by the addition of $NaHCO_3$ solution to reach pH 8, sparging with nitrogen gas, and finally, re-inoculating the new stage with an anaerobic seeding sludge at I/S ratio of 0.1.

After the start-up of the second stage, the VFA content began to slowly increase. From day 60 acetic acid showed a sharp increase which was associated with a decrease in other higher chain length volatile fatty acids such as caproaic and butyric acids (Figure 7-15). This is a clear indication of the activity of acetogenic microorganisms, which convert the higher VFAs into acetic acid.

This increase in acetic acid is associated with an increase in methane production (Figure 7-2). The acetic acid is now being used in methanogenesis but not at a rate that outstrips its production (Figure 7-15).

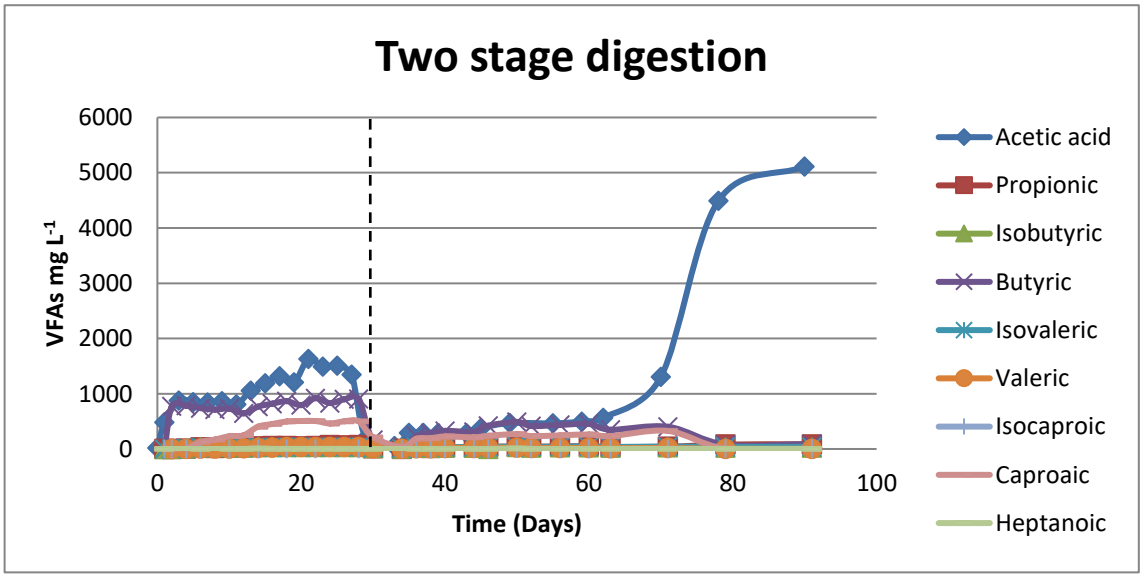

Figure 7-15: Volatile fatty acid behaviour from the two stage anaerobic reactor.

X axis represents time in days; Y axis represents the amount of VFAs in mg L^{-1}. The dashed line on day 29 represents the start of the second stage with pH control and reseeding steps in the case of the two stage setups.

Control No. 1 (the up-flow column control reactor) and control No. 2 (the re-inoculated control reactor) show similar overall results to the single stage reactors (Figure 7-16 and Figure 7-17). Both reactors show an overall increase in volatile fatty acids until they reach a stationary phase. This can be an indication of the inhibition of both acid producers (acidogenecis) as increasing acid production was stopped, and acid consumers (acetogenesis and methanogenesis) as there was no marked decrease in VFAs. This was also associated with a lack of active biogas production and almost steady pH, which all indicates a poor activity of anaerobic microorganisms.

In the re-inoculated control No.2, a slight decrease in acetic acid was observed from day 62 until the end of the experiment on day 91 (Figure 7-17). This might indicate some initial methanogenic or syntrophic acetate oxidation activity. However, this is not supported by evidence of active biogas production.

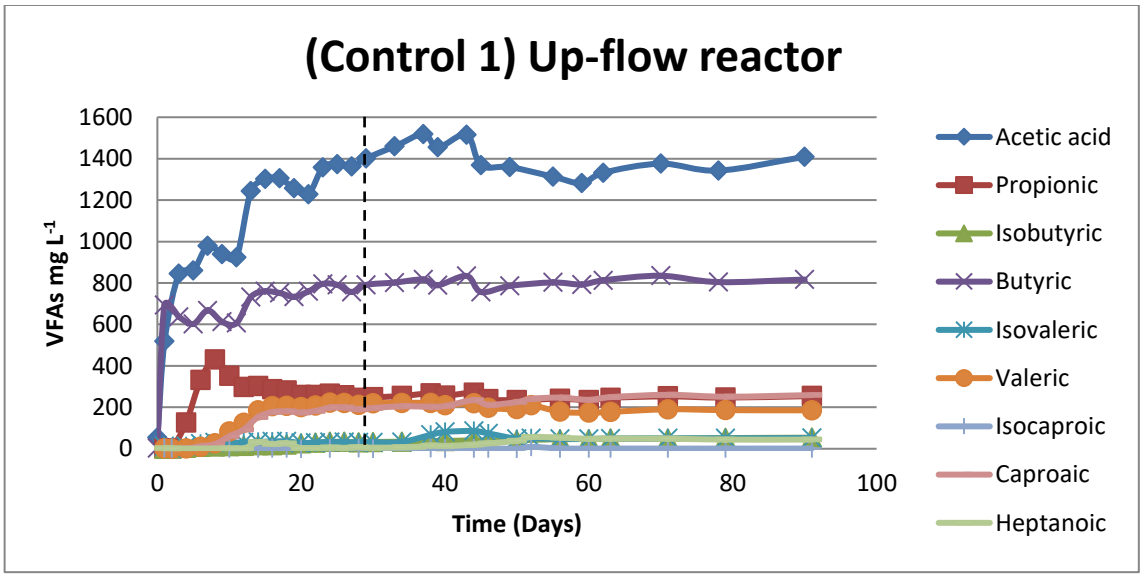

Figure 7-16: Volatile fatty acid behaviour from the up-flow anaerobic reactor (control 1).

X axis represents time in days; Y axis represents the amount of VFAs in mg L^{-1}. The dashed line on day 29 represents the start of the second stage with pH control and reseeding steps in the case of the two stage setups.

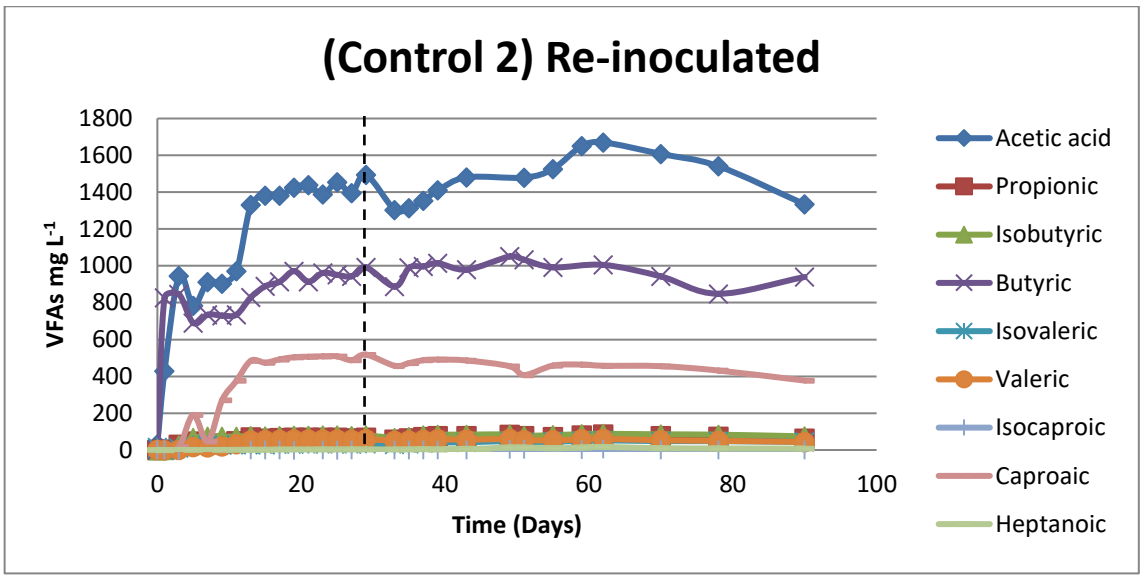

Figure 7-17: Volatile fatty acid behaviour from the Re-seeded (re-inoculated) (control 2) anaerobic reactor.

X axis represents time in days; Y axis represents the amount of VFAs in mg L^{-1}. The dashed line on day 29 represents the start of the second stage with pH control and reseeding steps in the case of the two stage setups.

In control No.3 (pH control), the VFA content shows a continuous increase from day 1 until day 19. After day 19, the level of acetic acid sharply decreased. This was associated with an increase in methane production.

A lower activity of the acetogenic microorganisms would be expected as a result of this decrease. The decrease may be due to lower productivity of acetic acid in front of higher consumption by the methanogens. The reason could be an inhibition of the acetogenic microorganisms or a lack in the availability of the proper intermediate for a fast acetic acid conversion. Methane gas stopped on day 32, when the acetic acid reached its lowest concentration.

There is no effect of starting up of the second stage (by changing the pH value to 8) on most of the VFA levels. However, the acetic acid concentration showed a slight and slow increase until the end of the experiment (Figure 7-18).

It is believed that this slight increase in acetic acid production could be due to the recovery of some acetogenic microorganisms, which then start converting the higher volatile fatty acids into acetic acid. No methane gas was observed during this slight and slow increase in acetic acid. The absence of methane could be related a possible inhibition of methanogens by accumulated LCFA (Figure 7-13).

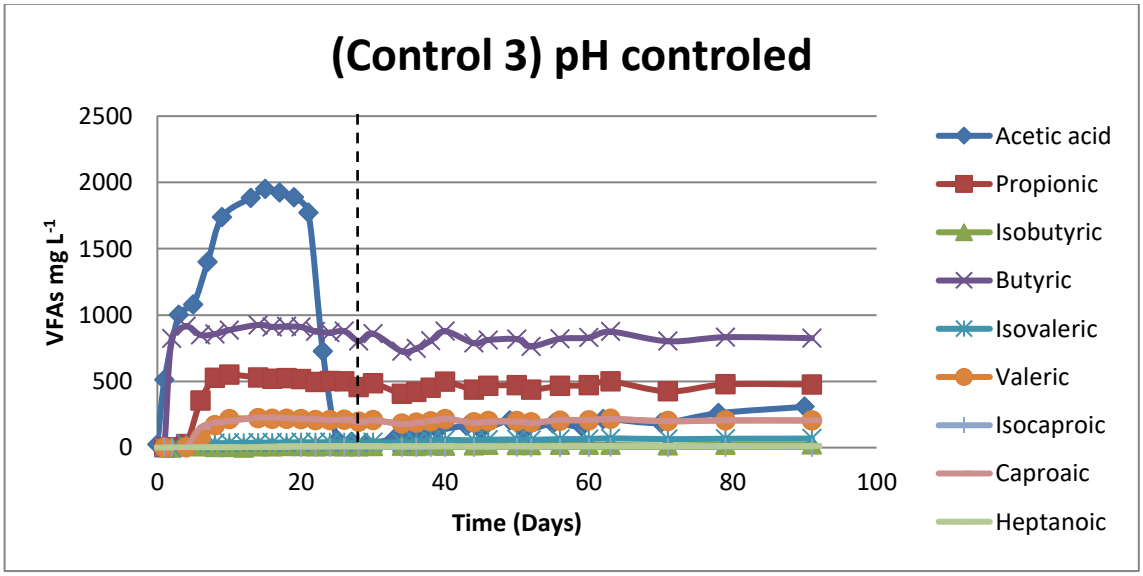

Figure 7-18: Volatile fatty acid behaviours from the automatic pH controlled (control 3) anaerobic reactor.

X axis represents time in days; Y axis represents the amount of VFAs in mg L^{-1}. The dashed line on day 29 represents the start of the second stage with pH control and reseeding steps in the case of the two stage setups.

7.3.5 Chemical oxygen demand

The initial dissolved COD values for all the experimental reactors were between 4150 and 4700 mg L^{-1}. These dropped to around 3000 mg L^{-1} during the first three days of the experiments. The drop may be due to hydrolysis activity (acidogenesis and acetogenesis), and is associated with the generation of hydrogen and a drop in pH. After this, COD values increased until reaching a plateau around day 20 in the single stage CSTR, control No.1 (up-flow reactor), and control No.2 (the re-seeded control reactor). These three reactors showed no effective methane production and show similar trends in pH and volatile fatty acids to the COD. This supports the theory that these three reactors were unable to successfully deal with the fat substrate under their current operational conditions.

However, in case of the pH controlled reactors (the two stage reactor, and control No.3), COD behaviour was directly related to methane gas production and volatile fatty acids behaviour.

In control No.3, the dissolved COD shows a continuous decrease starting from day 19. This is associated with the decrease in the acetic acid concentration (Figure 7-18), and the increase in methane gas generation from the reactor (Figure 7-2) and thus removal of carbon from the system as biogas. However, on day 32, the decrease in COD stopped and the level remained steady (Figure 7-19). This was associated with a cessation of methane gas production when the acetic acid content reached its lowest concentration inside the reactor.

A similar decrease in dissolved COD is seen in the first stage of the two stage reactor when methane gas was produced before the start-up of the second stage. This was also associated with a decrease in acetic acid level in the reactor (Figure 7-15). The COD concentration started to increase again after the second stage was started in the two stage reactor (Figure 7-19). This can be an indication of degradation of the organic substrate into a soluble form.

From day 62, the dissolved COD showed a continuous increase in the second stage reactor. This was associated with a similar rate of increase in acetic acid and methane gas production.

The increase in soluble COD along with increases in acetic acid content and methane gas production are good indicators for an active anaerobic digestion process of the feed substrate.

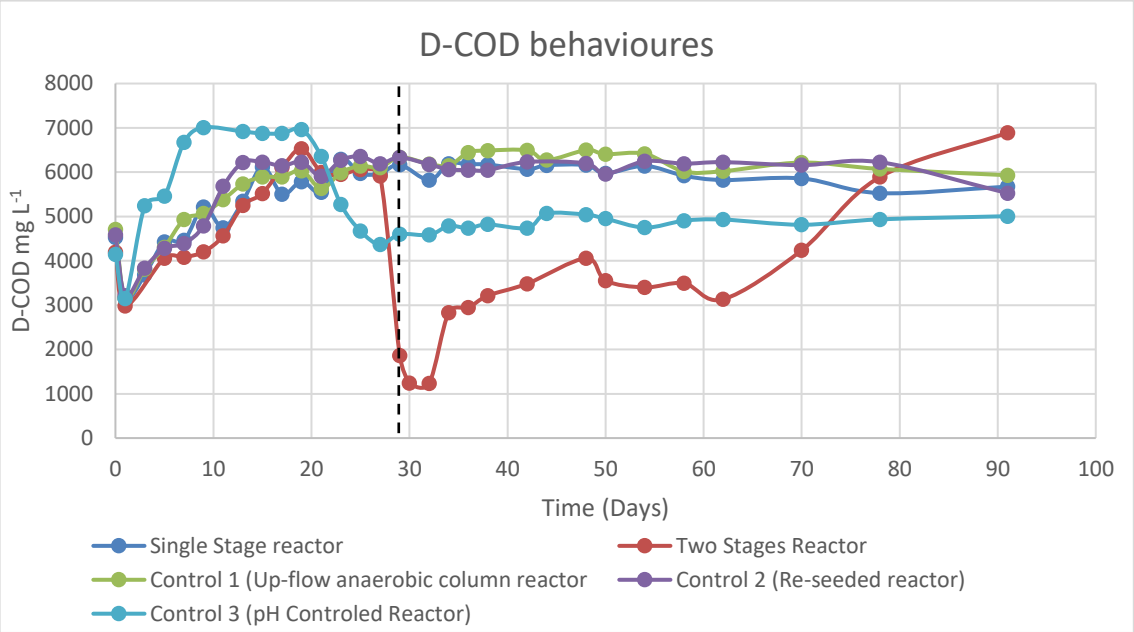

Figure 7-19: Chemical oxygen demand behaviour of the experimental reactors during the experimental run.

X axis represents time in days; Y axis represents the amount of dissolved COD in mg L^{-1}. The dashed line on day 29 represents the start of the second stage with pH control and reseeding steps in the case of the two stage setups.

7.4 Overall discussion:

7.4.1 The effect of re-inoculating low I/S ratio reactors:

The 1L working volume control reactor No.2 (with only re-seeding steps), was re-inoculated one time only with I/S ratio of 0.1 on day 29. This showed no successful start-up or any significant production of methane gas (Figure 7-2). The failure of this reactor could be due to several reasons. First, the overall I/S ratio was very low and would not provide enough suitable microorganisms to the reactors. Similar results were observed and discussed earlier in chapter 5 under the effect of using low I/S ratio. Second, the accumulation of the initial by-products of fat degradation (volatile fatty acid and LCFAs) might be the reason through their inhibitory effect on the anaerobic microorganisms.

7.4.2 Multi-stages reactors with pH controls and re-inoculating steps:

From the overall results, the two stage reactors showed higher methane and hydrogen gas production and greater ability to treat the fat substrate. Similar results of the advantage of two stage reactor over single stage reactor are reported by Akobi *et al.* (2016) in a comparison study between single stage and two stage anaerobic digestion of extruded lignocellulosic biomas.

The highest methane production was obtained from the two stage reactor with a total methane production of 927.82 mL (Figure 7-3). Reactor control No.3 with only pH control, showed nearly three times lower methane production of 304.67 mL compared to the two stage reactor. However, the methane production of control No.3 is still massively higher than the single stage, control No.1 (the single column reactor), and control No.2 (only re-seeding step two stage control CSTR).

Control No.3 (only pH controlled two stage CSTR), showed an increase in methane production during the first month, however, the process inside the reactor seems to be interrupted as the reactor stopped producing any gas after 38 days. The rest of the experimental reactors (single stage CSTR, control No.1 (up-flow single stage reactor), and control No.2 (re-seeding only two stage CSTR), did not generate much methane and were not able to effectively start-up.

An interesting observation from the two stage reactor and the control No3 reactor is that the increase in methane production started before the start of the second stage for both reactors.

The only difference in starting up procedure between these two reactors and the rest of the experimental reactors was the pH at the starting up point. The starting up pH value for all of the single stage CSTR, control No.1, and control No.2, was pH 8. This was due to the natural pH of both the fat substrate and the seeding sludge, no external interference was applied or any buffers added to change the pH at the starting up point in these reactors. Whereas in case of the first stage of the two stage reactors and the control No.3 reactor, the start-up pH value was lowered to pH 6 by the addition of 1 molar HCl through the pH controlling system before seeding the reactors with the inoculum. This was applied from day 0 in order to provide a separation of the two stages through pH control.

It appears that the lowering of the pH at start-up is the reason for the fast start up of the reactor. The initial low pH value might play a role in enhancing both hydrolytic and acidogenic microorganisms. It has been reported by Yu and Fang (2002), that the optimum pH for hydrolytic and acidogenic bacteria is between pH 5.5 and pH 6.5. Solera *et al.* (2002), give a similar optimum pH range for acidogenic bacteria of between pH 5.2 and 6.5.

Enhancing the hydrolytic and acidogenic bacteria by providing optimum initial pH conditions can result in better fat degradation. The increase in hydrolysis and acidogenesis activity is indicated by the hydrogen production, as well as by the increase in volatile fatty acid content in the reactor. The highest production of hydrogen gas was obtained from the two pH controlled reactors (control No.3 and the first stage of the two stages reactor) with 366.11 mL and 426.25 mL respectively, compared to between 219.89 to 239.97 mL from the other, non-pH controlled, reactors.

Better hydrolysis and acidogenesis processes will lead to higher availability of simpler organic substances (simpler volatile fatty acids including the sequence of splitting that results eventually in acetic acid). The availability of these simpler organic substances can enhance the activity of acetogenic and methanogenic microorganisms during the digestion process which is evident by the increase in methane production. Appropriate availability of volatile fatty acids will stimulate

acetogenic bacteria, responsible for converting these into acetic acid, hydrogen, and carbon dioxide. The availability of acetate, hydrogen, and carbon dioxide will stimulate the activity of acetotrophic and hydrogenotrophic methanogens and thus result in an increase in methane gas production.

Towards the end of the first stage (day 29), acetogenesis activities were decreasing in both the first stage of the two stage reactors and the control No. 3 reactor evident by the decrease in acetic acid. The decrease in acetic acid is possibly related to a shortage of acetogenic activity and to the consumption of the available acetic acid by methaneogenic microorganisms. A limitation of acetogenic activity as a result of inhibition by accumulated LCFAs released from the ongoing hydrolysis of fat is believed to be a limiting step in the progress of the reactors.

On day 29, the second stage was started in the two stage reactors in addition to control No. 2 (the reseeding only control), and control No. 3 (the pH-controlling only control). The pH was set to 8.5 for these reactors. After changing the pH to pH 8.5, the second stage of the two stages reactors and control No.2 (re-seeding only control) were re-inoculated with seeding sludge at an I/S ratio of 0.1. After the second stage was started, cumulative methane production increased to around 300 mL in control No.3, before it stopped producing methane from day 34 until the end of the experiment on day 91. However, in case of the second stage in the two stage reactor, no active methane production was observed from the setting up of the second stage until day 70, where a continuous increase in methane production began. The start of active methane production from day 70, was associated with an increase in acetic acid. This is a clear indication of higher activity of both acetogenesis (acetic acid production), and methanogenesis (methane gas production).

Providing an initial optimum pH condition would enhance the hydrolytic and acidogenic microorganism activity, and therefore better fat hydrolysis and degradation would be expected. The increase in hydrolysis and acidogenesis is indicated by hydrogen production from the experimental reactors as well as by the increase in volatile fatty acid content in the reactor.

To further enhance the digestion process and minimise the inhibitory effect of LCFAs, three and four stages reactors will be studied in the next chapter.

Chapter 8 Three and Four stage anaerobic reactors:

8.1 Objective:

The objective of this chapter is to investigate into the possibility of improving the digestion process further and minimising the inhibitory effect of LCFAs through physically separating the AD process into three and four phases. This is to further enhance on the initial findings of Chapter 7, in which a two stage reactor proved beneficial when compared to a single stage reactor. The separation strategy included pH control, re-inoculating, as well as, in the case of four stages, the introduction of an acetogenic stage seeded with *Thermosyntropha lipolytica*, a bacterium that can degrade LCFAs longer than 12 carbons (Long *et al.*, 2012). Up-flow column reactors were used for the final methanogenic stages (Figure 8-1).

The experimental set-up consisted of twelve separate reactors. Three reactors (2 CSTR and 1 Up-flow) were used for the three stage experiments. Four reactors (3 CSTR and 1 Up-flow) were used for the four stage experiments. In addition, another five reactors (4 CSTR and 1 Up-flow) were used as controls to get better understanding of the factors in this study.

8.2 Experiment design and operation conditions:

The same experimental reactors used in chapter 7 were used in this chapter with some modifications. New reactors were introduced to the experimental set and different operation conditions were used.

Continuously stirred tank reactors were used for the early stages of the multi-stage reactors, while up-flow reactors were chosen for the final methanogenic stage. In the three stage design, the initial processes of hydrolysis and acidogenesis are expected to take place in the first stage CSTR. Acetogenesis is expected to take place in the second (CSTR) stage and the methanogenesis step is expected to take place in the third and final stage, the up-flow column reactor. In the four stage design, the initial processes of hydrolysis and acidogenesis are expected to take place in the first stage CSTR. The first thermophilic acetogenesis seeded with *Thermosyntropha lipolytica* bacteria leads to the second stage (CSTR). The second normal mesophilic acetogenesis

(inoculated with anaerobic seeding sludge) is expected to take place in the third stage (CSTR). Methanogenesis is expected to take place in the fourth and final stage, the up-flow column reactor.

Five separate reactors were set as controls. Control No. 1 was an up-flow single stage column reactor to be compared with the multi-stage results under the current experimental conditions and the up-flow design. Control No. 2 was a single stage CSTR to be compared with the multi-stage results under the current experimental conditions. Control No. 3 was a CSTR reactor to study the effect of the re-inoculating (re-seeding) step without controlling the pH in order to establish the second, third, and four stages. Control No. 4 was a single CSTR to study the effect of only pH control to establish the second, third, and fourth stages without the re-inoculating process. Control No. 5 was a single stage thermophilic CSTR to study the effect of only thermophilic conditions on the digestion progress (Figure 8-3).

The experimental feed condition was 20g L^{-1} VS derived from 100% bovine fat powder for all experiments. The experiments were run at 35 ˚C (mesophilic), except for the control No.5 and the second stage of the four stages reactor, which were run at 60 ˚C (thermophilic) conditions. The initial working volume was 1L for all the reactors at start up. Inoculum to substrate ratio was 0.1 in terms of the volatile solids content for all the reactors. At re-inoculation, the same I/S ratio of 0.1 was used, except in the second stage of the four stage reactor and the control No.3, where 50 mL (equal to 0.05 v/v inoculum to substrate ratio) of active *Thermosyntropha lipolytica* culture was used. For the multi-stages reactors, first and second stages were set to 10 days each, whereas the third stage was 59 days in case of the three stage reactors (as a methanogenic stage in the 3 stages set), and 10 days in case of four stage reactors (as a second acetogenic phase). The fourth stage (considered as methanogenic stage) in the four stage reactors was run for 48 days. The start-up of each stage is clearly marked with dashed lines in the results graphs. For pH controlled reactors (three stages, four stages, and the control No.4) different pH conditions were selected. In case of the three stage reactor, the pH was 5 for the first stage (with the controller being set at a lower pH of 5 and higher pH of 5.5), and 7 for the second stage (with the low and high values of the controller

set at pH 6.5 and 7.5 respectively). Whereas it was 8 the third stage (with the low and high values of the controller set at pH 7.5 and 8.5 respectively).

However, in case of both the four stage reactors and control No.4, the pH was 5 for the first stage (with the controller being set at a lower pH of 5 and higher pH of 5.5), and 8.5 for the second stage (with the controller being set at a lower pH of 8 and higher pH of 9). In the third stage, the pH was 7 (with the low and high values of the controller set at pH 6.5 and 7.5 respectively). Whereas in the fourth stage, the pH was 8, with the low and high values of the controller set at pH 7.5 and 8.5 respectively. In order to keep to the minimum amount of the added buffer solutions to the reactors, the initial pH for the first stages (in pH controlled reactors) were left to decrease naturally without any acid addition.

pH was controlled by two automatic pH controllers equipped with alkaline and acid dosing pumps and combination glass pH electrodes (Figure 8.2). Hydrochloric acid (HCl) was used at a concentration of 1 M to lower the pH and a saturated solution of sodium bicarbonate ($NaHCO_3$) was used to raise the pH. The dosing rate was set at 0.1 mL min^{-1} for both acid and base, and waiting time between doses was set to 5 minutes.

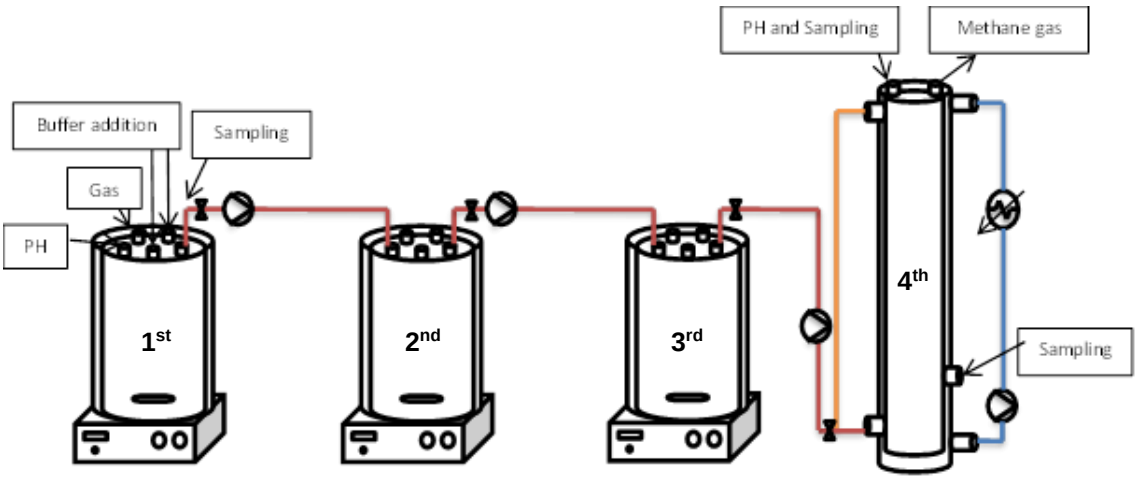

Figure 8-1: Four stage anaerobic reactor set.

1st: Hydrolysis & Acidogenesis CSTR 35 ˚C (mesophilic) reactor, pH 5.

2nd: LCFA Bio-treatment using (*Thermosyntropha lipolytica*) CSTR 60℃ (Thermophilic) reactor, pH 8.5.

3rd: Acetogenesis CSTR 35 ˚C (mesophilic) reactor pH 7.

4th: Methanogenesis Up-flow 35 ˚C (mesophilic) reactor pH8.

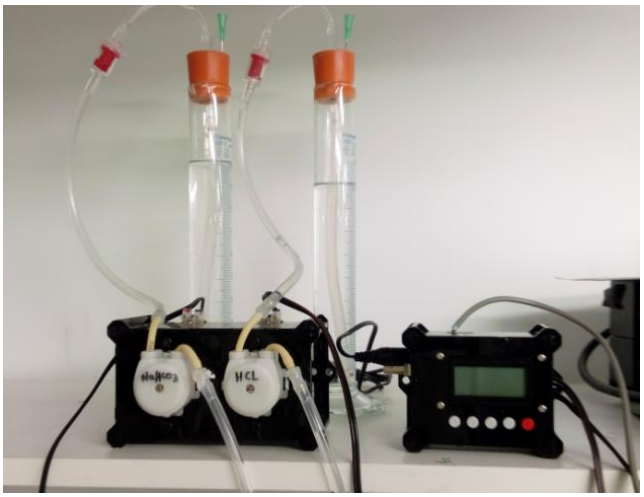

Figure 8-2 : pH controller with acid and alkaline dosing pumps.

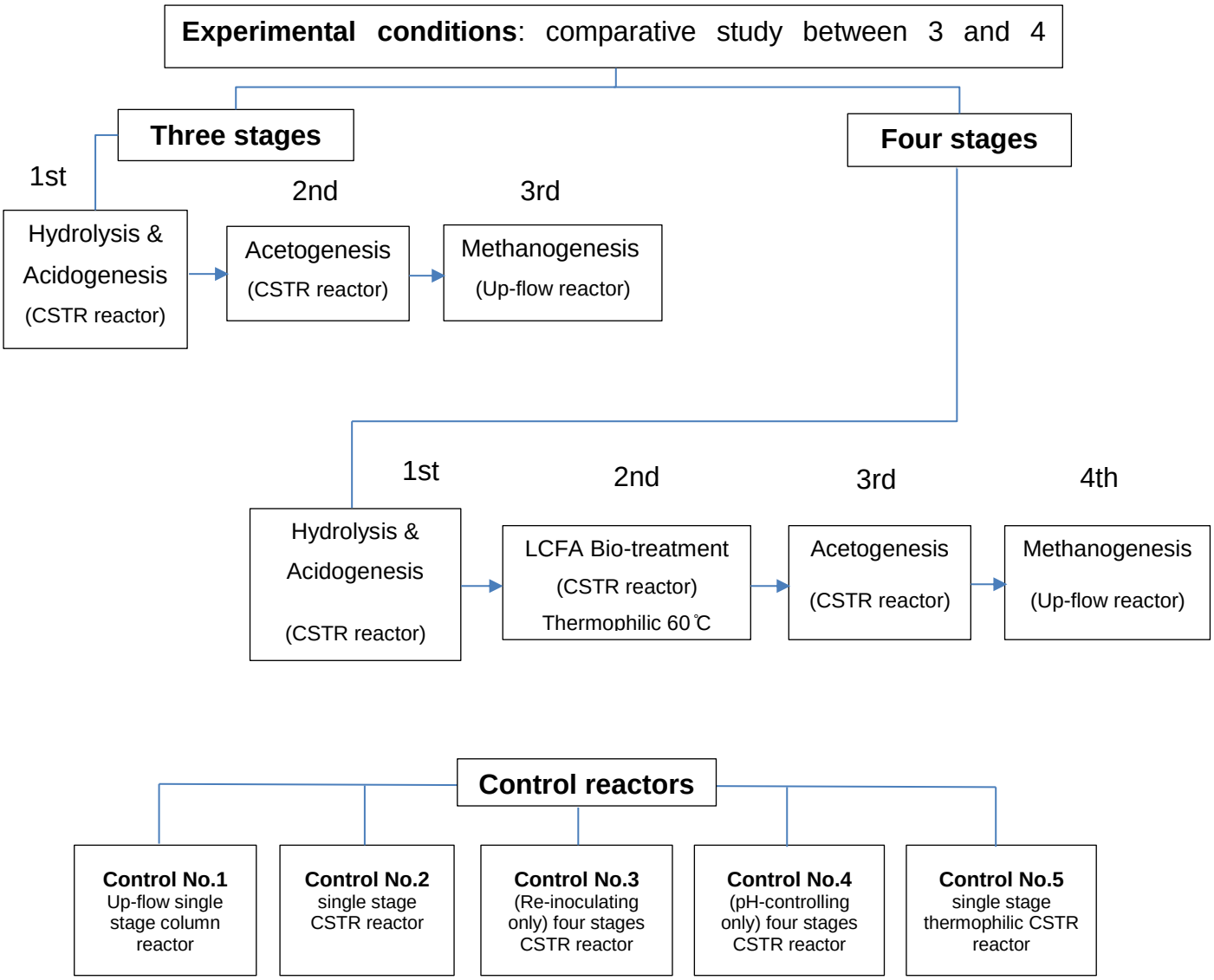

Figure 8-3: Experimental condition for the three and four stage anaerobic reactors

8.3 Results and discussion

8.3.1 Biogas production:

From the overall results, control reactor No.3, with only re-inoculating steps, showed higher and earlier methane production compared to the other reactors, as it started active methane production from day 25 (Figure 8-4). The three stage reactor showed a later increase in methane production after day 51, the rest of the experimental reactors did not generate any methane and were not able to effectively start up.

8.3.1.1 *Methane production:*

As can be seen from Figure 8-4 and Figure 8-5, the highest methane production was obtained from control No.3 (the re-inoculated control reactor), with a total methane production of 744.28 mL. The three stage reactor showed a 3.8 times lower methane production of 195.04 mL than control No.3. However, this was still much higher than the four stage reactor and the other control reactors.

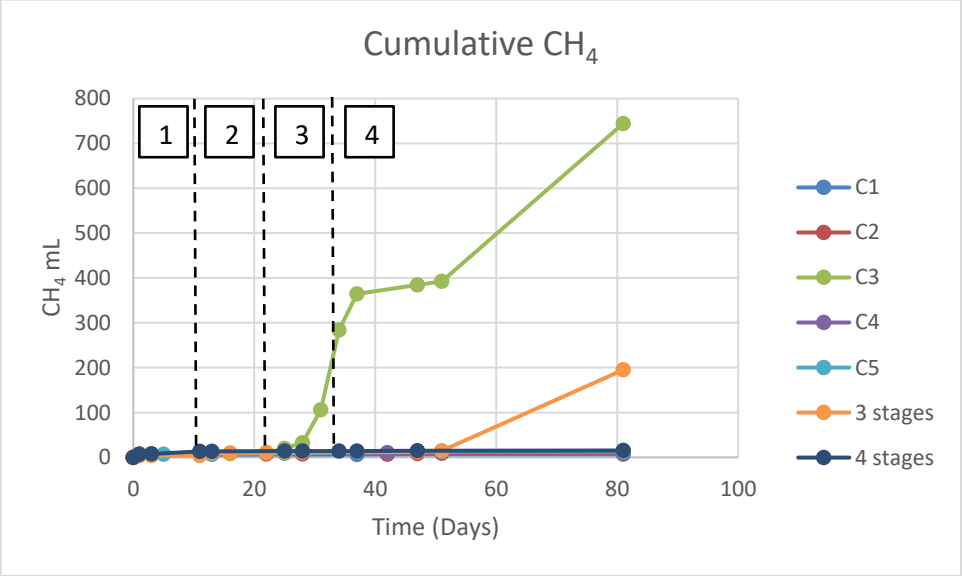

Figure 8-4: Cumulative methane production from the experimental reactors.

X axis represents time in days; Y axis represents cumulative methane in millilitres. The dashed lines on days 11, 22, and 33 represents the start of the next second, third, and fourth stages respectively, with pH control and/or reseeding.

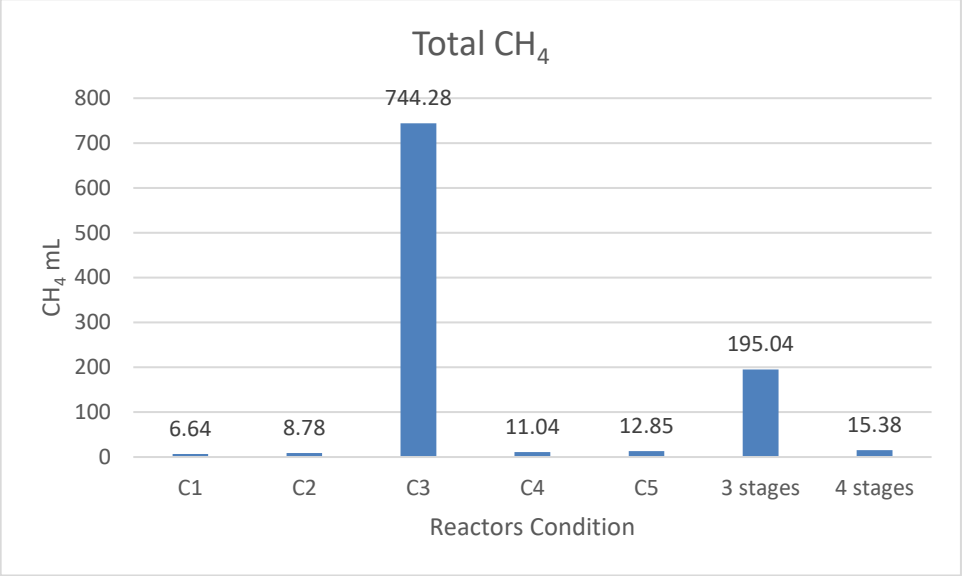

Figure 8-5: Total methane production from the experimental reactors.

X axis represents the time duration in days; Y axis represents the total methane in millilitres.

The first active methane gas production was observed from control No.3 (the re-inoculated control reactor) from day 25, during its third stage (Figure 8-4). This methane production was associated with an increase in CO_2 production and a decrease in acetic acid concentration (Figure 8-8 and Figure 8-17). The increase in CH_4 and CO_2 and associated decrease in acetic acid concentration are an indication of acetoclastic methanogenesis activity, generating CH_4 and CO_2 from acetate.

pH in control No.3 reactor showed an increase during the active methane production after day 25 (Figure 8-11). This could be due to the removal of acetic acid from the reactor (Figure 8-8 and Figure 8-17).

In case of the three stage reactor, active methane production started from day 47, 25 days from the start of the third stage. This increase in methane production was associated with an increase in CO_2, again indicating activity of acetoclastic methanogenesis. However, this methane production was also associated with an increase in acetic acid and a slight increase in propionic acid concentrations during the third stage of the three stage reactor. At the same time higher VFAs such as caproaic and butyric acids decreased inside the reactor (Figure 8-13). The increase in acetic acid associated with a decrease in other higher volatile fatty acids can indicate the activity of acetogenic microorganisms, which convert the higher VFAs into acetic acid. As this

increase in acetic acid was associated with an increase in the methane production (Figure 8-4 and Figure 8-13). It is believed that the acetic acid is now being used in methanogenesis but not at a rate that outstrips its production.

In case of the single stage control reactors No. 1 (up-flow column control reactor), No.2 (CSTR control reactor), and No.5 (thermophilic single stage CSTR control reactor), all showed very low methane production and could not effectively start up the digestion process. This is believed to be due to the low I/S ratio of 0.1 VS, which may not be enough to start up anaerobic digestion as reported in chapters 5 and 6.

However, four stage reactors showed no significant methane production as well. This could be due to several reasons; one is the large amount of buffer being added to the reactor in order to change the pH in each stage. The fast shift of temperature and pH, in addition to the possible high amount of salt that might be generated during acid and alkaline additions, could stress the microorganisms and cause reactor failure. Saponification of fat by the high amount of alkaline additions could be another reason, increasing the solubility of the fat resulting in higher contact between the microorganisms and inhibitory fatty products.

8.3.1.2 *Hydrogen production:*

As can be seen from Figure 8-6, most production of hydrogen gas was observed in the early days of the experiments. The variation in hydrogen production could be due to different hydrolysis and acetogenesis rates (possibly due to seeding sludge homogeneity limitations) and/or consumption of the hydrogen among the mixed microorganism culture. As an example, the highest total production of hydrogen gas was obtained from control No.4 (Figure 8-7). This could be due to fast hydrolysis in this reactor, as acetic acid and LCFAs showed a greater increase compared to the other reactors (Figure 8-12 and Figure 8-18).

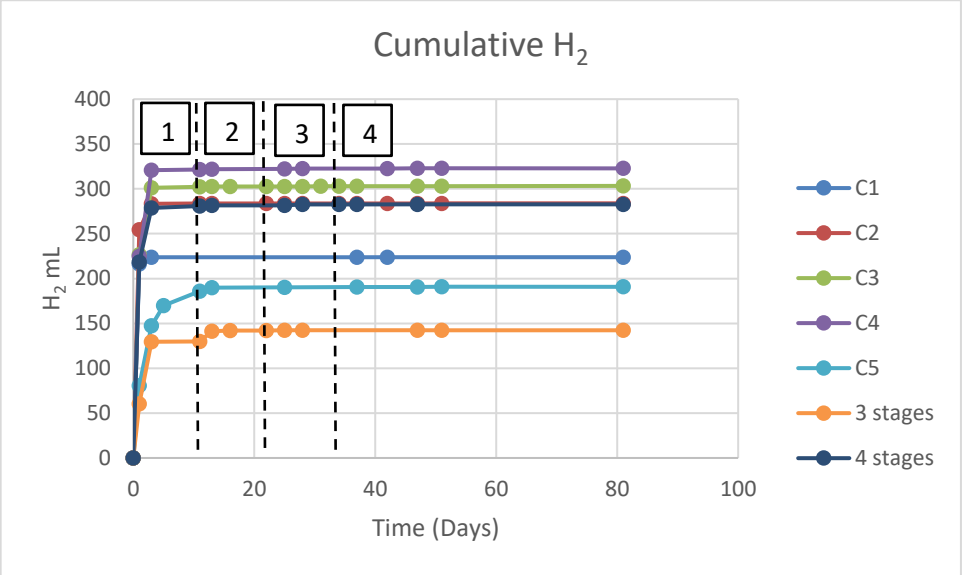

Figure 8-6: Cumulative hydrogen production from the experimental reactors.

X axis represents the time in days; Y axis represents the cumulative hydrogen in millilitres. The dashed lines on days 11, 22, and 33 represents the start of the next second, third, and fourth stages respectively, with pH control and/or reseeding.

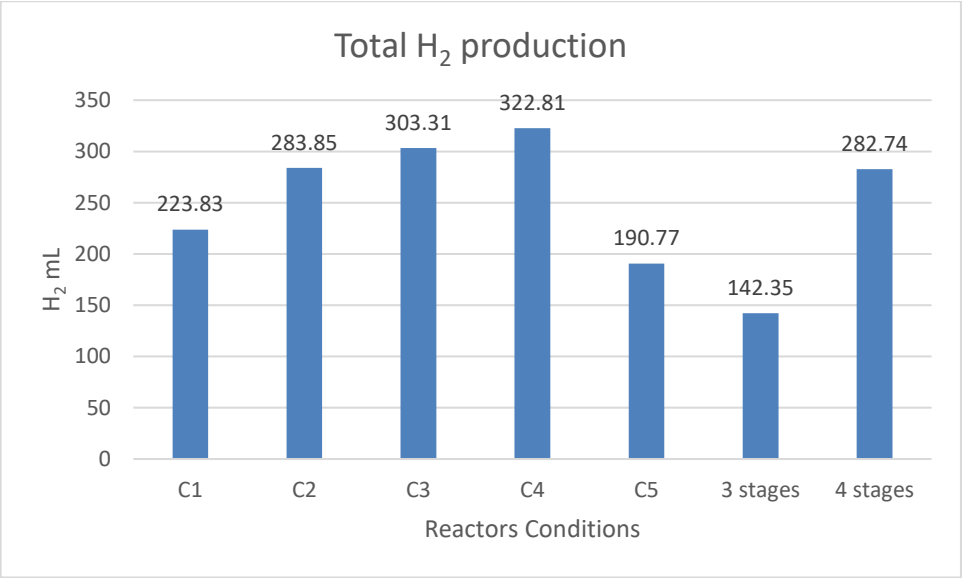

Figure 8-7: Total hydrogen production from the experimental reactors.

X axis represents the time duration in days; Y axis represents the total hydrogen production in millilitres.

8.3.1.3 *Carbon dioxide:*

Carbon dioxide shows similar behaviour to methane. Increasing carbon dioxide from both control No.3 and the three stage reactor, shown in Figure 8-8, can be an indication of acetoclastic methanogenesis activity, since the increase in carbon dioxide was associated with an increase in methane from these reactors (Figure 8-4). The situation would be different if the majority of the active methanogens were hydrogenotrophic microorganisms, as a decrease in carbon dioxide would be expected due to the conversion of carbon dioxide and hydrogen into methane.

Since there was no methane production during the increase in carbon dioxide from the control reactor No.4 and the four stage reactor after day 22, this increase is believed to be due the reaction between the hydrochloric acid and the saturated sodium bicarbonate during the pH control process (Figure 8-4 and Figure 8-8).

The highest total carbon dioxide production was from the active biogas reactor control No.3 (Figure 8-8 and Figure 8-9).

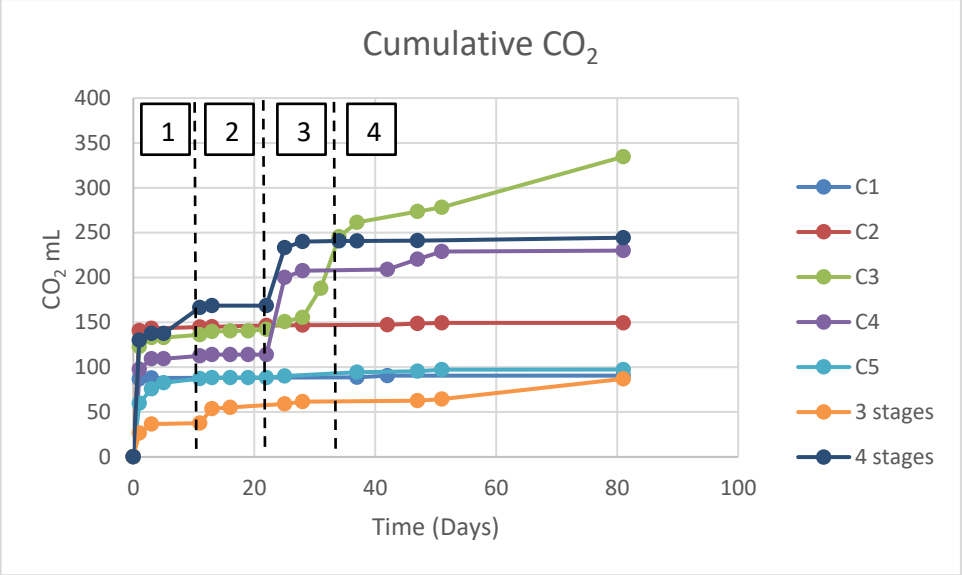

Figure 8-8: Cumulative carbon dioxide generation from the experimental reactors.

X axis represents time in days; Y axis represents the cumulative carbon dioxide in millilitres. The dashed lines on days 11, 22, and 33 represents the start of the next second, third, and fourth stages respectively, with pH control and/or reseeding.

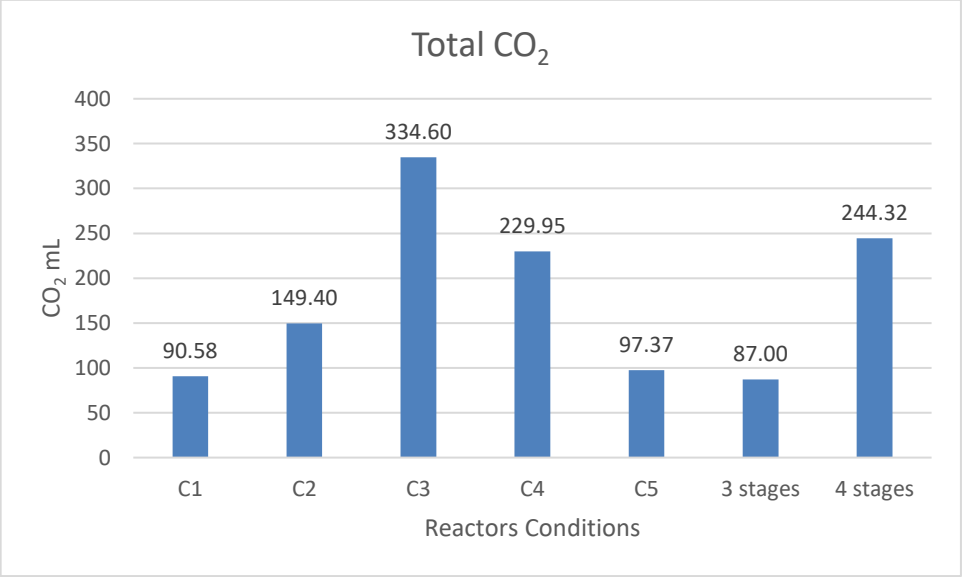

Figure 8-9: Total carbon dioxide generated from the experimental reactors.

X axis represents the time duration in days; Y axis represents the total carbon dioxide production in millilitres.

8.3.2 **pH behaviour:**

pH measurements were made both manually and with the automatic pH controllers. This was to overcome possible errors or drifts in the internal pH sensors, which might result from clogging of the ceramic reference junction or an accumulation of fat around the electrode's glass membrane, leading to wrong automatic readings and control of pH. Manual pH measurements were used to correct any drift in the automatic controller in order to maintain the required experimental pH conditions.

As can be seen from Figure 8-10 and Figure 8-11, the start point of the pH was around pH 8 for all reactors. This was due to the natural pH of both the fat powder substrate and the seeding sludge, no additions were made to change the pH at the start-up of experimental reactors.

The pH naturally dropped as a result of hydrolysis and acidogenesis during the first few days. The pH decreased from around pH 8 on day 0, to around pH 5 after only one day. Then it remained around pH 5 without noticeable change for the whole period of the experimental work in the case of single stage controls reactors No.1 and No.2 (Figure 8-10). In the case of the single stage thermophilic control reactor No.5, the pH was slightly higher but remained between pH 5 and pH 6. However, in the case of the multi stage reactors, the pH remained around pH 5 during the first stage of the control No.4 reactor (the pH controlled multistage control reactor), the three stage reactor, and the four stage reactor. pH remained around pH 5 during the first two stages of the control reactor No.3 (the reseeding only multistage control reactor).

As can be seen from Figure 8-11, on day 11, the pH was controlled from pH 5 to around pH 8.5 in both control reactor No.4 and the four stage reactor. It was also controlled to around pH 7 for the three stage reactor. The increase in the pH was performed through the addition of saturated $NaHCO_3$ via the automatic pH controller in order to provide the pH conditions of the second stages.

From the beginning of the third stage, the pH of the control reactor No.3 (with only re-seeding steps) showed a natural and continuous increase after re-inoculation. This increase is believed to be due to methanogenic activity, as the increase in pH was associated with an increase in methane gas production and

a decrease in acetic acid concentration (Figure 8-4, Figure 8-11, and Figure 8-17).

At the beginning of the third stage in both control reactor No. 4 and the four stage reactor, the pH values were lowered to pH 7 through the addition of 1M HCl via the pH controller's dosing pumps. In contrast to start the third and final stage in the three stage reactor, the pH value was set to pH 8 via the automatic pH control unit.

The fourth stage was started in the control reactor No.4 and in the four stage reactor by setting the pH to pH 8 through the pH control units and re-inoculating the four stage reactor with an anaerobic seeding sludge.

In the case of control No.3 the fourth stage was started by re-inoculating the reactor without any buffer addition. This caused a natural increase in the pH to pH 7.73 and was associated with an increase in methane and carbon dioxide production and a decrease in acetic, butyric and caproaic acids. The decrease in these higher VFAs indicates acetogenic activity. The increase in methane and carbon dioxide and the decrease in acetic acid indicates acetoclastic methanogenesis, the generation of CH_4 and CO_2 from acetate.

As can be seen from Figure 8-11, some fluctuations in pH occurred in the three pH controlled reactors after day 33. This is most probably due to drift and re-correction of the measurements of the pH controlling units. Drift in pH measurement and slower response are common limitations with long exposer of the pH electrode to the reactor's substrate. Another reason for the fluctuation could be natural pH changes due to the microorganisms' activities and correction of this pH by the controllers.

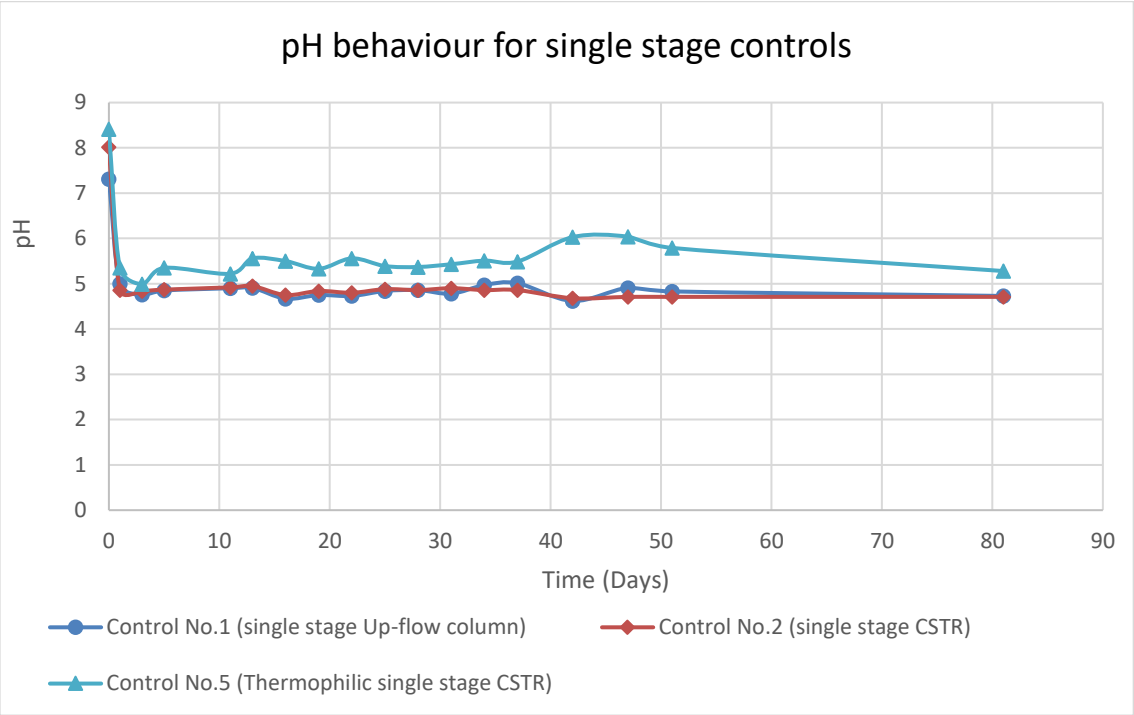

Figure 8-10: pH behaviour for the anaerobic single stage controls reactors.

X axis represents time in days; Y axis represents the pH.

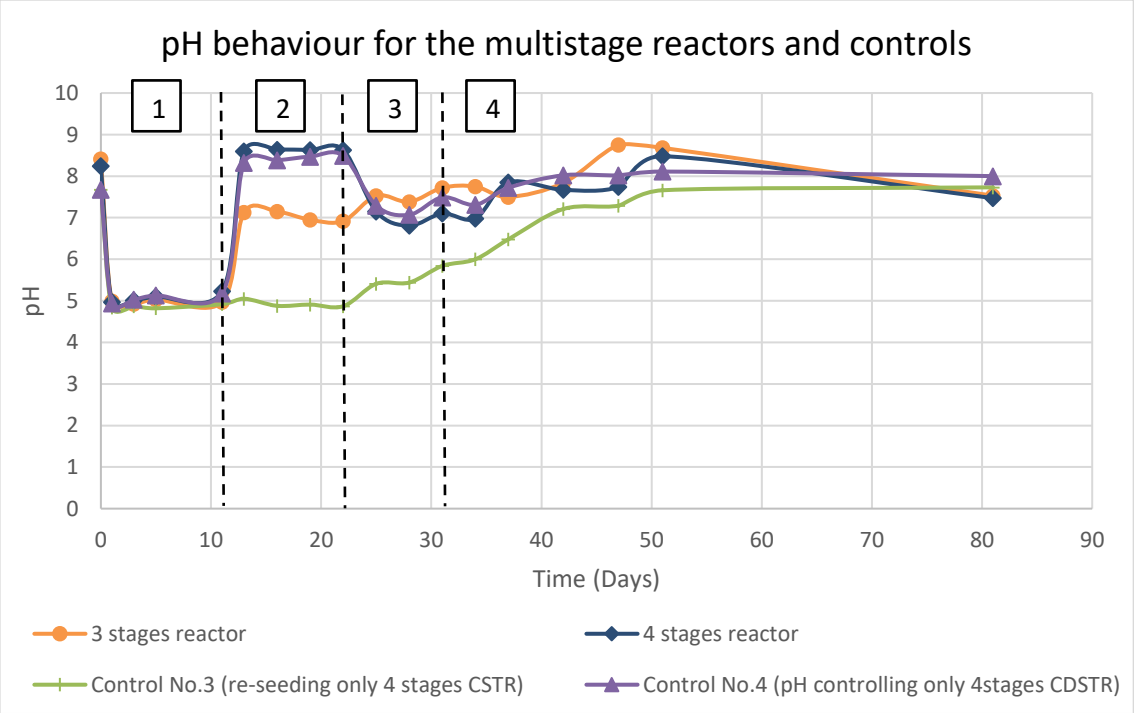

Figure 8-11: pH behaviour of the multi stage (3 and 4 stages) anaerobic reactors and the multi stage control reactors No. 3 and No.4.

X axis represents time in days; Y axis represents the pH. The dashed lines on days 11, 22, and 33 represents the start of the second, third, and fourth stages respectively, with pH control and/or reseeding.

8.3.3 Long chain fatty acids

The total LCFA concentration showed variations according to the experimental condition of each reactor. In addition, possible limitations were discussed in chapter seven.

The limitation could be due to physical limitations in the homogeneity of the samples. For example, some semisolid fat particles were sticking to the pipe wall and the sampling syringe during sampling which may lead to a reduction in fat content of the sample. Another possibility is the physical separation of some solidified fat in the single stage mesophilic CSTR and up-flow reactors. The partial separation of fat could be due the hydrophobicity of the fat and the circular mixing action in CSTR, leading to some spherical solidified fat particles on the single reactor surface. In the single stage up-flow column reactor (control No.1), both solidified fat and fat foam can occur.

As whole, non-filtered samples were used to determine the LCFAs in the reactor environment, solidification and separation of fat will have a direct effect on the LCFA results. On the other hand, the addition of alkaline buffer was found to prevent the separation and solidification of fat. This could be due to the alkaline hydrolysis of the fat that results in more homogenised samples. The re-seeded reactor showed less formation and separation of solid fat particles after the start of the second stage. This may indicate better degradation due to more active anaerobic microorganisms provided by the re-seeding step. In addition, no fat foam or solidified fat was observed in the single stage thermophilic reactor No.5, as the melting point of beef tallow is lower than the 60 °C. However, an oily liquid layer can be observed on top of the reactor contents.

Overall results of the LCFAs, illustrated in Figure 8-12, show an increase in LCFA concentration during the first 10 days of the experiment. This could be due to the hydrolysis process of the raw fat.

Between day 11 and day 22, the three single stage control reactors No. 1, 2, and 5 showed different LCFA behaviour. In the control reactor No. 1, (the up-flow control column reactor), the total LCFAs showed a continuous decrease without any related gas production. Therefore, the decrease in total LCFAs in this reactor is believed to be due to the separation and flotation of solidified fat

and foam on the reactor surface as a result of the up-flow circulation. In the control reactor No.2, the LCFAs remained nearly the same. In the thermophilic control No.5, the total LCFAs showed an increase between day 11 and day 22. The relatively high temperature (60 °C) in the thermophilic condition could be the reason as it prevents the solidification and separation of fat particles inside the reactor, but it does cause a liquid fat layer which still limits in the sample homogeneity.

In case of the multi stage reactors and controls, control No.3 (the re-inoculating control reactor) showed an increase in total LCFAs after day 22, associated with an increase in methane production and a sharp decrease in the acetic acid. This is an indication of the hydrolysis process of some solidified fat particles, resulting in an increase in the LCFAs. The increase in LCFA concentration might inhibit acetogenic activity and lead to a lower LCFA degradation and acetic acid production. After the fourth and last re-inoculating of control No.3, total LCFAs decreased while acetic acid increased along with methane production. The re-inoculation step would have provided the reactor with more anaerobic microorganisms to overcome the inhibition and dilute the inhibitory concentration of LCFAs at the same time.

Both three and four stage reactors showed similar behaviour in their final stages (up-flow mthanogenic) to the control reactor No.1. The total LCFAs showed a continuous decrease in this last stage, due to the same physical limitations that affected control No.1 (fat separation, flotation and foam formation). This could result in lower fat content in the samples and, therefore, lower LCFA results.

Separation of fat and the formation of solidified fat particles are expected to cause a decrease in the LCFAs results, whereas the breakdown of solidified fat particles during the stirring process will lead to an increase in detected LCFAs.

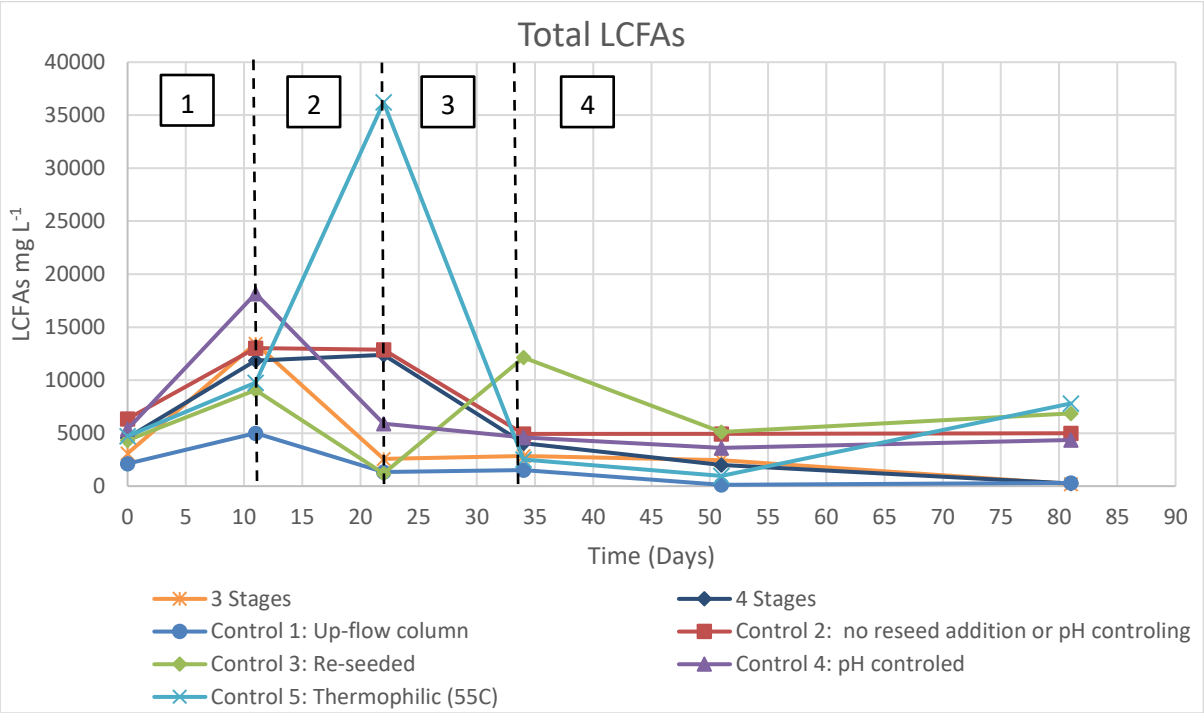

Figure 8-12: Total LCFA behaviour for the experimental reactors.

X axis represents time in days; Y axis represents the total LCFAs in mg L^{-1}. The dashed lines on days 11, 22, and 33 represents the start of the second, third, and fourth stages respectively, with pH control and/or reseeding.

8.3.4 **Volatile fatty acids**

All the reactors showed an increase in volatile fatty acids from early in the experiment. This increase can be directly related to the acidogenesis and acetogenesis activity of microorganisms. Acetic acid levels are one of the clearest indications of the digester progress, especially when combined with the methane and carbon dioxide production and the overall pH behaviour.

The three stage reactor showed an increase in acetic, butyric, and caproaic acids during the first 10 days of the experiment. However, as can be seen from Figure 8-13, between days 10 and 16 after the second stage was started, the VFAs showed another increase in these acids and in propionic and valeric acids as well. This could be due to higher acetogenesis activity, as it was associated with both a decrease in total LCFAs (Figure 8-8 and Figure 8-12), and a slight increase carbon dioxide in hydrogen gas production (Figure 8-6). Changing the pH at the beginning of the second stage from pH 5 to around pH 7 (Figure 8-11), and the re-inoculating step could be the reason for the enhanced acetogenesis process. On day 22, after the third and last stage was established in the up-flow column reactor, the acetic, propionic and caproaic acids showed a drop in concentration.

This decrease could be due to the start-up of the third stage. The reactor contents were transferred into a column reactor, followed by the addition of saturated $NaHCO_3$ solution to adjust the pH to 8, sparging with nitrogen gas, and finally, re-inoculating the new stage with an anaerobic seeding sludge at I/S ratio of 0.1.

Propionc acid and caproaic acid show a slight increase whereas butyric decreases between day 22 and 28. There were then no significant changes until day 51. Between days 51 and 81, acetic acid shows sharp and continuous increase, with a slight increase in propionic acid, and decrease in all of the higher VFAs (Figure 8-13). The drop in higher VFAs and the continuous increase in acetic acid between day 51 and day 81 (Figure 8-13) was associated with a decrease in the total LCFAs as well (Figure 8-12). This could be an interesting indication of the acetogenesis process, responsible for converting higher fatty acids into acetate. The increase in acetic acid between days 51 and

81, is associated with an increase in methane production (Figure 8-4) indicating that the acetic acid is now being used in methanogenesis but not at a rate that outstrips its production (Figure 8-13).

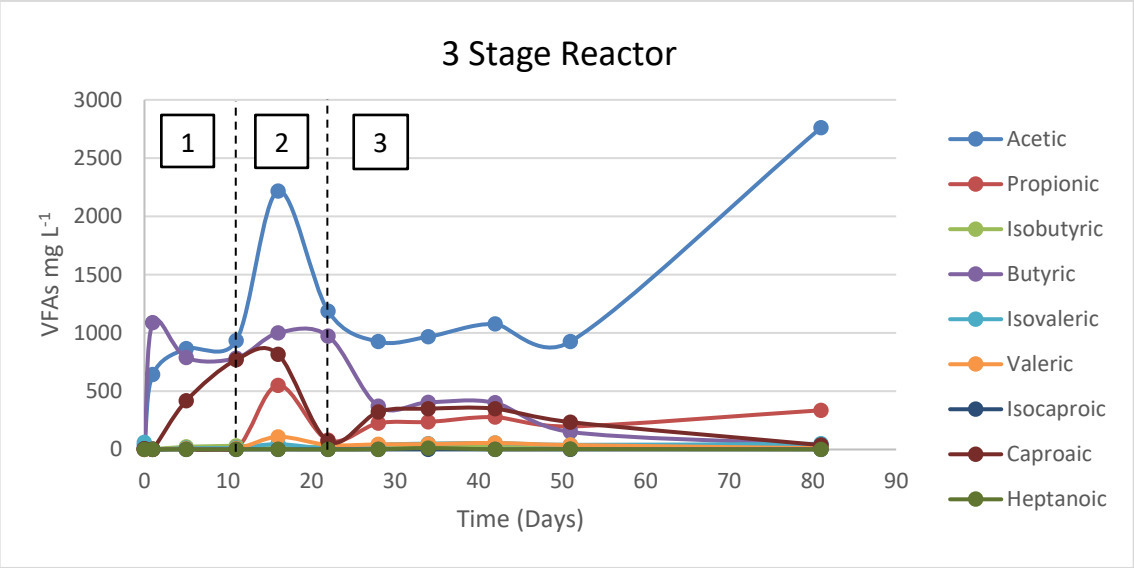

Figure 8-13: Volatile fatty acid behaviour from the three stage anaerobic reactor.

X axis represents time in days; Y axis represents the amount of VFAs in mg L^{-1}. The dashed lines on days 11, and 22, represents the start of the second, and third stages respectively, with pH control and reseeding

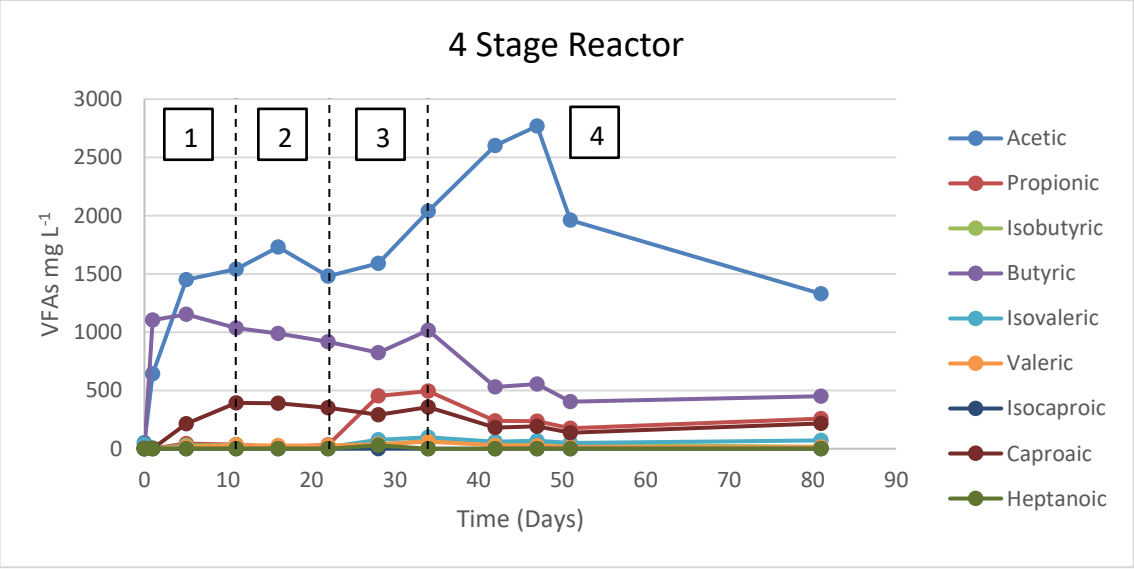

Figure 8-14: Volatile fatty acid behaviour from the four stage anaerobic reactor.

X axis represents time in days; Y axis represents the amount of VFAs in mg L^{-1}. The dashed lines on days 11, 22, and 33 represents the start of the second, third, and fourth stages respectively, with pH control and reseeding steps.

In case of the four stage reactor, acetic acid shows an increase at the beginning of the experiment and after the start-up of the third and fourth stages (Figure 8-14). No significant changes in VFAs were observed during the second stage, with conditions of 60 °C, pH 8.5, and *Thermosyntropha lipolytica* inoculation. During the fourth stage, acetic acid shows some decrease from day 47 until the end of the experiment. This might indicate some initial methanogenic or syntrophic acetate oxidation activity. However, this is not supported by evidence of active biogas production.

Looking at the VFA behaviour for control No.1 and control No.2 (Figure 8-15 and Figure 8-16), along with their methane, carbon dioxide and hydrogen gas production (Figure 8-4, Figure 8-6, and Figure 8-8), and the their pH behaviour (Figure 8-10), it is clear that the process of anaerobic digestion has been interrupted in these reactors as there are no active changes in these parameters.

The failure of these reactors could be due to several reasons. First, the inoculum to substrate ratio (I/S ratio) in term of the volatile solids (vs/vs) was very low and may not provide enough anaerobic microorganisms to the reactor. Second, the accumulation of the initial by-products of fat degradation (VFAs and LCFAs) might have an inhibitory effect on the microorganisms. The drop in pH during the first few days of the experiment could also affect the growth and activity of the acetogenic and methanogenic microorganisms.

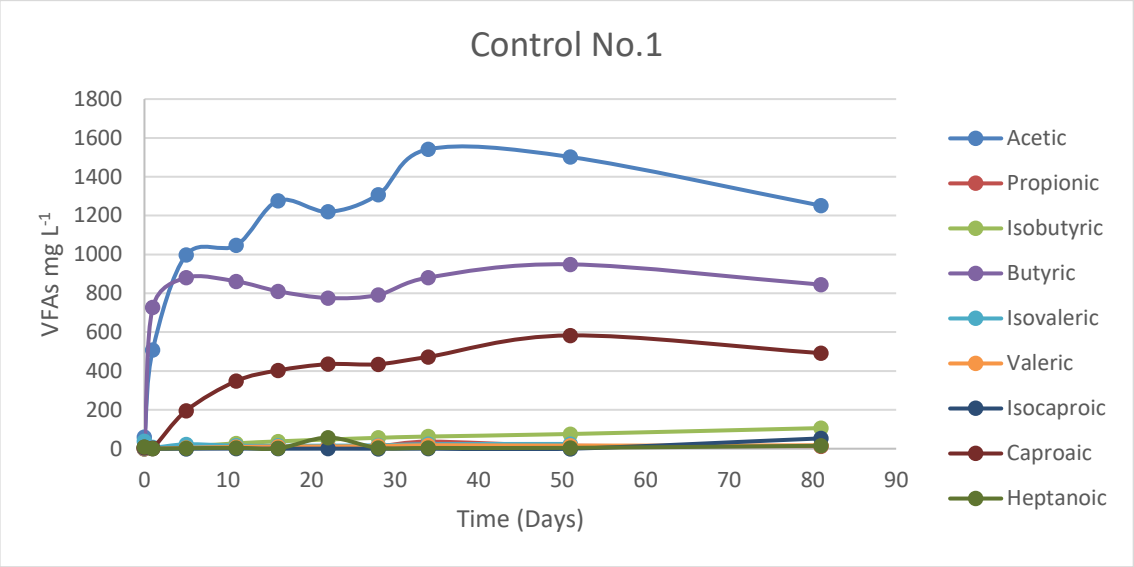

Figure 8-15: Volatile fatty acid behaviour from the single stage control No.1 up-flow column reactor.

X axis represents time in days; Y axis represents the amount of VFAs in mg L^{-1}.

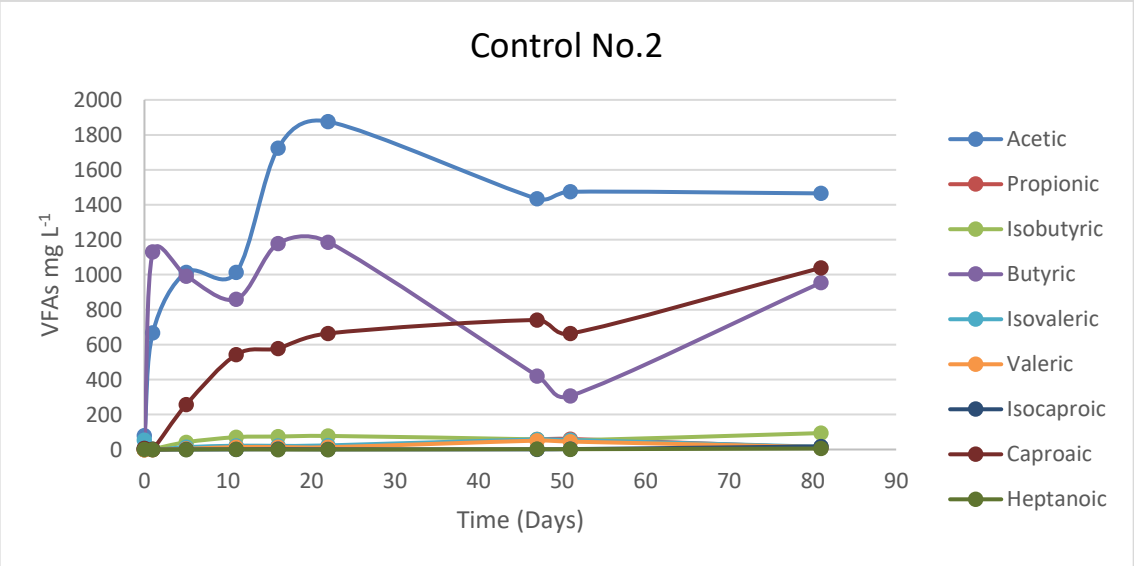

Figure 8-16: Volatile fatty acid behaviour from the single stage control No.2 CSTR anaerobic reactor.

X axis represents time in days; Y axis represents the amount of VFAs in mg L^{-1}.

In case of the control No.3 reactor (re-inoculating only), the VFAs show a continuous increase during the first and second stages from day 0 until day 22. After the start-up of the third stage on day 22, the concentration of acetic acid starts to decrease and this is associated with an increase in methane gas production, which indicates activity of methanogenic microorganisms. The decrease in acetic acid can also indicate a lower activity of acetogenic microorganisms due to inhibition or a lack in the availability of the proper intermediate for a fast acetic acid conversion.

After the start-up of the fourth stage, butyric and caproaic acids start to decrease while acetic acid increases until day 51. This is a clear indication of the activity of acetogenic microorganisms, which convert the higher VFAs into acetic acid. Between day 51 and day 81, acetic acid shows a sharp decrease associated with a sharp increase in methane gas production and carbon dioxide (Figure 8-17 and Figure 8-4). The decrease in acetic acid with an increase in methane and carbon dioxide could be due to acetoclastic methanogenesis, converting acetate into methane and carbon dioxide.

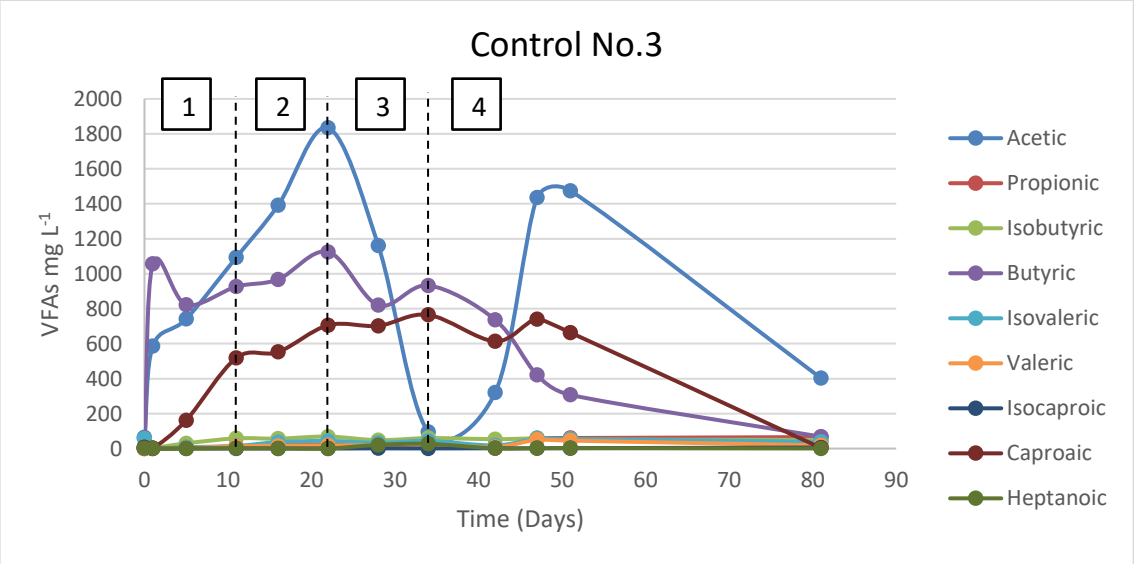

Figure 8-17: Volatile fatty acid behaviour from the multi stage control No.3 (re-inoculating only) anaerobic reactor.

X axis represents time in days; Y axis represents the amount of VFAs in mg L^{-1}. The dashed lines on days 11, 22, and 33 represent the start of the second, third, and fourth stages respectively, with only reseeding steps.

In case of the control reactor No.4 (multi-stage with pH control only), acetic and caproaic acids show an increase from the beginning of the experiment. However, both acids show fluctuations in between and after the automatic pH adjustment process (Figure 8-18). The increase in acetic and caproaic acids are believed to indicate activity of acidogenesis and acetogenesis microorganisms as the increase is associated with an increase in hydrogen and carbon dioxide at the beginning of the experiment (Figure 8-6 and Figure 8-8). The fluctuations of VFAs in between and after the automatic pH control process, could be a shock effect, especially at this low I/S ratio and fast change in pH from pH 5 to pH 8.5, 7, and 8 respectively. Acetic acid shows a decrease after the fourth stage, indicating some initial methanogenic or syntrophic acetate oxidation activity. However, this is not supported by evidence of active biogas production.

The lack of biogas production and reactor failure could be due to several reasons, one of them is the large amount of buffer being added to the reactor through the pH controller in order to change the pH according to each stage. The fast shift in the pH, in addition to the possible high amount of salt that might be generated during acid and alkaline addition could stress the microorganisms and cause reactor failure. Saponification of fat by the high amount of alkaline addition, could be another reason where an increase the solubility of fat results in higher contact between the microorganisms and the inhibitory fatty products.

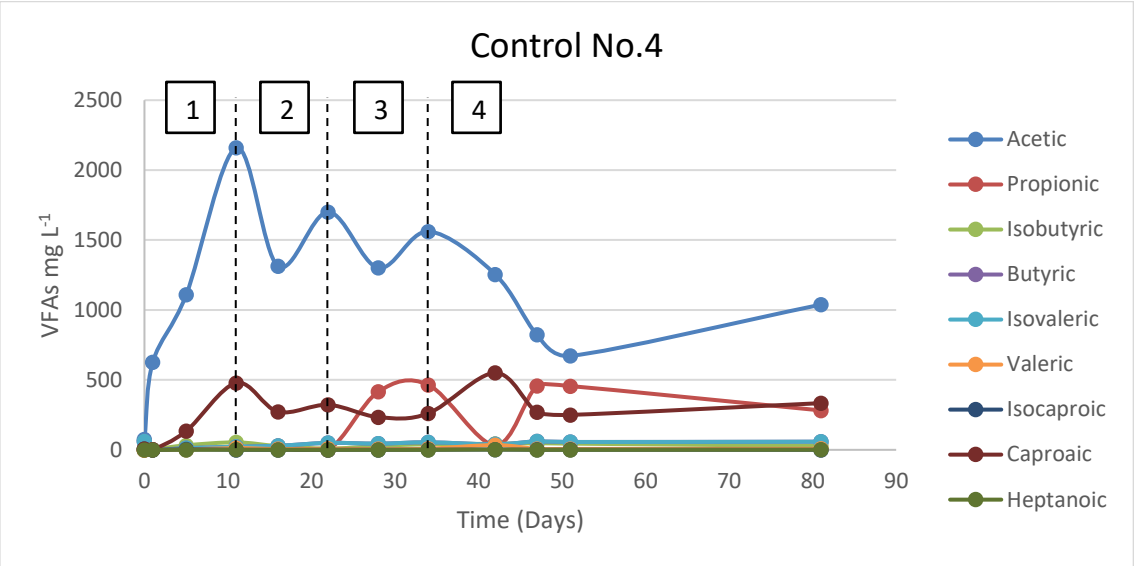

Figure 8-18: Volatile fatty acid behaviour from the multi-stage control No.4 (pH controlling only multi-stage control reactor).

X axis represents time in days; Y axis represents the amount of VFAs in mg L^{-1}. The dashed lines on days 11, 22, and 33 represent the start of the second, third, and fourth stages respectively, with only pH control steps.

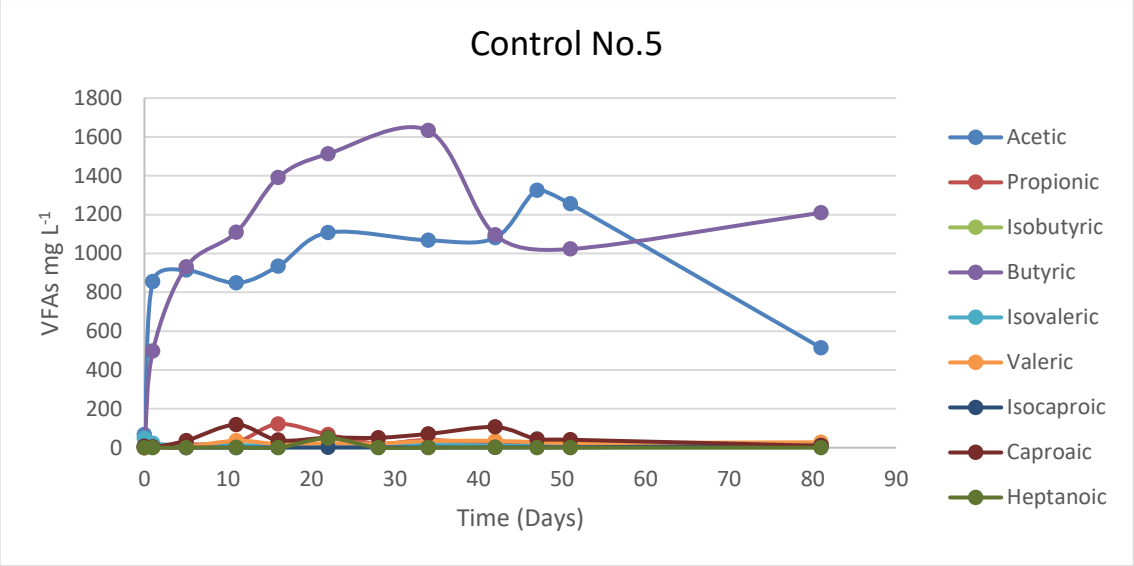

Figure 8-19: Volatile fatty acid behaviour from the single stage control No.5 thermophilic CSTR anaerobic reactor.

X axis represents time in days; Y axis represents the amount of VFAs in mg L^{-1}.

In case of the thermophilic control reactor No.5, acetic and butyric acids show an increase from the beginning of the experiment until days 34 and 47 respectively after which they start to decrease (Figure 8-19). The increase in acetic and caproaic acids is believed to indicate activity of acidogenesis and acetogenesis microorganisms associated with an increase in hydrogen and carbon dioxide at the beginning of the experiment (Figure 8-6 and Figure 8-8). However, the decrease in butyric and acetic acids might indicate some initial methanogenic or syntrophic acetate oxidation activity. However, this is not supported by evidence of active biogas production.

8.3.5 **Chemical oxygen demand**

As can be seen from Figure 8-20, the initial dissolved COD values for all the experimental reactors were between 5234 and 5566 mg L^{-1}. These dropped to around 3560 mg L^{-1} during the first three days of the experiments. The drop may be due to hydrolysis activity (acidogenesis and acetogenesis), and is associated with the generation of hydrogen and a drop in pH. After this, COD values increased until reaching a plateau around day 20 in the single stage controls No.1 (up-flow reactor), No.2 (the single CSTR reactor), and No.5 (thermophilic CSTR reactor). These three reactors showed no effective methane production and show similar trends in pH to the COD. This supports the theory that these three reactors were unable to successfully deal with the fat substrate under their current operational conditions.

However, in case of the multistage reactors and controls, (three stage, four stage, control No.3, and control No.4), COD behaviour was directly related to methane gas production, buffer addition for pH control, and volatile fatty acid behaviour.

In all of the automatic pH reactors, control No.4, three stage, and four stage reactors, there was an increase in dissolved COD after the start of the second stage on day 11. This increase was associated with the addition of alkali (saturated $NaHCO_3$) to raise the pH, therefore, a possible explanation of the increase in COD could be the increase in fat solubility through alkaline saponification.

From day 35, the behaviour of COD was steady in most of the reactors, especially in the ones with no active methane production. However, in case of control reactor No.3 (re-inoculating control), the soluble COD shows a slight and continuous decrease after day 35, which is associated with an increase in methane and carbon dioxide production (Figure 8-4 and Figure 8-8), a natural increase in the pH (Figure 8-11) and a decrease in acetic acid and other higher VFAs (Figure 8-17).

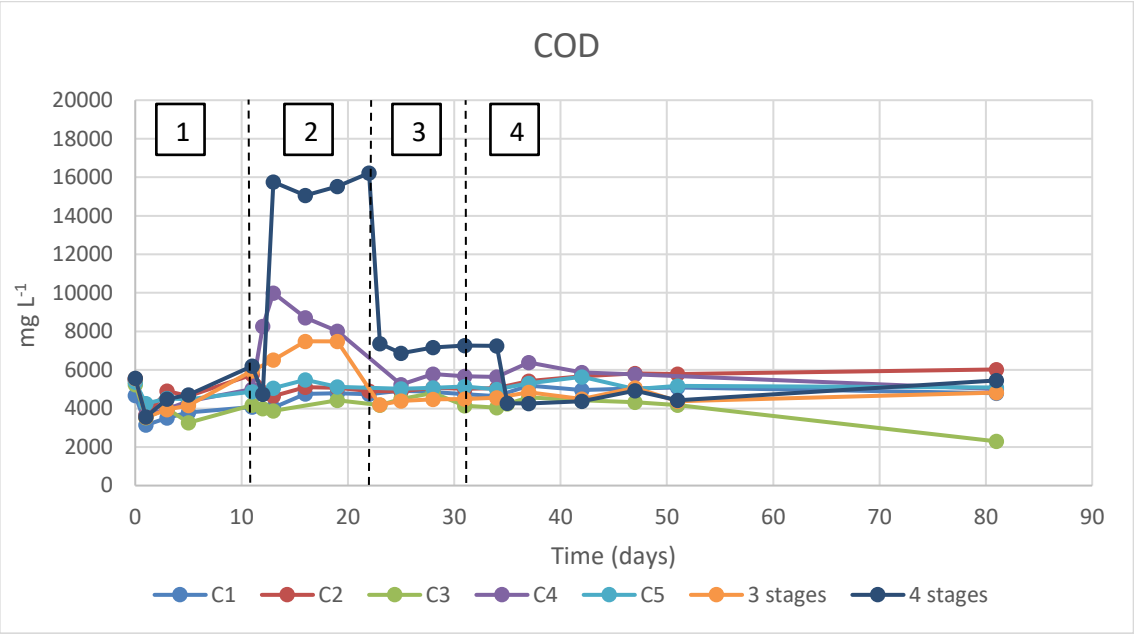

Figure 8-20: Soluble COD behaviour in the experimental reactors.

X axis represents time in days; Y axis represents the amount of dissolved COD in mg L^{-1}. The dashed lines on days 11, 22, and 33 represent the start of the second, third, and fourth stages respectively, with pH control and reseeding steps

8.4 Overall discussion:

8.4.1 The effect of re-inoculating low I/S ratio reactors:

The 1L working volume control reactor No.3 (with only re-seeding steps), was re-inoculated three times with an I/S ratio of 0.1 each time, on day 11, day 22, and day 33.

Re- inoculation steps showed a gradual and successful start-up of the digestion process at high fat concentrations, by re-introducing the anaerobic bacteria to the reactor. Similar finding was reported by Palatsi *et al.* (2009), in a study of anaerobic reactors recovering from LCFA inhibition through diluting the reactor content by re-inoculating steps. These re-inoculations increase the bacterial biomass and are found to be one of the best strategies to recover a reactor from LCFA inhibition.

From the overall results, control reactor No.3, with only re-inoculating steps, showed higher and earlier methane production compared to the other reactors. This reactor started active methane production from day 25 during its third stage, and reached a total methane production of 744.28 mL by the end of the experiment. Comparing to this the three stage reactor, shows a later increase in methane production after day 51, 3.8 times lower methane production of 195.04 mL than control No.3.

8.4.2 Multi-stages reactors with pH controls and re-inoculating steps:

From the overall results, control reactor No.3, with only re-inoculating steps (with no pH controlling), showed higher and earlier methane production compared to the other reactors, as it started active methane production from day 25. The three stage reactor, showed a later increase in methane production after day 51, the rest of the experimental reactors (including the four stage set and the rest of control reactors) did not generate methane and were not able to effectively start-up.

The control No. 3, with only re-inoculating steps was already discussed under “The effect of re-inoculating of low I/S ratio” earlier in this chapter.

In case of the three stage reactor, active methane production started from day 47, 25 days from the start of the third stage (Figure 8-4). This increase in methane production was associated with an increase in CO_2, indicating activity of acetoclastic methanogenesis. However, this methane production was associated with an increase in acetic acid and a slight increase in propionic acid concentration during the third stage of the three stage reactor (Figure 8-8). At the same time, higher VFAs such as caproaic and butyric acids decreased inside the reactor (Figure 8-13). The increase in acetic acid associated with a decrease in other higher volatile fatty acids can indicate the activity of acetogenic microorganisms, which convert the higher VFAs into acetic acid. As this increase in acetic acid is associated with an increase in methane production it is believed that the acetic acid is now being used in methanogenesis but not at a rate that outstrips its production. Overall, the reactor showed 3.8 times lower methane production of 195.04 mL than control No.3. However, this was still much higher than the four stage reactor and the other control reactors.

In case of the single stage control reactors No. 1 (up-flow column control reactor), No.2 (CSTR control reactor), and No.5 (thermophilic single stage CSTR control reactor), all showed very low methane production and could not effectively start-up the digestion process. This is believed to be due to the low I/S ratio of 0.1 VS, which may not be enough to start up anaerobic digestion as reported earlier in chapters 5 and 6.

However, in case of the four stage reactor, no significant methane production was observed although it was re inoculated and pH controlled. The failure in this reactor could be due to several possible reasons, one of them is the large amount of buffer being added to the reactor through the pH controller in order to change the pH in each stage. The fast shifting of temperature and pH, in addition to the possible high amount of salt that might be generated during acid and alkaline additions, could stress the microorganisms and cause reactor failure. Saponification of fat by high alkaline additions, could be another reason, increasing the solubility of the fat resulting in higher contact between the microorganisms and inhibitory fatty products.

Chapter 9 Conclusions, recommendations and future work

9.1 Conclusions:

9.1.1 General:

Anaerobic digestion of high fat concentrations (of 20g L^{-1} VS) is found to be possible when sufficient amount of anaerobic inoculum (I/S ratio of 1 or more vs/vs) are used. However, it is difficult to obtain this I/S ratio without using a very large volume of seeding sludge due to its naturally low volatile solids content. This will lead to the disadvantage of wasting most of the reactor volume and therefore reducing the capacity to receive fresh organic waste. However, it does suit the likely operation mode of large scale industrial reactors, especially in a two-stage system.

Minimising the added volume of the seeding sludge to increase the efficient use of a batch reactor's capacity, without interrupting the digestion process, is an option. However, lowering the I/S ratio generally results in instable and slower, or even failure of, the digestion process, especially when treating a substrate with a high fat concentration.

Therefore, different strategies were studied including different concentrations of fat in co-digestion with vegetable waste, different I/S ratios, different operating conditions of single stage reactors (mesophilic CSTR, thermophilic CSTR, and mesophilic up-flow), in addition to multi-stages reactors (two, three, and four) to optimise each phase in the digestion process were investigated. The aim was to achieve a good digestion process for high fat concentration with a minimum amount of seeding sludge in the reactor.

Two stage reactors (with pH controlling and reseeding steps), and a three time re-seeding strategy showed the best ability in treating high fat concentrations of 20g L^{-1} VS and minimising the required seeding sludge for the process at the same time. Use of a two stage reactor with two seeding steps of 0.1 I/S ratio each time was found to successfully treat the 20g L^{-1} VS fat substrate with 80% less seeding sludge volume comparing to the I/S ratio of 1. re-seeding a single reactor with four seeding steps of 0.1 I/S ratio each, was able to successfully treat the same amount of fat substrate with 60% less seeding sludge volume compared to the I/S ratio of 1.

Due to the complexity and overlap between the digestion processes, it was found to be very difficult to physically separate each stage in a separate reactor through optimising the pH condition and it can be concluded that three or more stages are difficult to set up and probably not worth the capital and running costs involved. The reactors in a two stage system can be easily separated by temperature, pH or a combination of both.

9.1.2 Fat concentration:

- At an inoculum to substrate ratio (I/S ratio vs/vs) of 1, more fat in the feed results in more methane production.
- Late start-up of active biogas production is associated with higher fat concentrations in the feed substrate.
- The total methane gas production is directly proportional to the amount of fat. In other words, the higher the proportion of fat contributing to the 20 gL^{-1} VS feed, the higher the total methane production. This is another motivation to convert the problematic slaughterhouse waste in Makkah during Hajj into an environmentally efficient energy source.

9.1.3 Inoculum to substrate ratio:

- controlling the I/S ratio to 1, while difficult in laboratory studies and requiring a large working volume of the reactor due to the naturally very low volatile solid content in the digested seeding sludge comparing to the feed substrate, will be more suitable in an industrial two stage system. ≥80% of the reactors' working volume is occupied by the seeding sludge when an I/S ratio of 1 is applied.
- Higher I/S ratios result in faster and much more stable start-up of the digester and higher methane production with lower initial hydrogen gas production.
  Lower I/S ratios result in instable and slower start-ups, with higher hydrogen gas production during the start-up time. An I/S ratio of 0.5 extended the methane production lag phase to between 31 days and 46 days for 75% and 100% fat conditions respectively. An I/S ratio of 0.1 was insufficient to actively treat the fatty substrate anaerobically. An I/S

ratio of 0.25, appears to be on the border line between sufficient and insufficient.

- There is a direct correlation between lower I/S ratios and hydrogen gas production during the early days of the experiments. Results show that lowering the I/S ratio below $\leqslant$0.5 leads to higher hydrogen production. 100% fat condition produces more hydrogen than the 75% one for all I/S ratios with highest hydrogen at the lowest (0.1) I/S ratio.

- Starting up a mesophilic anaerobic reactor with a low seeding sludge concentration has been shown to require more time and more careful management compared to the use of a high concentration of seeding sludge.

9.1.4 Acclimatised and not acclimatised seeding sludge:

- pre-acclimatised seeding sludge showed a better ability to treat fat waste than non-acclimatised sludge after several re-inoculating steps on both pathways.

9.1.5 pH control

- Providing an initial optimum pH condition enhances the hydrolytic and acidogenic microorganism activity, and therefore better fat hydrolysis and degradation are expected.

9.1.6 Re-inoculation

- Re-inoculation steps show a gradual and successful start-up of the digestion process at high fat concentration, by re-introducing anaerobic bacteria to the reactor.

9.1.7 Multi-stage reactors

- Two stage reactors show higher methane and hydrogen gas production and better ability to treat the fat substrate. Two stage reactor systems are likely to be good candidates for industrial systems.

- In case of the three stage reactors, active methane production started from day 47, 25 days from the start of the third stage. This increase in methane production was associated with an increase in CO_2, which probably indicates activity of the acetoclastic methanogenesis.

- A four stage reactor system failed and is unlikely to be a feasible in an industrial situation. The changes required between each stage probably do not allow enough time for any one stage to settle.

9.2 Research recommendations and suggestion for the anaerobic digestion of slaughterhouse waste generated during Hajj (pilgrimage) in Makkah:

Several recommendations can be proposed according to the results and observations during this study.

In case of Makkah situation, where tons of fatty slaughterhouse waste are generated (during the last four days of al-hajj) every year, anaerobic digestion treatment is suggested to be applied through a combination of two stage digester and high I/S ratio in the second stage.

This can be achieved through building two types of tank. First, large storage tanks (first stage), where the organic waste can be stored and hydrolysed after a grinding pre-treatment. Second, medium methanogenic (second stage) tanks that can be fed regularly with small amounts of the substrate from the first stage storage tanks in a semi-continuous mode over the year. This will offer a high I/S ratio in the second stage which can deal with the high concentration and inhibitory of fat according to the findings in this research. Furthermore, introducing other types of high nitrogen waste like sewage and other organic waste from Makkah city in a co-digestion with the slaughterhouse waste can be beneficial in reducing the inhibitory effects of the high fat concentration in the digester substrate.

9.3 Future Work:

- Different (closer) reseeding schedules can be studied to investigate the possibility to shorten the required time for the overall digestion process.

- Study co-digestion of the 20g L^{-1} VS fat with small percentages of other organic waste like (vegetable waste, food waste, or sterilised sewage sludge) with the two stage reactor in order to reduce the digestion time by providing simpler organic materials in the co-substrate to enhance the process and reduce the inhibitory of fat.

- Study a semi-continuous feeding condition on a two stage reactor.

- Test a scaled-up two stage system for treating sterilised fresh fatty slaughterhouse waste.

- Study pilot-scale trials on homogenised slaughterhouse waste in conjunction with solar thermal energy as temperature controlling option (At Hajj and Umrah Research Institute - Makkah - Saudi Arabia). This would be to see the applicability of implementing this on industrial scale during the annual Hajj period in Saudi Arabia, to deal with the slaughterhouse waste.

- Study the use of a thermophilic first stage in a two stage system, as it may prevent the accumulation and solidification of fat.

- Further develop the small anaerobic pH probe chamber to make new versions with oxygen and samples sensors with semi or full automated flushing and sampling process. Adding a sterilising option can be beneficial with the single cultures and sterilised experiments.

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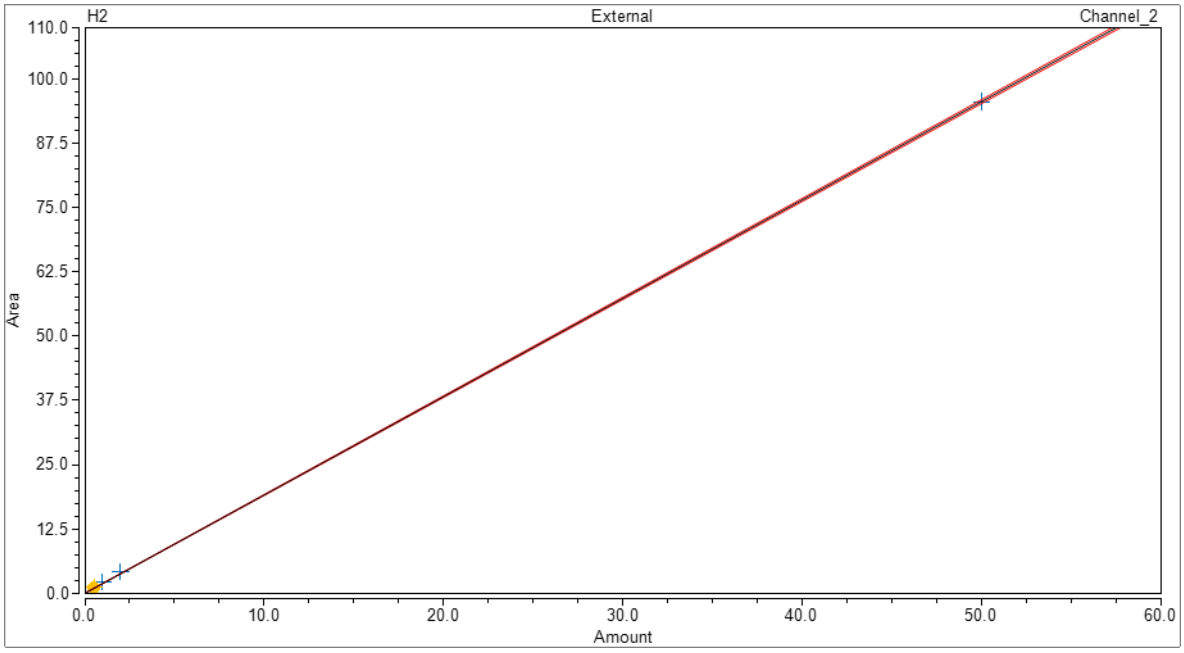
Appendix

Appendix

Appendix - A: (calibrations cuves).

A-1: calibrations curves for the gas analysis GC (THERMO 1310 TCD-GC).

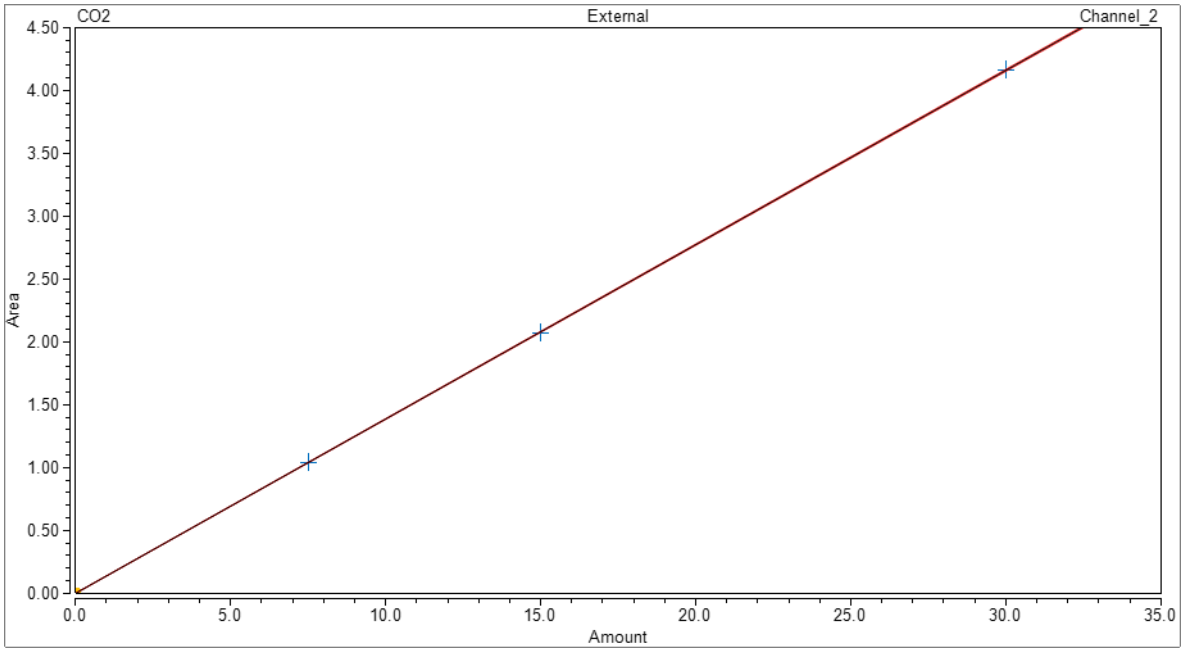
A-1-1: Hydrogen calibration:



Calibration Details	H2					
Calibration Type	Lin			Offset (C0)		0
Evaluation Type	Area			Slope (C1)		1.9125
Number of Calibration Points	4			Curve (C2)		0
Number of disabled Calibration Points	0			R-Square		1

Calibration Results		H2					
No.	Injection Name	Calibration Level	X Value	Y Value	Y Value	Area mV*min	Height mV
			Channel_2 H2	Channel_2 H2	Channel_2 H2	Channel_2 H2	Channel_2 H2
1	Calibration STD (1)	1	2	4.1194	4.1194	4.119	84.818
2	Calibration STD (2) * 0.5 concentration	2	1	2.0697	2.0697	2.07	40.488
3	Calibration STD (3) * 0.25 concentration	3	0.5	1.0818	1.0818	1.082	20.383
10	Calibration STD (4) 50% H2	10	50	95.611	95.611	95.611	678.486

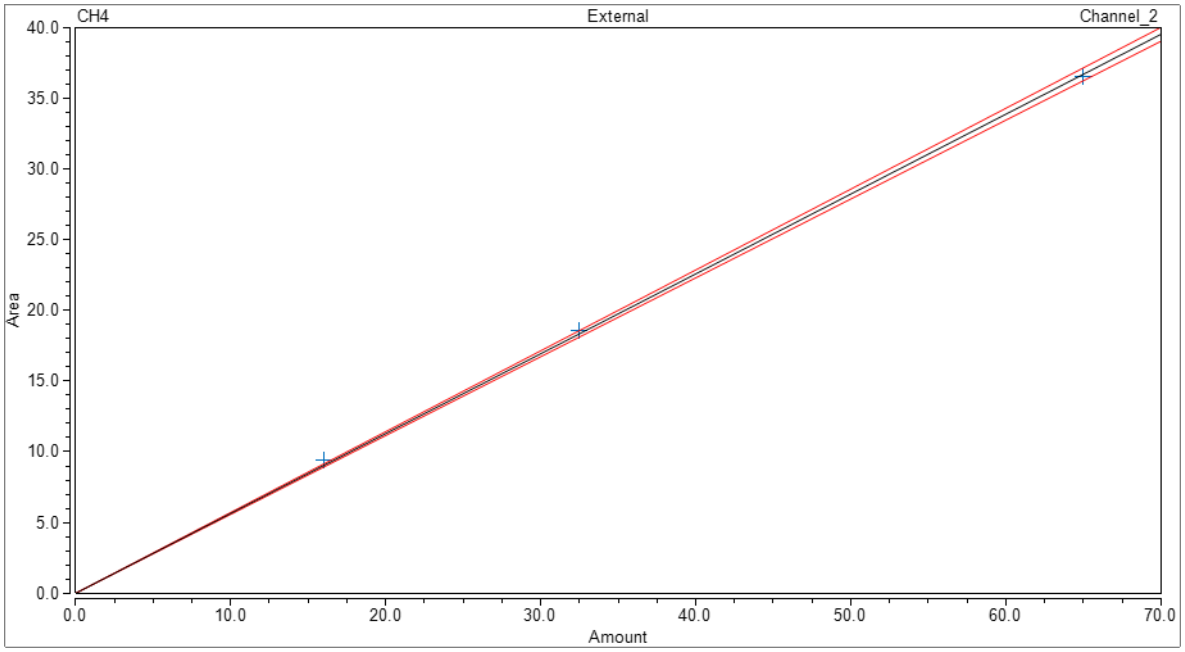
A-1-2: Carbon dioxide calibration:



Calibration Details	CO2					
Calibration Type	Lin			Offset (C0)		0
Evaluation Type	Area			Slope (C1)		0.1387
Number of Calibration Points	3			Curve (C2)		0
Number of disabled Calibration Points	0			R-Square		1

Calibration Results		CO2					
No.	Injection Name	Calibration Level	X Value	Y Value	Y Value	Area	Height
						mV*min	mV
			Channel_2 CO2	Channel_2 CO2	Channel_2 CO2	Channel_2 CO2	Channel_2 CO2
1	Calibration STD (1)	1	30	4.1639	4.1639	4.164	21.864
2	Calibration STD (2) * 0.5 concentration	2	15	2.0736	2.0736	2.074	12.783
3	Calibration STD (3) * 0.25 concentration	3	7.5	1.0419	1.0419	1.042	7.29

A-1-3: Methane calibration:

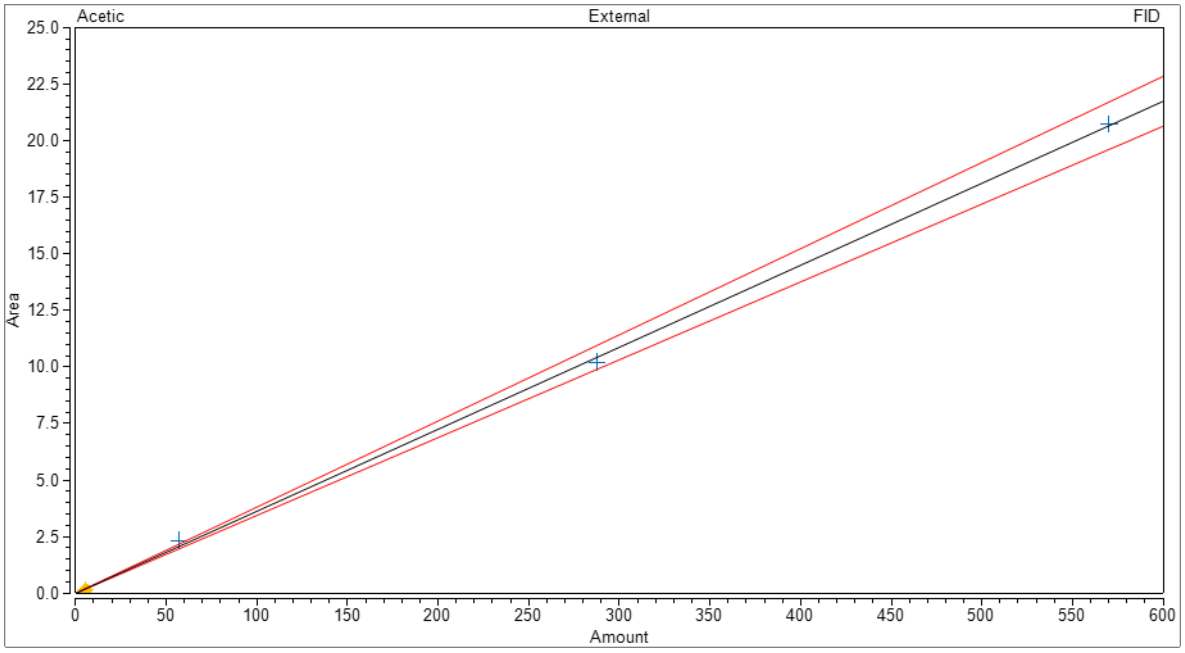


Calibration Details	CH4					
Calibration Type	Lin			Offset (C0)		0
Evaluation Type	Area			Slope (C1)		0.5647
Number of Calibration Points	3			Curve (C2)		0
Number of disabled Calibration Points	0			R-Square		0.9994

Calibration Results		CH4					
No.	Injection Name	Calibration Level	X Value	Y Value	Y Value	Area	Height
						mV*min	mV
			Channel_2	Channel_2	Channel_2	Channel_2	Channel_2
			CH4	CH4	CH4	CH4	CH4
1	Calibration STD (1)	1	65	36.5157	36.5157	36.516	103.926
2	Calibration STD (2) * 0.5 concentration	2	32.5	18.5436	18.5436	18.544	57.059
3	Calibration STD (3) * 0.25 concentration	3	16	9.4329	9.4329	9.433	30.665

A-2 calibrations curves for the volatile fatty acid GC (Trace 1300 Gas Chromatograph).

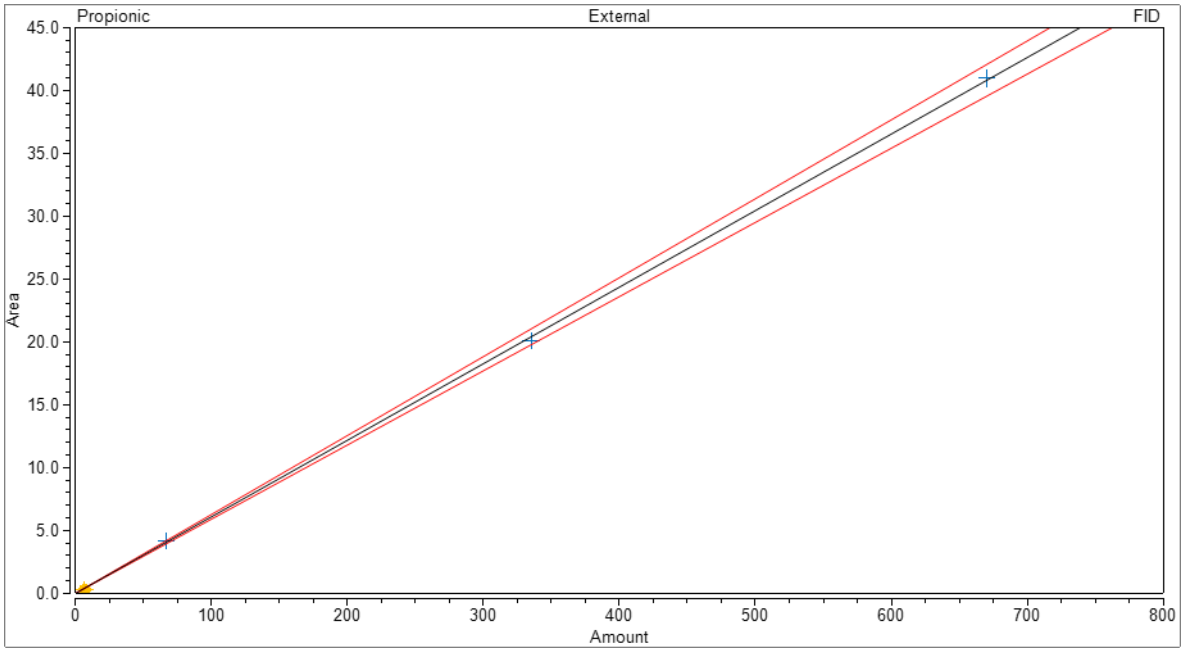
A-2-1: Acetic acid calibration:



Calibration Details	Acetic					
Calibration Type	Lin			Offset (C0)		0
Evaluation Type	Area			Slope (C1)		0.0362
Number of Calibration Points	4			Curve (C2)		0
Number of disabled Calibration Points	0			R-Square		0.9995

Calibration Results		Acetic					
No.	Injection Name	Calibration Level	X Value	Y Value	Y Value	Area	Height
						pA*min	pA
			FID	FID	FID	FID	FID
			Acetic	Acetic	Acetic	Acetic	Acetic
1	Cal 1	1	5.7	0.127	0.127	0.127	0.822
2	Cal 2	2	57.0475	2.3253	2.3253	2.325	15.123
3	Cal 3	3	287.5	10.2141	10.2141	10.214	67.498
4	Cal 4	4	570	20.7422	20.7422	20.742	135.851

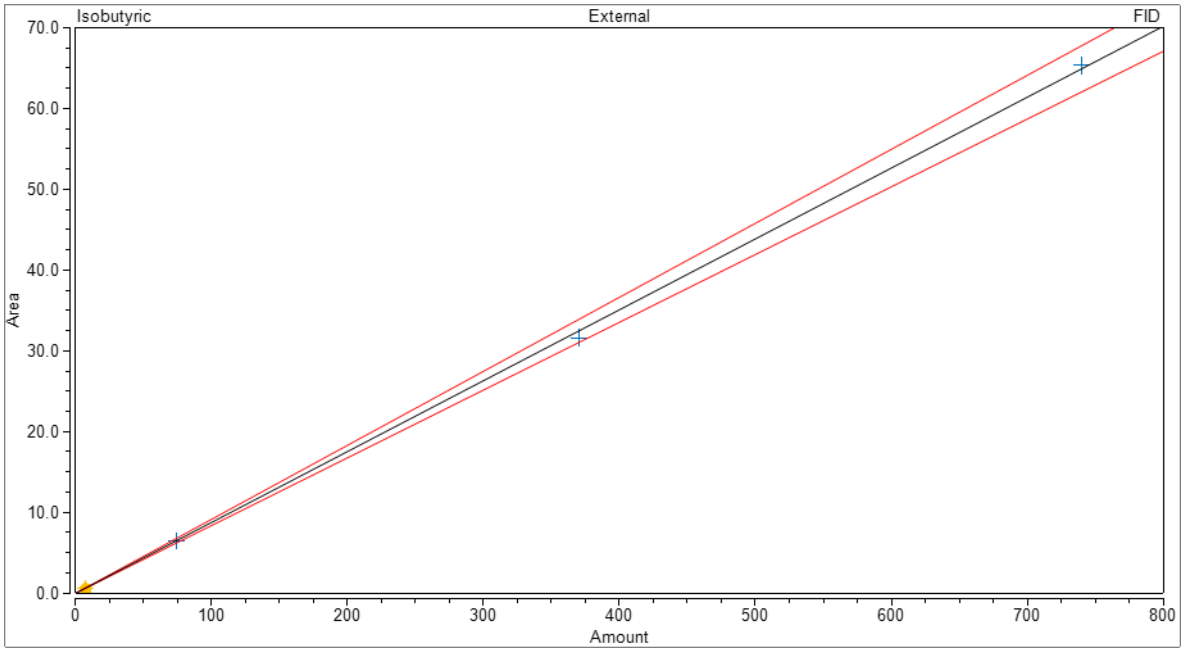
A-2-2: Propionic acid calibration:



Calibration Details	Propionic					
Calibration Type	Lin			Offset (C0)		0
Evaluation Type	Area			Slope (C1)		0.0609
Number of Calibration Points	4			Curve (C2)		0
Number of disabled Calibration Points	0			R-Square		0.9998

Calibration Results		Propionic					
No.	Injection Name	Calibration	X Value	Y Value	Y Value	Area	Height
		Level				pA*min	pA
			FID	FID	FID	FID	FID
			Propionic	Propionic	Propionic	Propionic	Propioni
1	Cal 1	1	6.7	0.2675	0.2675	0.267	2.031
2	Cal 2	2	67	4.197	4.197	4.197	30.837
3	Cal 3	3	335	20.0597	20.0597	20.06	147.645
4	Cal 4	4	670	40.9643	40.9643	40.964	295.605

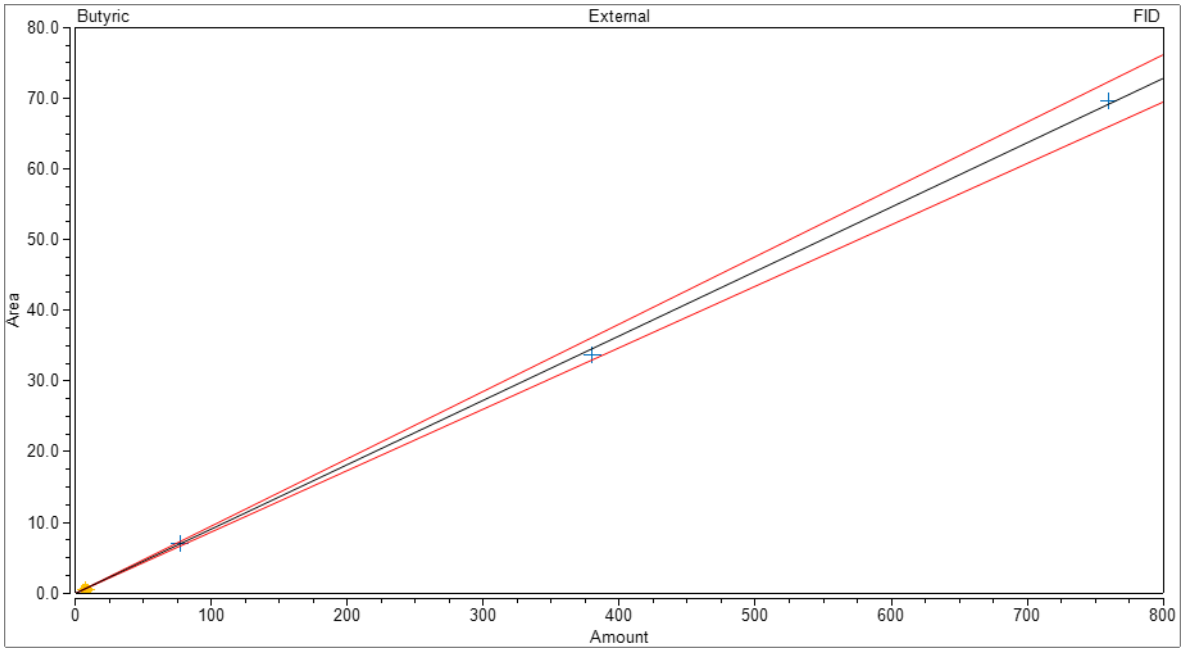
A-2-3: Isobutyric acid calibration:



Calibration Details	Isobutyric					
Calibration Type	Lin			Offset (C0)		0
Evaluation Type	Area			Slope (C1)		0.0877
Number of Calibration Points	4			Curve (C2)		0
Number of disabled Calibration Points	0			R-Square		0.9997

Calibration Results		Isobutyric					
No.	Injection Name	Calibration Level	X Value	Y Value	Y Value	Area	Height
						pA*min	pA
			FID	FID	FID	FID	FID
			Isobutyric	Isobutyric	Isobutyric	Isobutyric	Isobutyric
1	Cal 1	1	7.4	0.5145	0.5145	0.515	3.581
2	Cal 2	2	74.0124	6.4988	6.4988	6.499	43.042
3	Cal 3	3	370	31.6128	31.6128	31.613	207.464
4	Cal 4	4	740	65.3329	65.3329	65.333	417.788

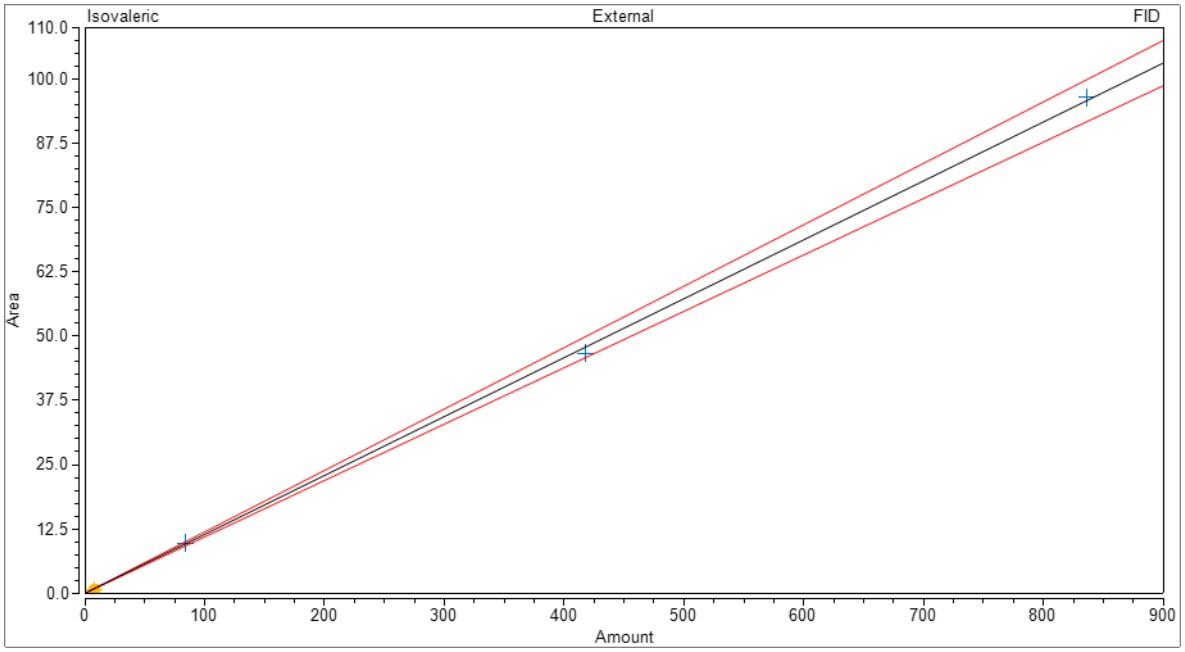
A-2-4: Butyric acid calibration:



Calibration Details	Butyric					
Calibration Type	Lin			Offset (C0)		0
Evaluation Type	Area			Slope (C1)		0.0911
Number of Calibration Points	4			Curve (C2)		0
Number of disabled Calibration Points	0			R-Square		0.9996

Calibration Results		Butyric					
No.	Injection Name	Calibration Level	X Value	Y Value	Y Value	Area	Height
						pA*min	pA
			FID	FID	FID	FID	FID
			Butyric	Butyric	Butyric	Butyric	Butyric
1	Cal 1	1	7.6	0.4967	0.4967	0.497	3.749
2	Cal 2	2	76.6557	7.0033	7.0033	7.003	50.762
3	Cal 3	3	380	33.6693	33.6693	33.669	247.809
4	Cal 4	4	760	69.6634	69.6634	69.663	498.615

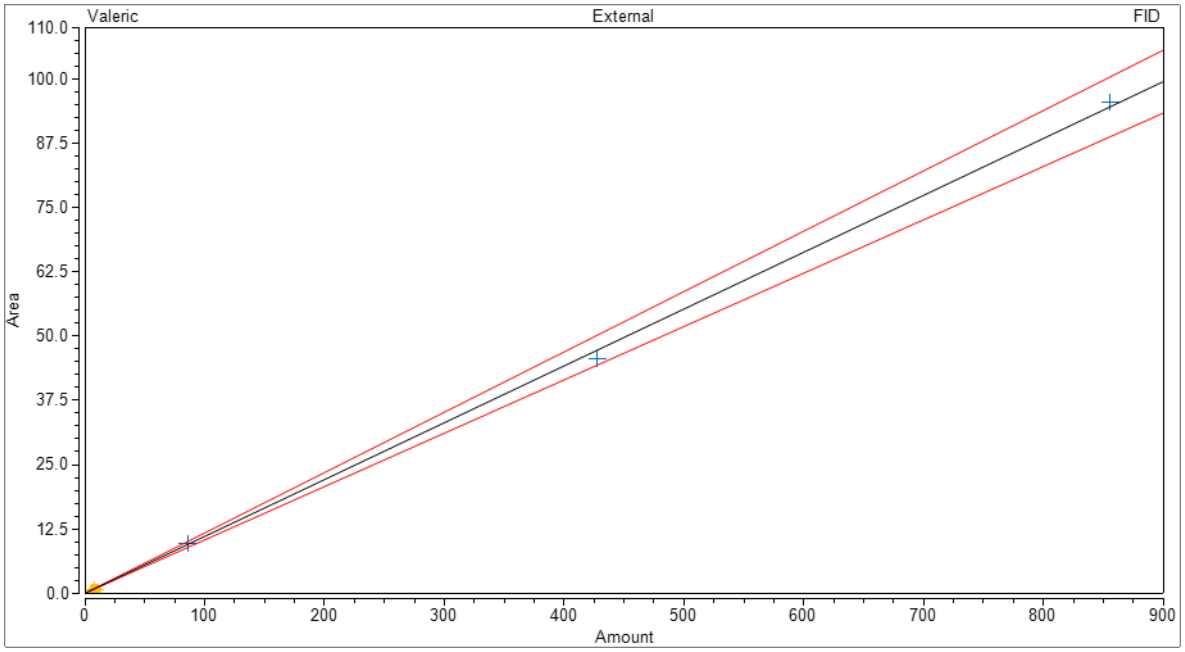
A-2-5: Isovaleric acid calibration:



Calibration Details	Isovaleric					
Calibration Type	Lin			Offset (C0)		0
Evaluation Type	Area			Slope (C1)		0.1146
Number of Calibration Points	4			Curve (C2)		0
Number of disabled Calibration Points	0			R-Square		0.9997

Calibration Results		Isovaleric					
No.	Injection Name	Calibration Level	X Value	Y Value	Y Value	Area	Height
						pA*min	pA
			FID	FID	FID	FID	FID
			Isovaleric	Isovaleric	Isovaleric	Isovaleric	Isovaleric
1	Cal 1	1	8.3	0.7606	0.7606	0.761	5.726
2	Cal 2	2	83.6461	9.7596	9.7596	9.76	70.997
3	Cal 3	3	418	46.6953	46.6953	46.695	343.212
4	Cal 4	4	836	96.3773	96.3773	96.377	691.069

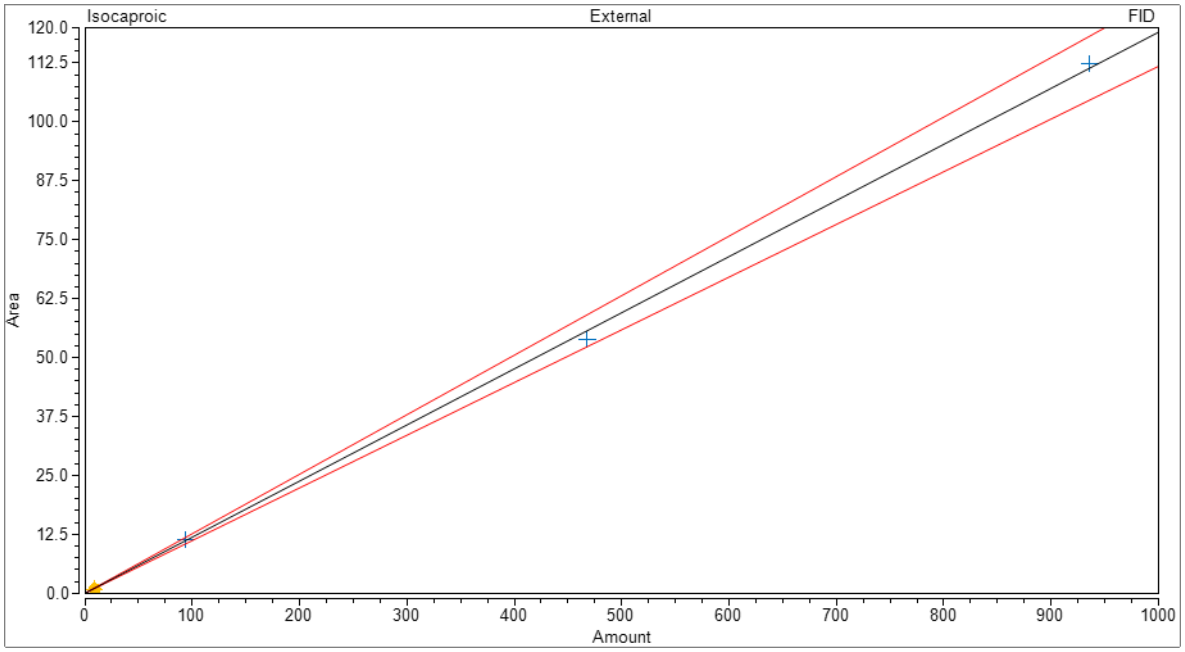
A-2-6: Valeric acid calibration:



Calibration Details	Valeric					
Calibration Type	Lin			Offset (C0)		0
Evaluation Type	Area			Slope (C1)		0.1106
Number of Calibration Points	4			Curve (C2)		0
Number of disabled Calibration Points	0			R-Square		0.9993

Calibration Results		Valeric					
No.	Injection Name	Calibration Level	X Value	Y Value	Y Value	Area	Height
						pA*min	pA
			FID	FID	FID	FID	FID
			Valeric	Valeric	Valeric	Valeric	Valeric
1	Cal 1	1	8.5	0.6965	0.6965	0.696	5.244
2	Cal 2	2	85.6871	9.6479	9.6479	9.648	71.196
3	Cal 3	3	428	45.6049	45.6049	45.605	342.875
4	Cal 4	4	856	95.492	95.492	95.492	695.726

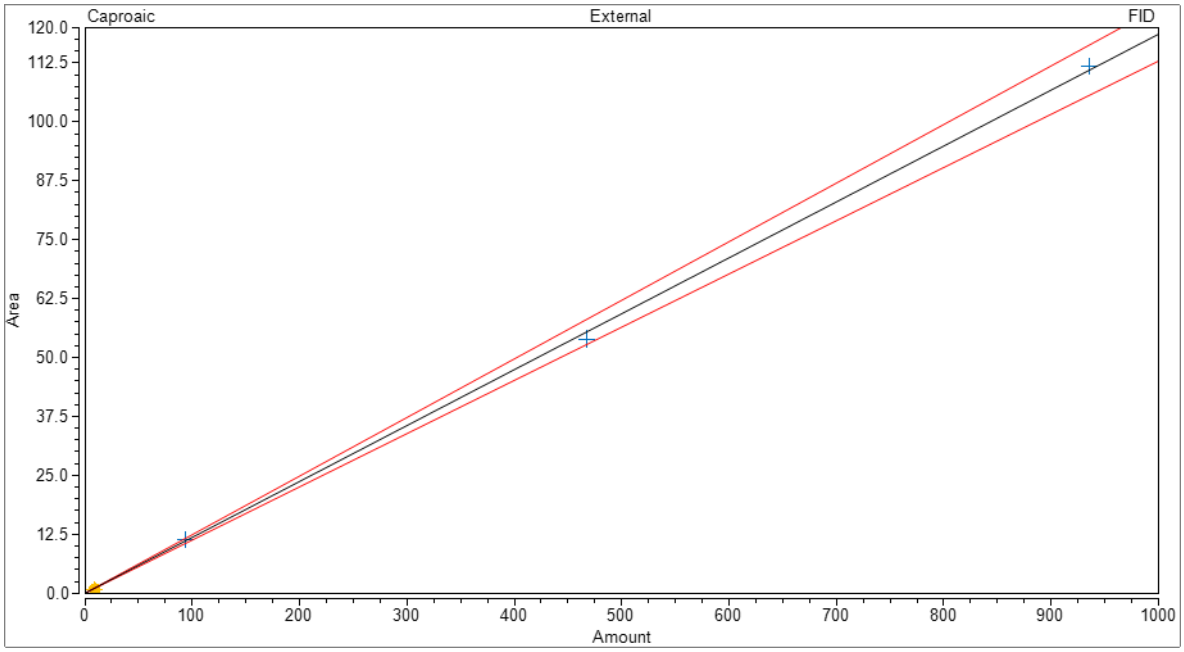
A-2-7: Isocaproic acid calibration:



Calibration Details	Isocaproic					
Calibration Type	Lin			Offset (C0)		0
Evaluation Type	Area			Slope (C1)		0.1191
Number of Calibration Points	4			Curve (C2)		0
Number of disabled Calibration Points	0			R-Square		0.9993

Calibration Results		Isocaproic					
No.	Injection Name	Calibration Level	X Value	Y Value	Y Value	Area	Height
						pA*min	pA
			FID	FID	FID	FID	FID
			Isocaproic	Isocaproic	Isocaproic	Isocaproic	Isocaproic
1	Cal 1	1	9.3	0.8568	0.8568	0.857	5.812
2	Cal 2	2	93.6236	11.3382	11.3382	11.338	76.065
3	Cal 3	3	468	53.7182	53.7182	53.718	365.133
4	Cal 4	4	936	112.4362	112.4362	112.436	749.823

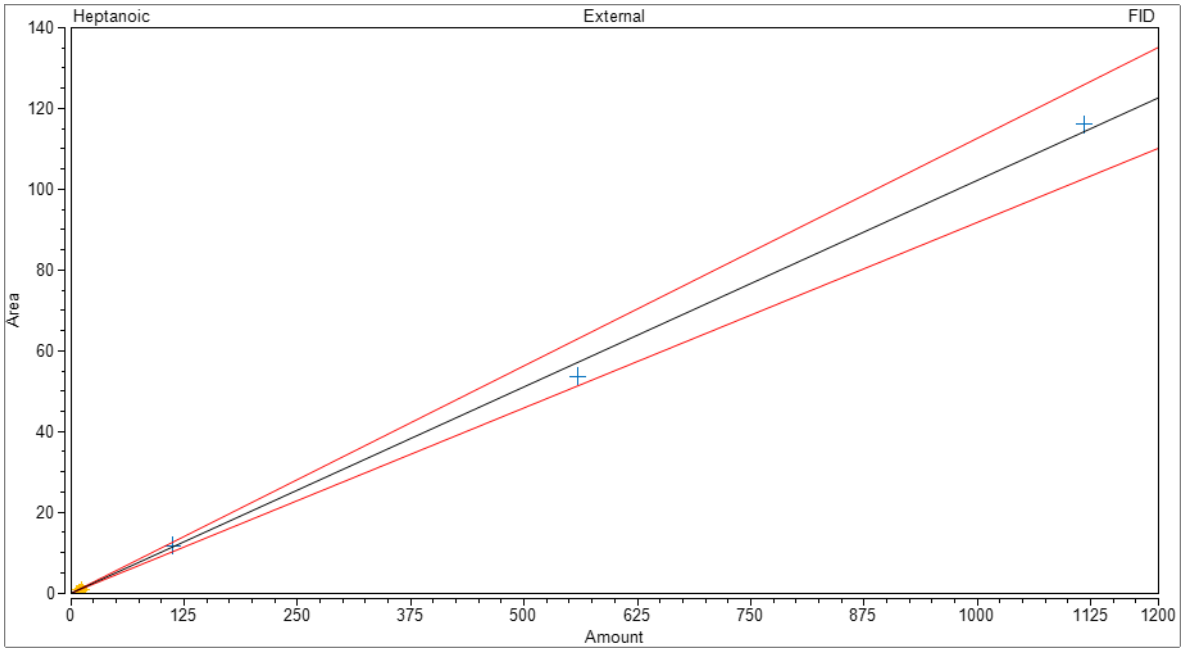
A-2-8: Caproaic acid calibration:



Calibration Details	Caproaic					
Calibration Type	Lin			Offset (C0)		0
Evaluation Type	Area			Slope (C1)		0.1186
Number of Calibration Points	4			Curve (C2)		0
Number of disabled Calibration Points	0			R-Square		0.9996

Calibration Results		Caproaic					
No.	Injection Name	Calibration Level	X Value	Y Value	Y Value	Area	Height
						pA*min	pA
			FID	FID	FID	FID	FID
			Caproaic	Caproaic	Caproaic	Caproaic	Caproaic
1	Cal 1	1	9.3	0.8365	0.8365	0.836	5.405
2	Cal 2	2	93.5072	11.3331	11.3331	11.333	72.54
3	Cal 3	3	467.5	53.9012	53.9012	53.901	352.007
4	Cal 4	4	935	111.6889	111.6889	111.689	714.624

A-2-9: Heptanoic acid calibration:

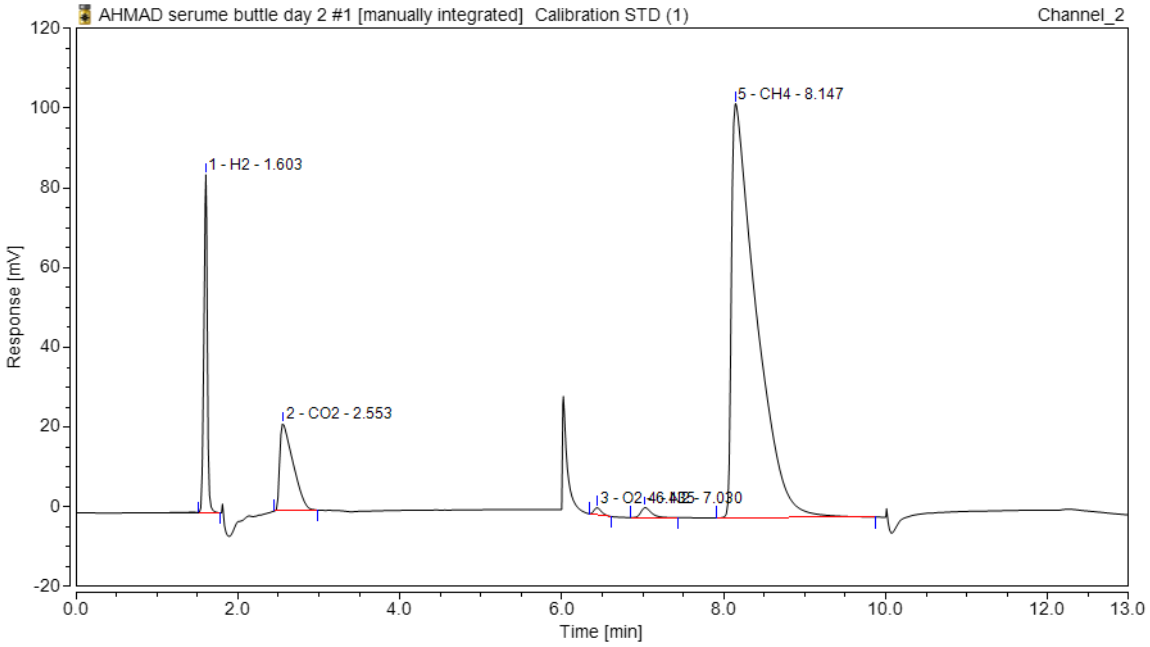


Calibration Details	Heptanoic					
Calibration Type	Lin			Offset (C0)		0
Evaluation Type	Area			Slope (C1)		0.1022
Number of Calibration Points	4			Curve (C2)		0
Number of disabled Calibration Points	0			R-Square		0.9982

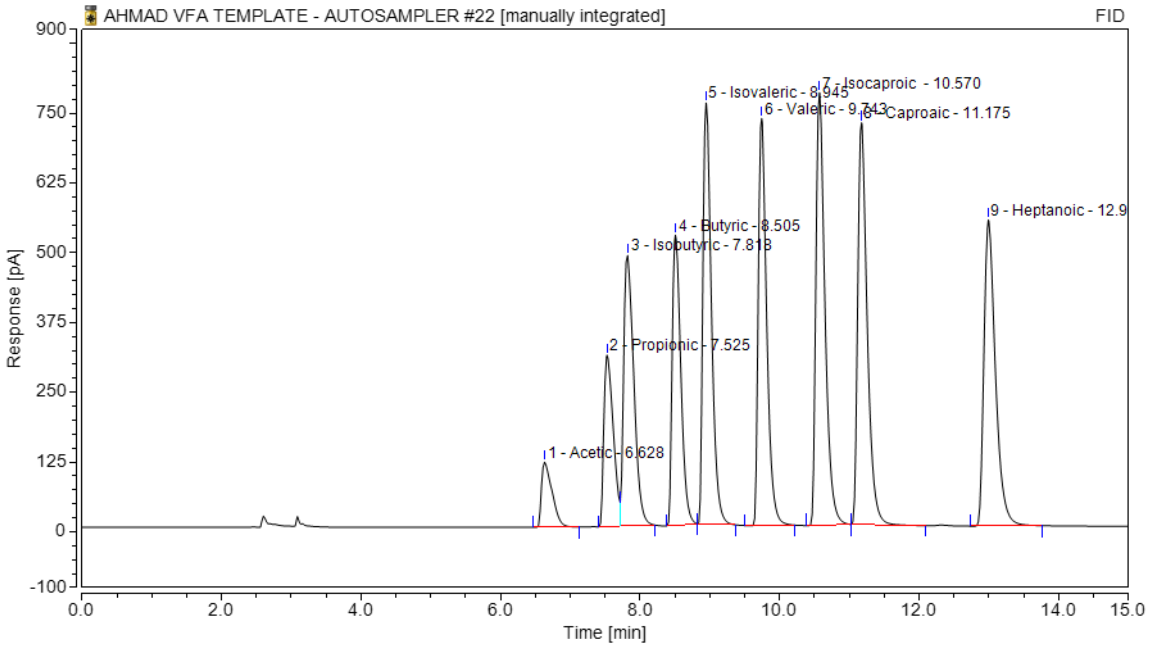
Calibration Results		Heptanoic					
No.	Injection Name	Calibration Level	X Value	Y Value	Y Value	Area	Height
						pA*min	pA
			FID	FID	FID	FID	FID
			Heptanoic	Heptanoic	Heptanoic	Heptanoic	Heptanoic
1	Cal 1	1	11.1	0.8679	0.8679	0.868	4.538
2	Cal 2	2	111.8289	11.7618	11.7618	11.762	61.367
3	Cal 3	3	559	53.6862	53.6862	53.686	280.126
4	Cal 4	4	1118	115.9841	115.9841	115.984	606.359

Appendix – B: (Chromatogram analysis– peaks):

B-1 Gas analysis chromatogram:



B-2 VFA analysis chromatogram:



B-3 FAME (LCFAs analysis) chromatogram.