# Application of $CO_2$ Microbubble to Enhance Methane Production in Anaerobic Digestion of Food Waste


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# Declaration

I, Wahyunanto Agung Nugroho, declare that I am the sole author of this thesis and that the research presented within is the result of my own efforts, unless acknowledged otherwise in the text. I confirm that this work has not been submitted for any other degrees.

# Abstract

Anaerobic digestion (AD) is a key technology for treating organic solid waste. Besides producing a significant amount of energy, the overall cost of the treatment is relatively low. It makes the technology become technically and economically feasible for treating organic solid waste. Many efforts have been carried out to enhance the performance of anaerobic digestion, with the main goal of maximizing methane production at a lower cost.

One way to enhance the methane production is by adding $CO_2$ into the digester. Many papers have reported the significant increase in the methane production after $CO_2$ addition. Despite most hypothesis that exogenous $CO_2$ provides an additional carbon source for the methane production, few have discussed how the system gaining the $H_2$ gas, as $CO_2$ cannot stand alone as the substrate for methane production, especially when no $H_2$ is added from any external source.

This research clarifies how the addition of exogenous $CO_2$ into the AD process boosts methane production. This process is then used to analyse the effectiveness of using $CO_2$ microbubble to enhance AD with landfill leachate addition. A comprehensive analysis using physical and chemical parameters as well as microbial community analysis are discussed in this thesis. All the discussions and conclusions are drawn based on three experimental constraints applied in this research: 1) mesophilic treatment, 2) batch operational mode, 3) $CO_2$ injection into the system through the use of microbubble technology.

Besides the observed methane enhancement after periodic dosing of $CO_2$, a higher methane yield than the theoretical methane potential was observed. In the

second study, landfill leachate is added into the medium with the aim to give additional micronutrients from a less utilized substrate. Landfill leachate is widely known to contain a significant number of toxic chemicals besides its essential trace elements. The injection of $CO_2$ microbubbles boosts the biogas production. The highest increase is shown when $CO_2$ microbubble is combined with landfill leachate addition. However, a significant decrease is shown by this treatment method using landfill leachate but without the $CO_2$ microbubble injection.

In the last study, a microbial study is performed to observe how the $CO_2$ microbubble may change the microbial community structure that leads to biogas enhancement. Even though it was expected that both bacterial and archaeal community structure could be revealed, the analysis can only identify the bacterial community. An additional primer that fits to the archaeal community should have been used for the PCR.

The results show that periodic injection of $CO_2$ microbubbles into the system increase the methane yields and methane production rates by up to 196% and 400%, respectively. In this study, a higher substrate degradation rate (140%) was also observed in the early stage of the treatment after $CO_2$ microbubble injection. This study concludes that the periodic dosing of $CO_2$ microbubbles increase the methane production in several ways: 1) increase substrate degradation rate, 2) provide additional carbon source in the form of exogenous $CO_2$, 3) alter the environment to a favourable conditions for the growth of the microbial community inside the digester. A favourable condition includes a toxicants reduction from the medium, which result a higher abundance of hydrolytic and acetogenic bacteria in the treatment with $CO_2$ microbubble injection.

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# Chapter 1

# Introduction

## 1.1. Background

In 2018, the global energy demand rose by 2.3%, with China, India and US accounting for around 70% of the global energy needs (IEA, 2019). Fossil fuel dominates the global energy consumption (70%). In contrast, the energy demand in UK fell by 0.7 % from the previous year, mainly due to the decreasing domestic energy consumption (Dept. Business UK, 2018).

The growth of carbon dioxide emissions is currently a consequence of the rise of energy consumption. Globally, the energy-related sector contributed up to 33 Gt of carbon dioxide emissions in 2018, the highest ever recorded in history (IEA, 2019). Around two thirds of these emissions occurred in Asian countries, where high economic growth was predicted to boost the rise of energy demand. However, the rise of carbon dioxide emissions did not apply universally. The fall of carbon dioxide emissions in the United Kingdom in the range of 2%-6% was an example of this (Dept. Business UK, 2019). An energy efficiency in the power system as well as the growth of renewable energy supply were some reasons behind this decrease.

The growing concern of the risk associated with carbon dioxide has motivated the use of energy from renewable resources. Renewable energy sources include solar energy, biomass, wind, ocean, hydro-electric and geothermal. When producing renewable

energy, feasibility study should be applied in the decision-making process. Electricity generation by photovoltaic collectors can be done by installation on the home rooftops without causing any land conversion. Wind turbines can be built either off- or on-shore, where agriculture should be least affected. Biomass is an abundant renewable resource for energy. It can be in the form of wood chips, agricultural waste, crop energy cultivated in marginal land and solid waste, especially organic.

With increasing population and national wealth in general, the volume of food waste is expected to keep increasing (Melikoglu et.al., 2013). Biodegradable waste is a significant fraction of solid waste, of which food waste dominates. In the European Union, around 88 million tons of food waste is generated annually with the value estimated at 143 billion euros (Stenmarck et al., 2016) -- around 10 million tonnes in UK alone with value of over £20 billion (WRAP, 2018), expected to rise every year. Crop residue is another massive waste generated globally as the result of increasing agricultural. Crop residue is a part of agricultural residue excluding animal manures and slurries. Such crops include cereal straw and grass planted in marginal land.

Anaerobic digestion (AD) is a widely used technology to convert biologically organic waste into energy. Since invented more than a century ago, the technology is increasingly utilised as it can reduce total operational cost by skipping the requirement of external electron acceptor, i.e. oxygen, in the process. Besides benefits from low-cost operation, anaerobic digestion (AD) provides surplus energy for the whole process by producing biogas (methane and hydrogen) and some by-products such as compost. The produced carbon dioxide even can be used for many purposes inside and outside the plant. In terms of greenhouse gas emissions, it should be noted that methane is 20-30 more detrimental than carbon dioxide, so as long as the methane is used, it prevents substantial fugitive methane emissions from natural degradation of the biomass. Hence AD is substantially GHG “negative” in fact. This fact is lost in conventional life cycle analysis (LCA), as LCA rates all biomass utilisation as simply carbon neutral, ignoring the conventional fate of the biomass in absence of the intervention.

AD is not only practical and economically feasible, but also a hub to address some issues related to energy demand -- waste generation and carbon dioxide emission. Regarding the $CO_2$, several attempts have been made to utilize the gas. One of the ways is to add the

$CO_2$ back into the digester to enhance the methane production. Several reports indicate a significant increase in the methane production *rate* (11% to 138%) after $CO_2$ was added into the reactor (Al-Mashhadani et al., 2016; Bajón Fernández et al., 2015). Substrate that has higher biodegradability showed a higher increase in the methane production rate as well after $CO_2$ addition.

Several hypotheses have been made regarding the mechanism of the methane increase after exogenous $CO_2$ addition into the digester. Those hypotheses can be briefly written as follows. Firstly, the $CO_2$ promotes a higher degradation rate of the substrate. This higher degradation rate was suggested due to the attack by the hydroxyl radical generated from the collapse of pure $CO_2$ microbubbles (Al-Mashhadani et al., 2016). The generation of hydroxyl radical due to the collapse of microbubbles was firstly reported by Takahashi et al. (2007). Zeta potential of the bubble surface plays a significant role in the bubble collapse. When pure $CO_2$ was injected, it will easily dissolve in the liquid and make the micro bubble size even smaller. With the smaller surface area of the bubble, the zeta potential per area become bigger and may leads to more rapid collapse of the bubble.

Secondly, this increase could be triggered by a more favourable environmental condition for the biochemical reaction to happen after the $CO_2$ addition. Al-Mashhadani et al. (2016) suggested that Le-Chatelier principles might apply in the process (equation 1.1). Briefly, the principle stated that any reduction at the product side will increase the reaction rate to generate more product. In accordance with this principle, an increase in the methane production can be done by stripping off the methane present in the reactor (Eq. 1.2). This principle seems to apply only if the $CO_2$ is injected into the medium rather than only adding it into the head space.

$$nA + mB \leftrightarrow xC + yD \quad (1.1)$$

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \quad (1.2)$$

Thirdly, an induction of $CO_2$ could encourage several microbes to increase their activity which will ultimately increase the rate of methane production. More specifically, an addition of $CO_2$ promotes the acetogenic bacteria to produce more acetic acid following the Wood–Ljungdahl pathway (Bajón Fernández et al., 2015; Francioso et al., 2010). The more acetic acid means the more available source for methane formation via acetoclastic

pathway. As more hydrogen production was also observed for the injected treatment, methanation via hydrogenoclastic pathway is another possible reason for the increase in the methane production rate.

## 1.2. Objective of the study

In general, this study aims to explain how exogenous $CO_2$ is used during the AD process that results in an increasing methane production rate. An analysis should be carried out more deeply using more measurable parameters those will be discussed in each Chapter. More specifically, the objectives of this study are:

1. Evaluate how the injection of $CO_2$ microbubbles can enhance the methane production. A co-digestion process, i.e. food waste and leachate, was done to observe how the chemical compounds inside the leachate might affect the performance.
2. Investigate how the microbial population might be affected by the treatment, that might lead to the alteration of the biogas generation pathway, liquid composition inside the digester and total methane production.

## 1.3. Hypothesis of the study

The hypothesis of the study is summarized in this chapter while the thinking which underlies the hypothesis can be read in the literature review.

1. The injection of $CO_2$ microbubbles will significantly increase the methane production rate. Regarding the addition of leachate, it should increase the methane production rate even more as it contains a significant amount of trace elements. It is because trace elements act as a co-enzyme in the biochemical process inside the cells
2. Stripping to remove some 'inhibitor' and product gases is the main mechanism intensified by $CO_2$ microbubble injection. This stripping initiates more favourable conditions for the biochemical reaction to produce methane more rapidly.
3. The dissolved $CO_2$ uptake is dominated by hydrogenoclastic methanogens over the autotrophic acetogens in the treatment with $CO_2$ microbubble.
4. An increase in the hyrogenoclastic activity occurs after $CO_2$ injection. Microbial abundance is used as the analysis tool for this hypothesis.

This hypothesis is specifically made for the following constraints:

1. The $CO_2$ added to the process is in the form of microbubbles.
2. The process temperature is mesophilic.
3. The mode of operation is batch.

## 1.4. Contribution of the research

1. This study gives a comprehensive discussion about the mechanism of how the injection of $CO_2$ microbubble into an AD system can enhance methane production.
2. As this study uses larger volume of reactors than commonly used in most published articles related to the $CO_2$ addition into AD, it can act as a steppingstone to study such application in a full-scale reactor. For additional information, up to recently, there is no single full-scale AD treatment those are adding $CO_2$ into the system to enhance methane production.
3. This study shows a new opportunity to use abundant landfill leachate along with microbubble technology to improve the AD performance in treating organic solid waste.
4. While most studies related to $CO_2$ addition only reported on the archaeal community dynamics, this study gives a picture of the bacterial community dynamics after $CO_2$ addition.

## 1.5. Organization of chapters

This thesis is organised in six chapters. Chapter 1 is the introductory chapter outlining the background for the study and scope and objectives of the research. The hypotheses are stated in this chapter. Chapter 2 is a literature review. The literature review opens with a brief discussion about anaerobic digestion, highlighting the treatment stage and some chemical equations that are useful for discussion in later chapters. This chapter also discusses some findings related to the carbon dioxide utilisation underpinning anaerobic digestion.

Chapters 3, 4 and 5 are the main chapters, discussing the result of the experiment. Chapter 3 discusses the mechanism of how the injection of $CO_2$ microbubbles can enhance

the methane production rate. Chapter 4 highlights the application of $CO_2$ microbubble in simultaneously enhancing the methane production rate and reducing the toxicity (inhibition by) of the medium. The potency of leachate to increase the performance is also discussed in Chapter 4. Chapter 5 presents the microbial dynamics during the treatment (with and without $CO_2$ injection) with metagenomic sequencing 16s rRNA as the major analysis approach.

Conclusions are drawn in Chapter 6. This chapter also suggests some future works.

# Chapter 2

# Literature Review

Anaerobic digestion (AD) is a widely applied technology for treating organic waste, including those in wastewaters, by converting them into more stable forms. It has also been reported that AD can decrease the number of pathogens in the effluent as well as less odorous biosolid coming out from the anaerobic digester (Parkin and Owen, 1986). Since the process is mostly obligatory anaerobic, no aeration is needed, resulting in a lower operational cost.

The utilization of exogenous $CO_2$ in AD has drawn attention due to a significant increase in the methane production rate after the addition of $CO_2$ into the system. The type of substrate and mode of operation likely influence how much methane enhancement could be achieved after the $CO_2$ addition. Many hypothesizes have been stated regarding the mechanism on how the exogenous $CO_2$ has significantly increase the methane production rate. This difference of opinion seems to be influenced by the differences in the way $CO_2$ is added into the system. In this review, a highlight is given to the injection of microbubbles as the technique to add the $CO_2$ because the same technique is applied in this study. Some basic information about AD needs to be discussed at the beginning of the review before going further to discuss some hypotheses about the mechanism of increasing methane after adding $CO_2$ into the system. Section 2.3 critically reviews some hypotheses in the published papers. The hypothesis of this study as well as the rationale behind it is explained in the last section (2.4).

## 2.1. Anaerobic digestion: general operational condition.

Regardless the type of waste, biogas is considered as the main product of AD. It is dominated by methane, carbon dioxide and nitrogen, besides several residual gases, such as ammonia, carbon monoxide, hydrogen, and hydrogen sulfide. Several factors are suggested to influence the volume and composition of the produced gas.

### 2.1.1. Feed substrate and inoculum

Feed substrate and seeding sludge/inoculum are likely the dominant factors to shape the gas composition and volume. The methane production is believed to be the best when the C/N ratio is maintained in the range of 20-30, even though this is still debated. A higher nitrogen composition in the feed substrate tends to produce more ammonia in the biogas. It should be noted that the presence of free ammonia at a certain level is harmful to some microbial communities, especially methanogens (Rajagopal et al., 2013). On the other hand, a higher C/N ratio may lead to a pH drop if the buffering capacity is not sufficient. This pH drop is due to a rapid increase of volatile fatty acids (VFA). The pH drop may cause more severe impact to the system than the presence of free ammonia since it affects most microbial communities within the digester (Angelidaki and Ahring, 1993).

To use the substrate efficiently, co-digestion is often applied. C/N ratio of the substrate is mainly considered for this co-digestion (Li et al., 2009; Zhang et al., 2014a). Maintaining this ratio is essential to ensure the microbial community receives satisfactory nutrition for the whole process (Cuetos et al., 2008; Yen and Brune, 2007). Gaining trace elements from the augmented substrate is another focus of co-digestion. Trace elements work by acting as a co-enzyme in the biochemical process, as insufficient trace elements could cause the AD to fail (Banks et al., 2012; Choong et al., 2016; Climenhaga and Banks, 2008). Supplementing trace elements to an optimum amount has been proven to give positive impact in the degradation of organic matter as well as total methane production, regardless the source of substrate fed into the system (Pobeheim et al., 2010; Schmidt et al., 2014; Wei et al., 2014). Iron is the most studied of all trace elements, followed by nickel and cobalt. It has been reported that the addition of trace elements into AD has been proven to reduce the hydrogen sulphide, regulate VFA concentration, increase substrate degradation, as well as methane production (Meng et al., 2013; Pobeheim et al., 2010).

The digestibility of the substrate is another important factor to be considered for the AD operation. The substrate needs to pass through the hydrolysis stage before being converted into biogas in the final stage. Food waste and sewage sludge are some easily biodegradable substrates. Agricultural waste is often a recalcitrant substrate because of its high cellulose and lignin content. A pre-treatment is often performed to recalcitrant

substrates to make them more accessible for the microbial community to degrade further into end products.

### 2.1.2. Temperature

Providing a suitable temperature for the microbial community inside the digester to accomplish the process is essential for a successful AD operation. A feature of AD employs highly variable microbial community. Each might require a different optimum temperature. Compromising a suitable temperature for all is important, especially when a single-stage treatment is chosen. A failure to achieve a suitable temperature for all could lead to operational failure (Chae et al., 2008; Song et al., 2004).

Two temperature regimes have been widely applied for a full scale of AD treatment, i.e. thermophilic (55$^{o}$C) and mesophilic (35-37$^{o}$C). There is also an increased study on low-temperature AD (Abram et al., 2011; Alvarez and Lidén, 2009; Massé et al., 2010)). Thermophilic is more favorable in terms of productivity as it gives a higher substrate degradation rate. Besides its obvious higher operational cost, the problem with this system might arise when the rate of substrate degradation far exceeds the VFA consumption rate (Chae et al., 2008). An accumulation of VFA in the system might lead to pH drop as has been discussed earlier. A single-stage treatment seems to be more susceptible to this pH drop than the two-stage one. No system failure due to pH drop has been reported for the two-stage process. Splitting the fermentation process from the methanogenic process might permit each microbial community to their own optimum environmental conditions.

### 2.1.3. Inhibitory substances

AD employs a number of different types of microorganisms with various sensitivities to their environment. Inhibitory substances are often present in the system either as a metabolite or carried by the feed substrate or seeding sludge. Among several inhibitors known to AD, ammonia and fatty acids are two dominant substances. Both are metabolites produced by microbial action. The presence of some organic materials and metal ions in the medium has been reported to inhibit the microbial process as well.

Ammonia. Ammonia is a fermentation product of an anaerobic process of nitrogen-containing substrates. Ammonia mainly exists in aqueous solution as the ammonium ion ($NH_4^+$). It is a nutrient for the microbial community and considered to be harmless if the concentration is relatively low (Liu and Sung, 2002). However, when its quantity exceeds the acceptable concentration, it becomes toxic to the microorganism. There is no exact value at what concentration ammonia becomes harmful. The studies observing the ammonia toxicity were carried out in the concentration of above 1.2 $gL^{-1}$ (Liu and Sung, 2002; Rajagopal et al., 2013). There are several proposed mechanisms of how ammonia is toxic to microbes. Passive diffusion into the cells is probably the most widely accepted. Once within the microbial cells, it causes proton imbalance and potassium deficiency (Gallert et al., 1998; Sprott and Patel, 1986). Ammonia in the gaseous form (free ammonia/$NH_3$) is even more toxic than the ionic form since it has a higher diffusivity into microbial cells.

The formation of free ammonia in the liquid is governed by several means as expressed in Equation 2.1.

$$NH_3 = \frac{TAN \ \frac{K_a}{[H]}}{\frac{K_a}{[H]} + 1} \quad (2.1)$$

Where $NH_3$ is the concentration of free ammonia nitrogen (mg/L), TAN is the total ammonia nitrogen concentration (mg/L), $K_a$ is the temperature-dependent dissociation constant ($0.564 \times 10^{-9}$ at 25 °C, $1.097 \times 10^{-9}$ at 35°C and $3.77 \times 10^{-9}$ at 55 °C) and [H] is the concentration of hydrogen ion ($10^{-pH}$). The equation (2.1) shows that the amount of free ammonia dissolved in the liquid is pH and temperature-dependent (Kayhanian, 1999).

According to equation (2.1), a decrease in pH and temperature can reduce free ammonia formation. In other words, the formation of free ammonia in the mesophilic process is lower than in the thermophilic process ones (Braun et al., 1981). This decrease in pH is mostly due to the formation of VFA during the fermentation process. Injecting $CO_2$ into the system might be a way to maintain the pH (Al-Mashhadani et al., 2016; Ying et al., 2013). Once it is dissolved, it will form carbonic acid that may lead to a pH drop in a liquid without an adequate buffering capacity. When ammonium is present, it acts as the buffer

to oppose a pH change. Instead of using a pipe gas injector, microbubble injection can boost the $CO_2$ dissolution efficiency. It is because the smaller the size of the bubbles, the higher the surface to volume ratio that leads to a better gas transfer rate.

Several techniques have been applied to reduce ammonia, which one of them is air stripping. Air stripping is a technique where the use of chemicals can be minimized. Different from the popular chemical coagulation, this technique is feasible to do on-site as no sediment is formed during the process (Kabdaslı et al., 2000; Ozturk et al., 2003). It works by transforming the ammonia from the aqueous phase (ammonium ion) into the gas phase (ammonia). So far, the technique has been successfully carried out by injecting air bubbles into the reactor. The ammonia removal varies from 10% to 95% (Calli et al., 2005; Campos et al., 2013). Several factors are suggested to affect the ammonia removal by air stripping, such as airflow rate, pH of the liquid as well as the temperature of the inlet air used for the stripping treatment (volatility). Most air stripping is done in alkali conditions (pH 10- 12), where most ammonia is in the form of ammonia gas (Campos et al., 2013; Kabdaslı et al., 2000). When applied to AD, oxygen-free gas should be used. Injection of $CO_2$ microbubble is expected to remove some ammonia from the liquid, while at the same time, carbon dioxide is dissolved, as illustrated in Figure 2.1.

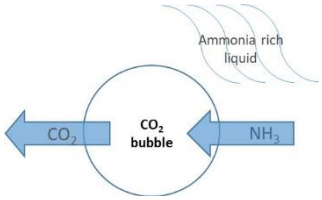



Figure 2.1. $CO_2$ and $NH_3$ gas transfer in the stripping process of ammonia using pure $CO_2$ microbubble.

Trace elements. Trace elements in the medium can be divided into two types, i.e. light ions and heavy metals (Chen et al., 2008a). Light ions are commonly introduced as salts, and mostly present as a cation when it is associated with toxicity for the microbial cells (McCarty and McKinney, 1961). As an aggregate, the salt content is measured as salinity. In anaerobic digestion, these salts comes from the feed substrate and are released when the organic matter decomposes ( Grady Jr et al., 1991). Such salts of concern are those of Na, K, Mg, Ca, and Al. In a relatively low concentration, salt is required by the microbes to support their metabolic activity. Each salt has a different role in the biological process.

However, in a higher concentration, these salts can be toxic for the microbes. The level of concentration when it becomes toxic is defined as $IC_{50}$ and is different for each salt (Mouneimne et al., 2003). Another mechanism of cell disruption by salt is cell dehydration. A high concentration of salt in the medium may cause cells to dehydrate, driven by the osmotic pressure due to the concentration gradient between the inside and outside of the cell membrane.

Similar to light ions, heavy metals are required by the microbial community for metabolism. They can be considered as micronutrients and metalloenzymes in AD (Choong et al., 2016). They work by acting as a co-enzyme in the biochemical process inside the cells. Several publications have reported that the presence of heavy metals in a sufficient quantity in the medium can increase the efficiency of organic substrate decomposition. AD process failure could occur if their amount is not sufficient (Banks et al., 2012; Climenhaga and Banks, 2008). Conversely, an excessive concentration of these elements in the digester can be toxic for the microorganism and can lead to the process failure as well. In fact, not all the heavy metals in the digester are toxic. Only if they are in the soluble form are toxic for the microorganism (Chen et al., 2008b; Lawrence and McCarty, 1965; Oleszkiewicz and Sharma, 1990). To run the optimum AD process, it is essential to maintain the level of heavy metals in an acceptable range.

Some heavy metals are an environmental concern since their toxicity can occur in relatively low concentration, including chromium, iron, cobalt, copper, zinc, cadmium, and nickel (Jin et al., 1998). Their concentration can be higher when waste containing these toxic pollutants enter the disposal site and then undergoes a natural anaerobic decomposition in the landfill site. This is the reason why the landfill leachate normally contains a high level of heavy metals (Baun and Christensen, 2004). The distinguishing feature of heavy metal is they can accumulate inside the microbial cells. In the anaerobic digester, methanogenic archaea are suggested to be the most prone microorganisms to heavy metal toxicity (Zayed and Winter, 2000). A certain level of heavy metal could be toxic to archaea but may not be to fermentative bacteria. Pre-treatment is sometimes required when any waste containing a high concentration of heavy metal will be treated biologically, especially if methane is desired to be the main product.

Organic Inhibitors. Toxic organic compounds that enter the digester come from several sources, such as agricultural waste and domestic waste, either directly or indirectly (Bunzel et al., 2013). Some of them are less degradable by any biological process. There are three dominant groups of organic toxins present in the waste stream, i.e. chlorophenol, halogenated compounds, and long-chain fatty acids (Chen et al., 2014a). Chlorophenolic compounds can be found as an active compound in pesticide and non-food preservatives. Besides highly resistant to any biological degradation, it is toxic to most microbial communities and even to humans due to its carcinogenicity. The inhibitory effect of these compounds to anaerobic digestion has been reported in many articles (Hernandez and Edyvean, 2008; Li et al., 2015). The degree of chlorination of the compounds holds a strong role in their toxicity (Chen et al., 2008b). For some chlorophenolic compounds, at a concentration of only 10 mg/L, or sometimes lower, it can induce a significant toxic effect on some acidogenic bacteria and methanogenic archaea. To prevent the component from entering the digester, chemical dechlorination is often applied to the waste containing chlorophenol. However, it was reported that in some chlorophenol compounds, the degradation products are even more toxic than their parent compounds (Wu et al., 1989).

Halogenated aliphatic compounds can be found as an industrial solvent, insecticide and pharmaceutical. They enter the digester in various ways, such as by disposal or washed away from households and industries. The compounds are considered to be highly recalcitrant and toxic to the environment as well (Cappelletti et al., 2012). Chloroform is probably the most widely studied among these compounds, in terms of its toxicity and biodegradability in the environment. It was reported that chloroform could give a toxic effect on the methanogenic community when its concentration is as low as 0.09 mg/L (Yu and Smith, 2000). As has been proposed by Yu and Smith (2000), the toxicity mechanism of chlorophenol is by dissolving into the microbial cells. The compound then binds to the methanogenic enzymes resulting in the inhibition of the activity of the methanogenic microbes. Similar to chlorophenol, chemical, and physical removal of these compounds are preferred over biological degradation.

Long-chain fatty acid (LCFA) is a type of fat, which includes both saturated and unsaturated. It is found mainly in food-related waste. These compounds are relatively slowly degraded during the biological treatment due to their long-chain structure and high

hydrophobicity. Its hydrophobicity induces the compounds to aggregate, separating from the bulk liquid containing the microbial community. Even though LCFA still can be considered as biodegradable, an excessive amount of these compounds in the digester could lead to treatment failure. Preliminary treatment to reduce their number is always suggested to avoid any treatment failure.

## 2.2. Biochemistry and Microbiology of Anaerobic Digestion

Anaerobic digestion is a complex process employing various types of microbial communities. The microbial community is dominated by those working in the methane production pathway, with some of them in non-methane production pathways (O'Flaherty et al., 2006). With the more advanced and more affordable cost of DNA analysis, more species of the microbial community are identified. Nowadays, nearly two hundred microbial species are recognized to work in the system (Abram et al., 2011). A number of protozoa are also identified in the digester besides bacteria and archaea, but little is known about their role in the anaerobic digestion process.

In the digester, each type of microbial community mediates a different activity, with different products and sometimes requiring a different optimum environmental condition to run the process effectively. It is also necessary to know that a by-product of a particular microbe might be the inhibitor of other microbes, especially when its level is higher than the tolerable limit. By comparing some reports from various operational conditions, the microbial composition of each digester can be highly variable. This variation sometimes makes quantitative information about the interaction among the microbes less reliable to use in general. A failure to provide acceptable conditions may result in operational failure. The lower economic value of the product gas and less stable bio-solid are probably the least impacts when optimum conditions are not imposed. This literature review will address the biochemical process and the diversity of the microbial community involved in the methane generation pathway only.

### 2.2.1. The biochemical process of anaerobic digestion

In the methane production pathway, the microbial community can be categorized into two main groups, i.e. fermentative bacteria and methanogenic archaea. The first group are involved in the hydrolysis, acidogenesis and acetogenesis, while the archaea work in

the downstream step using the products of the previous step to produce methane and other gases (Fig. 2.2).

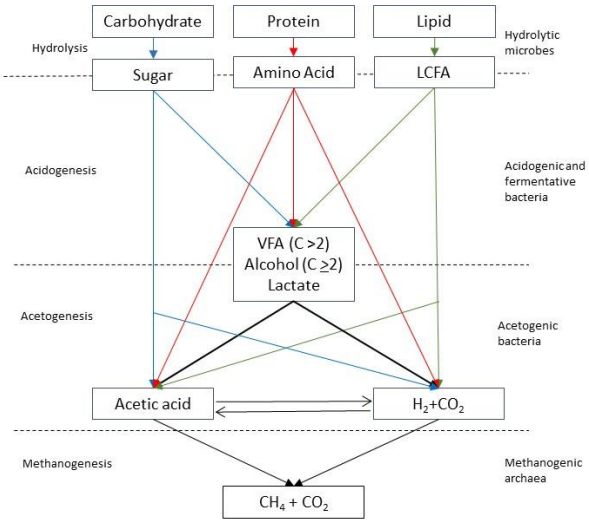



Figure 2. 2. The general process of anaerobic digestion

Hydrolysis is the first step in the digester to degrade gross organic compounds. This process occurs extracellularly with the help of enzymes. Hydrolysis is often considered as the rate-limiting step since the subsequent processes are highly dependent on the substrate provided by the hydrolysis process. In hydrolysis, polysaccharide and protein are converted into sugars and amino acids, respectively. The lipids are converted into long-chain fatty acids and glycerol using lipase secreted by the lipolytic bacteria (Cirne et al., 2007). The next step is the acid generation process or acidogenesis where the hydrolysate is converted into volatile fatty acid (VFA). The monosaccharide is converted into VFA through a typical Embden-Meyerhof-Parnas (EMP) glycolysis pathway, where two molecules of pyruvate are produced as the intermediate products (Elefsiniotis and Oldham, 1994). Amino acids are also converted into VFA via one of these pathways: reductive deamination of aliphatic amino acid or oxidation-reduction between pairs of amino acids, known as the Stickland reaction. The products of amino acid degradation are organic acids, ammonium, carbon dioxide, hydrogen, and some sulphuric compounds (Ramsay and Pullammanappallil, 2001). Hydrogen sulphide and ammonia are product gases of amino acid degradation and are toxic to the microbial community (Chen et al., 2008a).

Acetogenesis is the third step, where long-chain VFAs and long-chain alcohol are degraded into acetic acid. Besides acetic acid, butyrate and propionate are the prominent VFAs produced in acidogenesis. The fermentation of butyrate and other VFAs longer than a three-carbon chain into acetic acid and hydrogen is done in the mitochondria through a β-oxidation pathway. The propionate is fermented through the methylmalonyl-CoA (MMC) pathway (D G Cirne et al., 2007; Hagen et al., 2017).

Equation 2.2 shows a thermodynamic consideration of conversion of glucose into acetic acid, while equation 2.3 to 2.5 show the acetic acid production from glucose with propionate as the intermediate product. Conversion of glucose into acetic acid via ethanol production was simplified in equation 2.6 to 2.8 (Pipyn and Verstratete, 1981). The Gibbs free energy shows that all the pathways are thermodynamically favourable with no significant differences among the processes. Carbon dioxide and hydrogen gases are produced in all pathways of the acetogenic process.

$$C_6H_{12}O_6 + 4H_2O \rightarrow 2CH_3COO^- + 2HCO_3^- + 4H^+ + 4H_2 \qquad \Delta G^{0\prime} = -206.3\ kJ \ (2.2)$$

$$\mathrm{C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COO^- + 2H_2O + 2H^+} \quad \Delta G^{0'} = \text{-}358.1\ \mathrm{kJ}\ (2.3)$$

$$2CH_3CH_2COO^- + 6H_2O \rightarrow 2CH_3COO^- + 2HCO_3^- + 2H^+ + 6H_2 \quad \Delta G^{0\prime} = +152.2\ kJ \ (2.4)$$

$$\mathrm{C_6H_{12}O_6 + 4H_2O \rightarrow 2CH_3COO^- + 2HCO_3^- + 4H^+ + 4H_2} \quad \Delta G^{0'} = \text{-}205.9\ \mathrm{kJ}\ (2.5)$$

$$C_6H_{12}O_6 + 2H_2 \rightarrow 2C_2H_5OH + 2HCO_3^- + 2H^+ \quad \Delta G^{0\prime} = -225.9\ kJ \ (2.6)$$

$$\mathrm{2C_2H_5OH + 2H_2O \rightarrow 2CH_3COO^- + 2H^+ + 4H_2} \quad \Delta G^{0'} = +19.2\ \mathrm{kJ}\ (2.7)$$

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COO^- + 2HCO_3^- + 4H^+ + 2H_2 \quad \Delta G^{0\prime} = -206.7\ kJ \ (2.8)$$

Beside via those dominant pathways, acetic acid could also be produced by reductive Wood-Ljungdahl pathway, where carbon dioxide is fixated. In this process, hydrogen act as an electron donor and carbon dioxide is the electron acceptor (Eq. 2.9). The pathway starts with the reduction of carbon dioxide into carbon monoxide and formic acid, followed by its conversion into a methyl group. The methyl group, combined with carbon dioxide and CoA, generates Acetyl-CoA. The Acetyl-CoA is later used for the acetic acid production. Considering its Gibbs free energy, this process seems less favourable compared to the acidogenesis.

$$2CO_2 + 4H_2 \leftrightarrow CH_3COO^- + H^+ + 2H_2O \quad \Delta G^{0\prime} = -95\ kJ/mol \quad (2.9)$$

Regardless of the production pathway and the type of microbial community involved in the process, acetogenesis occurs in obligate anaerobic conditions with the favourable hydrogen partial pressure less than $4.6 \cdot 10^{-4}$ atm. The rate of acetogensis becomes slower when the hydrogen partial pressure is above this critical value. Since the solubility of the hydrogen gas is only about 1.4. $10^{-3}$ g/kg water in 35°C, naively, it should escape readily into the headspace once it reaches saturation. However, there are many possible mechanisms for hydrogen to remain in the medium. As it is practically insoluble, there is no easy mass transfer route out. The most possible way of this low-soluble gas stay in the liquid is by attaching on the surface of a substrate or microbial cell, which isolates the gas from the liquid (Al-Mashhadani et al., 2016). In other words, the distribution of this gas does not occur evenly throughout the liquid, but rather occurs in micro-structures, which are on heterogeneous solid surfaces. They will stay on the solid surface until they have enough buoyance force and rise up to the reactor headspace.

To maintain the hydrogen partial pressure at a low level, it is necessary to remove the hydrogen continuously as it is produced by the fermentation bacteria continuously as well. There are two possible mechanisms to maintain this low partial pressure naturally. Firstly, by syntrophic interaction between hydrogen producing microorganism and hydrogen consuming microorganisms. This mutual interaction could maintain a balanced environment for both microbes as long as the production and up-taking process of hydrogen occurs at the same rate. Secondly, by leaving the attached surface as it has sufficient buoyant force to detach from the solid surface.

Methanogenesis is the last step in the anaerobic digestion pathway. In some digestion plants, this process is done in a separated reactor from the fermentation tank with the aim is to provide optimum conditions for each process. The methane generation occurs in two different pathways, which are acetoclastic and hydrogenoclastic. The acetoclastic pathway occurs by converting acetic acid into methane while the hydrogenoclastic pathway occurs by utilising carbon dioxide and hydrogen to produce methane. Those pathways clearly show that the only digestible substrates for the methanogenic archaea are acetic acid, carbon dioxide and hydrogen.

De Vrieze et al., (2015) suggested that the methane generation pathway is influenced by many factors, including the composition of the substrate, loading pattern and operational conditions. For instance, feeding substrate with high protein content tends to shift the acetoclastic pathway to hydrogenoclastic. This process was initiated by the ammonia generation from degraded protein. With a high ammonia level, acetate oxidizing bacteria dominate the acetate utilisation with carbon dioxide and hydrogen as the products. This results in the more available substrate for the hydrogenoclastic archaea to produce methane (Karakashev et al., 2006). The same pattern was also reported in the thermophilic process. A report by Jing et al. (2017) showed that the presence of magnetite in the medium increases the efficiency of a symbiotic relationship between two species of microorganism. It resulted in an increase of nearly 50% of methane production from both pathways.

$$nA + mB \leftrightarrow xC + yD \quad (2.10)$$

$$CH_3COOH + H_2O \rightarrow CH_4 + H^+ + HCO_3^- \quad \Delta G^{0'} = -31.0\ kJ/mol \quad (2.11)$$

$$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O \quad \Delta G^{0'} = -135.6\ kJ/mol \quad (2.12)$$

Considering chemical reaction kinetics, removing the gas product increases the reaction rate in accordance with Le Chatelier's principle. The principles state that when equilibrium is disturbed, the system will adjust to the new equilibrium (Eq. 2.10) by opposing the change. Based on this principle, lowering the concentration of the methane likely will increase methane production from both pathways (Eq. 2.11 & 2.12).

Since the stripping of the product gases (e.g. methane) occurred extracellularly, while the gas production occurs intracellularly, the question would be how the stripping process affects the enzymatic process inside the cell so that the Le Chatelier's Principle is applied. Methane is produced inside the archaeal cells, indeed. Once it is produced, it will diffuse out through the cell membrane pores and attach to the outer surface of the archaea until it reaches a certain buoyance force before detaching from the microbial surface and rising up to the liquid surface. Based on "Thin Film Mass Transfer Theory", the rate of the diffusion of a thin surface is proportional to the concentration gradient between the two mediums separated by that surface. The microbubble sparging is expected to accelerate the detachment process of the attached gases so that the concentration gradient between the two mediums becomes higher. Since the rate of removal of the produced methane is higher, the methane production process is expected to be higher as well.

In AD, an oxygen-free gas should be used for the gas stripping (Al-Mashhadani et al., 2016). However, it is necessary to note that the gas stripping will not selectively remove the methane only, but also other gases, including ammonia, carbon dioxide and hydrogen. While removing ammonia gas may give a positive impact, removing the other two might affect negatively as they are feedstock for methane production via the hydrogen utilisation pathway. A significant increase is shown when carbon dioxide was used as a stripping gas, instead of nitrogen. While the removed $CO_2$ could be replaced by injecting the $CO_2$, replacing the hydrogen using its exogenous gas seems less economical.

### 2.2.2. Microbiology of an anaerobic digestion

Microbial community composition can be determined by several techniques, of which the most popular one is by analysing the 16s rRNA of the microbes, which is the most conserved region inside the microbial DNA. The recently advanced sequencing technique has created the opportunity to analyse more rapidly with a greater number of samples. The lower cost in using the analysis beneficially provides a number of samples that are statistically more relevant (Vanwonterghem et al., 2014). Along with other techniques, i.e. transcriptomics, metaproteomics and metabolomics, a more detailed microbial activity has been discovered and has given new insight into how AD system works. Beside strengthening support for some hypotheses believed to happen, studies revealed that the microbial community and its activity is unique regarding their condition of life. Using multi-omics techniques has also given a more detail insight into how the microbial community builds a mutual partnership to complete the process effectively (Hattori et al., 2000; Schnürer et al., 1999).

The study of microbial composition and its activity in the AD have been reported to be carried out under various AD conditions, such as operational temperature, substrate composition, substrate loading rate, pre-treatment method and number of stages in the AD system. From this data, it is suggested that the composition and the activity of the microbial community are significantly influenced by those conditions.

In general, the AD process can be divided into four simplified steps, where the first three are carried out mostly by the bacterial community, while the last one by the archaeal community (De Vrieze et al., 2015). The amount of the bacterial community in the digester is typically far in excess of the archaea (Hanreich et al., 2013, 2012; Heyer et al., 2013; Lü

et al., 2013). In some cases, it could occupy around 99% of the total microbial population, which means the number of archaea is around 1% or less of the total (Abram et al., 2011; Hagen et al., 2017). The rationale behind this is the bacteria mainly plays the role in the degradation of raw substrate, which some of them might be recalcitrant. It is followed by several biochemical processes to provide the archaea with ready substrate for methane formation. Obligate anaerobic conditions are required by most of the microbial community. Even though there are some bacteria which are believed to be facultative, but their number and role in AD process are insignificant compared to the whole microbial community in the AD (Toerien and Hattingh, 1969).

*Hydrolysis*

Firmicute is in the top list of the bacterial phyla to present in the anaerobic digester. They are detected in most anaerobic digesters regardless of the operational condition. It is followed by bacteria from phyla Bacteriodetes and Thermotogae (De Vrieze et al., 2015; Hanreich et al., 2013; Heyer et al., 2013; O'Flaherty et al., 2006). Based on their activity revealed in some -omic studies, these bacterial phyla can be considered as the key players in the AD system (Cantarel et al., 2008; Heyer et al., 2015; Zverlov et al., 2005). Some other bacteria from other phyla, such as bacilli and proteobacteria were also reported as less numerous.

*Clostridium thermocellum*, which belongs to phyla firmicute, is probably one of the most studied bacteria as an individual species among all bacterial community in the AD. Its availability in anaerobically digesting the cellulose and related materials has been reported by in some studies. Zverlov et al. (2005) have reported that 71 enzymes have been detected from the *Clostridium thermocellum* alone, with the main function is related to the polysaccharide and protein degradation. *Clostridium thermocellum* has shown its ability to grows rapidly in the medium contains high cellulosic compounds. Its activity to release enzyme complex named 'cellulosome' is the reason behind it. Cellulose 1,4-beta-cellobiosidase, xyloglucanase and endo-1,4-beta-xylanase, which are responsible for the hydrolysis of cellulose, hemicellulose and xylan, respectively, are among the enzymes released by *Clostridium thermocellum*. However, these enzymes are not exclusively associated to *Clostridium thermocellum*. Some other hydrolytic bacteria, such as

*Caldicellulosiruptor saccharolyticus*, also are active in releasing the same enzymes (Hanreich et al., 2013; Heyer et al., 2015).

The preferred living condition for each bacterial varies, which means to optimise the work, each microbe might require a unique condition as well. In a study by Kohrs et al. (2014), two anaerobic digesters operating in mesophilic and thermophilic condition are compared. Both digesters were fed continuously with agricultural biomass, which is expected to contain a high composition of cellulose, hemicellulose and lignin (Yu et al., 2007). The result shows that hydrolytic activities were mainly associated with Clostridiales and Thermoanaerobacteriales in the thermophilic process. In contrast, activity associated with Bacteroidales and Burkholderiales was shown more in mesophilic treatment, besides some activities from the previous two bacterial order as well. In overall, the hydrolytic activity was still higher in the thermophilic condition compared to that of the mesophilic. The superiority of thermophilic treatment in degrading substrate makes the process more preferable, especially when treating agricultural residue and energy crops (Hanreich et al., 2012; Tang et al., 2011; van Lier et al., 2001).

In fact, the population number of a particular species of bacteria is not always proportional to its activity. A substrate availability could be another factor shaping the bacterial community as well as its activity inside the digester. A study by Hanreich et al. (2013) shows how the abundant and activity of the microbe changes following the substrate availability and type. Their study was conducted in a mesophilic condition with agricultural residue as the feedstock. Similar to most studies in the AD, especially when the feed substrate comes from cellulosic-related compounds, Firmicute was the dominant phyla with Clostridiales is the dominant order, followed by Bacteriodetes phyla with its population far below the Clostridiales (Klocke et al., 2007; Schlüter et al., 2008). In the first week, when the cellulose was abundant, Clostridiales showed higher activity than that of Bacteriodetes. This is indicated by the detection of a significant amount of cellulase enzyme family associated with Clostridiales. After 30 days of the process, the amount of the cellulose in the medium decreased. At the same time, the number of Clostridiales population slightly decreased while the Bacteriodetes slightly increased. However, based on the protein analysis, higher activity was demonstrated by Bacteriodetes compared to that of Clostridiales in this day-30. This might show that Clostridiales is the main degrader of cellulosic compounds, while other hydrolytic bacteria might act more in degrading

other polymeric substance or as a fermenting bacterium following the hydrolysis step. This is supported by a study by Hagen et al. (2017) that uses food waste as the main substrate. Clostridiales is still detected but in an insignificant number. Instead, Coprothermobacter and Anaerobacullum together occupied more than 80% of the entire microbial community in the digester with the significant release of proteolytic enzyme.

*Acidogenesis and acetogenesis*

Following the hydrolysis is the acid generation step. The process is often given two separate names, i.e. acidogenesis and acetogenesis. However, since the expected end product of this sequential step is acetic acid, some authors simply name it as acetogenesis. Acetic acid is the most abundant intermediate in the anaerobic digestion process, followed by propionic acid (Toerien and Hattingh, 1969). Acetic acid is the energy source for some bacteria as well as the methanogenic group. Both acetic acid and propionic acid are the essential precursors for methane formation. However, an acid accumulation in the digester could happen if its production rate is higher than its consumption rate, or the buffer capacity of the system is not sufficient. It may lead to serious consequences for the digester, such as system failure due the decay of some microbial community.

In a two-stage treatment, this step is performed in the acidogenic reactor, which is separated from the methanogenic reactor. The purpose of this separation is to give the optimum condition for each group of the microbial community so that they can achieve their best performance in each reactor (Merlino et al., 2013). Bacteria are the main agents in acetogenesis, although some methanogenic archaea are also reported to take part in the process.

There are two pathways for acetate formation, i.e. via the heterotrophic process and the autotrophic process. In the heterotrophic pathway, the acetogens use organic substrate as the carbon and energy source. Some grow on a one-carbon compound media, such as CO, formate and methanol (Ljungdahl, 1986). The autotrophic bacteria grow by taking carbon dioxide and hydrogen as the only substrate. Homoacetogen is a type of acetogenic microbes that convert substrate(s) with acetate is the only products. Fourteen homoacetogens have been reported by Ljungdahl (1986) with most of them are

autotrophic acetogen. They come from five different genera and three of them are thermophilic. Three of them have been widely studied for autotrophic acetogen, i.e. *C. thermoaceticum*, *C. formicoaceticum*, *Acetobacterium woodii*. Figure 2.3 shows the simplified process of the two acetogenic pathways in the anaerobic digester.

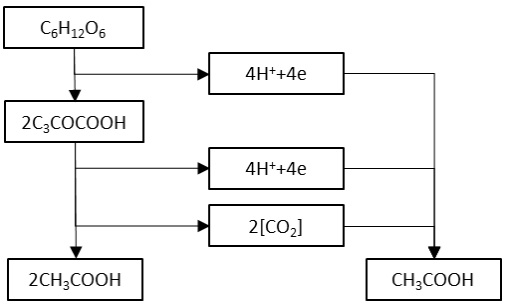



Figure 2. 3. Two types of acetogenic pathways in anaerobic digestion.

Heterotrophic acetogenesis begins with the utilization of hydrolysate from the previous process, e.g. glucose, amino acid, LCFA and long alcoholic compounds. Glucose is often the dominant hydrolysate in the digester, especially when cellulosic material was the dominant feeding substrate for the digester with a cellulosic substrate, glucose can be the dominant hydrolysate in the digester (Abram et al., 2011). As well, glucose is often used as the model substrate since it is the most available substrate for the heterotrophic microbes. *Clostridium acetylbotulicum* is probably the most studied to produce acetic acid from glucose (Heyer et al., 2015; Toerien and Hattingh, 1969).

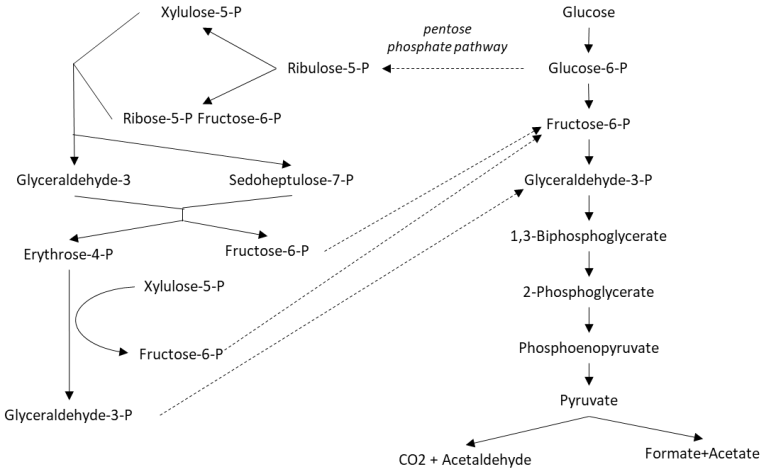



Figure 2. 4. Proposed model of glucose degradation by Abram et al. (2011). This cited model does not include the methane formation as in the original version. The right side is the glycolysis pathway and the left one is the pentose phosphate pathway.

Figure 2.4 illustrates a sequential glycolytic process proposed by Abram et al. (2011), where glucose was digested into acetic acid in the psychrophilic process. The conversion of glucose into acetic acid can be carried out in two different ways, i.e. glycolysis and pentose phosphate pathways. Glycolysis is preferred by most microbes as it gives a shorter route and lower energy. It converts the glucose into pyruvate with the formation of several intermediate products in between. The pyruvate is then converted into acids, aldehyde, format and carbon dioxide. Via the pentose phosphate pathway, the glucose is consumed with the end products are fructose-6-phosphate and D-glyceraldehyde-3-phosphate. Fructose-6-phosphate and D-glyceraldehyde-3-phosphate are also generated in the glycolysis pathway as intermediate products. To finish the process, these two compounds are introduced into the glycolysis pathway, which makes the whole process longer. Because of this, glycolysis is the more common pathway and is performed by most glycolytic bacteria. *Pelobacter propionicus* is among few bacteria to perform glycolytic process via the second route in low-temperature AD.

Ramsay and Pullammanappallil (2001) categorised amino acid-degrading microbes into five groups based on its fermentation pathway and the type of fermented amino acid. The fermentation products are various short-chain organic acids, ammonia, carbon dioxide and hydrogen and a small number of sulphuric compounds. As has been described in the early paragraph, the conversion of amino acid into acetic acid can be done in two pathways. The Stickland reaction is the common pathway, where it requires a pair of amino acid to act as an electron donor and receptor. This pathway is done by Group I bacteria with Clostridial species as the only bacteria reported in mediating the pathway. Another pathway is the digestion of a single amino acid with the presence of hydrogen-utilizing bacteria. Group II to V are all bacteria fermenting amino acid not using the Stickland reaction. These groups are categorized based on the amino acids digested and the metabolites produced in the fermentation. Clostridial species also dominate these groups, followed by *Peptostreptococcus sp*. While it is believed that bacteria solely take part in this process, some archaea have been reported to be involved in this process. This is indicated by the detection of a significant amount of amino acids-degrading enzyme associated with *Methanosarcinales* and Methanobacteriales (Heyer et al., 2013).

LCFA is a hydrolysate of fat, lipid, and grease in a hydrolytic process. In anaerobic treatment, it is converted into acetic acid and hydrogen through the β-oxidation process (Kim et al., 2004; Long et al., 2012). The process is started when the fatty acid is activated by coenzyme A that leads to the formation of acetyl-CoA and short-chain fatty acid. The acetyl-CoA is oxidised in a citric acid cycle repeating β-oxidation. LCFA is a serious inhibitor for anaerobic treatment (Rasit et al., 2015). Its hydrophobic character makes it separate from the aqueous phase, tending to float on the surface (Stoll and Gupta, 1997). Because of its long-chain structure, only a few bacteria are reported to be able to digest it, especially when the carbon number is more than twelve. *Syntrophomonas sapovorans*, *Syntrophomonas curvata*, *Syntrophomonas zehnderi*, and *Thermosyntropha lipolytica* are among the few bacteria are able to digest LCFA (Long).

Regardless of the substrate type, hydrogen is produced in heterotrophic acetogenesis. Interestingly, heterotrophic acetogenesis becomes unfavourable under a certain level of hydrogen partial pressure. For instance, conversion of propionic acid and butyric acid into acetic acid is an endergonic process with hydrogen as one of the products. The Gibbs free energy for this process is +48.1 kJ/mol and +76.1 kJ/mol for butyric acid and propionic acid, respectively. However, the process can be altered to become spontaneous by decreasing the hydrogen partial pressure, which is around $10^{-4}$ Pa. This agrees with Le Chatelier's Principle as discussed earlier. The principle states that by reducing the concentration of the product, the direction of the reaction will lead to the formation of more products. In a conventional digester, this hydrogen removal is performed in a syntrophic interaction between acetogen and hydrogen utilising microorganism. This syntrophic interaction will be discussed later in this chapter.

The autotrophic acetogenic pathway is performed by the bacteria with carbon dioxide and hydrogen as the substrates. The pathway was discovered first when *Moorela thermoacetica* was able to convert one mole of glucose into exactly stoichiometrically three moles of acetic acid (Ragsdale and Pierce, 2008). It was considered as an unusual behaviour by the time it was discovered since the typical acetogen could only convert one mole of glucose into two moles of acetic acid with carbon dioxide and hydrogen (Eq. 2.2). Another mole of acetic acid was found to be produced from the $CO_2$ and $H_2$ generated earlier in the previous step. This autotrophic pathway is then more popularly known as the Wood-Ljungdahl pathway (Ljungdahl, 1986). The pathway is based on the oxidative

and reductive direction of $CO_2/H_2$ utilisation (Ragsdale and Pierce, 2008). In this pathway, hydrogen acts as the electron donor while the carbon dioxide is the acceptor. *Clostridium thermoaceticum* is an example of this acetogen and has been widely studied for decades. It has also been reported that these bacteria can also grow in one-carbon compounds (Ljungdahl, 1986).

*Methanogenesis*

Methanogenic archaea are the microbial community responsible for methane production. Their presence in the digester is very small, less than 5% of the total population. In general, methane is formed in two pathways, i.e. acetoclastic and hydrogenoclastic. The name refers to the main substrate utilized in the formation, i.e. acetate and hydrogen. Acetoclastic is believed to be the dominant pathway regardless of the operational condition, simply because of the amount of acetic acid in the anaerobic digester dwarves hydrogen, which is used in another pathway. The acetate is produced either from an organic substrate or from $CO_2$ fixation (Ferry, 1999; Schnürer et al., 1999; Simpson and Whitman, 1993). Methane generation is a complex process, where acetic acid and hydrogen, along with carbon dioxide, are not the only substrate for the methanogenic archaea. Instead, methane can be generated from other substrates, such as formate, methanol and propionate (Ferry, 1999; Heyer et al., 2013; Jing et al., 2017a). The use of term 'hydrogenoclastic' to name one of the methanogenic processes probably is not quite right as the 'real' substrate is $CO_2$, while hydrogen is only such an electron donor or carrier in this process, and even can be replaced by formate (Morris et al., 2013). The methanogenic pathway is also condition-dependent since the microbial community and its activity is a subject to change according to the process condition (Conrad, 2005). When the operational condition changes, the dominant generation pathway might change as well.

The methanogenic pathway of a particular anaerobic reactor likely depends on the available species in the digester (Kotsyurbenko et al., 2001). Each type of methanogen is suggested to use only a specific methane generation pathway, either acetoclastic or hydrogenoclastic. The type of pathway in which the methanogen uses can be tracked from the enzymes they produce. In an acetoclastic process, some enzymes such as acetyl-CoA decarbonylase/synthase and energy converting hydrogenase are responsible for the

methanogenesis to support the acetate conversion. In hydrogenoclastic process, 5,10-methylenetetrahydromethanopterin reductase, tetrahydromethanopterin S-methyl-transferase, and coenzyme F420-reducing hydrogenase are such enzymes responsible for the process (Heyer et al., 2013).

*Methanosarcinales* and *Methanosaeta* are often associated with the acetoclastic methanogen based on the enzymes released for the methane production. These two methanogens have been reported to present in the most variety of treatment conditions and feed substrate. However, based on many reports, their population is likely only dominant in the mesophilic condition with an insignificant amount of inhibitor present in the reactor (Hanreich et al., 2013; Heyer et al., 2013; Kohrs et al., 2014). Complying with this condition, a study by Nettmann et al. (2010) showed that there is a strong correlation between the absence of *Methanosaeta*ceae in the biogas reactors and high concentrations of total ammonia.

*Methanomicrobiales*, *Methanobacteriales* and *Methanoculleus* are among the popular methanogens to mediate the hydrogenoclastic pathway based on their presence and activities in the anaerobic digester. The hydrogenoclastic tend to dominate the archaea inside the digester when the thermophilic condition applied and where agricultural biomass is the main feedstock (Bergmann et al., 2010; Hanreich et al., 2012; Krakat et al., 2010; Nettmann et al., 2010). Since hydrogenoclastics are relatively more resistant to inhibitors than the acetoclastics, their presence is seen to predominate in digesters with high levels of inhibitors, such as ammonia salt (Schnürer et al., 1999). In some cases, acetoclastic methanogen shows to dominate the archaeal community, but their activity was less significant compared to the hydrogenoclastic (Hanreich et al., 2012). In this report, the microbial activity is observed using metaproteomics along with the microbial abundance. It concurs with Kohrs et al. (2014) that while *Methanosarcinales* seems to dominate the methanogen community in mesophilic condition, the methanogenic activity is dominated by *Methanomicrobiales*. The domination of the hydrogenoclastic methanogen is also reported for most of the full-scale mesophilic reactors (De Vrieze et al., 2015; Nettmann et al., 2010). Since the reactors used in the study by De Vrieze et al. (2015) could be categorised by a high rate of treatment, this finding highlights that hydrogenoclastic pathway is a very important pathway in the high rate treatment. In other

words, it can be stated that hydrogenotrophic is the real dominant pathway for most methane production, unless the digester is in a perfect mesophilic condition.

Acetoclastic methanogen is highly susceptible to any disturbance. The change of loading rate, high level of ammonia, salt, VFA and temperature could potentially disrupt their population as well as their activity (Conrad, 2005; Nettmann et al., 2010; Schnürer et al., 1999). These disturbances could directly lead to systemic failure, resulting in no methane production. In some cases, the microbial community acclimatise and shift to a new microbial composition. When this happens, the number of hydrogenoclastic could surpasses the acetoclastic. In other instances, this disturbance only affects the acetoclastic activity without significantly changing the composition of the whole archaea, and then the hydrogenoclastic activity becomes dominant over the acetoclastic. Regardless of the mode of operation, acetic acid and propionic acid are the most abundant carbon sources in the anaerobic digester. When hydrogenoclastic dominates the methanogenic activity, how the VFA is utilised is questionable. At the same time, the amount of hydrogen and carbon dioxide produced from the previous step might be stoichiometrically too low compared to the produced methane. The key to this question is the presence of an oxidation pathway mediated by syntrophic acetate oxidizing bacteria (SAOB) that convert the acetic acid into $CO_2$ and $H_2$ (Morris et al., 2013; Nettmann et al., 2010; Schnürer et al., 1999). When the amount of $CO_2/H_2$ is abundant, hydrogenoclastic methanogens do most of the work.

*Syntrophic Interaction*

Syntrophic interaction is an essential process when two or more microbes work together in a mutualistic metabolism (Morris et al., 2013). While microbial syntrophic interaction may happen in any environmental condition, this phenomenon is mostly discussed in an anoxic system. In this interaction, a mixed microbial population works to support each other to survive in a certain environment by digesting a common substrate without the presence of molecular oxygen. This interaction is typically interphylum, where one microbe releases its metabolite which can inhibit itself when it reaches a certain level. In the other side, the partner microbes take the secreted metabolites of the first microbes as their substrate. The removal of the inhibitor favours the first microbes to continue its process. As an example, the syntrophic interaction between ethanol-producing bacteria

and hydrogenoclastic methanogen *Methanobacterium bryantii* in an anaerobic digestion (Bryant et al., 1967). Hydrogen is a by-product of the ethanol-producing bacteria, where it is only favourable in low hydrogen pressure. *Methanobacterium brayantii* uses the hydrogen to reduce $CO_2$ to produce methane, which resulting to a favourable condition to its microbial partner. Despite the fact that a syntrophic interaction means a joint activity between two or more microbes, some species have a speciality more in serving others and are thus called a syntrophic microorganism. Syntrophic microorganisms are very adaptive with their living environment. They are able to survive in either partner-free or partner-dependent modes. Some of them even have the ability to produce hydrogen in one time and utilise hydrogen in a different time (McInerney et al., 2008, 2007; Orphan, 2009).

A principle behind syntropy is the conservation of available chemical energy in the system (Morris et al., 2013). This process is expressed in the electron transfer between species. Stams et al. (2006) suggested that there are three possibilities of electron transfer:

(i) Firstly, by soluble compound from one microbe to another. In this case, hydrogen is the most utilised gas in the process, termed interspecies hydrogen transfer (IHT).
(ii) Secondly, by organic/inorganic transformation to inorganic materials. Humic substance and some vitamins are examples of the electron carrier. However, this type of electron transfer process is hardly discussed in the anaerobic digestion process.
(iii) Finally, by direct inter-species electron transfer (DIET), either by direct contact between two microbes or using electro-conductive material.

An alteration of electron transfer type is possible if the condition is shifted. Jing et al. (2017) demonstrated how the addition of conductive material (magnetite) alters the electron transfer process from IHT to DIET through a direct methanation of propionate.

Molecular hydrogen is the dominant electron carrier since it has small size and readily diffuses. Hence, hydrogen becomes the centre of a syntrophic interaction in the system (Morris et al., 2013). The important role of IHT in degrading organic substrate has been widely studied in anaerobic digestion (Stams et al., 2006; Stams and Plugge, 2009). A piece of evidence about the dependency of some organic degrading bacteria with hydrogen-

producing bacteria have been reported by several studies (Fennell et al., 1997; Shelton and Tiedje, 1984). Syntrophical oxidising bacteria play the role of hydrogen producers, with the substrate mostly in the form of short-chain VFA (i.e. acetate and propionate). At an elevated temperature, the diffusivity of hydrogen becomes higher, expected due to a higher electron transfer rate (de Bok et al., 2004). Such conditions may lead to a higher rate of substrate conversion. However, this theory faces a question since the aqueous solubility of hydrogen is very low.

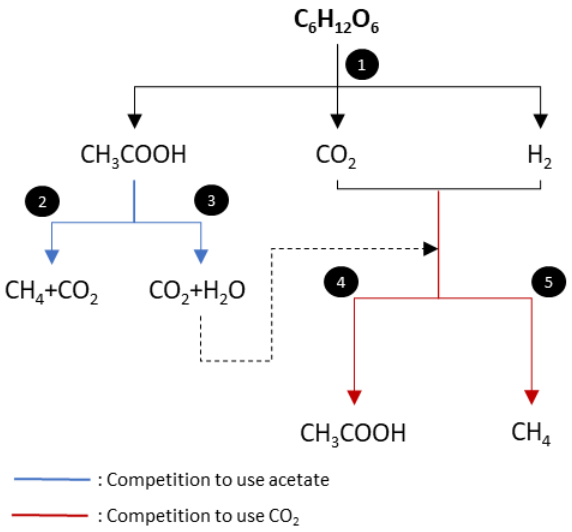



Figure 2. 5. Competition among microbe in utilising the same substrate derived from glucose. The number shows the microbial type: 1. Heterotrophic acetogen, 2. Acetoclastic methanogen, 3. Syntrophic Acetate Oxidizing Bacteria, 4. Autotrophic acetogen, 5. Hydrogenoclastic methanogen.

Formate is another possible electron carrier in a syntrophic interaction along with hydrogen (de Bok et al., 2004; S Hattori et al., 2000). The evidence is reported on the study of formate-oxidising bacteria *Syntrophobacter fumaroxidans* that are able to grow syntrophically with formate-utilising methanogen. While formate-utilising methanogen can survive syntrophically with the bacteria, the opposite result is obtained when the hydrogen-only-utilising methanogen is set as the partner. Many syntrophic bacteria are able to produce both hydrogen and formate (de Bok et al., 2004).

In anaerobic digestion, the main purpose of syntrophic interaction is to convert substrate into methane. At this point, the interface between C1 and C2 compounds (acetate, methanol, formate) become the focus of the syntrophic interaction. While the purpose of syntrophic interaction is to get the benefit for both microbes, the competition to utilize the same substrate is unavoidable. These competitions carry the potential to change the

pathway of methane formation. Figure 2.6 shows an example of the competition among microbes for utilizing the products of heterotrophic acetogen. In this model, glucose is used as the model substrate by the heterotrophic acetogen, where $CO_2$ and $H_2$ are the side products. A syntropy between heterotrophic acetogen and hydrogen-utilizing methanogen has become a standard interaction in anaerobic digestion since the acetate generation process is only favourable with a lower partial pressure of hydrogen. The utilisation of hydrogen to reduce the carbon dioxide by the methanogens allow the acetogen to sustain the process. With the presence of formate-utilizing bacteria and autotrophic homoacetogens in the digester, the competition to utilise carbon dioxide is unavoidable (Dolfing et al., 2008). Another competition is between the acetoclastic methanogen and SAOB to utilize acetate. In general, the acetoclastic methanogenic process is more favourable thermodynamically. However, in some cases where the thermophilic was applied or the pH was relatively low, the SAOB activity outperformed the methanogen (Morris et al., 2013; Nüsslein et al., 2001; Shigematsu et al., 2004). In the excess of sulphate in the digester, sulphate-metabolizing bacteria might outcompete the methanogen to utilize both hydrogen and acetate (Morris et al., 2013). In the presence of methane-oxidizing bacteria, this sulphate excess might become detrimental with the objective of the man-made anaerobic digestion process (Stams and Plugge, 2009).

## 2.3. $CO_2$ utilisation for enhancing methane production

The increasing level of $CO_2$ in the atmosphere has triggered more effort for storing and utilising $CO_2$. In fact, $CO_2$ is also a main component in the biogas produced from an AD process, along with methane and nitrogen. Despite the fact that $CO_2$ is produced internally by the microbial community, the addition of exogenous $CO_2$ has received more attention since it demonstrates a significant increase in the methane production rate. The amount of increase depends on the feed substrate and operational conditions (Alimahmoodi and Mulligan, 2008; Fernández et al., 2015; Salomoni et al., 2011). The idea of adding the $CO_2$ into the AD was firstly presented in 1994, where the $CO_2$ was added into the head space of the reactor and then the concentration was controlled (Bajón Fernández et al., 2017). Another trend of using the $CO_2$ is by coupling it with $H_2$ injection (Morris et al., 2014; Tao et al., 2019) with the aim was to give additional substrate for the hydrogenoclastic methanogen.

Rather than repeating these success stories, this section discusses more about the rationale behind the enhancement of the methane production due to the addition of $CO_2$ into the system. There are some proposed mechanisms on how $CO_2$ addition might have affected the methane production. This includes some physical-chemical effects on the media, such as affecting the pH and removing toxic inhibitors. A highlight is given to the microbial process which may have been altered due to the addition of $CO_2$. Until recently, there have been only few papers discussing this phenomenon, in the sense of mechanism by which exogenous $CO_2$ is able to increase methane production, especially when injection is an applied technique. Several hypotheses have been proposed from previous papers. They can be summarised in the following points.

1. The $CO_2$ injection has been suggested to increase the degraded fraction of the substrate (Al-Mashhadani et al., 2016). The hypothesis based on the mass balance analysis, which concluded that there should be more molecular hydrogen produced in the system to complement the $CO_2$ added into the system to make the hydrogenoclastic methanogen more possible to occur. It was supported by the presence of more hydrogen in the off-gas, which was also reported by Fernández et al. (2015). Since no external $H_2$ was added, an additional amount of the hydrogen gas should have been produced biologically in the system. Al-Mashhadani et al., (2016) suggested that higher degradation of the feed substrate might be the reason for this higher hydrogen gas production. This additional degradation is predicted as a result of the physico-chemical process that occurs as a result of the addition of $CO_2$.

   There are two possible ways of how $CO_2$ might support this hypothesis. **Firstly**, if only the $CO_2$ was in the microbubble, hydroxyl free radicals might have been generated in the collapse of the bubble (Takahashi et al., 2007b). This bubble collapse is initiated by the diffusion of carbon dioxide to the bulk liquid, driven by the concentration gradient. As the bubble becomes smaller, the ratio of the zeta potential to the bubble surface area becomes bigger. In a sub-saturated liquid environment, a pure carbon dioxide microbubble can even dissolve completely (Al-Mashhadani et al., 2016). This leads to the localized rapid increase of the temperature and pressure that may cause the bubble collapse. As the microbubble collapse, hydroxyl free radical generated. Hydroxyl radical is a strong oxidant with the ability to degrade organic compounds (Ikeura et al., 2011; Khuntia et al., 2015). This condition might apply for the study by

Al-Mashhadani et al. (2016) where the microbubbles are injected, but probably not for the study by Fernández et al. (2019, 2015) as the $CO_2$ is injected conventionally.

**Secondly,** the size of the microbubble causes a lower buoyancy force that causes longer residence time in the medium, either in the bulk liquid or attach onto the surface of any substrate. As the $CO_2$ dissolves in the liquid, it forms bi/carbonic acid and is followed by a pH drop. A localized pH shock may occur on the surface of the attached substrate due to $CO_2$ dissolution. This localized pH shock can attack more substrate and lyse cells, for instance. The potential of pure carbon dioxide in degrading the substrate was also demonstrated by Mulakhudair et al. (2017). Using pure $CO_2$ microbubbles, a significant reduction of *Pseudomonas putida* content is achieved. It also opens a new potential for low-temperature disinfection, which should require less cost. Another report by Mulakhudair et al. (2016) demonstrated the potential for lignin degradation supported by the microbubbles treatment under acidic conditions. However, this report in lignin degradation applied a longer duration of $CO_2$ injection than implemented in the AD process, which was around five minutes.

However, it should be noted that whichever the proposed degradation mechanism, it potentially will affect the microbial community inside the system. It is because both hydroxyl radical and pH shock will also attack the microorganism unselectively as well as the substrate. This disruption can potentially induce a negative response by the system as a whole. If this happens, it would not militate against methane production, but also to substrate decomposition. In the reality, the opposite occurs, especially an increase in methane production. If indeed a higher substrate degradation has occurred, it should be supported by a higher removal of either COD or TVS. While the report by Al-Mashhadani et al. (2016) did not mention this, the study of Fernández et al. (2014) shows that no difference in the removal of COD and TVS had been demonstrated, except for one treatment that was fed with sewage sludge and injected with a concentration of 30% $CO_2$. However, the methane production by this treatment was lower than that which was treated with 90% $CO_2$ in the same study.

If either hydroxyl radical or localized pH shock has been generated, it may only have an effect on the acetoclastic methanogens, instead of the whole system. There are two reasons for this. Firstly, acetoclastic methanogens are the most sensitive

microbes to any disruption precursor. Any disruption against the community has the potential to decrease its activity. It may cause an alteration in the methanogenic pathway if only the hydrogenotrophic methanogens are present in sufficient quantity. Otherwise, methane production may decline. Secondly, the amount of either generated radical or pH shock was too small compared to the whole system. An only small fraction of the microbubble was less than 100 microns in size (Al-Mashhadani et al., 2016). In different study those do not use microbubble, both *Methanosaeta* and *Methanosarcina*, acetoclastic methanogens, maintained as the most abundant archaea in the system over the hydrogenoclastic (Fernández et al., 2019). More detail on the microbial side will be discussed later in this section.

2. The injection of $CO_2$ has stripped off a number of product gases that ultimately make the chemical process more favourable to form new products (Al-Mashhadani et al., 2016). Le Chatelier's Principle is the rationale behind the proposed mechanism. According to the principle, any disruption applied to any equilibrium, the system will counteract by adjusting it to a new equilibrium. Based on the principle, the removal of any product of a chemical reaction will make the system to form more products. The same condition result is achieved if the concentration of the reactants is increased.

   In anaerobic digestion, once being hydrolysed, the produced monomers will undergo an acidification process to become fatty acid. Acetogen converts the longer chain of fatty acid into acetic acid, carbon dioxide, hydrogen and sometimes a little formic acid. Equation 2.13 and 2.14 shows some examples, where propionic acid and butyric acid are converted into acetic acid by heterotrophic acetogen. Propionic acid is the most dominant fatty acid after acetic acid, followed by butyric acid. Both are converted into acetic acid by heterotrophic acetogens. However, this process is endergonic and will only be favourable under very low pressure of hydrogen gas ($4.6 \cdot 10^{-4}$ atm). To sustain the process, a syntrophic interaction must be done between the acetogen and hydrogenoclastic methanogen. In this symbiosis, the hydrogen is consumed by the methanogen to be converted into methane.

$$CH_3CH_2COH + H_2O \rightarrow CH_3COOH + CO_2 + H_2 \quad \Delta G^{0\prime} = +76.1\ kJ/mol \quad (2.13)$$

$$CH_3CH_2CH_2COOH + H_2O \rightarrow CH_3COOH + H_2 + CO_2 \quad \Delta G^{0\prime} = +48.1\ kJ/mol \quad (2.14)$$

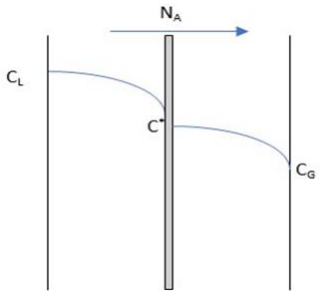
Instead of allowing the methanogen to naturally remove the hydrogen, removal could be performed more rapidly using a gas stripping technique. As the environment becomes more favourable after stripping, it allows the acetogen to continue the process resulting in more acetate produced in the system. However, since stripping is an unselective process, not only hydrogen is removed, but also some other gases include $CO_2$. It may give a negative impact due to the loss of $CO_2$ since it is one of the precursors of methane. This condition may lead to a decrease in the methane production. The reduction of the methane production rate is reported by Al-Mashhadani et al. (2016) when nitrogen was used as the injected gas, instead. This contrasts with the increase of methane production after $CO_2$ was injected into the system, replacing nitrogen, increasing with $CO_2$ composition in the microbubble, the maximum increase found with pure $CO_2$.

In fact, there are dozens of conversion processes that occur in the reactor at the same time and they might be affected as well by this stripping action. Another gas that is likely to be removed is methane, either produced via hydrogenoclastic (Eq. 2.11) or acetoclastic (Eq. 2.12). Based on Le Chatelier's Principle, stripping of the methane potentially will increase the rate of methanogenesis (Al-Mashhadani et al., 2016). It may lead to a higher conversion rate from either $CO_2/H_2$ or acetate into methane. The same condition applies for methanation from formic acid if it is available in the digester. In the case where methane oxidising bacteria are present in the AD, the increase of the methane production might raise their activity, consuming more methane. Rather than benefiting the process, this type of bacteria will decrease the methane concentration. In such circumstances, stripping of the methane faster than the catabolism by methane oxidising activity could be a fortuitous outcome. Other toxic gases, such as free ammonia might potentially be removed as well by this stripping process (Serna-Maza et al., 2014; Walker et al., 2011). Hence, it seems obvious that the $CO_2$ injection has played several roles at once, e.g. stripping of some gases to make the process more favourable in producing more products and giving some additional substrate for some microorganism inside the AD.

Regarding the gas transfer in the liquid phase by the injection process, several factors are suggested to affect it: transfer coefficient of the specific gas, concentration gradient between two-phase and interface area between the liquid and gas phase. It

is expressed in a "Thin Film Mass Transfer Theory" as presented in equation 2.15. $K_L$ $a$ can practically be calculated using equation 2.16.

$$N_A = K_L a(C_1 - C_e) \quad (2.15)$$




$$K_L a = \frac{1}{T} ln\left(\frac{C_L^o - C_e}{C_L - C_e}\right) \quad (2.16)$$

Figure 2. 3. Illustration for thin film gas liquid mass transfer theory

Where $N_A$ is the mass transfer flux, $K_L$ is the liquid side mass transfer coefficient from the gas to liquid, $a$ is the bubble surface area per unit volume, and $C_L$-$C_e$ is the concentration gradient of a certain gas between two different phases. The equation explains that the surface area of the bubble and concentration gradient drive the transfer rate between the gas and liquid phase. It then becomes obvious that the injection of microbubbles enhances the efficiency of gas removal as well as dissolution of $CO_2$.

There is a question left regarding this proposed mechanism: where the molecular hydrogen comes from for the autotrophic acetogen and hydrogenotrophic methanogen if the hydrogen has been removed from the liquid. As has been discussed, the gas stripping is carried out only for a few minutes a day. Once the gases are removed, especially hydrogen, it will make the condition to be more favourable for the acidogenic activity to happen. It results in more acetate, hydrogen, and carbon dioxide available in the system. The time lag between the periodic stripping gives time for the hydrogen-consuming microorganism to utilize the 'freshly produced' hydrogen before it accumulates again and begins to re-create a condition that is unfavourable. This is probably one of the reasons for higher hydrogen content in the off-gas. Despite the fact that hydrogen has a higher calorific value per unit

mass than methane, a higher content of hydrogen in the off-gas can be seen as an energy loss if methane is meant for the desired product.

Using chemical analysis, the evidence of higher acetogenic activity could be tracked using the amount of acetate in the system. As the rate of acetogenic activity becomes higher, the acetate concentration should be higher as well. In turn, it might reduce the concentration of the propionic and butyric acid as a result of acetogenic transformation. This fits well with the result of Francioso et al. (2010) showing that higher acetic acid was available in the medium after $CO_2$ injection. However, this assumption is not totally correct since the presence of acetic acid in the liquid is very dynamic. It can be produced by the acetogen while at the same time it is converted by either the SAOB or acetoclastic methanogen. Any un-converted VFA left in the effluent can be meant as an energy loss since it is one of the methane precursors. A study on the microbial population and its acetogenic activity should help to answer the question.

While the addition of exogenous $H_2$ along with $CO_2$ has demonstrated an increase in methane production (Morris et al., 2014; Tao et al., 2019), it potentially faces some drawbacks. Firstly, either produced biologically or electrically, the production process of hydrogen gas consumes energy. Adding exogenous hydrogen means spending more energy into the process. Furthermore, hydrogen can be categorized as a ready-to-use gas without necessarily being converted into another form of energy. An energy loss due to the low efficiency of hydrogen utilization during the process should be taken into account since hydrogen solubility in water is very low, which is $1.6 \times 10^{-6}$ g $H_2$/g water. The safety issue related to hydrogen storage and utilization is another consideration.

Secondly, since the hydrogen is added from an external source and may increase the hydrogen pressure in the medium, it potentially reduces the activity of heterotrophic acetogen as the environment becomes less favourable. If this happens, the system potentially will face a material loss due to a lower conversion rate of VFA into acetate. A higher acetate concentration found in the medium, as has been reported by Tao et al. (2019), does not necessarily mean a high conversion of longer chain VFA into acetate. It is because acetic acid can also be produced via Wood-Ljungdahl pathway

when $H_2/CO_2$ are available in the medium (Eq. 2.9). Since $H_2/CO_2$ become abundant in the system after $H_2/CO_2$ injection, it might encourage the activity of hydrogen consuming bacteria, i.e. autotrophic acetogen and hydrogenotrophic methanogen, leading to more production of acetate and methane. The domination of *Methanomicrobiales* in the archaea community, as reported by Tao et al. (2019), supports the occurrence of $H_2/CO_2$ utilization. Nevertheless, since the study did not measure the removal of some chemical parameters in the effluent, especially VFA other than acetate, TVS and/or COD, as well as the acetogenic activity, this hypothesis cannot be confirmed more convincingly. It was even reported that an increase of volatile solids in the digester are observed several times.

3. The exogenous $CO_2$ may have been utilised by acetogen to produce acetic acid via Wood-Ljungdahl pathway by autotrophic acetogen (Fernández et al., 2019; Francioso et al., 2010; Mohd Yasin et al., 2015). The increase in the concentration of acetic acid is then followed by an increase in the methane production rate. Following the increase in acetate production, acetoclastic methanogen is suggested to remain as the dominant pathway in the methane production. It is supported by a study by Fernández et al. (2019) where acetoclastic methanogen occupied more than 90% of the total archaea in the system. The study was also observed a significant increase of *Methanosaeta* composition. However, this increase could not be confirmed as to whether it was because of the increase of available acetate or reduction in the free ammonia level as both parameters are experienced simultaneously and commonly affect the archaea community. As an additional information, this study is done in a high level of ammonia (>3 g/L), a level that is considered to be toxic for most of the microbial community (Rajagopal et al., 2013), especially for acetoclastic methanogen. In addition, it is a fact that the concentration of acetoclastic methanogen is not always proportional to its activity (Hanreich et al., 2012; Kohrs et al., 2014).

   Different from the study by Fernández et al. (2019), the study by Francioso et al. (2010) uses two-stage treatment, where acetogenic and methanogenic tanks are separated. In this study, the $CO_2$ was continuously injected into the acetogenic tank only. To track the $CO_2$ utilization, $C^{13}$ isotope was used for the $CO_2$ injected. The result shows that more acetate was produced in the tested acetogenic tank than that of

the control, and it was associated with the $C^{13}$ isotope. This result was not surprising as the $CO_2$ injection is applied in the acetogenic tank only, where it is a favourable condition for the acetogen, but not for the methanogen. The dominant mechanism might be different in the one-phase treatment where a free competition in utilizing the $CO_2$ may occur more intensively between the autotrophic acetogen and hydrogenoclastic methanogen in the reactor.

4. The exogenous $CO_2$ may have been utilised by the hydrogenoclastic methanogen to form methane using $H_2$ as the electron donor (Al-Mashhadani et al., 2016; Alimahmoodi and Mulligan, 2008). In the presence of formate in the system, the electron transfer could also be accomplished by the formate without neglecting the role of the hydrogen in another the transfer process as well (Morris et al., 2013). A thermodynamic consideration is probably the main reason that the hydrogenoclastic methanogenesis might have dominated the $CO_2$ utilization over the autotrophic acetogenesis. It is demonstrated with their Gibbs free energy, which is -135.1 kJ/mole and -95 kJ/mole for the methanogen and acetogen, respectively.

   Regarding the increase of acetate level after $CO_2$ injection, it was not necessarily attributed to the domination of the Wood-Ljungdahl pathway over the heterotrophic acetogen. Since the environment becomes more favourable for the heterotrophic acetogen due to the periodic removal of $H_2$, more acetate can be produced via this pathway. Considering Le Chatelier's Principle, the autotrophic acetogenesis is less likely to happen in the early process when acetate is available abundantly as a result of the higher substrate degradation rate. Instead, SAOB might utilize the acetate. This is followed by the methanation of produced $CO_2/H_2$ by the hydrogenoclastic methanogen, which the process can be seen as an indirect methanation of acetate. Despite the fact that the oxidation of acetate is thermodynamically less favourable than the direct methanation of acetate, some findings show that the process is preferable in the medium with high salt, high ammonia, and high VFA (Morris et al., 2013; Nüsslein et al., 2001; Shigematsu et al., 2004).

   In fact, the presence of a certain pathway does not contradict with the occurrence of another pathway in the utilization of the common substrate since they can occur simultaneously in the same reactor. A certain pathway might be preferable in the

system in some reactor conditions. To ensure which route might be dominating the process as well giving a better result, a less biased study is necessary to be conducted to answer the question. With the constraint of the nature of the feed substrate and time constraint, promoting a certain pathway might be economically and energetically preferable.

## 2.4. Explanation of the hypothesis

The hypothesis of the study is summarized in this section while the thinking underlies the hypothesis can be read in the literature review.

1. The injection of $CO_2$ microbubble will significantly increase the methane production, in general. Regarding the addition of leachate, it should increase the methane production as it contains a significant amount of trace elements.

   The benefit of adding trace elements in AD has been discussed in many literatures. However, leachate has also been reported to contain a significant amount of toxic chemicals that may outweigh the benefit of the trace elements. With the injection of $CO_2$ microbubbles, the toxicity of the leachate can be reduced, yielding a better AD performance. There are several possible mechanisms for the reduction of leachate toxicity. One possibility is the $CO_2$ strips of some toxic chemicals (Bloor and Banks, 2005; Quan et al., 2010; Shibin et al., 2007). In this mechanism, only chemicals that have quite high volatility can be removed. Another possible way is that $CO_2$ promotes biological process that lead to high biodegradation of toxic chemicals. The biodegradation of some organic chemicals in AD system have been reported in some literature as well (Battersby and Wilson, 1988; Carballa et al., 2007).

2. Stripping off some product gases by the injected microbubbles is predicted to be the main mechanism, as has been discussed in the previous section. This is initiated with the hydrogen removal by the microbubbles which result in a higher heterotrophic acetogenic rate. It gives a 'domino effect' to several other processes as discussed earlier. After the acetic acid is available more abundantly in the medium, it may trigger higher rates of acetic acid utilisation by acetate-consuming microorganisms, either from bacterial or archaeal species. By stripping off the methane, more methane will be produced, based on Le Chatelier's Principle. Indeed, the solubility of $H_2$ and $CH_4$ are

very low in the water. One of the possibilities is they stay longer by attaching on to the surface of the substrate or organism that produce the gases. Once they have enough buoyance force, they will detach from the surface. Stripping is suggested to accelerate this detachment. Logically, the microbubble application will give a more significant impact on accelerating the detachment process than fine bubbles. However, this study will not cover this comparison.

3. The utilisation of dissolved $CO_2$ is dominated by hydrogenotrophic methanogenesis over the autotrophic acetogenesis. The main reason is the hydrogenoclastic methanogenic activity is thermodynamically more favourable than the autotrophic acetogenic activity, which is -135.6 kJ/mol (methanogen) over -95 kJ/mol (acetogen). Moreover, following the Le Chatelier's Principle, a high concentration of acetic acid in the medium (produced by acetogenesis) makes it less favourable to produce more acetic acid using a different pathway.
4. The methanogenic activity will be dominated by hydrogenoclastic methanogenesis over the acetoclastic. Besides being more favourable thermodynamically, the hydrogenoclastic route is more resilient to any disruption that may occur as a result of $CO_2$ addition. Such potential disruptions are locally pH shock attributed to $CO_2$ microbubble dissolving and high VFA concentration due to higher acetogenesis rate. As there will be a significant increase of the VFA, acetate oxidation by SAOB is likely to happen significantly. It may lead to the indirect methanation of acetate. Some findings about indirect methanation have been reported in several articles when an unusual condition occurred in the reactor (Morris et al., 2013; Nüsslein et al., 2001; Shigematsu et al., 2004).

# Chapter 3

# $CO_2$ Microbubble Promotion of a Substrate Degradation Rate and Efficiency in AD of Food Waste

## 3.1. Introduction

The addition of $CO_2$ enhances the methane production rate in anaerobic digestion (AD). In practice, there are various techniques to add $CO_2$ into a digester. Some of those are by adding $CO_2$ into the reactor headspace and by injecting it into the liquid. To increase the dissolution rate, the $CO_2$ is injected in the form of microbubbles. The increase in dissolution rate is due to increasing the interface between gas and bulk liquid. $CO_2$ microbubble injection increases methane production rate up to 109% when food waste is used as the feed stock (Al-Mashhadani et al., 2016). A lower increase of methane production rate between 11% to 25% is reported when fine bubbles are injected in anaerobic digestion of food waste (Fernández et al., 2015; Fernández et al., 2014).

Many hypothesises are proposed for how $CO_2$ addition enhances methane production rate, especially when the mode of delivery is by injection of microbubbles. Firstly, $CO_2$ microbubbles increase the substrate degradation rate. Substrate is firstly degraded into a

soluble fraction before being utilised by the next processes. Increasing substrate degradation results in enhancement of methane production rate. This higher substrate degradation rate is predicted due to generation of hydroxyl radicals after $CO_2$ microbubbles collapse. Hence, this mechanism likely to occur only when pure $CO_2$ is injected in the form of microbubbles. Takahashi et al. (2007) report the mechanism for microbubble collapse to generate free radicals.

However, it is important to note that free radicals attack unselectively in the digester. While the generation of free radicals may increase the degradation rate, it may also harm microorganisms inside the digester, for which methanogenic archaea are the most sensitive (Conrad, 2005; Nettmann et al., 2010; Schnürer et al., 1999). Any disruption to this archaeal community likely will decrease the methane production rate. In fact, the methane production rate increases after $CO_2$ microbubble injection. Al-Mashhadani et al. (2016) reported that only a small fraction of bubbles generated during their study can be categorized as microbubbles less than 100 microns. Consequently, the number of free radicals that are generated are quite small. Therefore, there could other mechanisms for this enhancement besides free radical attack. However, Al-Mashhadani et al. (2016) found that although methane production rate rose monotonically with $CO_2$ composition in the microbubble phase, a very large increase occurs with pure $CO_2$ microbubbles. The other component used is $N_2$, which prevents all but pure $CO_2$ microbubbles from complete dissolution, due to the establishment of equilibrium – $N_2$ has very low solubility in aqueous solutions.

Secondly, $CO_2$ provides an additional carbon source. Most of the hypotheses state that introducing exogenous $CO_2$ increases acetate generation via Wood-Ljungdahl pathway. In this pathway, monoacetogens play the main role to convert $CO_2$ and hydrogen into acetate. In fact, there are two possible pathways for exogenous $CO_2$ utilization in AD. The second is the methanation of $CO_2$ via the hydrogenoclastic pathway. The hypothesis for the first pathway is supported by increasing acetate concentration after $CO_2$ addition and followed by increasing methane production rate (Francioso et al., 2010; Yasin et al., 2015). Fernández et al. (2019) also report the domination of acetoclastic methanogen after $CO_2$ injection, which supports the claim. However, increasing hydrogenoclastic methanogen is also observed in their study after $CO_2$ injection. Sometimes, an injection of hydrogen gas is performed along with $CO_2$ to enhance the overall methane production rate. Tao et al.

(2019) report a significant increasing of hydrogenoclastic methanogen after simultaneous injection of $CO_2$ and $H_2$ into the digester. Both $CO_2$ utilization pathways are possible simultaneously in the digester, however. Nevertheless, there may be a dominant pathway with energetically more efficient methane production.

Thirdly, the addition of $CO_2$ creates a suitable environment to generate more products. This suitable environment may occur when the injected gas removes some inhibitors from the medium or creates a suitable microenvironment for the microorganism (Al-Mashhadani et al., 2016). Acetate is the dominant methane precursor. It is generated in acidogenesis. This step is non-spontaneous except while hydrogen partial pressure is under $4.6 \cdot 10^{-4}$ atm (Ahring and Westermann, 1988). Naturally, hydrogen is reduced in a syntrophic interaction by hydrogenoclastic methanogen. This removal can be accelerated using gas stripping, which in this case is done by $CO_2$ microbubbles. As stripping removes gases unselectively, some product gases, such as methane, are also removed. According to Le Chatelier's principle, removing a product from a medium at equilibrium will increase a reaction rate to generate more products. Of course, metabolism is inherently a non-equilibrium process, yet quite commonly, a species excreted, extracellular metabolites inhibit its own metabolism, creating a similar effect.

In practice, the methane yield of any substrate is lower than its theoretical value. The actual methane yield ranges from 30% to 90% from the theoretical value, depending on the nature of the substrate and operational conditions (Angelidaki and Sanders, 2004). The removal of inhibitors by microbubble injection is also expected to increase substrate conversion efficiency into methane.

The objective of this study is to explore the mechanisms for $CO_2$ microbubbles to enhance methane production rate, with an emphasis on examining the current hypotheses presented above. In this study, only chemical and physical properties are used for analysis. No microbial analysis is performed. The investigation is carried out with three constraints: the $CO_2$ is injected through microbubbles; the operational mode is batch; the operational temperature is mesophilic. The mechanism of how the test reactor generates additional methane after $CO_2$ microbubble injection is explored in the 'Result and Discussion' section.

## 3.2. Method

### 3.2.1. Feedstock and seeding sludge

The feedstock used in this experiment is reproducible artificial food waste (see Appendix 3 for the recipe). To obtain the substrate for the AD, all food waste is crushed with an electrical kitchen chopper while no sieving is applied. To help the crushing process, water is added to the food waste with the ratio 3:1 for the food waste and water, respectively. The seeding sludge is collected from a full-scale anaerobic digestion plant treating municipal organic waste (Stockport, England). For a longer duration of storage, the seeding sludge is stored under temperature 4$^{o}$C. Before being used, the seeding sludge is degassed at 35$^{o}$C for few days to ensure no digestible substrate remains in the seeding sludge (Angelidaki and Sanders, 2004) as well as for acclimatizing the inoculum.

The characteristics of the seeding sludge are demonstrated in Tab 3.1. The pH of the raw seeding sludge is quite an alkali (8.5). This confirms a relatively high TAN in the liquid (3238 $mgL^{-1}$) with lower total volatile fatty acid (TVFA) in it (110.56 $mgL^{-1}$). The solid content in the seeding sludge is high (11.985 $gL^{-1}$), but it is in the range of typical values for seeding sludge.

Table 3 1. Characteristics of the raw seeding sludge*

| **Parameters** | **Unit** | **Seeding sludge** |
|---|---|---|
| pH | | 8.5 |
| Total Solid | g/L | 11.985 |
| TDS | g/L | 8.36 |
| TSS | g/L | 3.63 |
| TVS | g/L | 3.59 |
| TCOD | g/L | 7.61 |
| sCOD | g/L | 6.97 |
| DOC | ppm | 103.33 |
| Ammonium | ppm | 3237.99 |
| TVFA | mg/L | 110.58 |

*before degassed

The physical and chemical properties of the feedstock are presented in Table 3.2. The total solid content of the feedstock is 29.5% on a wet basis, which is slightly above the average of the typical solid content of food waste (Zhang et al., 2014b, 2007). More than 90% of the solid fraction is volatile, which means it contains a high convertible portion of solid.

For each reactor, 20 L of seeding sludge is used. Each the reactor is fed with 2 kg of food waste in a wet basis. Neither pH buffer nor additional micronutrients are added into the reactor.

Table 3 2. Proximate analysis of the feedstock

| Parameters | Unit | Value |
|---|---|---|
| Carbohydrate [a] | gram | 18.49 |
| Fibre [a] | gram | 2.47 |
| Protein [a] | gram | 4.75 |
| Fat [a] | gram | 1.1 |
| Total Solid (TS) | % | 29.5 |
| Volatile Solid (VS) | % | 28.19 |
| VS/TS ($f_{VS}$) | % | 0.95 |
| C[b] | % | 82.07 |
| H[b] | % | 8.98 |
| N[b] | % | 5.92 |
| S[b] | % | 3.03 |
| C/N | | 13.88 |

[a] determined per 100 grams of non-dried sample
[b] value from dry weight

### 3.2.2 Chemical analytical method

The gas composition and volume are analysed daily. The composition of several gases ($H_2$, $CO_2$, $N_2$, $O_2$, $CH_4$) are analysed using gas chromatography equipped with TCD detector (Thermo Scientific TRACE 1310) with two columns employed: Restek Packed MS-5a 60/80 and Restek Packed HS-Q60/80. The gas volume is measured in room temperature (20$^o$C). It is carried out by releasing the gas from the collection bag using an electric air pump (DC 12V, 3A) and the flow rate is set using rotameter (Fig. 3.1). The time to empty the gasbag is measured using digital time counter with the accuracy of 0.01 second. The total volume is calculated simply by multiplying the time and the flowrate. The total volume of the biogas production of each treatment is its cumulative volume collected in the gasbag plus the volume in the headspace at the end of the treatment.

The pH of the liquid is measured using a pH probe (Hanna instrument). Total ammonium nitrogen (TAN) is registered using Cole-Parmer Ammonia selective ion probe. The volatile fatty acid (VFA) analysis is performed in a Gas Chromatography equipped with FID detector (Thermofischer Scientific TRACE 1310) using column Thermo TR-FFAP 50m x 0.32

mm, 5 µm. For the preparation of the VFA measurement, the liquid is filtered using a syringe filter (Sartorius Minisart 0.22 um). Dissolved organic carbon is measured for the filtered samples using GE Instruments Sievers 5310 C Portable TOC Analyser.

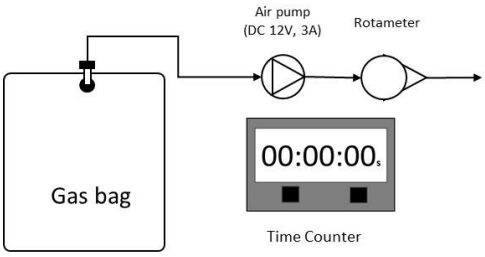



Figure 3 1. Set of equipment for measuring the biogas volume. The biogas volume is measured in the room temperature, which is around 20°C.

The total chemical oxygen demand is measured using COD cuvette test kit (HACH LCK014) by following the instruction given by the company. The reading is done on HACH DR3900 Laboratory Spectrophotometer. Total solid (TS) and total volatile solid (TVS) are measured using the standard APHA method with some necessary adjustment. The nutrition content of the food waste is estimated from the food information label, while the elemental composition is measured using Flash 2000 CHNS Elemental Analyzer.

### 3.2.3. Experimental set-up

The study is conducted in three cycles. In each cycle, the treatment is performed until there is no biogas being produced by the reactors, or the biogas that is produced is insignificant. Two digesters are used in this study: one for the $CO_2$ microbubble treatment and another one for the control. The digester volume is 30 litres with only 20 litres of working volume (Fig.3.2). Each reactor has three ports on the top, which are for liquid sampling, gas outlet and gas inlet. Three ceramic diffusers (Point Four® MBD 75) are placed on the bottom of test reactor, and only one diffuser for the control with the purpose is only for flushing with nitrogen before the experiment started. The average bubble size generated by the diffusers in a steady flow ranges between 350 µm to 450 µm (Desai et al., 2018). The study is carried out in an atmospheric pressure and mesophilic regime with temperature at 35$\pm$2°C.

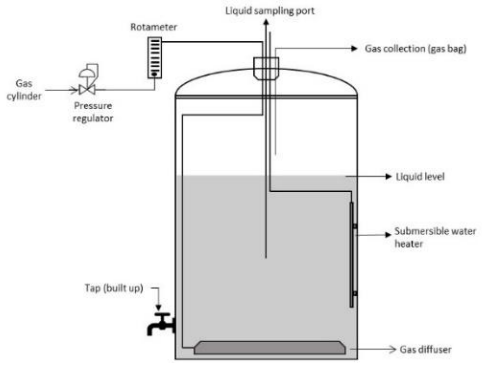



Figure 3 2. Set-up for each AD reactor

## 3.3. Result and Discussion

### 3.3.1. Methane production

All treatments are carried out in three cycles, in which the duration of each cycle varies. In the first cycle, both test and control finish in less than 35 days. A longer duration is shown for all treatments in the second and third cycle, which are more than 40 days. The cumulative methane profile is presented in Fig.3. 3. Since degassing is carried out before each cycle, all the gases produced in this study can be considered as products of only the fed substrate. The trace substrate leftover in the liquid may also give some effects but should be insignificant.

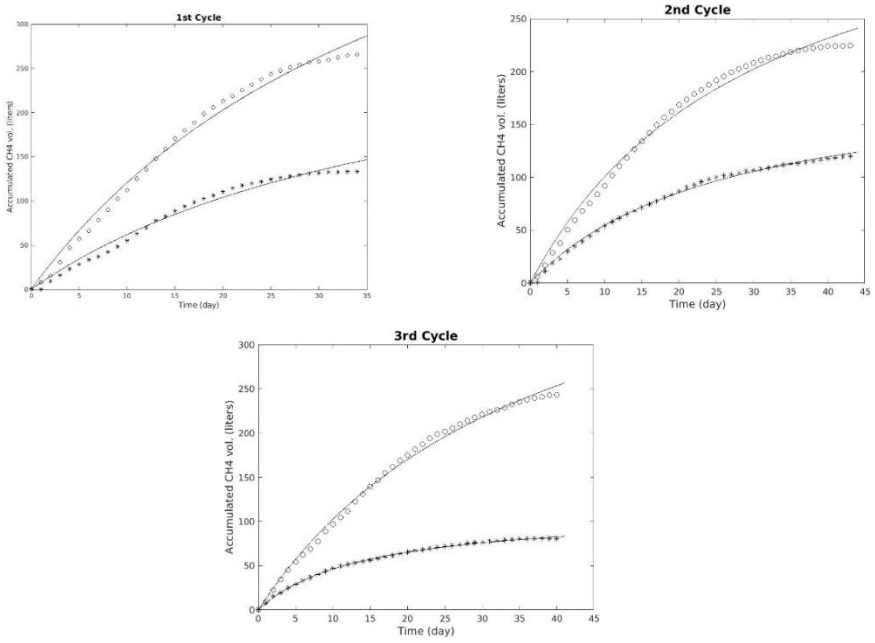



Figure 3. 3. Cumulative methane volume of each treatment, where 'o' is the test, '*' is the control and '-' is the nonlinear regression plot using Monod model (Eq. 3.1).

The test reactor also demonstrates significantly higher volumetric methane production, which is around 2.1 times (on average). A similar increase is also reported by Al-Mashhadani et al. (2016), in which food waste is used as the feedstock. When using food waste, a lower increase is reported by Fernández et al., (2014), ranging between 11–16%. Within the same study, a higher increase is shown when sewage sludge is fed (96–138%). When hydrogen gas is supplied into the reactor simultaneously with $CO_2$, around three-fold higher methane production rates could be achieved with the maximum yield around 550 $L(kgCOD)^{-1}$ (Tao et al., 2019). The latest study is carried out in a continuous process.

$$y = \frac{b(1)\, t}{b(2) + t} \quad (3.1)$$

The methane accumulation data from the experiment is fitted using a Monod functional form (Eq. 3.1). The fitting is performed using a nonlinear regression algorithm in MATLAB (`nlinfit`) with additional calculation of the residuals and estimated variance-covariance coefficients `([beta,R,J,CovB,MSE,ErrorModelInfo])`. While the fitting curve is presented in Fig. 3.3, the residual of the fitting is presented in Fig.3.4. The kinetics parameters and coefficient of correlations ($R^2$) of the fitting line is presented in Table 3.3. The Monod model shows a good fitting with all correlation coefficients greater than 0.99 ($R^2 \geq 0.99$). The $R^2$ of the control's fit is slightly higher than that of tests showing that this is a slightly better fit than that of the test. This is also shown by the residuals plot of both treatments where the control's shows smaller residuals than that of the test. The intersection between the data and the fitting curves for both treatments occurs at relatively the same time, which are at day-15 and day-25. It can be seen in both the fitting curve and residuals plot.

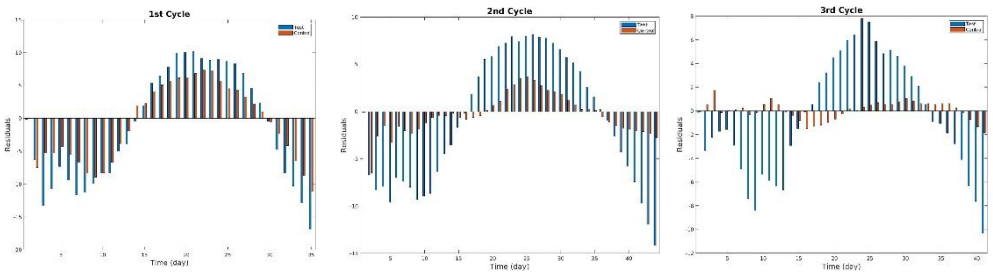



Figure 3 .4. Residual plot of the Monod model for each treatment

Table 3 3. Model parameter and correlation coefficient

| Para-meter | 1st | | 2nd | | 3rd | | Average | |
|---|---|---|---|---|---|---|---|---|
| | Test | Cont'l | Test | Cont'l | Test | Cont'l | Test | Cont'l |
| b(1) | 639.646 | 323.773 | 408.754 | 197.568 | 497.04 | 111.642 | 515.147 | 210.994 |
| b(2) | 43.042 | 42.196 | 30.661 | 26.204 | 38.419 | 14.121 | 37.374 | 27.507 |
| $R^2$ | 0.99 | 0.983 | 0.991 | 0.997 | 0.996 | 0.999 | 0.992 | 0.993 |

Monod kinetics is typically used to model microbial growth as well as substrate utilization by the microorganism in the batch process. The main objective is to estimate the maximum growth rate as well as the half-saturation constant. Originally, the model is plotted using growth rate and substrate concentration for the y-axis and t-axis, respectively. However, in this study, the two parameters are replaced by methane accumulation volume (b(1)) and time (b(2)) with the aim is to acquire the best fitting. In general, this model can be used for predicting the cumulative methane production in respect of time and the rate of methane production. The specific rate of methane production can be estimated by subtracting the methane volume at a certain time by the methane volume previously. It can be expressed as $dy/dt=(y_n-y_{n-1})/t$. For estimating the ultimate methane production as well as considered time to finish the treatment, an extrapolation is needed. However, with any value of b(1) that is much higher than the actual value, any extrapolation should not be done using this model. Furthermore, no more or an insignificant amount of methane is produced at the end of each cycle.

By considering the b(1) parameter, it shows that the test reactor has an average methane production rate around 2.4 times as much as the control's. This is close to the actual value that shows the production rate around 2.1 times as much as the control. An advanced model, such as ADM1, is needed to accurately estimate production rate and the ultimate methane production for upscaling, which is not covered by this study.

To estimate the maximum methane yield that can be achieved by a particular substrate, theoretical methane potential (TMP) is calculated using Eq. 3.2 to 3.4. The TMP can be predicted if the elemental composition is known (Lier et al., 2008). Eq. 3.2 shows the formula for calculating the TMP of a substrate with known elemental composition.

$$C_nH_aO_b + \left(n - \frac{a}{4} - \frac{b}{2}\right)H_2O \rightarrow \left(\frac{n}{2} + \frac{a}{8} - \frac{b}{4}\right)CH_4 + \left(\frac{n}{2} + \frac{a}{8} - \frac{b}{4}\right)CO_2 \quad (3.2)$$

Using STP (0°C, 1 Atm) as a standard condition, the theoretical methane volume per gram of VS is estimated using Eq. 3.3.

$$Y_{CH4} = \frac{\left(\frac{n}{2} + \frac{a}{8} - \frac{b}{2}\right) 22.4}{12n + a + 16b} \left(\frac{LCH_4}{g\ VS}\right) \quad (3.3)$$

The composition of the substrate used in this study is presented in Table 3.2. Unfortunately, since the composition of oxygen from the elemental analysis could not be measured, the TMP cannot be directly calculated using the listed elemental composition. Instead, the calculation is carried out using the composition of carbohydrate, protein, and lipid in the substrate using the general formula of each compound. Table 3.4 presents the theoretical methane that can be produced by a typical substrate that is calculated using Eq. 3.4.

Table 3. 4. Theoretical characteristics of typical substrate components and their methane yield (Angelidaki and Sanders, 2004)

| Substrate type | Composition | $CH_4$ yield $L(gVS)^{-1}$ |
|---|---|---|
| Carbohydrate | $(C_6H_{10}O_5)_n$ | 0.415 |
| Protein* | $C_5H_7NO_2$ | 0.496 |
| Lipid | $C_{57}H_{104}O_6$ | 1.014 |

* The nitrogen is converted into $NH_3$

With the known composition and value of TVS, the TMP can be estimated using Eq. 3.4, where $M_s$ is the mass of the sample, $f_{vs}$ is the VS fraction in the sample. The composition of carbohydrate, protein and lipid is represented by $f_{carb}$, $f_{prot}$ and $f_{lip}$, respectively. $Met_{carb}$, $Met_{prot}$ and $Met_{lip}$ is presenting the TMP of the carbohydrate, protein and lipid, respectively.

$$V_{CH4} = M_s . f_{vs} \left(f_{carb} . Met_{carb} + f_{prot} . Met_{prot} + f_{lip} . Met_{lip}\right) (LCH_4) \quad (3.4)$$

The result is presented in Table 3.5, in which the value is compared with the actual methane yield and volume achieved in this study. The theoretical methane yield and volume of the feedstock is 472.95 (L $CH_4$ (kg VS)$^{-1}$) and 241.72 litres, respectively. Theoretically, the methane yield of food waste should be slightly higher than the yield of carbohydrate (415 LCH4(kgVS)$^{-1}$). This is due to the typical composition of the food waste where carbohydrate is often the dominant fraction. The actual yield and total volume of methane that are produced by the control are approximately around 60% and 50% of the

TMP value, respectively. Substrate characteristics and environmental factors are suggested to affect the actual methane production. Environmental factors may include temperature, pH, and inhibition by other compounds, while the substrate characteristic is mainly about its structural characters, such as the crystallinity index and degree of polymerization (Chang and Holtzapple, 2000; Flory and Vrij, 1963; Hall et al., 2010).

Interestingly, the yield and volume of the methane produced by the test reactor are significantly higher than those are produced by the control and even slightly higher than those of the theoretical value. Usually, the actual methane yield that can be achieved by any substrate rarely exceeds 90% of the TMP. This begs the question of how $CO_2$ microbubbles increase methane production in AD while the substrate degradation is only around 75% of the volatile solid (Table 3.5).

Table 3. 5. Theoretical methane potential (TMP) compared to the actual methane production (AMP)

| Treatment | $CH_4$ yield (L $CH_4$ (kg VS)$^{-1}$) | | | $CH_4$ volume (L) | | |
|---|---|---|---|---|---|---|
| | STP | 20°C# | AMP:TMP | STP | 20°C# | AMP:TMP |
| TMP | 440.66 | 472.95 | 100% | 225.22 | 241.72 | 100% |
| Test* | - | 501.76+37.5 | 106.09% | - | 246.81±22.2 | 102.10% |
| Control* | - | 245.13+69.3 | 51.8% | - | 116.76±27.2 | 48.30% |

*mixed of food waste and seeding sludge
#calculated using ideal gas law

Based on the three main hypotheses, the following section discusses how $CO_2$ microbubbles actuate the additional methane production rate.

### 3.3.2. $CO_2$ microbubble increase substrate degradation rate.

Hydrolysis is the ultimate constraint on the rate of biological treatment – the final product cannot be produced faster than the release of the sugary materials. The overall hydrolysis performance is measured as a solid reduction in the whole process. Table 3.6 shows the reduction of TS, TVS and COD. This shows only slight difference in the reduction of all solid parameters between the test and control reactors and is not significant. The TVS reduction of both treatments is ranging from 73% to 75%, while the TS and COD reduction of both treatments ranging from 57% to 59% and 67% to 69%, respectively. An insignificant difference in the solid removal between the test and control after $CO_2$ fine bubble injection is also reported by Fernández et al. (2015). It shows that injection of $CO_2$

microbubble does not affect significantly in increasing the degradable fraction of substrate.

Table 3. 6. Reduction of solid parameters

| Parameters | Unit | $CO_2$ Treatment | Control |
|---|---|---|---|
| TS | % | 57.08±2.8 | 58.40±3.7 |
| TVS | % | 74.68±4.1 | 73.52±3.8 |
| COD | % | 68.89±2.3 | 67.41±2.5 |

Although it does not affect the number of degradable fractions, injection of $CO_2$ microbubble is thought to enhance substrate degradation rate that result in higher methane production rate. This higher degradation rate can be assessed using several parameters.

The first step is measuring solid content in a timely basis, typically daily. The data is then calculated using first order reaction kinetics as shown in Eq. 3.5.

$$\frac{dS}{dt} = -k_{dis}S \quad (3.5)$$

where $K_{dis}$ is the disintegration rate, S is substrate concentration, and t is the duration of the measured hydrolysis. The data used for the calculation must be obtained from the parameter measurement (e.g. COD) in a daily basis.

Unfortunately, the COD measurement is only performed in the first cycle and discontinued in the next two cycles. The reason for discontinuing is because it is less useful due to inconsistency. Typical data of solid degradation should show a continuous reduction until it reaches a certain point, and should not fluctuate non-monotonically, i.e. the solid content of a certain day is higher than that of the previous day. This inconsistency is due to irregular mixing applied in the reactor, while at the same time 20 L working volume is used in the study. An effort to mix the liquid via microbubble gas sparging is done when taking the samples.

Alternatively, the disintegration rate can be estimated using data of the final product, i.e. methane (Angelidaki and Sanders, 2004; Astals et al., 2013). To calculate the rate, the Monod equation is used as first-order kinetics (Eq. 3.6). This equation is usually used to measure microbial growth against the substrate availability, where S is the substrate

concentration, $K_s$ is the half-saturation constant, X is the microbial concentration in the medium. In assessing the hydrolysis rate, the disintegration rate constant ($K_{dis}$) is used instead of the $\mu_{max}$, and the X is replaced by *B* (cumulative methane volume). In a condition where the amount of substrate is abundant, Eq. 3.6 can be derived into Eq. 3.7.

$$\frac{dX}{dt} = \frac{\mu_{max}.S}{K_s+S}.X \quad (3.6)$$

$$k_{dis} = \frac{lnB_t - lnB_0}{t} \quad (3.7)$$

Since Eq. 3.7 is derived for a condition where the substrate is abundant, the $K_{dis}$ value is calculated using the early stages of the cumulative methane curve when the level of substrate is considered abundant. It is done by plotting a straight line into the methane cumulative curve with the correlation coefficient of no less than 0.98 ($R^2 \geq 0.98$). In this study, the data that is used to calculate the $K_{dis}$ is the first seven days of the methane data without including the lag phase. The result shows that the $K_{dis}$ of the test reactor is 0.37 $day^{-1}$, which is around 42% higher than that of the control (0.261 $day^{-1}$). This shows clearly that the injection of $CO_2$ microbubbles increases the substrate degradation rate.

Secondly, a higher degradation rate can be confirmed by comparing the VFA concentration between the test and control reactors. Increasing VFA concentration after $CO_2$ addition has been reported by several articles (Francioso et al., 2010; Salomoni et al., 2011; Tao et al., 2019). Fig.3.5 shows the daily VFA concentration in the medium of the test and control reactors. In AD, it is typical to observe a drastic increase of VFA within the first 24 hours of treatment, which is also shown by Fig. 3.5 (Cysneiros et al., 2012). Increasing VFA concentration does not only show a rapid acidogenesis rate, but also high hydrolysis rate. However, this study does not show a significant difference in the VFA concentration between the test and the control in general. In the first cycle, the daily VFA concentration of the control is mostly slightly higher than the test, while in the third cycle, the condition is reversed. In fact, VFA presence in the medium is dynamic. VFA is an intermediate product, which may be produced and utilized at the same time. It means that lower VFA concentration does not always show lower degradation performance but may also mean more rapid VFA utilization. This phenomenon sometimes makes the concentration of VFA in the medium cannot be used as the main parameter for comparing the degradation rate between two different treatments.

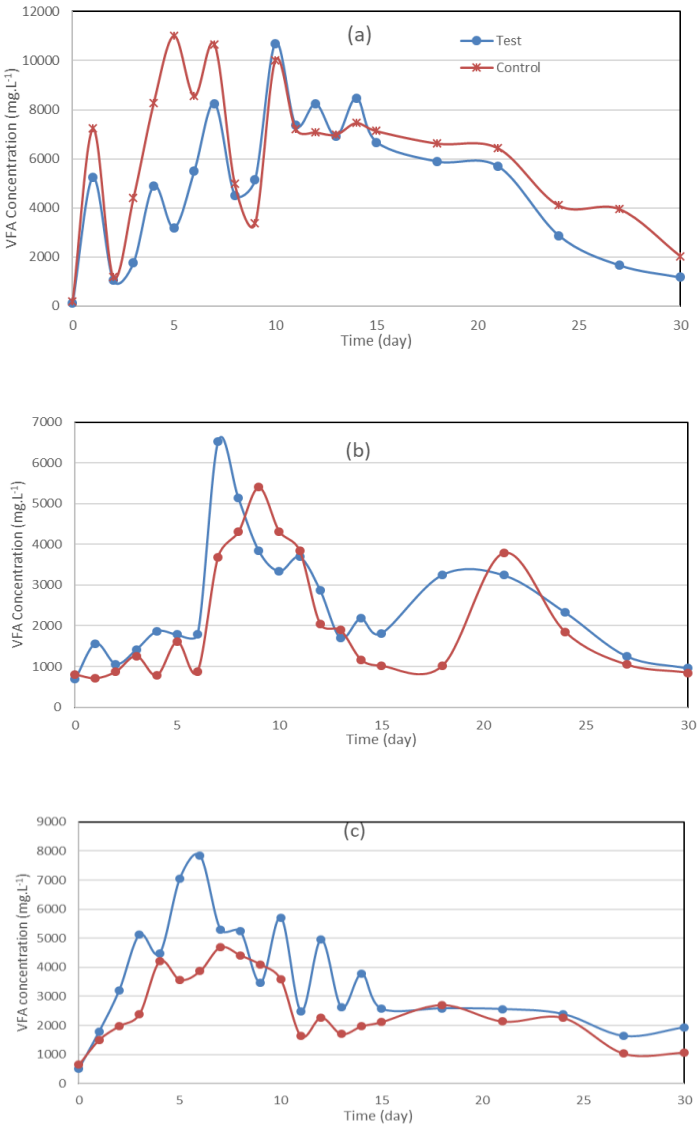



Figure 3. 5. VFA concentration of the medium during the treatment, with (a) is the 1st cycle, (b) is the 2nd cycle and (c) is the 3rd cycle

Thirdly, a higher degradation rate after $CO_2$ microbubble injection can be explained by comparing the $CO_2$ and $H_2$ volume those are produced by each treatment. While $H_2$ presence in biogas is discussed in several reports, $CO_2$ presence is rarely discussed intensively, in terms of assessing substrate degradation. Looking at the $CO_2$ and $H_2$ volume in the gas collection bag, especially in the early-stage, may give different insights into what phenomenon is occurring. In AD, $CO_2$ can be considered as an intermediate product, even though it is also produced in methanogenesis. So is $H_2$, if methane is intended to be the main product. Fig. 3.6 shows the steps in which $CO_2$ and $H_2$ are produced during AD. $CO_2$ is produced in various steps including alcoholic fermentation, acidogenesis, acetogenesis,

and methanogenesis, while $H_2$ is produced only in acidogenesis and acetogenesis. Since all of those processes occur intracellularly, substrate degradation should have already occurred.

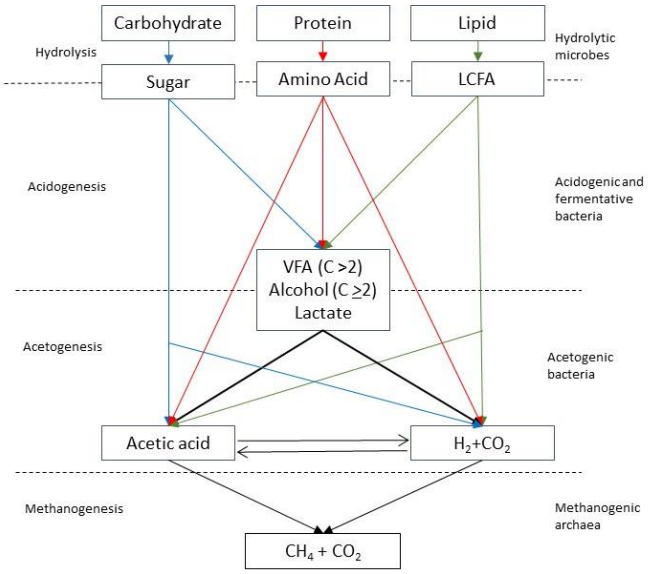



Figure 3. 6. The general process of anaerobic digestion

Fig. 3.7 shows the daily $CO_2$ volume collected in the gas bag, while the daily $H_2$ volume is shown in Fig. 3.8. This data is presented only for thirty days, which is a standard duration for anaerobic treatment. For the test reactor, the $CO_2$ volume presented includes that is produced by the microorganism and an amount of $CO_2$ that is injected but may not be absorbed. The $CO_2$ daily volume of the test reactor is mostly above 10 liters, except in a few days in the second cycle. Unfortunately, an absorption study is not performed here. Significant absorption of exogenous $CO_2$ (49-88%) is reported by Alimahmoodi and Mulligan (2008) when $CO_2$ is added into the reactor's headspace. A lower absorption (3-34%) is reported when $CO_2$ is injected as fine bubbles (Fernández et al., 2014). For the $H_2$, its daily volume collected from the test reactor is also significantly higher than that of the control in all cycles, ranging from 2 to 20 times as much, excluding the first three days, in which it is much higher.

The daily $CO_2$ graph also shows a significantly higher declining rate of the control's $CO_2$ than that of the test. It is expressed with the average rate constant of the control, which

is 0.242 $day^{-1}$, while the rate constant of the test is 0.0433 $day^{-1}$. This higher declining rate constant is due to no exogenous $CO_2$ added into the control reactor.

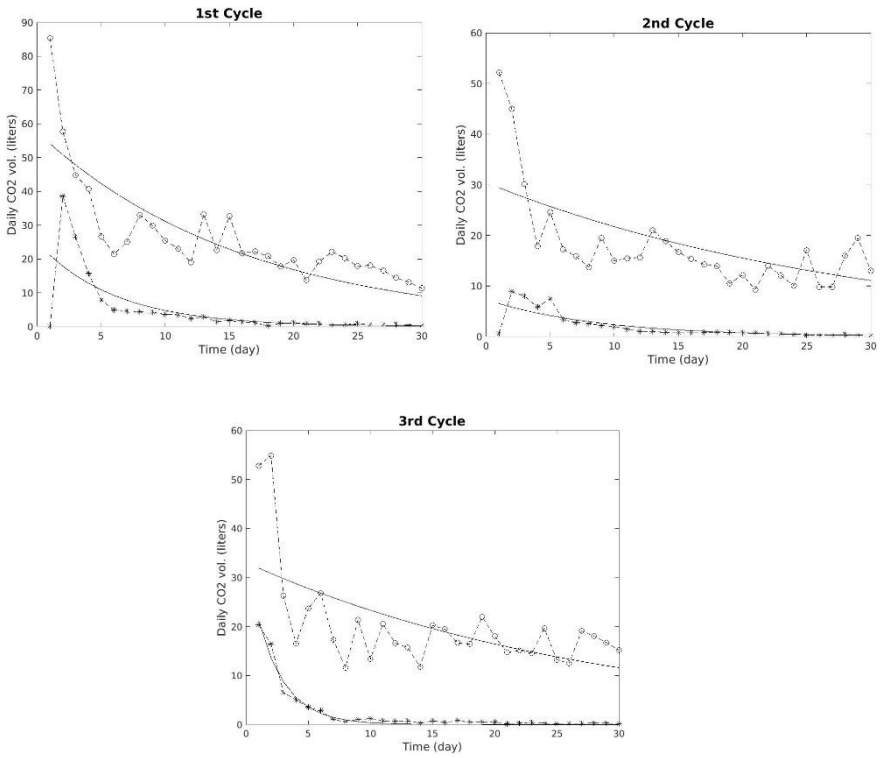



Figure 3. 7. Daily volume of the CO2 in the collection gas bag, where '--o' is test, '--*' is control, and '-' is the regression. The regression is plotted with linear regression using equation $yt=y_0.exp(-k.t)$.

The most interesting part is shown in the first three days by each cycle when an appreciable volume of $CO_2$ and $H_2$ are produced by the test reactor. The $CO_2$ volume could reach 83 liters in only 24 hours after the first $CO_2$ injected into the system. It then drops drastically in the following days with some fluctuation for the rest of the process. In fact, the volume of $CO_2$ injected daily is around $10\pm0.1$L only, which is much lower than the daily $CO_2$ volume shown by the test reactor. A massive $CO_2$ production is also demonstrated by the control, but it began in the second day except for the last cycle. However, the volume is much lower than that of the test reactor. It concludes that massive $CO_2$ production by the test reactor shows a significantly higher substrate degradation rate in the early stages.

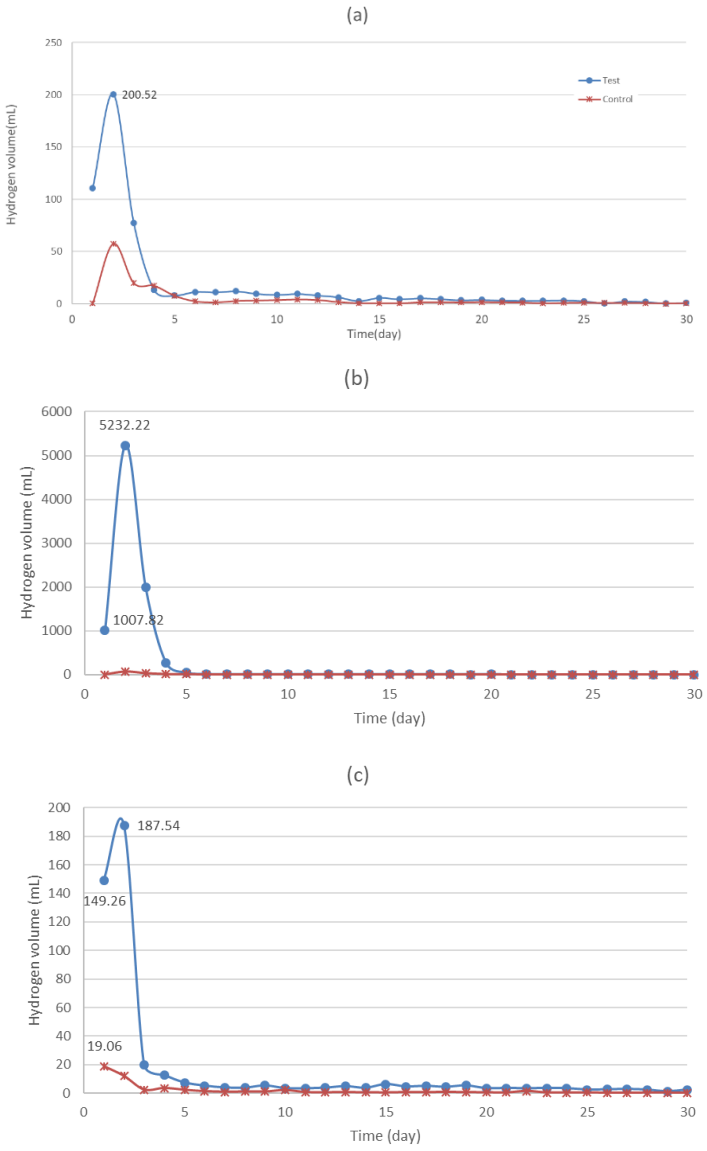



Figure 3. 8. Daily volume of the H2 in the collection gas bag of each treatment in the first cycle (a), second cycle (b) and third cycle (c).

Similar to $CO_2$, a significantly high volume of $H_2$ is shown in the first three days, especially in the second cycle when it reached 5 liters. On the previous day, the volume reaches 1 L. On the same day, the volume of methane produced is only about 8 liters, which amounted to only 10% of the total biogas produced on that day. With dark fermentation, $H_2$ is produced mainly in the acidogenic and acetogenic stages using substrates that are solubilized from the hydrolysis step (Levin et al., 2004). In the presence of syntrophic acetate oxidation bacteria (SAOB), $H_2$ gas can also be formed by oxidation of acetate into

$CO_2$ and $H_2$ gas. Whichever the formation process, high $H_2$ availability can be interpreted as a high degradation process that occurred previously.

It is also interesting to see in the early stages when the methane volume is relatively low, but the volume of $CO_2$ and $H_2$ are quite high. Since methane is intended as the main product, the presence of $H_2$ in the output gas can be considered as residue and energy loss. An imbalance between $CO_2$/ $H_2$ production and utilization may be the reason for this phenomenon. In short, the daily presence of appreciable $CO_2$ and $H_2$ in the gas collection bag may explain that injection of $CO_2$ microbubble injection increases the substrate degradation rate.

There are some possible mechanisms for how $CO_2$ enhances the substrate degradation rate, which occurs especially in the early stage. Al-Mashhadani et al. (2016) suggested there are two possible phenomena when $CO_2$ is injected in the form of microbubbles. Firstly, the microbubble collapse that leads to free radical generation. Free radicals are highly reactive and may disrupt the substrates resulting in substrate degradation. The hypothesis is based on the reports by Takahashi et al. (2007) that shows a free radical generation without dynamic stimuli after the injection of microbubbles in water. Bubbles diameter less than 50 µm is the prerequisite for the microbubbles to generate free radicals when they collapse. In this study, the size of the microbubbles is estimated ranging between 350 µm to 400 µm (Desai et al., 2018). Although there is a possibility that the diffuser may generate some bubbles with a size smaller than 50µm, but it should be very little.

Secondly, Al-Mashhadani et al. (2016) also suggested that when $CO_2$ microbubble dissolved, a microenvironment that has a unique property from the whole system may occur. Microbubbles have low buoyancy force resulting in longer residence time in the liquid medium, either suspended in the liquid or attached onto the surface of the solid particle. When $CO_2$ microbubbles dissolve, they could generate a local pH shock that attacks the substrate which can help the enzymatic process so that the degrading bacteria may work more easily.

Either mechanism -- free radicals or local pH shocks -- attacks unselectively and potentially disrupts the microbial communities, where methanogen is the most sensitive. If this

happens, it contradicts the fact that higher methane is produced after $CO_2$ microbubble injection. Considering the hypothesis about the formation of the microenvironment, the microbubbles may also have adjusted the pH locally to a condition that is more favorable for the hydrolytic microbial community so that biological hydrolysis could occur more rapidly. The hypothesis of the formation of the microenvironment is proposed since the injection of the $CO_2$ only decreases the medium pH at most by 0.3 from the initial value. This pH stability can occur in the medium probably because of a good buffering capacity by the high ammonia content dissolved in the medium. In fact, the total ammonia nitrogen (TAN) of the medium is higher than $3gL^{-1}$ (Table 7). A suitable environment is another possible reason that makes the hydrolytic microorganism work more rapidly.

In short, $CO_2$ microbubble injection shows a positive impact on the substrate degradation rate but does not increase the substrate degradation fraction. As free radical generation and local pH shocks may give only little impact on the degradation rate, there should be another explanation for this phenomenon. This will be discussed in the next section.

### 3.3.3. $CO_2$ microbubble promoting suitable environmental conditions for efficient substrate utilization

Injection of $CO_2$ microbubbles may promote the methane production rate by making a favorable environment for the biochemical process to happen. With favorable conditions, substrate can be utilized more efficiently, resulting in more methane production. To assess the efficiency, three parameters are used: acidogenesis (%A), methanogenesis (%M) and biodegradability (% BD) (Field et al., 1988). Acidogenesis (%A) and methanogenesis (%M) measures the amount of substrate that is converted into VFA and methane, respectively. Biodegradability (%BD) assesses the amount of substrate that is converted biologically in the system.

$$\%A = 100.\frac{(COD_{CH_4}+COD_{VFA_f})}{COD_0} \quad (3.8)$$

$$\%M = 100.\frac{COD_{CH_4}}{COD_0} \quad (3.9)$$

$$\%BD = \%A + \frac{Y_A}{1-Y_A}.\left(\%A - 100.\frac{COD_{VFA_0}}{COD_0}\right) + \frac{Y_M}{1-Y_M}.\%M \quad (3.10)$$

Where $COD_{CH4}$ and $COD_{VFA}$ is the amount of CH4 and VFA those are converted as COD, respectively. Eq. 3.11 is a general formula to convert a certain compound into COD (Lier et al., 2008). The VFA conversion is carried out by calculating all types of VFA compound presences in the samples. The yield of acetogen ($Y_A$) and methanogen ($Y_M$) is 0.05 g COD $g^{-1}$ COD and 0.029 g COD $g^{-1}$ COD, respectively.

$$CH_aO_b + \frac{1}{4}(4n + 1 - 2b)O_2 \rightarrow nCO_2 + \frac{a}{2}H_2O \quad (3.11)$$

The results of these calculations are presented in Table 3.7. The control shows acidogenesis and methanogenesis efficiencies around 63% and 53%, respectively. The methanogenesis efficiency of the control is close to the percentage of its actual methane yield compared to the theoretical yield (AMP:TMP), which is 51.8% (Table 5). The biodegradability parameter (%BD) is also close to its COD removal. This means that nearly a hundred percent of the degraded substrate in the control is converted biologically in the system while only an insignificant fraction is converted by non-biological means.

Table 3. 7. Efficiency parameters

| Parameter | Test (%) | Control (%) |
|---|---|---|
| Acidogenesis | 115.98+7.9 | 62.71+7.2 |
| Methanogenesis | 108.67+5.8 | 53.02+9.5 |
| Biodegradability | 124.12+10.2 | 66.89+8.5 |
| Parameter | Test (%) | Control (%) |

On the other hand, all test parameters show efficiencies higher than 100%, which in conventional treatment is not possible. If only the efficiency is under 100%, increasing utilization of substrate could be the sole mechanism. However, since the values are higher than 100%, an additional carbon source may explain this phenomenon.

There are at least two possible ways how $CO_2$ microbubbles can promote suitable conditions that increase the substrate utilization efficiency. Firstly, they remove some inhibitors from the medium. Secondly, dissolved $CO_2$ creates a suitable pH regime for the microbial communities.

Acetate is the main methane precursor that is generated during acetogenesis (Eq. 3.12 & 3.13). This endogenic process does not occur spontaneously. Only under low partial pressure of $H_2$ ($<4.6 \cdot 10^{-4}$ atm), can the process occur. In a conventional process, $H_2$ is removed by syntrophic interaction with hydrogenoclastic methanogen (Morris et al.,

2013). Stripping the gas off can accelerate hydrogen removal, so that the environment become more favorable for the acetogenic process (Al-Mashhadani et al., 2016).

$$CH_3CH_2COH + H_2O \rightarrow CH_3COOH + CO_2 + H_2 \quad \Delta G^{0\prime} = +76.1\ kJ/mol \ (3.12)$$

$$CH_3CH_2CH_2COOH + H_2O \rightarrow CH_3COOH + H_2 + CO_2 \quad \Delta G^{0\prime} = +48.1\ kJ/mol (3.13)$$

In fact, $H_2$ has very low solubility in the liquid while at the same time it is produced biologically in the system. It is possible that once it is produced by the microorganism, it is attached to the surface of its producers until it reaches sufficient buoyancy force to detach and rise to the liquid surface. A higher hydrophobicity characteristic of $H_2$ makes it possible to attach to the solid surface even longer before detachment. An injection of microbubbles may accelerate this detachment process. Once the $H_2$ concentration is kept to the minimum, acetogenesis may occur more rapidly by converting the longer chain of fatty acids and alcohol into acetic acid. As a result, more acetate, $CO_2$, and $H_2$ will be produced, while on the other hand, the concentration of longer chain-VFA is lowered. As acetic acid becomes more abundant, it triggers a higher rate of acetate utilization, either by syntrophic acetate oxidation bacteria (SAOB) or hydrogenoclastic methanogen.

Since VFA concentration between test and control does not show a significant difference (Fig. 3.4), this hypothesis can be confirmed by the daily $H_2$ volume collected from the test reactor. Because $H_2$ is produced only after the process initiated (day-0), the $H_2$ removal process occurs from day-1. While a notable $H_2$ volume in the early stage shows a high substrate degradation, its daily volume until the end of the process may show the $H_2$ detachment that is accelerated by the $CO_2$ microbubbles. $H_2$ volume that is measured from the gas bag of the test reactor is always higher than that of the control. After day-3, the $H_2$ volume in the test's gasbag drops drastically but remains significantly higher than that of the control. This volume ranges from two times to 27 times as much as the control. For instance, when the $H_2$ volume of the control is 264.14 mL (cycle-2, day-4), the $H_2$ volume of the control is only 10.44 ml. The $H_2$ volume gap between the test and control tends to narrow towards the end of process. Using $CO_2$ fine bubbles, the average $H_2$ concentration of the test reactor is around four times as much as that of the control, as reported by Fernández et al. (2015). In fact, $H_2$ fraction in the biogas is typically much less. This is because $H_2$ is an intermediate. Its availability is dynamic, depending on its production and utilization rates by microbial communities.

As the stripping gas scrubs the gas unselectively, it does not only remove $H_2$ but also other gases including methane. Regarding the increase of methane production rate, Le Chatelier's principle may explain this phenomenon (Al-Mashhadani et al., 2016). The principle simply states that when any equilibrium is disturbed, a system will adjust to a new equilibrium, by opposing the change imposed. Metabolic pathways are not equilibrium systems, but most secreted metabolites are inhibitors, so the removal acts like a Le Chatelier "pull". Based on this principle, if the concentration of methane in the media is reduced, it will result in an increase in the methane production rate, regardless of the pathway (Eq. 3.14 & 3.15). For this purpose, gas stripping techniques using oxygen-free microbubbles can be performed (Al-Mashhadani et al., 2016). It is important to note that a gas stripping technique will not specifically remove the methane alone, but also other gases, such as carbon dioxide, $H_2$, and even toxic gases, such as ammonia. While removing ammonia gas can have a positive effect, removal of $CO_2$ and $H_2$ will impact negatively because both gases are methane precursors via hydrogenoclastic pathway and acetate precursor via homoacetogenic pathway.

$$CH_3COOH + H_2O \rightarrow CH_4 + H_2 + CO_2 \quad \Delta G^{0\prime} = -31.0\ kJ/mol \quad (3.14)$$

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \qquad \Delta G^{0\prime} = -135.6\ kJ/mol \quad (3.15)$$

Similar to $H_2$, methane solubility is low in the liquid. At STP conditions, its solubility varies from 2.47 to 0.36 mmol $kg^{-1}$, depend on the impurity content of the water (Duan and Mao, 2006). Once it is produced, it will attach to the surface of the microorganism and partially dissolved into the liquid media. Methane will only dissolve into the liquid media until it reaches saturation. After that, the methane will accumulate in the form of bubbles until it reaches a certain buoyancy force to detach and rise to the liquid surface. The injection of $CO_2$ microbubbles is expected to accelerate the detachment process, as well as to remove the dissolved methane in the bulk liquid. However, because methane is only available after the AD process begins, this principle can only be applied from the day-1 onwards. Hence, the acceleration of methane production after the first day is likely the result of regular methane removal by $CO_2$ microbubbles sparging.

Regarding $CO_2$ removal by microbubble sparging, it does negatively affect the system. A study by Al-Mashhadani et al., (2016) reported that a significant increase in methane production is only demonstrated when pure $CO_2$ microbubbles are injected. A negative

impact (lower methane production) is demonstrated when pure nitrogen microbubbles are injected, instead. However, when nitrogen sparging is followed by $CO_2$ injection, higher methane is produced as has been shown relative to only $CO_2$ injection. It seems that the injected $CO_2$ is also acted to replenish the scrubbed $CO_2$ -- making the negative impact of gas scrubbing minimal.

Ammonia is another inhibitor in AD. Ammonia concentration of more than 3 $gl^{-1}$ is considered to be toxic, especially for the methanogenic archaea (Rajagopal et al., 2013). In the gas phase, ammonia is more harmful to the methanogenic community (Angelidaki and Ahring, 1993). Some factors affect the composition of the gas phase of the dissolved ammonium, which is expressed by Eq. 3.16, where $NH_3$ is the concentration of free ammonia nitrogen (mg/L), TAN is the total ammonia nitrogen concentration (mg/L), $K_a$ is the temperature dissociation constant ($0.564 \times 10^{-9}$ at 25 °C, $1.097 \times 10^{-9}$ at 35°C and $3.77 \times 10^{-9}$ at 55 °C) and [H] is the concentration of hydrogen ion ($10^{-pH}$). The equation shows that the amount of free ammonia dissolved in the liquid is pH and temperature-dependent (Kayhanian, 1999).

$$NH_3 = \frac{TAN \frac{K_a}{[H]}}{\frac{K_a}{[H]} + 1} \quad (3.16)$$

Table 3. 8. Initial and final concentration of VFA, TAN and COD in the medium in each cycle

| Parameters | Sequence | Unit | $CO_2$ | | Control | |
|---|---|---|---|---|---|---|
| | | | Initial | Final | Initial | Final |
| VFA | 1st Cycle | $mgL^{-1}$ | 117 (39) | 887 (nd) | 184 (55) | 486 (68) |
| | 2nd Cycle | $mgL^{-1}$ | 687 (30) | 846 (102) | 801 (172) | 769 (91) |
| | 3rd Cycle | $mgL^{-1}$ | 519 (68) | 409 (112) | 652 (88) | 466 (71) |
| TAN | 1st Cycle | $gL^{-1}$ | 3.28 | 4.50 | 3.09 | 4.06 |
| | 2nd Cycle | $gL^{-1}$ | 4.05 | 5.00 | 4.27 | 5.00 |
| | 3rd Cycle | $gL^{-1}$ | 4.91 | 4.09 | 4.64 | 4.12 |
| COD | 1st Cycle | $gL^{-1}$ | 29.26 | 9.57 | 28.97 | 10.95 |
| | 2nd Cycle | $gL^{-1}$ | 29.36 | 10.63 | 28.57 | 10.49 |
| | 3rd Cycle | $gL^{-1}$ | 29.60 | 11.73 | 28.75 | 11.90 |

nd= not detected

number in bracket is acetic acid

While the temperature is controlled to be the same (Table 8), a higher pH in the control reactor that starts in the second week may increase the presence of the free ammonia in the reactor (Fig. 3.3). Moreover, with the periodic gas stripping imposed on the test

reactor, it may induce the periodic removal of free ammonia from the liquid. This further supports the rationale of how the injection of $CO_2$ microbubbles improves the efficiency of substrate utilization by reducing its inhibitor.

Fig. 3.9 shows the amount of free ammonia (FA) presents in the medium per 1000 mg of TAN, which is calculated using Eq. 3.9. The two colors show the pH of the test and control starts in the second week where a significant pH gap between them is shown. After the first week, the test has pH ranging between 7.6 to 7.9, while the control has pH ranging from 8.0 to 8.5 (Fig. 3.10). Both treatments experience a pH drop within 24 hours after the process is initiated. It gradually increases. However, the control's pH shows higher increases until it reaches pH 8.0 in the third week and keep increasing until it reaches pH 8.5. The same trend happens as well in the test reactor but the pH the maximum pH it reaches is 7.9, which occurs in the fourth week. This higher pH makes the FA concentration of the control can be three times as much as the test and may have decreased the methane production. This already lower free ammonia in the test reactor may be further improved by a daily free ammonia removal by microbubble sparging.

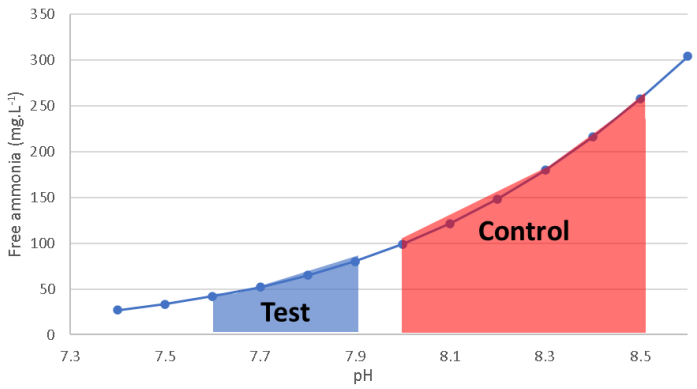



Figure 3. 9. Free ammonia concentration per 1000 mg of TAN in different pH. The colour shows free ammonia of the treatment which starts in the second week.

It was hypothesized that the lower pH of the test is due to higher VFA concentration. However, since there is no difference in the VFA concentration (in average) between the treatment, a lower pH due to higher dissolved $CO_2$ in the test's may be the reason. Based on the preliminary study, 10 liters of $CO_2$ microbubble injection into the digested can decrease the pH at most by 0.3 from the initial value. A good buffering capacity by the high TAN may explain a remain relatively higher pH in the digester.

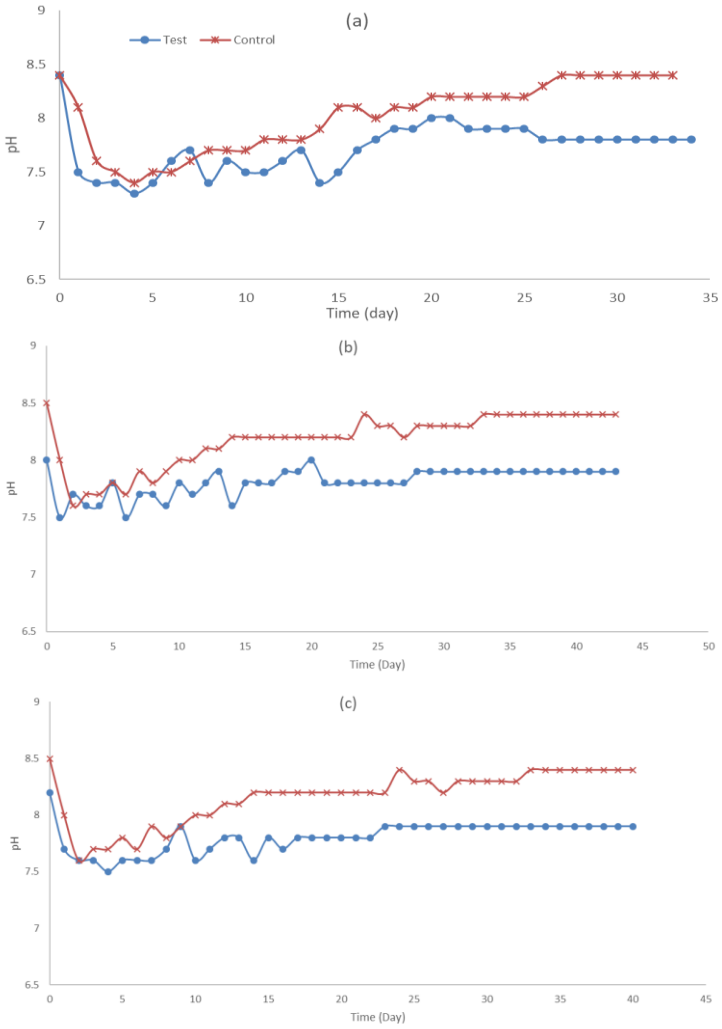



Figure 3. 10. Medium pH during the treatment, with (a) is the 1st cycle, (b) is the 2nd cycle and (c) is the 3rd cycle

In short, $CO_2$ microbubble injection can improve the environmental conditions in several ways: accelerating $H_2$ removal that increases acetogenesis process, removing methane that increases methane production based on Le Chatelier's principle, removing toxic gas (free ammonia) and lowering pH.

### 3.3.4. $CO_2$ microbubble injection provides extra carbon source to the process

A significant methane enhancement is demonstrated by the test reactor. Its methane yield and volume are even higher than those of the theoretical value (TMP). As has been discussed, $CO_2$ microbubble injection does not increase degradation fraction of the

substrate. Moreover, the theoretical value is calculated using the whole volatile solid content in the substrate, which is higher than the actual degraded volatile solid (75%). Since an efficient substrate utilization will only give a maximum 75% of the TMP, an additional carbon source from the $CO_2$ microbubble injection should be the reason of how the test reactor achieve additional methane.

Unfortunately, a $CO_2$ absorption study is not conducted here. The daily $CO_2$ injected into the reactor is around $10\pm0.5$ liters, while daily $CO_2$ volume collected in the gas bag is always above 10 liters, except for in few points in the second cycle. In fact, $CO_2$ is produced biologically inside the digester. It means that if only all the injected $CO_2$ is not absorbed at all, the net volume of $CO_2$ produced by the microorganism can be estimated by subtracting the presented $CO_2$ volume by 10 L. However, an absence of absorption may not be the right conclusion. $CO_2$ is biologically produced by the microorganisms inside the reactor. Once the gas is produced and excreted from the cell, it should attach on the microbial surface while some of it dissolves in the liquid. In fact, the solubility of the $CO_2$ in pure water under 35$^{o}$C is around 1g/L. A higher solubility (2.2 to 6.1 $gL^{-1}$) has been reported to be achieved in the liquid sludge by transforming it into an ionic form or bonded to other mineral or compounds (Alimahmoodi and Mulligan, 2008). Using the ideal gas law, this value is equal to 1.12 L $CO_2$/L to 3.11 L $CO_2$/L under atmospheric pressure. For 20 liters of sludge, as is used in this study, such saturation will only be achieved by injecting at least 22.4 liters of $CO_2$ in a condition where no initial $CO_2$ is dissolved in the liquid. Otherwise, the injected $CO_2$ will only fill the concentration gap between the initial and the saturated condition.

The only time that $CO_2$ is expected to be absorbed more efficiently is at the starting time (Day-0) after the seeding sludge is flushed using nitrogen. A significant absorption of exogenous $CO_2$ (49-88%) is reported by Alimahmoodi and Mulligan (2008). In their study, the injection is performed on the first day only followed by maintaining the $CO_2$ pressure in the headspace. A wider range of $CO_2$ absorption (3-34%) is reported by Fernández et al., (2014), where food waste and sewage sludge is used as the feeding substrate.

Two scenarios are made to compare how much exogenous $CO_2$ is required to close the methane production gap between the test reactor and control. One scenario uses the methane volume of the control as the volume benchmark and another one is using 90%

of TMP volume as the benchmark. A 90% volume is taken because it is impossible to convert 100% of the TVS into methane since substrate is also required for microbial growth and maintaining the condition beside the non-degradable fraction. If the 90% of TMP is used (217.55 litres), only around 30 L of more methane is needed for the entire process to reach the methane volume by the test reactor (246.81 litres). By using Eq. 8, this volume can be fulfilled by adding the same volume of $CO_2$ using assumption that the $CO_2$ is 100% utilized in the process (Table 9). To accomplish, four times as much $H_2$volume is needed. This gas can be fulfilled by both internally (from acidogenesis, acetogenesis, or SAOB process) and externally by adding the $H_2$ in the reactor. However, if this scenario is used, it would raise another question on how the system could reach the 90% of TMP volume from the actual volume (control).

Table 3. 9. Scenario on the volume of CO2 and H2 required to fulfil the gap of methane volume between the test reactor (246.81 litre) and the rest.

| Parameter | Unit | | 90% TMP | Control |
|---|---|---|---|---|
| Volumetric production | Litre | | 217.55 | 116.76 |
| Volume gap | Litre | whole process | 29.26 | 130.81 |
| Additional $CO_2$ required | Mole | whole process | 1.31 | 5.84 |
| | | per day | 0.04 | 0.19 |
| | Litre | whole process | 31.40 | 140.39 |
| | | per day | 1.05 | 4.68 |
| Additional $H_2$ required | Mole | whole process | 5.23 | 23.36 |
| | | per day | 0.17 | 0.78 |
| | Litre | whole process | 115.63 | 561.57 |
| | | per day | 4.19 | 18.72 |

The volume and mole are calculated for 20°C
The amount per day is calculated based on standard 30 days of operation

$$C_6H_{12}O_6 + H_2O \rightarrow 2CH_2COOH + 4H_2 + 2CO_2 \quad (3.17)$$

For another scenario using volume of the control as the benchmark, the biggest question would be how the system will provide a massive amount of $H_2$ required for the process to occur. In fact, no exogenous $H_2$ is induced into the system. Theoretically, the internal system could not provide that much of additional $H_2$. By using sugar as a model substrate (Eq. 3.17) the volume of $H_2$ can be produced if the entire fed substrate utilized in the acetogenesis process is only around 346.72 liters (20°C, 1 atm). It does not include any possibility of existing biomass (microorganism) degradation during the anaerobic process.

Therefore, combination of additional substrate in the form of $CO_2$ and increased substrate utilization efficiency is suggested to be the reason for this enhancement. An enhancement due to increasing substrate degradation is also possible but seems to be minor in this study.

### 3.3.5. Utilization pathway of the exogenous $CO_2$

To determine the $CO_2$ utilization pathway with less bias, a study using 'non-bias' analysis, such as metagenomics and metabolomics should be performed. Since this project does not cover any omic study, the hypothesis by which pathway $CO_2$ is utilized dominantly is only based on the literature.

There are two major pathways suggested for the $CO_2$ utilization in enhancing methane production. The first one is the utilization by homoacetogens to form acetic acid via Wood-Ljungdahl pathway (Eq. 3.18). Another pathway is the direct methanation by hydrogenotrophic methanogenesis using $H_2$ as the reducer (Eq. 3.8). Both pathways are possible to occur in the system simultaneously. However, there may be some alteration as an effect of environment change by $CO_2$ microbubble.

An increase of acetic acid has been reported when $CO_2$ is added into the headspace of the reactor (Alimahmoodi and Mulligan, 2008). It is then suggested that production of acetic acid via Wood–Ljungdahl pathway is the reason for that. Different from the previous study, Tao et al., (2019) reported that when $CO_2$ is introduced into the reactor along with $H_2$, there is an increase in the acetate concentration as well beside the domination of the hydrogenotrophic methanogenesis.

$$2CO_2 + 4H_2 \leftrightarrow CH_3COOH + 2H_2O \quad \Delta G^{0\prime} = -95\ kJ/mol \quad (3.18)$$

Based on the thermodynamics, the $CO_2$ utilization by hydrogenotrophic methanogenesis is more favorable to happen than that by autotrophic acetogenesis. It is demonstrated with their Gibbs free energies, which is -135.1 kJ/mole and -95 kJ/mole for the methanogenesis and acetogenesis, respectively. Regarding the increase of acetate level after $CO_2$ injection, it is not necessarily attributed to the domination of the Wood-Ljugdahl pathway over the heterotrophic acetogenesis. Since the environment becomes more favorable for the heterotrophic acetogen due to the periodic removal of $H_2$, more acetate

can be produced via this pathway. Considering Le Chatelier's Principle, autotrophic acetogen is less likely to happen in the early process when acetate is available abundantly as a result of the higher substrate degradation rate. Instead, SAOB might take the role to utilize the acetate. It is followed by the methanation of produced $CO_2/H_2$ by the hydrogenoclastic methanogenesis, which the process can be seen as an indirect methanation of acetate. Despite the fact that the oxidation of acetate is thermodynamically less favorable than the direct methanation of acetate, some findings show that the process is preferable in the medium with high salt, high ammonia and high VFA (Morris et al., 2013; Nüsslein et al., 2001; Shigematsu et al., 2004).

Regarding the microbial studies related to the $CO_2$ injection (without $H_2$), a study by Fernández et al., (2019) shows that the domination of acetoclastic methanogenesis (*Metanosaetaceae* and *Methanosarcinales*) is shown over hydrogenoclastic methanogenesis (*Methanobacteriaceae*). After a single injection of $CO_2$, the decrease in the relative abundance of acetoclastic methanogens is shown in the sewage sludge treatment, while it increases in the food waste treatment. On the other hand, the relative abundance of hydrogenoclastic methanogens increased in the food waste but decreased in sewage sludge. When the $H_2$ is added simultaneously with $CO_2$, hydrogenoclastic methanogens is dominant over the homoacetogens (Tao et al., 2019). In fact, the abundant of acetoclastic methanogen is not always proportional to its activity as has been reported in other studies (Hanreich et al., 2012; Kohrs et al., 2014). It is supported by other studies that in the presence of methanogen, the competition in utilizing $CO_2$ is less possible for the homoacetogenic bacteria (Kotsyurbenko et al., 2001; Stams and Plugge, 2009). Moreover, the TAN level is relatively high. Hydrogenoclastic methanogens are more resilient in such conditions compared to acetoclastic methanogens that may result in the indirect methanation of acetate.

## 3.4. Conclusions

This study discusses the mechanism for how $CO_2$ microbubbles enhance methane production rate using food waste as feed stock. There is no significant difference in solid removal between the test and control reactors, which is 57% to 59% for the TS, 73 to 75% for the TVS and 67% to 69% for the COD. A periodic injection of $CO_2$ microbubble results

in higher substrate degradation rate (42%), methane yield (104%), and methane volume (112%).

In the first few days, the test reactors always produce a massive amount of $CO_2$ and $H_2$up to 85.31 liters and 5.3 liters, respectively. Since both gases are produced using only soluble substrate, a much higher production of both gases in the early stage meaning a higher substrate degradation rate in that stage.

Increasing methane yield and volume after $CO_2$ microbubble injection is thought because of two reasons. Firstly, $CO_2$ microbubbles injection provided additional carbon source and secondly, promoting an efficient utilization of substrate. This efficiency increasing could be due to removal of free ammonia by microbubble stripping that results in higher biochemical reaction rate.

While there is more than one possible pathway of $CO_2$ utilization, hydrogenotrophic methanogen seems to be preferable than the homoacetogen. Based on Gibbs free energy, utilisation of $CO_2$ by hydrogenotrophic methanogens needs -135.6 $kJ.mol^{-1}$ energy while homoacetogens needs -95 $kJ.mol^{-1}$. Since this study does not perform a microbial analysis, the dominant $CO_2$ utilization pathway is predicted using thermodynamics considerations only.

# Chapter 4

# Simultaneous Methane Enhancement and Reduction of Landfill Leachate Toxicity by Carbon Dioxide Microbubbles in an Anaerobic Co-digestion of Food Waste and Landfill Leachate

## 4.1. Introduction

Co-digestion of substrates is a practice to optimize the anaerobic digestion (AD) performance by effectively using different sources of substrates with a particular objective, such as balancing the nutrient or increasing the buffer capacity inside the digester (Li et al., 2009; Zhang et al., 2013). While maintaining a carbon-nitrogen ratio (C/N) is the most frequent practice in co-digestion (Cuetos et al., 2008; Yen and Brune, 2007), gaining trace elements from another substrate is another focus. Trace elements act as co-enzymes in the biochemical process inside the cells to sustain the AD process (Choong et al., 2016; Climenhaga and Banks, 2008). Supplementing trace elements could give a positive impact on substrate degradation, substrate utilization as well as total methane production (Pobeheim et al., 2010; Schmidt et al., 2014). It also has been proven to reduce the hydrogen sulphide formation and regulate volatile fatty acid (VFA) concentration (X. Meng et al., 2013; Yu et al., 2015).

Landfill leachate contains significant amounts of trace elements (Barrantes Leiva et al., 2014; Liao et al., 2014; Pastor et al., 2013). The main source of the leachate is a mixture of municipal waste containing organic matter, salt, alkalinity, and trace metal (Baun and

Christensen, 2004). Utilizing the leachate to support other biological processes seems to give more value to the leachate itself. Moreover, leachate from long-operated landfills has relatively stable properties and tends to be less tractable to treatment biologically alone (Kjeldsen et al., 2002). Several reports have also highlighted the positive impact of adding landfill leachate in the anaerobic digestion when it is well managed (Hombach et al., 2003; Liao et al., 2014).

One of the biggest challenges in using landfill leachate in biological processes is its toxicity. Even though some metals are essential for cell growth, some have can denature protein intracellularly (Gadd and Griffiths, 1977). The ammonia content in leachate seems to be another concern and is discussed intensively in some published articles. In fact, high ammonia levels are not only found in landfill leachate, but also in seeding sludge of AD itself which can be even higher (Shahriari et al., 2012; Walker et al., 2011). Landfill leachate also contains a significant number of recalcitrant compounds and volatile organic compounds (VOC) that are toxic (Baun et al., 2004; Koshy et al., 2007; Plotkin and Ram, 1984). Such toxicants come to the landfill site in several ways, such as from domestic and industrial waste as well as agricultural practices. Removing toxic chemicals, or at least minimizing their toxic effects, is another challenge when introducing landfill leachate in the AD process. This will be even more challenging within the AD reactor, rather than doing it separately. The main reason for doing it in one vessel, e.g. in a bioreactor, is that it is expected to reduce space and time for the treatment compared to separate units.

Ozonation and advanced oxidation processes (AOP) are among the successful techniques to reduce some toxic chemicals in landfill leachate (Abu Amr et al., 2013; Tizaoui et al., 2007). For ammonia removal, adsorption/absorption and coagulation-flocculation have shown effectiveness and economic feasibility applied to leachate (Dia et al., 2018; Halim et al., 2010). However, these techniques seem only feasible outside the anaerobic reactor, since applying such techniques inside the reactor has the potential to disrupt the microbial community. Reducing the toxicity of the heavy metals inside the reactor vessel is the most promising prospect for some heavy metals of concern. Most HMs cannot be degraded into different compounds so that they likely remain in the reactor until the process finishes.

Gas stripping can be an alternative to remove some toxic components in landfill leachate. It can be performed inside the AD reactor. The basic principle of gas stripping is to transform the toxicants from the aqueous phase to the gas phase, either by chemical reaction or increasing its volatility. Ammonia removal by using air stripping achieves up to 95% of total ammonia removal after around four hours of treatment (Campos et al., 2013; Kurniawan et al., 2006 Ozturk et al., 2003). The application of air/gas stripping to remove VOCs from the water has also been successful (Juang et al., 2005; Lamarche and Droste, 1989; Nirmalakhandan et al., 1987). To apply it for anaerobic digestion, an oxygen-free gas should be applied instead of air.

As oxygen-free gas should be used for gas stripping for AD applications, circulating biogas that is produced by the system itself can be one way to apply the stripping technique (Walker et al., 2011). The circulated biogas contains methane, $CO_2$, nitrogen, and trace amounts of hydrogen gas, hydrogen sulphide and ammonia. The latter two gases have the potential to disrupt the performance of microorganisms if they exceed tolerable limits. With some of the success stories of applying $CO_2$ in anaerobic treatment, pure $CO_2$ injection can be a simultaneous method for removing several toxic compounds while increasing biogas production. Introducing $CO_2$ into AD process has not only proven to increase significantly the methane yield but also opens an opportunity to reduce the carbon footprint by recycling $CO_2$ from the biogas (Al-Mashhadani et al., 2016; Alimahmoodi and Mulligan, 2008; Salomoni et al., 2011). When the liquid medium of the AD contains a significant number of toxicants, such as when it is induced with landfill leachate, applying gas stripping may give several benefits simultaneously: enhancing the biogas production, reducing carbon footprint and eliminating/reducing the toxicity of the organic pollutants.

This study is to investigate the ability of $CO_2$ microbubbles to reduce the toxicity of leachate while enhancing biogas production. A daily injection is carried out, instead of continuous $CO_2$ injection. It is hypothesized that the injection of $CO_2$ microbubbles could reduce the toxicity effect of the landfill leachate added in the anaerobic digester in several ways, such as to increase the volatility of the toxicants and to increase the degradation rate to degrade the toxicants. The discussion about the increase in the degradation rate is covered in the previous chapter.

## 4.2. Materials and method

### 4.2.1. Feedstock, leachate and seeding sludge

Reproducible artificial food waste (Appendix 3) is used in this experiment. The composition of food waste is the same as that is used in Chapter 3. Each reactor receives 2 kg of food waste.

Table 4. 1. Proximate analysis of the feedstock

| Parameters | Unit | Value |
|---|---|---|
| Carbohydrate [a] | gram | 18.49 |
| Fibre [a] | gram | 2.47 |
| Protein [a] | gram | 4.75 |
| Fat [a] | gram | 1.10 |
| Total Solid | % | 29.50 |
| Volatile Solid | % | 28.19 |
| VS/TS ($f_{VS}$) | % | 0.95 |
| C[b] | % | 82.07 |
| H[b] | % | 8.98 |
| N[b] | % | 5.92 |
| S[b] | % | 3.03 |
| C/N | | 13.88 |

[a] determined per 100 grams of non-dried sample
[b] value from dry weight

The characteristics of the inoculum/seeding sludge that is used is the same as in Chapter 3. For landfill leachate, it is collected from a sanitary landfill site in Northern England, the United Kingdom. Table 4.2 shows the properties of landfill leachate and seeding sludge that is used in this study.

Table 4. 2. Properties of the leachate and seeding sludge (inoculum)

| Parameters | Unit | Landfill Leachate | Seeding sludge |
|---|---|---|---|
| pH | | 8.1 | 8.5 |
| Total Solid | g/L | 10.54 | 11.99 |
| TDS | g/L | 6.35 | 8.36 |
| TSS | g/L | 4.19 | 3.63 |
| TVS | g/L | 3.20 | 3.59 |
| COD | g/L | 5.31 | 7.61 |
| sCOD | g/L | 4.86 | 6.97 |
| TOC | ppm | 1367.74 | 103.33 |
| TAN | ppm | 1424.13 | 3237.99 |
| TVFA | mg/L | 360.67 | 110.58 |

The pH of both leachate and seeding sludge is alkali, which may due to high total ammonia nitrogen (TAN). The level of TAN of the seeding sludge is higher than that of the landfill leachate added. A quite low concentration of VFA is probably a result of the degassing

process that is carried out before the treatment. The low VFA can be associated with low readily substrate for methane production. To represent toxic content (other than ammonium) in the raw landfill leachate and seeding sludge, TOC used as the general parameter. Tab. 2 shows that the leachate had a much higher TOC level than the seeding sludge and potentially more toxic to the microbial population inside the AD.

Table 4. 3. Concentration of the trace metals in the raw landfill leachate

| HM | $\mu gL^{-1}$ | HM | $\mu gL^{-1}$ | HM | $\mu gL^{-1}$ |
|---|---|---|---|---|---|
| Ag | 1.2 | Co | 156.8 | Ni | 323.9 |
| Al | 399.2 | Cr | 750.5 | Pb | 104.1 |
| As | 155.3 | Cu | 1732.9 | Rb | 793.3 |
| B | 19482.7 | Fe | 816 | Se | 232.9 |
| Ba | 58.6 | Ga | 1.4 | Sr | 134.2 |
| Be | 0.1 | Li | 1061.2 | Te | 0.1 |
| Bi | 0.7 | Mg | 11611.1 | Tl | 0.7 |
| Ca | 11142 | Mn | 403.1 | U | 0.8 |
| Cd | 3 | Mo | 440.6 | V | 240.3 |
| Ce | 0.8 | Nd | 0.1 | Zn | 2570.9 |

The trace metal analysis is carried out for raw landfill leachate only (Table 4.3). There are 30 metals are detected from ICP-MS analysis. Most metals had concentration under 1mgL-1, except for B, Ca, Mg, Cu, Li and Zn. Calcium and Magnesium content shows the highest concentration among other metals. The presence of high Mg and Ca in the liquid may increase the possibility of interaction with other free metal that lead to reduction in the toxicity (Gadd and Griffiths, 1977). In the application, the landfill leachate is added to only 10% of the total medium. This ensures dilution of the metal to around one tenth its typical concentration, before considering the metal content in the seeding sludge.

### 4.2.2. Analytic Chemistry Methods

The gas composition, and characteristic of the solid and liquid is carried using the same methods and equipment as in Chapter 3. Different from Chapter 3, landfill leachate is added to some treatments in this study, with the emphasis of providing trace metals for the AD. The heavy metal content is measured for the raw landfill leachate only using ICP-MS.

### 4.2.3. Experimental set-up

Four lab-scale cylindrical digesters made of stainless steel are used in the study. The reactors are the same as those are in Chapter 3.

These are the treatments applied in this study: (I) combined treatment of $CO_2$ microbubbles with additional leachate (LL-$CO_2$), (II) $CO_2$ microbubbles without leachate ($CO_2$), and (III) leachate addition without $CO_2$ injection (LL). No leachate and no $CO_2$ injection is applied as the control (Con). For the treatments without landfill leachate, 20 L of seeding sludge is used for each reactor. For the treatment using landfill leachate, about 18 L of seeding sludge is used with an additional of 2 L of landfill leachate (10% v/v) making the total liquid volume is 20 L. The reason to add 10% of landfill leachate is based on the result on the preliminary study that uses landfill leachate addition in various concentrations: 5%, 10%, 15% and 20% (Appendix 2). While 5% leachate addition gives no difference in the methane results (one higher and another one lower than the control), the addition of 10% leachate starts to give a significant decrease in the methane production. In fact, landfill leachate may have different characteristics regarding the source and time of collection. This makes checking the chemical composition of landfill leachate before deciding the volume to add is important if it is not collected at the same time and place. The injection of $CO_2$ microbubbles is applied on a daily basis, 10 minutes a day, making the total gas injected per day $10 \pm 0.5$. All treatments performed in a suspended batch system where no mixing is performed.

The author is aware that the different results between the treatment with and without $CO_2$ injection might be affected by the un-intentional mixing done by the periodic gas sparging. A report by Karim et al., (2005) shows, in a relatively diluted liquid medium (5% slurry), a regular mixing (24 hours a day) does not affect the gas production. An increase of methane production (10%-30%) after a regular mixing is shown only in a thicker mixture ($\geq$ 10%). Mixing intensity seems to affect the biogas production. A report by Ma et al., (2019) shows that a regular mixing with 6o rpm or less does not affect significantly to the biogas production. In this study, since the sparging is carried out only for ten minutes a day with the gas flowrate of only 1 litre per minute, the effect of mixing due to the sparged gas can be neglected.

| Table 4 4. Treatment matrix | | |
|---|---|---|
| Treatment | $CO_2$ microbubble | Landfill leachate addition |
| I. Combined Treatment (LL-$CO_2$) | Yes | Yes |
| II. $CO_2$ only ($CO_2$) | Yes | No |
| III. Leachate without $CO_2$ injection (LL) | No | Yes |
| IV. Control | No | No |

All the reactors are fed with 2 kg of food waste (wet basis). Neither pH buffer nor additional micronutrients are added into the reactor. Each experiment is performed until the cumulative methane production reaches a stationary state, with the aim to observe the maximum gas yield as well as the possible highest degraded fraction of the feedstock that could be achieved during the treatment. The study is carried out for three cycles.

## 4.3. Result and discussion

### 4.3.1. General operational condition

The treatment is carried out in mesophilic mode, in which the temperature is maintained to be 35$\pm$1$^{o}$C.The pH is monitored daily by taking 5ml samples out before measuring it. The daily pH is shown in Fig. 4.1. Fig. 4.1 indicates that the pH of all treatments are in an acceptable range (7.3-8.5), even though slightly higher than the optimum value (6.8 to 7.5) (Liu et al., 2008). This higher pH is possibly due to high total ammonia nitrogen (TAN) content in the medium (Fig. 2). The TAN concentration of the medium is high, ranging from 3000 to 5300 mg.$L^{-1}$. A low C/N ratio (13.88) of the feedstock may also have affected the increase in pH due to high ammonium release from protein degradation. This high pH and also high TAN may portend that the system would not give an optimum result if these conditions persist.

All treatments begin with relatively high pH (8.4), then drop to around 7.3 to 7.5 within 24 hours. In a batch system, rapid fermentation of the substrate typically occurs in early days resulting in a notable increase of VFA production in that period (Cysneiros et al., 2012). A significant decrease in pH due to increasing VFA level can cause system failure if the media does not have good buffering capacity. Over the next few days, the pH is quite stable for all treatments and no significant difference is recorded among them. A gradual pH increase occurs in the second week and reaches pH 8.0 and even higher for the treatments without $CO_2$ microbubbles injection. The slowdown in the VFA production along with the increase in the VFA utilization may be the reason of this phenomenon as

have been reported by some studies (Luo et al., 2012; Tao et al., 2019; Wang et al., 2013). In this point, an obvious difference in the pH value is recorded between the treatment with and without injection of $CO_2$ microbubbles.

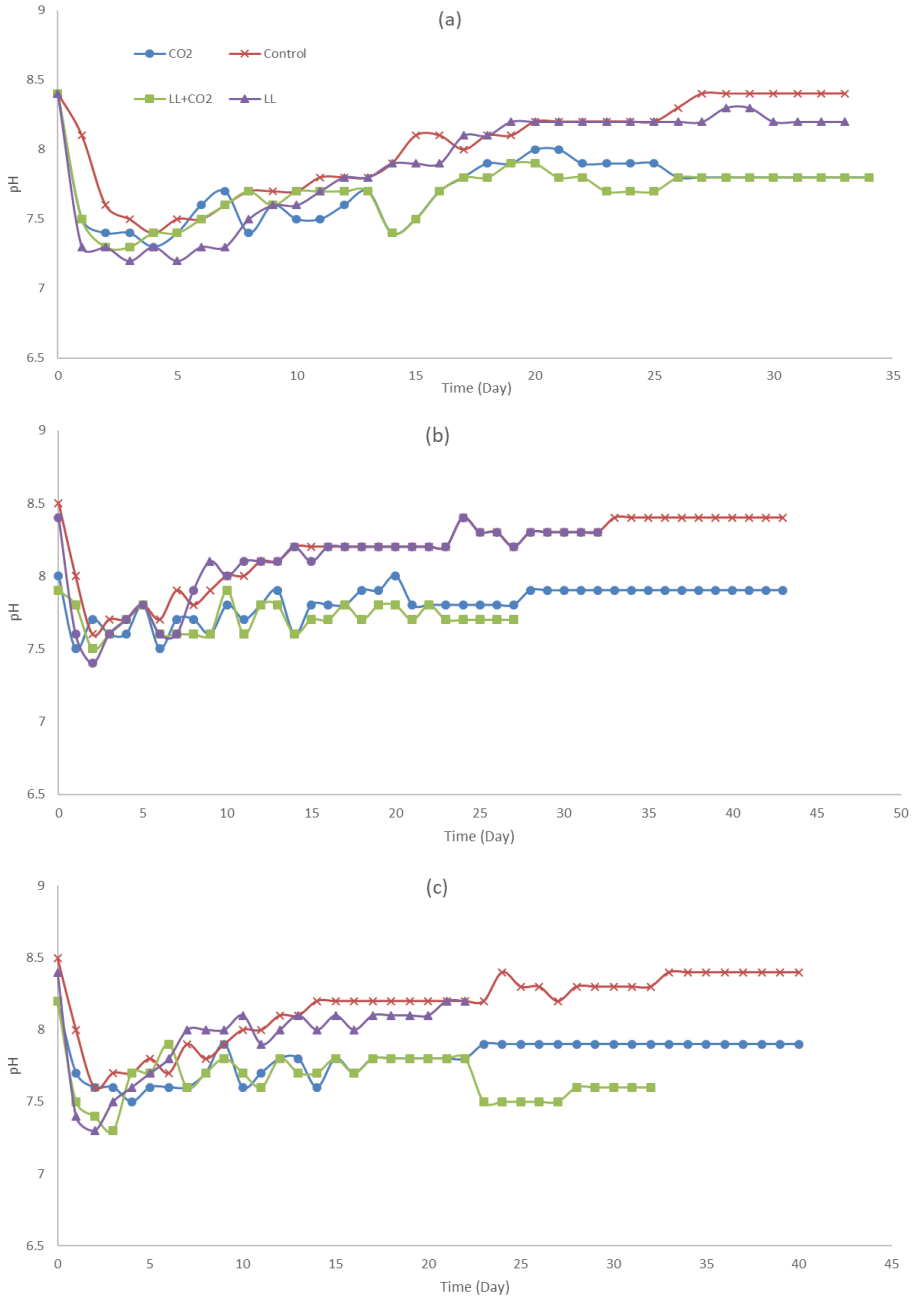



Figure 4. 1. pH value of the treatment. Picture a, b and c are showing the 1st, 2nd, and 3rd cycle respectively.

The total VFA concentration before and after the treatment is presented in Fig. 4.3. A highlight is given to acetic acid and propionic acid, which are the main precursors of

methane. The first cycle started with a low VFA concentration as a result of inoculum degassing prior to starting the process. The treatment with leachate (LL-$CO_2$ and LL) shows a higher initial VFA concentration in the first cycle compared to the treatment without leachate addition. This higher VFA is suggested due to the leachate addition that contains an amount of VFA, especially acetic acid.

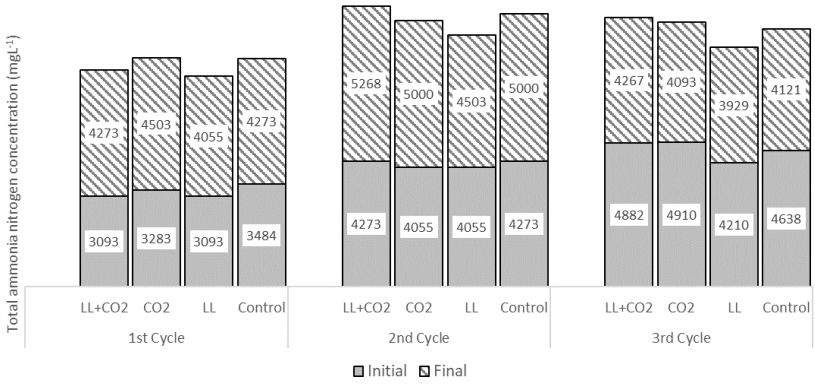
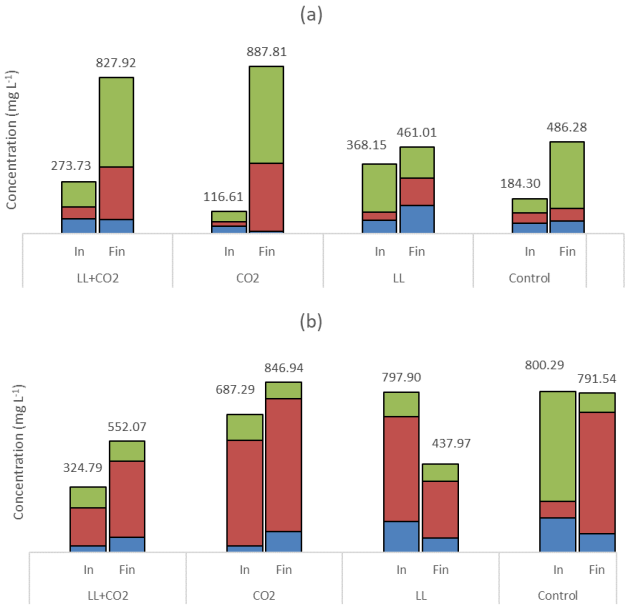



Figure 4. 2. The concentration of total ammonia nitrogen (TAN) in the initial and final stage of each treatment.



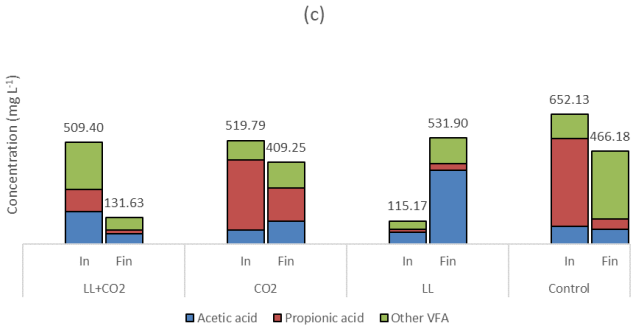



Figure 4 1. Initial and final concentration of total VFA of each treatment. (a) First cycle, (b) second cycle and (c) third cycle.

By the end of the first cycle (day 33), the VFA content, especially acetic acid and propionic acid, for all treatment are higher than the initial value, which is close to 500 $mgL^{-1}$ or even more for some treatments. The decrease of the pH in the early stage indicates that a higher production of VFA occurred as a result of the degradation process of the feedstock. It is suggested that the total VFA should be maintained below 1.5 $gL^{-1}$ to optimize the microbial activity (Pohland and Ghosh, 1971). A high VFA concentration has been reported to negatively affect the degradation of the substrate in AD (Siegert and Banks, 2005). However, a much higher concentration of total VFA (>4 $gL^{-1}$) is reported by Cysneiros et al., (2012) and is remaining perform high substrate degradation (83%-93% of VS) as long as the pH is maintained to be in the optimum state.

### 4.3.2. Substrate degradation

Table 4 shows the average reduction of the total solid (TS), volatile solid (VS) and total COD from each treatment in this study. All treatments showed approximately 60% for TS removal and the VS removal is between 70% to 80%. The COD reduction occurred in the range between 60% and 70%. By using ANOVA test (0.05), there is no significant difference in the removal of those parameters. However, by comparing the values numerically, it shows that the treatment with the leachate addition had slightly higher TVS reduction, which is between 6-9%, regardless of microbubble injection. A similar trend is also shown by TS removal. A higher solid removal in the treatment with leachate addition indicates an increase in the number of degraded fractions of the substrate. Landfill leachate contains a significant amount of trace metals those may boost the substrate degradation (Espinosa et al., 1995; Pobeheim et al., 2010).

Table 4. 5. The average removal of total solid (TS), total volatile solid (TVS) and COD

| Parameter | Unit | LL+$CO_2$ | $CO_2$ | LL | Control |
|---|---|---|---|---|---|
| TS | % | 63.12±5.4 | 60.08±2.8 | 62.81±6.5 | 58.40±3.8 |
| TVS | % | 79.32±5.4 | 74.68±3.9 | 78.40±4.6 | 73.52±3.6 |
| TCOD | % | 68.30±2.7 | 63.82±3.5 | 69.18±4.1 | 61.32±2.5 |

The injection of $CO_2$ microbubbles is unlikely to affect the degraded fractions of the substrate. This agrees with the report by Fernández et al., (2014) which shows no significant difference in the total solid and volatile solid reduction between the test and control, regardless the type of substrate. Even though it does not increase the degraded fraction of substrate, injection of $CO_2$ microbubbles shows its ability in enhancing substrate degradation rate, as has been discussed in Chapter 3.

An AD performance to degrade substrate is variable, in which the nature of substrates plays the most important role besides the operational set-up (Astals et al., 2013; Mata-Alvarez et al., 2000; Neves et al., 2009). Two factors are suggested to affect the biodegradability of the substrate, i.e. structural characteristics of the degraded substrate (Cirja et al., 2008; Fan et al., 1980) and the accessible area of the substrate for the attachment of the extracellular enzyme (Sun and Cheng, 2002). Since the degradation process is performed by microorganisms, the working environment in which the microorganisms live significantly affects the degradation process itself. Of all environment factors, the most intensively studied are temperature, pH and inhibitory compounds. The temperature can be stated as an independent factor and is easily to control, while two other environmental factors (pH and inhibitory compounds) are affected mainly by feedstock, inoculum and sometimes the operational temperature (Khalid et al., 2011).

### 4.3.3. Methane production

The cumulative methane production of each cycle during the study is presented in Fig.4. 4. While the duration to finish the first cycle is the same for all treatments, a notable variation is shown in the next two cycles. In general, any treatment without landfill leachate addition shows relatively longer duration. Even though taking place in a shorter period, a combined treatment (LL+$CO_2$) always shows the highest methane production among all. The combined treatment also shows a significant increase in the production rate in the second and third cycle. This contrasts with the LL treatment, in which the

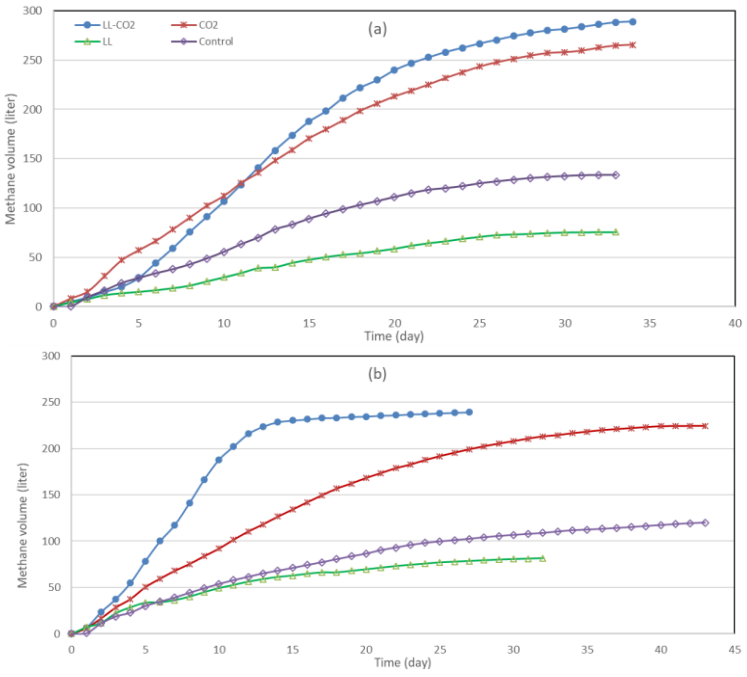
methane production is significantly the lowest. The treatment with landfill leachate only (LL) shows a constantly reduction in the methane production in the second and third cycle.

To calculate the methane rate ($\mu_{CH4}$), Monod equation is used (Eq. 4.1). The equation is then transformed into Eq. 4.2 using assumption that the substrate is available abundantly.

$$\frac{dX}{dt} = \frac{\mu_{max} \cdot S}{K_s + S} \quad (4.1)$$

$$\mu_{CH4} = \frac{lnB_t - lnB_0}{t} \quad (4.2)$$

To get the condition with abundant substrate, the calculation is carried out for the methane data in the first eight days. A straight-line plot is done for the methane data excluding the lag phase. The coefficient of correlation is set to be 0.98 ($R^2 \geq 0.98$) unless for treatment LL, which the value could not be obtain. Instead, the 0.95 is used for the correlation coefficient of treatment LL. In fact, such equation can also be used for estimating the disintegration phenomenon inside anaerobic digestion if the solid sample is not provided (Angelidaki et al., 2009; Astals et al., 2013). However, it seems only best applied in a more controlled environment without any significant inhibitors present in the reactor. It is shown by the treatment with LL, where the TVS removal is slightly higher than that of the control but had significantly lower methane production.



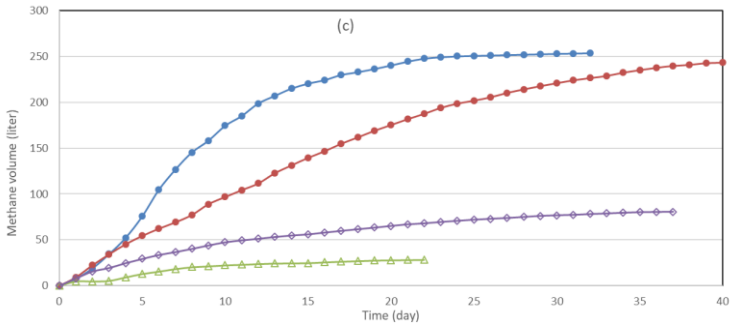



Figure 4. 2. The cumulative methane production of 1st cycle (a), 2nd cycle (b), 3rd cycle (c).

Fig. 4.5 shows that the highest $\mu_{CH4}$ is demonstrated by LL+$CO_2$ followed by treatment with $CO_2$ only. The $\mu_{CH4}$ of both treatments increase in the second cycle and then decrease in the third cycle. The $\mu_{CH4}$ increase of the LL+$CO_2$ in the second cycle is fit with the cumulative methane data (Fig. 6) that shows a rapid increase of the methane production in the early days. For the $\mu_{CH4}$ of the treatment without $CO_2$ microbubble, a slightly different value is shown between them with the value of the LL treatment is lower. If this value is used to estimate the rate of disintegration (as discussed in Chapter 3), the conclusion would be: the rate of disintegration is only influenced by the $CO_2$ microbubble. In fact, the solid reduction of the LL treatment is higher than that of the $CO_2$ treatment and control (Table 4.4). Due to some reason, an addition of landfill leachate has shown a negative effect in the methane production if no $CO_2$ microbubbles is injected, but not for the solid reduction. Therefore, this value cannot be used as a tool for measuring the disintegration rate of the feedstock.

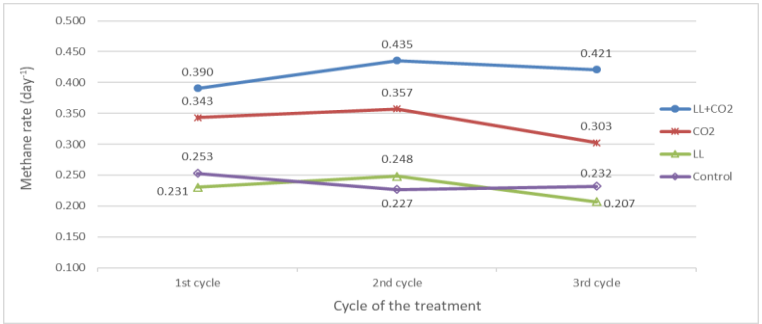



Figure 4. 3. The methane rate of each treatment

Other tools to measure AD performance are methane yield ($Y_{CH4}$) and methane production rate (MPR). The methane MPR is intentionally differed from the methane rate ($\mu_{CH4}$). While $\mu_{CH4}$ is derived from the graph of cumulative methane volume that shows the

highest rate period as well as can be used to estimate the substrate degradation rate (Chapter 3), MPR is calculated using the total volume of the methane divided by the amount of volatile solid digested per day (Eq. 4.3). $V_{95\%}$ represents 95% of total volumetric methane production, dTVS is the utilized volatile solid, and $t_{95\%}$ is the time to achieve 95% of the total methane production. The reason for using 95% of total methane volume is typically the methane production decrease significantly after it reaches 95% of the total methane that can be produced in the whole process (batch) and it is also not feasible economically to continue the process, especially when performed in a full-scale plant. In fact, MPR is more widely used as the parameter to assess the methane production rate in an anaerobic digestion.

$$MPR = \frac{V_{95\%}}{dTVS.\, t_{95\%}} \quad (4.3)$$

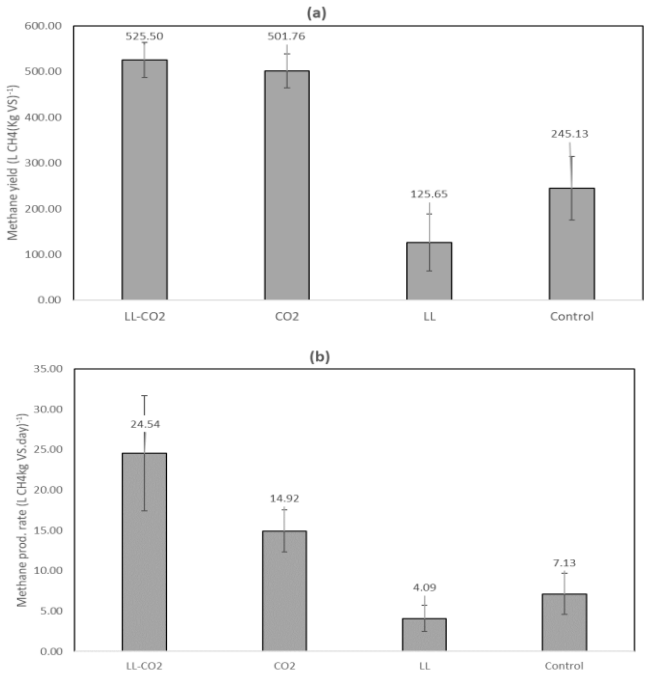



Figure 4. 4. Average methane yield (a) and methane production rate (b) of each treatment.

Fig. 4.6 summaries the $Y_{CH4}$ and MPR of each treatment. In the average, the $Y_{CH4}$ of LL+$CO_2$ and $CO_2$-only treatment are 525.5 and 501.76 $LCH_4(Kg\ VS)^{-1}$, respectively. It is more than twice as much of the value of the control, which is 245.13 $LCH_4(Kg\ VS)^{-1}$. The typical methane yield of food waste is in the range between 100 to 400 $LCH_4(Kg\ VS)^{-1,}$ which

depends on the substrate characteristic and environmental factors. This value is in fact lower than the theoretical methane yield, which is estimated based only on the substrate composition (Lier et al., 2008). Using Eq. 4.4 and the substrate composition (Table 4.1), the theoretical methane yield of the feedstock is 472.95 $LCH_4$(Kg VS)$^{-1}$. The yield of the carbohydrate, protein and lipid is 0.415, 0.496 and 1.014, respectively. All value is in STP (0$^o$C, 1 atm) (Angelidaki and Sanders, 2004). All the values need to be converted in room temperature (20$^o$C) for the estimation of yield.

$$Y_{CH4} = \frac{\left(\frac{n}{2} + \frac{a}{8} - \frac{b}{2}\right) 22.4}{12n + a + 16b} \left(\frac{LCH_4}{g\ VS}\right) \quad (4.4)$$

The same trend is shown for the MPR. For this parameter, the MPR value for the LL+$CO_2$ and $CO_2$ treatment are 24.54 L $CH_4$(kgVS.day)$^{-1}$ and 14.92 $LCH_4$ (kgVS.day)$^{-1}$, respectively. The MPR of LL-$CO_2$ is nearly triple of the MPR of the control, while the $CO_2$ treatment had MPR double of the control. A significantly higher value of MPR for the combined treatment is due to the shortest time to finish the treatment with no significant difference in the volumetric methane produced.

In contrast, the lowest values for $Y_{CH4}$ and MPR are shown by the treatment with landfill leachate only (LL) that occur in all cycles. Besides its achieving lowest in the methane volume, LL treatment also shows the shortest treatment duration. This is in contrast with Liao et al., (2014) where the addition of landfill leachate in the anaerobic digestion of food waste shows a more stable methane yield than the treatment without landfill leachate addition. A co-digestion of landfill leachate with other substrates has also been reported to demonstrate significant increase in the methane production and COD removal (Hombach et al., 2003; Montusiewicz and Lebiocka, 2011).

### 4.3.4. Discussion

A notably higher performance of all treatments with injection of $CO_2$ microbubbles over the treatments without $CO_2$ microbubbles is demonstrated clearly in this study. In contrast, the addition of leachate without $CO_2$ microbubbles (LL) shows the lowest methane production, which is only 60% of the methane production of the control and it continues to decline over the next two cycles. This low performance is predicted due to the toxicity effect of the added landfill leachate. The toxicants seem to accumulate in the

digester due to the addition of leachate into it at the beginning of each cycle. Interestingly, this low performance is not proportional to the reduction of solids inference in LL treatment, which is even slightly higher than the control. In other words, the toxicants seem to only affect the methane production process but do not affect the substrate solubilization.

On the other hand, the highest methane enhancement is demonstrated by the combined treatment (LL+ $CO_2$), which is explicable due to several reasons. Firstly, an increase in substrate degradation translates into a higher supply of solubilized substrate to the system. Compared to the treatment without leachate addition, the TVS reduction of this treatment increases ranging from 6% to 9%. It is suggested that the presence of the micronutrients (trace metals) supplied by the landfill leachate has contributed to this increase as previously discussed. This is supported by other studies on the effect of heavy metal on AD performance. The added trace metals enhance the AD process in several mechanisms, such as increasing substrate solubilization, improving acetogenesis, and catalyzing the direct conversion of propionate to methane via direct interspecies electron transfer (Banks et al., 2012; Jing et al., 2017b). The overall result shows that the addition of landfill leachate accelerates the methane production when it is combined with injection of $CO_2$ microbubbles. It is shown by the MPR of the LL+$CO_2$ treatment that is nearly three times as much as the control's, and 1.6 times of MPR compared to treatment with $CO_2$ only.

Secondly, the injection of $CO_2$ microbubbles into the system. In general, the increase of methane production after the addition of $CO_2$ is reported widely. There are several proposed mechanisms of how $CO_2$ addition can increase the methane production, which most articles mention it supplies additional carbon source for the system. This exogenous carbon source is either utilized by homoacetogen or hydrogenoclastic methanogen (Fernández et al., 2019; Tao et al., 2019). When it is injected by microbubbles, it improves the environmental conditions for the microorganism that result in increasing of methane production (Al-Mashhadani et al., 2016). An improving environmental condition includes reduction the medium toxicity, which in this study may primarily come from landfill leachate. An obvious difference is shown by the methane production of the combined treatment (LL+$CO_2$) and the treatment with only landfill leachate (LL).

*4.3.4.1. Landfill leachate toxicity*

The toxicant content in the landfill leachate can be in two forms: inorganic and organic. The inorganic toxicants are mainly metals and ammonia, while the organic toxicants mainly come from aromatic, halogenated and phenolic compounds. The metals themselves may exist in the landfill leachate in various forms, i.e. light metals (Al, K, Na, Mg), and heavy metals (As, Ba, Be, Cd, Cr, Cu, Fe, Hg, Ni, Pb, Zn). Some of the organic toxicants reported to be present in the landfill leachate are ethylbenzene, dichloro- and trichloro-ethane and n-chlorophenol (Alkalay et al., 1998; Baun et al., 2004).

Biological methane potential (BMP) test and anaerobic toxicity assessment (APA) are two common assessments to measure the toxicity of specific toxicants into the anaerobic digestion process. The assessment is carried out by adding particular compounds to an AD process and observing their effects on methane production. IC-50 is a common parameter used for assessing the toxicity of certain compounds, which shows the inhibitory effect of the compounds in reducing methane production by 50% (Blum and Speece, 1991). A decline in methane production is mostly a result of methanogenic activity declining since methanogenic archaea are the most sensitive microbes in AD against any disruption (Conrad, 2005; Nettmann et al., 2010; Schnürer et al., 1999). A lower IC-50 value of a compound shows a higher toxic effect.

Ammonia is the most common toxicant in AD. Its toxicity to the microbial community has been discussed intensively in many literatures. In this study, the TAN level of the landfill leachate is much lower than that of the seeding sludge (Table 2). This concentration is even lower than that considered toxic to most microbial communities inside the anaerobic digester. Therefore, if it comes to the ammonia toxicity in this study, the seeding sludge should contribute more than the landfill leachate.

$$NH_3 = \frac{TAN \ \frac{K_a}{[H]}}{\frac{K_a}{[H]} + 1} \quad (4.5)$$

In a liquid medium, ammonia may exist in two forms, i.e. ionic and free ammonia (FA) dissolved in the liquid media. Different from its ionic form, free ammonia is more harmful to the microbial community since it has higher diffusivity to infiltrate the cells membrane

and disrupt the activity inside the microbial cell cause proton imbalance and potassium deficiency (Gallert et al., 1998; Sprott and Patel, 1986). The level of FA in the medium is driven by concentration of TAN, temperature and pH of the medium as shown in Eq. 4.5 (Kayhanian, 1999). [H] is the concentration of hydrogen ion that the value can be calculated as $10^{-pH}$, while Ka is dissociation constant that the value depends on the temperature. Each factor seems to give positive correlation to the presence of free ammonia. Since no significant difference in the TAN concentration and temperature is demonstrated among the treatments in this study, the amount of FA mainly depends on the pH. Hence, the treatments without injection of $CO_2$ microbubbles are expected to have higher FA than that of treatments with $CO_2$ microbubbles injection. A periodic injection of $CO_2$ microbubbles may not only reduce the medium pH, it also eliminates completely the FA by stripping before it is accumulated to a certain level that results in significant decrease in methane production. The methane yield of the control that is only around 50% of the theoretical value may support the possibility of ammonia toxicity that affects the methanogenic activity.

Regarding the content of metals, they are mainly derived from the landfill leachate. In general, metals can act as micronutrients for enzymatic processes. The addition of certain metals in the anaerobic digestion process has an advantage in increasing methane production and disintegration rate (Banks et al., 2012). However, if the concentration is exceeds the tolerance limit, they can be toxic for microbes (Chen et al., 2014b; Mouneimne et al., 2003). The free form of metals makes them more accessible to the biological process, which means more harmful for the microorganisms. There is no exact value for which concentration of certain metals is toxic to the methanogens. For instance, Harris et al. (1990) mentioned that Cu, Zn, and Pb are toxic at the concentration of 5, 10 and 64 $mgl^{-1}$, respectively. More specific effects using the IC-50 parameter are mentioned in different reports. For the same elements, the IC-50 values are 12.5, 16 and 67.2 $mgl^{-1}$ for Cu, Zn and Pb, respectively (Lin, 1992). In this study, the concentration of the metals, especially heavy metals, are under toxic limits (Table 3). For instance, the concentration of Cu, Zn and Pb are only, 1.73, 2.57 and 0.10 $mgl^{-1}$, respectively. Moreover, the leachate that is added into the digester in this study is only 10% of the total working volume which ensures the overall concentration are significantly lower and more tolerable for most microbial communities inside the digester.

For organic toxicants, their presence in the landfill leachate has been reported widely (Baun et al., 2004; Öman and Hynning, 1993). The composition of the organic toxicants in landfill leachate varies depending on the collection time and the source of the landfill leachate itself, since local policy related to the waste disposal is also variable. The organic toxicants in the landfill leachate mainly consists of compound from three groups, i.e. aromatics, halogenated and phenolic compounds. Some refractory compounds may exist, but they are less toxic since they are less available biologically for the microorganism (Alkalay et al., 1998). Among the organic toxicants, chlorophenolic compounds are probably the most toxic to the methanogens, which is indicated from its low IC-50 value (Alkalay et al., 1998; Dienemann et al., 1990). The inhibitory effect of these compounds to the anaerobic process has been reported in some articles and can come in a wide variety of concentrations (Hernandez and Edyvean, 2008; Li et al., 2015).

In this study, analysis to detect specific organic toxicant in raw landfill leachate is not performed. Instead, dissolved organic carbon (DOC) is used as a general parameter of organic content in the raw leachate. This parameter has also been used in several publications to measure the performance of biological treatment in reducing organic toxicants (Dienemann et al., 1990; Millot et al., 1987). It is true that TOC does not only show the toxic compounds in the medium, but also any other natural and synthetic compounds. This parameter does not provide specific measurements of certain compounds, indeed. It at least gives a general information of what may be present in the liquid. Table 2 shows the TOC of the land fill leachate that is far higher (1367ppm) than that of the seeding sludge (103ppm). Since the TVFA concentration of the landfill leachate is only a quarter the TOC, it hints that the landfill leachate may contain a significant quantity of toxic chemicals. The landfill leachate added into the LL treatments is 10% of the total working volume and is given in each cycle. This means that if the toxic compounds are not removed completely in the first cycle, they can accumulate in the next two cycles. This is probably what happens with LL treatment. The data shows that the methane production of this treatment keeps decreasing in the next treatment. Accumulation of toxic compounds may explain this.

Interestingly, the LL treatment shows a normal level solid reduction, which it is even slightly higher than the control and treatment with $CO_2$ only. This may show that the toxic compounds only significantly affect the methanogenic archaea but not the hydrolytic

bacteria. This agrees with some literature that report methanogenic archaea is the most affected microbial communities inside anaerobic digestion against any disruption. The effect to the activity of acidogenesis and acetogenesis bacteria cannot be confirmed in this study as the VFA concentration is highly dynamic.

With regards to which toxicants significantly affect methane reduction relative to the control, this study indicates that organic toxic chemicals seem to be have the greatest influence, despite the fact that the ammonia toxicity decreases methane production as well. This is shown by the lowest methane production occurring in LL-treatment, in which landfill leachate is added but without any $CO_2$ injection. Since the heavy metal and ammonia concentration of the landfill leachate is relatively low, a significant reduction in methane production by LL treatment must have a strong correlation with the toxicity of organic compounds. In short, a treatment with landfill leachate addition receives two toxic streams: high ammonia from seeding sludge and toxic organic chemicals from landfill leachate.

#### *4.3.4.2. Injecting $CO_2$ microbubbles and reduction of landfill leachate toxicity*

As has been discussed, ammonia and organic compounds are the most studied toxicants here. Two mechanisms may contribute to this toxicity reduction -- firstly, by gas stripping and secondly, by biodegradation. The biodegradation process may have been boosted by the periodic injection of $CO_2$ microbubbles into the system.

Ammonia can be removed by gas stripping. For anaerobic conditions, oxygen-free gas should be used. Recycling the biogas or injecting $CO_2$ or $N_2$ are options for the gas. Factors potentially affecting ammonia removal during the stripping process include temperature, pH and duration of the stripping (Campos et al., 2013; Cheung et al., 1997; Kurniawan et al., 2006; Walker et al., 2011). A reduction of total ammonia concentration up to 95% has been reported using air stripping (Calli et al., 2005; Campos et al., 2013). In this study, periodic gas injection, which can also act as a stripping gas, is performed using $CO_2$ microbubbles for 10 minutes a day, equivalent to 300 minutes in 30 days. However, the study shows that there are no significant differences in the final ammonia concentration between all treatments. In liquid medium, ammonia has two different forms, in which free ammonia (dissolved gas) is more toxic to the microorganism. The presence of free ammonia is driven by several factors, such pH and temperature. Since the temperature is

set to be the same, the pH increases in the presence of more ammonia in the LL-treatment. In addition, by using $CO_2$ injection, the free ammonia can be removed periodically that may have reduced the toxicity risk associated with dissolved free ammonia.

Regarding the organic compounds in the landfill leachate, the removal can be carried out either by stripping or enhanced biodegradation. Most of the detected organic toxicants in the landfill leachate can be categorized as volatile organic compounds (Först et al., 1989; Öman and Hynning, 1993; Sabel and Clark, 1984). Some significant removal of toxic chemicals, such as methylene chloride, trichloroethylene, and methyl isobutyl ketone by gas stripping has been reported by many articles (Ghoreyshi et al., 2014; Nirmalakhandan et al., 1987; Soltanali and Shams Hagani, 2008). Volatility of the compound has an important role in the stripping process (Soltanali and Shams Hagani, 2008).

For the external factors, rate of removal in the stripping process is driven by several factors as is explained in Eq. 4.6 (Juang et al., 2005; Nirmalakhandan et al., 1987), in which J is the rate of transfer of the solute (mole $(L.s)^{-1}$), $K_L$ is overall mass transfer coefficient (fps $(ms^{-1})$), a is interfacial area per unit volume of tower ($m^2.m^{-3}$), C* is equilibrium concentration of solute in aqueous phase ($mole.L^{-1}$), and C is concentration of solute in aqueous phase ($mole.L^{-1}$). Based on the equation, the interfacial area between the gas and liquid has a strong correlation with the stripping process. The application of microbubbles in the stripping process is expected to increase the rate of VOC removal.

$$J = K_L a(C^* - C) \quad (4.6)$$

Besides the physico-chemical treatment, some organic toxicants can be removed by means of biological remediation. A study by Bouwer et al. (1981) shows how some halogenated organic compounds could be removed either partially or completely in anaerobic treatment. Some later studies also reported the ability of anaerobic digestion in degrading aromatic and phenolic compounds (Boyd et al., 1983; Field et al., 1995). In few cases, the metabolites of the degraded compounds are more toxic than their parent compounds. The injection of $CO_2$ microbubbles into the system has shown an overall higher degradation rate, which occurs especially in the early days of treatment. This higher degradation is predicted to affect the reduction of organic toxicant as well. Although no analysis is specifically done to measure the toxicants of concern, there are

two factors which may support it. Firstly, a significantly high methane production in all cycles occurs with the combined treatment (LL+$CO_2$) over all treatment, while the treatment with landfill leachate addition only (LL) shows the lowest methane production. Secondly, there is an increase in the solid degradation in the combined treatment (LL+$CO_2$) compared to the treatment without landfill leachate addition. While the availability of more metals is thought to be the main reason to increase the degraded fraction of substrate, $CO_2$ microbubbles contribute more in boosting the biodegradation rate using several mechanisms that has been discussed in Chapter 3.

## 4.4. Conclusions

In general, the addition of landfill leachate into anaerobic digestion of food waste can increase the degradation of substrate ranging from 6% to 9%. This increase is suggested due to an increase of the micronutrients, which is contained in the landfill leachate. This substrate degradation enhancement gives positive impact on the methane production when it is combined with the injection of $CO_2$ microbubbles, which results in the highest performance among all treatments. This is shown by the increase of methane volume, methane yield and methane production rate as high as 125%, 114% and 244%, respectively. A lower increase than the combined treatment is shown by the treatment with $CO_2$ addition only, with the increase in the methane volume, methane yield and methane production rate are 111%, 104% and 109%, respectively. The increase of methane production rate after landfill leachate addition may increase an economic feasibility to apply landfill leachate in AD.

On the contrary, when landfill leachate addition is not combined with a daily dosing of $CO_2$ microbubbles, the lowest performance among all scenarios occurs. A significant *decrease* in the methane volume, methane yield and methane production rate are shown to be around 40.90%, 48.74% and 43%, respectively. Two types of toxicants are suggested to impact detrimentally the methane production: organic toxic compounds and ammonia, especially in the free form.

Regarding to the reduction of landfill leachate toxicity, $CO_2$ microbubbles play a role in two possible mechanisms. Firstly, the daily gas stripping removes the volatile compounds (ammonia and organic toxicants) from the liquid. Secondly, it enhances the biological degradation that also affect in degrading some organic toxicants in the landfill leachate.

Since there is a bias whether the toxicants from the landfill leachate is removed dominantly either by the gas sparging or microbial biodegradation, a study to examine this question should be carried in the future. A microbubble gas sparging into the landfill leachate prior adding it into the AD is one of the possible methods to test it.

Chapter 5

# Microbial Community Dynamics in Anaerobic Digestion under $CO_2$ Microbubble Treatment

## 5.1. Introduction

Anaerobic digestion (AD) is one of the oldest technologies to treat organic waste and is economically feasible. Many efforts have aimed to increase AD performance. The addition of $CO_2$ is promising as it already increases methane production rate significantly. The idea of adding the $CO_2$ into the AD was firstly reported in 1994 before attracting further study to develop the approach (Bajón Fernández et al., 2017). The amount of increased methane depends on the feeding substrate and operational condition (Alimahmoodi and Mulligan, 2008; Fernández et al., 2015; Salomoni et al., 2011). The initial practice simply added $CO_2$ into the reactor headspace. An injection technique was later introduced either using a fine bubble diffuser or injecting microbubbles by fluidic oscillation (Al-Mashhadani et al., 2016; Bajón Fernández et al., 2015). Another technique to apply $CO_2$ is by coupling it with $H_2$ injection (Morris et al., 2014; Tao et al., 2019) with the aim is to give additional substrate for the hydrogenoclastic methanogen.

Anaerobic digestion is a complex process that employs various types of microbial communities. Besides being reported to increase methane production rate, very little literature discusses how $CO_2$ addition affects the microbial community inside the digester. In general, the microbial community inside the digester is dominated by those working on the methane production pathway, with only a few working outside this pathway (O'Flaherty et al., 2006). With more affordable costs for advanced DNA analysis, more species of microorganisms in the digester have been found. Until now, it is estimated that

there are more than two hundred species of microorganisms that have been known to co-exist in AD (Abram et al., 2011). Apart from bacteria and archaea, small amounts of protozoa are also discovered within the digester. However, their role in AD is still not well known.

The study of microbial composition and its activity in the AD has been reported to be carried out in various conditions, such as temperature, substrate loading rate, number of stages in the AD system, and chemical composition inside the digester. By comparing the data of those reported, it is suggested that the composition and the activity of the microbial community are significantly influenced by their operating conditions (De Vrieze et al., 2015; Krakat et al., 2010; Shigematsu et al., 2004; Zamanzadeh et al., 2016). The type of feeding substrate likely affects the formation of certain chemical composition, such as ammonia and VFA concentration. It means that the type of feeding substrate also affects the microbial composition as well.

For the archaeal community, Fernández et al., (2019) reported that acetate utilizing methanogen remains dominant in a treatment with and without $CO_2$ injection. Despite the domination of acetoclastic methanogens, an increase of hydrogenoclastic methanogen composition in the community has been shown for several treatments after $CO_2$ injection. A different phenomenon was demonstrated when hydrogen was injected along with $CO_2$, in which hydrogenoclastic methanogens dominate the archaeal community (Tao et al., 2019). Unfortunately, for those reported, the focus of the discussions is more on the archaeal community with very little discussion about the bacterial community. In fact, a massively higher degradation in the early process has been demonstrated after $CO_2$ microbubble injection in this study. Hence, it is thought to be necessary to explore the bacterial community as well, especially in the early stage of the process.

Besides achieving a higher methane production rate, our previous study discovers a notable substrate degradation rate within 24 hours since the AD receives its first $CO_2$ injection. This is shown by high $CO_2$ and $H_2$ volume collected in the gas bag in the first three days of operation and is confirmed with a higher value of degradation rate compared to the control. Based on this finding, we hypothesize that $CO_2$ microbubble injection increases the activity of hydrolytic bacteria in the early days of operation. Since

the test reactor achieves significantly higher methane volume but without significantly increasing the degraded fraction of the substrate, it is hypothesized that there are at least two possible explanations. Firstly, the system receives an additional carbon source in the form of $CO_2$ that is added using microbubbles injection. Secondly, $CO_2$ microbubbles injection promotes efficient substrate utilization. The latter occurs as the environment becomes favorable after $CO_2$ injection.

It is debatable how $CO_2$ is utilized by the microorganism, either dominated by homoacetogen bacteria or hydrogenothropic methanogens. Since the utilization efficiency is thought to be initiated by a higher intensity of acetogenesis, the microbial community related to that step should be more abundant after the injection of $CO_2$ microbubbles.

The objective of this study is to observe how the $CO_2$ microbubble injection affects the microbial community dynamics inside the anaerobic digester. The change in the microbial community structure against the time is observed more comprehensively based on the current hypotheses. The microbial dynamics data may provide some useful information to engineer microbial communities with respect to increase AD performance in general. This study is performed with these constraints: microbubbles are used for injecting $CO_2$ and the operational mode is a mesophilic batch process.

## 5.2. Material and Method

### 5.2.1. Feedstock seeding sludge

Artificial food waste is used in this experiment. Before adding the substrate into the digester, the food waste was crushed with an electrical kitchen chopper and no sieving was applied. To help the crushing process, water was added to the food waste with the ratio 3:1 for the food waste and water, respectively. Two kilograms of food waste was added into each reactor. Different from the artificial food waste that is used in the previous chapter, this study uses food waste with higher protein content and carbohydrate per dry weight. However, the C/N ratio is bit lower than that of the previous study.

Each reactor used about 20 litres of inoculum/seeding sludge. The inoculum was collected from a full-scale anaerobic digestion plant in Stockport, The United Kingdom. For longer

time storage, the seeding sludge was stored under temperature 4°C. Before being used, the seeding sludge was degassed in 35°C for few days to ensure no digestible substrate left in the seeding sludge (Angelidaki and Sanders, 2004) as well as for acclimatising the inoculum. Each reactor was flushed with nitrogen gas (1 lpm) for 20 minutes before the experiment began.

Table 5 1 . Physical and chemical properties of food waste and seeding sludge/inoculum

| Food waste | | Seeding sludge | |
|---|---|---|---|
| Parameter | | Parameter | |
| Total Solid (TS) * | 28.98 | pH | 8.5 |
| Total Volatile Solid (TVS)* | 25.07 | TS ($gL^{-1}$) | 11.98 |
| TVS/TS | 0.87 | TVS ($gL^{-1}$) | 8.36 |
| Carbohydrate* | 19.98 | TS/TVS | 0.71 |
| Sugar* | 2.58 | TSS ($gL^{-1}$) | 3.62 |
| Fibre* | 3.60 | TVSS ($gL^{-1}$) | 3.59 |
| Protein* | 5.32 | TCOD ($gL^{-1}$) | 7.61 |
| Fat* | 1.38 | sCOD ($gL^{-1}$) | 6.97 |
| C# | 81.07 | Total VFA ($mgL^{-1}$) | 110.58 |
| H# | 8.21 | TAN ($mgL^{-1}$) | 3237.99 |
| N# | 6.02 | | |
| S# | 2.90 | | |
| C/N | 13.46 | | |

*per 100 gram of wet sample
# Percentage from dry samples, excluding the oxygen content.

### 5.2.2. Experimental set up

The experiment was done in mesophilic condition (35±1°C) in batch process for maximum 30 days. Stainless steel reactors are used in this study are the same as the reactors being used in Chapter 3. To maintain the temperature, a submersible heater is placed in the reactor. For the additional heating maintenance, all the reactors are placed in an incubator as shown in Fig.5.1.

There are two types treatments performed in this study: with CO2 microbubble injection (test) and without $CO_2$ microbubble injection (control). The study is done in duplicate.

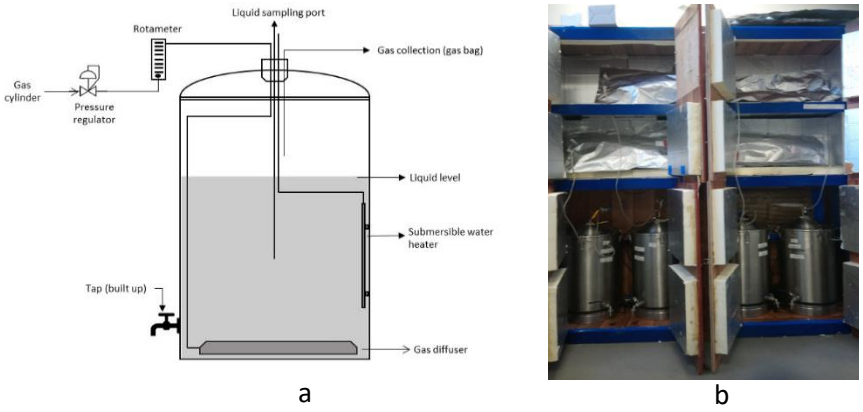



Figure 5. 1. The experimental set-up with (a) is the batch reactor and (b) is the incubator to place all the reactors

### 5.2.3. Analysis of chemical and physical properties

All the chemicals analysis those are performed in this study is the same as that of the Chapter 3. In short, the liquid parameters those are measured are pH, VFA and total ammonia nitrogen (TAN). While for the solid content, the measure parameters are total solid (TS), total volatile solid (TVS), chemical oxygen demand (COD) and elemental composition. The macronutrient composition is estimated based on composition on the food label displayed. Five gases are measured using GC. They include: $CH_4$, $H_2$, $CO_2$, $N_2$ and $O_2$.

### 5.2.4. Microbial analysis

#### 5.2.4.1. Sample collection

The samples for microbial analysis were taken in five different times from each reactor: starting time, one hour after it was started, 24 hours from the starting time, the day when the have the highest rate of methane production and the 30$^{th}$ day. The samples were taken from the tap at the bottom of the reactor. Before a sample was taken, gas mixing was applied to each reactor for about 10 minutes. $CO_2$ was used for the gas for the tested reactors, while nitrogen was used for the control.

For the sample, 45 mL of liquid media was taken from each reactor and filled in a sterile 50 mL Falcone® tube. It was then centrifuged using 5000g RCF for 15 minutes. After the supernatant was removed, another 45 mL of deionized water was put into the tube and

the centrifuged again. The similar process was repeat twice before finally we got the final solid sample. All these samples were stored under -80$^{o}$C before being analysed.

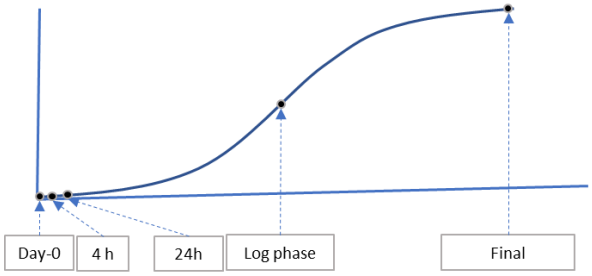



Figure 5. 2. Time when the sample for microorganisms were collected

### 5.2.4.2. DNA extraction

The DNA of the solid samples was extracted using extraction kit from Qiagen (DNeasy PowerSoil Kit). About 0.29-0.30 gram of sludge sample was taken from each treatment to be extracted. All the procedure of extracting followed the guidance from the kit. The quantity and quality of the extracted DNA was firstly carried out using NanoDrop (ThermoFisher Scientific, Waltham, MA). Qbit fluorimeter (Life Technologies, Carlsbad, CA) were used to evaluate the quantity of the extracted DNA after PCR.

### 5.2.4.3. 16S Nanopore Sequencing and Data Analysis

Starting sample was quantified using the Qubit High Sensitivity double stranded DNA kit. 10ng of sample was combined with 1ul of primers with a unique barcode, 25µl of NEB Long Amp Taq 2X master mix, and nuclease free water to a final volume of 50 µl.

PCR was performed to amplify the 1500 bp 16S rRNA gene. PCR product was purified using a 0.6X Ampure bead clean up. PCR products were quantified using the Qubit HS dsDNA kit. The 16S barcoding kit is provided by Oxford Nanopores (SQK-RAB204). A detail barcode of each sample can be seen more detail in Appendix The 3' flanking sequence of the forward primer contains a wobble base (denoted by M; in the primer the base is either an A or a C) in a variable region of the 16S gene. The forward 16S primer is 5' - ATCGCCTACCGTGAC - barcode - AGAGTTTGATCMTGGCTCAG - 3' and the Reverse 16S primer is 5' - ATCGCCTACCGTGAC - barcode - CGGTTACCTTGTTACGACTT - 3'.

An equal amount of each sample was pooled and combined with 1 µl of the Oxford Nanopore rapid sequencing adapter and incubated for 5 minutes before the pool was loaded on to Oxford Nanopore R9.4.1 flow cell and sequenced on a GridION sequencing platform. The sequencing data is analysed using an online Nanopores pipeline to acquire the taxonomy id (taxid) of each gene. The taxid is then processed using 'Taxize' package in R to retrieve the microbial identity and its lineage from **NCBI database**.

## 5.3. Result and Discussion

### 5.3.1. Digestate characterization

All the treatments were carried out for a maximum 30 days, despite the possibility that the test reactors can still produce the gas for a few more days. On the other hand, the control reactors show a shorter treatment duration seen by no more gas that could not be produced after day-21 and day-20 for Test-1 and Test-2 reactor, respectively. All the treatments were started at a high pH (8.3). High total ammonia nitrogen (TAN) is suggested to be the main reason of the high pH. As shown in Fig. 5.5, the initial TAN was 3238 and ended with slightly higher TAN (3425-3530).

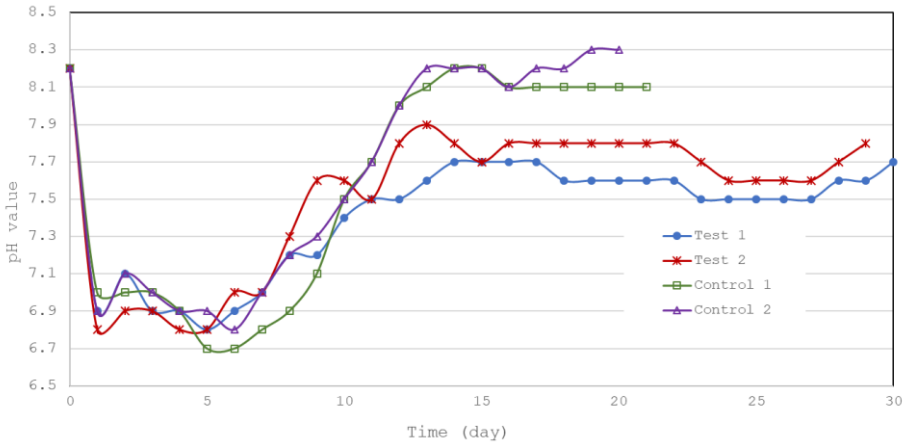



Figure 5. 3. pH value of each treatment

Within 24 hours, the pH dropped to be below 7.0 for all treatments (Fig. 5.3). A rapid increase of VFA is suggested to be the reason of this pH drop. This increase in VFA is shown in Fig. 5.4. No significant difference in the VFA concentration is shown between test and control reactors although the test reactors show higher degradation rate (Fig. 5.4). This is

because VFA is intermediate products, which the presence is very dynamics because they are produced and utilized at the same time.

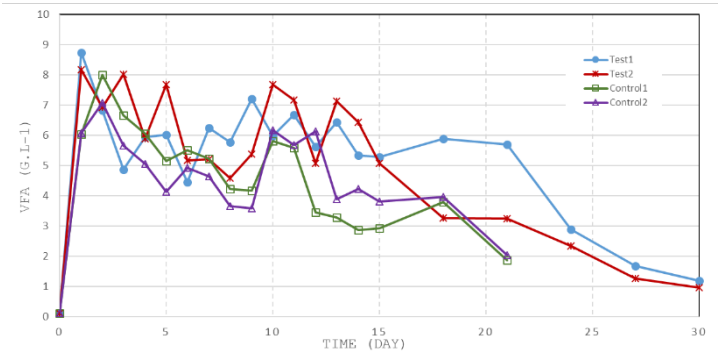



Figure 5. 4. Total VFA concentration of each treatment.

The pH values in all reactors show a rapid increase after day-10 and the value was higher than the optimum condition. While the test reactor shows an increase in pH which is only slightly higher than 7.5, the control reactor reaches a pH of up to 8.3. Since TAN concentration of all treatments did not show a significant difference, this pH difference is suggested due to VFA concentration and injection $CO_2$.

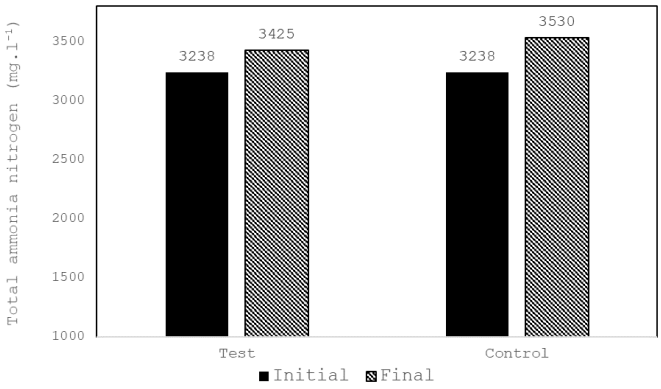



Figure 5. 5. The average value of total ammonia nitrogen (TAN) of test and control reactor in the initial and final time.

The average reduction of TS, TVS and total COD observed in the study is presented in Table 5.2. There is no significant difference in the reduction of all solid parameters between the test and the control reactors. It means that the injection of $CO_2$ microbubbles did not affect the degradation fraction of the substrate.

Even though there is no significant difference in the degraded fraction of the, it affects the substrate degradation rate. An intensive discussion about this degradation read can be read in Chapter 3.

Table 5 2. Summary of digester performance

| | Solid Reduction | | | | Average $CH_4$ production | |
|---|---|---|---|---|---|---|
| | TVS (%) | TS (%) | COD (%) | Volume (litres) | Yield ($LCH_4$. $kgTVS^{-1}$) | Prod. Rate ($L\ CH_4$. $kgTVS^{-1}\ day^{-1}$) |
| Test | 79.1 | 63.12 | 66.11 | 235.96 | 473.16 | 15.77 |
| Control | 78.24 | 62.75 | 66.25 | 62.01 | 124.45 | 5.93 |
| Test/Cont. | 1.01 | 1.01 | 1.00 | 3.81 | 3.80 | 2.66 |

### 5.3.2. Biogas production

The production of methane, $CO_2$, and $H_2$ is presented in Fig. 5, 6 and 7, respectively. The figure for methane is shown in a cumulative volume, while the $H_2$ and $CO_2$ are in a daily volume. The treatment for the test reactors was stopped in the day-30, even though the reactor might still be able to produce biogas in the next few days if the process is continued. All control reactors were completely finished earlier (21 days) with significantly low methane volume. Inhibition of free ammonia is suggested to be the main reason since the medium TAN concentration is considered to be toxic for the methanogen. It is different from the test since periodic gas stripping is performed daily that may result in regular removal of free ammonia.

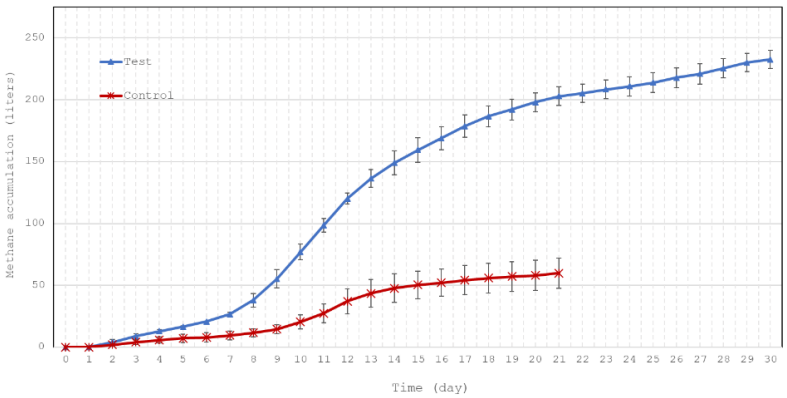



Figure 5. 6. Cumulative methane production

All the reactors began to produce biogas on the first day, without any methane detected in the biogas in all treatments. On the first day, the composition of the biogas was

dominated by $CO_2$ and followed by $N_2$ and $H_2$ (Appendix 5). The highest volume of methane produced in the test reactors occurred on the day-9. The volume was 17 liters and 12 liters for the test-1 and test-2 respectively. For the control, the highest methane production was on the day-10 with only 5.5 liters and 4 liters for the control-1 and control-2, respectively. In the third week, the methane production by the control was started to flatten and totally finished on the day-21. Even though the treatment is stopped on day-30, the test reactor seems to still be able to produce methane if the treatment is continued. This can be seen from the methane curve that is still not flattening. On average, the test reactor is able to produce methane with a volume and yield 3.8 times compared to that produced by the control (Table 5.2). The test reactors also demonstrate a higher methane production rate, which is around 2.66 times as much as the control.

In contrast with the methane production in the early days, $CO_2$ is produced in a high volume by most treatments. A notably higher $CO_2$ is produced by both test reactors, which reached 68 liters and 47 liters on the first day for the test-1 and test-2, respectively. For the control-1, the highest daily $CO_2$ production was on the second day, which reaches nearly 20 liters. In fact, the daily $CO_2$ that is injected into the test reactors is only $10\pm0.1$ liters. It means the rest of the $CO_2$ volume is biologically produced by the system. Since $CO_2$ is an intermediate product in AD, the actual $CO_2$ that is biologically produced by the system can be higher than that is measured from the gas collection bag.

In an AD, $CO_2$ is produced by many steps, i.e. alcohol generation, acid generation and methane generation via acetolactic pathway. Since there was no methane produced in the first day, it can be assumed that the produced $CO_2$ on that day comes only from the alcoholic and acid generation process. A high presence of $CO_2$ in the biogas also means a high rate of the alcoholic and acid generation process. In fact, hydrolysis is the first process to degrade the substrates. It means that massively higher $CO_2$ produced in the early days has a significantly higher degradation of the substrate.

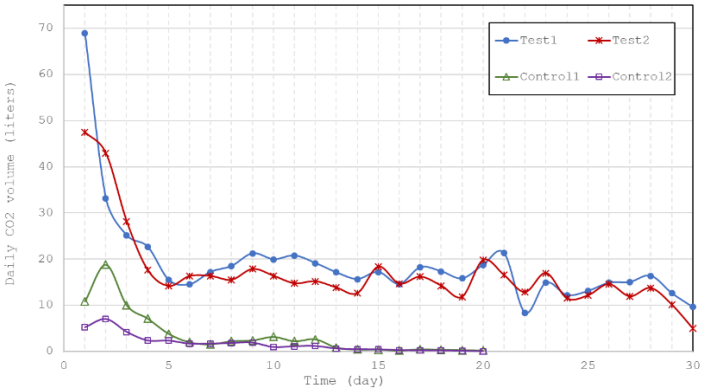



Figure 5. 7. Daily volume of CO2 in the gas collection gas

A notable $H_2$ volume by the test reactors is also observed in the gas collection bag. It started at about 1.1 liters on the first day and reached 5 liters on the second day for the Test-2 reactor (Fig. 5.7). Lower methane production is shown by the Test-1 reactor. This high volume is the result of the high volume of biogas and a high concentration of $H_2$ in the biogas that reached 15% on that day. As for the control, high $H_2$ production is shown on the first day only by the Control-1 reactor with the volume was 1.2 liters. Much lower $H_2$ is produced by the Control-2 reactor. In this AD system, $H_2$ is considered as an intermediary since it is one of the precursors of methane. The presence of this gas in the biogas can be assumed as energy loss. Hence, a relatively high energy loss occurs in the first two days of treatment by the Test-1 and Test-2 reactors.

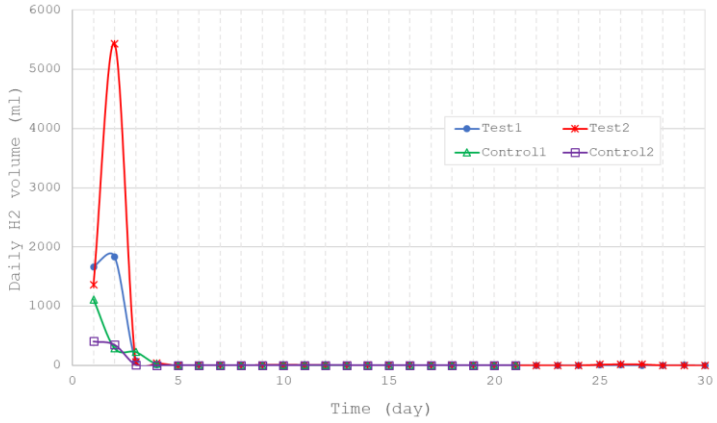



Figure 5. 8. Daily volume of the $H_2$ in the gas collection bag

In the AD process, $H_2$ is mainly produced during acidogenesis and acetogenesis. $H_2$ can also be produced by syntrophic acetate oxidation bacteria (SAOB) by up-taking acetate to

produce $H_2$ and $CO_2$. As relatively high hydrogen volume occurs on the first two days, it likely is produced more by the acidogenesis and acetogenesis process. This is supported by a high pH drop that means a high VFA formation in that early stage. As this process needs feeding of soluble substrate only, a disintegration of the substrate should have happened prior to these processes. This may provide an idea of how $CO_2$ microbubbles increase the degradation rate of the substrate.

## 5.3.3. Microbial community analysis

### 5.3.3.1. General result

The nucleic acid concentration that is extracted from each sample may provide an indication of the microbial abundance in respect of sampling time (Fig. 5.9). For this microbial analysis, only samples from Test1 and Control1 are carried out. About 290mg of solid is extracted from each sample. Initially, both treatments show a relatively same amount of nucleic acid, which is about 110 $ngL^{-1}$ to 120 $ngL^{-1}$. Four hours later, a decline in the microbial abundance is shown by the control. On the other hand, the microbial abundance of the test is relatively stable with only a slightly increase.

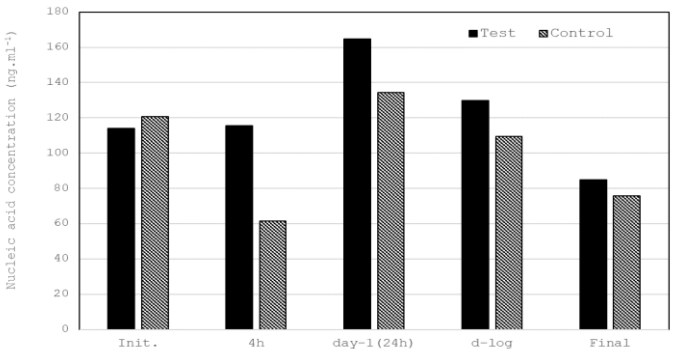



Figure 5. 9. Concentration of the nucleic acid extracted from each samples.

Normally, the microbial community experiences an adaptation period (lag phase) that is demonstrated by a constant number of microbial populations. Population decline is also possible during this adaptation period. The duration it takes for the lag phase varies. Internally, the microbial ability to adapt to their environment is the most significant factor, while how significant the environment changes from the initial condition is the external factor. As the microbial community of both treatments comes from the same source, the

environmental difference between the two treatments may the sole factor for this population difference.

The highest abundance for both treatments (among five sample points) is shown on day-1, in which their nucleic acid reaches nearly 180ng$L^{-1}$ and 140 ng$L^{-1}$ for the test and control, respectively. There is a possibility that this abundance keeps increasing in the next few days. The highest microbial abundance on day-1 may show the highest microbial activity in that particular time. The $d_{log}$ samples are taken in the day when the reactors reach their highest methane production rate. It occurred in day-9 for the test reactor and day-10 for the control reactor. On $d_{log}$, the nucleic acid concentration of both treatments shows a decrease in the number of nucleic acids. This contrasts with the fact that the highest methane production occurs on a particular day. A decrease in the bacterial activity may take place, while at the same time, the methanogenic activity may increase. In fact, the microbial abundance is not always proportional to their activity (Hanreich et al., 2013). A lower abundance is also shown by both reactors at the end of the treatment.

A PCR is performed to amplify the DNA with the universal primer provided by Oxford Nanopores. Each sample shows a high read, which is between 50k to 100k reads. Unfortunately, since the PCR primer is only fit for amplifying bacterial genes, the archaeal community is only identified in one sample with a very low quantity. Among the ten samples, those are analyzed, only the $d_{log}$ sample of the test show a number of archaeal communities. However, it is very insignificant with 11 reads of a total of 90,800 read, or only about 0.012%. These identified archaea belong to *Methanosarcinales* order, in which nine of them from the family *Methanosarcinaceae*. *Methanosarcinaceae* is a robust and versatile methanogen that can produce methane from various carbon sources, such as $CO_2/H_2$, acetate and formate (De Vrieze et al., 2012). An insignificant number of protozoa is also detected in almost all samples. The presence of some protozoa has also been reported by Abram et al., (2011) with their role in anaerobic digestion is remain unknown. Normally, the archaeal community constitutes at least 1% of the total microbial community inside an anaerobic digester. A different primer should be used for the archaeal community. It can be sequenced simultaneously with bacterial primer, or separately using real-time PCR (De Vrieze et al., 2015; Kong et al., 2018).

Between 50,000 to 100,000 readings are obtained from each sample. This led to the identification of up to 1,500 species that belong to 11 different phyla. The richness of species is different in each sample. The maximum richness is shown at the starting point, in which around 1500 is obtained. It decreases within 24 hours with only around 500 species identified. This decrease does not necessarily mean those species are no longer present, but only cannot be identified as their abundance are relatively low. This is shown by identification of some species, which do not appear from the previous sample.

### 5.3.3.2. Bacterial community structure

At the phylum level, *Firmicute*s dominates the bacterial community ranging from 48% (initial) to 95% on day-1. It is followed by *Bacteriodetes* and *Proteobacteria* (Fig. 10). Some bacteria from phyla *Actinobacteria, Spirochaetes, Synergistetes,* and *Chloroflexi* are also identified but in small quantities. Interestingly, *Thermotogae,* which is normally identified in most AD digestate, does not appear in any sample within this study. Both the test and control show the same trend in how *Firmicutes* composition increase within 24 hours after the treatments are started. Its composition declines in the $d_{log}$, with the test reactor, shows slightly higher Firmicutes composition compared to the control's.

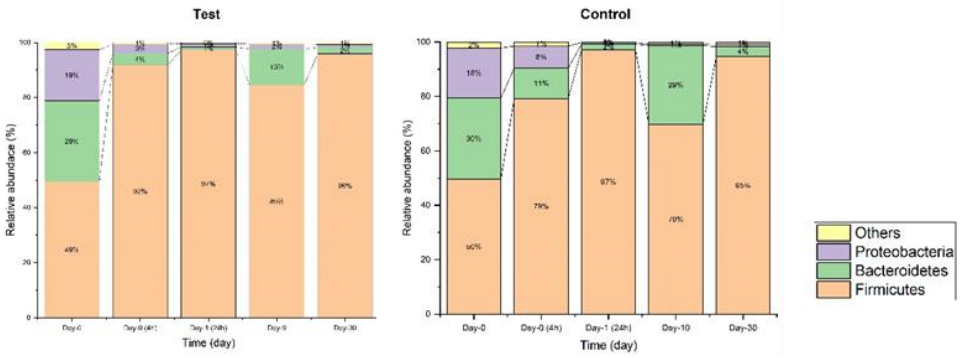



Figure 5. 10. Bar graph showing the bacterial composition in a phylum level.

At the order level, *Clostridiales*, which belong to phyla *Firmicutes,* is the dominant bacteria. It starts with relative abundance (RA) less than 20%. Their abundance increases dramatically within 24 hours in both treatments. In the test reactor, their RA reaches 90% within 24 hours. It remains the dominant order until the end of the process with some decrease of RA in $d_{log}$. In the control, the *Clostridiales*' RA reaches 60% after 4 hours since the treatment started but it then declines to only about 40% in the day-1. The increase

during the first four hour in the controls is suspected due to the decrease of some other bacterial order. It is shown by the decrease in the gene's reading as well as the decrease in the nucleic acid concentration extracted from the sample (Fig. 9).

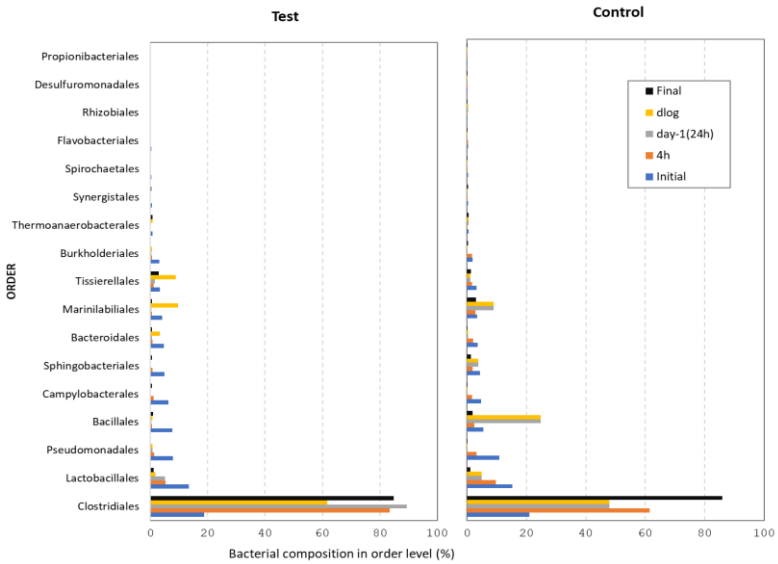



Figure 5. 11. Bacterial order that comprise more than 90% abundance in the reactor, with exclusion for the initial sample, in which it comprises only 76-78% of the total identified bacteria.

*Clostridiales* are well known for their ability to use a wide range of substrate, including complex polysaccharides, such as cellulose and hemicellulose (Chen et al., 2005; Hanreich et al., 2013; Zverlov and Schwarz, 2008). Normally, they share their role in degrading substrate with *Bacteriodales*, which their number is insignificant in this study. Following *Clostridiales, Bacillales* shares about 20% of the total bacterial abundance in the control's reactor. It is followed by *Lactobacillales*, which order is the second most abundant bacteria in the test reactor (Zverlov and Schwarz, 2008). This data shows that the bacterial community is dominated by those involved in the substrate degradation process.

The *Clostridiales* starts with around 20% abundance. They increase rapidly within 24 hours of treatment. Fig. 11 shows the *Clostridiales* abundance of the test reactor is significantly higher for the sample 4h and day-1. Since *Clostridiales* is a substrate degrader, a high abundance of *Clostridiales* may the reason for higher substrate degradation of the test reactor in the early stage.

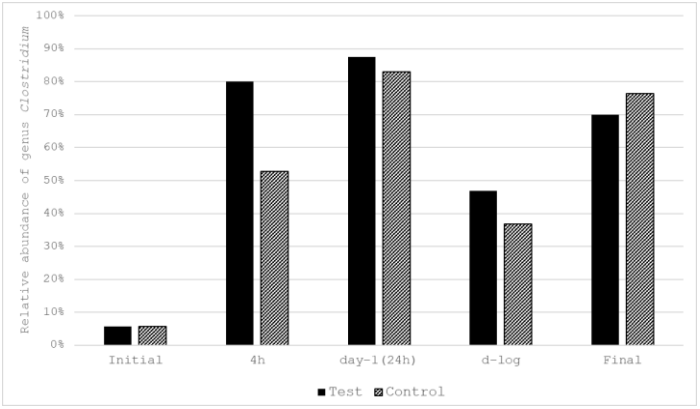



Figure 5. 12. Relative abundance of bacterial community belongs to genus Clostridium in respect of sampling time.

Except for the initial sample, *Clostridium* is the most abundant genus in the reactor (Fig. 12). It grows rapidly after the treatments started. They begin from a relatively low population and reach their dominance among the bacterial community after then. In fact, *Clostridum* is the top-listed bacteria, playing their essential role in acidogenesis and acetogenesis. A significant increase of such genus in the digester may reflect a high acidogenesis and acetogenesis activity in the reactor. Some Clostridium species, such as *C. butyricum* and *C. paraputrificum* are detected with significant abundance in the samples. *C. butyricum* is an effective producer of $H_2$ in fermentative hydrogen production. It is also well known as producers of butyric acid and isopropanol (Kim et al., 2006; Yokoi et al., 1998; Zeng, 1996). The high presence of genus *Clostridium* can be the reason behind high $H_2$ and $CO_2$ volume in the early stage of treatment.

The availability of syntrophic bacteria in anaerobic digestion is essential to create a favorable environment for all microbial communities. A decrease in the adaptation period is another benefit that is provided by syntrophic bacteria (Müller et al., 2016). In this study, some syntrophic bacteria from the genus *Syntrophomonas* are identified as well, especially during the log phase. However, the number is insignificant compared to the bacteria that work in hydrolysis and acidogenesis-acetogenesis step. Besides being the primary producer of $H_2$ and $CO_2$ in the system, *C. butyricum* is reported for their role in extracellular electron transfer in a syntrophic interaction (Kong et al., 2018). The presence of an electron transfer agent makes a high possibility to convert substrate into methane more effectively.

### 5.3.4. Discussion

In this study, the bacterial community structure is observed. The composition changes with the treatment and sampling time. While it is clear that the injection of $CO_2$ microbubbles shows a significant impact on methane production, the data about the microbial composition may provide additional insight into what is occurring microbiologically during the treatment. Since archaea are only identified in one sample, the discussion will focus on the bacterial community.

In general, the bacterial community is dominated by *Firmicute* phylum, with *Clostridiales* showing the dominant bacteria in genus level. Since such bacteria is an effective substrate degrader, its significantly higher abundance in the early stage can be the reason for the higher substrate degradation rate after injection of $CO_2$ microbubbles. A high substrate degradation in the early stage is discussed extensively in Chapter 3. By the end of the treatment, both treatments do not show a significantly different *Clostridiales* composition. This may answer a significant increase in the substrate degradation rate In the test reactors.

Besides the higher methane production rate, another distinction in the performance of the test is its higher $CO_2$ and $H_2$ volume, especially in the first few days of the treatment. Both gases are actually fermentation products resultant from substrate conversion into alcohol and acetic acid, in which *Clostridium* is a well-known agent. A high abundance of *Clostridium* may confirm a higher fermentation activity inside the digester. Since there is no significant difference in the Clostridium composition between the treatments, this begs the question of how the test reactor achieves higher fermentation activity.

The first possible reason is the actual number of such bacteria in the test reactor may be higher than that in the control, even though with similar relative abundance. Relative abundance sometimes does not directly reflect the actual number of microorganisms since the amplification during the PCR may affect a certain microorganism to be amplified more than the others. A simple example is the absence of the archaeal community in most samples. Since the nucleic acid from each sample is extracted from the same number of solids, then the amount of nucleic acid can be used to provide a comparison of the relative abundance of microbes from a certain sample to the other samples. Using this argument,

Fig. 5.9 may show a relatively high number of *Clostridiales* during the early stage of treatment compared to the control.

Secondly, the Clostridium number between the treatments may be the same, but they may have different activity rates per bacterium. In fact, the number of microorganisms is not always proportional to their activity in the digester (Hanreich et al., 2013). A notable volume of $H_2$ and $CO_2$ in the early stage clearly illustrates this phenomenon. VFA is another product of fermentation. An increase in the VFA has been reported in several studies after $CO_2$ addition. Even though there is no significant difference in the VFA concentration between the treatments in this study, it does not mean that the VFA generation rate is low. VFA is considered an intermediate product in methane production. Their presence in the digester is highly dynamically variable. Since $H_2$ and $CO_2$ are two major fermentations (acidogenesis-acetogenesis) by-products, this can be alternative evidence of high fermentation rate after in the early stage after $CO_2$ microbubble injection. Several studies suggest a multiple -omics analysis to minimize any bias in analyzing the microbial activity at the molecular level.

Regarding which pathway dominates the methane production, the current result of the microbial analysis cannot provide clearer evidence since no archaeal community can be identified in most samples. With a high acetogenesis rate in the system, the additional production by autotrophic acetogens, which use $H_2/CO_2$, seems to be minor, based on Le Chatelier's principle. Moreover, a high ammonium concentration in the medium may result in higher activity by syntrophic acetate oxidation bacteria (SAOB) than the autotrophic acetate formation by homoacetogen (Morris et al., 2013; Nüsslein et al., 2001). As for the syntrophic bacteria, they mostly have an ability to change the process direction due to the variation of the environmental conditions (Morris et al., 2013). Thus, a deeper analysis at the molecular level should be performed to reveal a non-biased analysis of the activity of the syntrophic bacteria during the treatment.

Normally, the addition of $CO_2$ into AD can enhance the methane production around twice as much when $H_2$ is not supplemented along with $CO_2$. Relatively low methane yield is shown by the control. A theoretical methane potential presented in Chapter 3 can be the standard of methane yield in this study since both have a similar composition. Free ammonia toxicity can explain this low methane yield. However, this toxicity seemingly

does not affect the bacterial community, in the sense of abundance. Methanogenic archaea should be affected the most since they are sensitive to any inhibition. However, the result of the current analysis cannot prove the hypothesis with certainty.

## 5.5. Conclusions

Injection of $CO_2$ microbubbles enhances methane production in anaerobic digestion of food waste. A methane volume and yield up to 3.8-fold is shown by the treatment with $CO_2$ microbubble injection. *Clostridiales* and *Clostridium* are the dominant order and genus, respectively observed in the samples regardless of the treatment. A significantly higher *Clostridiales* abundance in the test reactor in the early stage explains higher substrate degradation of the test. As well, high Clostridium abundance is found consistent with high $H_2$ and $CO_2$ daily volume in the first few days of treatment. Since archaea cannot be identified by the current analysis, the possibility of pathway alteration cannot be explored.

# Chapter 6

# Conclusion and Future Work

## 6.1 Conclusion

This research is motivated by seeking numerous possibilities to optimize anaerobic digestion (AD) performance in treating food waste, especially using $CO_2$ microbubble. The main objective of this study is to gain a comprehensive understanding of the mechanisms of $CO_2$ microbubbles in enhancing methane production. This report covers three main specific objectives, which all of them are laboratory-based study.

The first part of this study examines the hypothetical mechanism of how $CO_2$ microbubble injection can increase methane production. The study concludes that periodic injection of $CO_2$ microbubble into the AD treatment can boost methane production in three mechanisms. Firstly, it increases the substrate degradation rate. A high degradation rate estimated from the methane data supports this hypothesis. A notable daily volume of $CO_2$ and $H_2$ indicates a higher substrate degradation as well as acetogenesis process, especially in the early stage of treatment. Secondly, it provides an additional carbon source for the system. This is proved by the methane yield that is higher than that of the theoretical methane potential. Lastly, $CO_2$ microbubble promotes more efficient substrate utilization that leads to higher methane production. This is shown by the high efficiency of acetogenesis and methanogenesis process. Regarding the last hypothesis, there are at least three mechanisms that promote a higher efficiency of substrate utilization. Decreasing hydrogen partial pressure is the first one. The second mechanism is based on Le Chatelier's principle, in which the gas injection promotes the removal of products that

results in a higher production rate of methane. Removing some toxic chemicals from the medium is the third mechanism.

The ability of $CO_2$ microbubbles to reduce the toxicity effect is highlighted in the second part of this study. In this study, landfill leachate is added as the source of toxic chemicals, besides it also gives the benefit of micronutrient into the medium. In this study, periodic injection of $CO_2$ microbubbles shows its ability to simultaneously enhance methane production while at the same time, it reduces the toxicity effect of the added landfill leachate.

In the third study, a microbial outlook is observed. The microbial community structure between the test and control is compared in regard to five different sampling time: initial, four hours and 24 after the treatment starts, the time when each treatment reaches the highest production rate and at the end of the process. In general, the phyla are dominated by Firmicutes, in which Clostridiales is the most abundant order. A significantly higher abundance of this order in the test reactor within the first 24 hours of the treatment may answer a higher degradation rate of the test reactor. A high daily volume of $CO_2$ and $H_2$ shows a higher acetogenesis process, which is also indicated by the relatively higher abundance of Clostridium genus in the test reactor.

## 6.2. Future Work

While three hypotheses are answered in this study, a lab-scale treatment is remaining the scale of the study. For an application on an industrial scale, a study using a higher reactor volume is essential. A modelling study that involves an industrial standard model (ADM1) may be necessary before deciding for industrial-scale application. The challenge for this modelling might be how adding the $CO_2$ in the substrate parameter.

In Chapter 4, there is a left question whether the toxicant is dominantly removed by the gas sparging or biodegradation. It is necessary to do a future study to reveal it. A suggested study would be doing the gas sparging to the landfill leachate before adding it into the AD process.

Since no archaeal community is identified during the sequencing process, it may be necessary to do an additional sequence or real-time PCR that uses a primer that fits

archaea. This analysis may answer how $CO_2$ microbubble affects the archaeal structure in the digester. It is important as the last study shows the methane production of the control in this study is much lower than that of the control of the previous study. In contrast, for the test, the methane volume shows no significant difference from that of the previous study.

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# Appendix

# Appendix 1. Preliminary study: Exploring the potency of $CO_2$ microbubble to chemically/physically degrade the substrate.

**1. Objective:**

Observing the capability of $CO_2$ microbubble to do an oxidative hydrolysis. It was hypothesized that $CO_2$ microbubble might have potency to produce hydroxyl radical, without acid solution or dynamic stimuli.

**2. Experimental Set-up**

The equipment set-up is presented in Figure A2.1. The $CO_2$ microbubble was induced to the reactor for 15 minutes for each repetition. Every 5 minutes, 2 ml of liquid was taken from the rig to analyse the sCOD. The flowrate of the pure $CO_2$ was set to be between 0.1 to 0.2 LPM with the pressure was 2.2 bar.

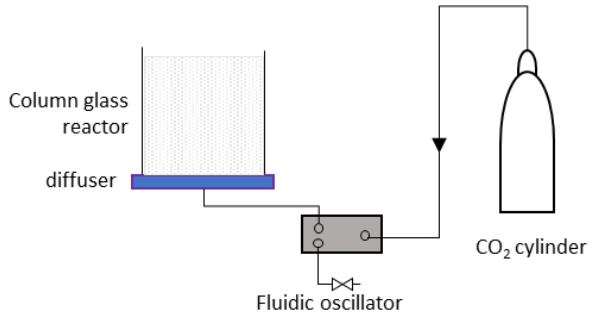



Fig.A2.1. Experimental set-up for chemical/physical degradation of substrate

For each treatment, the crystalline cellulose was fed as the substrate. The cellulose slurry is prepared in 100 ml working volume using deionized water as a medium. The prepared concentration for cellulose slurry is 5% w/v. Since pure crystalline cellulose is insoluble, the soluble COD (sCOD) was the only chemical analysis to be carried out to see if there was any degradation due to the $CO_2$ microbubble sparging. The analysis was done before and after the treatment.

**3. Result**

| | Total sCOD at sampling time ($mg.L^{-1}$) | | | |
|---|---|---|---|---|
| | Initial | 5 minutes | 10 minutes | 15 minutes |
| Repeat-1 | 66.66 | 69.99 | 71.04 | 68.56 |
| Repeat-2 | 62.01 | 61.33 | 62.13 | 59.77 |
| Repeat-3 | 78.06 | 77.67 | 77.86 | 77.94 |

The above table shows the soluble COD in each sampling time in all repetition. It shows a slight fluctuation in the sCOD. Even though it was thought that there would be no sCOD initially as crystalline cellulose is insoluble, but the reading still shows small number of sCOD in the initial samples. Reading error there might be some impurity in the cellulose powder. This data also shows that there was no substrate disintegration take place during the microbubble treatment.

# Appendix 2. Preliminary experiment: Effect of leachate addition in the anaerobic digestion (AD) of food waste.

**1. Objective:**

The preliminary experiments were performed in two different reactor size, i.e. 1 L glass reactor and 20 L stainless steel reactor. The objective of the experiment in the 1 L reactors was to observe how the leachate may affect the anaerobic performance of food waste. Different leachate to seeding sludge ratio was applied to determine which composition should be taken for the bigger scale with $CO_2$ microbubble. The experiment in the 20 L reactor was to initially observe how $CO_2$ microbubble might affect the AD performance, especially with the leachate addition.

**2. Experimental Set-up and Result**

2.1. Experiment with 1 L glass bottle

In this experiment, three different composition of leachate were observed, i.e. 5%, 10%, 15%, and 20%. One control was carried out. The set-up of the experiment is shown in Fig A1. This experiment was carried out with 1 L glass reactors (Duran® glass) with 500mL working volume. Each reactor has three outlets made of metal bulkhead fitting (4mm), which was for gas output, gas flushing and liquid sampling. All the reactors were placed in the water bath. The water bath was laboratory-made. It consists of one plastic container (16 L), multi-position magnetic stirrer (RT 15 IKAMAG) and an immersion circulator heater. Plastic gas bag was used to collect the gas. The experiment was carried out under mesophilic conditions for a maximum 20 days. Two experiments were carried out with different feeding substrate.

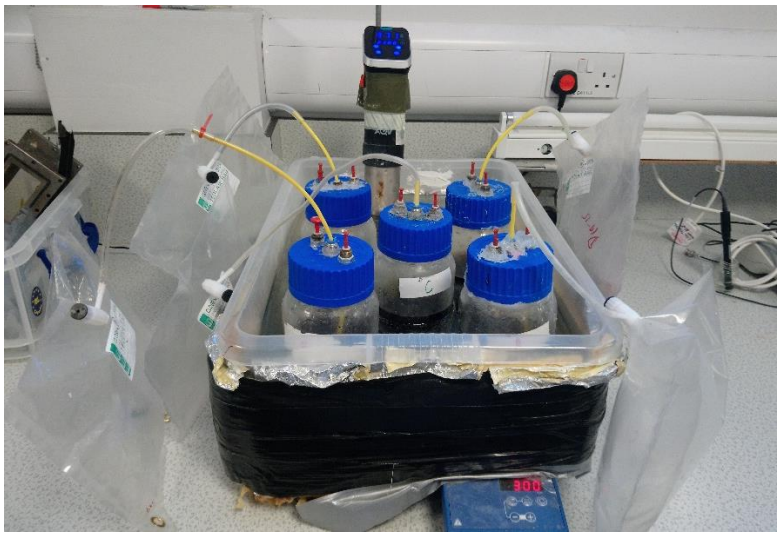
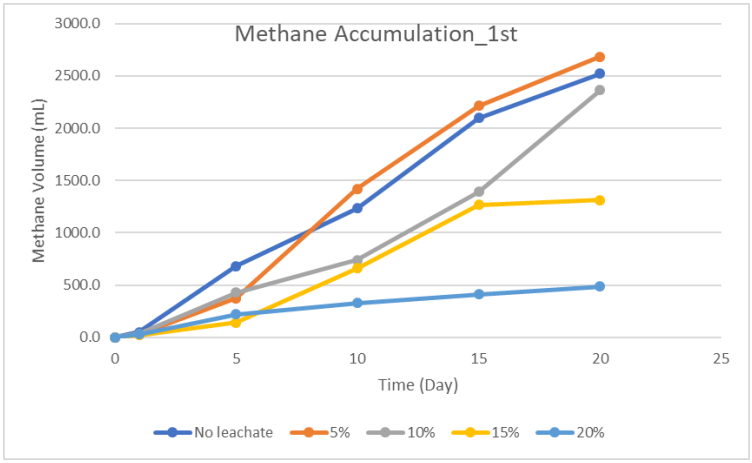


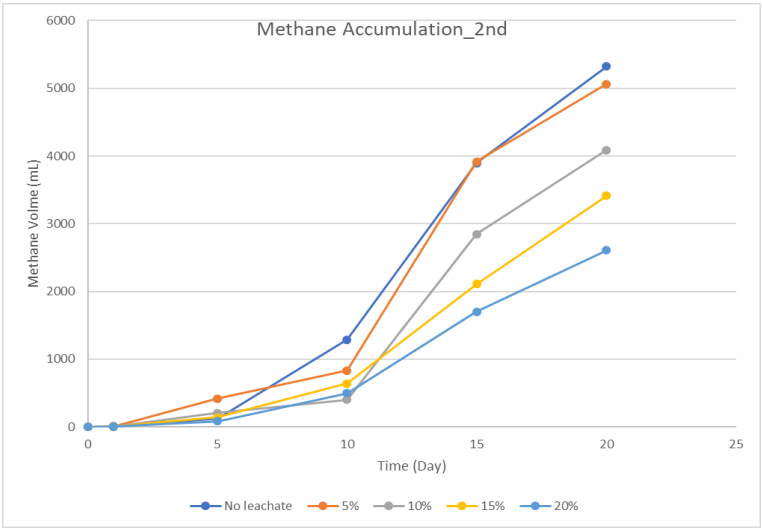
Fig. A1.1. Experiment set-up of 1 L bottles

For the first run, each reactor was fed with 50 gram of real food waste, while for the second run, 100 gram was fed into each reactor. The food waste was collected from some cafes located in the University of Sheffield. For the control, 500 mL of seeding sludge was

used in each reactor. The seeding sludge was degassed by placing it in the water bath (37°C) for few days until no biogas produced by all the reactors. The amount of leachate added into the test reactor depend on the targeted ratio of leachate to seeding sludge, which was 5%, 10%, 15%, and 20%. All chemical analysis was done using the same method as describe in the method chapter.

**Result**




A.1.2. Methane accumulation of the 1L reactor test

2.2. Experiment with 20 L reactors

The experiment set up for the 20 L reactor was exactly the same as the set-up for the main experiment that have been written in the main chapter. Instead of using a synthetic waste, this experiment used a real waste. This experiment was carried out in three cycle.

**Result**

The result shown in Fig A.1.2 is the methane accumulation during the preliminary study. In the first cycle, the temperature control for the submersible heater in two reactors (treatment B and C) were broken, making the temperature of both reactor reach 50°C. It was suspected that the temperature control failed in the 22th day. It resulted in the significant temperature difference from the other two. The biogas production also stopped in the 28th day for both the failed reactors.

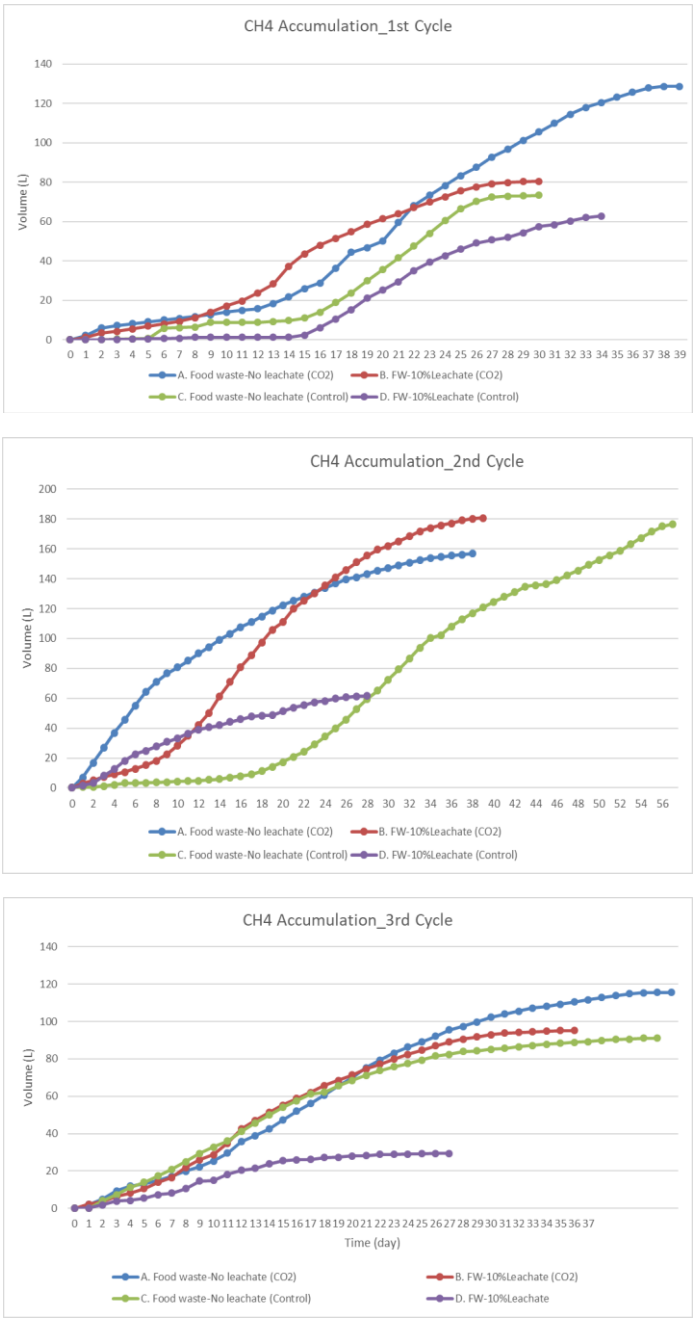



Fig. A1.3. Methane accumulation during the preliminary study.

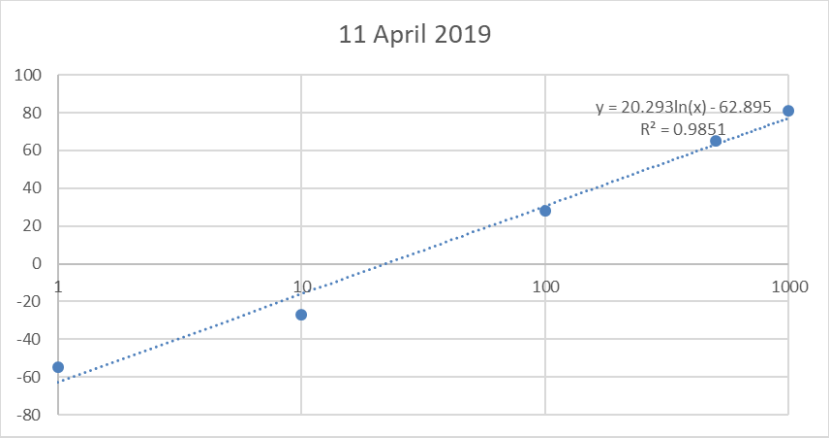
## Appendix 3. Recipe for making the reproducible food waste

| No. | Material | Amount (gram) |
|---|---|---|
| 1 | Softwhite bread (Tesco Brand) | 400 |
| 2 | Wholewheat bread (Tesco Brand) | 400 |
| 3 | Baked bean (Tesco Brand) | 420 |
| 4 | Mixed salad | 150 |
| 5 | Water | 630 |
| | Total Mass | 2000 |

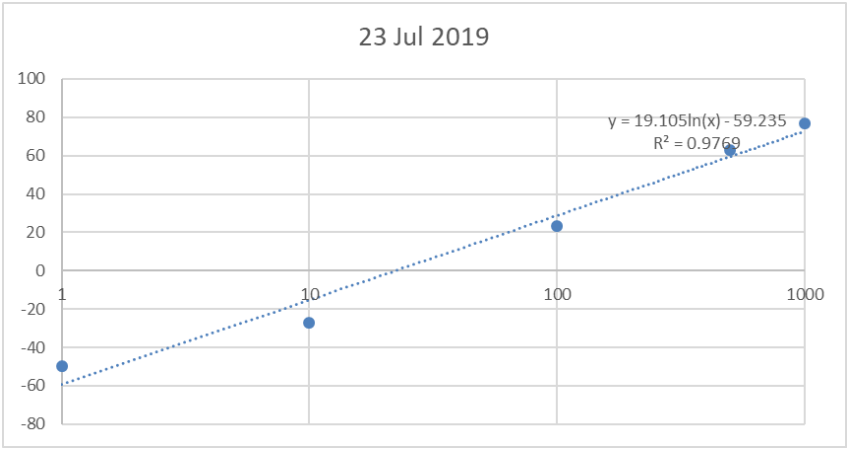
## Appendix 4. Examples of calibration for the total ammonia nitrogen (TAN) probe

The TAN probe was calibrated following the company guidance. Instead of four different concentration, five concentration was carried out to increase the accuracy. Those concentrations are: 1ppm, 10ppm, 100 ppm, 500 ppm and 1000 ppm. The calibration result could only be used for the same day measurement. Therefore, more than 10 calibration has been done for measuring all the TAN. In the following picture, only three calibration are shown to give a rough idea of the calibration method. The correlation coefficient ($R^2$) should be maintained to be higher than 0.95.

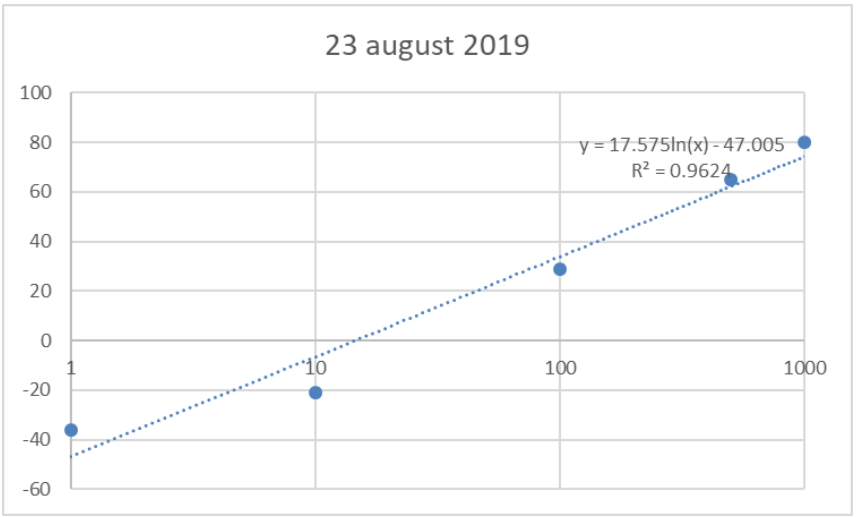
| 11-Apr-19 | |
|---|---|
| Conc. (ppm) | Reading (mV) |
| 1 | -55 |
| 10 | -27 |
| 100 | 28 |
| 500 | 65 |
| 1000 | 81 |




| 23-Jul-19 | |
|---|---|
| Conc. (ppm) | Reading (mV) |
| 1 | -50 |
| 10 | -27 |
| 100 | 23.5 |
| 500 | 63 |
| 1000 | 77 |




| | 23-Aug-19 |
|---|---|
| Conc. (ppm) | Reading (mV) |
| 1 | -36 |
| 10 | -21 |
| 100 | 29 |
| 500 | 65 |
| 1000 | 80 |



Appendix 5. Barcode of each sample for 16S Nanopores sequencing.

| Sample ID | Nanopore Barcode | Barcode Sequence |
|---|---|---|
| A0 | 1 | AAGAAAGTTGTCGGTGTCTTTGTG |
| A04 | 2 | TCGATTCCGTTTGTAGTCGTCTGT |
| A1 | 3 | GAGTCTTGTGTCCCAGTTACCAGG |
| A9 | 4 | TTCGGATTCTATCGTGTTTCCCTA |
| AF | 5 | CTTGTCCAGGGTTTGTGTAACCTT |
| CO | 6 | TTCTCGCAAAGGCAGAAAGTAGTC |
| C04 | 7 | GTGTTACCGTGGGAATGAATCCTT |
| C1 | 8 | TTCAGGGAACAAACCAAGTTACGT |
| C10 | 9 | AACTAGGCACAGCGAGTCTTGGTT |
| CF | 10 | AAGCGTTGAAACCTTTGTCCTCTC |
| neg control | 11 | GTTTCATCTATCGGAGGGAATGGA |
| post control (log) | 12 | CAGGTAGAAAGAAGCAGAATCGGA |

## Appendix 6. Biogas composition for study in Chapter 5

| Day | Test1 | | | Test 2 | | |
|---|---|---|---|---|---|---|
| | $CH_4$ (%) | $H_2$ (%) | $CO_2$ (%) | $CH_4$ (%) | $H_2$ (%) | $CO_2$ (%) |
| 0 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 1 | 0.00 | 1.83 | 107.77 | 0.00 | 2.44 | 117.42 |
| 2 | 11.29 | 10.31 | 81.39 | 11.30 | 9.78 | 76.18 |
| 3 | 19.15 | 0.28 | 94.25 | 15.02 | 0.22 | 97.09 |
| 4 | 19.23 | 0.05 | 92.79 | 15.58 | 0.19 | 79.54 |
| 5 | 21.39 | 0.02 | 77.43 | 15.71 | 0.00 | 70.59 |
| 6 | 26.13 | 0.03 | 76.32 | 17.62 | 0.02 | 86.84 |
| 7 | 32.57 | 0.02 | 75.83 | 22.04 | 0.02 | 82.05 |
| 8 | 44.58 | 0.03 | 52.67 | 31.46 | 0.02 | 64.92 |
| 9 | 51.27 | 0.02 | 48.21 | 49.41 | 0.04 | 53.86 |
| 10 | 54.19 | 0.02 | 44.60 | 57.83 | 0.03 | 36.21 |
| 11 | 51.81 | 0.03 | 52.26 | 61.10 | 0.03 | 41.78 |
| 12 | 57.17 | 0.02 | 51.67 | 63.03 | 0.03 | 42.29 |
| 13 | 62.54 | 0.03 | 55.60 | 42.38 | 0.02 | 43.84 |
| 14 | 54.76 | 0.03 | 57.31 | 41.37 | 0.02 | 50.88 |
| 15 | 40.13 | 0.02 | 64.68 | 34.02 | 0.01 | 60.11 |
| 16 | 42.18 | 0.02 | 69.37 | 42.72 | 0.02 | 63.61 |
| 17 | 35.80 | 0.02 | 69.31 | 38.90 | 0.02 | 62.75 |
| 18 | 29.45 | 0.02 | 68.63 | 38.17 | 0.02 | 62.39 |
| 19 | 26.69 | 0.02 | 77.49 | 26.69 | 0.02 | 59.08 |
| 20 | 24.93 | 0.01 | 91.97 | 27.35 | 0.01 | 78.92 |
| 21 | 20.70 | 0.01 | 90.98 | 22.39 | 0.01 | 77.20 |
| 22 | 20.28 | 0.01 | 83.51 | 16.38 | 0.01 | 85.37 |
| 23 | 18.47 | 0.01 | 81.27 | 16.34 | 0.01 | 87.52 |
| 24 | 18.80 | 0.01 | 81.66 | 15.19 | 0.01 | 84.38 |
| 25 | 22.03 | 0.02 | 92.28 | 19.59 | 0.10 | 80.87 |
| 26 | 21.46 | 0.01 | 79.23 | 21.25 | 0.12 | 82.22 |
| 27 | 17.21 | 0.01 | 83.31 | 20.61 | 0.12 | 77.80 |
| 28 | 21.61 | 0.01 | 81.88 | 27.10 | 0.02 | 76.33 |
| 29 | 24.66 | 0.01 | 75.51 | 34.19 | 0.01 | 68.20 |
| 30 | 22.43 | 0.01 | 77.27 | 29.00 | 0.01 | 71.15 |

| Day | Control1 | | | Control2 | | |
|---|---|---|---|---|---|---|
| | $CH_4$ (%) | $H_2$ (%) | $CO_2$ (%) | $CH_4$ (%) | $H_2$ (%) | $CO_2$ (%) |
| 0 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 1 | 0.00 | 5.38 | 31.84 | 0.00 | 3.91 | 50.34 |
| 2 | 9.21 | 10.33 | 66.79 | 11.29 | 2.90 | 58.36 |
| 3 | 22.69 | 1.64 | 71.18 | 8.81 | 0.05 | 34.84 |
| 4 | 26.62 | 0.26 | 73.42 | 12.14 | 0.00 | 30.97 |
| 5 | 32.21 | 0.03 | 68.50 | 10.57 | 0.00 | 38.50 |
| 6 | 34.39 | 0.02 | 62.72 | 10.58 | 0.03 | 32.75 |
| 7 | 38.57 | 0.02 | 57.17 | 44.88 | 0.02 | 37.25 |
| 8 | 44.40 | 0.02 | 52.82 | 40.96 | 0.02 | 22.55 |
| 9 | 50.19 | 0.02 | 35.35 | 42.40 | 0.02 | 18.77 |
| 10 | 65.08 | 0.03 | 27.34 | 56.19 | 0.02 | 12.96 |
| 11 | 67.37 | 0.03 | 17.50 | 65.08 | 0.02 | 14.22 |
| 12 | 87.20 | 0.03 | 19.69 | 72.71 | 0.02 | 11.06 |
| 13 | 76.38 | 0.02 | 8.08 | 56.24 | 0.02 | 7.17 |
| 14 | 79.76 | 0.03 | 8.12 | 86.17 | 0.01 | 9.19 |
| 15 | 84.48 | 0.03 | 13.65 | 84.99 | 0.03 | 13.71 |
| 16 | 86.52 | 0.04 | 8.71 | 69.73 | 0.02 | 10.55 |
| 17 | 83.45 | 0.03 | 13.34 | 61.66 | 0.02 | 10.55 |
| 18 | 69.09 | 0.02 | 15.59 | 52.04 | 0.02 | 6.06 |
| 19 | 67.45 | 0.02 | 15.59 | 50.43 | 0.02 | 6.06 |
| 20 | 63.73 | 0.02 | 9.25 | 26.40 | 0.02 | 0.72 |
| 21 | 42.96 | 0.02 | 9.25 | 26.40 | 0.02 | 0.72 |
| 22 | 42.96 | 0.02 | 9.25 | 26.40 | 0.02 | 0.72 |