IMPROVEMENT OF CO-DIGESTING SWINE MANURE AND WASTE KITCHEN OIL

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APPROVAL

The undersigned, appointed by the dean of the Graduate School, have examined the dissertation entitled

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presented by Cuong Manh Duong,

a candidate for the degree of doctor of philosophy,

and herby certify that, in their opinion, it is worthy of acceptance.

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DEDICATION

This dissertation is dedicated to my beloved family in Vietnam because of their unconditional supports for me.

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LIST OF ABBREVIATIONS

AD	: Anaerobic digester
ANOVA	: Analysis of variance
BMP	: Biomethane potential
BP	: Biogas potential
C/N ratio	: Carbon/Nitrogen ratio
CH_4	: Methane
CO_2	: Carbon Dioxide
H_2S	: Hydrogen Sulfide
HRT	: Hydraulic retention time
MP	: Methane potential
NH_3-N	: Ammonia Nitrogen
O/M ratio	: Waste kitchen oil/ Swine manure ratio
OLR	: Organic loading rate
SM	: Swine manure
TAN	: Total Ammonia Nitrogen
TS	: Total solids
VFA	: Volatile fatty acid
VS	: Volatile solids
WKO	: Waste kitchen oil

ABSTRACT

Anaerobic co-digestion of swine manure (SM) and waste kitchen oil (WKO) was conducted to evaluate the effect of substrate loading rates on biogas production efficiency. Biogas yields of M4O2 (4 g-VS_{SM} L^{-1} d^{-1} + 2 g-VS_{WKO} L^{-1} d^{-1}) and M2O1 were 917 ± 43 and 909 ± 37 mL g-VS-fed^{-1}, which were 25.7% and 24.6% higher than the mono-digestion of M2, respectively. However, higher OLRs of SM and WKO did not increase biogas yield. A significant increase of bacterial alpha-diversity was observed in M2O1, at 233.0 ± 3.6 compared with 218.7 ± 5.1 of M2. Less bacterial alpha-diversity and volatile fatty acids accumulation were observed in M4O1 and M4O2. When the digesters were fed with M2, introduction of more than 1.2 g-VS_{WKO} L^{-1} d^{-1} did not increase biogas yield per VS-fed compared to M2O1, and might cause system imbalance. It took up to 84 days to observe system failure.

Biogas potential (BP) study showed significantly higher of biogas yield produced from WKO compared to SM. Therefore, addition of WKO into SM digesters resulted in the increase of biogas production, from 9.0 ± 3.2% to 22.6 ± 3.1%, compared with digestion of only SM. However, BP of M4O1 and M4O2 were 93.2 ± 6.6% and 95.6 ± 4.8%, compared with BP of separate substrates, which indicated that the synergistic effects of co-digestion were due to the increase of organic loading rate and nutrient balance during the process. Co-digesting SM and WKO at ambient or thermophilic working temperature resulted in the low biogas productions compared to mesophilic condition. Application of M2O1 in the on-farm ADs could result in the highest biogas production while maintaining system stability. However, water consumption and digester size were both higher than when M4O1 or M4O2 was applied.

A polynomial regression model with variable interaction was developed which showed the effectiveness in terms of biogas production from SM and WKO. The differences of biogas yield estimated by the model and the lab results were in the range of 0.2% to 8.6%, except only one OLR, in which 15.9% difference was observed. However, only the selected dataset with removal of zero biogas values was utilized for the model improvement, and model application was limited in the ranges of each key variable. Two decision support tools were developed based on the model to estimate biogas production and other operating factors of on-farm ADs.

Chapter 1: Introduction

1. Biogas and its application

Energy consumption per capita is increasing worldwide due to expansion of industrial, personal and agricultural activities. While carbon-based fossil fuel, such as coal and oil, are main sources, the exploitation and overuse of these non-renewable resources may lead to their rapid exhaustion in near future (Chiari & Zecca, 2011; Whiting et al., 2017). The discovery of other energy types is crucial for sustainable development of mankind. Renewable fuels like ethanol, biodiesel or biogas are becoming attractive as a solution for cutting human's energy demands. Among renewable energy sources, biogas is promising because its production can be based on abundant different organic wastes while technologies are improving and gaining popularity.

Operation of swine commercial barns generate huge amount of solid and liquid manure which, without proper treatment, could lead environmental pollution and negatively affect quality of water source (US-EPA, 2013). Unfortunately, swine manure (SM) is not being widely used as an energy-rich substrate for anaerobic digestion to produce biogas. Waste from swine barn can be used alone in mono-digestion process or combined with other substrates in co-digestion systems (Kougias & Angelidaki, 2018). Production of biogas in the U.S. agriculture sector is receiving more attention due to policy changes. It is considered as an attractive approach to help reduce the human's dependence of fossil-derived fuels, thus there is an urgent need to investigate methods to improve on-farm biogas production.

1.1. Key steps of biogas production

Biogas is a mixture of several gases generated during degradation of organic substrates without oxygen (Mao et al., 2015). Biogas can be produced in natural conditions like in landfills in the absence of oxygen or in anaerobic digestors (AD). Diversity and balance of bacterial community in biogas reactors is the key for degradation of different organic compounds to produce biogas. During this process, biochemical reactions occur by activities of different microorganisms to transform complex organic materials to methane, carbon dioxide and other gases (Weiland, 2010). The conversion of complicated substrates to methane in anaerobic digesters is categorized into four key steps, including hydrolysis, acidogenesis, acetogenesis and methanogenesis (Drosg, 2013).

In the first step, carbohydrates, proteins and lipids are utilized by microorganism to be transformed to simple molecules such as amino acid, sugar and peptides. Next, fatty acids are created by acid-forming bacteria during the consumption of above compounds. During acetogenesis, the formations of hydrogen, carbon dioxide (CO_2) and acetate are taken place due to activity of acetate bacteria. In the final step, the performance of methanogenetic microorganisms produces methane by using acetate and hydrogen as their substrates (Figure 1).

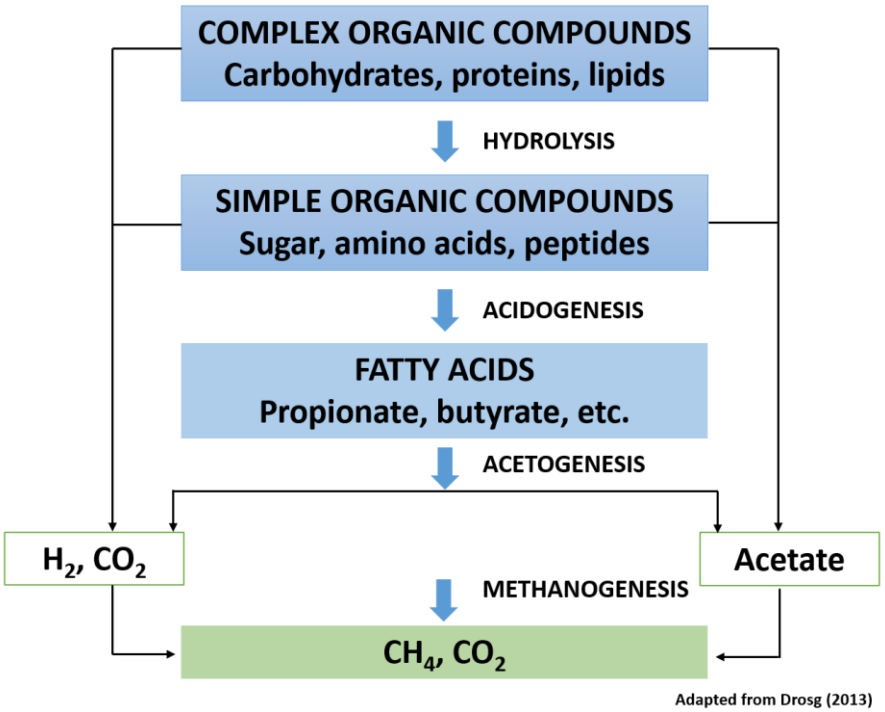

Figure 1: Key steps of biogas production

1.2. Upgradation of biogas

Methane (CH_4) and CO_2 are two main gases in biogas which determine quality of gas mixture after anaerobic digestion. Concentrations of CH_4 and CO_2 account for 50-75 percent and 25-50 percent in the gas mixture released from anaerobic reactors, respectively (Kougias & Angelidaki, 2018). Small amounts of other compounds, like nitrogen, ammonia, hydrogen sulfide (H_2S) or water vapors can also be found in biogas, which consist of less than 1 percent of the mixture. Because methane is the flammable gas, providing energy when burnt, presence of other gases is unwanted (Mulu et al., 2021). Carbon dioxide and H_2S can form acid compounds when exposing to water, and cause corrosion of metal equipment, especially at high temperature. Therefore, presence of

these gases or ammonia negatively affect biogas cleanliness. Purification is usually performed to raise methane concentration and, as a result, increase biogas value (Drosg, 2013; Zhao et al., 2021).

Several approaches have been applied to remove CO_2 or H_2S in the biogas production. Removal of CO_2 can be performed by using absorption techniques, filter membrane or applying biological approach, while H_2S can be removed during or after digestion by supplement of air, iron chloride or iron oxide, etc. (Ryckebosch et al., 2011; Wu et al., 2021). Upgradation of biogas results in methane-rich gas called biomethane which consists of more than 95 percent of CH_4 (Abatzoglou & Boivin, 2009). This final product can be used for heating and cooking, or for generating electricity (Figure 2). Methane gas derived from biogas mixture can be injected into natural gas network for further consumption, such as for usage as a type of fuel for bus or truck (Achinas & Willem Euverink, 2020).

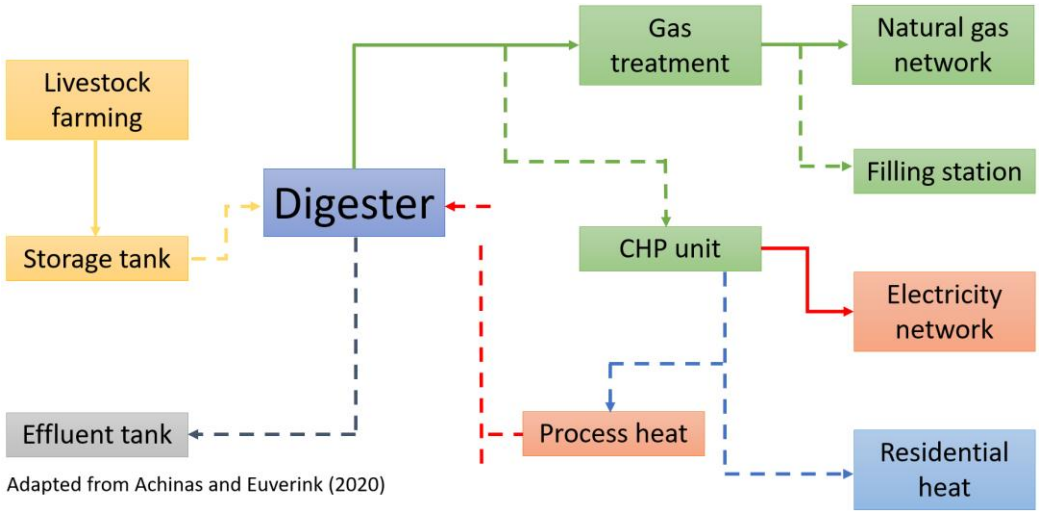

Figure 2: Scheme of biogas production and utilization

2. Factors effecting biogas production

Substrates and C/N ratio

Organic materials like agricultural by-products, animal or human wastes can be used as substrates for producing of biogas. Chemical components of the feedstocks can affect the stability of biogas production. For example, optimal ratio of carbon to nitrogen in anaerobic reactors should be in range of 20 to 70 (Mata-Alvarez et al., 2011). Nitrogen-rich materials like SM can inhibit the

performance of methanogenetic microorganisms (Chen et al., 2008). When nitrogen concentration in digesters is too high, microbial community can be stressful or vulnerable, leading to low biogas production or even performance failure (Ahring et al., 1995). Meanwhile, more oily substrates can lead to accumulation of volatile fatty acid, the factor which decreases pH value and significantly damages bacterial consortium in digester, causing failure of AD performance.

Co-digestion of different substrates is a solution to overcome that issue. The combination of different substrates can adjust carbon to nitrogen ratio of feedstock and balance bacterial activity in anaerobic reactor. For example, nitrogen-rich substrates are often combined with other materials containing high amount of carbon to balance C/N ratio in ADs. Mixture of these two substrates can show high potential in stabilizing nutrient value and increasing buffer capacity of reactors (Astals et al., 2012).

Temperature

Anaerobic digestion can be conducted under mesophilic, thermophilic, or even psychrophilic conditions (Tian et al., 2018). Generally, anaerobic performance is more stable in mesophilic reactors with temperature range from 30 to 40 °C (Bouškova et al., 2005). The fluctuation of daily temperature in thermophilic systems should be less than 1°C while the variation in mesophilic digesters can be around 2-3 °C (Drosg, 2013).

Operation of AD plants under thermophilic conditions gains several benefits, such as increasing digestion rate and improving accessibility of bacteria to substrates which results in higher biogas yield. Another benefit of thermophilic digestion is the deactivation of harmful bacteria and parasites in agricultural substrates (Ahring, 1995). By-product released from thermophilic AD can be categorized and Class A digestate, which can be used directly as a fertilizer source in agriculture without any further treatment. However, some studies showed low biogas production obtained from thermophilic condition compared with the mesophilic digestors. In the studies conducted by Astals et al. (2012, 2013) and by Hashimoto (1983), biogas yield of SM collected from thermophilic digesters were much lower than from reactors working at mesophilic condition.

While low biogas volume is recorded in reactors run at ambient or psychrophilic condition, anaerobic digesters performed at low temperatures are still being applied worldwide, especially in

small farms or household scale when ambient temperature is not too low, and insulation or heating equipment are not available. Simple construction without the need of reactor heating are main reasons for the popularity of ambient temperature digestion.

Hydraulic retention time

Hydraulic retention time (HRT) is the length of time which substates are kept in the reactor. HRT is determined by division of reactor's working volume by daily loading volume. HRT should be between 15 and 30 days (Mao et al., 2015). HRT is associated to working volume and flow rate of digestate in digester. Low HRT means substrate amount is loaded more frequently which can cause hydraulic overload and result in the wash out of bacterial community. Consequently, biogas performance is disturbed or diminished. Meanwhile, high HRT happens when feedstock is added less frequently into reactor, resulting in low biogas production and decreasing digester productivity (Drosg, 2013).

pH

The growth of bacteria in anaerobic digester is linked closely to pH change (Chen et al., 2008). Optimal pH of anaerobic performance is in range between 7 and 8 to maintain stable performance of bacteria (Drosg, 2013). pH lower than 6.0 is not suitable for activity of methanogenetic microorganisms. Many reasons can result in drop of pH during biogas production, such as sudden changes of temperature or organic loading rate. Imbalance of substrate compounds can lead to accumulation of volatile fatty acid which decreases pH value. The decrease of pH links closely to the system failure. Therefore, stability of pH value can be a factor affecting AD performance.

Organic loading rate

The common organic loading rate (OLR) in anaerobic reactors operating under mesophilic temperature is around 3.0 kg-VS m^{-3} d^{-1} or 3 g-VS L^{-1} d^{-1} (Drosg, 2013). Duan et al. (2019) showed optimal methane yield was achieved when OLR of SM was 1.89 g-VS/L.d, at 483.38 ± 12.81 mL g-VS^{-1}, and the increase of OLR resulted in lower methane production. Significant changes of OLR may disturb bacterial consortium and result in failure of biogas production. The increase of OLR should be performed slowly and gradually when starting the anaerobic systems so that microbial organisms can adapt effectively to the change of working environment (Boušková et al., 2005).

3. Failure of anaerobic digester

Significant changes made to AD operation can endanger the stability of biogas production (process perturbation). Process imbalance can be caused by several factors, such as substrate overloading, high HRT, presence of toxic compounds or sudden changes of substrates or temperature (Chen et al., 2008). Many indicators were proposed to determine the instability of biogas reactors, such as concentration of volatile fatty acids (VFAs), pH change or microbial analysis. Among them, VFAs concentration is a reliable tool to analyze the system performance.

VFAs are short chain molecular consisting of 2-8 carbon atoms which are key intermediates for determining the stability of AD systems. Common types of VFAs generated in ADs are acetic acid, propionic acid, butyric acid (Weiland, 2010). The formation and consumption of VFAs during substrate digestion keep the concentration of these intermediates at a suitable level. When the digester is stressed, VFAs are formed at a higher rate than the amount bacteria can utilize. As a result, accumulation of VFAs lead to decrease pH and inhibit the activity of microbial community. Therefore, VFAs level can be a good indicator of process stability. Drosg (2013) suggested that VFAs concentration below 1 g L^{-1} representing digester stability. Meanwhile, the concentration higher than 4 g L^{-1} may be an indicator of unstable performance of digester. Combination of butyrate and its isoform, isobutyrate, is also a good indicator for determining the process failure (Ahring, 1995). The normal levels of VFAs vary between systems and are determined by types of substrate and working conditions like temperature or HRT (Angelidaki & Ahring, 1993).

4. Co-digestion and its benefits

Anaerobic co-digestion is a process in which two or more substrates are combined and loaded into biogas digesters for the purposes of increasing biogas production and maintaining stability of system performance. Benefits of co-digesting different organic materials were presented in many studies (Nogueira et al., 2019a; Wang et al., 2020). Mixing different substrates can increase organic loading rate, reduce ammonia concentration and balance carbon-to-nitrogen ratio (Astals et al., 2013). Other benefits of co-digestion include increasing buffer capacity, adjusting pH (Hidalgo et al., 2015) or diluting toxic compounds (Chen et al., 2008).

Zhang et al. (2013) reported the highest ORL of co-digestion between food waste and cow manure

is 15 g-VS L^{-1} d^{-1} during the semi-continuous digestion while digestion is not efficient when food waste is digested alone at the concentrations exceed 12 g-VS L^{-1} d^{-1}. Both VFAs and ammonia concentrations in co-digestion digesters are higher compared to mono-digestion mode. However, the co-digestion performance is more stable because buffer capacity is strengthened even without monitoring of pH (Nayono et al., 2010).

5. Research objectives

This study aimed to investigate the effectiveness of combining SM and waste kitchen oil (WKO) for anaerobic co-digestion propose and to evaluate several approaches to improve biogas production. In chapter 2, different ratios of SM and WKO were studied to identity the combination that works best in terms of biogas produced per volatile solid (VS) fed or VS destroyed, as well as the threshold of organic materials loaded to AD. Promising candidates were selected for replicated study to deeply evaluate biogas production and other factors including volatile fatty acid concentrations and bacterial diversity. Metagenomic studies were performed to evaluate changes of bacterial community in digestates and to explain process performance when different organic loading rates were applied. Oil concentrations were discovered to find out limit of oil threshold which should be added to maintain the process balance.

Chapter 3 focused on the efficiency of biogas production of SM and WKO in different temperature conditions, and recommendations of loading rates applied in on-farm ADs. First, biogas potential study was conducted to investigate the maximum volume of biogas produced from substrates alone and there promising combinations. Then, performances of digesters in three temperature conditions – ambient, mesophilic, and thermophilic (30 °C, 40 °C and 55 °C) – were evaluated and compared to determine effects of loading rates and system efficiency in each temperature conditions. Finally, scenarios of different loading rates at the most efficient temperature were discussed for farm application, focusing on biogas production, digester's size, water consumed or stability of process.

In chapter 4, key variables, including manure loading rate, ratio of oil to manure, and temperature, were selected to establish a regression model for determination of biogas production from SM and WKO. Correlations between gas produced and other variables were calculated to determine the

importance of each factor in terms of biogas prediction. Different approached were utilized to improve accuracy of model, including application of polynomial regression model and variable interaction. A user-friendly, excel-based tool was created for easy application of model which facilitate the use of the model for AD managers on the farms without trained skills of statistics.

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Chapter 2: Optimization of Co-digesting Swine Manure and Waste Kitchen Oil

1. Introduction

1.1. Swine manure and biogas production

Nearly 1 billion swine heads worldwide produce about 1.55 billion tons of manure annually (World Biogas Association, 2019). Mostly this is due to the expansion of large pig farms at certain regions and number of swine operations over the last few decades. Concentrated, large pork production results in much manure that is needed for crop fields and land application (Szogi et al., 2015). Proper ways to manage manure is essential for sustainable development of pig farms and its application without treatment. On-farm biogas production is becoming an attractive manure management alternative due to advantages such as reducing solids, creating renewable energy, and most importantly, additional income (World Biogas Association, 2019). The use of swine manure (SM) as a common substrate for biogas production has been applied worldwide (US-EPA, 2018). In the U.S., a significant growth in pork production occurred in the last three decades, with the production increasing from around 7.2 million heads in 1980 to about 9.5 million in 2020 (USDA-ERS, 2021). Potential biogas production from SM is very high.

Missouri ranks 11th for biogas potential among the U.S. states with more than 200 thousand tons of methane gas can be produced (US-NREL, 2013). The state also ranks 7th for swine production which makes SM a promising candidate substrate for anaerobic digesters (ADs). Missouri is among top 10 states for potential of methane production from SM with approximately 3.45 billion ft^3 each year (US-EPA, 2018). Application of biogas approach in the state was estimated to reduce 34,000 tons of methane emission annually with potential of electricity generated around 301,000 MWh per year. Therefore, using different approach to improve with manure management and to create renewable energy and preserve organic fertilizer is promising for farm operation in Missouri.

1.2. Co-digestion of swine manure and waste kitchen oil

Anaerobic digestion of pig manure as mono substrate mostly generates a low level of biogas yield due to imperfect characteristics of SM. Carbon to nitrogen (C/N) ratio of this substrate is far lower

than the optimal range required for digestion. A high concentration of nitrogen in pig manure may also accumulate ammonia nitrogen, which can inhibit anaerobic digester (AD) performance (Chen et al., 2008). Many approaches were investigated to increase productivity of biogas process, such as co-digestion or pre-treatment (González-Fernández et al., 2008; Kasinath et al., 2021). Co-digestion of manure and other organic wastes is a popular way to overcome this issue, while also increasing the amount and quality of biogas production. Pig manure can be co-digested with agricultural residuals, such as food scraps or kitchen waste (Cuetos et al., 2011; Dennehy et al., 2018; Tian et al., 2018). Different mixtures balance the C/N ratio which is a main reason for improvement of biogas and methane production. Among substrates for co-digestion with SM, oil-rich wastes have been shown to improve AD performance (Fierro et al., 2014; Hidalgo et al., 2014; Marchetti et al., 2019).

Waste kitchen oil (WKO) is one of the promising additives for co-digestion with SM since it has a high energy potential and ability to balance the C/N ratio. Used cooking oil is an abundant waste material. For example, Teixeira et al. (2018) estimated that 320 kg of used oil are released when each ton of cooking oil is consumed. Inappropriate discharge of used oil can create environmental pollution due to its high organic content. Theoretically, one gram-volatile solid of lipids degraded can release 1,014 mL of methane, making it a valuable substrate for producing biogas (Wan et al., 2011). Moreover, nutrient balance has been improved by combining these substrates. While pig manure showed a poor carbon content of C/N ratio of 12.3, a much higher ratio (53.4) was observed in oily waste (Hidalgo et al., 2014). Therefore, mixing pig manure and cooking oil can balance the C/N ratio in feedstock, thus increasing buffer capacity of digestion and improving biogas yield while also reducing environmental pollution.

Balancing nutrient components of substrates is critical to avoid system failure during biogas production of SM and oil. Ahring et al. (1995) reported that overloading of oil-rich materials can be problematic due to accumulation of volatile fatty acids (VFAs). High concentration of VFAs could decrease pH value in AD, therefore, negatively affecting activities of the bacterial community. Consequently, a decrease of biogas production can occur if the digestion process is disturbed. Therefore, determining the optimal organic loading rate (OLR) and threshold of oil concentration is

critical for maintaining productivity and stability of the AD system.

1.3. Research objectives

In this study, we examined the effectiveness of OLRs in co-digesting SM and WKO. Different loading rates of SM and WKO were investigated to evaluate optimal conditions for biogas production and to determine the loading limit of substrates for ensuring system performance. The WKO threshold level was evaluated by loading manure feedstock with different concentrations of oil to establish an oil limit which could be fed into the digester to avoid overloading and failure. Bacterial communities of selected digesters were analyzed for comparison to understand the relationship between substrate loading rates and changes in microorganisms.

2. Materials and methods

2.1. Experimental setup

2.1.1. Sample collection

The study was conducted at the Agricultural Engineering Building laboratory, University of Missouri, Columbia, MO, USA. Solid manure (23 – 30%VS) was collected from a central Missouri, commercial pig farm and kept at – 20 °C. Sample was thawed at 4 °C for 24 hours before loading. Oil (99.5%VS) was collected from the campus dining service and stored in 22.7-L (5-gal) buckets at room temperature (24 °C) until use.

2.1.2. Total solid and volatile solid measurement

Total solid (TS) and volatile solid (VS) of manure and oil were measured using EPA Method 1684 (US-EPA, 2001). In summary, sample was weighed to 40 grams and dried at 105 ˚C in an oven for at least 12 hours for measurement of TS. Next, residue was dried at 550 ˚C in a muffle furnace for two hours to determine VS. Calculation of TS and VS is based on following formulas:

$$\%TS = \frac{W_{total} - W_{dish}}{W_{sample} - W_{dish}} \times 100 \qquad \text{(Eq.1)}$$

$$\%\ VS = \frac{W_{total} - W_{volatile}}{W_{total} - W_{dish}} \times 100 \qquad \text{(Eq. 2)}$$

where W_{dish}, W_{sample}, W_{total}, $W_{volatile}$ are weights of dish, sample and dish, residue after 105 °C heating, and residue after 550 °C heating, respectively.

2.1.3. Substrate loading and digester maintaining procedure

Length of the time which substates are kept in reactor is called hydraulic retention time (HRT). HRT is determined by dividing reactor's volume by daily loading volume, following below equation:

$$HRT = \frac{V}{DLV}$$

where V and DLV are working volume (L) and daily loading volume (L/d), respectively.

HRT of 21 days and working volume of 1.375 L were selected based on stability and optimal biogas production recorded from different HRTs derived from previous studies in the lab (Nogueira et al., 2019). Initial digestate was inherited from previous lab research (Wang et al., 2020). From above equation, daily feedstock volume of 65.5 mL was calculated which included substrates and water. Since substrates were fed every two days, double amount of feedstock volume (131 mL) was added to each digester during each loading. Determination of actual weights of each substate was based on desired VS loading rate. For example, calculation of feedstock including 2 g-VS_{WKO} L^{-1} d^{-1} (M2) and 1 g-VS_{WKO} L^{-1} d^{-1} (O1) in each loading time was:

- VS-Weight of manure = 2 g-VS L^{-1} d^{-1} x 2 days x 1.375 L = 5.5 g-VS.
- VS-Weight of oil = 1 g-VS L^{-1} d^{-1} x 2 days x 1.375 L = 2.75 g-VS.

Actual substrate's weights were calculated by dividing VS-Weight by %VS. For example, if VS of manure and oil are 23.4% and 99.5%, respectively, actual amounts of substrates in each loading are:

- Weight of manure = 5.5 g-VS/23.4% = 23.5 grams.
- Weight of oil = 2.75 g-VS/99.5% = 2.76 grams.

Finally, water was added to mixture of manure and oil to reach total volume of 131 mL. When feedstock was loaded into digester, an equal amount of digestate was withdrawn to maintain stable working volume. After addition of feedstock, nitrogen gas was injected into headspace of each glass jar at the rate of 25 L min^{-1} in 15 – 20 seconds to establish low-oxygen environment. The reactors were kept in incubator with temperature set up at 40 °C (mesophilic condition). All digesters were swirled manually three times per day to increase interaction between bacteria and substrates. Each jar was connected to a 10-L Tedlar bags for biogas storage and production measurement. Biogas volume was measured every two or four days depending on volume collect

and when the biogas production was high, measured daily.

Value of pH was measured every four days by using pH meter (Pinpoint, American Marine Inc, Ridgefield, CT, USA). Carbon dioxide (CO_2) concentration was measured every eight days by CO_2 analyzer (Fyrite, Bacharach, New Kensington, PA, USA) to estimate CH_4 concentration indirectly, similar to previous studies (Wang et al., 2020) (Figure 3). Records of pH and CO_2 were conducted as routine measurements to evaluate the stability and performance of each digester.

Figure 3: Carbon dioxide analyzer

2.1.4 Establishment of model for biogas measurement

Indirect method was applied to determine gas production released using a model created to determine bag's volume (mL) based on bag's height (mm), following method reported in previous study (Wang et al., 2020). Height of the bag was measured by a custom-designed device built with melamine boards, joined by two ball bearing slide set, which could move vertically when the bag was put inside (Figure 4). Before the height measurement, fresh air was put into bags by using a precision syringe (Magnum VICI, Baton Rouge, LA) which allowed accurate air volume injected. Three bags were used to reduce error, and measurement was started after 2,500 mL of air was injected. Then, corresponding heights were measured after each 500 mL of air was added. Air was injected at the pace of 200 mL each time when air accumulation exceeded 11,000 mL until maximum bag's capacity was reached.

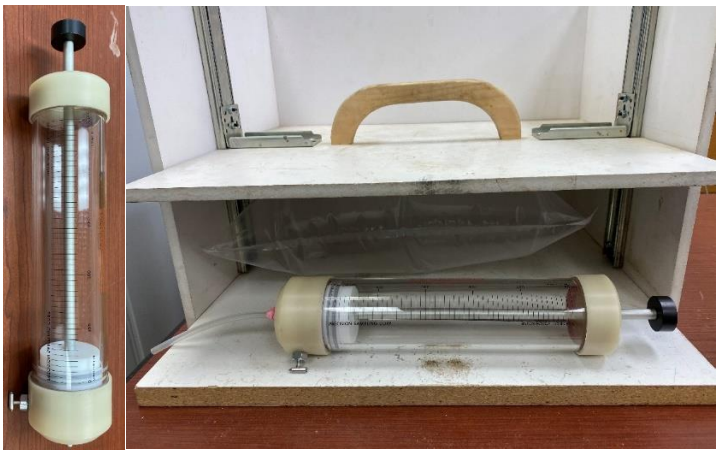


Figure 4: Precision syringe and height measurement device

Six different models, including exponential, logarithmic, power, linear, second order polynomial and third order polynomial regression, were fitted using Excel's built-in functions (Microsoft, Redmond, WA). Selection of model for further use was based on R-squared (R^2) values which determined how well the data fit the models. For example, R-squared of 99% means there are 99 percent of data points can be explained by the model.

2.2. Experiment 1

Selection of optimal combinations of SM and WKO were conducted in a preliminary experiment, in which ten different loading rates were evaluated to investigate effects of dosages to biogas production, while considering AD productivity based on biogas produced per VS-fed or VS-destroyed (VS-des). To avoid substrate overload when starting up the reactor, which can negatively affect bacterial activity, gradual increase of loading rates was followed (Wang et al., 2020). Before desired loading rates were applied in the main study, digesters went through adaptive process including three phases.

2.2.1. Adaptive phases

When starting the new study, all ten jars were loaded with M2 until biogas production became stable after four weeks (Phase 1). Then, one jar was kept loading with M2 while loading rates of two other digesters was changed to M4 in Phase 2. The other seven jars were loaded with M2O1. In the Phase 3, loading rate of a M4 jar was changed to M6, of three M2O1 jars to M4O1, and of another M2O1 jar to M2O2 while the feedstocks of other digesters were kept the same. While Phase 2 lasted for 32 days, a period of 112 days (5 HRTs) was set for Phase 3 because several

disturbances were recorded during the study.

Feedstocks and water were added to each digester every two days and equal amount of digestates were replaced. Measurements of pH and CO_2 were conducted every four and eight days, respectively, as routine measurements to evaluate the stability of digester's performance. Biogas volumes were measured every day, two days or four days, depending on amount of biogas produced by each group.

2.2.3. Final SM and WKO comparisons

In the main study, ten 1.89 L (half-gallon) jars with a working volume of 1.38 L were originally used and each jar was assigned to one of the combinations between 2, 4 or 6 g-VS_{SM} L^{-1} d^{-1} (M2, M4 or M6) and 0, 1 or 2 g-VS_{SM} L^{-1} d^{-1} (O0, O1 or O2) after completing three adaptive phases. M2O3 was fed to the last jar to decide limit of oil loaded. Moving from adaptive phase to main study were performed by choosing dosages closest to the desired OLRs so that disturbance by changing OLR can be minimized (Table 1). For example, among three M2O1 digesters, two were changed to M2O2 and M6O1, respectively. New loadings of two M4O1 jars were M4O2 and M6O2, respectively. M2O2 of adaptive phase was moved to M2O3. OLRs of other jars were kept constant. The M2O2 was observed to have inconsistent biogas production after loading rate was changed from M2O1 during adaptive phases. Since the reason of gas drop was unclear during that time, digestates from other working jars was added to this digester to reinforce bacterial consortia before loading rate was changed to M2O3 again.

Table 1: Changes of organic loading rates in each digester from Adaptive phase 1 to Experiment 1

Digester / Phase	1	2	3	4	5	6	7	8	9	10
Adaptive 1	M2	M2	M2	M2	M2	M2	M2	M2	M2	M2
Adaptive 2	M2	M4	M4	M2O1	M2O1	M2O1	M2O1	M2O1	M2O1	M2O1
Adaptive 3	M2	M4	M6	M2O1	M4O1	M2O1	M2O1	M4O1	M4O1	M2O2
Experiment 1	M2	M4	M6	M2O1	M4O1	M6O1	M2O2	M4O2	M6O2	M2O3

Due to foaming and clogging issues observed in jars with high organic loading rates (OLRs), 3.78-L (1-gallon) jars were used to replace the 1.89-L (0.5-gal) digesters to minimize the issue. The

experiment was conducted for 12 HRTs (252 days) because two loading dosages (M2O3 and M2O2) failed after 68 and 112 days of the test due to high amount of oil added. The time extended was to ensure that no further failure occurred.

At the end of Experiment 1 or when the failures were determined, digestates from each digester were measured (TS, VS, ammonia, and alkalinity tests). Measuring TS and VS is to evaluate VS reduction rate and biogas production per VS-des, which are important factors for comparing the effectiveness of AD systems. Determination of total alkalinity and total ammonia nitrogen (TAN) concentrations was done by using Alkalinity Test Kit (Model AL-AP, MG-L, Hach, Loveland, CO) and Nitrogen, Ammonia Test Kit (Model NI-SA, Hach, Loveland, CO), respectively. Calculation of VS-destroyed (VS-des) and biogas per VS-destroyed were done by following equations.

- VS-des (g/d) = $VS_{input} - VS_{output}$
- Biogas/VS-des (mL g-VS^{-1}) = $\frac{\text{Biogas (mL/d)}}{\text{VS−destroyed (g/d)}}$

where VS_{input} and VS_{output} are amounts of volatile solid in feedstock and digestate taken out of digester in each loading (g-VS d^{-1})

2.3. Experiment 2

At the end of the Experiment 1, relationships between gas productions and VS-fed and VS-destroyed was evaluated to determine overall effectiveness of the co-digestion. Four loading rates with highest ratio of biogas volumes and biogas yield per VS-fed were selected for a detailed study in triplicate, including M2 (baseline), M2O1, M4O1 and M4O2.

Twelve new 1.89-L glass jars with working volume of 1.375 L were set up for replicated study. Jars were assigned into four loading groups. At the beginning, M2 or baseline group was supplemented by 2 g-VS_{SM} L^{-1} d^{-1} while 2 g-VS_{SM} L^{-1} d^{-1} and 1 g-VS_{WKO} L^{-1} d^{-1} (M2O1) were fed into other jars following semi-continuous procedure described above. After each jar had stabilization, designed loading rates were maintained for each group. In case of severe foaming issue, 3.78-L digesters were utilized as replacement (Figure 5).

Figure 5: Set up of biogas digesters

This study was conducted in 4 HRTs (84 days) to determine the stability of digestion. Digestates were collected at the end of the experiment for measurement of TS, VS, VFAs and for metagenomic study. TS and VS of digestates were measured to calculate VS reduction rate and biogas production per VS-fed and VS-des. Concentration of the main VFAs, including acetic, propionic, butyric and isobutyric, was measured as a factor to evaluate strength of digestate in each reactor (Fierro et al., 2014; Weiland, 2010). Measurement of VFAs content followed the method described by Meeroff et al. (2004).

2.4. Waste kitchen oil threshold

Digesters fed with 4 g-VS_{SM} L^{-1} d^{-1} showed several issues in the previous experiment, so a lower VS loading rate of SM (M2) was considered in the oil threshold experiment. In contrast, M2O2 caused system interruption in the previous experiment while digesters fed with M2O1 were stable until the experiment was completed. Therefore, amounts of oil lower than 2 g-VS L^{-1} d^{-1} were examined to determine the threshold level of WKO that could be added to reactor. Three groups, including M2O1.2, M2O1.4 and M2O1.6, were set up in replicate at 40 °C. The loading dosage of M2O1.8 was not included because a pilot experiment had shown a significant decrease of biogas production and pH for this dosage and M2O1.6 (unpublished data). Before desired loading rates were applied, all digesters were loaded with M2 in at least 1 HRT to ensure normal performance of digesters and the bacterial community. Feedstocks were fed every two days, following procedure

described above. After application of the desired loading rates, jars were maintained for 4 HRT (84 days) to ensure enough time for evaluation of each group with stabilization. Digestates were collected at the end of the experiment or when failure was obtained to measure TS, VS and for metagenomic study.

2.4. Bacterial analysis

Digestate samples of Experiment 2 and of oil threshold experiment were collected for analysis of the microbial community. Samples were sent to Metagenomics Center, University of Missouri. DNA extraction was followed for metagenomics test (Ericsson et al., 2015). 16S RNA genes were amplified with universal primers (U515F/806R) and then sequenced. Identification of Operational Taxonomic Unit (OTU) and Taxonomy was based on methods presented by Hosseini Taleghani et al. (2020) and Wang et al. (2020). Alpha-diversity was determined using the Shannon diversity index, representing the number and distribution of unique Amplicon Sequence Variants – ASVs (Bolyen et al., 2019; Shannon, 1948).

2.5. Data analysis

Raw data was analyzed and graphed by Excel 2019 (Microsoft Corporation). Data were presented as mean ± standard deviation. One-way ANOVA was performed by R Software, Version 4.0.0, followed by Tukey post hoc test (p <0.05) for comparison of significant difference between groups (R Core Team, 2021).

3. Results

3.1. Selection of model for biogas volume measurement

Different regression models were established to evaluate the ones which accurately describe relationships between biogas volume (mL) and bag height (mm). Among six equations developed, exponential model showed the lowest accuracy with R^2 = 0.8573. The R-squared increased significantly, to 0.9563 and 0.9749 when linear and power models were applied, respectively. Moreover, logarithmic equation and second or third order polynomial models came up with even better fitting, with R-squares ranging from 0.9914 to 1. (Table 2).

Table 2: Model selection for biogas volume measurement based on bag height

Type of relationship	Regression model	R-squared

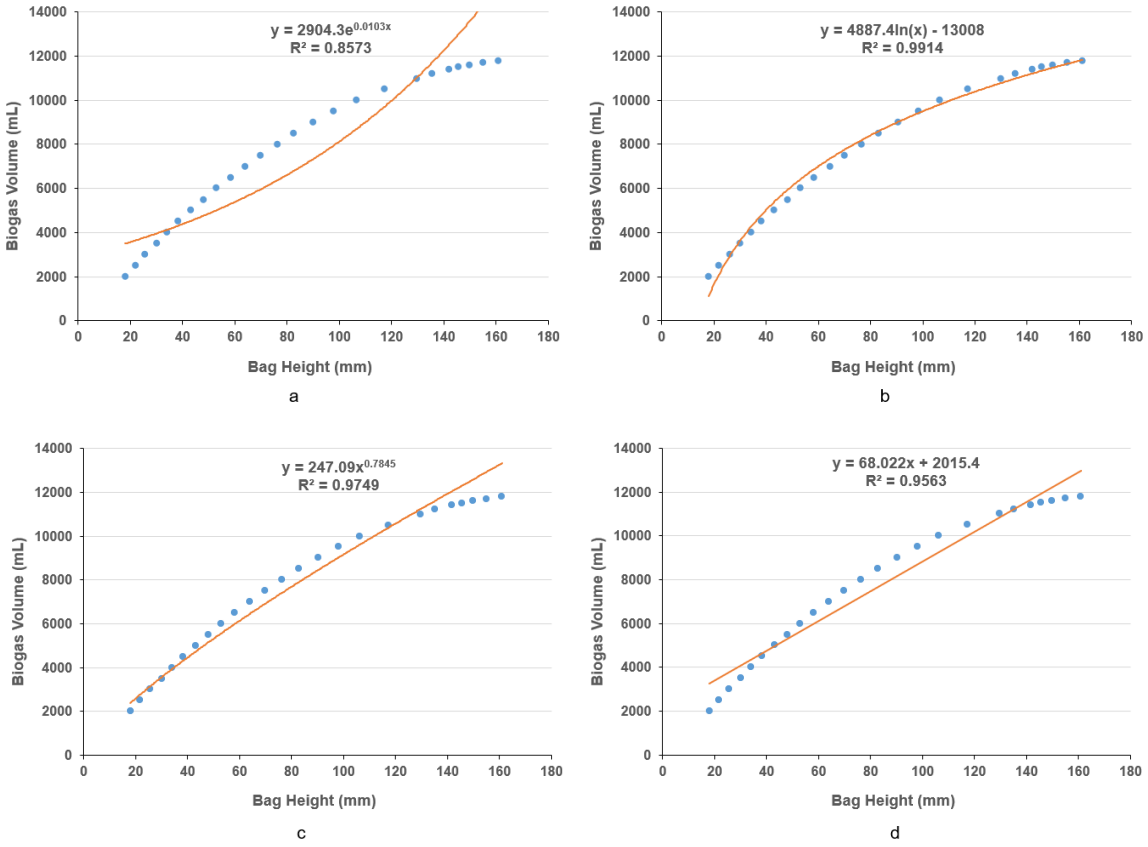
Exponential	$y = 2904.3e^{0.0103x}$	0.8573
Logarithmic	$y = 4887.4\ln(x) - 13008$	0.9914
Power	$y = 247.09x^{0.7845}$	0.9749
Linear	$y = 68.022x + 2015.4$	0.9563
Second order polynomial	$y = -0.4047x^2 + 140.13x - 320.23$	0.9999
Third order polynomial	$y = 0.0005x^3 - 0.534x^2 + 149.77x - 508.45$	1

y and x represent biogas volume (mL) and bag height (mm).

Figure 6 showed the six regression models, in which logarithmic, second and third order polynomial regressions relatively fit data than others. The curves of the last two models fitted all data points which demonstrated higher accuracy. Third order polynomial model was selected due to its best performance in terms of describing variable relationship ($R^2 = 1$). The following equation was used for determination of biogas volume based on bag height.

$$y = 0.0005x^3 - 0.534x^2 + 149.77x - 508.45$$

where y and x are biogas production (mL) and biogas height (mm).



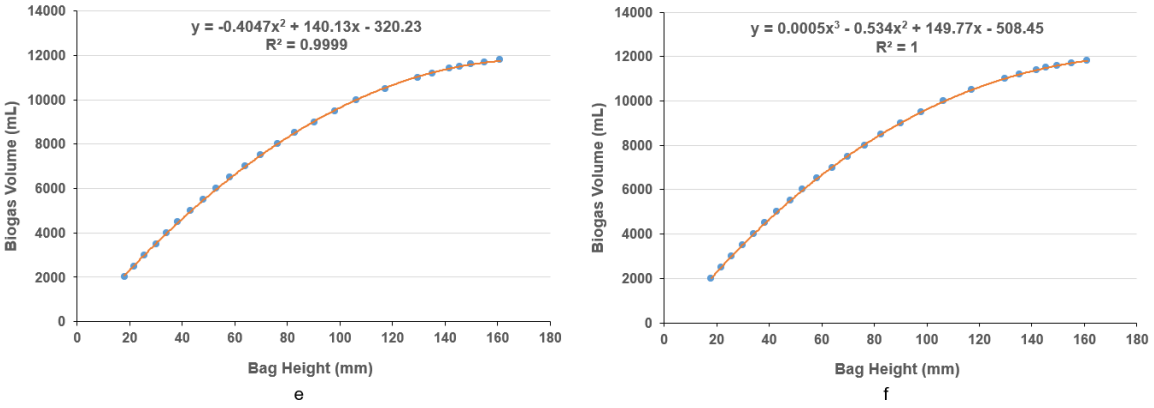


Figure 6: Different regression models for biogas volume measurement

(a). Exponential, (b). Logarithmic, (c). Power

(d). Linear, (e). Second order polynomial, (f). Third order polynomial

3.2. Experiment 1

3.2.1 Adaptive phases

Ten jars were going through three continuous adaptive phases which lasted for 28, 32 and 112 days, respectively, before desired loading rates were applied in the Experiment 1 (Figure 7). The third phase was longer than others due to the instability of biogas productions recorded in jars loaded with high amount of oil. In the phase 1, when all jars were loaded with M2, average of biogas production in the last 12 days is 1,963 ± 105 mL d^{-1}. Stability of biogas production in Phase 1 demonstrated the balance of AD system when small amount of substrate is loaded regularly.

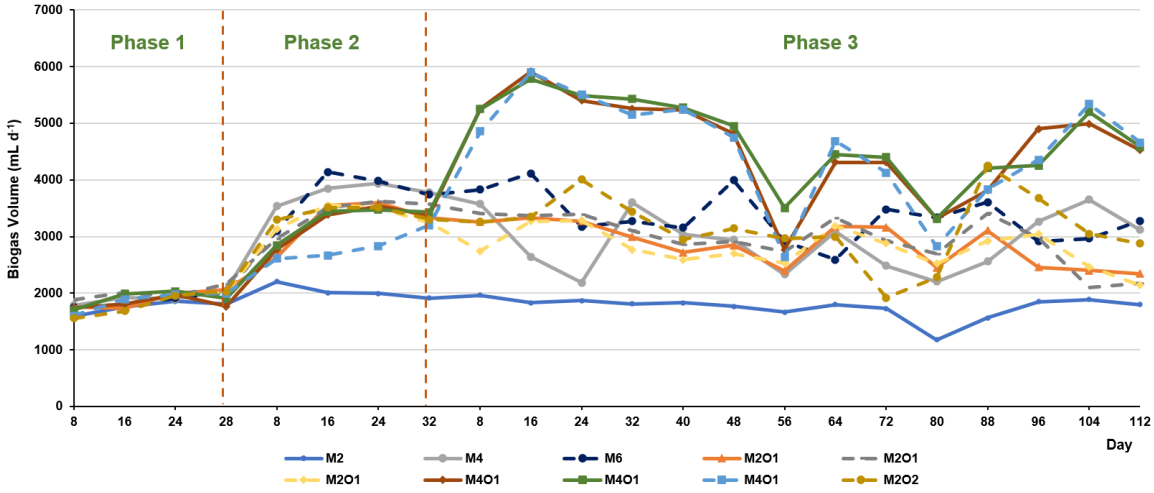


Figure 7: Biogas production in three adaptive phases

Biogas production of M4 group was two times higher than of M2 digesters in Phase 2 which

demonstrated efficiency of AD system when doubling SM as the sole substrate (Table 3). Group M2O1 produced 3,398 ± 134 mL biogas d^{-1}, which was 11.1% lower than production of M4 loading rate. Therefore, it could be assumed that biogas potential of 1 g-VS_{WKO} is mostly equal to of 2 g-VS_{SM}. Significant drops of biogas production in all groups were observed in Phase 3 and disturbance occurred frequently due to unstable temperature in incubators. However, some interesting trends were still observed. Biogas yield of digester fed with M6 was not statistically significantly higher than M4 jar, showing the OLR limit of manure should be less than 4 g-VS L^{-1} d^{-1}. More biogas volume was produced from group M4O1, average 4,816 ± 477 mL d^{-1}, compared to gas production from M4 or M2O1, which demonstrated efficiency of combination of oil and high ratio of manure. The frequent disturbance recorded from jars loaded with M2O2 suggested that equal VS loading rate of WKO at 2 g-VS L^{-1} d^{-1} and above is not suitable for AD stability.

While pH range of all other jars were stable around 7.0 to 7.8, significant drop was observed in M2O2 reactor. The pH value was as low as 6.7 before digestates from other jars were added to this digester. Moreover, instability of CO_2 concentration was also recorded in this group, which were supporting factors suggesting the inefficiency of digester fed with high amount of oil.

Table 3: Performance of anaerobic digesters during adaptive phases

	Phase 1	**Phase 2**		
	M2	**M2**	**M4**	**M2O1**
Biogas (mL d^{-1})	1,963 ± 105	1,947 ± 64	3,822 ± 141	3,398 ± 134
pH	7.4 ± 0.0	7.5 ± 0.0	7.8 ± 0.0	7.5 ± 0.0
CO2 (%)	26.8 ± 1.3	28.0 ± 1.4	29.5 ± 0.6	26.9 ± 0.5

	Phase 3					
	M2	**M4**	**M6**	**M2O1**	**M4O1**	**M2O2**
Biogas (mL d^{-1})	1,831 ± 74	3,360 ± 373	3,108 ± 213	2,359 ± 358	4,816 ± 477	3,069 ± 494
pH	7.2 ± 0.0	7.3 ± 0.1	7.7 ± 0.1	7.0 ± 0.1	7.3 ± 0.1	6.9 ± 0.1
CO2 (%)	25.3 ± 0.6	21.7 ± 1.2	22.0 ± 0.0	27.2 ± 0.4	29.2 ± 2.0	19.3 ± 7.1

3.2.2. Extended monitoring of Experiment 1

From day 68 of Experiment 1, biogas productions of digesters without disturbance could be

categorized into four levels from lowest to highest productions (Figure 8). Lowest biogas volume was represented by the production of M2 digester (baseline). The second and third levels with moderate biogas production included M4, M6 and M2O1 or M4O1 and M6O1, respectively. Loading rates of M4O2 or M6O2 belonged to the last group which resulted in highest amount of biogas production. Data showed that loading AD at 6 grams VS of SM per liter per day did not result in higher biogas production compared with AD fed with 4 g-VS_{SM} L^{-1} d^{-1} even in mono-digestion or when oil was supplemented. Interestingly, addition of 1 gram VS of oil resulted in biogas production similar to mono-digestion of 2 g-VS of SM (Table 4). It is important to note that volatile solid of WKO is more than four times higher than of SM, at 99.5% compared to around 21 – 23% in this study. Therefore, when a reasonable small amount of oil was added into digester, biogas production increased by several times compared to the same amount of SM fed.

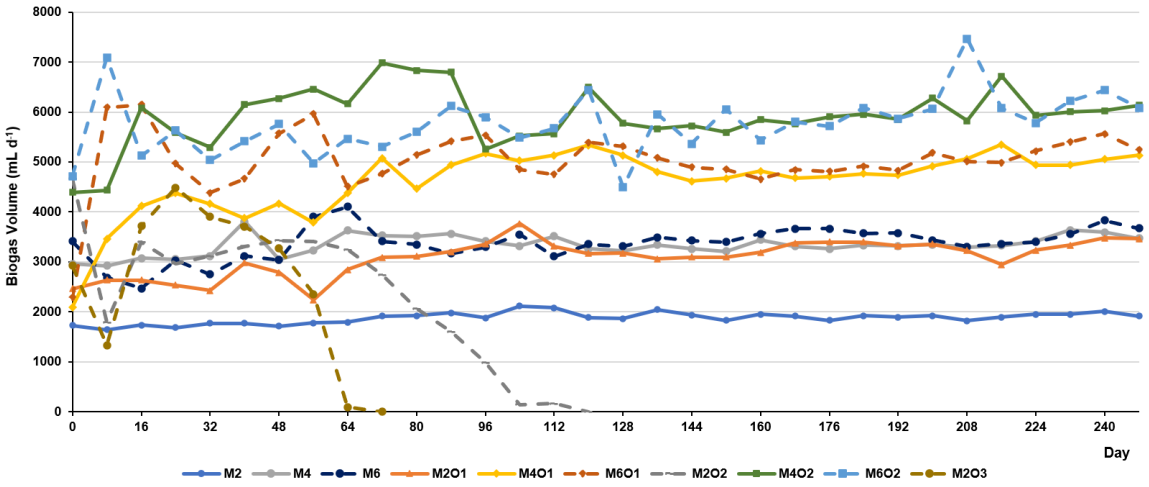


Figure 8: Trend of biogas production of each loading dosage in Experiment 1

When more oil was added to the M2 AD, however, bacterial community in the digesters seemed to be stressed. Biogas production sharply decreased to zero after 112 days when M2O2 was introduced to digester. Biogas production in M2O3 treatment stopped even earlier, after 72 days, which suggests that higher oil ratio in M2-fed AD might result in sooner AD failure. In another experiment conducted including two digesters fed with M2O2 or M2O3 to repeat testing the effect of high oil concentration, biogas volumes could not be measured after day 88 and day 52, which again confirmed the hypothesis of ineffectiveness of high oil dosage. On the other hand, when 2 g-VS_{WKO} L^{-1} d^{-1} was added to AD fed with 4 g-VS_{SM} L^{-1} d^{-1}, no disturbance was observed during the

252 days of the study. The findings suggest that concentration of oil should not be greater than the addition of SM and raising OLR of manure (but not more than 4 g-VS L^{-1} d^{-1}) increased oil threshold. Moreover, ratio of oil to manure seemed to be critical to determine amount of each substrate can be added into digester.

During the study, pH values of all functioning jars were stable, ranging from 7.4 ± 0.1 to 7.9 ± 0.1, which were in the optimal range of pH (Drosg, 2013). It is interesting to note that the drop of pH in digesters M2O2 and M2O3 occurred earlier than the decrease of gas production for the AD jars that ceased biogas production. Values of pH in each jar were recorded lower than 7.0 after 52 and 84 days for M2O3 and M2O2, respectively, and the values dropped continuously to 5.5 and 5.4 in the next 24 and 40 days, respectively (Figure 9). Same observation was recorded when these two loading dosages were tested again and drops of pH lower than 7.0 were recorded after 40 and 76 days, respectively. Decrease of pH was observed again before the drop of biogas production occurring, which reaffirmed that pH could be a good indicator to determine stability of AD performance.

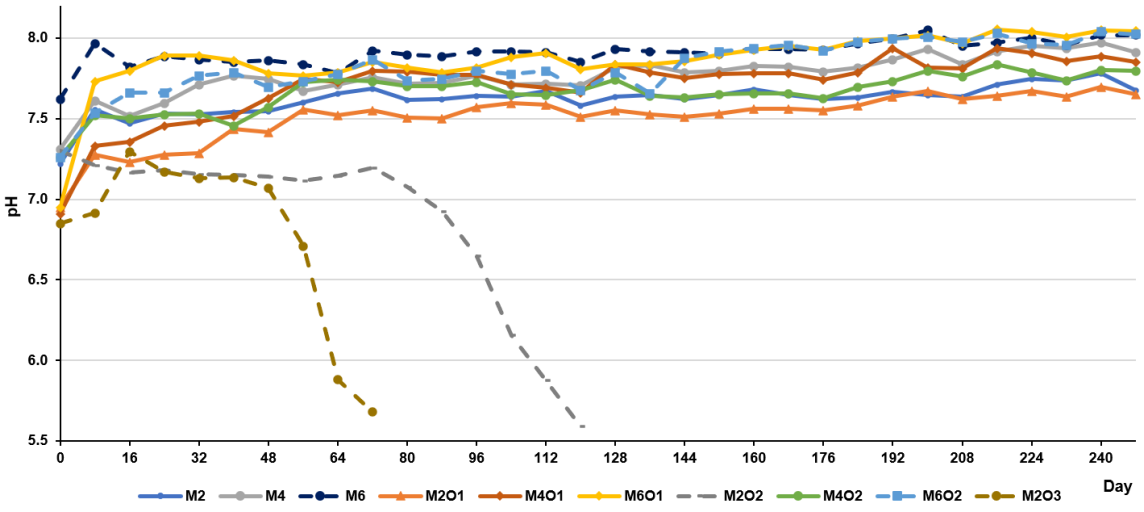


Figure 9: Trend of pH of each loading dosage in preliminary study

Averages at biogas volumes from day 72, when production rates were stable, until the end of the experiment (day 252) were summarized to evaluate the effects of the different dosages (Table 4). In digesters without oil addition, biogas production was 1,931 ± 76 mL d^{-1}, and increased to 3,386 ± 124 and 3,455 ± 171 mL d^{-1} when SM loading was changed from 2 to 4 and 6 g-VS L^{-1} d^{-1}, which are equal to 75.4% and 79.0% increase. If methane percentage was supposed to account for 60%

of the gas mixture like data reported in other studies, methane yield in M2 group was around 419 mL/g-VS_{added} (Duan et al., 2019; Fierro et al., 2014; Ye et al., 2013). This production was similar to result reported by Duan et al. (2019) and higher than production reported by others (Lymperatou et al., 2018; Marchetti et al., 2019; Ye et al., 2013).

Similarly, increase of gas productions were observed when suitable amounts of WKO were introduced into digester. Addition of 1 g-VS L^{-1} d^{-1} of WKO into M2, M4, M6 digesters resulted in increased amount of gas by 69.10%, 45.69% and 46.83% compared to mono-digestion of SM, respectively. When O2 was added, biogas productions of M4 and M6 jars increased by 77.75% and 70.44% compared to digestion of sole SM. M4O2 resulted in highest volume of biogas compared to other loading rates, at 6,019±461 mL d^{-1}. If biogas yield per VS-fed was considered, three groups, including M2O1, M4O1 and M4O2 were the most promising candidates, at 792, 718, and 730 mL/g-VS_{fed}, respectively.

Table 4: Summary of biogas and other variables in the preliminary study from day 72 to day 248

Parameter	Unit	M2	M4	M6	M2O1	M4O1	M6O1	M4O2	M6O2
VS-feedstock	g d^{-1}	2.75	5.50	8.25	4.13	6.88	9.63	8.25	11.00
VS reduction	%	61.9 ± 1.2	56.8 ± 0.7	42.2 ± 1.3	63.0 ± 0.6	58.1 ± 0.4	46.0 ± 0.3	53.1 ± 0.1	51.1 ± 0.1
Biogas									
• Volume/day	mL d^{-1}	1,931 ± 76	3,386 ± 124	3,455 ± 171	3,266 ± 178	4,933 ± 231	5,073 ± 271	6,019 ± 461	5,888 ± 546
• Volume/Digester volume	mL L^{-1} d^{-1}	510 ± 20	895 ± 33	913 ± 45	863 ± 47	1,303 ± 61	1,340 ± 72	1,590 ± 122	1,556 ± 144
• Volume /VS-fed	mL/g-VS_{fed}	702 ± 27	616 ± 23	419 ± 21	792 ± 43	718 ± 34	527 ± 28	730 ± 56	535 ± 50
• Volume /VS-destroyed	mL/g-VS_{des}	1,134 ± 44	1,084 ± 40	992 ± 49	1,257 ± 68	1,235 ± 58	1,147 ± 61	1,374 ± 105	1,047 ± 97
pH	-	7.7 ± 0.1	7.8 ± 0.1	7.9 ± 0.1	7.5 ± 0.1	7.7 ± 0.2	7.9 ± 0.1	7.7 ± 0.1	7.8 ± 0.1
CO_2 concentration	%	16.7 ± 0.7	22.5 ± 1.0	23.0 ± 2.2	20.4 ± 0.8	24.5 ± 0.8	23.6 ± 1.8	25.2 ± 0.8	24.0 ± 2.0
Total Alkalinity	g L^{-1}	13.9 ± 0.5	27.7 ± 0.9	37.9 ± 0.9	13.9 ± 0.9	26.7 ± 0.9	39.5 ± 0.9	26.4 ± 2.9	38.7 ± 1.2
TAM	g L^{-1}	1.8 ± 0.0	3.6 ± 0.0	6.4 ± 0.0	2.1 ± 0.4	3.4 ± 0.2	6.0 ± 0.1	3.6 ± 0.0	5.8 ± 0.2

TAN: Total Ammonia Nitrogen; mean ± sd.

Other variables were measured for determining effects of substrates and AD performance. pH in digesters with same manure substrates were very similar, and the increase of manure loading seems to be associated with the higher pH. Concentrations of CO_2 in gas released from M2 digesters were the lowest, at 16.7 ± 0.7% while only slight difference was observed from other dosages, ranging from 20.4 ± 0.8% to 25.2 ± 0.8 %. Concentrations of CO_2 were lower than other studies (Deepanraj et al., 2015; Li et al., 2017). It could be explained by the fact that the use of 3.79-L digesters created large headspace volume (around 2.42 L) which finally resulted in presence of nitrogen and other gases in the biogas bags. Total Alkalinity and Ammonia concentrations were directly proportional to the additions of SM. Correlations of these concentrations with SM are 0.99 and 0.92, respectively, significantly higher than with WKO (0.23 and 0.17).

Volatile solid reductions were in range of 42.2% - 63% (Figure 10). Three loading rates, M2O1, M4O1, M4O2 came with highest volume of biogas produced per amount of VS destroyed, at 1,257 ± 68, 1,235 ± 58 and 1,374 ± 105 mL/g-VS-des which was an important criterion to evaluate AD performance in term of reducing solid waste. Based on the summary, four loading dosages, M2O1, M4O1 and M4O2 plus M2 as the baseline, were selected to perform replicated experiment. They were loading rates resulted in with biogas yields per VS-added or per VS-destroyed (Figure 10).

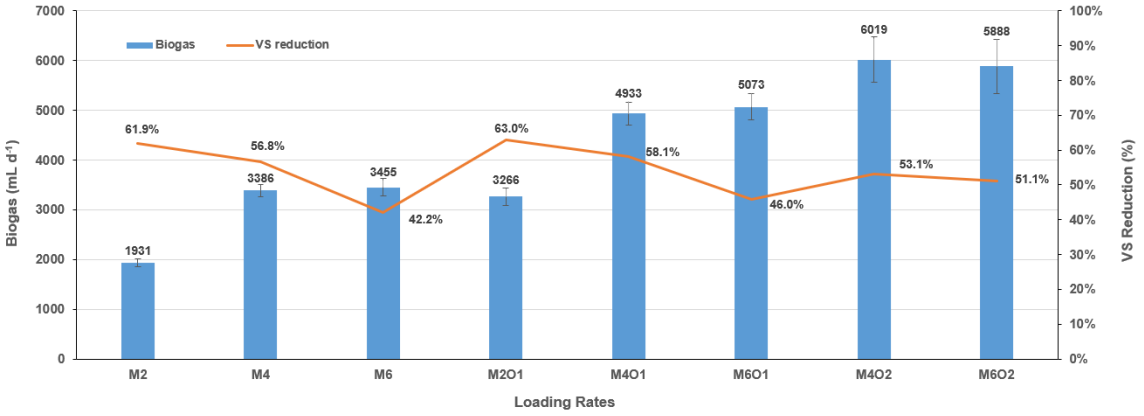


Figure 10: Average of biogas production and VS reduction in each digester from day 72 to day 248.

3.3. Experiment 2

During 4 HRTs of the study (84 days), biogas productions of M2 and M2O1 groups were more stable than of other loading rates, averaged 2,006 ± 64 and 3,753 ± 152 mL d^{-1} (Figure 11). When the OLRs of two last groups were changed from M2O1 to M4O1 and M4O2, biogas yields increased

significantly after 4 days with M4O1 and 24 days with the other. However, several fluctuations were recorded, indicating the instability of AD performance of these two dosages. Biogas produced by each group averaged 5,456 ± 125 and 7,565 ± 352 mL d-1 for M4O1 and M4O2, respectively, which were higher than in the previous Experiment (Table 4).

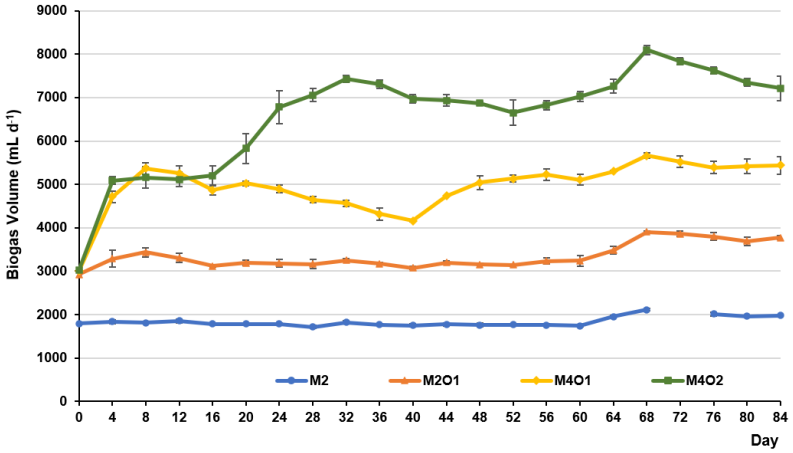


Figure 11: Average of biogas production in each group in Experiment 2.

One datapoint of M2 group was mis-recorded in day 72.

Averages of pH for the four loading rates were 7.5 ± 0.0, 7.5 ± 0.0, 7.7 ± 0.1 and 7.7 ± 0.1 (Figure 12). Values of pH seems to be correlated with VS loading of SM. ANOVA followed by Tukey test showed no difference in pH value between M2 and M2O1 groups or between M4O1 and M4O2, while significant difference was observed between groups loaded with 2 g-VS_{SM} L^{-1} d^{-1} and these loaded with 4 g-VS_{SM} L^{-1} d^{-1} ($p < 0.05$).

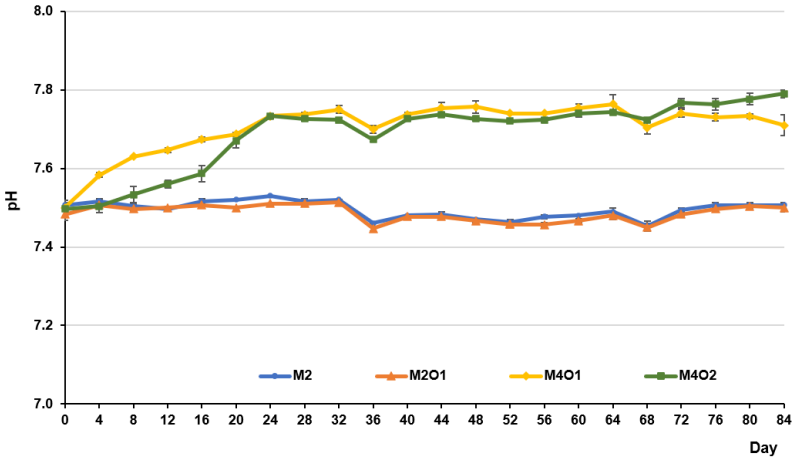


Figure 12: Average of pH in each VS loading group in Experiment 2.

VS reductions ranged from 61.4 ± 0.7% to 67.6 ± 0.6% and highest reduction was obtained in M2O1 group (Table 5). Groups M2O1 and M4O2 were the most promising when considering factors, such as volume of biogas per VS-fed or per VS-destroyed. However, concentration of VFAs in the group fed with high OLRs were significantly higher than in other groups. Accumulation of VFAs is one of common factors leading to failure of AD reactors. Therefore, high VFAs concentration in groups M4O1 and M4O2 indicated that the stability of these loading rates may not be as good as of digesters fed with M2 or M2O1

Carbon dioxide contents were stable in the study, ranging from 26.6 ± 0.5% to 29.5 ± 0.9%, which were higher than in the Experiment 1 due to the use of 1.89-L digesters (Table 5). When ORL changed from 2 g-VS_{SM} to M4, concentrations of Alkalinity and Ammonia doubled. It suggested that both Alkalinity and Ammonia seems to correlate strictly with loading rate of SM.

Table 5: Summary of biogas and other variables in Experiment 2

Parameter	Unit	M2	M2O1	M4O1	M4O2
n		3	3	3	3
VS-feedstock	g d^{-1}	2.75	4.13	6.88	8.25
VS reduction	%	66.9 ± 0.5a	67.6 ± 0.6a	61.4 ± 0.7c	63.6 ± 0.5b
Biogas					
• Volume/day	mL d^{-1}	2,006 ± 64a	3,753 ± 152b	5,456 ± 125c	7,565 ± 352d
• Volume/AD volume	mL L^{-1} d^{-1}	1,060 ± 34a	1,982 ± 80b	2,882 ± 66c	3,996 ± 186d
• Volume /VS-fed	mL/g-VS_{fed}	729 ± 23a	909 ± 37c	793 ± 18b	917 ± 43c
• Volume /VS-destroyed	mL/g-VS_{des}	1,090 ± 35a	1,343 ± 54c	1,291 ± 30b	1,443 ± 67c
pH	-	7.5 ± 0.0a	7.5 ± 0.0a	7.7 ± 0.1b	7.7 ± 0.1b
CO_2 concentration	%	26.6 ± 0.5a	27.7 ± 0.5a	29.5 ± 0.9b	29.1 ± 0.8b
Total Alkalinity	g L^{-1}	10.7 ± 0.5a	9.6 ± 0.0a	19.2 ± 0.0b	19.2 ± 0.0b
TAN	mg L^{-1}	1.6 ± 0.0a	1.6 ± 0.1a	3.2 ± 0.2b	3.2 ± 0.0b
VFAs	mg L^{-1}	1,223.7 ± 318.4a	1,017.7 ± 97.3a	3,372.7 ± 962.7b	1,851.3 ± 418.0a

TAN: Total Ammonia Nitrogen; mean ± sd.

3.4. Bacterial analysis

Firmicutes and Bacteroidetes are two most abundant phyla in all digesters, contributing from 81.5% to 88.0% among bacterial community (Figure 13). Addition of oil increased Firmicutes consortium, from 56.0% in mono digestion to 63.8, 73.2 and 75.9% when co-digestion was applied. Percentage of Bacteroidetes decreased from 28.3% to 17.7% when 1 g-VS_{WKO} was added and to 13.3% and 12.0% in digesters with higher OLRs.

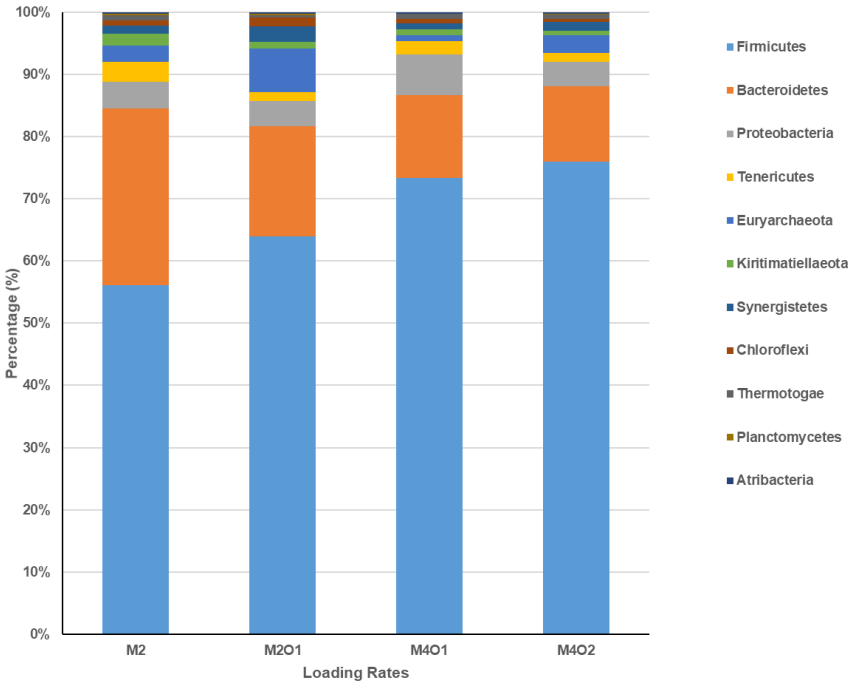


Figure 13: Bacterial community in digesters with different loading dosages

It is noteworthy that adding 1 g-VS_{WKO} L^{-1} d^{-1} raised alpha diversity of bacterial community to 233.0 ± 3.6, compared to 218.7 ± 5.1 in reactor with mono-digestion of SM (Table 6). However, significant diversity decrease was observed in digester loaded with M4O1 or M4O2, at 183 ± 7 and 166 ± 4, respectively. All the differences were statistically significant ($p < 0.005$). No significant difference was observed in alpha diversity of methanogens between all groups, with the range from 4.3 ± 0.6 to 5.0 ± 1.0. However, percentages of Euryarchaeota which most methanogens belong to were 2.7%, 7.1%, 0.9% and 2.8% in groups M2, M2O1, M4O1 and M4O2, respectively. This indicates that the addition of small amount of oil dramatically increased percentage of Methanogens, which relates directly to the increase of biogas production. On the other hand, when more manure and oil were added, the present of methanogens in digesters tended to be lower. That was another

indicator showing that digester fed with higher OLR seems to be less stable even biogas production was observed.

Table 6: Bacterial diversity in different groups

Group	M2	M2O1	M4O1	M4O2
Alpha-diversity				
Microorganism	218.7 ± 5.1a	233.0 ± 3.6b	183.0 ± 7.0c	166.0 ± 4.4d
Methanogens	4.7 ± 0.6	5.0 ± 1.0	4.3 ± 0.6	5.0 ± 1.0
Percentage (%)				
Firmicutes	56.0 ± 0.9	63.8 ± 3.4	73.2 ± 0.9	75.9 ± 0.8
Bacteroidetes	28.3 ± 0.9	17.7 ± 4.5	13.3 ± 0.9	12.0 ± 0.7
Proteobacteria	4.3 ± 0.2	4.0 ± 0.3	6.6 ± 1.3	4.0 ± 0.1
Tenericutes	3.2 ± 0.2	1.4 ± 1.1	2.1 ± 0.5	1.4 ± 0.1
Euryarchaeota	2.7 ± 0.2	7.1 ± 1.8	0.9 ± 0.3	2.8 ± 0.2
Kiritimatiellaeota	1.9 ± 0.2	1.1 ± 0.2	1.0 ± 0.1	0.7 ± 0.1
Synergistetes	1.3 ± 0.1	2.5 ± 0.1	0.8 ± 0.1	1.4 ± 0.2
Chloroflexi	0.9 ± 0.0	1.4 ± 0.9	0.7 ± 0.4	0.5 ± 0.2
Thermotogae	0.8 ± 0.1	0.5 ± 0.3	0.8 ± 0.3	0.8 ± 0.3
Planctomycetes	0.2 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	0.0 ± 0.1
Atribacteria	0.2 ± 0.0	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.0
Armatimonadetes	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Total (%)	**100**	**100**	**100**	**100**

3.5. Waste kitchen oil threshold

After 1.2, 1.4 and 1.6 g-VS_{WKO} L^{-1} d^{-1} was supplemented into the M2 reactors, biogas productions were significantly increased after 8 days (Figure 5). In the last HRT, from day 64 until the end of the test (day 84), average of biogas production in groups M2O1.2 and M2O1.4 were 4,006 ± 85 and 3,710 + 178 mL d^{-1}. Biogas production of M2O1.2 was 6.7% higher than of M2O1 while biogas

yield per amount of VS-fed was at 910 ± 19 mL g-VS-fed^{-1}, which was similar to of M2O1. Interestingly, decrease of biogas production was observed with statistical difference reported (p < 0.05), when changing loading dosage from O1.2 to O1.4. Average of pH from day 64 to day 84 in two groups were very similar, at 7.5 ± 0.0.

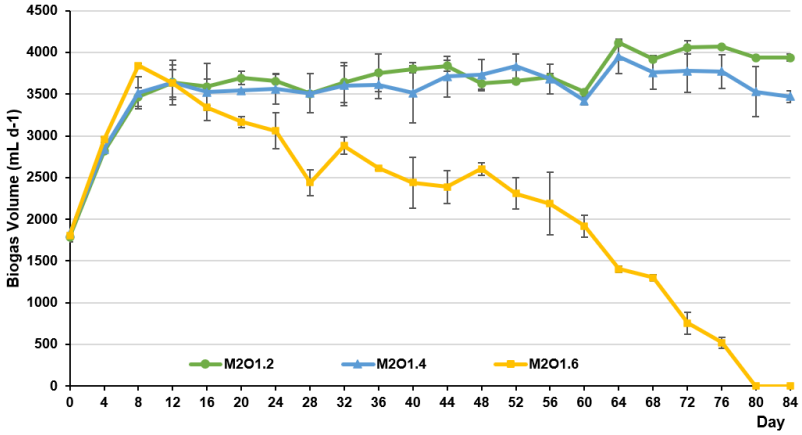


Figure 14: Trend of biogas production in oil threshold experiment

In the group supplemented with 1.6 g-VS$_{WKO}$ L^{-1} d^{-1}, reduction of biogas volume was recorded from day 16 and no biogas was measured at day 84. Decrease of pH value was observed more clearly from day 64 when pH dropped less than 7.0 (Figure 15). At the end of the test, the pH dropped to 5.9 ± 0.2 which indicated the failure of AD productivity. Because no extra benefit was obtained when amount of oil introduced to M2 reactors was increased from 1.2 to 1.4 g-VS L^{-1} d^{-1}, oil threshold should be less than or equal to 1.2 g-VS L^{-1} d^{-1} when M2 was chosen for digester operation.

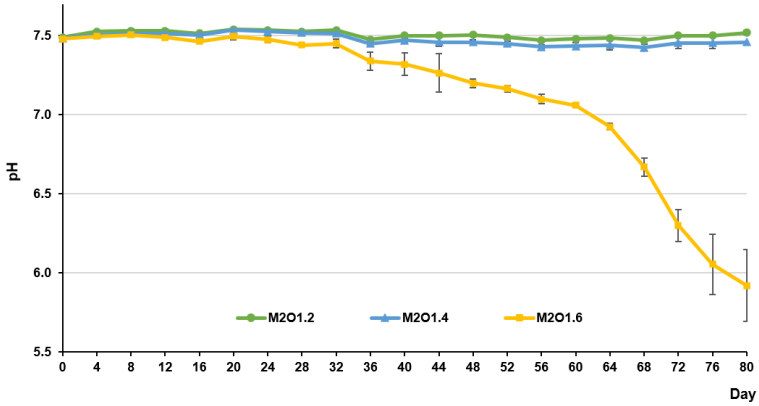


Figure 15: Trend of pH in oil threshold experiment

Data of microbial analysis showed that instability of biogas production resulted in the decrease of

Phylum Bacteroidetes, Proteobacteria, Tenericutes, Euryarchaeota, Kiritimatiellaeota and Chloroflexi (Figure 16). Meanwhile, counts of Firmicutes, Synergistees, Thermotogae and Actinobacteria was higher in digester added with 1.6 g-VS_{WKO} L^{-1} d^{-1}. Significant increase of Actinobacteria was observed in failure digester (M2O1.6) at 11.3% while it mostly did not present in other groups.

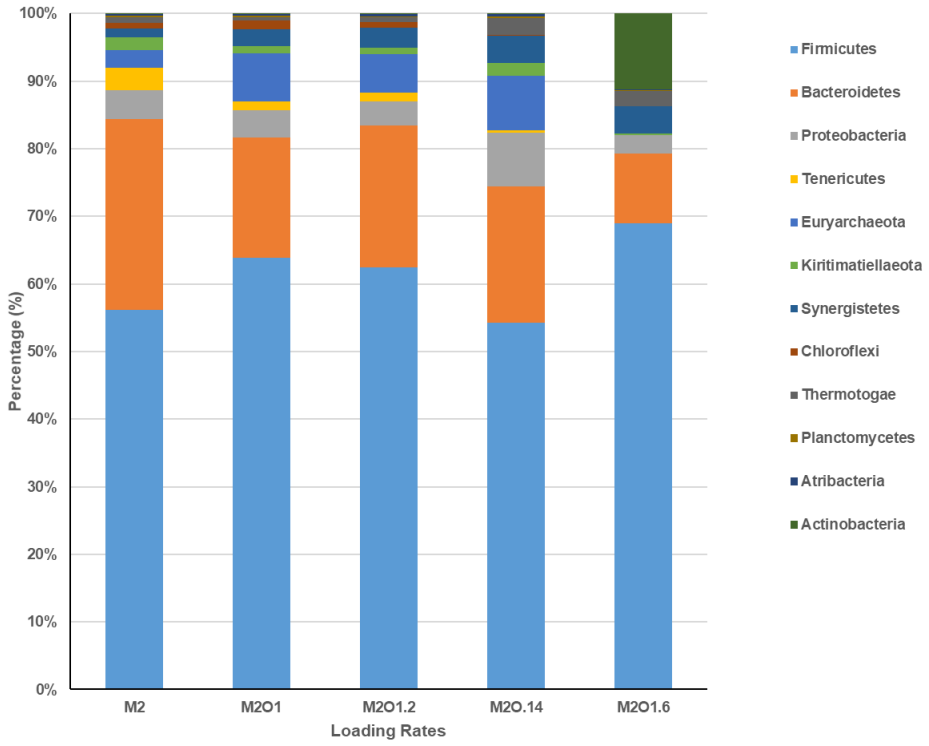


Figure 16: Comparison of bacterial community among groups loaded with M2 and other OLRs of WKO

Similar to Experiment 2, introduction of 1 or 1.2 g-VS L^{-1} d^{-1} of oil to M2 reactors resulted in increasing alpha diversity, from 217.8 in group with mono-digestion of manure to 233.0 and 232.0 in group M2O1 and M2O1.2, respectively (Table 7). This indicates that bacterial richness was enriched during co-digesting SM and WKO. However, when increasing OLR of oil to 1.4 g-VS L^{-1} d^{-1}, alpha diversity slightly dropped to 209.0. The addition of 1.6 g-VS_{WKO} L^{-1} d^{-1} not only caused disturbance of AD digester but also lessened alpha diversity, which was significantly lower than other groups, at 163.0.

Similar to changes of alpha diversity, percentages of Euryarchaeota increased form 2.7% in digester fed with only M2 to 5.6% and 8.0% when 1.2 or 1.4 g-VS_{WKO} L^{-1} d^{-1} was introduced.

Increase of Euryarchaeota richness is an important reason leading to increase of biogas production. On the other hand, severe disturbance was observed in M2O1.6 group where percentage of Euryarchaeota presented around 0.1%. Decline of the methanogens, the species responsible for producing methane in AD reactor, due to accumulation of VFAs, led directly to system failure.

Table 7: Change of bacterial community in different oil concentrations

Group	M2	M2O1	M2O1.2	M2O1.4	M2O1.6
Alpha diversity					
Bacteria	217.8	233.0	232.0	209.0	163.0
Methanogens	4.7	5.0	5.0	6.0	4.0
Percentage (%)					
Firmicutes	56.0	63.8	62.3	54.3	69.0
Bacteroidetes	28.3	17.7	21.0	20.1	10.3
Proteobacteria	4.3	4.0	3.6	8.0	2.7
Tenericutes	3.2	1.4	1.3	0.3	0.0
Euryarchaeota	2.7	7.1	5.6	8.0	0.1
Kiritimatiellaeota	1.9	1.1	1.0	2.0	0.1
Synergistetes	1.3	2.5	2.9	4.0	4.1
Chloroflexi	0.9	1.4	0.8	0.1	0.0
Thermotogae	0.8	0.5	0.7	2.5	2.2
Planctomycetes	0.2	0.2	0.2	0.2	0.1
Atribacteria	0.2	0.3	0.3	0.4	0.1
Actinobacteria	0.0	0.0	0.0	0.0	11.3
Total	**100**	**100**	**100**	**100**	**100**

4. Discussion

4.1. Effects of organic loading rates to biogas production and bacterial community

Addition of proper amount of WKO to SM digester was proven to increase biogas production. Similar results were reported in other studies, which indicated WKO could be a promising candidate for co-digestion with manure (Fierro et al., 2014; Hidalgo et al., 2015; Marchetti et al., 2019).

Nogueira et al. (2019) reported the changes of biogas production and bacterial community during co-digestion of cattle manure and WKO. Biogas volume increased by 203% without any adverse effect when 2% addition of WKO was introduced. The decrease of Bacteroidetes came with increase of Clostridiales and Synergistates were recorded when higher amount of WKO was used. Small amount of oil not only resulted in higher biogas productivity but also in richness of bacterial community and methanogens which are main reasons for the enhance most of productivity of AD system. The increase of hydrogen-consuming methanogens when oily substrates was introduced into ADs was reported by Ahring (1995) and Marchetti et al. (2020).

However, several issues recorded when loading rate was increased to 4 g-VS_{SM} L^{-1} d^{-1}, even when biogas production was higher during such co-digestion. For example, accumulation of VFAs was more obvious with significant increase of fatty acid concentrations. Both alpha diversity of bacteria and percentage methanogens altered negatively. Bacterial diversity of M4O1 or M4O2 was lower than M2, which seems to indicate the fluctuation of biogas productions. Several studies suggested that high TAN contents due to addition of SM could become an inhibition factor and negatively affect methanogens and bacterial acclimation (Hashimoto, 1986; Chen et al., 2008). Longer observation than 4 HRTs might be necessary to evaluate the bacterial changes. On the other hand, increase of OLRs to 6 g-VS_{SM} L^{-1} d^{-1}, showed lower effectiveness of co-digestion with less biogas production per VS-destroyed or VS-added. Therefore, ceiling limit of SM loading should be less than 4 g-VS_{SM} L^{-1} d^{-1}, and lower VS loading amount should be selected for the stability of AD system.

In some cases, increase of OLR did not result in higher biogas yield, and lower biogas production was recorded, even when AD performance was maintained. For example, less biogas was produced by M6O2 digester compared with M4O2, or lower biogas yield was obtained in M2O1.4 than in M2O1. This phenomenon was explained by Hansen et al. (1998) which was called “inhibited steady state”. This is an abnormal activity in biogas digester where AD performance is kept stable, but biogas production is lower than the system’s expectancy. Main reason of decrease of biogas yield was explained by interaction of three factors: NH_3, VFA and pH. Free ammonia inhibits methanogenic activity, resulted in accumulation of VFA. High concentration of VFA reduces the pH

value and then free ammonia concentration is decreased.

Other reasons contributing to the poor performance of digester containing high VS loading were foaming and clogging issues. Foaming inside digester may block the gas pipes and lead to collapse in some cases (Long et al., 2012). In our study, clogging issue was recorded frequently in digesters fed with 6 g-VS_{SM} L^{-1} d^{-1} in both mono and co-digestion even reactors were swirled manually three times per day. In addition, explosion was obtained from M6O2 digester several times during the experiment due to severe clogging issue (Figure 17). Efforts including the use of magnetic stirrer or change of digester's size did not result in any positive result. In large, scale application, the use of recirculating and spraying the digestate, impeller or anti-foaming agent may be necessary to avoid the problem (Kougias et al., 2016; Teng et al., 2014).

Figure 17: Explosion of biogas digester with high organic loading rate

4.2. Determination of waste kitchen oil limit

While addition of oil helped increase of biogas yield, high amount of oil fed into digester might resulted in severe imbalance in bacterial community and decreased biogas production. Decrease of biogas yield when higher concentration of oil was added into digester was described in a study conducted by Lymperatou et al. (2018). While feeding of 1% w/w crude glycerol (CG) to reactor containing SM increased methane yield, the addition of 3% CG showed the decrease of methane production. Causing of imbalance of microbial activity and failure of AD performance due to the accumulation of VFAs was reported in previous studies (Ahring et al., 1995; Awe et al., 2018;

Beccari et al., 1998). In our study, group fed with M2O1.6 resulted in significant drops of pH and biogas production while lower oil loading remained stable, and no extra benefit was obtained when OLR of oil was changed from 1.2 g-VS L^{-1} d^{-1} to 1.4 g-VS L^{-1} d^{-1}. Therefore, ceiling limit of oil should be less than 1.2 g-VS L^{-1} d^{-1} when 2 g-VS_{SM} L^{-1} d^{-1} was chosen for digester operation.

Loading rate of oil can be increased by raising amount of manure fed. However, even more biogas was obtained in this case, richness of methanogens might be negatively affected. Therefore, balancing OLR of oil is critical to maintaining the health of bacterial community as well as the stability of AD performance. It is important to note that significant decrease of biogas was observed obviously after two or four HRTs (42 or 84 days), depending on concentration of oil introduced into digester. Complete stop of biogas production could take even longer time. Therefore, long-time study with proper maintenance is necessary to fully evaluate effectiveness and stability of co-digestion when oil was used as a substrate. It is noted that change of pH or decrease of gas produced should not be indicators for early recognition of system deterioration because they usually occur when the process failure already happens (Drosg, 2013). Instead, accumulation of VFAs concentration or increase of hydrogen gas should be taken into account since they occur before reactor's vulnerability. Among of them, measurement of VFAs is recommended to recognize process perturbation (Ahring et al., 1995). However, pH measurement could be considered as a simple indicator to determine the stability of AD performance.

5. Conclusion

Co-digestion of SM and WKO was demonstrated to increase biogas production as well as efficiency of AD system when appropriate ratio of oil was maintained. Higher biogas potential of WKO compared with of SM and the increase of OLR during co-digestion are reasons supporting the increase of biogas yield. Other advantages were showed when combining these two substrates for co-digestion. For example, higher VS reduction and more biogas volume per VS-fed or VS-destroyed were recorded when applying M2O1, M4O1 or M4O2, which were essential factors to determine the effectiveness of AD system. Richness of bacterial diversity and percentage of methanogens were enhanced when small amount of oil was introduced.

Loading more than 4 g-VS_{SM} L^{-1} d^{-1} did not result in any extra benefit, and even more forming and

clogging issues were recorded in digesters with high OLR. Increase of biogas production was obtained in digester fed with 4 g-VS_{SM} L^{-1} d^{-1} and 1 or 2 g-VS_{WKO} L^{-1} d^{-1}, compared with those fed with M6O1 or M6O2, respectively. However, using M4O1 or M4O2 resulted in more fluctuations and accumulation of VFAs during the process, along with less diversity of bacterial and less richness of methanogens, compared with digesters fed with only 2 g-VS_{SM} L^{-1} d^{-1} and 1 g-VS_{WKO} L^{-1} d^{-1}. Introducing more than 1.4 g-VS_{WKO} L^{-1} d^{-1} into digester performed with 2 g-VS_{SM} L^{-1} d^{-1} caused significant imbalance of bacterial and AD system. A long time period, up to 84 days, might be necessary to record digester failure during co-digestion. Therefore, choosing appropriate loading rate and combination of SM and WKO is essential to maintain stability of bacterial community and AD performance.

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Chapter 3: Biogas Production of Swine Manure and Waste Kitchen Oil at Different Temperatures

1. Introduction

Improvement of biogas production and productivity of anaerobic digestion (AD) are important tasks to ensure biogas a valuable energy source. Different approaches have been applied to increase biogas production, such as co-digesting different substrates, changing digester's designs, applying two-phase digestion, pretreating organic materials, optimizing working conditions, etc. (Duan et al., 2019; Hidalgo et al., 2015; Mao et al., 2015; Patinvoh et al., 2017). Among them, co-digestion is a promising approach as it does not require the change of digester's design while several substrates can be treated at the same time. However, each organic material has different degradation rate or needs different working condition. Therefore, determination of biogas potential (BP) or biochemical methane potential (BMP) of substrates is a key for evaluation of co-digestion system efficiency. BMP is the maximum of methane volume which can be obtained during anaerobic process of substrates. Determination of BMP is usually based on batch study in where feedstocks were fed into digester one time (Angelidaki et al., 2009; Weinrich et al., 2018). Biogas or methane production is measured until no gas is released from reactor, then accumulation of gas obtained determines BMP of substrates (Holliger et al., 2016). Efficiency of digestion can be evaluated based on percentage of biogas recorded during the process per BMP of substrates.

In our previous study, waste kitchen oil (WKO) was demonstrated to be co-digested effectively with swine manure (SM) to increase biogas production when proper amount of oil was introduced into digester during semi-continuous AD performance at mesophilic condition. Mixing manure and WKO helped balance carbon-to-nitrogen ratio of feedstock, a factor shown to maintain the stable condition of AD system. Adding oil also resulted in more diversity of bacterial community and increase of methanogens in reactors, which were essential to remain the consistence of methane yield (Chapter 2). However, co-digestions of SM and WKO at different temperatures, including ambient and thermophilic conditions, had not been widely evaluated. Thus, it is necessary to determine efficiency of co-digesting the two substrates in terms of biogas production, in comparison

with maximum potential of individual substrates. Another question is how data obtained from the pilot study can be transferred to farm scale operation for further decision-making, such as for determination of digester volume or estimation of biogas production.

In this project, we conducted a batch study to determine biogas potential of two substates alone (SM and WKO) as well as biogas potentials of their combination (in co-digestion process). Next, efficiencies of semi-continuous co-digestion at three temperature settings (ambient, mesophilic and thermophilic) were evaluated by comparing biogas production with BP of individual substrates. Other factors were considered to evaluate AD efficiency, including bacterial diversity and volatile solids (VS) reduction. Finally, calculations of digester volume, oil and water usage, and biogas production were performed based on data of a typical commercial swine barn to discuss different scenarios for decision-making purposes.

2. Materials and methods

2.1. Sample collection

Manure sample was collected from a central Missouri commercial finishing pig farm while WKO was provided from a university campus dining service in Columbia, Missouri. Manure was kept in 5-gal (18.93-L) buckets at -20 ºC and was thawed at 4 ºC for at least one day before each loading. Oil was kept in a glass container at room temperature (24 - 25 ºC). Total solid (TS) and VS of substrates were measured to determine substrate volume of SM and WKO introduced into digesters.

2.2 Biogas potential of swine manure and waste kitchen oil

2.1. Experiment 1: pilot study without replication

Batch AD approach was used to determine BP of each substrate as well as potential of co-digestion of the two substrates. The digestates used to conduct BP studies were taken from digesters following semi-continuous AD method in two scenarios: both SM and WKO were utilized for co-digestion (Experiment 1A); only SM was used (Experiment 1B).

Five 1.89-L (0.5-gal) jars were loaded with feedstock containing 2 g-VS_{SM} L^{-1} d^{-1} and 1 g-VS_{WKO} L^{-1} d^{-1} in Experiment 1A while loading rate of 2 g-VS_{SM} L^{-1} d^{-1} was introduced into five other digesters in the next experiment, 1B. Substrates were added every two days following the semi-continuous

AD approach described in Chapter 2. Nitrogen gas was flushed into each digester headspace to create anaerobic environment after loading. All digesters were swirled three times per day to enhance the contact between bacteria and substrates. Biogas volumes were indirectly measured by using height-to-volume model based on the method described by Nogueira et al. (2019) and Wang et al. (2020). Digesters in all groups were kept at 40 ºC for one hydraulic retention time (HRT) to ensure the stability of biogas performance in each system.

When the Experiment 1 was set up, digestate from five jars were mixed and divided into each digestion jar for homogenization purpose. Then, part of digestate was withdrawn from jars so that the working volume of each reactor was maintained at 1.25 L before new substrate was added. No organic material was supplemented into the control group while manure or oil or their combinations were diluted with tap water to the volume of 131 mL, before divided into four test groups, including 4 g-VS_{SM} L^{-1} (M4), 4 g-VS_{WKO} L^{-1} (O4) and the two substrate mixtures (M4O2 and M4O4). Because final working volumes were 1.381 L, organic loading rates of each studied group above were 5.5, 5.5, 8.3 and 11.0 g-VS, respectively.

After added with respective feedstocks, digesters were flushed with nitrogen gas, then capped and incubated at 40 ºC. Jars were manually swirled three times per day, biogas volume was measured and recorded daily until no biogas was produced from each digester or biogas accumulation of each digester in three continuous days was less than 1 percent of total biogas production (Holliger et al., 2016). The purposes of the pilot study were to evaluate effect of digestate (with and without oil) to biogas production and biogas potential before the replicated experiment (Experiment 2) was conducted.

2.2. Experiment 2: Replicated study

For more insight of biogas potential of the different substates, replicated study was conducted in triplicate with five groups, including control and four testing loading rates at 40 ºC (mesophilic condition). Digestate with manure and oil was selected because pilot study showed slightly higher biogas potential per VS added were obtained, from 7.2% to 35.9%, when using digestates collected from reactors loaded with two types of substrates instead of from mono-digestion.

Before the study, fifteen 1.89-L (0.5-gal) glass digesters with working volume of 1.375 L were

loaded with 2 g-VS_{WKO} L^{-1} d^{-1} and 0.5 g-VS_{WKO} L^{-1} d^{-1} using semi-continuous approach described above for one HRT. Then, digestate from all jars were mixed and separated for homogenization purpose. Similar to Experiment 1, part of digestate was withdrawn so that the AD working volume remained 1.25 L. While no substrate was introduced into the control digesters, other studied groups included 4 g-VS_{SM} L^{-1} (M4), 4 g-VS_{WKO} L^{-1} + 1 g-VS_{WKO} L^{-1} (M4O1), 4 g-VS_{WKO} L^{-1} + 2 g-VS_{WKO} L^{-1} (M4O2) and 2 g-VS_{WKO} L^{-1} (O2), which corresponded to loading rates of 5.5, 6.9, 8.3 and 2.8 g-VS, respectively. The first three dosages were similar to the amount of substrates added in each loading time into M2, M2O0.25 and M2O1 digesters in the semi-continuous experiment where feedstocks were added every two days.

Digesters were capped after loading and no further addition of substrate was performed. Biogas productions were measured every day to determine biogas accumulation. Biogas potentials were calculated by subtracting average of biogas production in each group to the biogas production observed in the group without substrate (control group). Measurement of carbon dioxide (CO_2) was conducted on day 7 when more than 70 percent of biogas potentials were obtained based on pilot study. Concentration of CO_2 was analyzed to evaluate quality of biogas because it is a major gas in the biogas mixture, beside methane (CH_4). Measurement was terminated when no more biogas produced or when accumulation of biogas obtained in the last three days was less than total accumulation of biogas. Total solid (TS), volatile solid (VS) and pH were measured before and after the study to determine the changes of digestate and to evaluate biogas potentials per amount of VS added.

2.3. Efficiency of biogas production in different temperature conditions

Four organic loading rates (ORLs), M2, M2O1, M4O1 and M4O2 were selected for evaluation of biogas production of different conditions, including ambient, mesophilic and thermophilic temperatures following semi-continuous AD approach described above. These OLRs were proved to be highly efficient in term of biogas production per VS-fed or VS-destroyed in pervious experiment conducted in the lab in which different combinations of SM and oil were examined (Chapter 2). Temperatures of ambient, mesophilic and thermophilic conditions were set up at 30, 40 and 55 °C, respectively, which are typical temperatures of these conditions (Boušková et al.,

2005; Ziganshin et al., 2013).

2.3.1. Mesophilic and thermophilic studies

Glass digesters with capacity of 1.89 L (0.5 gal) were used during the studies, except in case of severe forming issue when larger, 3.79-L (1-gal) jars were used for replacement. Data of biogas production, TS and VS measurements at 40 °C (mesophilic condition) were following those from the previous experiment, in which substrates were loaded every two days, following continuous approach described above.

After mesophilic study completed, temperature was raised from 40 °C to 55 °C in 30 days, at a rate of around 2 °C per day to determine biogas productivity in thermophilic temperature (Bouškova et al., 2005). Digesters with bigger size (3.79 L) were used as a replacement when foaming and clogging issues were observed frequently. The study was then conducted for 3.5 HRTs (72 days) to fully evaluate biogas production. Digestate samples of each reactor were collected at the end of the study to measure TS and VS and to perform metagenomics analysis which was followed procedure described in Chapter 2.

2.3.2 Ambient temperature study

Because digester failures were observed in two among four groups after the thermophilic study, the new four groups were set up at 40 °C again in replicate before the study AD performance at ambient temperature started. After AD reactors had a chance to stabilize for at least one HRT, temperature was decreased from 40 °C to 35 °C in 15 days and then from 35 °C to 30 °C in the same period of time. Significant drops of biogas productions in groups M4O1 and M4O2 were recorded when temperature was decreased to 35 °C. Therefore, 35 °C condition was upheld for more evaluation.

After 3 HRTs, the reduction was still observed in group loaded with M4O2 while digesters with M4O1 recovered and biogas of other OLRs (M2 and M2O1) were consistent. Then, digesters fed with 4 g-VS_{SM} L^{-1} d^{-1} were continued at 35 °C while additional four jars were setup with digestates from other working digesters for replacement of those jars to start the 30°C study. Data were collected during a period of five HRTs (104 days) to fully evaluate gas production of co-digesting SM and WKO for determination of biogas efficiency. Digestate samples were collected when the

study was completed for measurement of TS and VS. Because failures were observed in all oil-containing digesters, no sample collection for metagenomics study was performed.

2.4. Comparisons of digester setup for decision-making purpose

Different scenarios were considered in terms of biogas production, digester' capacity and AD stability when different organic loading dosages were applied. Four loading rates containing SM alone and with WKO were evaluated, including M2, M2O1, M4O1 and M4O2. The VS loading of SM in these scenarios were 2 g-VS L^{-1} d^{-1} and 4 g-VS L^{-1} d^{-1} (or 2 kg-VS m^{-3} d^{-1} and 4 kg-VS m^{-3} d^{-1}). respectively. Ratio of oil to manure (O/M ratio, g-VS/g-VS) was 0.5 for M2O1 and M4O2, and 0.25 for M4O1 (Table 8).

Table 8: Organic loading rates and biogas yields of four feedstocks combinations

	Unit	M2	M2O1	M4O1	M4O2
Manure loading rate	kg-VS m^{-3} d^{-1}	2	2	4	4
Oil loading rate	kg-VS m^{-3} d^{-1}	0	1	1	2
Total OLR	kg-VS m^{-3} d^{-1}	2	3	5	6
Biogas yield	m^3 kg-VS^{-1}	0.729 ± 0.023	0.909 ± 0.037	0.793 ± 0.018	0.917 ± 0.43

SM production of a swine farm is assumed at 560 kg pig^{-1} 120-day^{-1} or 4.67 kg pig^{-1} day^{-1}, and VS production of SM (VSP_{SM}) is 45 kg-VS pig^{-1} 120-day^{-1} or 0.375 kg-VS pig^{-1} day^{-1} (ASABE Standard, 2019). Therefore, VSP_{SM} of a typical commercial swine farm with 10,000 heads is estimated at 3,750 kg-VS day^{-1}. Digesters' working volumes were supposed to account for 70 percent of reactors' capacity.

Calculations of working volume, digester volume and loading rates were based on following equations:

$$\text{Working volume } (m^3) = \frac{VSP_{SM}\ (\text{kg-VS d}^{-1})}{MLR\ (\text{kg-VS m}^{-3}\ \text{d}^{-1})}$$

$$\text{Digester volume } (m^3) = \frac{\text{Working volume (m3)}}{70\%}$$

$$\text{Manure loading } (m^3\ d^{-1}) = VSP_{SM}$$

$$\text{Oil Loading } (m^3\ d^{-1}) = \frac{\text{Oil loading rate x Working volume}}{\text{VS-WKO}}$$

Water consumption (m^3 d^{-1}) = Working Volume – Manure Loading – Oil Loading

Biogas production ($m^3\ d^{-1}$) = Biogas yield x Total OLR x Working volume,

where VSP_{SM} and MLR are volatile solids production of SM (kg-VS d^{-1}) and manure loading rate (kg-VS $m^{-3}\ d^{-1}$), respectively.

2.5. Data analysis

Data analysis was processed by using Excel (Microsoft Corporation, Redmond, WA, USA). Graphs and mean comparisons were performed using built-in functions in Excel.

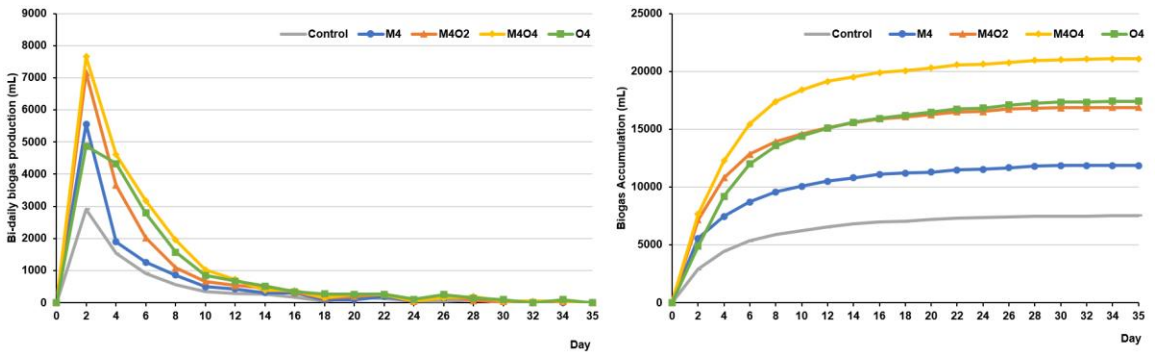
3. Results

3.1. Biogas potential of swine manure and waste kitchen oil

3.1.1. Experiment 1: Pilot study without replication

The experiment took 30 days to collect all biogas production when using manure digestate while 35 days was required when manure-oil digestate was used (Figure 18). Day 2 recorded highest biogas production rate in the two measurement periods, with an exception of O4 group in Experiment 1B where highest rate of biogas released was obtained in day 4. This suggests that when only oil was fed, slightly longer time is necessary for the adaption of bacterial community to the new substrates.

In Experiment 1A, biogas production obtained after 7 days accounted for more than 70 percent of total gas produced for every group, and more than 90 percent of biogas production was recorded after 15 days. Total gas volume was recorded after 32 days. Meanwhile, it took 9 days for biogas production in each group to be beyond 70 percent of total biogas production. However, total biogas produced was obtained after only 27 days, 5 days sooner than when manure-oil digestate was used. Biogas production of control group was only 3,703 mL in Experiment 1B, significantly lower than production in Experiment 1A, which was 7,530 mL. Biogas productions of all other groups were significantly higher when manure-oil digestate was used. This could be explained by the use of digestate. Using digestate collected from co-digestion of SM and WKO resulted in more undigested nutrient-rich substrates where biogas production could be produced in a longer period even without adding new substrates. As a consequence, the increases of both biogas production and time to perform the study were observed.



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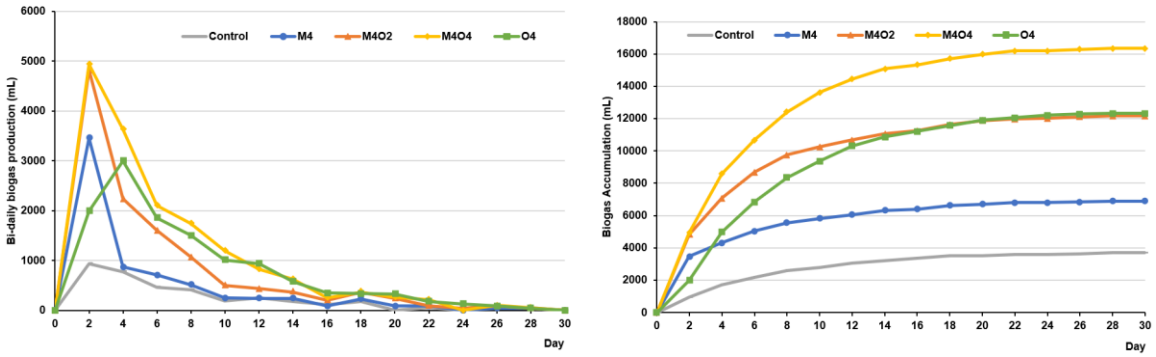


b

Figure 18: Biogas production and accumulation in pilot study using co-digesting manure-oil digestate (a) and mono-digesting swine manure digestate (b)

Biogas potential of O4 was similar to of M4O2 in both experiments, which suggested gas potential of 2 g-VS of WKO is equal to of 4 g-VS of SM. Biogas potential of M4 in Experiment 1A was higher than in Experiment 1B, at 4,326 mL compared to 3,185 mL in second experiment (Table 9). Meanwhile, only slight differences of biogas potential were observed in group M4O2 and O4. Potential of gas volume produced from O4 was around 14.6% higher when digestate from manure-oil AD was used instead of from manure-fed AD only. Biogas potential from each gram VS_{WKO} added was 2.29 to 2.71 times higher than of SM in both experiments. As a consequence, potential of biogas obtained per VS_{added} of M4O4 was slightly higher than of M4O2, ranged from 8.8% to 12.2% higher. Meanwhile, significant increase of biogas/VS was recorded when comparing M4O4 with M4, from 56.8% to 98.5% in two experiments. The results confirm the benefits of co-digesting SM and WKO in terms of increasing biogas production while a small amount of oil was added. The pH values were similar between the OLRs in each experiment, at 7.7 when using M-O digestate

and 7.6 when SM digestate was used.

However, it should be noted that these experiments were conducted without replication. Therefore, the differences might not come from their performance or digestate characteristics. Instead, error or leakage might occur and negatively affect the accuracy of the study.

Table 9: Biogas potential of substrates in two pilot studies

		Control	M4	M4O2	M4O4	O4
Loading rate	g-VS	0	5.5	8.3	11.0	5.5
M-O digestate						
Biogas production	mL	7,530	11,856	16,877	21,094	17,425
Biogas potential	mL	-	4,326	9,347	13,564	9,895
BP/VS	mL/g-VS		787	1,133	1,233	1,799
pH		7.7	7.7	7.7	7.7	7.7
SM digestate						
Biogas production	mL	3,703	6,888	12,156	16,348	12,337
Biogas potential	mL	-	3,185	8,453	12,645	8,634
BP/VS	mL/g-VS		579	1,025	1,150	1,570
pH		7.6	7.6	7.6	7.7	7.7

3.1.2. Experiment 2: Replicated study

Experiment with three digesters in each group were conducted until day 50 to make sure all biogas produced was captured. Similar to the pilot study, highest biogas rate was recorded in the first two days, accounting for 27 ± 0% to 39 ± 0% of total biogas production (Figure 19). More than 69% of total biogas production was collected within one week and was as high as 85% within the first two weeks. Biogas produced during the period of one HRT (21 days) accounted for 90 ± 5% to 94 ± 3% of total gas recorded. Gas volumes of three groups M4, M4O1, M4O2 obtained in day 1 were similar to each other, from 3,656 ± 68 to 3,773 ± 68 mL, and significantly higher than gas productions of control and WKO groups ($p < 0.05$). The differences of daily biogas production between these three groups became more obvious from day 2 with lowest and highest productions observed in M4 and M4O2 groups, respectively. While biogas volume of M4 digesters was higher

than of O2 group in day 1, more biogas was produced by digesters fed with only oil compared to mono-digestion of SM from day 2, resulting in similar biogas potential between these two groups when the experiment was completed.

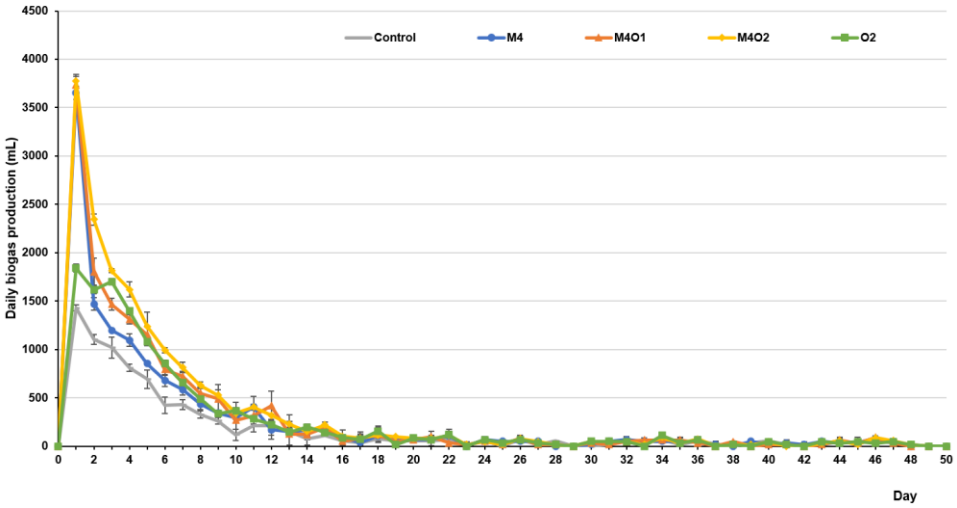


Figure 19: Daily biogas production of the Experiment 2, replicated study

It is interesting to note that the different of biogas accumulations between testing groups came from total of daily biogas released in the first 10 days of the study, with volumes of biogas accumulated in the M4, M4O1, M4O2 and O2 groups were 10,613, 12,286, 14,083, and 10,341 mL, respectively (Figure 20). There was no obvious gap in biogas recorded between M4, M4O1 and O2 groups from day 11 until day 50 (*p-value* > 0.05), from 2,453 to 2,515 mL while only slight increase was observed for the from M4O2 loading, at 2,875 mL. However, the difference was not statistically significant (*p-value* > 0.05).

Total biogas accumulated from manure-containing digesters (M4) were about 2.73% higher than of oil group (O2), at 13,128 ± 599 compared to 12,779 ± 360 mL. However, the difference was not statistically significant (*p-value* > 0.05). It again confirmed that biogas potential of 2 g-VS_{KWO} was similar to potential of 4 g-VS_{SM}. Adding 1 and 2 g-VS of oil in co-digestion increased total biogas production by 12.6% and 29.1%, respectively.

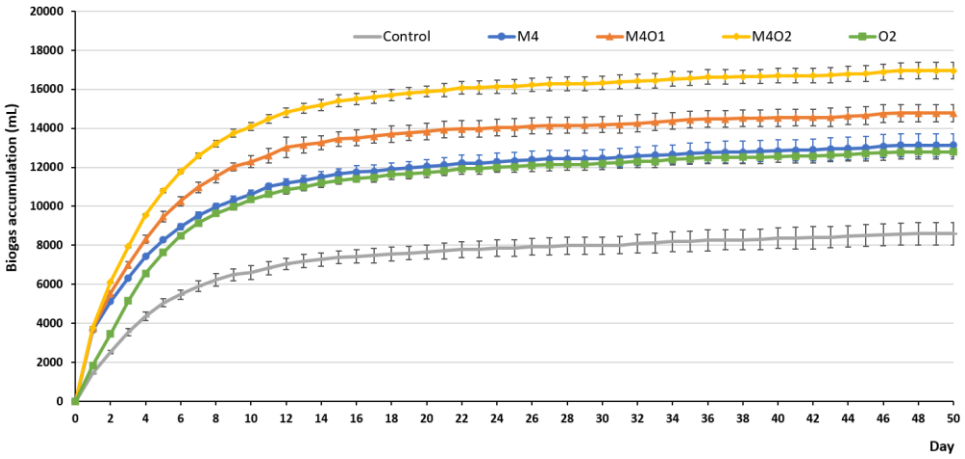


Figure 20: Biogas accumulation in replicated experiment

Biogas potentials of 4 g-VS_{SM} and 2 g-VS_{WKO} were 4,550 ± 208 and 4,200 ± 118 mL, respectively, with only 8% increase of gas released from M4 digester compared to the O2 group (Table 10). However, biogas obtained from one VS gram of oil fed was 1,527 ± 43 mL/g-VS_{fed}, which was significantly higher than 827 ± 38 mL biogas release from each gram VS of manure. If supposed methane accounted for 55% of total gas, biochemical methane potential of SM was approximated at 455 ± 15 mL/g-VS_{fed}, which was slightly higher than data reported by Sun et al. (2015). The results confirm that WKO is an energy-dense material and addition of oil at proper amount will result in significantly higher biogas production. As a result, biogas potentials per VS-fed were 902 ± 27 and 1,014 ± 25 mL/g-VS-fed when 1 and 2 g-VS of oil were introduced, which were equal to 9.0 ± 3.2% and 22.6 ± 3.1% increase compared to mono-digestion of SM. On the other hand, the VS-destroyed rate of SM was at 78.8 ± 5.8%, which was significantly lower than of WKO, at 97.0 ± 6.9%. This was in agreement with the study reported by Astals et al. (2013), which suggested that oily substrate was more attractive for bacterial activity than SM. Biogas potential per VS-destroyed of WKO was also higher than of SM, at 1,600 ± 45 and 1,038 ± 47 mL g-$VS^{-1}_{destroyed}$, respectively.

Biogas potentials of M4O1 and M4O2 were 6,201 ± 183 and 8,365 ± 208 mL, which were equal to 93.2 ± 6.6% and 95.6 ± 4.8% of sum of potentials of separate materials. Therefore, it could be concluded that co-digestion does not proportionally increase biogas yield compared to total potentials of each substrate. Synergistic benefits actually were caused by the increase of maximum OLR which can be added to digester and higher diversity of bacterial community when co-digestion

is applied. Values of pH in each group were similar, at 7.7 ± 0.1, while only slight difference of CO_2 concentration was observed between the VS loading rates, suggesting quality of ultimate biogas production were the same.

Table 10: Comparison of biogas potentials and other factors in replicated BMP study

	Unit	Control	M4	M4O1	M4O2	O2
Loading rate	g-VS	0	5.5	6.875	8.25	2.75
VS-destroyed	%	-	78.8 ± 5.8	84.4 ± 9.1	82.0 ± 6.3	97.0 ± 6.9
Biogas production	mL	8,578±555	13,128±599	14,779±437	16,944±422	12,779±360
Biogas potential	mL	-	4,550 ± 208	6,201 ± 183	8,365 ± 208	4,200 ± 118
BP/VS-fed	mL $g\text{-}VS^{-1}$		827 ± 38	902 ± 27	1014 ± 25	1527 ± 43
BP/VS-destroyed	mL $g\text{-}VS^{-1}$		1,038 ± 47	1,077 ± 32	1,245 ± 31	1,600 ± 45
Co-digestion efficiency	%	-	-	93.2 ± 6.6	95.6 ± 4.8	-
pH	-	7.7 ± 0.0	7.7 ± 0.0	7.7 ± 0.0	7.7 ± 0.0	7.7 ± 0.0
CO_2	%	24.7 ± 0.6	27 ± 0.5	26.3 ± 0.8	26.2 ± 0.6	25.5 ± 0.9

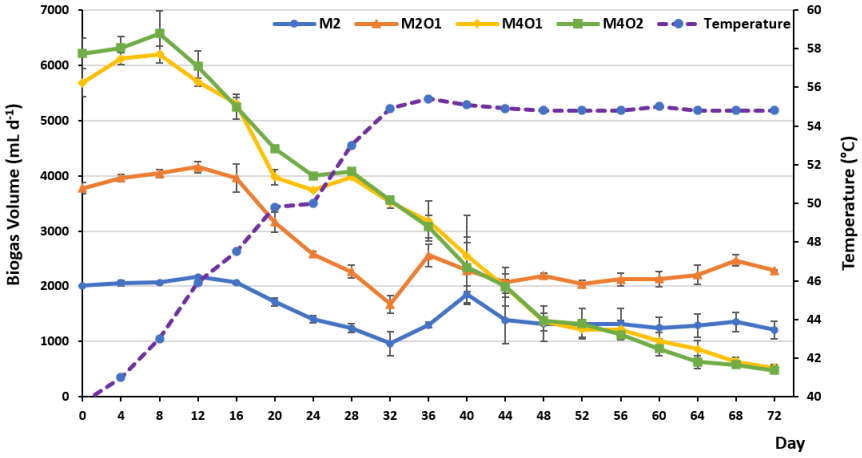
3.2. Performance of anaerobic digestion at different temperatures

3.2.1. Mesophilic and thermophilic conditions

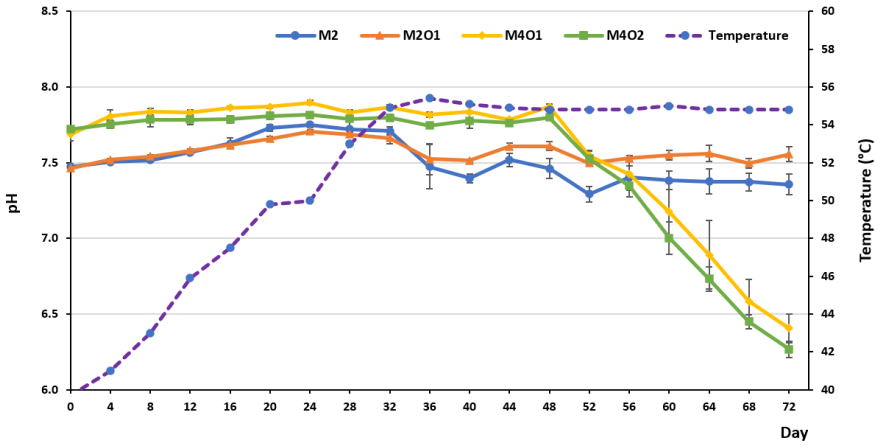
Data from previous experiment showed consistence of biogas production when co-digesting SM and WKO at mesophilic condition (40 °C). However, the change of temperature from 40 °C to 55 °C resulted in significant drop in biogas production in all four loading rates (Figure 21a). While biogas reductions of M4O1 and M4O2 were recorded in day 12, obvious drop of biogas yield in M2 and M2 digesters were obtained from day 20. This suggests that high loading rate resulting in more vulnerable characteristic of AD performance. Biogas production of M2 and M2O1 became more stable from day 44 until the end of the study with gas yields averaging 1,718 ± 68 and 3,161 ± 182 mL d^{-1}, respectively. These biogas productions were about 10 – 15% lower than data collected in the mesophilic condition. Meanwhile, increase of temperature first resulted in slightly higher level of pH in M2 and M2O1 groups, from 7.6 ± 0.0 at day 16 to 7.7 ± 0.0 day 32 (Figure 21b). Then, drops of pH in these two groups were observed from day 36. Finally, pH values of these two loading

rates became consistent quickly, around 7.4 ± 0.1 and 7.6 ± 0.1, respectively.

On the other hand, the decrease of biogas yield when digesters were fed by M4O1 or M4O2 was continuing from day 12 until the end of the study. Final biogas productions were below detectable level at day 72. The reductions of pH were also observed from day 52 and final pH values were less than 6.5 when the measurement was concluded. It should be kept in mind that the drop of pH followed reduction of biogas production, which is the reason why pH should not be considered solely as an indicator for early detection of digester imbalance. However, the drop or consistence of pH could be a good factor to confirm the fluctuation in stability of AD performance.



(a)



(b)

Figure 21: Change of biogas production (a) and pH (b) when temperature was increased from 40 °C to 55 °C, Experiment 3

Similar observations were reported in previous studies (Astals et al., 2012, 2013; Hansen et al.,

1999). Methane yield from SM-fed biogas system reported by Hansen et al. was 188 mL/g-VS at 37 °C, significantly higher than the production at 55 °C (67 mL/g-VS). In the study conducted by Astals et al. (2012), supplement of oily matter increased biogas production to 0.74 L g-VS-fed^{-1}, compared to 0.45 L g-VS-fed^{-1} of mono-digestion of pig manure. However, mono-digestion of SM in thermophilic condition resulted in only 0.17 L g-VS-fed^{-1} while co-digestion of manure with glycerol increased biogas production to 0.47 L g-VS-fed^{-1}, which was still lower than yield recorded at mesophilic temperature (Astals et al., 2013). Compared to mesophilic digestion, higher NH_3 and VFA concentration were observed in thermophilic condition, which lead to higher instability risk.

Changes of bacterial community

Increasing temperature from mesophilic to thermophilic condition resulted in dominance of *Thermotogae*, the hyperthermophilic bacterial phylum (Figure 22). While the present of *Thermotogae* was less than 1% in all loading rates at 40 °C, this phylum accounted for 59.93% to 66.22% in digesters working at 55 °C, leading it the most populous phylum. Abundance of thermophilic bacteria in anaerobic digesters at high temperature was reported in previous studies (Stolze et al., 2016, Shaw et al., 2017). Meanwhile, abundance of the four most popular phyla in mesophilic digesters, including *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Tenericutes* was significantly reduced with the increase of temperature. While Firmicutes accounted for 56.06% to 75.97% of total bacteria in reactors at 40 °C, its percentage at thermophilic digestion ranged from 30.52% to 37.88%. Similarly, while Bacteroidetes' abundance in mesophilic condition was between 12.04% to 28.35%, it accounted for 0.08% to 1.36% in digesters working at 55°C. *Proteobacteria* and *Tenericutes* accounted for less than 1% of total bacteria in thermophilic reactors.

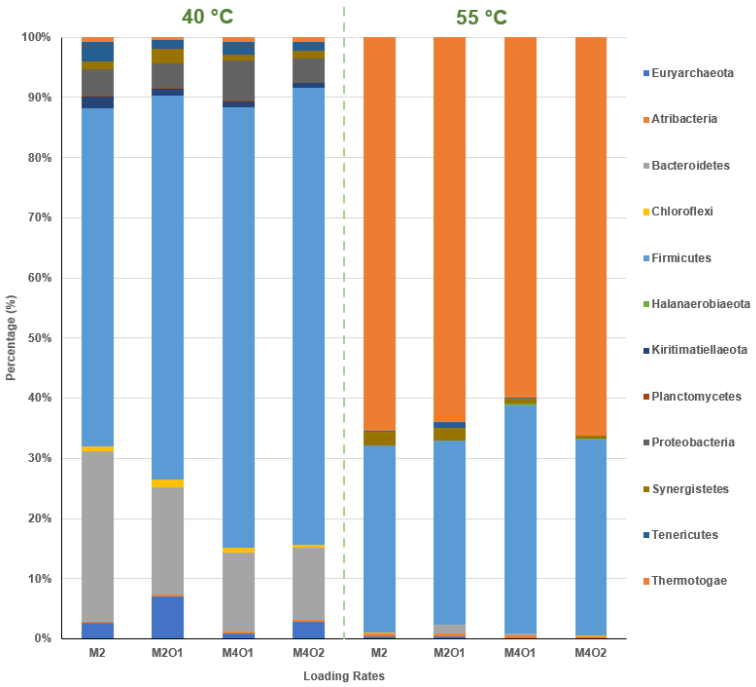


Figure 22: Changes of bacterial community from mesophilic to thermophilic condition

Thermophilic digestion was also seen link closely to the decrease of bacterial diversity in digesters. Alpha-diversities of M2, M2O1, M4O1 and M4O2 at 55 °C were 91, 109, 101, 62, respectively, significantly lower than the diversities of corresponding loading rates at 40 °C (216.3 ± 4.0, 233.0 ± 6.0, 183.3 ± 7.6, 166.0 ± 4.4). Meanwhile, *Euryarchaeota* accounted for 0.36%, 0.49%, 0.06%, 0.02% of total bacteria in M2, M2O1, M4O1 and M4O2 groups at thermophilic condition, which showed significant decreases compared to 2.7%, 7.0%, 0.9%, 2.8%, respectively, at 40 °C (Table 11). The significant drop of *Euryarchaeota's* abundance in digesters fed by M4O1 and M4O2 seems to be the reason for the drop of biogas production.

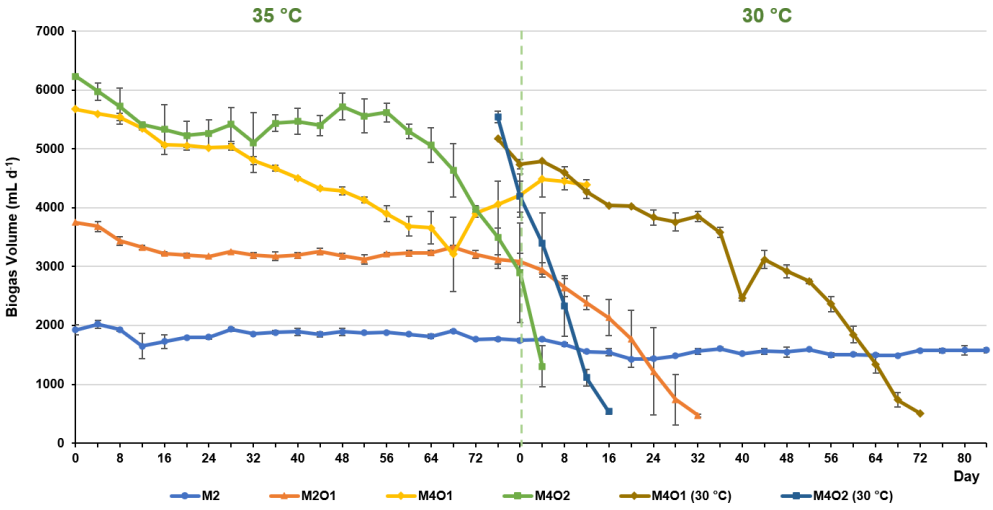
Table 11: Changes of bacterial community from mesophilic to thermophilic condition

	Mesophilic				**Thermophilic**			
	M2	**M2O1**	**M4O1**	**M4O2**	**M2**	**M2O1**	**M4O1**	**M4O2**
Euryarchaeota	2.66%	7.01%	0.91%	2.77%	0.36%	0.49%	0.06%	0.02%
Firmicutes	56.06%	63.72%	73.23%	75.97%	31.16%	30.52%	37.88%	32.63%
Bacteroidetes	28.25%	17.84%	13.20%	12.04%	0.12%	1.36%	0.21%	0.08%
Proteobacteria	4.26%	3.97%	6.74%	3.99%	0.00%	0.00%	0.10%	0.09%
Tenericutes	3.24%	1.41%	2.12%	1.42%	0.27%	0.84%	0.04%	0.02%

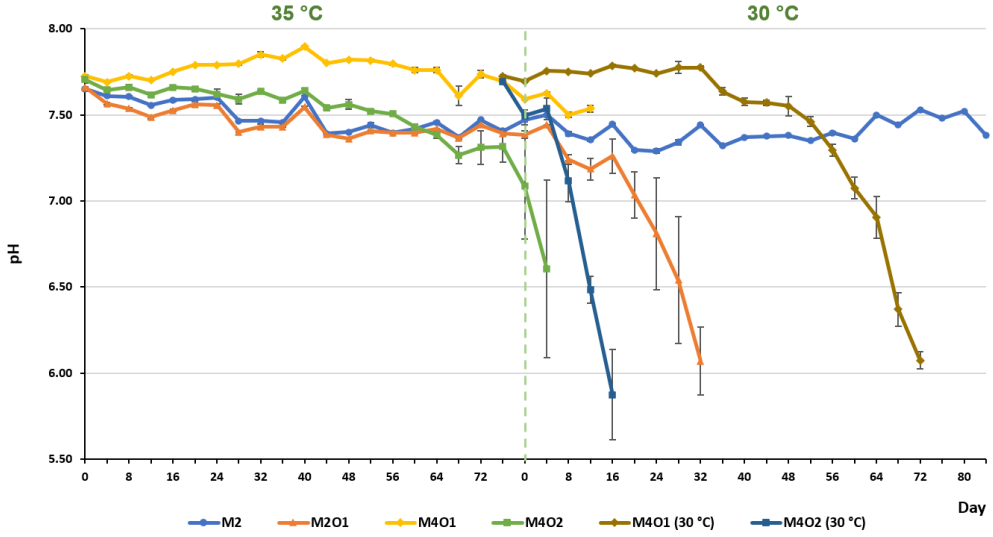
Kiritimatiellaeota	1.89%	1.10%	0.98%	0.75%	0.00%	0.00%	0.00%	0.00%
Synergistetes	1.25%	2.48%	0.84%	1.40%	2.15%	2.18%	0.80%	0.41%
Chloroflexi	0.86%	1.33%	0.75%	0.47%	0.03%	0.07%	0.06%	0.02%
Thermotogae	0.84%	0.47%	0.83%	0.77%	65.36%	64.01%	59.93%	66.22%
Atribacteria	0.24%	0.30%	0.27%	0.34%	0.48%	0.46%	0.58%	0.38%
Planctomycetes	0.24%	0.18%	0.05%	0.02%	0.01%	0.03%	0.02%	0.01%
BRC1	0.04%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Actinobacteria	0.04%	0.04%	0.05%	0.02%	0.00%	0.00%	0.04%	0.04%
Acidobacteria	0.03%	0.02%	0.02%	0.03%	0.00%	0.00%	0.00%	0.00%
Armatimonadetes	0.02%	0.06%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Halanaerobiaeota	0.00%	0.00%	0.00%	0.00%	0.05%	0.03%	0.26%	0.09%

3.2.2. Ambient condition

When temperature was decreased to 30 °C, significant disturbance was recorded in groups fed with higher oil-to-manure ratio. More fluctuation and followed by decrease of biogas production occurred in M4O1 and M4O2 group at 35 °C (Figure 23a). Average of daily biogas yield released from digesters fed M4O1 dropped from 5,678 ± 12 mL at day 0 to 3,209 ± 636 mL at day 68. Then, recovery of AD performance in this group was recorded from day 72. Meanwhile, biogas volume released from M4O2 decreased continuously from day 60 and was under measurable level at day 84. When other digesters were setup and fed with M4O1 and M4O2 to start 30 °C study, biogas productions of these groups dropped even faster and cannot be measured after 72 days and 16 days, respectively.



a



b

Figure 23: Change of biogas production (a) and pH (b) when decreasing temperature from 40 °C to 30 °C

Group loaded with M2O1 also recorded the decrease of biogas production from 3,754 ± 0 mL d^{-1} at day 0 to around 3,217 ± 56 mL d^{-1} at 35 °C and consistence of AD performance was noted from day 16 to day 80. However, setting the working condition at 30 °C caused negative consequence to biogas yield of M2O1 and resulted in failure of AD system after 32 days. It is interesting to note that even biogas yield of M2 group dropped from 2,006 ± 64 mL d^{-1} at 40 °C to 1,859 ± 45 mL d^{-1} at 35 °C and then to 1,538 ± 35 mL d^{-1} at 30 °C, no significant fluctuation ($p > 0.05$) was recorded during each temperature level. These data suggests that high loading rates with high oil-to-manure

ratio are not suitable for anaerobic digestion at ambient temperature. Similar to the previous studies, pH value of working digesters showed consistency while severe drops of pH were observed in the failure groups (Figure 23b). It again suggested that even not being a good indicator to detect AD imbalance, stability of pH value could be considered as an important factor to determine the consistent performance of AD system.

3.3. Efficiency of biogas production in different temperature conditions

Highest biogas yield per VS fed was observed when ratio of oil to manure is around 0.5 compared to other testing groups at 40 °C (Table 12). Biogas yields of four loading rates in the study (M2, M2O1, M4O1 and M4O2) were 729 ± 23, 909 ± 37, 793 ± 18 and 917 ± 43 mL g-VS-fed^{-1} respectively, at mesophilic condition. Efficiency of AD performance in mesophilic temperature was very high, ranging from 88.2 ± 2.8% to 90.4 ± 4.2% of total biogas potential of substrates. Both groups of M2O1 and M4O2 showed the most efficient biogas production, around 90%, while slightly lower efficiencies were observed in other groups. Biogas production observed in semi-continuous ADs were always lower than biogas potential due to part of digestate was withdrawn during loading, which contained un-digested organic materials.

Increasing temperature to 55 °C reduced productivity of AD system. Significant drops were recorded in all loading rates at thermophilic condition. Biogas yields of M2 and M2O1 were only 470 ± 19 and 536 ± 37 mL g-VS-fed^{-1}, corresponding to 56.8 ± 2.3% and 52.9 ± 3.6% efficiency. Meanwhile, AD performance in M4O1 and M4O2 digesters showed severe imbalance, implying the insufficiency of co-digesting with high loading rates and high ratio of oil to manure. Higher temperature also resulted in lower VS destroyed rate. VS reduction rates of M2 and M2O1 at 55 °C were 59.5% and 65.7%, which were slightly lower than at 40 °C. Significant drop of VS destroyed was recorded in M4O1 and M4O2 groups, at 34.0% and 29.7%, which suggest large amount of organic materials were not used and transformed by bacterial community.

When temperature was reduced to 30 °C, biogas production of group fed with 2 g-VS_{SM} L^{-1} d^{-1} was showing a significant dropped to 559 ± 13 mL d^{-1}, which was equal to efficiency rate of 67.6 ± 1.6%. These biogas production and efficiency rate were lower than at 40 °C, but higher than at 55 °C. VS reduction rate of M2 at 30 °C was 59.7%, similar to at 55°C while lower VS destroyed rates were

recorded in other failure groups. However, all other combinations of SM and WKO in the study showed imbalance at ambient temperature. These suggest that mesophilic condition is the most suitable temperature for co-digestion of SM and WKO.

Table 12: Efficiency of anaerobic digestion at different temperatures

		M2	M2O1	M4O1	M4O2
Biogas potential	mL g-VS-fed^{-1}	827	1014	902	1014
Mesophilic (40 °C)					
Biogas yield	mL g-VS-fed^{-1}	729 ± 23a	909 ± 37c	793 ± 18b	917 ± 43c
Efficiency	%	88.2 ± 2.8	89.6 ± 3.6	87.9 ± 2.0	90.4 ± 4.2
VS reduction	%	66.9	67.6	61.4	63.6
Thermophilic (55 °C)					
Biogas yield	mL g-VS-fed^{-1}	470 ± 19	536 ± 37	133 ± 42	101 ± 40
Efficiency	%	56.8 ± 2.3	52.9 ± 3.6	14.7 ± 4.7	10.0 ± 4.0
VS reduction	%	59.5	65.7	34.0	29.7
Ambient (30 °C)					
Biogas yield	mL g-VS-fed^{-1}	559 ± 13	0	0	0
Efficiency	%	67.6 ± 1.6	0	0	0
VS reduction	%	59.7	47.4	40.4	56.3

3.4. Scenarios of selecting organic loading rates.

Calculations were based on a commercial swine farm with 10,000 heads in four different loading rates, M2, M2O1, M4O1 and M4O2 at 40 °C. VS production was estimated at 3,750 kg per day. In the case AD performance followed M2 or M2O1 scenario, the loading rate of manure would be 2 kg-VS m^{-3} d^{-1}. Then, working volume was calculated by dividing VS yield (3,750 kg-VS d^{-1}) by manure daily loading rate (2 kg-VS m^{-3} d^{-1}) or was equal to 1,875 m^3 (Table 13). In the next scenario where M4O1 or M4O2 was applied, manure loading rate was 4 kg-VS m^{-3} d^{-1} and working volume would be 937,5 m^3. Digester volume (which was 133% larger than working volume) in these two cases would be 2,679 and 1,339 m^3, respectively. Determination of digester capacity is essential in terms of operating costs while maintaining system stability. If capital cost was assumed at $11.24 ± 4.13 per ft^3 for complete mix digesters (Gloy, 2011), average costs for establishing digesters in

these two scenarios would be at $1,063,394 ± 368 and $531,495 ± 184, respectively. Therefore, choosing higher SM loading rate would significantly reduce capital cost.

Calculation of daily feedstock loading was performed by dividing working volume by the HRTs of 21 days or was equal to 89.3 m^3 when M2 or M2O1 was fed or 44.6 m^3 when digester was operating at loading with M4O1 or M4O2. If the estimated VS of SM was 23.4% which was similar to substrate used in the laboratory study, loading amount of manure would be 16,026 kg d^{-1} or 16.0 m^3 d^{-1}. VS loading rates of oil in M2, M2O1, M4O1 and M4O2 were 0, 1, 1, 2 kg-VS m^{-3} d^{-1}, or were equal to 0, 1,875, 937.5, 1,875 kg-VS d^{-1}, respectively. If supposing volatile solids of WKO was 99.5 %, oil loading rate of M2O1 or M4O2 would be 1,884 kg d^{-1} or 1.88 m^3 d^{-1}. Similarity, loading rate of oil in M4O1 digester would be 942 kg d^{-1} or 0.94 m^3 d^{-1}.

Total OLRs in the four scenarios were 3,750, 5,625, 4,687.5 and 5,625 kg-VS d^{-1}. When biogas yields in each case were 0.729, 0.909, 0.793 and 0.917 m^3 kg-VS_{fed}^{-1} which were similar to data reported in the laboratory study, daily biogas productions were predicted to be 2,733.8, 5,113.1, 3,717.2 and 5,158.1 m^3 d^{-1} for each scenario, respectively. Amount of water added into digester in four cases could be estimated by subtracting daily feedstock loading and loading rates of manure and oil, which were at 73.3, 71.4, 27.7 or 26.7m^3 d^{-1}, respectively.

Table 13: Different scenarios of choosing organic loading rates at 40 °C

	Unit	M2	M2O1	M4O1	M4O2
Daily volatile solids	kg-VS d^{-1}	3,750	3,750	3,750	3,750
Working volume	m^3	1,875	1,875	937.5	937.5
Digester volume	m^3	2,679	2,679	1,339	1,339
	ft^3	94,608	94,608	47,286	47,286
Feedstock Loading	m^3 d^{-1}	89.3	89.3	44.6	44.6
Manure					
• VS Loading Rate	kg-VS m^{-3} d^{-1}	2	2	4	4
	kg-VS d^{-1}	3,750	3,750	3,750	3,750
• VS	%	23.4%	23.4%	23.4%	23.4%
• Loading rate	kg d^{-1}	16,026	16,026	16,026	16,026

	$m^3\ d^{-1}$	16	16	16	16
Oil					
• VS Loading Rate	kg-VS/$m^3\ d^{-1}$	0	1	1	2
	kg-VS d^{-1}	0	1,875	937.5	1,875
• VS	%	99.5%	99.5%	99.5%	99.5%
• Loading Rate	kg d^{-1}	0	1,884	942	1,884
	$m^3\ d^{-1}$	0.0	1.88	0.94	1.88
Water Loading Rate	$m^3\ d^{-1}$	73.3	71.4	27.7	26.7
Total OLR	kg-VS d^{-1}	3,750.0	5,625.0	4,687.5	5,625.0
Biogas yield	$m^3\ \mathrm{kg-VS}_{\mathrm{fed}}^{-1}$	0.729	0.909	0.793	0.917
Biogas production	m^3/d	2,734	5,113	3,717	5,158
	$m^3\ m_{\mathrm{working}}^{-3}\ d^{-1}$	1.46	2.73	3.97	5.50
	$m^3\ m_{\mathrm{digester}}^{-3}\ d^{-1}$	1.02	1.91	2.78	3.85
	$ft^3\ d^{-1}$	96,541.5	180,568.3	131,271.2	182,157.5

It can be observed from Table 13 that by applying only small amount of oil (1.88 $m^3\ d^{-1}$) into field-scale digester which was fed with 2 kg-VS_{SM} $m^{-3}\ d^{-1}$, biogas production increased by approximately 87.0%, from 2,734 to 5,113 $m^3\ d^{-1}$. However, largest volume of digester and highest amount of water used daily were required in these two scenarios with daily water usage was 73.1 and 71.4 $m^3\ d^{-1}$, respectively.

When the two loading rates M4O1 and M4O2 was selected, digester's volume and water used could be reduced by half compared to the previous scenario. Digester capacity was only 1,339 m^3 while only 26.7 – 27.7 m^3 water was necessary to add to the feedstock. If M4O1 was followed, amount of oil loaded daily was lowest, compared to other combinations, at 0.94 $m^3\ d^{-1}$. However, biogas production was 3,717 $m^3\ d^{-1}$, which increased only by 36.0% compared to M2 and reduced by 27.3% compared to M2O1. On the other hand, choosing M4O2 resulted in biogas production at 5,158 $m^3\ d^{-1}$, which was similar to productivity of M2O1. It can be explained by the same OLRs (5,625 kg-VS d^{-1}) and similar biogas yield (0.909 and 0.917 m^3 kg-VS^{-1}) between these two scenarios. However, it should be noted that when M4O2 was applied, digester volume and water

used were reduced by half compared to M2O1. Therefore, biogas production per working volume or digester volume was highest in the last scenario when M4O2 was followed, at 5.50 and 3.85 m^3 m^{-3} d^{-1}, respectively. It would result in reduction of construction cost when building digester as well as increase of digester's usage value. Similarly, M4O1 required the least water usage or reactor's capacity while producing high amount of biogas per digester volume, at 2.78 m^3 m^{-3} d^{-1}. Gas yields estimated in two scenarios which only 2 kg-VS of manure per $m^3_{working}$ was loaded daily without or with oil were only 1.02 and 1.91 m^3 $m^{-3}_{digester}$ d^{-1}, significantly lower than productions obtained in other scenarios. It was interesting to note that adding small amount of oil when M2O1 was applied resulted in almost doubling the biogas production per volume of digester compared to mono-digestion of sole manure.

4. Discussion

4.1. Biogas potential and effect of temperature to AD efficiency

Biogas potential study showed much higher production rate obtained from WKO compared to SM. The BP of substrates in co-digestion was not as high as the combinations of each substrate's potential in mono-digestion. However, understanding maximum biogas which could be produced from each scenario is essential to evaluate AD performance in different loading rates or working conditions.

Changing temperature from ambient to mesophilic condition was proved to increase biogas yields in this study which was in agreement with previous reports. Deng et al. (2014) investigated effects of temperatures, from 15 °C to 35 °C, to biogas production rate using different loading rates, and data showed obvious increase of gas produced when increasing temperature. On the other hand, less oily substrates could be digested by AD reactors at low temperature. Lansing et al. (2010) conducted a low-temperature AD experiment using SM and different volume of cooking grease, including 2.5%, 5% and 10% by volume. Significant drop of methane yield was reported, from 0.31 to 0.12 m^3 kg-VS^{-1} day^{-1}, when increasing grease loading rates from 2.5% to 10% by volume. It suggests that the decrease of oil is necessary to maintain high biogas production. All combinations of SM and WKO in this study failed at the ambient temperature, and significant drop of pH values in the failure digesters supposed that bacterial community might not be able to acclimate with the

new environment. Therefore, higher temperature or reduction of oil loading rate is crucial for maintaining stability of biogas performance

Meanwhile, thermophilic condition has been applied in many anaerobic digesters with several advantages such as increase of digestion rate, improvement of biogas yield or elimination of harmful pathogen in substrates (Ahring, 1995). However, biogas production in our study was significantly hampered when temperature was increased from 40 °C to 55 °C. “Inhibited steady state” was observed in digesters with M2 or M2O1 where gas produced was stable but lower than digester’s potential. Inhibition of anaerobic performance was recorded in groups supplemented with M4 and O1 or 2O, which seemed to be the factor responsible for process perturbation. Hashimoto (1983) reported the increase of methane productions in anaerobic digesters working at 55 °C with HRT of 15 or 5 days, compared to the system set up at 35 °C. However, longer HRT (25 days) with feedstock of 62.5 kg-VS m^{-3} resulted in instability of AD performance at thermophilic condition due to the increase of ammonia concentration and content of influent VS. This suggests that the decrease of HRT (or increase of feedstock volume) and OLR might be a promising approach to keep co-digestion of SM and WKO stable. However, it should be kept in mind that the decrease of HRT also comes along with higher water usage and lower VS reduction rates, which could cause the increase in operating cost as well as potential environmental issues.

4.2. Decision-making scenarios

Volume of water usage, digester’s capacity or biogas production varied a lot when different VS loading rates and manure-oil ratio were applied to co-digestion of SM and WKO. Application of oily materials contributed to the increase biogas volume was demonstrated in previous studies (Baba et al., 2013). In our estimation, application of 1 kg-VS_{WKO} m^{-3} d^{-1} could lead to increase of biogas yield by 87.0% in comparison with mono-digestion of manure. Using M4O2 instead of M2O1 did not result in more biogas production. However, choosing M4O2 came with reduction of digester’s volume by half as well as decreasing water usage, which were important factors relating to operating costs. The capital cost of M4O2-fed digesters also reduced by half, compared with ADs fed with M2O1. Meanwhile, M2 and M2O1 can result in more stable performance and higher microbial diversity even biogas productions per digester’s volume were significantly lower than

other loading rates in the study.

It should be noted that several factors could affect AD performance and biogas production in commercial scale which might contribute to the deviation between results estimated based on lab study and actual biogas production. Nsair et al. (2019) reported different stirring approaches could lead to change of biogas yield in large scale AD plant. Teng et al. (2014) discussed about factors including digester design, heating, stirring or insulation, which were important to maintain balance of AD system and biogas production. Therefore, more on-farm studies are necessary to improve the accuracy of biogas estimation.

5. Conclusion

Biogas potential of oil was significantly higher than of SM, suggesting that application of small amount of WKO could dramatically increase total biogas production in AD system. Introducing oil at VS loading rates of 1 or 2 g-VS L^{-1} d^{-1} increased biogas potential of feedstocks from 9.0 ± 3.2% to 22.6 ± 3.1%, compared to digestion of only SM. When comparing to separate potential of two substrates, maximum biogas produced from two combinations of manure and oil (M4O1 and M4O2) were 93.2 ± 6.6% and 95.6 ± 4.8%, indicating that synergistic effects of co-digestion came from the ability of increasing OLR and balancing carbon-to-nitrogen ratio rather than from increasing total biogas potential.

Co-digestion of SM and WKO at mesophilic condition resulted in highest efficiency and highest percentage of VS reductions. When changing temperature to thermophilic or ambient conditions, AD performance was negatively affected and system imbalance was recorded in all testing treatments, except for M2O1 at 55 °C. During thermophilic digestion, bacterial activity was hampered significantly, resulting in lower diversity as well as percentage of methanogens. System failure also came with the significant drop of VS destroyed, which could be a potential threat to the environment due to organic pollution.

Selection of different loading rates should be based on availability of water usage and intention of capital using. Applying M4O1 or M4O2 could directly result in smaller digester's volume or water required (which were equal reducing construction cost) while biogas production reduces slightly or even unchanged. However, high loading rates of manure seem to come with potential threats due

to accumulation of volatile fatty acid or less bacterial diversity, which might result in poor performance when the system faces technical issues such as temperature drop or OLR changes. If M2O1 is introduced, high biogas production and more stable system might be achieved. However, reactor's volume and daily water usage has to be doubled compared with other loading rates.

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Chapter 4: Prediction Model of Biogas Production from Co-digestion of Swine Manure and Waste Kitchen Oil

1. Introduction

1.1. Biogas production and the need of biogas prediction

Popularity of manure-fed anaerobic digesters (ADs) in the United States is becoming obvious in recent years with total number of operational digesters increased from 141 in 2010 to 273 in 2021 (US-EPA, 2021). ADs also provide an alternative to treat manure while producing biogas as a renewable fuel. Biogas production generated from on-farm ADs can be used for different purposes, such as heating or generating electricity. US-EPA (2018) estimated potential of energy generated from ADs fueled by SM could be 6,341,527 MWh per year, which could be a significant factor for reducing farm operating cost. The state-run programs, such as the Low Carbon Fuel Standard (LCSF) in California from 2009, have played an important role in the development of many ADs by paying credits to operators (Greene, 2019; Jaffe & Dominguez-Faus, 2016). Due to those benefits, more on-farm digesters are being constructed (US-EPA, 2021). However, if the estimation of biogas yield could not be performed before ADs operation, the production might be higher than the demand which might result in emission of methane (CH_4) into the atmosphere (Reinelt et al., 2016). For more useful application of biogas and to avoid potential environmental thresh, accurate prediction of biogas production is necessary before constructing new AD plants or even during operation of current ADs.

Biogas production efficiency of ADs is affected by many factors. Different type of substrates, loading rate, temperature or hydraulic retention time (HRT) lead to different levels of methane productivity (Weiland, 2010). The Anaerobic Digestion Model No 1 was introduced by International Water Association (IWA) task group as one of the earliest tools developed for prediction of biogas produced from ADs (Batstone et al., 2002). Since then, there have been more models established (Lhanafi et al., 2018; C. Mao et al., 2017; Wang et al., 2012). The models can be applied to estimate biogas production from different substrates at different working conditions. However, biogas calculation based on these tools are generally complicated, making it difficult for on-farm

application. Moreover, the changes of feedstocks, loading rates, or temperature can affect biogas production, leading to the fluctuation of the biogas yields among digesters. Therefore, a simple model which can be used to estimate biogas production based on the feedstock and working conditions is necessary to improve the efficiency of biogas application.

Results presented in Chapter 2 and Chapter 3 illustrated the efficiency of combining swine manure (SM) and waste kitchen oil (WKO) as substrates for co-digestion in AD systems. The data also confirmed that organic loading rate (OLR), substrate ratio, and temperature were the main factors affecting biogas production, while ratio of oil to manure was essential to maintain the balance of AD activity. However, biogas production was proven to have low efficiency in thermophilic condition when manure was fed at the VS loading rates between 2 to 4 g-VS L^{-1} d^{-1}. Meanwhile, co-digestion of SM and WKO was more promising at 40 °C in terms of biogas productivity compared with thermophilic condition. On the other hand, operation of AD plant in mesophilic condition, ranging from 30 to 40 °C, is easier and requires less energy cost, compared with in thermophilic temperature (US-EPA, 2016). Co-digestion of SM and oily substrates were reported in several studies (Hidalgo et al., 2015; Long et al., 2012; Marchetti et al., 2020). However, previous studied mostly focused on a specific temperature. Therefore, biogas study conducted at different mesophilic temperatures is crucial for the accurate evaluation of biogas production from co-digesting SM and WKO. Moreover, a user-friendly tool is needed so that the prediction of biogas yield could be performed conveniently, which is a supporting factor for making decision of AD plant design and operation.

1.2. Regression model and its application for biogas production

1.2.1. Introduction about regression model

Regression model is a useful tool to predict a dependent variable (an outcome or a response variable) using other independent variables (or predictors). The most popular type of regression model is linear regression, which can be used to estimate value of a response variable based on changes of other predictors. When several predictors are used for prediction, linear regression can be given by:

$$Y = \beta_0 + \beta_1 X_1 + \ldots + \beta_n X_n + \varepsilon \qquad \text{(Eq.1)}$$

where Y and X are response variable (biogas prediction or biogas yield) and predictors, β is coefficients (James et al., 2013).

In simple regression model with one predictor, calculation of coefficients was performed by equations:

$$\widehat{\beta_1} = \frac{\sum_{i=1}^{n}(x_i-\bar{x})(y_i-\bar{y})}{\sum_{i=1}^{n}(x_i-\bar{x})^2}, \quad \text{(Eq. 2)}$$

$$\hat{\beta}_0 = \bar{y} - \hat{\beta}_1 \bar{x}, \quad \text{(Eq. 3)}$$

where $\bar{y} = \frac{1}{n}\sum_{i=1}^{n} y_i$ and $\bar{x} = \frac{1}{n}\sum_{i=1}^{n} x_i$ are sample means (James et al., 2013).

Polynomial relationship among predictors can be applied to improve accuracy of linear regression model. The use of quadric (second-order) model could result in a regression equation which fits data much better than the simple linear model. In some cases, the use of interaction between variables or cubic (third-order) model can generate a new model with higher accuracy (University of California, Irvine, n.d.). If two predictors are used, quadric model with interaction can be given by:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_1^2 + \beta_4 X_2^2 + \beta_5 X_1 X_2 + \varepsilon \quad \text{(Eq. 4)}$$ (University of California, Irvine, n.d.)

1.2.2. Application of regression model to predict biogas production

Application of mathematical models in terms of biogas production provided a reliable tool for biogas estimation. For example, ADM1 focused on biochemical and physico-chemical steps during the biogas production (Batstone et al., 2002), then CH_4 yield could be estimated based on several initial parameters, such as feedstock flowrate, total COD, Alkalinity or pH (Ozgun, 2019). Machine learning or statistical learning have been applied for the development of biogas models in which several factors and their relationships were considered. Study conducted by Wang et al. (2020) focused on biogas prediction based on machine learning algorithms. Eight parameters were selected for establishment of algorithms, including glucan content, temperature, C/N ratio, total nitrogen, total carbon, lignin content, xylan content, cellulose content. Biogas thermodynamic was predicted by applying of multi-layer perceptron neutral network and artificial neutral network (ANN) in the work proposed by Farzaneh-Gord et al. (2020). The use of neutral network models with ant

colony optimization algorithms was applied for prediction of biogas flow rate, which were presented by Beltramo et al. (2016). Applications of neural network or ant colony algorithm were reported in studies conducted by Dach et al. (2016), Nair et al. (2016) and Verdaguer et al. (2016). The use of machine learning in model establishment is a new approach with promising potential for the accurate prediction of biogas production based on different factors and conditions. However, appliation of these models is limited due to their complexity which normally required statistical skills and training.

The use of regression models provided an attractive alternative for biogas prediction due to their simple and effective (Mao et al., 2017). Several regression models were established based on common factors including type of substrates, feedstock loading rate, initial pH, etc. The development of these models usually resulted in regression equations, for example those reported in Table 14. Lhanafi et al. (2018) studied co-digestion of dairy wastes using batch digesters at mesophilic condition (38 ± 1 °C). Factorial experimental design was applied to investigate relationship between three factors (pH, loading rate and inoculum) and biogas yield. Another model by Mao et al. (2017) was developed to predict biogas production based on two variables, including initial pH and swine manure/corn straw (SM/CS) ratio. Data were collected from batch digesters with the use of 1L glass bottles under mesophilic condition with three SM/CS ratios and five initial pH settings. Dairy manure and chicken waste were co-digested in batch digestion study under mesophilic condition in the study conducted by Wang et al. (2012). Five level of DM/CM (14.6, 25, 50, 75, 85.1) and five levels of C/N (17.9. 20, 25, 30, 32.1) were selected for establishment of nine treatments. Predicted methane production were compared with actual CH_4 yield to evaluate model accuracy.

The main disadvantage of regression models is that they were based on specific conditions and input variables. Therefore, they cannot be used when substrate or conditions were changed. However, these models are generally simple and easy to use compared with other models generated by application of machine learning.

1.2.3. R software and its application for the model development

R was developed from the 1990s as a free software for applications in statistics and graphics

(Hornik, 2020). Since then, R has been used worldwide and became one of the most popular statistical software (Fox & Leanage, 2016; Ozgur et al., 2015). R is widely known for the statistical application and data analysis, and is applied in many fields, such as agriculture, biological science, or mathematics, etc. (Tippmann, 2015).

R provides powerful tools with many built-in functions, which can be used for development of linear and nonlinear regression models or analyzing classical statistics (R Core Team, 2020). The codes in R can be packed and distributed as “packages”. The packages can be downloaded and used for special purposes to help extend R’s capacity (Wickham & Bryan, n.d.).

1.3. Research objectives

This study was conducted to investigate relationship between biogas productions and several operating variables, including VS loading rate of SM, oil-to-manure ratio, OLR, temperature, and pH at mesophilic condition. Regression models were established to estimate biogas production based on three main variables – manure VS loading rate, oil-to-manure ratio and operating temperature specially for co-digestion of SM and WKO. Different approaches were applied to improve model accuracy. Finally, an excel-based program was developed to provide a user-friendly decision-making tool to help estimate biogas production based on different inputs of manure, oil and temperature, as well as to provide recommendations for AD construction and operation.

Table 14: Summary of some regression models for biogas prediction

Model	Description	References
$Y = 56.8+15.5X_1 +15.0X_2 -14.1X_3 +4.2X_1X_2 +9.7X_1X_3 +6.4X_2X_3$, where X_1, X_2, X_3 are pH, Inoculum, Organic load (g-VS).	Regression model based on batch digestion at mesophilic condition (38 ± 1 °C).	Lhanafi et al. (2018)
$Y = 52.67 + 7.73X_1 + 6.68X_2 – 6.8\ X_1X_2 – 12.54X_1^2 – 4.88\ X_2^2$, where X_1, X_2 are initial pH and SM/CS ratio.	Model developed from co-digestion of swine manure and corn straw with different substate ratio and pH initial.	Mao et al. (2017)
$Y = 240 – 11.93A + 19.02B + 6.75AB – 10.56A^2 – 15.06B^2$, where A, B are ratio of dairy manure to chicken manure and of carbon to nitrogen.	Model was developed to predict biogas production from dairy manure, chicken manure and wheat straw at mesophilic condition.	Wang et al. (2012)

2. Materials and methods

2.1. Substrate collection and co-digestion set-up

Substrate collection and biogas production were performed following procedure described in Chapter 2. In summary, SM was collected from a central Missouri pig farm and frozen at -20 °C before use. WKO was collected from university campus dining service and kept at room temperature. Loading rates of SM and WKO were calculated based on volatile solid (VS) which were measured by EPA Method 1684 (US-EPA, 2001). Glass jars with capacity of 1.89 L (0.5 gal) and working volume of 1.375 L were used as digesters, while 3.79-L jars were utilized when severe foaming and clogging issue was recorded in a loading rate.

Hydraulic retention time (HRT) of 21 days was followed, based on the similar study conducted in the laboratory (Nogueira et al., 2019). Substrates were loaded every two days while biogas productions were measure every two or four days, depending on the abundance of biogas collected. Value of pH was measured every four days using a pH meter (Pinpoint, American Marine Inc, Ridgefield, CT, USA). Measurement of pH was performed as a simple indicator to monitor AD performance.

2.2. Experimental design and data collection

Results in Chapter 2 suggests that biogas production and AD stability depends on VS-loading of SM, temperature and oil-to-manure ratio (O/M ratio). Therefore, these three factors were chosen as key variables for development of the regression model (Table 15). Previous chapters also prove that loading more than 4 g-VS of SM per liter per day did not result in significant improvement in term of biogas yield, O/M ratio should not exceed 0.5 and significant disturbance of biogas production was observed at thermophilic condition. Therefore, VS loadings of SM in this study were selected to be 2, 3 or 4 g-VS L^{-1} d^{-1}, temperatures in range between 30 °C to 40 °C were focused and three levels of O/M ratios (0, 0.25 and 0.5) were considered for the model development. HRT was not included in the model because previous study conducted by Nogueira et al. (2019) showed optimal HRT of 21 days, which was in agreement with common HRTs applied in completely mixed digesters (Holzem, 2015).

Table 15: Key variables and their levels in regression model

Variable	Notation	Description
Manure loading	X_1	VS loading of SM (2, 3, 4 g-VS L^{-1} d^{-1}).
Oil-to-manure ratio	X_2	Ratio between WKO and SM (0, 0.25, 0.5).
Temperature	X_3	Digester temperature (40 °C, 35 °C and 30 °C).

Nine essays were set up in replicate which were combinations between the three VS loading levels of SM (2, 3 and 4 g-VS L^{-1} d^{-1} or M2, M3 and M4) and three levels of O/M ratio (0, 0.25 and 0.5 or R0, R0.25 and R0.5) – Table 16. VS loading of oil was calculated based on specific VS contents of SM and O/R ratio in each essay. For example, M4R0.25 represents VS loadings of SM and WKO were 4 g-VS L^{-1} d^{-1}, and 4 x 0.25 = 1 g-VS L^{-1} d^{-1}, respectively.

Table 16: Experimental design

Essay	Factors		
	X1 (Manure Loading – g-VS/L/d)	X2 Oil-to-manure ratio	X3 (Temperature - °C)
1	2	0	40 °C, 35 °C, 30 °C
2	2	0.25	40 °C, 35 °C, 30 °C
3	2	0.50	40 °C, 35 °C, 30 °C
4	3	0	40 °C, 35 °C, 30 °C
5	3	0.25	40 °C, 35 °C, 30 °C
6	3	0.50	40 °C, 35 °C, 30 °C
7	4	0	40 °C, 35 °C, 30 °C
8	4	0.25	40 °C, 35 °C, 30 °C
9	4	0.50	40 °C, 35 °C, 30 °C

The study was started at 40 °C, then temperature was gradually decreased to 35 °C and 30 °C. In each temperature level, all essays were monitored for 3.5 - 4 HRTs so that stability of ADs were ensured for at least 2 HRTs. Data were collected from the last HRT by averaging biogas production of digesters in each group every four days to reduce error. Therefore, dataset included six datapoints per essay per temperature level, except when failure of digesters happened in which biogas production was assigned as 0 mL/d. Data of M2R0, M2R0.5, M4R0.25 and M4R0.5 at 40 °C were adapted from previous study conducted in triplicate.

2.3. Establishment and improvement of regression model

2.3.1. Creation of dataset in R

Dataset contained 137 observations with one response variables (biogas production) and three key variables (predictors) or feature variables (VS loading of SM, O/M Ratio and temperature). Five observations represented failure points during the study (M4R0.5 at 35 °C, M2R0.5, M3R0.5, M4R0.25 and M4R0.5 at 30 °C). Description of dataset (named “gas1”) is given as:

- Biogas: biogas production (mL/d) released from digesters.
- Manure: manure VS loading rate (g-VS/L/d).
- Ratio: ratio of oil to manure.
- Temperature: working temperature of digesters (°C)

Two other variables were included in the dataset, which were OLR and pH, which were used to evaluate the relationship with biogas production. OLR was the sum of VS loading of SM and WKO. For example, OLR of essay M4R0.25 is 4 g-VS L^{-1} d^{-1} + 1 g-VS L^{-1} d^{-1} = 5 g-VS L^{-1} d^{-1}.

- OLR: total loading rate (g-VS/L/d).
- pH: value of pH measured in digesters.

Dataset was created by using Excel (Microsoft Corporation, Redmond, WA, USA) and saved as a *.csv file (Figure 24).

Biogas	Temperature	Manure	Ratio	pH	OLR
1954	40	2	0	7.5	2
2111	40	2	0	7.5	2
2002	40	2	0	7.5	2
2018	40	2	0	7.5	2
1963	40	2	0	7.5	2
1983	40	2	0	7.5	2
2934	40	2	0.25	7.4	2.5
2922	40	2	0.25	7.5	2.5
2885	40	2	0.25	7.5	2.5
2990	40	2	0.25	7.5	2.5
2959	40	2	0.25	7.5	2.5
2878	40	2	0.25	7.6	2.5
3480	40	2	0.5	7.5	3
3906	40	2	0.5	7.5	3
3863	40	2	0.5	7.5	3
3801	40	2	0.5	7.5	3
3696	40	2	0.5	7.5	3
3772	40	2	0.5	7.5	3

Figure 24: Part of dataset created in Excel

The file was loaded into R using read.csv function (Willems, 2018), by following code:

```
> gas1=read.csv("~/Model1.csv")
```

where gas1 and Model1.csv are names of dataset created in R and of dataset csv file, respectively.

2.3.2. Calculation of correlation coefficients

Correlation coefficients (r) were calculated to document the relationship among variables, which represents linear agreement between each pair of variables, and is in range between -1 and 1 (The University of Texas at Austin, 2015). Correlation represents linear relationship between two variables (James et al., 2013). The higher the r value is, the stronger relationship between two variables. While positive value of r indicates that two variables are directly proportional, negative r value suggests inverse proportional relationship between two factors (U.S. Naval Academy, 2015). Correlations between biogas production and other factors, including VS loading of SM, O/M Ratio, Temperature, OLR and pH, were calculated to evaluate relationship between variables. Correlation between two variables X and Y is given by:

$$\text{Cor (X, Y)} = \frac{\Sigma_{i=1}^{n}(x_i-\bar{x})(y_i-\bar{y})}{\sqrt{\Sigma_{i=1}^{n}(x_i-\bar{x})^2}\sqrt{\Sigma_{i=1}^{n}(y_i-\bar{y})^2}} \qquad \text{(Eq. 5) (James et al., 2013).}$$

Coefficients and model development were performed by using R Software, Version 4.0.0 (R Core Team, 2020). Package “GGally” in R was utilized to determine correlations of the key variables (Briatte, 2015), by following syntax:

```
> install.packages("GGally")
> library(GGally)
> ggcorr(gas1,label = TRUE, label_round = 2)
```

2.3.3. Establishment of linear regression model

Linear regression models were developed by using function “lm” in R (Porras, 2018). Intercept and coefficients could be generated with *p-value* for determination of statistical significance of each variable. R-squared or adjusted R-squared was used to evaluate model accuracy, which determined percentage of data that can be explained by the model (IBM, 2005). Significance of model was evaluated by *p-value* of the model. The following syntax was used to establish the linear regression model:

```
> gas1.md1 = lm(Biogas ~ Manure + Ratio + Temperature, data = gas1)
```

The “zero points” in the dataset might negatively impact model performance. Therefore, another dataset named “gas2” was created by removing observations with biogas productions that equal to 0, using command “filter” in dplyr library (Nishida, 2016). Then, new regression model was developed for comparison with original model, using following syntax:

```
> install.packages("dplyr")
> library(dplyr)
> gas2 = filter(gas1, Biogas > 0)
> gas2.md1 = lm(Biogas ~ Manure + Ratio + Temperature, data = gas2)
```

2.3.3. Polynomial regression model with interaction

Application of second- and third-order (or quadratic and cubic) models could result in new regressions which fit data better than simple linear model (Helwig, 2017). Therefore, polynomial regression models with variable interaction were applied to increase model accuracy, using the following codes:

```
> gas2.md3 = lm(Biogas ~ Manure + Ratio + Temperature + I(Manure^2) + I(Ratio^2) + I(Temperature^2) +
Manure*Ratio*Temperature, data = gas2)
> gas2.md4 = lm(Biogas ~ Manure + Ratio + Temperature + I(Manure^3) + I(Ratio^3) + I(Temperature^3) +
Manure*Ratio*Temperature, data = gas2)
```

Significance of models were determined by *p-values* of the models. Adjusted R-squared was used to analyze the improvement of new models, compared with the original linear regression.

2.3.4. Stepwise selection procedure

Stepwise selection was applied to optimize number of variables included in model. The process is performed by adding and removing variables to observe the change of model (Kassambara, 2018; Z. Zhang, 2016). Akaike Information Criterion (AIC) is a criterion to determine model’s performance which is calculated by the following equation:

$$AIC = -2\log\text{-likelihood} + 2edf \quad \text{(Eq. 6)}$$

where edf is the equivalent degrees of freedom of fit (Venables & Ripley, 2002)

Stepwise algorithm was performed by using the command “step” (ETH Zurich, n.d.):

```
> summary(step(gas2.md3))
```

2.4. Development of user-friendly and on-farm ADs tools for the model application

For transferring experiment results from the lab study to on-farm ADs, biogas yield was assumed to be proportional with digester's working volume when loading rate was kept the same. For example, when VS loading rate of SM is 2 g-VS L^{-1} d^{-1} (or 2 kg-VS m^{-3} d^{-1}), if biogas production of a digester with working volume of 1.375 L is 2 L d^{-1} (or 1.45 L L^{-1} d^{-1} or 1.45 $m^3/m^3/d$), then biogas yield of an AD with working volume of 1,000 m^3 will be supposed to be 1000 x 1.45 = 1,450 m^3 d^{-1}. The model created was used to develop two Excel-based programs to predict biogas production and provide recommendations of on-farm ADs in two scenarios: before an AD is constructed or for an existed AD. The on-farm ADs tools include three main components: Key variable input, AD variables and Model output. The Key variable requires the input of some parameters, including number of pig, manure production, and VS of Manure and Oil. In the case that AD is already built, information of digester volume is necessary. The AD variables section provides recommendations about digester volume (or SM loading rate in the second scenario), temperature and WKO loading rate. After specific values of each variable are entered in the ranges recommended, Model output would provide information about biogas production, water loading rate or construction cost. However, the use of on-farm ADs tools needs to be based on some following model assumptions:

1) Complete-mix AD.
2) Pig manure as feedstock.
3) Co-digestion of swine manure and waste kitchen oil.
4) Hydraulic retention time of 21 days.
5) No issue in ammonia content.

2.4.1. Scenario 1: Recommendation of digester volume and working conditions

In the scenario 1, it was assumed that a farm manager wants to estimate AD capacity and biogas production before constructing an AD system. First, number of pig (head) or manure production (m^3 d^{-1}), VS of manure and VS of oil (%) would be entered into the tool. Then, manure VS production would be calculated as:

VS_{SM} production (kg-VS d^{-1}) = Manure production (m^3 d^{-1}) x Manure VS (%) x 1000.

(Eq. 7)

It is supposed that VS loading of SM in range of 2 to 4 g-VS L^{-1} d^{-1} (or 2 to 4 kg-VS m^{-3} d^{-1}). Then, recommendation of digester's working volume ($V_{Working}$) would be estimated based on VS_{SM} production (kg-VS d^{-1}):

$$V_{Working}\text{-max } (m^3) = \frac{VS_{SM} \text{ production } (\text{kg-VS } d^{-1})}{2 \ (\text{kg-VS } m^{-3} \ d^{-1})} \quad \text{(Eq. 8)}$$

$$V_{Working}\text{-min } (m^3) = \frac{VS_{SM} \text{ production } (\text{kg-VS } d^{-1})}{4 \ (\text{kg-VS } m^{-3} \ d^{-1})} \quad \text{(Eq. 9)}$$

Estimation of digester volume (m^3) is assumed to be 133% larger than $V_{Working}$.

In the next step, digester volume (m^3) and temperature (°C) would be determined among the recommendation ranges. Then, actual VS loading of SM would be calculated based on VS_{SM} production and chosen digester volume. While minimum of VS loading of WKO is 0 kg-VS m^3 d^{-1}, recommendation of maximum of VS_{WKO} loading (VS-fed) is determined by:

- If temperature is selected in a range between 35 to 40 °C, VS_{WKO}-fed is equal to 0.5 x VS_{SM}-fed because it was proven in Chapter 1 that adding oil beyond WKO/SM ratio of 0.5 did not result in extra biogas production per VS_{fed}.
- If temperature is selected at 35 °C and minimum of digester volume was chosen volume (or VS_{SM}-fed is 4 kg-VS L^{-1} d^{-1}), VS_{WKO}-fed is equal to 0.25 x VS_{SM}-fed because M4R0.5 was observed to be failure at 35 °C.
- If temperature is selected in range between 30 to 35 °C and digester volume is less than average of maximum and minimum recommended volume (or VS_{SM}-fed is less than 3 kg-VS L^{-1} d^{-1}), VS_{WKO}-fed is equal to 0.25 x VS_{SM}-fed. Otherwise, addition of WKO is not recommended to avoid digester imbalance. The reason is that M2R0.25 and M3R0.25 were only combinations shown to be effective at ambient temperature (30 °C).

After a feedstock volume of WKO is selected, daily loading rate (m^3) and daily water usage (m^3) can be calculated by:

$$\text{Daily loading rate } (m^3) = \frac{V_{Working} \ (m^3)}{\text{HRT of 21 d}} \quad \text{(Eq. 10)}$$

Daily water usage (m^3) = $V_{Working}$ – Manure production – Oil loading (Eq. 11)

Model equation would be applied to estimate biogas production per m^3_{woking} per day, then the result can be multiplied with working volume for calculation of biogas production per day.

2.4.2. Scenario 2: Recommendation of working conditions

In the second scenario, it is assumed that an AD plant already existed, and farm manager wants to estimate amount of SM and WKO should be fed into digester as well as temperature and water usage to optimize biogas production.

In the first step, digester volume (m^3), number of pig (head), manure production (m^3/d) and VS of manure and oil (%) should be entered. It should be noted that number of pig and manure production are for reference and can be left blank.

It is assumed that VS loading of SM is in a range of 2 to 4 g-VS L^{-1} d^{-1} (or 2 to 4 kg-VS m^{-3} d^{-1}). Then, recommendation of manure loading (SM_{fed}, m^3/d) would be estimated based on working volume (which is supposed to account for 70% of digester volume), using the following equations:

$$SM_{fed}\ min\ (m^3/d) = \frac{V_{working}\ (m^3)\ x\ 2\ kg{-}VS\ m^{-3}d^{-1}}{VS_{SM}(\%)\ x\ 1000\ (m^3/kg)} \qquad \text{(Eq. 12)}$$

$$SM_{fed}\ max\ (m^3/d) = \frac{V_{working}\ (m^3)\ x\ 4\ kg{-}VS\ m^{-3}d^{-1}}{VS_{SM}(\%)\ x\ 1000\ (m^3\ kg^{-1})} \qquad \text{(Eq. 13)}$$

When manure loading and temperature are selected, recommendation of maximum of VS_{WKO}-fed is determined as mentioned above. Finally, estimation of daily water usage and biogas production are similar to method discussed in scenario 1.

2.5. Data analysis

Calculation of mean and standard deviation were performed using Excel (Microsoft Corporation, Redmond, WA, USA). The *p-values* of models were performed by using built-in functions or packages in R. The significance was determined as statistical significance when *p-value* was less than 0.05.

3. Results

3.1. Biogas production and relationship between variables

3.1.1. Effects of loading rate and temperature to biogas production

Co-digestion of SM and WKO at 40 °C resulted in the stability of biogas productions of all loading rates, except for those with high VS loading of SM and WKO, including M4R0.25 and M4R0.5,

which was similar to results observed in the previous chapters (Figure 25). Reducing the temperature from 40 °C to 35 °C caused severe fluctuation of AD performance in M4R0.25 and M4R0.5 digesters. Decrease of biogas production from M4R0.25 group was recorded in the first 3 HRTs, then the digesters recovered and reached their normal biogas yield. However, imbalance of M4R0.5 was irreversible, and biogas production could not be measured after 4 HRTs as reported in Chapter 3. When temperature was maintained at 30 °C, interruption of AD performance was recorded in all digesters fed with O/M ratio of 0.5 and in M4R0.25 also. This suggested co-digestion of 4 g-VS_{SM} L^{-1} d^{-1} with WKO was not effective, or lowering of VS loading rate is necessary to maintain system balance.

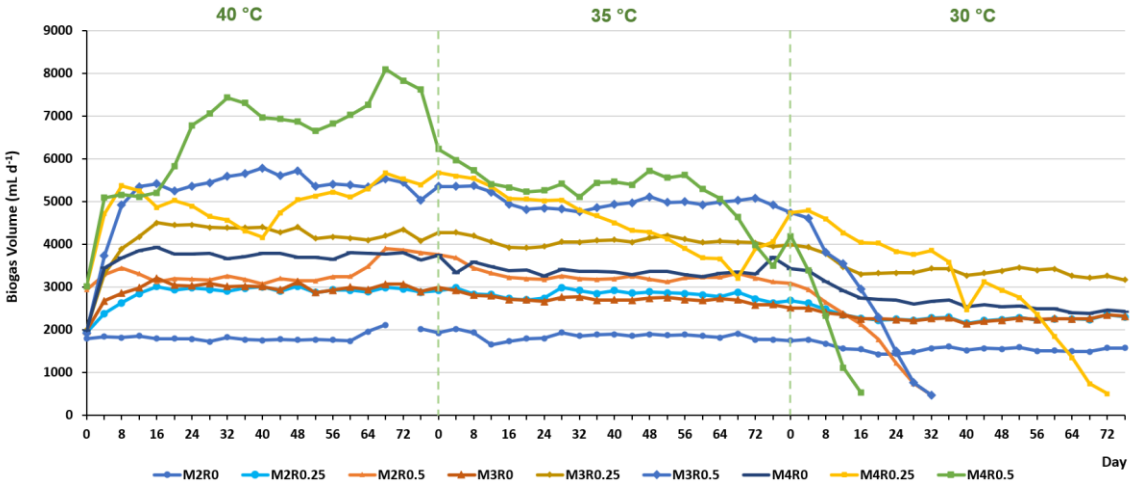

Figure 25: Average of biogas production (mL d^{-1}) in each essay in different temperature condition

3.1.2. Correlation between variables

Figure 26 shows the relationship between biogas production and four factors, including VS loading of SM, O/M Ratio, temperature and OLR. In general, increase of value of each variable in its range leads to higher biogas production. This suggests a positive correlation between biogas yield and each variable. Some outliers were observed in each figure, which represented failure groups in the study and might decrease the correlations (The Pennsylvania State University, n.d.a). Therefore, removal of these outliers would result in the better representation of the relationships between variables, which is important to increase of model accuracy.

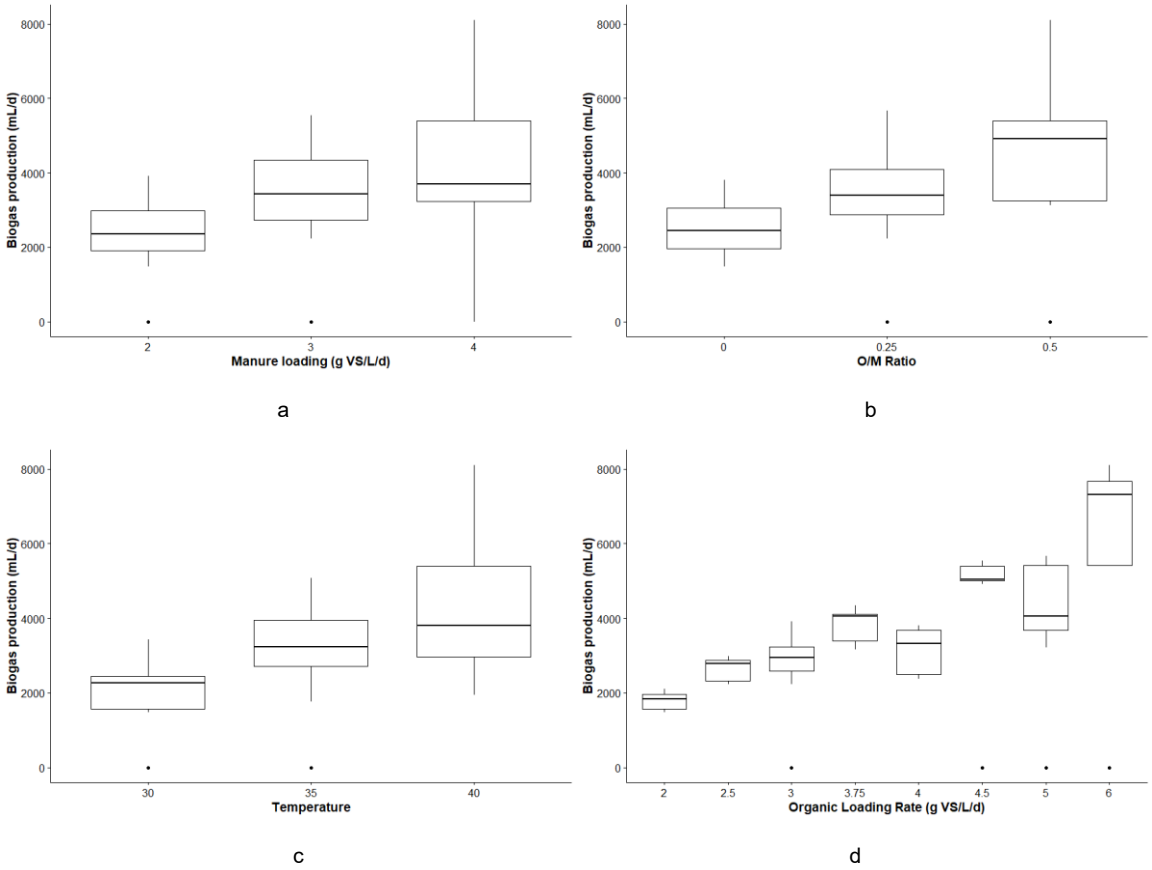

Figure 26: Biogas production at different VS loading of (a) SM, (b) O/M ratio, (c) temperature and (d) OLR

Correlation coefficients between biogas production and the key variables were in range from 0.42 to 0.64 when original data was applied (Figure 27). Moderate positive relationship among biogas yield and temperature or OLR was observed (r = 55 and 0.64, respectively), which suggested important roles of these factors in the model. In selected dataset, when zero values were removed, range of r changed, and was between 0.45 and 0.87. Higher r value between biogas production and OLR suggests interaction between SM and WKO might be necessary for model improvement. Low correlation between pH and biogas indicates that pH might not be a strong predictor to estimate biogas production. It is interesting to note that high correlation between VS loading of SM and pH was recorded in both cases (r = 0.87) which is in agreement with the study presented by Duan et al. (2019).

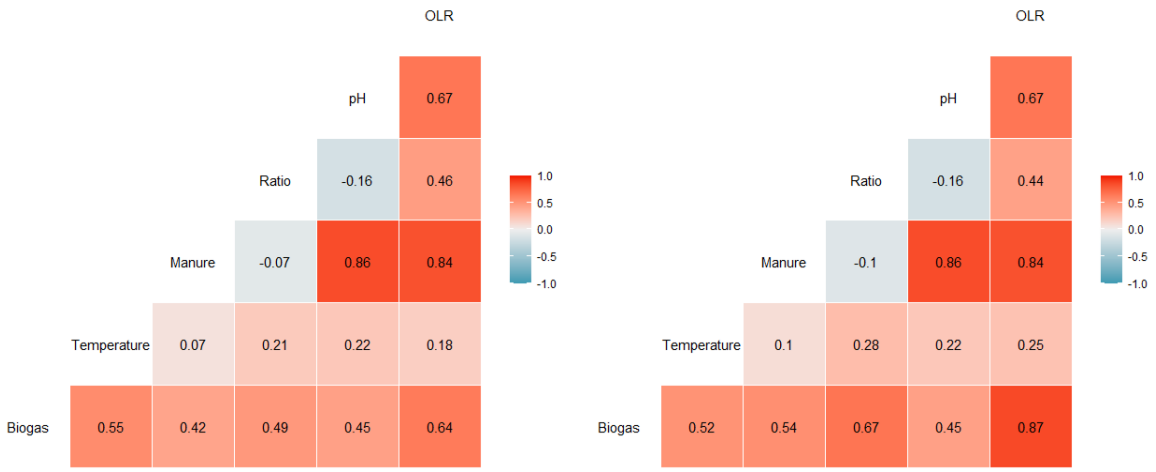

Figure 27: Correlation coefficients between the key variables in (a) original dataset and (b) in selected dataset

3.2. Establishment of linear regression model

A simple linear regression model was established based on the original dataset with 137 observations (Equation 14). The model created was statistically significant with *p-value* less than 0.05. However, the adjusted R-squared was not high, at 0.6155 (Figure 28). The zero values of biogas production included in the dataset might be the reason for moderate R-squared and adjusted R-squared.

```
lm(formula = Biogas ~ Manure + Ratio + Temperature, data = gas1)

Residuals:
    Min      1Q  Median      3Q     Max
-5015.6  -388.1    22.4   519.5  2255.3

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept) -5583.30     771.72  -7.235 3.32e-11 ***
Manure        795.30     101.24   7.855 1.19e-12 ***
Ratio        3269.73     418.39   7.815 1.48e-12 ***
Temperature   165.22      20.92   7.898 9.40e-13 ***
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 939 on 133 degrees of freedom
Multiple R-squared:  0.6237,    Adjusted R-squared:  0.6152
F-statistic: 73.49 on 3 and 133 DF,  p-value: < 2.2e-16
```

Figure 28: Model establishment based on original dataset, output of R software

All predictors were significant ($p < 0.05$) in the model. Based on estimations of intercept and predictor's coefficients listed in Table 17, the linear regression model was given as:

$$Y = -5583.30 + 795.30X_1 + 3269.73X_2 + 165.22X_3 \quad \text{(Eq. 14)}$$

where Y is biogas production (mL d^{-1}) and X_1, X_2, X_3 are manure loading (g-VS L^{-1} d^{-1}), O/M ratio and temperature (°C), respectively.

Table 17: Estimation and significance of predictors in model for prediction of biogas production

Variable	Estimation	Standard Error	*p-value*
Intercept	-5583.30	771.72	***
X_1 (Manure, g-VS L^{-1} d^{-1})	795.30	101.24	***
X_2 (O/R ratio)	3269.73	418.39	***
X_3 (Temperature, °C)	165.22	20.92	***

*Significance: ***p < 0.001, ** p < 0.01, * p < 0.05*

3.3. Improvement of linear regression model

3.3.1. Data pruning for improvement of linear regression model

Improvement of correlation coefficients between biogas production and O/M ratio or OLR suggests that the removal of zero biogas points might increase model accuracy (Figure 27). A new linear regression model created based on selected dataset (by removal of zero biogas values) resulted in significant increase of adjusted R-squared, at 0.8877 (Figure 29), compared with 0.6152 of original model (Figure 28). However, it should be kept in mind that after elimination, AD failures are not included in the new model and estimation of biogas production at these values would be considered as extrapolation. The prediction based on extrapolation may result in the significant bias (The Pennsylvania State University, n.d.)

```
lm(formula = Biogas ~ Manure + Ratio + Temperature, data = gas2)

Residuals:
     Min       1Q   Median       3Q      Max
-1480.00  -233.24    55.42   229.80  1748.34

Coefficients:
             Estimate Std. Error t value Pr(>|t|)
(Intercept) -3950.04     396.80  -9.955  < 2e-16 ***
Manure       1003.05      51.94  19.313  < 2e-16 ***
Ratio        4660.55     219.74  21.210  < 2e-16 ***
Temperature    98.91      10.99   9.002  2.6e-15 ***
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 466.2 on 128 degrees of freedom
Multiple R-squared:  0.8903,    Adjusted R-squared:  0.8877
F-statistic: 346.3 on 3 and 128 DF,  p-value: < 2.2e-16
```

Figure 29: Model establishment based on selected dataset, output of R software

3.3.2. Application of polynomial regression models and variable interaction

Application of quadratic (second-order) or cubic (third-order) term and interaction between key variables resulted in significant model improvement, compared with previous models. The new adjusted R-squared was 0.9655 in both cases (Figure 30), which was much higher than that observed in the original model, at 0.6152 (Figure 28). Several literatures suggested the use of polynomial regression increased the R-squared and the model accuracy (Helwig, 2017; Ostertagová, 2012; University of California, Irvine, n.d.).

The *p-values* showed statistical significance when applying both quadratic and cubic regression models. However, both R-squared and adjusted R-squared in the two cases were the same, and Anova algorithm showed no statistical difference between the two models. Therefore, a quadratic regression model with 10 predictors was selected for the next procedure of model improvement.

```
lm(formula = Biogas ~ Manure + Ratio + Temperature + I(Manure^2) +
    I(Ratio^2) + I(Temperature^2) + Manure * Ratio * Temperature,
    data = gas2)

Residuals:
     Min       1Q   Median       3Q      Max
-1117.17  -124.98   -15.88   160.58   805.51

Coefficients:
                          Estimate Std. Error t value Pr(>|t|)
(Intercept)              -2199.906   2756.005  -0.798 0.426305
Manure                     972.946    464.806   2.093 0.038417 *
Ratio                    18826.040   5381.653   3.498 0.000656 ***
Temperature                 63.498    147.930   0.429 0.668506
I(Manure^2)               -260.207     50.339  -5.169 9.41e-07 ***
I(Ratio^2)                 634.055    839.451   0.755 0.451524
I(Temperature^2)            -1.257      2.099  -0.599 0.550431
Manure:Ratio             -7453.646   1925.046  -3.872 0.000176 ***
Manure:Temperature          34.971     10.003   3.496 0.000661 ***
Ratio:Temperature         -503.397    142.657  -3.529 0.000591 ***
Manure:Ratio:Temperature   238.025     50.659   4.699 6.99e-06 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 258.4 on 121 degrees of freedom
Multiple R-squared:  0.9682,    Adjusted R-squared:  0.9655
F-statistic: 367.8 on 10 and 121 DF,  p-value: < 2.2e-16
```

a

```
lm(formula = Biogas ~ Manure + Ratio + Temperature + I(Manure^3) +
    I(Ratio^3) + I(Temperature^3) + Manure * Ratio * Temperature,
    data = gas2)

Residuals:
     Min       1Q   Median       3Q      Max
-1117.17  -124.98   -15.88   160.58   805.51

Coefficients:
                          Estimate Std. Error t value Pr(>|t|)
(Intercept)             -1.003e+03  1.994e+03  -0.503 0.615756
Manure                   2.212e+02  3.873e+02   0.571 0.568867
Ratio                    1.893e+04  5.368e+03   3.527 0.000595 ***
Temperature              1.982e+01  7.809e+01   0.254 0.800118
I(Manure^3)             -2.891e+01  5.593e+00  -5.169 9.41e-07 ***
I(Ratio^3)               8.454e+02  1.119e+03   0.755 0.451524
I(Temperature^3)        -1.197e-02  1.999e-02  -0.599 0.550431
Manure:Ratio            -7.454e+03  1.925e+03  -3.872 0.000176 ***
Manure:Temperature       3.497e+01  1.000e+01   3.496 0.000661 ***
Ratio:Temperature       -5.034e+02  1.427e+02  -3.529 0.000591 ***
Manure:Ratio:Temperature  2.380e+02  5.066e+01   4.699 6.99e-06 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 258.4 on 121 degrees of freedom
Multiple R-squared:  0.9682,    Adjusted R-squared:  0.9655
F-statistic: 367.8 on 10 and 121 DF,  p-value: < 2.2e-16
```

b

Figure 30: Model improvement by application of (a) quadratic and (b) cubic regression model

3.3.3. Variable selection

Using stepwise procedure for quadratic regression model resulted in the removal of two predictors, including O/M Ratio-squared and Temperature-squared. The procedure slightly decreased AIC, from 1476.86 to 1474.06. Only minor decrease of R-squared was observed, from 0.9682 in previous model to 0.9679, while adjusted R-squared was mostly unchanged (Figure 31). Again, *p-value* confirmed statistical significance of the new model (*p-value* < 0.05).

```
lm(formula = Biogas ~ Manure + Ratio + Temperature + I(Manure^2) +
    Manure:Ratio + Manure:Temperature + Ratio:Temperature + Manure:Ratio:Temperature,
    data = gas2)

Residuals:
     Min       1Q   Median       3Q      Max
-1119.58  -123.32   -13.36   154.16   818.55

Coefficients:
                          Estimate Std. Error t value Pr(>|t|)
(Intercept)               -758.215   1166.040  -0.650 0.516745
Manure                    1004.224    460.921   2.179 0.031258 *
Ratio                    19195.897   5328.285   3.603 0.000456 ***
Temperature                -23.030     30.793  -0.748 0.455942
I(Manure^2)               -260.863     49.750  -5.243 6.65e-07 ***
Manure:Ratio             -7505.724   1881.516  -3.989 0.000113 ***
Manure:Temperature          34.291      9.944   3.448 0.000773 ***
Ratio:Temperature         -503.831    142.076  -3.546 0.000554 ***
Manure:Ratio:Temperature   238.979     49.617   4.816 4.21e-06 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 257.4 on 123 degrees of freedom
Multiple R-squared:  0.9679,    Adjusted R-squared:  0.9658
F-statistic:   463 on 8 and 123 DF,  p-value: < 2.2e-16
```

Figure 31: Selection of model predictors using Stepwise procedure in R

Among eight predictors observed after stepwise procedure, seven showed statistical significance

in the model, except for Temperature (Table 18). Model equations with all predictors was represented as:

$$Y = -758.215 + 1004.224X_1 + 19195.897X_2 - 23.030X_3 - 260.863X_1^2 - 7505.724X_1X_2 + 34.291X_1X_3 - 503.831X_2X_3 + 238.979X_1X_2X_3 \quad \text{(Eq. 15)}$$

where Y is biogas production (mL d^{-1}) and X_1, X_2, X_3 are manure loading (g-VS L^{-1} d^{-1}), O/M ratio and temperature (°C), respectively.

Table 18: Estimation and significance of predictors in model for prediction of biogas production

Variable	Estimation	Standard Error	*p-value*
Intercept	-758.215	1166.040	
X_1 (Manure, g-VS L^{-1} d^{-1})	1004.224	460.921	*
X_2 (O/R ratio)	19195.897	5328.285	***
X_3 (Temperature, °C)	-23.030	30.793	
X_1^2	-260.863	49.750	***
X_1X_2	-7505.724	1881.516	***
X_1X_3	34.291	9.944	***
X_2X_3	-503.831	142.076	***
$X_1X_2X_3$	238.979	49.617	***

*Significance: ***p < 0.001, ** p < 0.01, * p < 0.05*

Comparison of biogas productions generated from the final biogas model with actual productions showed high similarity, with the difference ranging from 0.2% to 8.6%, except biogas yield of M4R0.25 at 30 °C, of which 15.9% difference was observed (Figure 32). The results confirm the model accuracy in terms of biogas prediction. Therefore, the model developed could be applied for estimation of biogas production based on VS-loading of SM, O/R ratio and temperature. However, it should be noted that AD failure could not be predicted by the model due to the extrapolation, after the removal of zero biogas values.

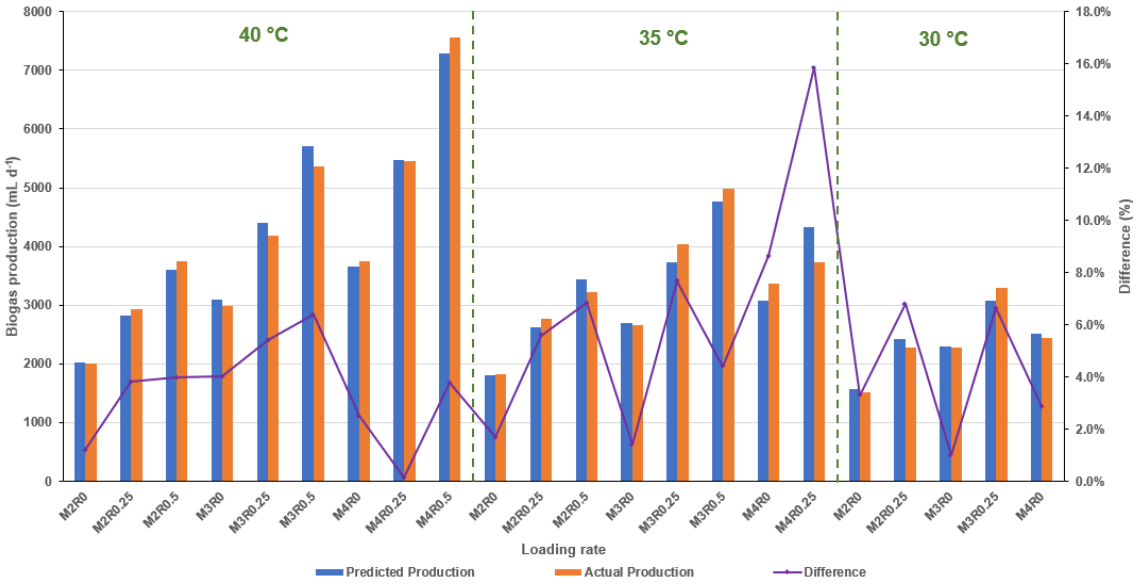

Figure 32: Comparison of predicted biogas production and actual production

3.4. Decision support tool for model application

3.4.1. Recommendation of digester volume and working conditions

In the first scenario in which the farm manager wants to estimate digester volume and biogas production, there are four key variable inputs with, including number of pig (head), manure production (m^3 d^{-1}), VS of SM and WKO (%) (Figure 33). In the case that manure production is unknown, VS_{SM} production (kg-VS d^{-1}) would be estimated based on data reported by ASABE standard. (2019), as supposed in Chapter 3. For example, a farm with 10,000 heads of finishing pig is estimated to generate 3,750 kg-VS d^{-1}. When manure production is known and provided, VS production would be calculated, and all the recommendations would be based on this value. For example, if SM production was 16 m^3 d^{-1} and VS_{SM} was 23.4%, daily VS production would be 3,744 kg-VS m^{-3} d^{-1}, which was similar to the estimation based on reference. Then, digester volume would be recommended in range of 1,337 to 2,674 m^3, or from 47,216 to 94,431 ft^3, which is similar to typical size of a complete mix digester (The Pennsylvania State University, 2012).

Increasing digester volume led to the decrease of VS loading of SM, and therefore, might increase the ranges of other key variables (temperature and VS loading of WKO). Choosing a larger digester capacity instead of minimum size leads to the decrease of VS loading of SM from 4 to 2 kg-VS m^{-3} d^{-1}. For example, if digester volume was selected to be 2,674 m^3 (maximum capacity),

temperature could be chosen between 30 to 40 °C, and addition of WKO loaded would be up to 2 kg-VS m^{-3} d^{-1}, or 1.88 m^3 d^{-1} if working temperature was set at 40 °C and VS_{WKO} was 99.5%, which was similar to data in this study.

RECOMMENDATION OF DIGESTER VOLUME AND WORKING CONDITIONS

KEY VARIABLE INPUT

No. of Heads	head	10000
Manure Production	m^3/d	0
VS of Manure	%	23.4
VS of Oil	%	99.5

AD VARIABLES

Variable	Unit	Recommendation		Input
		From	To	
Digester Volume	m^3	1339	2679	2679
Temperature	°C	30	40	40
Oil	m^3/d	0	1.88	1.88

MODEL OUTPUT

Working Volume (70% of digester's capacity)	m^3	1875
Predicted Manure VS Production	kg-VS/d	3750
Acutal Manure VS Production	kg-VS/d	0
Manure Loading Rate	kg-VS/m^3/d	2.00
Oil-to-Manure Ratio	-	0.50
Construction cost	**$**	**1062599**
Water recommended	**m^3/d**	**87.4**
Biogas Production	**$m^3/m^3_{working}/d$**	**2.62**
	m^3/d	**4909**
	m^3/kg-VS	**0.873**

INSTRUCTION

1. Enter number of heads, manure production (kg/d), VS of Manure and Oil (%).
Note: Please blank Manure Production if unknown.
2. Choose specific digester volume and Temperature (°C).
3. Select loading rate of waste kitchen oil (m^3/d).
Note: Oil recommendation will be changed based on each selected temperature.
4. Results of working volume, water recommended, biogas production (m^3/d) and biogas yield (m^3/kg-VS) will be available after all factors are set.
5. Model assumptions: (1) Complete-mix AD. (2) Pig manure as feedstock. (3) Co-digestion of swine manure and waste kitchen oil. (4) Hydraulic retention time of 21 days. (5) No issue in ammonia.

Figure 33: A user-friendly tool for recommendation of digester volume and working conditions

Biogas production (m^3 d^{-1}) would be calculated based on the model created in the previous section using the specific value of each factor listed above. For example, if maximum values of digester

volume, temperature and WKO were selected, which was equal to following OLR of M2O1 at 40 °C, biogas production was estimated at 4,905 m^3 d^{-1} and biogas yield was 0.874 m^3 kg-VS^{-1}. These predicted values were slightly different from estimations reported in Chapter 3, in which biogas production and biogas yield were 5,113 m^3 d^{-1} and 0.909 m^3 kg-VS^{-1}, respectively. The difference percentages were 4.1% and 3.9%, respectively. Dilution water was recommended was also in agreement when applying two approaches, at 71.3 m^3 when using model-based program and 71.4 m^3 if calculating manually.

3.4.2. Recommendation of working conditions

In the second scenario in which digester was already built, and biogas prediction and recommendation of VS loadings of SM, WKO and temperature were necessary, five key variables can be configured, including digester volume (m^3), number of pig (head), manure production (m^3), VS of SM and WKO (Figure 34). Two parameters – number of pig and manure production – were not used directly in the model. Instead, these data were used to compare with maximum of SM should be fed into AD, so that farm managers could divert part of the SM properly. For example, if manure production (23.4% VS) was 19 m^3 d^{-1} and maximum of SM recommended was 16 m^3 d^{-1} (based on actual digester capacity), then 3 m^3 d^{-1} (or 700 kg-VS d^{-1}) of SM should be diverted to other AD or composted rather than feeding into AD system to avoid overloading.

Choosing the minimum recommended SM loading rate instead of higher value would result in the decrease of VS loading of SM and led to the change of recommended temperature and loading rate of WKO, as discussed in the previous section. Results observed in this scenario was similar to the previous data if all factors were set up with similar value. For example, if digester volume was 2,675 m^3 (working volume was assumed to account for 70% of total capacity, or at 1,873 m^3), loading rates of SM (23.4% VS) and WKO (99.5% VS) were 16 and 1.88 m^3 d^{-1} (or OLR was M2O1), and temperature was set at 40 °C, the biogas production and biogas yield were estimated to be 4,904 m^3 d^{-1} and 0.873 m^3 kg-VS^{-1} (Figure 34). Water needed was recommended at 71.3 m^3 d^{-1}, which was similar to the estimation in the previous scenario.

RECOMMENDATION OF WORKING CONDITIONS

KEY VARIABLE INPUT

Digester Volume	m^3	2675
No. of Heads	head	12000
Manure Production	m^3/d	19
VS of Manure	%	23.4
VS of Oil	%	99.5

AD VARIABLES

Variable	Unit	Recommendation		Input
		From	To	
Manure	m^3/d	16.0	32.0	16.0
Temperature	°C	30	40	40
Oil	m^3/d	0	1.88	1.88

MODEL OUTPUT

Working Volume (70% of digester's capacity)	m^3	1873
Manure VS Loading	$kg\text{-}VS/m^3/d$	2.00
	kg-VS/d	3744
Oil-to-Manure Ratio	-	0.50
Water recommended	**m^3/d**	**71.3**
Biogas Production	**$m^3/m^3_{working}/d$**	**2.62**
	m^3/d	**4904**
	$m^3/kg\text{-}VS$	**0.873**

INSTRUCTION

1. Enter digester volume, number of heads, manure production (m^3/d), VS of Manure and Oil (%).
Note: Number of heads and Manure Production are for reference and can be left blank.
2. Choose specific Manure loading rate (m^3/d) and Temperature (°C).
3. Select loading rate of waste kitchen oil (m^3/d).
Note: Oil recommendation will be changed based on each selected temperature.
4. Results of working volume, water recommended, biogas production (m^3/d) and biogas yield ($m^3/kg\text{-}VS$) will be available after all factors are set.
5. Model assumptions: (1) Complete-mix AD. (2) Pig manure as feedstock. (3) Co-digestion of swine manure and waste kitchen oil. (4) Hydraulic retention time of 21 days. (5) No issue in ammonia.

Figure 34: Decision-making tool for recommendation of working conditions

4. Discussion

4.1. Relationship between biogas production and other variables

Biogas production had a strong relationship with other operating variables, including temperature and OLR. Removal of failure points (biogas production was zero) increased correlation coefficient of biogas and O/M ratio or OLR. This implies that using the selected dataset might be an appropriate approach to increase model accuracy. However, this is also the limitation of the model, in which the ranges of the key variables are restricted.

Working temperature and OLR showed strongly impact to biogas production of SM and WKO as discussed in the previous chapters. AD efficiency can be improved by changing the variables. For example, the decrease of OLR when co-digesting substrates at low temperatures is necessary to maintain the stability of ADs. Therefore, the application of the models before running the real on-farm experiments is an effective way to save time and efforts.

4.2. Model application and decision-making tool

Model created showed an effective way to predict biogas production based on the three main factors. Application of selected dataset and polynomial regression with variable interaction dramatically increased the adjusted R-squared value of the regression model to 0.9655, compared with a previously obtained lower value at 0.6152 of a simple linear regression model using original dataset. The *p-values* generated showed statistical significance of each model. Stepwise procedure was demonstrated as an efficient method to reduce number of predictors in the model. The application of other approaches, such as generalized linear models or Tweedie Generalized Linear Model might be other effective ways to generate regression models based on the original dataset for increasing R-squared value and model accuracy (Dunn & Smyth, 2001; Smyth & Verbyla, 1999)

The use of excel-based program made it convenient for farm owners to estimate biogas production when selecting different specific values of each factor. Digester size was estimated from 1,337 to 2,674 m^3 or from 47,216 to 94,431 ft^3. This estimation was in agreement with previous study (The Pennsylvania State University, 2012).

4.3. Model limitations

Accuracy of the model was validated by comparing biogas production estimated by model with lab AD biogas rates. However, the model could only estimate outputs following the limited ranges of each factor. For example, the study was conducted at only three levels of temperatures, and there were no data of biogas production for temperature less than 30°C or beyond 40 °C. Moreover, VS loading rate of SM less than 2 g-VS L^{-1} $d^{-1}$was not included in the study, while several large scale digesters are operated with low loading rate (Kougias & Angelidaki, 2018; Navickas et al., 2013). Using model to predict of biogas production based on the values out of the mentioned ranges of

each factor might be considered as extrapolation, which might result in significant bias (The Pennsylvania State University, n.d.b). Further studies should focus on more different working conditions so that prediction range of model could be expanded.

Even though the final model showed high R-squared and adjusted R-squared values, it was based on only laboratory study. No data had been collected from on-farm ADs for comparison of predicted results with actual biogas productions. Meanwhile, several factors of on-farm ADs could affect biogas production, such as design and type of digesters, HRT, fluctuation of temperature, change of animal diet, etc., which might lead to the reduction of model accuracy (Mao et al., 2015; Rajendran et al., 2012; Teng et al., 2014). Therefore, comparison of data generated by model with actual biogas production is necessary to improve model performance for on-farm application.

5. Conclusion

Temperature of AD and loading rate of SM and WKO showed significant impact to biogas production and digester stability. Decreasing temperature from 40 °C to 30 °C caused severe disturbance and even failure in all digesters with O/M ratio at 0.5 as well as at M4R0.25. Therefore, choosing proper OLR and low oil-to-manure ratio is crucial to maintain the consistent performance of ADs. High correlation between biogas production and OLR was observed, suggesting interaction between SM and WKO might play an important role in the model.

Several regression models were established to predict biogas production based on different variables, including VS loading of SM, O/M ratio and temperature with statistical significance. Removal of all AD failure observations in the dataset resulted in the increase of adjusted R-squared to 0.8877, compared with 0.6152 of model in which all observations were included. However, prediction of AD failure would be considered as extrapolation, which lead to poor estimations. Application of polynomial models and variable interaction increased the adjusted R-squared value to 0.9655, which was much higher than previous models. Stepwise procedure reduced numbers of predictors from 10 to eight in the quadric model with variable interaction while not alter adjusted R-squared value. Application of final model to original dataset resulted in the difference between predicted biogas productions and actual data from 0.2% to 8.6%, except biogas yield of M4R0.25 at 35 °C in which 15.9% difference was observed.

The Excel-based programs were developed as user-friendly and decision support tools to estimate digester volume and biogas production based on SM and WKO loading and temperature, using the model developed for on-farm application. The program could be used to provide recommendations of digester volume, oil volume and water usage before construction AD system or manure, oil loading and water usage for existed ADs. Digester volume recommended was in agreement with common capacity of completely mixed ADs, and biogas productions predicted were similar to data calculated manually in Chapter 3.

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Chapter 4 Appendix: Syntax codes for model developments

Load database into R (Willems, 2018)

```
gas1=read.csv("~/Model1.csv")
head(gas1)
summary(gas1)
```

Create histogram graph and summary biogas and pH based on different groups (Højsgaard, 2021)

```
hist(gas1$Biogas)
#install.packages("doBy")
library(doBy)
summaryBy(Biogas + pH~ Manure + Ratio + Temperature, data = gas1,
        FUN = function(n) {c(m = mean(x), s = sd(x)) } )
```

Create graphs of biogas production based on different variables (STHDA, n.d.)

```
#Graphs of biogas production by SM, O/M Ratio, Temperature or OLR
#install.packages("ggpubr")
library("ggpubr")
ggboxplot(gas1, x = "Manure", y = "Biogas",xlab="Manure loading (g-VS/L/d)", ylab="Biogas
production (mL/d)")+ theme(
  plot.title = element_text(color="Black", size=14, face="bold.italic"),
  axis.title.x = element_text(color="Black", size=14, face="bold"),
  axis.title.y = element_text(color="Black", size=14, face="bold")
)
ggboxplot(gas1, x = "Ratio", y = "Biogas",xlab="O/M Ratio", ylab="Biogas production (mL/d)")+
theme(
  plot.title = element_text(color="Black", size=14, face="bold.italic"),
  axis.title.x = element_text(color="Black", size=14, face="bold"),
  axis.title.y = element_text(color="Black", size=14, face="bold")
)
```

```
ggboxplot(gas1, x = "Temperature", y = "Biogas",xlab="Temperature", ylab="Biogas production
(mL/d)")+ theme(
  plot.title = element_text(color="Black", size=14, face="bold.italic"),
  axis.title.x = element_text(color="Black", size=14, face="bold"),
  axis.title.y = element_text(color="Black", size=14, face="bold")
)
ggboxplot(gas1, x = "OLR", y = "Biogas",xlab="Organic Loading Rate (g-VS/L/d)", ylab="Biogas
production (mL/d)")+ theme(
  plot.title = element_text(color="Black", size=14, face="bold.italic"),
  axis.title.x = element_text(color="Black", size=14, face="bold"),
  axis.title.y = element_text(color="Black", size=14, face="bold")
)
```

Generate correlation coefficient (Briatte, 2015)

```
#Correlation coefficient
#install.packages("GGally")
library(GGally)
ggcorr(gas1,label = TRUE, label_round = 2)
```

Generate selected dataset by excluding zero-biogas production values (Nishida, 2016)

```
library(dplyr)
gas2 = filter(gas1, Biogas > 0)
summary(gas2)
ggcorr(gas2,label = TRUE, label_round = 2)
```

Create regression models (Helwig, 2017)

```
gas1.md1 = lm(Biogas ~ Manure + Ratio + Temperature, data = gas1)
summary(gas1.md1)
gas1.md2 = lm(Biogas ~ Manure + Ratio + Temperature + I(Manure^2) + I(Ratio^2) +
I(Temperature^2) + Manure*Ratio*Temperature, data = gas1)
summary(gas1.md2)
```

```
gas2.md1 = lm(Biogas ~ Manure + Ratio + Temperature, data = gas2)
summary(gas2.md1)
gas2.md3 = lm(Biogas ~ Manure + Ratio + Temperature + I(Manure^2) + I(Ratio^2) +
I(Temperature^2) + Manure*Ratio*Temperature, data = gas2)
summary(gas2.md3)
gas2.md4 = lm(Biogas ~ Manure + Ratio + Temperature + I(Manure^3) + I(Ratio^3) +
I(Temperature^3) + Manure*Ratio*Temperature, data = gas2)
summary(gas2.md4)
```

Compare different model and stepwise algorithm (ETH Zurich, n.d.; Phillips, N. (2018).)

```
anova(gas2.md2,gas2.md3)
anova(gas2.md3,gas2.md4)
anova(gas2.md2,gas2.md4)
anova(gas2.md2,gas2.md3,gas2.md4)
summary(step(gas2.md3))
```

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Chapter 5: Conclusion

In this study, co-digestion of swine manure (SM) and waste kitchen oil (WKO) was investigated to optimize the process of biogas production and to create a user-friendly and decision-support tool for prediction of biogas yield based on different organic loading rate (OLR) of substrates and AD operating temperatures.

Experiments conducted in chapter 2 evaluated different VS loading rates of SM and WKO for co-digestion at mesophilic condition (40 °C). VS loading of SM more than 4 g-VS L^{-1} d^{-1} was shown to have very limited benefit while high biogas production and VS removal were recorded in four OLR settings, including M2, M2O1, M4O1 and M4O2. Study conducted in triplicate based on these promising OLRs resulted in biogas productions of 2,006 ± 64, 3,753 ± 152, 5,456 ± 125 and 7565 ± 352 mL_{biogas} d^{-1}, respectively, with VS reduction ranged from 62.4 ± 0.7% to 67.6 ± 0.6%. Higher alpha diversity of microbial community was observed when adding small amount of WKO to SM feedstock. However, high OLRs resulted in less bacterial diversity as well as the accumulation of VFAs which could be negative factors leading to AD failure. Adding more than 1.2 g-VS_{WKO} L^{-1} d^{-1} to digester fed with 2 g-VS_{SM} L^{-1} d^{-1} did not result in increase of biogas production, and might even lead to system imbalance.

In Chapter 3, biogas productions of promising candidates selected from Chapter 2 were evaluated in other temperature conditions, including ambient (30 °C) and thermophilic (55 °C). Both M4O1 and M4O2 stopped producing biogas when AD temperature was changed to thermophilic or ambient temperature. Compared with biogas potential, operation of ADs at 40 °C resulted in the system efficiency (by comparing with biogas potential of feedstocks) ranging from 87.9 ± 2.0% to 90.4 ± 4.2%, while highest efficiencies recorded in 55°C and 30°C were only 65.6% and 59.6%, respectively. Based on the efficiencies of AD performance at different temperatures, 40 °C was recommended for AD system to maximize biogas production. Different scenarios of OLR were discussed based on the operation of on-farm ADs. Recommendations were provided in terms of daily OLR of SM and WKO, digester volume, water usage, and prediction of biogas production.

Different regression models were developed in Chapter 4 as an approach to predict of biogas production based on manure VS loading rate (g-VS L^{-1} d^{-1}), oil-to-manure ratio, and temperature.

Selected dataset (excluding zero biogas production) and application of polynomial regression with variable interaction increased adjusted R-squared from 0.6152 of simple linear regression model to 0.9655 of the final quadric regression. Comparison of predicted biogas production based on the model with actual laboratory dataset showed the differences were from 0.2% to 8.6%, except only one OLR with 15.9% difference. The user-friendly tools were developed to provide recommendations of digester volume, OLR and predict biogas production for application of on-farm AD systems or to provide recommendations of ORL and estimate biogas yield for existed ADs. More studies are necessary to evaluate extended ranges of input factors and to conduct more combinations of key variables for model performance.

Appendix: Effectiveness of Manure Pit Additive in Reducing Emissions and Solids

1. Introduction

1.1. Swine operations and odor problem

One of the obvious trends in US animal feeding operations is the larger operations concentrating in certain areas. For example, the hog and pig sales of nine counties located in the top swine producing states of Iowa, North Carolina and Minnesota accounted for more than 13 percent of the nation's total (USDA-NASS, 2014). The average size of a typical farm in these regions has been increasing since 1950 (Lowder et al., 2016). Manure management is one of the most important challenges many farmers face due to regulations and operational requirements. For some animal farms – especially those without enough distances to neighbors – the odor released from manure storage pits can become a nuisance. In addition, a large amount of manure emission can be dangerous to the worker's and animal's health.

Ammonia (NH_3) and hydrogen sulfide (H_2S) are two common gases produced from manure storage; they are often measured as a surrogate of odor. These gases also have a relatively low permissible limit of exposure to ensure personnel safety. The Occupational Safety and Health Administration recommends exposure of H_2S and NH_3 not exceed 20 and 50 parts per million (ppm, ceiling), respectively (OSHA, 2011b, 2011a). High concentrations of these gases can cause health problems, ranging from eye irritation to a loss of consciousness or death (Donham, 2010; ASABE Standards, 2011).

1.2. Odor treatment approaches

Many approaches have been used to resolve problems related to animal manure and prevent potential sources of air pollution. Indirect methods include changing the diet, modification of ventilation systems or room temperature, and using biofiltration (Liu et al., 2014; Z. Ye et al., 2011). The separation of solid and liquid manure has been integrated into many farms so that additional treatment such as composting and anaerobic digestions can be used (Zubair et al., 2020). Manure can also be transferred to other farms for additional treatment (Ali et al., 2012). The economic

aspects, however, are often the major barrier to the successes of these management approaches. Because many of these mitigations require significant investment in infrastructure and/or operating costs, many farmers would consider utilizing pit additives as they can be simple and less expensive. The utilization of commercial pit additives for mitigating odors and reducing manure solids is another approach studied recently. These commercial additives can be biological or chemical products, and most of them claim odor abatement and other benefits like reducing solids or foaming (Duerschner et al., 2020; H. Mao et al., 2019). However, the effective dose of these additives is questionable, and empirical testing is needed to determine the products' effects and working conditions. Additives can be tested on site by spraying directly into the barn followed by odor measurements (Kim et al., 2008); the additives can also be distributed on the floor for future analysis (Provolo et al., 2016). This on-site approach, however, cannot be implemented in remote areas because the presence of odor experts is required. Unstable environmental conditions may also negatively alter the test results (Capelli et al., 2013). Another limitation of this approach is that the spraying was based on the manufacturer's recommended dosage without knowing the manure nutrient contents and gas concentrations, to adjust the dosage. As a result, optimal dose may not be achieved.

An alternative approach for evaluation of manure additives is the use of laboratory tests (that provide uniform testing conditions) instead of barn trials to confirm field effectiveness. Laboratory experiments are usually conducted using reactors or digesters that simulate manure storage conditions in a finishing barn (Hudson et al., 2001; Banhazi et al., 2009; B. Chen, 2019; B. Chen et al., 2020). Manure can be added once or periodically. The manufacturer's recommended dose or a variety of doses can be tested at the same time. Air ventilation might be applied for aeration or to collect odor samples for assessment. Some studies have used a glass bottle test for primarily assessment or field tests of the additive. One of the largest comprehensive pit additive effectiveness experiments was conducted in 2001 to evaluate 35 types of additives (Heber et al., 2001). Since then, there have been more additive products developed and marketed. Novel products such as biological-based additives can reduce the odors and solids while maintaining valuable nutrients for long-term manure storage.

1.3. Study objectives

This study aimed to evaluate the effectiveness of a biological pit additive that was marketed in Missouri (USA). The main components of the product are enzymes, bacteria and other chemicals which are claimed to reduce biochemical oxygen demand (BOD), chemical oxygen demand (COD), odor and solids of manure. However, component details are proprietary. The objectives of this study were to systematically evaluate effects of manure-treating product in term of reducing total and volatile solids as well as mitigating odor concentration.

2. Materials and Methods

2.1. Sample preparation

The laboratory experiments were conducted at the Agricultural Engineering Building Laboratory, University of Missouri, Columbia, MO (USA). Solid manure (27.3%TS, 23.4%VS) was provided by a commercial finishing farm located at central Missouri, and slurry manure (2.3%TS, 1.5%VS) was collected from University of Missouri Swine Research Center (Figure 1).

Figure 1. Manure and liquid collected at swine commercial finishing barn and University of Missouri Swine Research Center

Manure samples were stored in -20°C freezer until use. Solid and liquid manure was mixed using an 81-cm (32 in.) drywall mud mixer (Pro-Grade 32" Mixer, Amazon, Seattle, WA) driven by an electric drill to obtain mixture with 8-9%TS (pH 6.2 - 6.9) (Table 1).

Table 1: Characteristics of manure and mixture

	pH	Total solid (%)	Volatile solid (%)

Manure solid	-	27.3	23.4
Manure liquid	7.19	2.3	1.5
Mixture	6.56 ± 0.24	8.43 ± 0.46	6.70 ± 0.23

A manure (pit) additive, “PitStop Solution” was used to treat the manure (Figure 2). The product, granted US Patent 7,183,248, was provided by Treyco Supply, Inc. The additive (pH 12.1) was diluted following the manufacturer’s instructions using distilled water to a 5% solution (pH 10.9). One kg of the product was recommended to treat 10,000 kg of pit manure which was considered as 100% dosage.

Figure 2. Manure pit additive, “PitStop Solution, used in this study

Additive product was first tested in 3.79-L (1-gallon) glass jars (15.25 cm diameter, 25.4 cm in height) to optimize application dosage and to characterize the potential of products in reducing solids in 12 weeks (Experiment 1, short-term experiment). This experiment tested the null hypothesis that the manure additive does not reduce the solid contents and gas concentrations during a short-term manure accumulation and storage period. Based on these results, a subsequent long-term experiment (Experiment 2) was conducted to verify effectiveness of the additive in reducing manure solids and odor concentrations. The null hypothesis of the Experiment 2 was that the manure additive does not reduce manure solids and odors or alter manure nutrients.

All experiments were conducted in the laboratory at normal room temperature (25°C).

2.2. Experiment 1 (Short-Term Experiment)

Four treatments were conducted in triplicate, including 0 (control), 50, 100 and 200% dosage (Figure 3). All jars were started with 20% volume of pre-mixed manure (680 g) at the beginning week. Additive was added to the respective groups at day 3 after the manure had a chance to settle. Dosages were calculated based on current amount of manure in jars with ratio of manure to additive at 10,000:1. Manure was added weekly at the rate of 600 g during weeks 2 to 5, and 400 g during week 6, which the jars were full and filled with 3.48 kg of mixture. Manure pH was measured 3 cm from manure surface weekly using a pH meter (Pinpoint, American Marine Inc, Ridgefield, CT, USA).

Figure 3: Experiment 1 with 12 glass jars set up in laboratory.

All jars were kept together under room temperature and monitored for at least three months to mimic short-term manure storages. The glass jars were not ventilated but were capped. The plastic lids had a 1.3 cm (0.5 in.) diameter opening, allowing the manure to off-gas to the lab. The NH_3 and H_2S concentrations were measured from the headspaces every month by using individual chemical tubes (Draeger Gas Detection Tubes, Draeger Safety, Sugarland, TX) and a hand-held bellows pump (Draeger Accuro pump, Draeger Safety). Detection limits of H_2S and NH_3 tubes were 0.2 ppm and 5 ppm, respectively (Figure 4).

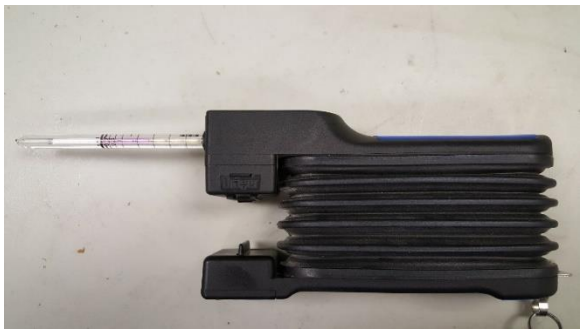

Figure 4: Hand-help below bump for detection of ammonia and hydrogen sulfide concentrations

Grab samples of the manure were collected at the end of the experiment for TS, VS, and pH measurements. The first short-term experiment was conducted from September 2017 to January 2018 (Experiment 1A) in a ventilation hood to maintain consistent airflow. A second experiment was started in April 2018 and completed in September 2018 (Experiment 1B) in another hood without ventilation.

2.3. Experiment 2 (Long-Term Experiment)

The long-term storage and treatment of manure was performed to mimic the deep pit storage of commercial finishing farms with the use of nine 15 cm ID x 152 cm long (6 in. ID x 5 ft. long) schedule-40 PVC tubes as reactors. Reactors were attached to a mobile woody rack for easy movements during the study and tied together by steel chains. All reactors were filled by tap water for leakage detection before usage (Figure 5). Petroleum jelly was used to fixed leakage when detected.

Figure 5. Use of tap water to detect leakage in reactors.

Three treatments consisted of dosages of 0, 100 and 200% with three replicates of each treatment. Dosage 50% was not included in the experiment because no significant effectiveness of this dosage was recorded in Experiment 1. All reactors were started with 20% volume of pre-mixed manure (5.6 kg) at the beginning of the experiment. Manure was added every week at a rate of 3.8 cm (700 g) per week to mimic the manure accumulation in a commercial finishing barn. Each reactor was filled with 28 kg of manure at the end of the experiment, and when filled, the average headspace was 6,088 ± 172 cm^3.The additive was added at week 6 after manure had a chance to settle. Dosage was calculated based on amount of manure in reactors when additive was introduced (9.1 kg) with the ratio of manure to additive at 10,000:1 (Figure 6).

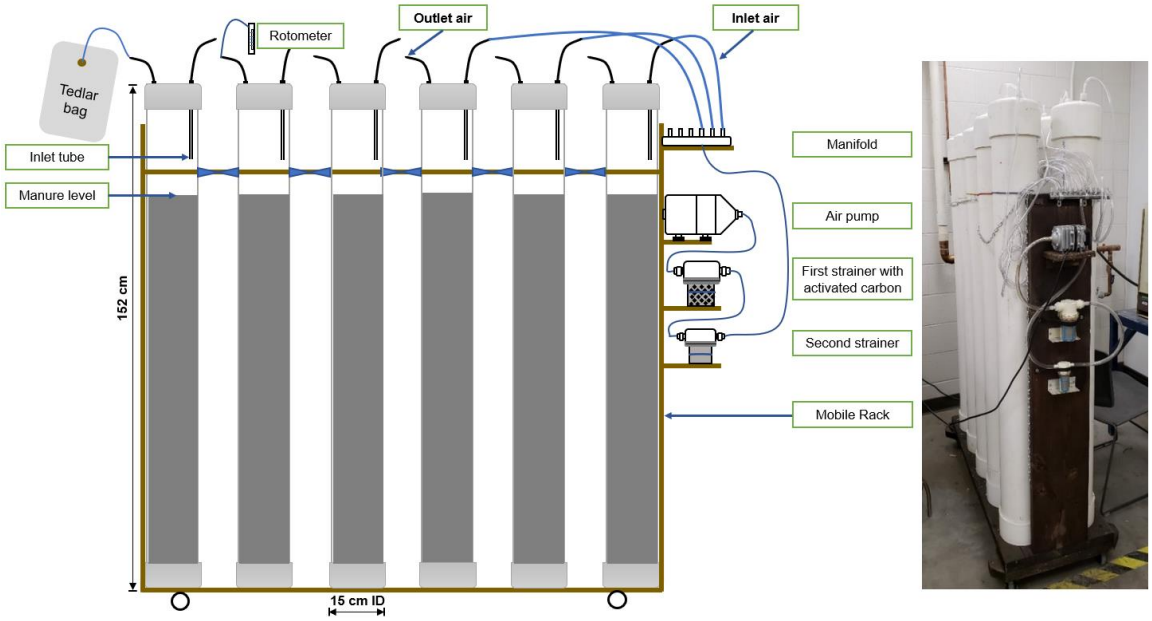

Figure 6: Scheme and photograph of reactors set up for Experiment 2.

Caps of reactors were attached (not glued) to PVC tubes and sealed with petroleum jelly to prevent air leakage so that they could be detached for weekly manure loading. Each cap was installed with straight-through wall connector (0.635 cm or ¼ in. polyethylene, McMaster-Carr, Elmhurst, IL) for air ventilation. Airflow was provided by a piston air pump (Eco Air1, EcoPlus, Vancouver, WA. Rated at 3,000 L/h, 18 W) that went into each reactor through a 12-outlet manifold. Room air was pumped through two in-line strainers (1.27 cm or 1/2 in. and 1.905 cm or ¾ in. Female NPT Strainer, VacMotion Inc., Plymouth, MA) with a 50-mesh stainless steel screen. The first strainer contained activated carbon pallets (Acurel LLC, Cranbury, NJ) to filter significant particulate matter and odors

before entering the reactors. The second strainer was installed in series to ensure filtering of potential materials from the first strainer.

Airflow was maintained at a rate of two liter per minute (2,000 cm^3/min) for each of the reactor. The air exchange rate (AER) calculated was 20 air exchanges per hour (AE/h) when the reactor was full and distance from reactor cap to manure level was 34 cm, which was within the range of AERs of deep-pit headspace in a 1,000-hd finishing pig barn. The estimated AERs of a deep-pit headspace ranged from 5 to 31 AE/h when one pit ventilation fan (assumed 0.61-m diameter fan, one pit fan operating in cold weather) was running at 155.7 m^3/min (5,500 ft^3/min, operating at 24.9 Pa or 0.1 in. water column, and 85% efficiency), for various manure levels (empty to 85% full of 2.4-m deep-pit). When the deep-pit was ventilated by three pit fans (warmer weather), the estimated AERs ranged from 16 to 95 AE/h at various manure levels. The 20 AE/h was also similar to the reported mean exchange rate of 22.8 AE/h, in commercial swine buildings (Banhazi et al., 2011). A rotameter (RMA-16, Dwyer, Waco, TX) was used to check airflow rate of each reactor daily. For more accurate measurements, an airflow calibrator system (Gilibrator-2, Sensidyne, LP, St. Petersburg, FL) was used to monitor the airflow of each reactor once per week (Figure 7). When the exhaust airflow fluctuated more than 200 cm^3/min (10%), the ventilation system was checked to verify no leakage and obstruction. The airflow was adjusted via the manifold valve when needed. Manure levels in the reactor were measured weekly using a laser tape measure (DW030PL, DEWALT, Baltimore, MD) to monitor the manure addition and potential leakages.

Figure 7: Airflow calibrator and rotameter for airflow measurement

Effectiveness of the additive in reducing odor was evaluated by comparing the treated and

untreated (control) odor and gas concentrations. Concentrations of NH_3 and H_2S were measured every month via individual chemical (Draeger) tubes and a hand-held bellows pump as described above. The exhaust air was released from each reactor and was sampled for odor evaluation using 10-L Tedlar bags after 3 months and 6 months from start of experiment. Samples were shipped overnight to St. Croix Sensory, Inc (MN) for odor concentration and hedonic tone evaluation as described by Lim et al. (2001).

Measurements of H_2S continued into the 7^{th} and 8^{th} months of the experiment because there was indication that the monthly and grab measurements could not monitor the fluctuations of the H_2S emissions. Additional gas samples from each of the reactor were collected three times per week (on Monday, Wednesday, and Friday) for two weeks in the 8^{th} month of measurement. Exhaust air samples of the reactors were collected using 25 L-Tedlar bags over 10 min. Bags were pre-flushed once with compressed air and conditioned once with the reactor exhausts before collection (Lim et al., 2003). Concentrations of H_2S were measured using a pulsed fluorescence analyzer (Model 450i, Thermo Scientific, Waltham, MA) (Figure 8). The analyzer was checked using zero air and calibration gas (2,348 ppbv) before and after the bag measurements to monitor the accuracy of the analyzer. The zero air readings of the analyzer ranged from 2 to 49 ppbv and averaged 24.2 ± 14.7 ppbv, while the calibration gas reading ranged from 2,153 to 2,330 ppbv and averaged 2,229 ± 59.8 ppbv. No adjustment of the measurements was made because the zero air and calibration gas readings were relatively consistent over the two weeks, and the differences were relatively low compared with the exhaust concentrations.

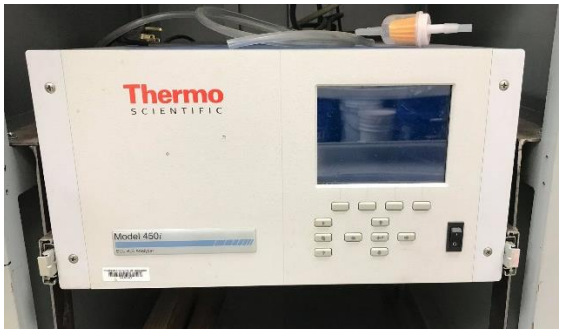

Figure 8. Measurement of hydrogen sulfide concentration using pulsed fluorescence analyzer in month 8

Manure nutrient variables, including TS, VS, total nitrogen, phosphorus, potassium, pH, total carbon, and electrical conductivity, were all sampled and measured at the end of the experiment. Sampling was conducted by emptying each of the reactors into a 76-L (20-gallon) container. Manure samples were mixed using the drywall mud mixer and frozen at -20°C immediately, then sent to the Soil and Plant Analysis Laboratory at University of Missouri for analysis (Nogueira et al., 2019a). This lab is a certificated lab following standard analysis methods.

2.4. Data analysis

Raw data were analyzed using R version 3.5.1 (Free Software Foundation, Boston, MA). Mean values among groups were compared using One-way ANOVA. Tukey's Honest Significant Difference test (Tukey Test) was applied to confirm significant differences between groups. All averages of odor concentrations were reported as geometric means because they typically exhibit lognormal distributions (CEN, 2003). A decimal logarithm (base 10) transformation was applied to odor concentrations to determine statistically significant differences between control and treatment groups. Means were considered significantly different when the *p-value* was less than 0.05.

3. Results

3.1. Experiment 1 (Short-Term Experiment)

3.1.1. Experiment 1A

Table 2 summarizes variables measured during the first short-term experiment. The pH of the manure at day 0 was 6.7 ± 0.0. Means of pH, after mixing manure at the end of the experiment ranged from 8.1 ± 0.3 to 8.4 ± 0.1, but no statistical difference was observed. Concentrations of NH_3 in the headspace of the 100% dosage treatment was the lowest when all other concentrations were relatively similar. However, there was no significant difference in NH_3 concentrations among the four groups ($p = 0.949$). Concentration of H_2S could not be measured by Draeger Gas Detection Tubes at the detection limit of 0.1 ppm. This may be caused by the jar's small volume and direct contact with the environment, and emission of H_2S could be released quickly to the air at certain occasions, and could be more variable than NH_3 as reported in the literature (Ni et al., 2010). Evaporation from 100% and 200% treatments was higher than others due to unbalanced airflow in the hood, which resulted in lower manure height in the jars and higher TS and VS after the

experiment.

Table 2: Parameters of samples in glass jars at the end of Experiment 1A.

Variables/Treatments	Unit	Control	50%	100%	200%
Number of Samples	-	3	3	3	3
pH	-	8.2 ± 0.1a	8.4 ± 0.1a	8.1 ± 0.3a	8.2 ± 0.1a
Ammonia	ppm	133.3 ± 62.9a	125.0 ± 43.3a	116.7 ± 56.9a	140.0 ± 40a
Hydrogen sulfide	ppm	N/D[a]	N/D[a]	N/D[a]	N/D[a]
Total solids	%	8.3 ± 0.2a	8.3 ± 0.0a	9.1 ± 0.3b	9.3 ± 0.2b
Volatile solids	%	6.0 ± 0.2a	6.0 ± 0.0a	6.6 ± 0.2b	6.8 ± 0.1b

[a] N/D = not detectable (below detection limit); different letters in the same row indicate significant difference ($p < 0.05$); mean ± standard deviation.

3.1.2. Experiment 1B

The initial pH of each group at the beginning of the experiment was 6.8 ± 0.0. After adding the manure weekly, pH values of all four groups generally increased, and the overall mean values ranged from 8.1 ± 0.1 to 8.2 ± 0.0 over the experiment, which were similar to those observed in the previous experiment (Figure 9). However, the difference between groups was barely distinguishable. The fact that the control and treatment groups had similar pH trends suggested that the additive did not alter the chemical characteristics of the manure storage.

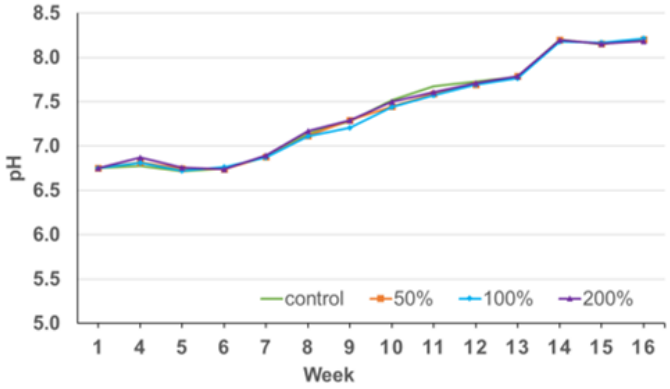

Figure 9: Mean pH of different groups in the Experiment 1B. Values reported are the means of three replicates.

Table 3 summarizes the variables of the second short-term experiment. Ammonia concentrations measured at the headspace of the glass jars were relatively high (>240 ppm). The concentration of the 50% dosage group was the lowest, and all NH_3 concentrations were from 240.0 ± 17.3 to

286.7 ± 20.8 ppm; there was no significant difference in NH_3 among the four groups (p = 0.246). The testing environmental without airflow like Experiment 1A seemed to be reason for increase in NH_3 concentrations between the first and second short-term experiment. Again, H_2S could not be detected at the 0.1 ppm level. Lowest TS and VS were observed in group treated with 200% additive dosage at 5.3 ± 0.2% and 3.4 ± 0.1% versus 5.7 ± 0.1% and 3.8 ± 0.1% of control, respectively. The differences of TS and VS were statistically significant with *p-values* equal to 0.007 and 0.003, respectively. In general, the additive reduced the amount of TS and VS with increasing dosage during the short-term experiment.

Table 3: Parameters of samples in glass jars at the end of the Experiment 1B.

Variables/Treatments	Unit	Control	50%	100%	200%
Number of Samples	-	n = 3	n = 3	n = 3	n = 3
pH	-	8.2 ± 0.0a	8.1 ± 0.1a	8.2 ± 0.0a	8.1 ± 0.1a
Ammonia	ppm	250.0 ± 36.1a	240.0 ± 17.3a	286.7 ± 20.8a	271.1 ± 33.1a
Hydrogen sulfide	ppm	N/D[a]	N/D[a]	N/D[a]	N/D[a]
Total solids	%	5.7 ± 0.1a	5.7 ± 0.2ab	5.4 ± 0.1bc	5.3 ± 0.2c
Volatile solids	%	3.8 ± 0.1a	3.8 ± 0.1a	3.5 ± 0.1b	3.4 ± 0.1b

[a] N/D = not detectable (below detection level); different letters in the same row indicate significant difference ($p < 0.05$); mean ± standard deviation.

3.2. Experiment 2 (Long-Term Experiment)

3.2.1. Reactor's Airflow Rate, Manure Level, and pH values

Airflow rates of each of the reactors were measured to analyze any abnormal performance of the ventilation system and reactors. Over the eight months of testing, the airflow rates fluctuated between 1,800 to 2,200 cm^3/min (Figure 10a). In general, they remained at 2,025 ± 122 cm^3/min throughout the testing period without major adjustment. Manure levels in the reactors were shown to have a steady weekly increase due to the consistent manure addition (Figure 10b).

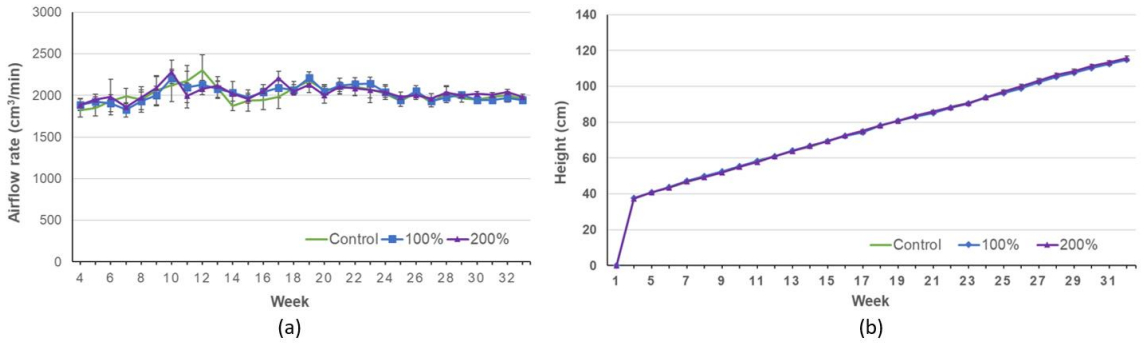

Figure 10: Airflow rate (a) and height of manure (b) of the three treatment groups over a 33-week period of Experiment 2. Values reported are the means of three replicates.

The pH recorded weekly was similar to the short-term experiment using glass jars (Figure 11). The difference of pH in each group was again relatively minor. Interestingly, the pH of all reactors were not over 7.0 during the first 23 weeks and were above 7.5 after five months or more of loading, while the pH in the glass jars was over 7.0 after only 6-7 weeks. This suggested that the airflow system may be critical to maintain the pH in the reactors and better represent the deep-pit manure storages.

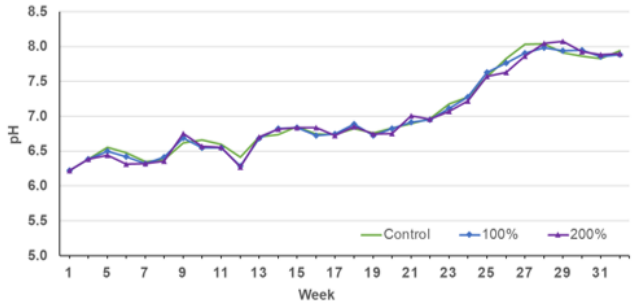

Figure 11: Mean pH during the long-term test of Experiment 2. Values reported are the means of three replicates.

3.2.2. Gas Concentrations

In general, the gas concentrations increased over the first four months most likely due to manure addition (Figure 12). There was a downward trend for both gases after the fourth month. The overall mean NH_3 concentrations for the control, 100%, and 200% treatment groups were 44.7 ± 13.4, 39.6 ± 11.3, and 37.8 ± 9.1 ppm, respectively, and there was no significant difference (p = 0.566) between the groups during the six months of monitoring. Manure added with 200% dosage released relatively lower NH_3 than others. The overall mean concentration for 200% treatment was

30.0 ± 5.2 ppm versus 66.0 ± 4.4 ppm from the control reactors and 48.0 ± 18.7 ppm from 100% dosage columns. The exhaust concentrations did not continue to increase during the last two months for both the NH_3 and H_2S concentrations. However, the gas concentration measurements were very limited and were likely not capturing the fluctuation over time.

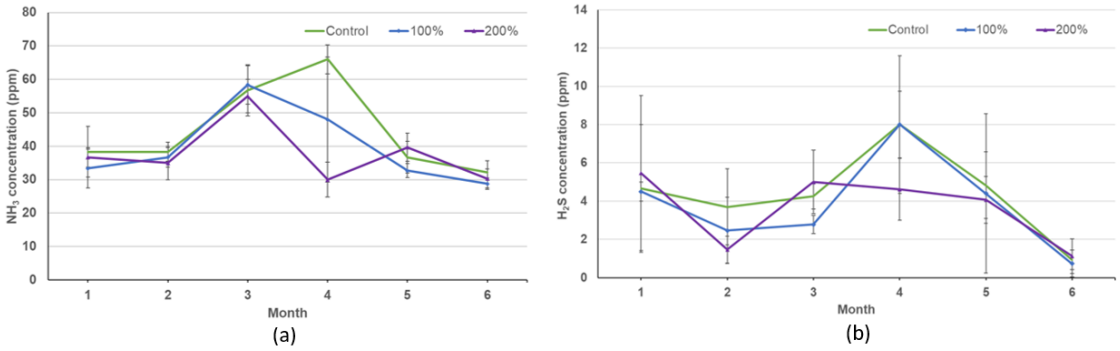

Figure 12: Concentrations (ppm) of ammonia (a) and hydrogen sulfide (b) in the exhaust air of the reactors over six months in Experiment 2.

The concentrations of H_2S appeared to be more variable and difficult to evaluate. This was likely due to the nature of how the gas was slowly released by the microbial activities (mostly anaerobic bacteria) and trapped at the bottom portion of the reactors. There has been research showing sporadic releases of H_2S from manure storages (Ni et al., 2009). We have conducted tests by manually tapping the glass jars (of the short-term experiment); the H_2S concentration of the jar headspace increased from below the detection limit to over 10 ppm, which confirmed the sudden H_2S release. The grab sampling could not monitor such releases. The overall mean H_2S concentrations for the control, 100%, and 200% treatments were 4.4 ± 2.3, 3.8 ± 2.5, and 3.6 ± 1.9 ppm, respectively. The treated groups of 100% and 200% dosages had 21% and 18% lower H_2S concentrations than the control reactors, respectively, but the concentrations were not significantly different from each other over the six months (p = 0.822). Due to the fluctuation of H_2S concentrations, the long-term experiment was extended into the eighth month to allow additional sampling and monitoring of the H_2S concentrations using a pulsed fluorescence analyzer (Table 4). Overall, the 10-minute sampling of H_2S concentrations showed higher concentrations after the manure loading (day 2); this was followed by a lower trend on days 4 and 6. Again, there was no significant difference in H_2S level released from each group (p = 0.933). The dataset showed an

improvement in the H_2S levels, but no conclusion could be made about the sporadic releases. More frequent monitoring of the exhaust concentrations is needed to better characterize the diurnal and other variations.

Table 4: H_2S concentrations (ppmv) measured by bag sampling and pulsed fluorescence analyzer during the eighth month of testing in Experiment 2.

Week	Day	Control	100%	200%
1	2	12.0 ± 4.4	10.6 ± 2.4	10.4 ± 4.0
	4	4.6 ± 2.7	4.0 ± 2.6	3.0 ± 1.0
	6	1.5 ± 1.1	1.1 ± 1.2	3.6 ± 1.8
	Average	6.0 ± 5.4	5.2 ± 4.8	5.7 ± 4.1
2	2	9.4 ± 1.6	12.0 ± 1.6	9.0 ± 4.4
	4	3.7 ± 1.4	5.4 ± 1.2	4.1 ± 3.7
	6	2.3 ± 2.7	4.8 ± 2.7	3.4 ± 2.7
	Average	5.1 ± 3.8	7.4 ± 4.0	5.5 ± 3.0
Average		**5.6 ± 4.2**	**6.3 ± 4.2**	**5.6 ± 3.2**

mean ± standard deviation.

3.2.3. Odor Evaluations

Odor concentrations and hedonic tones of the reactor exhaust samples were summarized in Table 5. Overall, lower concentrations were observed in the treatment groups versus the untreated reactors especially during the sixth month of sampling. After logarithmic transformation of odor detection threshold (log10 DT), no significant difference was observed for the odor concentrations between groups during the 3-month period ($p = 0.449$). However, after six months of treatment, the Tukey Test showed no difference between control and 100% dosage, but there was a significant difference between 200% treatment with other groups ($p < 0.05$) (Figure 13).

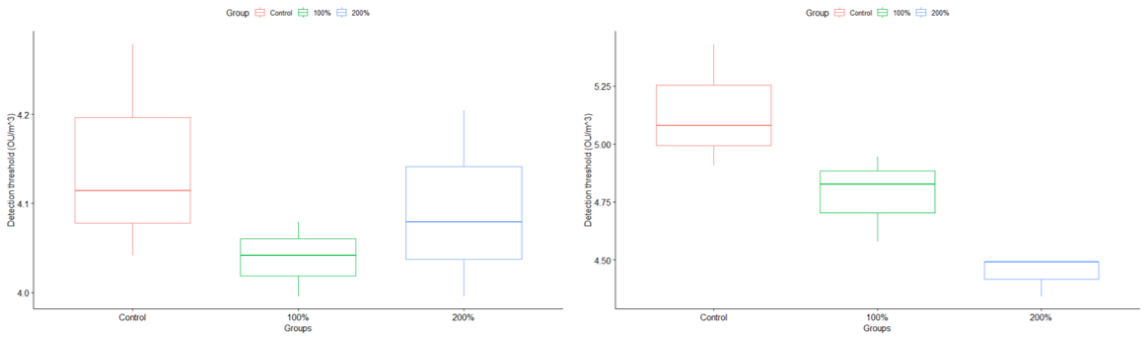

Figure 13. Means of detection threshold in each group after 3- and 6-month period

The odor concentrations of six-month-storage samples were so high that the olfactometry laboratory had to pre-dilute the three control and two treated samples before the olfactometer could make a measurement. Because of the dilution, no hedonic tone could be evaluated for the pre-diluted samples. The hedonic tones averaged -5.8, -5.4, and -5.6 for the 3-month control, 100%, and 200% groups, respectively, with no significant difference observed. All of the hedonic tone values were in the negative range indicating unpleasant characteristics. In the sixth month measurement, the hedonic tone of the control reactors and two reactors of the 100% group was not measured at a 10:1 dilution. The hedonic tone value for the last reactor treated by 100% dosage was -4.2, while the average value for the 200% group was -6.30 ± 1.66.

Table 5: Mean odor concentration and hedonic tone of exhaust samples after 3 and 6 months of storage in Experiment 2.

Odor Variables	Unit	Month	Control	100%	200%
Detection threshold (DT)	OU/m^3	3	13,945 ± 4,163a	10,933 ± 1,050a	12,387 ± 3,099a
		6	137,936 ± 99,785a	60,736 ± 25,106a	27,651 ± 5,196a
$Log_{10}DT$	OU/m^3	3	4.11 ± 0.09a	4.09 ± 0.16a	4.08 ± 0.62a
		6	5.14 ± 0.27a	4.78 ± 0.19a	4.44 ± 0.04b
Hedonic tones	-	3	-5.77 ± 0.35a	-5.43 ± 0.40a	-5.63 ± 0.42a
		6	N/M[a]	N/M[b]	-6.30 ± 1.66

[a] N/M = not measured at 10:1 dilution level; [b] Data observed for one reactor in the group, the other two were not measured at a 10:1 dilution level; different letters in the same row indicate significant difference ($p < 0.05$); mean ± standard deviation.

3.2.4. Manure Nutrient Evaluations

Manure stored inside the reactors for over eight months had nutrient levels, moisture, pH, and

electrical conductivity similar to each other (Table 6). The pH and TS/VS agreed with the measurements observed in the short-term experiment. There was a slight reduction in the concentration of nitrogen, ammonium, phosphorus, and potassium when double amount of additive was applied. However, the difference between three groups was not significant with *p-values* for each indicator ranging from 0.107 to 0.679. Only the *p-value* of electrical conductivity was lower than 0.05 but it was not an important criterion to evaluate the nutrient components. The results indicated that the manure nutrients were similarly preserved when the additive was added. Similar to the short-term experiments, reactors treated with a 200% dosage of additive showed a reduction in TS and VS. However, the statistical data analysis did not show significant differences with *p-values* of 0.145 and 0.182 for TS and VS comparisons, respectively.

Table 6: Manure nutrient factors and total solids/volatile solids of the three groups at the end of Experiment 2.

Variables/Treatments	Unit	Control	100%	200%
Nitrogen (N)	ppm	5,214± 472a	5,315 ± 116a	5,067 ± 319a
	lb/ac-in	1,180 ± 107a	1,204 ± 26a	1,147 ± 72a
	lb/1000 gal	43.4 ± 3.9a	44.3 ± 1.0a	42.2 ± 2.7a
Ammonium (NH_4)	ppm	4,462 ± 63a	4,522 ± 37a	4,184 ± 439a
Phosphorus (P)	ppm	1,064 ± 14a	1,082 ± 22a	954 ± 159a
	lb P_2O_5/ac-in	552 ± 7a	561 ± 11a	495 ± 83a
	lb P_2O_5/1000 gal	20.3 ± 0.3a	20.6 ± 0.4a	18.2 ± 3.0a
Potassium (K)	ppm	1,688 ± 9a	1,604 ± 81a	1,415 ± 215a
	lb K_2O/ac-in	459 ± 3a	436 ± 22a	385 ± 59a
	lb K_2O/1000 gal	16.9 ± 0.1a	16.0 ± 0.8a	14.2 ± 2.1a
Moisture	%	93.0 ± 0.1a	93.1 ± 0.3a	93.2 ± 0.4a
pH	-	7.9 ± 0.1a	7.8 ± 0.0a	7.9 ± 0.1a
Electrical conductivity	mmhom/cm	10.9 ± 0.7a	12.2 ± 0.6b	11.0 ± 0.2a
Total solids	%	6.9 ± 0.1a	7.1 ± 0.1a	6.7 ± 0.3a
Volatile solids	%	5.0 ± 0.1a	5.1 ± 0.1a	4.7 ± 0.3a

Different letters in the same row indicate significant difference ($p < 0.05$); mean ± standard deviation.

4. Discussion

Results of the Experiment 1 did not show significant difference in NH_3 and H_2S concentrations. This may be due to a combination of the lack of airflow, small working volumes, and relatively shallow storage depth. The TS and VS, however, showed a significant difference between the control and groups treated with a 200% dose of additive in the second setup of short-term study (Experiment 1B). Under this limited replication and test condition, this particular pit additive required high dosage to show its effectiveness. The jar tests in Experiment 1 were thus only recommended to verify additive dosage and long-term storage reactors should be used to better mimic the deep-pit air exchanges. Meanwhile, the Experiment 2 (long-term storage) showed more promising outcomes. The observed pH values were relatively stable perhaps due to the consistent ventilation within the reactors for long-term manure storage and treatments. However, Zhu et al. (2006) recorded a quick increase in pH from 7.8 to 8.8 after two days when aeration is applied to 91.6 cm H x 15.3 cm ID columns containing 15 L liquid swine manure. The differences of the aeration, headspace volume, and air exchange rate between the two studies (headspace of 1,832 cm^3 in Zhu's experiment compared with 12,500 cm^3 in this study at week 23, when pH increased above 7.0 and 23 kg manure was added in each reactor) may be the reason for the pH gap.

Similar to the short-term experiments, NH_3 and H_2S concentrations were not significantly reduced when additive was applied at either the recommended dosage or double the dosage. The decimal logarithm transformation of the detection threshold showed a significant difference between the 200% treatment with others in sixth month odor sampling, which confirmed that the biological additive could mitigate some odor although the gas concentrations measured were not affected. Similar observations were reported in other additive tests conducted by Banhazi et al. (2009), which demonstrated an odor reduction due to a manure additive but the reduction was not statistically significant. In a study reported by Provolo et al. (2016), the BACTY complex additive did not show effectiveness during the first four weeks but a TS reduction was observed after 155 days. Similar to this study, longer term incubation (six months or longer) might be needed to observe effectiveness of a certain additive. More frequent and semi-continuous measurements of the gas concentrations are recommended for future tests. For important manure nutrients and

characteristics, no significant differences were observed between the control and treated groups in our study. Therefore, the risk of nutrient loss because of additive applied can be eliminated. Experimental designs and dosage tested can greatly affect additive evaluation and sometimes lead to contradictory conclusions. For instance, More Than Manure® was reported to give a higher level of TS in manure compared to the untreated group in studies conducted by Sun et al. (2014) and Duerschner et al. (2020). However, Holly and Larson (2017) observed no difference in TS level when manure was treated with this same additive. Low rate treatment was effective in the additive study conducted by Banhazi et al. (2009) as well as Shah and Kolar (2012), while a high dose did not show any significant change. This has not been explained, but the data suggests that the evaluation of different dosages should be performed at the same time to find the optimal working rates. Interestingly, field tests in these studies did not show significant outcomes even when the additives had been proved to work in the laboratory test. Therefore, tests with different working conditions and dosages are critical to determine the consistence of manure additives because several variables in the field might have affected the consistency observed in the laboratory setting. Insight into the biological mechanisms underlying the bacteria's activity can lead to future development of specific products. For example, an additive for prevention of NH_3 emission during manure composting has been developed based on a *Bacillus* strain isolated from swine manure (Kuroda et al., 2004, 2017). Since the microorganism's characteristics were known, additive storage and working conditions could be specified, resulting in optimization of the product's effectiveness. Another interesting work was reported by Van der Stelt et al. (2007) when the effect of ammonia emission on four additives was studied. A combination of two additives was shown to mitigate the NH_3 volatilization, but no difference was observed when additives were applied separately.

Some additives were demonstrated to alter the bacterial diversities in swine manure to affect fertilizer properties or land applications (Z. Li et al., 2019; H. Mao et al., 2019). Therefore, once the effect of an additive is verified, the next step should be to study bacterial strains or enzymes in the products (McCrory & Hobbs, 2001) or the interaction between additive with microbial community in manure, for example, with *Eubacterium* and *Clostridium*—the bacterial genera that contributes the

most nuisance odors (Zhu, 2000). In this study, no field test of the additive was conducted. A future study may test additive application in the commercial barns where the interaction between additive and various environmental conditions can be fully evaluated. Metagenomics evaluation of the control and treated samples could also be performed to better analyze the microbial differences.

5. Conclusion

In this study, a relatively simple design was applied to simulate long-term manure storage with ventilation air, for pit additive effectiveness test. Odor reduction was observed in the sixth month sampling but not in the third month sampling, suggesting some additives take time to show effects. Low reductions of total solids and volatile solids were observed only for the 200% dosage group. Nutrient contents were not affected by the additive during the six-month treatment period, indicating the preservation of manure nutrient for agriculture purposes after application of additive.

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