Anaerobic Digestion Enhanced by Bacteria Microalgae Consortium

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By

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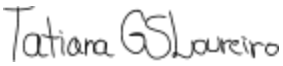

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Abstract

Anaerobic digestion of organic farm waste is widely used for methane production. However, several factors can affect the methane production yield, including inhibitory compounds. This paper provides a detailed summary of the small batch reactor research conducted on the enhancement of anaerobic digestion by microalgal bacteria. Organic farm waste (manure) was mixed with synthetic wastewater and microbial flocs in an attempt to increase methane production. The results outline how different factors relate and affect the methane production rate, including ammonia and chlorophyll, and offer headway for successfully increasing methane yields of microalgae bacteria enhanced digestion.

1 Introduction

Organic wastes pose a huge problem to the environment all around the world. As wastes are broken down through anaerobic digestion, they produce two greenhouse gases that contribute to global warming: methane and CO_2. However, when captured, methane gas can be used as a renewable, environmentally-friendly energy source. The leftover organic sludge from anaerobic digestion can even be utilized as fertilizers for crops.

Biogas, such as that mentioned above, has been used as a source of energy for centuries, most commonly as lighting and cooking fuels. Most recently, scientists have been trying to enhance anaerobic digestion in favor of methane production, so that its application can be widely used and economically efficient. The success of anaerobic digestion is dependent on many parameters including, but not limited to, the type of waste, temperature, and pH. Ideal conditions for anaerobic digestion are difficult to attain because of the varying parameters, but enhancements are being made through various additives, such as bacteria, or through co-digestion of various wastes.

This study focuses on the role of microalgae bacteria flocs in enhancing the production of methane during anaerobic digestion. As the start of a long term study, small batch reactors were used to digest a mixture of agricultural digestate, wastewater, and bacteria flocs at mesophilic conditions to monitor methane production, COD, phosphorus, ammonia and chlorophyll-a content. Through these preliminary tests, favorable parameters to increased methane production were explored.

2 Background

This chapter will explore anaerobic digestion and important factors that need to be taken into consideration when trying to create ideal conditions for biogas generation.

2.1 Anaerobic Digestion

Anaerobic digestion (AD) is a four step process that produces methane and carbon dioxide without the presence of oxygen. The first step, hydrolysis, converts organic polymers and lipids into monosaccharides, amino acids and long chain fatty acids (Kothari, Pandey, Kumar, Tyagi, & Tyagi, 2014). These products are then fermented into alcohols and volatile fatty acids (VFA) in a process called acidogenesis. Acidogenic microorganisms are most productive at a pH between 5.5 and 6.5 (Mao, Feng, Wang, & Ren, 2015). The alcohol and organic acids are then converted, through acetogenesis, into hydrogen, carbon dioxide, and acetic acids. The final step, methanogenesis, transforms the contents into the final products of methane and carbon dioxide (Ezebuiro & Körner, 2017). Methanogens, the bacteria that carry out methanogenesis, are most efficient in a pH range of 6.5 to 8.2, much higher than that of acidogenesis. With an optimal pH of 7.0, their growth rate is severely affected when the pH strays outside of the aforementioned range (Mao, Feng, Wang, & Ren, 2015). Ideally, hydrolysis and acidogenesis would be carried out at a lower pH range and the last two steps, acetogenesis and methanogenesis, would be carried out at a higher pH range. However, an overall pH range of

6.8-7.4 is reported as ideal for all four steps of anaerobic digestion (Mao, Feng, Wang, & Ren, 2015).

2.2 Important Components and Parameters

Successful methane production is dependent on various factors. This may include the types of organic waste being digested, the set temperature at which digestion is carried out, and the ability to maintain a balance between all the parameters involved in the process throughout digestion to ensure its successful completion.

2.2.1 Water and the Amount of Solid Matter

Various parameters and factors can affect the anaerobic digestion process. Water is an important component that not only controls the growth of the microbial population, but also works as a buffering agent to substrates and reactants present within the process. Based on the total amount of solid matter (TS) present, the process can be classified as either a wet (<15% TS) or dry system (>15%) (Kothari, Pandey, Kumar, Tyagi, & Tyagi, 2014).

Wet digestion has been classified as less feasible than dry digestion. The wet process not only consumes and wastes an enormous amount of water, but it also decreases the nutrient value of manure and requires a large amount of land and energy. For these reasons, dry digestion is usually favored, especially since this process yields much higher production rates (Kothari, Pandey, Kumar, Tyagi, & Tyagi, 2014).

2.2.2 Temperature

Another crucial component involved in anaerobic digestion is temperature. Variations can greatly affect the overall process, microbial growth and the decomposition of organic matter, thus either enhancing or inhibiting biogas yield. There are three main temperature ranges associated with AD processes: thermophilic (55-70℃), mesophilic (37℃) and psychrophilic (10-20℃). Thermophilic AD is usually favored during anaerobic digestion due to its faster reaction rate and higher load bearing capacity, which results in an overall higher productivity in comparison to a mesophilic AD process. However, thermophilic AD may cause acidification, a decrease in process stability, high toxicity, poor methanogenesis, and a high energy input. Mesophilic AD has better stability and higher richness in bacteria but also lower methane yield, poor biodegradability and nutrient imbalance (Mao, Feng, Wang, & Ren, 2015). Psychrophilic digesters were popular during the 1980s when biogas was used for heating, but they were soon replaced by mesophilic digesters, since no anaerobic psychrophilic bacteria were found below 20℃ (Kothari, Pandey, Kumar, Tyagi, & Tyagi, 2014).

Ideally, the optimal AD process would involve thermophilic AD during the hydrolysis and acidogenesis steps and mesophilic AD during methanogenesis, similar to a two-phase AD process (Mao, Feng, Wang, & Ren, 2015). Although this "combined temperature" process would provide the best methane yield, it is not economically favorable due to heating requirements.

2.2.3 C/N Ratio

The nutrient level of the digestion substrate, C/N ratio, is another important factor of anaerobic digestion that needs to be controlled. The ratio represents the relationship between carbon and nitrogen in organic matter (Kothari, Pandey, Kumar, Tyagi, & Tyagi, 2014). A higher

C/N ratio helps to avoid ammonia inhibition because it leads to low total ammonia nitrogen (TAN) and free ammonia (FA) concentrations in the system, while a low C/N ratio increases the risk of ammonia inhibition. However, this ratio cannot be too high, since that would result in nitrogen degradation, which would decrease the biogas yield. The optimal C/N ratio for AD has been found to be between 20 and 35 (Mao, Feng, Wang, & Ren, 2015).

2.2.4 Retention Time

The retention time is another parameter that needs to be regulated during the anaerobic digestion process. This period of time is the time required to complete the degradation of organic matter, and greatly depends on the temperature, substrate composition and amount of volatile solids continuously fed into the digester daily (OLR). The time required can be calculated as

$$RT = \frac{V}{Q} \quad (1)$$

where V is the reactor volume and Q is the flow rate (Mao, Feng, Wang, & Ren, 2015). An average RT for mesophilic AD is between 10-40 days, but only around 14 days during thermophilic AD in a dry process (Kothari, Pandey, Kumar, Tyagi, & Tyagi, 2014). Additionally, the RT is directly proportional to the degradation rate, and the optimal methane yield conditions have be found to be a combination of a low ORL with a high RT (Mao, Feng, Wang, & Ren, 2015).

2.3 Inhibiting Compounds

Throughout the digestion process, chemicals are consumed and produced, some are added to enhance the process, and some act as inhibitory compounds. While inhibitory

compounds are undesired, they do prove helpful when present in the proper quantities. Some elements are antagonistic to one another and others show synergistic effects. The key to methane production is finding a balance and maintaining it throughout digestion.

2.3.1 Ammonia

Ammonia can be found as the ammonium ion (NH4+) or free ammonia (NH3) during anaerobic digestion and is attributed with being one of the main reasons for inhibition. While anaerobic microorganisms feed on the nitrogen from ammonia, high levels of free ammonia are seen as inhibitors of anaerobic digestion. Overall, an ammonia level below 200 mg/L has been labelled as favorable for digestion (Charnier et al., 2017). However, inhibitory levels of total ammonia nitrogen have been cited from 1.7 to 14 g/L resulting in at least 50% less methane produced than peak conditions (Chen, Cheng, & Creamer, 2008). Hydrophobic free ammonia is toxic because it can diffuse through cell membranes causing imbalances that prevent cell growth. As pH increases within a reactor, the total concentration of ammonia shifts towards NH_3 from NH^{4+}, acting as a toxic agent. Reducing the temperature, from a thermophilic to mesophilic state, decreases the effect of ammonia inhibition. The addition of other ions, such as Na^{+}, Ca^{2+}, and Mg^{2+}, was found to be combative against ammonia inhibition during digestion up to 50 g/L of $NH^{4+}Cl$ (Chen, Cheng, & Creamer, 2008).

2.3.2 Volatile Fatty Acids

Volatile fatty acids (VFA) are mainly comprised of acetic acid, propionic acid and butanoic acid but also include methanoic and pentanoic acid. As early products in AD that will be converted to methane, they play a large role in the efficiency of AD (Geng et al., 2016). With

a buildup of ammonia inhibiting digestion, VFAs will also accumulate. However, this development will lead to a decrease in pH and free ammonia. The resulting oscillation produces an "inhibited steady state" where the process is producing small amounts of methane at a stable rate (Chen, Cheng, & Creamer, 2008). Of the three acids, acetic acid is most easily digested by methanogens to produce methane and carbon dioxide. As digestion begins, acetic acid is the most abundant component until a peak in VFA production is reached. At this point, propionic acid begins to increase in abundance and butanoic and acetic acid decreases gradually upon conversion (Geng et al., 2016). Levels of propionic acid were successfully reduced by using a Cao-ultrasonic pretreatment with acidification of waste activated sludge and additional seed sludge (grease). As a result, methane production increased by 69% (Geng et al., 2016).

2.3.3 Light Metal Ions

The presence of light metal ions in a reactor can be beneficial or toxic to the digestion process depending on the levels present. Aluminum, sodium, potassium, magnesium and calcium can be introduced to the system to adjust the pH or may occur naturally as organic matter is digested. While there are optimum levels of each ion, it has also been documented that toxicity of one ion is decreased by the presence of other ions when present in moderate quantities (Chen, Cheng, & Creamer, 2008).

Acetogenic and methanogenic microbes are inhibited by high levels of aluminum ions. Aluminum must compete with iron and manganese but also adheres itself to cell membranes or walls (Chen, Cheng, & Creamer, 2008). It was found that 1000 mg/L could decrease

methanogen's productivity by 50% and acetogen's by 72% (Cabirol, Barragan, Duran, & Noyola, 2003).

Sodium has been recorded as more toxic to microorganisms that feed on propionic acid than those that utilize acetic acid. However, acclimation of methanogens to high levels of sodium can shorten the lag phase of methane production (Chen, Cheng, & Creamer, 2008). Sodium is highly necessary for ATP and NADH formation. These molecules are intermediates in the multiple reactions used to produce methane and carbon dioxide (Dimroth & Thomer, 1989). An optimum level of sodium for methane production is dependent on the type of methanogenic microorganisms present, the temperature of the reactor and the amount of other ions present. Generally, an overall range of 100-350 mg Na+/L is best (Chen, Cheng, & Creamer, 2008).

Potassium ions are also a highly valuable ion to maintain within the digester fluid because they can remove metals that have attached to exchangeable sites in the digestive matter. In literature, low concentrations of potassium have been shown to significantly improve methane production for both mesophilic and thermophilic reactor conditions. Inhibition from large concentrations of potassium were evident under thermophilic conditions and appeared to affect mostly acetogenic microbes (Chen, Cheng, & Creamer, 2008).

High concentrations of magnesium have been documented as provoking single cell growth and an optimum level of 720 mg/L was reported for anaerobic bacteria (Chen, Cheng, & Creamer, 2008).

Calcium ions would optimally be around 200 mg/L as they are essential for many strains of methanogens. Too much could result in precipitation of carbonate and phosphate, a reduction in methanogenesis and a loss in buffer capacity (Chen, Cheng, & Creamer, 2008). However,

some literature calls out that concentrations up to 7000 mg/L had no inhibitory effects on methane production (Jackson-Moss, Duncan, & Cooper, 1989). These conditions would be dependent on the contents of the digester.

2.3.4 Heavy Metals

Heavy metals pose a significant risk to anaerobic digestion because they are non-biodegradable and highly toxic upon accumulation resulting in enzyme dysfunction and structure disruption. The metals of concern are iron, nickel, copper, zinc, and lead but only in their free ionic form. Iron is seen as least toxic for acetogenic and methanogenic microorganisms followed by lead, nickel, zinc and copper. In combination, these ions can have antagonistic or synergistic effects on the reactor system depending on which ions are present and in what concentrations (Chen, Cheng, & Creamer, 2008). Toxicity of these heavy metal ions can be counteracted by activated carbon, kaolin, bentonite, diatomite, other waste materials and sulfide (Ulamnu et al., 2003).

2.3.5 Sulfide

While sulfide can counteract heavy metal inhibition, it is also quite problematic for an AD system. One reason is that sulfide-reducing bacteria (SRB) compete with the microorganisms trying to carry out digestion for nutrients. Hydrolytic and acidogenic microbes do not typically compete with SRB because SRB do not digest sugars. They prefer to wait for the degradation products of the first two steps of AD. Acetogens and methanogens must compete with SRB which kinetically and thermodynamically has a better chance of utilizing the degradation products (Chen, Cheng, & Creamer, 2008). Under mesophilic conditions, SRB

dominated digestion but under thermophilic temperatures, methanogens consumed the nutrients (Colleran & Pender, 2002). Sulfide is toxic to both SRB and methanogens making it critical to keep levels under control. By diluting the wastewater or removing sulfide during digestion and maintaining a pH around 7.2, the toxicity of sulfide can be controlled (Chen, Cheng, & Creamer, 2008).

2.3.6 Organic Compounds

With a buildup of organic chemicals on cell membranes, the cell will eventually swell or leak from ion gradient disruption leading to cell lysis. While small levels of organic content can prevent inhibition, excess amounts of organics will cause AD inhibition. Younger cultures are more resistant to higher levels of organic matter but microorganisms can be acclimated to tolerate larger amounts. Halogenated aliphatics, lignins and long chain fatty acids are especially inhibitory to methanogenic microorganisms (Chen, Cheng, & Creamer, 2008).

2.4 Types of Waste

Various types of waste can be used for anaerobic digestion, and the different parameters associated with the process might vary depending on the chosen substrate. The most common waste types are characterized below, but this project focused on agricultural waste materials to produce biogas.

Municipal waste is one of the most abundant types of waste. Since more materials are being recycled every day, biowaste has presented a higher organic composition and a lower biotoxic composition (Chen, Cheng, & Creamer, 2008). However, the main problem associated

with it is ammonia inhibition. In this case, the dilution of the digester and the adjustment of the C/N ratio of the feedstock have successfully reduced ammonia inhibition (Kayhanian, 1999). Another challenge with this type of waste includes sludge production, since heavy metals tend to accumulate in it to potentially toxic concentrations, causing a reduction in gas production and methane content in biogas, as well as the removal of volatile suspended solids and chemical oxygen demand (COD) (Chen, Cheng, & Creamer, 2008).

Another common type is industrial waste, which include both food, paper and pulp, textile and petrochemical refinery industries. The food industry produces ideal waste for anaerobic digestion, since it is very rich in organic matter (Chen, Cheng, & Creamer, 2008). However, AD might be hindered due to different cations and anions that might be present, such as Na^+, Cl^- and SO_4^{2-} (Feijoo, Soto, Méndez, & Lema, 1995). Another challenge that might be faced when using food industry waste for anaerobic digestion, is the fact that the digestion of protein and lipids leads to the accumulation of ammonia and long chain fatty acids, which are strong inhibiting factors, but these challenges might be overcome by co-digestion (Chen, Cheng, & Creamer, 2008).

The waste produced by the paper and pulp industry is favorable for anaerobic digestion because it contains a high COD concentration in the effluents. On the other hand, this industry's waste has various common inhibitors, such as sulfide, halogenated compounds and long chain fatty acids, resulted from the pulping operations. Similarly, the textile industry does not produce waste that is very suitable for AD. The industrial waste produced from textiles usually contains dyes, surfactants and heavy metals, which create a great chemical complexity and can easily inhibit the anaerobic digestion process. However, a very promising industrial waste type comes

from petrochemical refineries. Aldehydes, esters, alcohols and acids have been found useful for methane production after prolonged acclimation. Not only would this result in large energy savings over AD processes, but methane would also be produced on a scale for use as fuel (Chen, Cheng, & Creamer, 2008).

As mentioned, this study focused on using agricultural waste. This type of waste refers to animal waste that comes from a farm, including livestock and poultry, wastewater, manure, slurry and feed. It often has a high total ammonia nitrogen concentration, and hence the biggest issue associated with it is ammonia inhibition. Additionally, swine manure has a high sulfate concentration, and the inhibition caused by both ammonia and sulfide influence each other (Chen, Cheng, & Creamer, 2008). Another important factor is the use of antibiotics and chemotherapeutics as feed additives, which may be highly inhibitory, even at 1 mg/L, and require pretreatments of the material before digestion (Varel & Hashimoto, 1981). A low biogas production could also be due to a high C/N ratio, high lignin content from crop residues or pesticide and herbicide residues. Acid or base hydrolysis are usually applied as pretreatment, but the byproducts from these reactions are also potential inhibitors and might affect process kinetics (Chen, Cheng, & Creamer, 2008).

2.5 Reactor Designs

The are many different types of reactors that can be used for anaerobic digestion. Highlighted below are the most commonly used reactors, including the ones that have been reported to yield the best biogas production results.

2.5.1 Conventional Anaerobic Reactors

There are three main types of conventional anaerobic reactors: an anaerobic sequencing batch reactor (ASBR), a continuous stirred tank reactor (CSTR) and an anaerobic plug flow reactor (APFR).

An anaerobic sequencing batch reactor consists of a single tank, fill-and-draw unit. Both the treatment steps and fermentation of the process occur inside the single tank. An ASBR is good for low-flow applications, as it poses high process control and efficiency. However, it can also be designed based on the range of influent volumes. Among its main advantages are efficient quality control of the effluent, flexibility, low requirements, cost-effectiveness and high biogas yields. On the other hand, however, this type of reactor poses insufficient settle-ability, channeling and clogging. Furthermore, an ASBR would need agitation to improve biomass retention, and additional scientific studies are required to improve its performance (Mao, Feng, Wang, & Ren, 2015).

Differently from an ASBR, a continuous stirred tank reactor is recognized for its reliability, high-rate and ability to treat wastewater with high levels of suspended solids. This type of reactor consists of suspended microorganisms that are intermittently and continuously mixed. However, this requires a lot of energy and labor. A two-phase CSTR is common during wet continuous digestion processes due to its simplicity and low costs, but the system's sensitivity leads to fewer alternatives for improvement in addition to the reactor's incapability of retaining a high concentration of microorganisms in the reactor. Also due to the constant stirring, volatile fatty acids are produced, which could lead to process inhibition. The latest studies have

shown that CSTRs could be combined either in series with a sedimentation tank or with a membrane bioreactor to improve its microorganism concentration (Mao, Feng, Wang, & Ren, 2015).

Another type of conventional process reactor is an anaerobic plug flow reactor (APFR). They are attractive due to their low investment cost, high efficiency and good bioconversion, which result in low concentrations of VFA in the effluent, sludge retention and stable performance. They consist of a single-phase reactor that has no internal agitation and can be loaded with thick solids, either at mesophilic or thermophilic conditions. However, in most cases, additional equipment is needed, such as storage and digestive tanks, homogenization and feeding systems, and cogeneration units, which may increase the overall process cost (Mao, Feng, Wang, & Ren, 2015).

2.5.2 Sludge Retention Reactors

One of the most successful sludge retention reactors is the internal circulation reactor (IC), which consists of two sets of 3-phase separation modules. This type of reactor is characterized by the ability to separate gas, liquid and biomass at the same time, which improves biomass retention and improves the quality of the final effluent. Its special features include the separation of biomass in two different stages and an internal effluent circulation, beside its lower cost and higher efficiency. Additionally, these reactors can treat low-strength wastewaters at higher HRT, and have proved successful in a variety of industries (Mao, Feng, Wang, & Ren, 2015).

2.5.3 Anaerobic Membrane Reactors

Two of the most widely used membrane reactors are the anaerobic fluidized bed reactor (AFBR) and the expanded granular sludge blanket (EGSB). The AFBR uses an inert medium, such as sand or alumina, for bacterial attachment and growth. This medium is kept in suspension by a strong upward flow of wastewater, which improves mass transfer efficiency and resistance to inhibiting factors, resulting in an higher overall process efficiency. Additionally, this set-up has a much lower cost and eliminates the possibility of clogging. On the other hand, membrane fouling is a common problem, especially when treating a protein-rich substrate. One solution to this problem would be to add some solid media, such as powder or granular activated carbon (Mao, Feng, Wang, & Ren, 2015).

The expanded granular sludge blanket is another preferred and successful process. It is generally used by small and medium-sized industries when the volumetric gas production rate is low. Among its various advantages are a smaller footprint, higher mixing due to higher upflow velocity and better mass transfer, biomass activity and transport of substrate into sludge aggregates. These advantages make this process more suitable for soluble pollutant treatments. However, a common problem is that suspended solids cannot be substantially removed (Mao, Feng, Wang, & Ren, 2015).

Among all the reactors mentioned above, the EGSB and IC are considered the most advanced and efficient. They both show higher loading capability, resistance to impact, up-flow velocity and attachment between the sludge and biomass. They also separate the gas, liquid and

biomass simultaneously in a 3-phase separator. However, due to its advanced technology, these reactors usually have a high investment and maintenance cost (Mao, Feng, Wang, & Ren, 2015).

2.6 Microalgae Bacteria

One way of enhancing AD is through the addition of microalgae bacteria. Results from prior experiments show that methane yield can be improved anywhere from 25-96% (Mao, Feng, Wang, & Ren, 2015). Microalgae bacteria prefer balanced ratios of carbon/nitrogen/phosphorus for growth but provide enhanced removal rates of these elements in reactors. When subject to alternating dark and light periods of time, the dissolved oxygen and pH decreases in the absence of light and increases under light exposure (Van Den Hende et al., 2014).

The bacteria used for anaerobic digestion of agricultural wastes is primarily from the species *Rhizobium* and the algae falls under species *Chlamydomonas* and *Scenedesmus*. Studies have shown that by mixing microalgae bacteria and agriculture waste, they interact in a manner that produces more methane than the sum produced by algae and bacteria separately (Wirth et al., 2015).

3 Methodology

Batch reactors consisted of synthetic wastewater, microalgal bacterial flocs (MaB-flocs), and agricultural digestate. Contents were subject to characterization testing beforehand and during the digestion process.

3.1 Synthetic Wastewater

To prepare the first round of batch reactor samples, two types of synthetic wastewater were mixed according to the compositions outlined in Table 1. The chemicals mixed in Table 1 and Table 2 were balanced to 1 liter with deionized water. The pH was then adjusted using sodium bicarbonate to be between 7.0 and 8.3.

Table 1 - Synthetic Wastewater Composition

Overall Concentration	450 mg/L	600 mg/L
Chemical	**Concentration (mg/L)**	
Glucose	223.8	298.4
Sodium Acetate	452.44	603.26
KH2PO4	10	10
NH4Cl	100	100
CaCl2	10	10
MgSO4 - 7H2O	5	5
FeSO4 - 7H2O	5	5
Nutritive Solution	1 mL	1 mL

Table 2 - Nutritive Solution Composition

Chemical	**Concentration (mg/L)**	
H3BO3	50	50
ZnCl2	50	50
CuCl2	30	30
MnSO4 - H2O	50	50
(NH4)6Mo7O24 - 4H2O	50	50
AlCl3	50	50
CoCl2 - 6H2O	50	50
NiCl2	50	50

3.2 Microalgal Bacterial Flocs (MaB-flocs)

Mab-flocs were pre-cultured in batch reactors with each type of synthetic wastewater, 450 mg/L and 600 mg/L. To obtain 1 L vessels, 800 mL of wastewater was mixed with 200 mL MaB-flocs taken from previous batch reactors. Each 1 L mixture was separated in two 500mL Erlenmeyer flasks. Flasks were placed in a 30℃ oven and subject to 16 hours of light followed by 8 hours of darkness with cycles of stirring. After 2.5 days, the like mixtures were combined in separate 2 L Erlenmeyer flasks and additional wastewater was added to obtain a working volume of 1.5 L. These flasks were subject to 16 hours in light, 8 hours of darkness and continuous stirring over a 6-7 day period.

3.3 Agricultural Digestate

The digestate used in this study is a mix of agricultural wastes including manure, corn silage, and grass. These ingredients are continuously collected at a nearby farm where they are first decomposed in a 400 m^3 tank for 1-2 months. Once decomposition in completed, contents are transferred to a 1000 m^3 post-digestion tank, and this product can be used as fertilizer for crops for up to six months. Batch reactors in this study utilize digestate from the first tank.

3.4 Batch Reactors

The first set of batch reactors consisted of four 250 mL jars. Each set of jars utilised one type of synthetic wastewater. One jar in each set contained 50 mL of MaB-flocs, 110 mL of synthetic wastewater, and 20 g of digestate. The other jar comprised of 50 mL of MaB-flocs and 150 mL of synthetic wastewater. These jars were held at 30℃ continuously stirred at 200 rpm for 8 days. A timer was set up to allow 16 hours of light and 8 hours of darkness. Measurements were taken at the start of the experiment and after 8 days.

Figure 1: All four 1-L reactors

A second batch of reactors was created in 1 L reactors (Figure 1). These reactors were made in a similar fashion as the first batch. Each pair used one type of wastewater and the contents of its corresponding first reactor. One pair was comprised of the first batch liquid (with digestate), another 100 g of digestate and 500 mL of fresh wastewater. The other pair had the first batch liquid (without digestate) and 600 mL of fresh wastewater. These reactors were not stirred during digestion but were subject to the same dark/light cycle as the first batch of reactors. Bottles were agitated by hand before any measurements were completed.

3.5 Characterization Testing

Various techniques were applied during this study in order to determine any variation of the organic matter before and after digestion. These techniques include total suspended solids (TSS) and volatile suspended solids (VSS) measurements, chlorophyll-a, ammonia and phosphorus contents, as well as gas chromatography analyses for a better product characterization. It was important to measure TSS and VSS because it provides information about the organic matter in the samples and how that is affected by the digestion process. Similarly, it was important to know the amount of ammonia and phosphorus present in the reaction because these compounds are strong inhibiting factors. Additionally, the chlorophyll-a content indicates any changes in the amount of algae present in the reactor after digestion. Lastly, gas chromatography provided more insight on the amount of methane and carbon dioxide produced during the process.

3.5.1 Total Suspended Solids (TSS)/ Volatile Suspended Solids (VSS)

Total suspended solids were measured prior to creating the reaction mixture and after the reaction was given time to proceed. To prepare, liquid samples of reaction ingredients, and later the reaction vessel, had to be centrifuged to separate solids from the liquid. Solid particles were then placed in crucibles and dried overnight in a 100℃ oven to obtain TSS measurements. After TSS measurements were completed, samples were placed in another oven at 550℃ for two hours so that volatile suspended solids could be measured. TSS and VSS were calculated using the following equations:

$$TSS = \frac{M1 - M0}{Vs} * 1000 \text{ and } VSS = \frac{(M1 - Mo) - (M2 - M0)}{Vs} * 1000 \qquad (2 \& 3)$$

Here, M1 stands for the mass of the crucible in addition to liquid sample dried at 105℃ for 24 hours, M0 stands for the mass of the empty crucible, M2 stands for the mass of the crucible in addition to liquid sample burned at 550℃ for 2 hours, and Vs stands for the sample volume.

3.5.2 Chlorophyll-a

To measure chlorophyll-a content of samples, 5 mL of reaction mixture were centrifuged at 4000 rpm for 20 minutes. The liquid was poured off the top of the sample leaving just the solid matter. 5 mL of methanol was added and the tube was agitated. The test tubes were placed in a water bath on a hot plate at 110℃ for 15-20 minutes. The liquid was then measured in a spectrophotometer (UV-Vis 2550 Anthelie Light, Shimadzu, Japan) at wavelengths of 652 and

665 nanometers. These readings were converted to chlorophyll-a content using the following equation:

$$Chl(a) = 16.29*A_{665} - 8.54*A_{652} \quad (4)$$

3.5.2 Ammonia and Phosphorus

In order to measure the amount of ammonia present in the solutions, 10 mL of each mixture were poured into separate vials, where two drops of polyvinyl alcohol, two drops of a mineral stabilizer and 400 µL of Nessler reactive were added to form the final solution. A light-yellow color was desired for each vial; if the tone was a warmer, orange color, the mixture was too concentrated and additional dilution was necessary.

Once the mixtures were ready, the ammonia content was measured using the "Nessler Method" in the Hach DR 5000. In the machine, ƛ was set to 425 nanometers, and four measurements were taken for each sample, rotating the vials by 90° between each data recording. By averaging all the results obtained for a single sample, the ammonia content was calculated using the following equation,

$$[NH3]\,(mg/L) = Avg\ Result * 3.446 * Dilution\ Factor \quad (5)$$

where the dilution factor was omitted in the cases that no dilution was necessary.

Similarly, the phosphorus concentration was measured by preparing two samples for each solution: one to serve as the control, and the other to serve as the reactive. 10 mL of each solution were poured into separate vials, and two drops of the reactant were added to the non-control test-tubes, where the reactive mix was finalized.

Once the solutions had been prepared, the Hach DR 5000 machine was used to measure the phosphorus concentration. Again, four measurements were done for each vial, rotating the tube by 90° between each recording, and average of the results was calculated. Finally, the concentration was determined by

$$[PO4]\,(mg/L) = Avg\ Result * Dilution\ Factor \tag{6}$$

and the dilution factor was once again omitted if no dilution was previously done to the solution.

3.5.3 Gas Chromatography

Gas chromatography (GC) was used to identify how much methane gas was being produced during the digestion. After various lengths of digestion, 1 mL samples of gas were drawn from the different mixtures and analyzed in the GC apparatus (Varian 430-GC, Varian, Inc., Palo Alto, California) for 7 minutes. The temperatures used in the chosen method were: 30℃ for the column oven, 50℃ for the injector (type 1041) and 180℃ for the detector (type TCD). The amount of methane produced was expected to be directly proportional to the amount of microalgae bacteria present in the solutions, since the bacteria was being used to enhance the methane production.

4 Results & Discussion

To better discuss the results and avoid confusion, reactors from the first batch will be referred to as A1,1, A2,1, B1,1 and B2,1. Similarly, reactors from the second batch of experiments will be referred to as A1,2, A2,2, B1,2 and B2,2. This notation identifies the type of reactor first, followed by its the batch number.

When comparing the same type of reactor in both batches (e.g., A1,1 and A1,2), it is possible to see from Table 3 and Table 4 that TSS and VSS results are higher for the first batch for all reactor types. This difference in total suspended solids and volatile suspended solids could be attributed to the difference in concentration in the reactors. Batch 1 is composed of 200mL reactors, while Batch 2 is composed of 1L reactors including a higher amount of wastewater in Batch 2. When calculating the wastewater (WW) to flocs (F) to digestate (D) ratio, it was found that Batch 1 contained 2.2WW:1F:0.4D, while Batch 2 had 12.2WW:1F:2.4D, resulting in a much less concentrated solution for Batch 2, and hence a lower overall organic matter content, which is represented by lower TSS and VSS results.

Table 3 - TSS, VSS, Chlorophyll, CO_2 and CH_4 Results for Small Reactors (200 mL) After 7 Days

Batch 1	TSS (g/L)	VSS (g/L)	CO_2 (mL)	Average CO_2/L of Mixture (mL/L)	CH_4 (mL)	Average CH_4/L of Mixture (mL/L)	Chl(a) (mg/L)
A1,1 (WW,450 +F+D)	5.483	4.115	0.399	2.215	1.288	7.156	620.55
A2,1 (WW,450 +F)	0.390	0.375	-	-	0.923	3.972	302.74
					0.666		
B1,1(WW ,600+F+ D)	5.422	4.023	-	9.050	0.070	10.680	554.17
			0.098		0.156		
			2.700		3.843		
			2.089		3.620		
B2,1 (WW,600 +F)	0.287	0.310	-	-	0.253	15.850	257.80
					6.087		

By focusing on the amount of CO_2 produced during the digestion process, it is possible to see that reactors A2,1 B2,1, A2,2 and B2,2 do not have a detectable amount of CO_2 left after digestion. The algae present in these samples consume CO_2 to produce CH_4 and, since these reactors do not contain digestate, no other carbon source is available for consumption. When comparing reactor A1,1 to A1,2, it is possible to see that the reactor from Batch 2 produces more carbon than the other with levels of 0.399 and 3.613 mL CO_2/L mixture, respectively. This can be explained by the greater amount of digestate present in the sample: reactor A1,1 contains 11% by volume of digestate, while A1,2 contains 15% by volume, but both reactors contain the same

amount of microalgal flocs. On the other hand, reactor B1,1 produces 9.05 mL CO_2/L mixture which is more CO_2 than B1,2 that only produced 2.741 mL CO_2/L mixture on average. Similarly to A1,1 and A1,2, the digestate concentration is higher in B1,2, but the opposite effect can be attributed to the more concentrated (600 mg/L) wastewater present in B1,2. The more concentrated wastewater provides more nutrients to the digestion process, which aids the bacteria and microalgae flocs in converting CO_2 to CH_4.

Methane production per liter of reaction was higher for all reactors from Batch 1 when compared to Batch 2. Although reactors A1,2 and B1,2 have a higher organic matter content than A1,1 and B1,1, their lower CH_4 production could be attributed to a need for more microalgal flocs in the mixture. Respectively, these reactors produced 1.839, 1.237, 7.156 and 10.680 mL of CH_4/L of mixture based on averages of several gas chromatography runs. As mentioned above, the component ratio in Batch 1 is 2.2WW:1F:0.4D and 12.2WW:1F:2.4D in Batch 2. This means that there is a lot more digestate to be digested, and hence CO_2 to be consumed in Batch 2, but the same amount of flocs is present in both batches, so the methane production could have been hindered by the level of flocs in the reactors. Similarly, reactors A2,2 and B2,2 produced less CH_4 than A2,1 and B2,1 with 1.413, 2.571, 3.972 and 15.850 mL CH_4/L mixture respectively. Although these reactors do not contain any digestate, the difference in methane production could also be attributed to the greater amount of wastewater present in the reactors from Batch 2. When comparing reactors from the same batch, B2,1 produced 15.850 mg CH_4/L and B2,2 produced 2.571 mg CH_4/L. This is contradictory to the expected results of the type A reactors where digestate enhanced methane production. The outcome of B2,1 and B2,2 is most likely linked to the higher wastewater concentration and the amount of nutrients it provides to the bacteria and

microalgae in the digestion process. Since there was no digestate for the flocs to consume, the only available source of carbon would come from CO_2.

Table 4 - TSS, VSS, Chlorophyll, CO_2 and CH_4 Results for Large Reactors (1 L) After 7 Days

Batch 2	TSS (g/L)	VSS (g/L)	CO_2 (mL)	Average CO_2/L of Mixture (mL/L)	CH_4 (mL)	Average CH_4/L of Mixture (mL/L)	Chl(a) (mg/L)
A1,2 (WW,450 +F+D)	2.652	2.170	1.390	3.613	0.829	1.839	409.08
			4.246		2.040		
A2,2 (WW,450 +F)	0.315	0.265	-	-	1.393	1.413	438.92
					0.867		
B1,2 (WW,600 +F+D)	3.728	3.090	1.698	2.741	0.886	1.237	453.39
			2.578		1.044		
B2,2 (WW,600 +F)	0.228	0.205	-	-	2.455	2.571	642.48
					1.658		

Chlorophyll-a content results also presented some discrepancies. Reactors A1,1 and B1,1 had 620.55 mg Chl(a)/L and 554.17 mg Chl(a)/L, a higher content than A1,2 and B1,2 having 409.08 mg Chl(a)/L and 453.39 mg Chl(a)/L. While methane production and chlorophyll-a are related, the process of producing biogas involves not only green algae from the microbacterial flocs, but also green and brown bacteria. Chlorophyll-a measurements only measure the amount of green algae in the sample. They do not give a measurement of the total biomass in the reactor,

which contains both brown and green bacteria and algae. Since Batch 2 reactors contained more wastewater than Batch 1 reactors with a constant amount of flocs, the lower Chl(a) content of A1,2 and B1,2 could be associated to the lower flocs concentration in the reactor. On the other hand, reactors A2,2 and B2,2 presented a higher Chl(a) content than A2,1 and B2,1. Respectively, each reactor had 438.92, 642.48, 302.74, 257.80 mg Chl(a)/L. Since Chl(a) is based on the amount of green algae in the reactor, this growth would most likely be due to oxygen left in the reactor that algae uses to multiply. Although the 1-L reactors were sealed, it was not ensured that oxygen gas was absent from inside the bottles. An absence of carbon dioxide would also allude to more methane production, but since this is not the case, it is plausible that the algae and bacteria used the carbon dioxide and nutrients in the wastewater to multiply instead of produce more methane gas.

Table 5 - Amount of Ammonia in Large Reactors (1L) Before and After Digestion

Reactor	**A1,2**	**A2,2**	**B1,2**	**B2,2**
Before Digestion (mg/L)	127.74	100.05	127.74	100.05
After Digestion (mg/L)	453.41	27.74	479.6	14.30

Ammonia content was measured before and after the second batch of reactors was allowed to digest. The before digestion calculation is a combination of the ammonia added to the wastewater and the ammonia measured in the decoction of pure digestate. During the digestion process, consumption of digestate produces ammonia and it can be seen that the ammonia level

in reactors A1,2 and A2,2 increased significantly after one week from 127.74 mg/L to 453.41 and 479.6 mg/L, respectively. The bacteria in the reactors without digestate, A2,2 and B2,2, utilized the ammonia present in the wastewater alone to produce methane, hence the decrease in ammonia content in those reactors, from 100.05 mg/L to 27.74 mg/L and 14.30 mg/L, respectively.

This decrease in ammonia could also be attributed to the growth of bacteria in the reactors. The bacteria feed on the nitrogen component of ammonia for nucleic acid synthesis to multiply. Ammonia is a strong inhibitory component and, even though the levels of ammonia are smaller than those labelled inhibitory in scientific literature, there could be synergistic effects between ammonia and other organic compounds within the wastewater or digestate that amplify inhibition. The addition of bacterial flocs was meant to eliminate nutrients, like ammonia, during anaerobic digestion. Measurement of this component was only conducted once methane production was no longer desired because it was necessary to remove the reactor bottle caps.

Table 6 - Methane Production at days 3 and 7 of Large Reactors (1L)

Batch 2	**Avg. Methane Produced after 3 days (mL)**	**Avg. Methane Produced after 7 days (mL)**	**Percent Difference (%)**
A1,2 (WW,450+F+D)	3.96	1.43	63.88
A2,2 (WW,450+F)	2.71	1.13	58.30
B1,2 (WW,600+F+D)	2.68	0.97	63.81
B2,2 (WW,600+F)	3.19	2.06	35.42

During anaerobic digestion of Batch 2, gas chromatography measurements were carried out on the fourth and eighth days of digestion. While some of the gas was removed for gas chromatography measurements on the fourth day, this does not explain the large decrease in methane observed in the reactors on the eighth day. In Table 6, methane levels decrease by more than 50% for samples A1,2, A2,2, and B1,2 while sample B2,2 sees a much smaller percent difference of about 35%. This significant difference could be due to the ratio of wastewater flocs and digestate used. In both batches, the average methane produced per liter of mixture was highest with 600 mg/L wastewater and no digestate, which can be seen in B2,2, since it had the highest chlorophyll(a) content, smallest TSS and VSS, and produced the most methane.

5 Conclusions & Recommendations

This preliminary study of anaerobic digestion enhanced with microalgae bacteria flocs has proven that methane production is possible at the given formulations but that it is a complex process with many parameters that require attention before ideal conditions can be determined. For further analysis and improvements to be made, the experiments should be repeated to see if a similar result is produced. B2 reactors producing the most amount of methane may be an anomaly. If it is not, many more experiments will need to be run to determine the cause for its high methane yield without the presence of digestate. To decrease the amount of carbon dioxide in the reactors with digestate and hopefully increase methane production, higher levels of bacterial flocs should be added. Reactors in each batch should also have one specific total volume or amount of wastewater added to allow for better comparison between bottles and batches. Methane measurements should also be carried out each day to monitor when the methane in each reactor begins to decrease and when the carbon dioxide level increases.

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Appendix A

Ammonia Data

Wastewater Measurements

Reading	WW 450 mg/L	WW 600 mg/L
1	0.229	0.172
2	0.229	0.167
3	0.228	0.172
4	0.225	0.167
Average	0.228	0.170
[NH3], 450 (mg/L)	39.241	
[NH3], 600 (mg/L)	29.205	

After 7 days Digestion - Batch Reactors (Scale Up, 1L)

Reading	A1 (WW,450+F+D)	A2 (WW,450+F)	B1 (WW,600+F+D)	B2 (WW,600+F)
1	1.323	0.082	1.393	0.038
2	1.311	0.067	1.388	0.051
3	1.313	0.068	1.394	0.039
4	1.316	0.105	1.392	0.038
Average	1.316	0.081	1.392	0.042
[NH3] (mg/L)	453.407	27.740	479.597	14.301
Dilution Factor	100			

Phosphorus Data

Wastewater Measurements

Reading	WW 450 mg/L	WW 600 mg/L
1	0.04	0.13
2	0.03	0.13
3	0.04	0.09
4	0.04	0.12
5	-	0.13
Average	0.04	0.12
[PO4], 450 (mg/L)	1.88	
[PO4], 600 (mg/L)	6.00	

After 7 days Digestion - Batch Reactors (Scale Up, 1L)

Reading	A1 (WW,450+F+D)	A2 (WW,450+F)	B1 (WW,600+F+D)	B2 (WW,600+F)
1	2.41	0.1	2.59	0.08
2	2.39	0.08	2.57	0.09
3	2.42	0.09	2.56	0.08
4	2.41	0.07	2.59	0.08
Average	2.41	0.09	2.58	0.08
[PO4 (mg/L)	240.75	8.50	257.75	8.25
Dilution Factor	100			

Gas Chromatography

Small Reactor, 200mL							
After 7 Days Digestion *Continuous stirring							
Mixture	**CH4 (area)**	**CO2 (area)**	**H2O (area)**	**Comments**	**CO2 (mL)**	**CH4 (mL)**	**Average**
A1	**25296**	**8103**	**3201**	**-**	**0.399**	**1.288**	**1.288**
A2	**18079**	**-**	**-**	**-**	**-**	**0.923**	**0.794**
A2001	**13011**	**-**	**-**	**-**	**-**	**0.666**	
B1	**1243**	**-**	**-**	**Stopped before the end**	**-**	**0.070**	**0.113**
B1001	**2931**	**1086**	**-**		**0.098**	**0.156**	
B1002	**75763**	**61762**	**9893**		**2.700**	**3.843**	**3.732**
B1003	**71356**	**47521**	**6663**		**2.089**	**3.620**	
B2	**-**	**-**	**-**	**Stopped before the end**	**-**	**-**	**3.170**
B2001	**-**	**-**	**-**		**-**	**-**	
B2002	**4851**	**-**	**-**		**-**	**0.253**	
B2003	**120074**	**-**	**1580**		**-**	**6.087**	

Batch Reactor, Scale-up 1L							
After 3 Days Digestion *No continuous stirring							
Mixture	**CH4 (area)**	**CO2 (area)**	**H2O (area)**	**Comments**	**CO2 (mL)**	**CH4 (mL)**	**Average**
A1	**97503**	**42822**	**4201**	**CH4 and CO2 peaks were combined**	**1.888**	**4.944**	**3.963**
A1,2	**58755**	**17057**	**4636**	**CH4 and CO2 peaks were combined**	**0.783**	**2.982**	
A2	**34509**	**-**	**1334**	**-**	**-**	**1.755**	**2.712**
A2,2	**72312**	**-**	**1295**	**-**	**-**	**3.669**	
B1	**61725**	**24773**	**15004**	**CH4 and CO2 peaks were slightly combined**	**1.114**	**3.133**	**2.681**
B1,2	**43903**	**45995**	**8358**	**-**	**2.024**	**2.230**	
B2	**96607**	**-**	**1772**	**-**	**-**	**4.899**	**3.190**
B2,2	**29120**	**-**	**-**	**-**	**-**	**1.482**	

Batch Reactor, Scale-up 1L							
After 7 Days Digestion *No continuous stirring							
Mixture	**CH4 (area)**	**CO2 (area)**	**H2O (area)**	**Comments**	**CO2 (mL)**	**CH4 (mL)**	**Average**
A1	**16226**	**31218**	**6026**	**Reactors were not continuously stirred (broken stirrer)**	**1.390**	**0.829**	**1.434**
A1,2	**40141**	**97812**	**19688**		**4.246**	**2.040**	
A2	**27358**	**-**	**-**		**-**	**1.393**	**1.130**
A2,2	**16973**	**-**	**-**		**-**	**0.867**	
B1	**17345**	**38391**	**6718**		**1.698**	**0.886**	**0.965**
B1,2	**20469**	**58918**	**10035**		**2.578**	**1.044**	
B2	**48348**	**-**	**1265**		**-**	**2.455**	**2.057**
B2,2	**32604**	**-**	**-**		**-**	**1.658**	

Total Suspended Solids (TSS) and Volatile Suspended Solids (VSS)

WW = wastewater

F = flocs/microalgae

D = digestate

Mo = mass of empty crucible

M1 - mass of crucible + flocs, before ignition

M2 = mass of crucible + flocs, dried in for 24hrs @ 105C

M3 = mass of crucible + flocs, dried for 2hrs @ 550C

TSS = total suspended solids

VSS = volatile suspended solids

After 7 Days Digestion - Small Reactor (200mL)									
Sample	**Mo (g)**	**M1 (g)**	**M2 (g)**	**M3 (g)**	**Vs (mL)**	**M2 - Mo (g)**	**TSS (g/L)**	**M3 - Mo (g)**	**VSS (g/L)**
A1 (WW,450+F+D)	**20.6055**	**40.1646**	**20.8248**	**20.6602**	**40**	**0.2193**	**5.483**	**0.0547**	**4.115**
A2 (WW,450+F)	**21.0162**	**40.1589**	**21.0318**	**21.0168**	**40**	**0.0156**	**0.390**	**0.0006**	**0.375**
B1 (WW,600+F+D)	**22.6428**	**40.1359**	**22.8597**	**22.6988**	**40**	**0.2169**	**5.422**	**0.056**	**4.023**
B2 (WW,600+F)	**21.1417**	**40.1443**	**21.1532**	**21.1408**	**40**	**0.0115**	**0.287**	**-0.0009**	**0.310**

After 7 Days Digestion - Batch Reactors (Scale Up, 1L)									
Sample	**Mo (g)**	**M1 (g)**	**M2 (g)**	**M3 (g)**	**Vs (mL)**	**M2 - Mo (g)**	**TSS (g/L)**	**M3 - Mo (g)**	**VSS (g/L)**
A1 (WW,450+F+D)	**21.0165**	**25.1370**	**21.1226**	**21.0358**	**40**	**0.1061**	**2.652**	**0.0193**	**2.170**
A2 (WW,450+F)	**22.6481**	**29.4467**	**22.6607**	**22.6501**	**40**	**0.0126**	**0.315**	**0.002**	**0.265**
B1 (WW,600+F+D)	**21.1415**	**25.5678**	**21.2906**	**21.167**	**40**	**0.1491**	**3.728**	**0.0255**	**3.090**
B2 (WW,600+F)	**20.6062**	**28.3047**	**20.6153**	**20.6071**	**40**	**0.0091**	**0.228**	**0.0009**	**0.205**

Chlorophyll

After 7 Days Digestion - Small Reactors (200mL)			
Sample	**A665**	**A652**	**Chl(a) (mg/5mL)**
A1 (WW,450+F+D)	**0.357**	**0.317**	**620.55**
A2 (WW,450+F)	**0.133**	**0.077**	**302.74**
B1 (WW,600+F+D)	**0.308**	**0.262**	**554.17**
B2 (WW,600+F)	**0.108**	**0.055**	**257.80**

After 7 Days Digestion - Batch Reactor (Scale Up - 1L)			
Sample	**A665**	**A652**	**Chl(a) (mg/L)**
A1 (WW,450+F+D)	**0.227**	**0.193**	**409.08**
A2 (WW,450+F)	**0.213**	**0.150**	**438.92**
B1 (WW,600+F+D)	**0.252**	**0.216**	**453.39**
B2 (WW,600+F)	**0.308**	**0.212**	**642.48**