Comparison of conventional and electro-selective fermentations of swine manure to produce short and medium chain volatile fatty acids

by

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Abstract

Volatile fatty acids (VFAs) are produced through the microbial fermentation process. This process occurs during anaerobic digestion and VFAs are a byproduct during anaerobic degradation of complex organics. This process can be utilized and enhanced for sustainable VFA production and valorize the carbon, instead of channeling it to methane in anaerobic digesters. VFAs are valuable precursors in pharmaceuticals, food industry, biorefinery, and as electron donors in wastewater treatment. This study will specifically utilize high strength swine wastewater from a Confined Animal Feeding Operation (COD range 1000 – 20,000 mg/L) focused on the enhancement of VFA production through fermentation at varying hydraulic retention times (HRT) at three different pHs (5, 7, or 9), implemented with and without a bioanode.

In this study, swine wastewater fermentation was conducted using anoxic sludge inoculum from a wastewater facility. Performance monitoring was done through VFA quantification and current density production using high-performance liquid chromatography (HPLC) and a potentiostat, respectively. At the 10-day HRT operation, both pH 5 and 9 fermenters produced acetate and propionate (34 mM and 14 mM) as the predominant VFAs, later shifting to butyrate and isocaproate (7.8 mM and 7.7 mM) and overall, a fermentation efficiency of 52% and 57.7%, respectively. Fermentation efficiency is essentially the ratio of VFA accumulation as COD to the influent COD. Acetate and isocaproate remained predominant VFAs in both pH 5 and 9 for both 5 and 2-day HRT operation. The 5-day HRT exhibited the highest fermentation efficiency at both pH 5 and 9 at 69.8% and 83.2%, respectively. During the 2-day operation, washout occurred dropping the fermentation efficiencies to 24.5% and 36.6%.

When a bioanode was employed in an MEC serving as the fermentation reactor, with a poised anode potential of -300 mV (vs) Ag/AgCl, the fermentation efficiencies remained low as the anode respiring bacteria (ARB) utilize the VFAs to produce a high current density (0.5-2.2 A/m^2) eventually channeled to hydrogen gas. Shorter chain VFAs such as acetate, are more readily available for the ARB to utilize which selectively enriched the dominant VFAs in the MEC to be longer chain acids such as isovalerate, valerate, and isocaproate which were more prevalent in all 10-, 5-, and 2-day operations.

At the phylum level, significant enrichment of *Firmicutes* was observed at both pH 5 (81% at 10d; 74% at 5 d; and 64% at 2d HRTs) and pH 9 (82% at 10d; 81% at 5d; 77% at 2d). There were significant differences in selective enrichment at the genus level between the two pH conditions. While *Clostridium_sensu_stricto_1* was the predominant genus at pH 5 (44% at 10d; 40% at 5d; and 23% 2 d) and pH 9 (34% at 10d; 30% at 5 d; 23% at 2d), the second predominant genus was *Terrisporobacter* and *Turicibacter* at pH 5, while it was *Fastidiosipila* at pH 9.

The research of VFA production in wastewater treatment and implementing microbial electrochemistry techniques is becoming an increasingly important topic within the context of wastewater resource recovery. Wastewater is a huge resource to recover energy and valuable byproducts which gives waste a new life and promotes a circular economy.

Table of Contents

List of Figures

List of Tables

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Dedication

I wanted to give dedication to the National Science Foundation for allowing me to be a part of the NRT and having funding to continue my research (NSF NRT grant #1828571). I also wanted to thank the Department of Energy for its contribution to funding the research as well (DoE-DEEE00009504).

Chapter 1 - Introduction

Global populations have been estimated to rise to 9.8 billion by 2050 (UN, 2017). With increasing populations, a higher demand of natural resources will also increase. Implementing reusing and recycling is becoming crucial and commonly known as resource recovery. Resource recovery can be a way to move away from the typical societal production systems of the extraction of raw materials and using industrial techniques for transformation of these raw materials into products (Puyol et al., 2017; Lovins, 2008). For the extraction of raw products, this requires the use of non-renewable inputs such as fossil fuels for commonly a one-time use item along with a significant amount of energy and effort required. Resource recovery techniques can be implemented to recover these raw materials and products from waste streams they enter, to be used again. Wastewater treatment is a viable source to recover and reuse products due to 50-100% of lost product resources being contained in wastewater streams (Puyol et al., 2017). These lost products are stored energy which consists of recoverable resources like nutrients, carbon, and clean water. Most municipal wastewater treatment plants apply energy intensive aeration to treat wastewater and generate one product, namely clean water, while a significant amount of sludge is generated in this process which is either land applied or discarded to a landfill. The current linear process for wastewater treatment also adds to the greenhouse gas footprint (both life cycle and actual emissions).

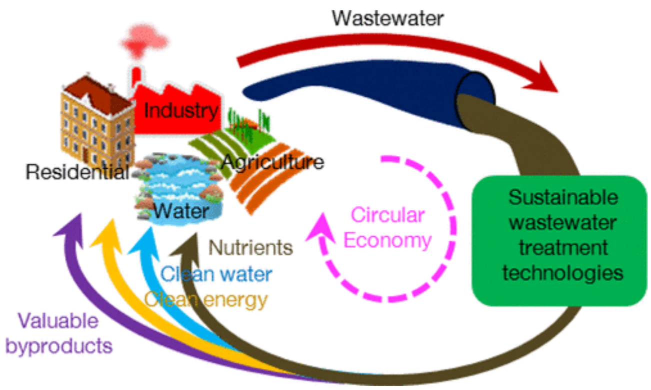


Figure 1: Resource Recovery opportunities in municipal wastewater Treatment (Ghimire et al., 2021)

A circular economy is the concept that products and materials should remain in use for as long as possible, with waste being considered a secondary stream of materials that can be recycled, reused, or recovered (Ghisellini et al., 2016). During wastewater treatment, resources (especially solids) removed to produce treated water are sent to landfills or land-applied for inefficient reuse, the nutrients (N and P) are removed rather than recovered for beneficial reuse, and the valuable resources never get used again. Implementing resource recovery within wastewater treatment can give materials another life, keep waste out of landfills, sustainably produce energy for the treatment facility operation, and produce clean water to put back into the environment in the process. Value added products that have opportunity to be regenerated and reused from wastewater treatment typically include renewable energy captured as biogas, nutrients for fertilizers, valorized carbon sources such as organic acids or alcohols, and water for reuse (Chrispim et al., 2020). Developing technology platforms for a circular economy needs to keep the product market in mind, as it will not be advantageous for people to use if there is not an economical value as well (Ramos-Suarez., 2021). Figure 1 shows that these recovered resources can be beneficially used by manufacturing industries, agricultural-production systems, residential communities, and natural resources.

Research Objectives

The goal of this research was to demonstrate efficient recovery of carbon as volatile fatty acids from swine wastewater through fermentation. The research objectives to accomplish these goals are:

- Study the profiles of short chain and medium chain fatty acids produced during swine wastewater fermentation and how differing pH and hydraulic retention times may optimize the production through fermentation efficiency analysis.
- Analyze electro-selective fermentation as an alternative resource recovery platform for organic acids sequestration, especially C3 – C6 acids through selective consumption of acetate to yield high current densities by the electroactive microbial communities.

Chapter 2 - Literature Review

Anaerobic Treatment and/or Digestion

In developed countries, approximately 80% of municipal wastewater is treated using aerobic processes such as the activated sludge process which requires aeration to the tanks to remove organic carbon (McCarty et al., 2011). These aeration processes are highly energy intensive using roughly 0.5 kWh per m^3 of wastewater treatment for activated sludge process alone (Shen et al., 2015). Activated sludge processes are activated because they build up to high concentrations which leads to significant growth of biomass that must be wasted and discarded (Rittman and McCarty, 2020). While activated sludge processes accomplish reduction of biological oxygen demand (BOD) and chemical oxygen demand (COD), these processes alone are not capable of being net positive in energy production because of the extensive aeration requirements and metabolic pathways that exist in aerobic environments that promote higher sludge yield and hence significant sludge production. Additionally, aerobic treatment processes enable nutrient removal without an option for nutrient recovery and beneficial reuse. One way to achieve a net positive energy production within wastewater treatment is implementing anaerobic treatment and/or side-stream anaerobic digestion. Anaerobic treatment can allow for metabolic pathways to utilize the organic matter present in wastewater, to convert to energy (as methane) or other higher Carbon compounds with greater economic value (such as organic acids), enable efficient nutrient recovery, along with water for indirect reuse.

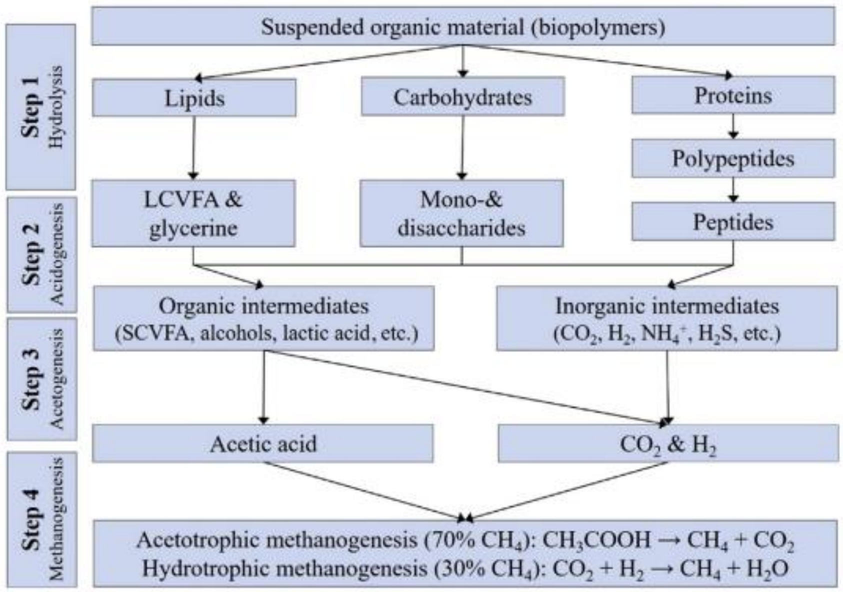


Figure 2: Methane Production from Anaerobic Digestion (He et al., 2018)

Currently, the most common way energy is produced from wastewater treatment is from biogas production from the anaerobic digestion process. The biogas produced is about 50-75% methane with the remainder being carbon dioxide and traces of other gases like hydrogen sulfide and water vapor (Korbag et al., 2020). This occurs in four steps which are hydrolysis, acidogenesis, acetogenesis, and methanogenesis, which is shown in Figure 2 and each byproduct of the previous step becomes the substrate for the following one. Hydrolysis is the most rate limiting step as it is a slow process for the microbes to break down the complex organic material in wastewater, which is in the form of proteins, carbohydrates, and lipids, and gets converted to amino acids, sugars, and fatty acids, respectively. Once the complex material has been broken down into simpler substrates, the acidogenic bacteria can utilize these to produce fatty acids commonly containing 2-6 carbons with acetic acid being the most readily available substrate for the methanogenesis process (Tshemese et al., 2023). Bacteria convert the fatty acids to carbon dioxide and hydrogen gas and acetate can be directly used to produce methane. This is split into two different pathways of methanogenesis of acetolactic and hydrogenotrophic. In the acetolactic pathway, acetate is utilized by methanogens and contributes to roughly two-thirds of the total methane formation (Fenchel et al., 2012). Hydrogenotrophic methanogens use hydrogen gas as an electron donor to reduce carbon dioxide to methane and water (Arif et al., 2023).

Carbon Valorization in the Anaerobic Food Web

Conventional VFA Production routes

In current anaerobic digestion methods for waste sludge, the methanogenesis process is only utilized for the production of methane as the final product, which leads to greater solids stabilization. However, we can manage these microbial pathways for sustainable methods of valuable chemical production that can add significant economic value to an intermediate product that would otherwise be microbially transformed, from wastewater treatment. An intermediate product of the anaerobic microbial food web is volatile fatty acids (VFAs). VFAs are essential chemicals that are extensively used for several industrial industries and are currently generated primarily through petrochemical processes (Agnihotri et al., 2022). Table 1 shows the general processes required for VFA production of VFAs ranging from 2 to 6 carbons, also known as short-chain fatty acids (SCFAs). All of these processes require extensive treatment with high temperatures, high pressures, expensive catalysts, and polishing steps to remove impurities.

Table 1: VFAs Current Petroleum Feedstock Production

VFA	Chemical Production Process	Reference
Acetate CH_3COOH	Nearly all acetate is produced through vapor-phase reaction using ethylene over a palladium catalyst. Carried out at 175-200 ^oC and 5-9 bar. Demand of 6000 kt/year.	(ACS, 2020)
Lactate $CH_3CHCOOH$	Ethene oxidation using palladium catalyst to form acetaldehyde, which under high pressure is converted to lactonitrile with an additional catalyst. Sulfuric acid is used on lactonitrile to produce lactic acid.	(Ojo and Smidt, 2023)
Propionate CH_3CH_2COOH	Carboxylation of ethylene at 180 ^oC and 700 atm using a complex palladium catalyst. Also, can oxidize propionaldehyde where several separation steps occur after.	(Lapidus et al., 1979; Intratec, 2020)
Butyrate/Iso-Butyrate $CH_3(CH_2)_2COOH$	Oxidation, hydrogenation, and esterification of butyraldehyde, which is obtained from crude oil derived propylene, using aluminum complex catalysts, requiring lower temperatures at 0-20 ^oC.	(Varma and Madras, 2008; Agnihotri et al., 2022)
Valerate/ Iso-Valerate $CH_3(CH_2)_3COOH$	Syngas and 1-butene implemented with platinum or palladium catalysts promotes hydroformylation to form valeraldehyde at high temperatures around 200 ^oC. This is then oxidized to form valeric acid.	(Riemenschneider, 2000)
Caproate/Iso-Caproate $CH_3(CH_2)_4COOH$	Hexane undergoes oxidation with strong oxidizing agents carried out at 60 ^oC, where methyl groups present in hexane form to a carboxylic acid group which forms caproate as one resulting product.	(Kruse et al., 2006)

VFAs have several applications and play many important roles in industries and have significant market value for these industries. Acetic acid is a commercial acid heavily used in industries for plastic production, food, cosmetics, industrial, pharma, and household uses (Onissiphorou, 2023). For 2023, the global acetic acid market is approximately USD 23.23 billion (EMR, 2023). For lactic acid, this chemical is also highly valuable in chemical, food, pharma, and cosmetic industries (Yankov, 2022). In these industries lactic acid is used for pH neutralization, preservatives/flavoring agents, in surgical sutures, and an anti-acne agent, along with several other purposes within these industries. The global market value for lactic acid has been evaluated at USD 3.37 billion in 2023 (GVR, 2023). Propionate is primarily used for food preservatives, herbicides, pharmaceuticals, and plastic production and is estimated to have a global market value of USD 330 million in 2022 (Agnihotri et al., 2022; GM, 2023). Butyric acid is valuable in similar industries being used in polymer fibers, medications for antianxiety and inflammatory suppressant, enhance animal feeds, and bactericides in cosmetics (Jiang et al., 2018). The global market for butyric acid in 2023 is estimated to be around USD 389.2 million (Precedence 2023). Valeric acid has value in consumer industries with being known to add a fruity flavor or scent to food and cosmetics because of its esters, with additional use as a plasticizer and in pharmaceuticals (Goldberg and Roken, 2009). Valeric acid had a global market of USD 16 million due to its attributes for food and cosmetics demands (EMR 2022). Typically, caproic acid is used for biofuel precursors, flavoring additives, and antimicrobial agents with an estimated global market of USD 191.8 million in 2022 (Chen et al., 2017; R&M 2023).

Microbial VFA Production

Due to the current production of VFAs being incredibly energy intensive and causing environmental implications, microbial pathways have been studied for the production of VFAs for more sustainable methods. For acetic acid being utilized in plastic production, it is estimated for that alone, it contributes to nearly 3 million metric tons per year (Nicholson et al., 2021). For context, that is roughly the annual emission for 650,000 passenger vehicles (Bressler, 2021). VFAs are produced from pure-culture fermentation systems if specific VFAs are being targeted, or mixed-culture microbial systems, similar to anaerobic digestion, for a mixed VFA profile end product (Ramos-Suarez et al., 2021). Research has been extensively growing for acidogenic fermentation to produce VFAs as this is derived from the earlier steps in the methanogenesis process during anaerobic digestion, seen in Figure 2 the VFAs are an intermediate byproduct produced. Mixed culture over pure culture fermentation for VFA production has advantages because of the capability for a wide range of feedstocks that can be used including agricultural waste, food waste, and municipal wastewater solids (Lee et al., 2014; Ramos-Suarez et al., 2021). A common mixed culture that is used to study VFA production through microbial fermentation is waste activated sludge, which is chosen due to its ubiquitous availability from municipal wastewater treatment facilities (Jiang et al., 2007). Many microbial strains can exist in sludge, including *Acetobacter, Acetomicrobium, Clostridium, Acetothermus,* and *Thermoanaerobacter* which have been studied for the production of acetic acid (Pal and Nayak, 2016; Dietrich et al., 1988). Microbes part of *Propionibacteria* genus primarily produce propionic acid, isovaleric acid, and some acetic acid as well (Coral et al., 2008; Thierry et al., 2004). For butyric acid production, several bacteria have been noted for production, including microbes from genuses *Butyrivibrio, Butyribacterium, Clostridium, Eubacterium, Fusobacterium, Megasphera,* and *Sarcina* (Bhatia and Yang, 2017). Not many studies have been able to determine the microbial pathways of isobutyrate production, however one reported that some strains of *Pseudomonas* may be capable of degrading amino acids to isobutyryl-CoA, which may produce isobutyrate (Lang et al., 2014). Bacteria from *Oscillibacter* are bacteria present in human intestine microbiota and produce valerate (Rosés et al., 2021). One study noticed the enrichment of bacteria from *Bifidobacterium, Caproiciproducens, Actinomyces*, and *Clostridium_sensu_stricto_12* when studying caproic acid production in anaerobic fermentation from sugars (Wang et al., 2022). Using these mixed cultures will require feedstocks for the microbes to utilize the carbohydrates, sugars, and lipids to convert into VFAs.

Fermentation Feedstocks

During anaerobic digestion, the feedstock for the methanogens to go through the methanogenesis process is the organic matter present in wastewater which contains carbohydrates, proteins, and lipids. When selecting a feedstock for VFA production, sustainable and renewable sources that have no competition with food are highly preferred, which leads to wastewater being a commonly used substrate for fermentation processes (Pandey et al., 2022). As previously mentioned, municipal wastewater is not always compatible with anaerobic treatments as it is not always high enough in organic matter for the processes to be efficient. Waste streams that have commonly been studied for VFA production that are high in carbohydrates and sugars are agricultural wastes and food wastes (Pandit et al., 2021). However, several food waste streams contain lignin (11-23%) which requires pretreatment processes that are energy intensive and costly for chemical additions (Pandit et al., 2021). Animal manure is a viable feedstock option as it is high in organic matter and very carbon rich and does not require pretreatment processes. Millions of tons of animal manure is produced yearly and goes untreated or unmanaged which causes significant risk for environmental pollution (Guo et al., 2010).

While studies on animal manure streams for VFA production are limited, one study found when performing anaerobic digestion on cow manure compared to municipal wastewater, the cumulative biogas yield was significantly greater in the cow manure feedstock (Macias-Corral et al., 2008). This was due to the additional nutrients supplied to the fermentative microbes, which created byproducts methanogens could use for biogas production. Another study investigated acidogenic fermentation in an anaerobic membrane bioreactor (AnMBR) of chicken manure with a pH control of 6 and uncontrolled. The uncontrolled reactor increased in pH towards pH of 8 and yielded higher VFAs (up to 19 g/L-d total VFAs) than the maintained pH 6 reactor (up to 12 g/L-d) in the study (Yin et al., 2022). A study using heat-shock (75 ^{o}C for 5 hr) pretreated swine manure for methanogen inhibition, observed the SCFA production during a batch operation system with initial pHs being set up to 6, 8, and 10 in three separate reactors. They observed at a pH of 10 the SCFA production was the most efficient with the highest carbon to VFA conversion of 71.2% and primarily produced acetate and propionate during the 20-day operation (Zhang et al., 2020). The research on swine manure producing VFAs is limited, and this study deemed this a good feedstock for fermentation as it is high in carbohydrates, protein, and low in lipids at 43.75%, 20.4%, and 6.5%, respectively (Zhang et al., 2020).

VFA Optimization

Different operational conditions of anaerobic fermentation can allow for an enhancement of VFA production, while keeping a sustainable and low-cost approach. The factors studied most and seen as important for the optimization of VFA production are pH, temperature, C/N ratio, and retention times. The operating pH is one of the most significant factors for influencing VFA production because of its effect on hydrolysis and the preference of pH by desired microbes for the process (Fang et al., 2020). One study studied the effect of pH on acidogenic fermentation in a batch mode operation of sewage sludge at differing pH values of 12 to 3, adjusted every 12 hours to a decreasing pH unit. They determined pH played a big role in hydrolysis with a soluble protein concentration was 67.9% higher at pH 11 than pH 3, which allowed for a greater use of the soluble organic compounds by the microbes for an increased VFA production (Liu et al., 2011). Acetic acid and propionate were the dominant VFAs in all pH values tested, however at pH 9 they produced the most total VFAs as mg COD per gram of volatile solids (VS) present in the reactors, as high as about 600 mg COD as VFA/g VS (Liu et al., 2011). The effects of temperature have been limited and contradictory in studies for the production of VFAs, however mesophilic conditions (20-50 ^oC) have the most favorability overall (Fang et al., 2020). One study using food waste for VFA production determined the high solubilities rates of carbohydrates and proteins were under mesophilic conditions at 35 ^oC and 45 ^oC at 70% and 72.7%, respectively (Komemoto et al., 2009). This study had also determined the high production of butyric acid in the mesophilic temperature range whereas at higher temperatures, lactic acid was the primary VFA (Komemoto et al., 2009). An appropriate C/N ratio is important because an excess of ammonia is desirably avoided as this can cause inhibition of key fermentative microbes (Zhang et al., 2014). One study had shown a gradual butyrate increase and an acetate decrease when the C/N ratio had jumped from 5 to 30 (Liu et al, 2008). While there have been difficulties concluding the direct relationship of C/N ratios and the VFA distribution of specific VFAs, we know a high carbon content will play an important role for a mixed VFA stream production (Fang et al., 2020).

Selecting proper hydraulic and solids retention times (HRT and SRT) can avoid washout of key bacteria and give bacteria more time to react with the organic matter in the waste streams. Studies have shown a longer SRT may contribute to the growth of methanogens, which can decrease the VFA production efficiency in a system (Fang et al., 2020). However, a study on acidogenic fermentation of waste activated sludge saw a 44% increase in VFA concentrations when the SRT was increased from 4 days to 12 days (Feng et al., 2009). In literature, long SRTs have not been widely studied and the influence of SRT has been shown to vary from one study to another. One study studying the effects of HRT on acidogenesis determined a VFA yield increase when the HRT was increased from 4 to 12 hours, although only slightly increased when changing from 16 to 24 hours (Fang and Yu, 2000). Another study noticed an increase in VFA production after each HRT increase when increased from 4 to 8 to 12 days, with dominant VFAs being acetate and propionate, with butyrate hitting a maximum during the 8-day HRT operation (Lim et al., 2008). HRT has also shown differing results between studies as differing parameters are used during operations.

Table 2: Operation Conditions Optimal for Production of Mixed VFA Streams

Parameter	Optimal Condition	Reference
pH	A pH range of 8-11 is most optimal when sludge is used because of enhanced hydrolysis and is within acidogens preferred environment range. Enrichment of fermentative microbes studied in alkaline conditions for increased VFA production.	(Liu et al., 2011; Lee et al., 2014)
Temperature	Mesophilic conditions (20-50 ^{o}C) are most ideal for VFA production as this keeps a low toxic risk for the bacteria and key VFA forming enzymes maintain highest productivity compared to thermophilic conditions (50-60 ^{o}C).	(Komemoto et al., 2009; Zhuo et al., 2012)
C/N Ratio	For anaerobic fermentation a C/N of 20-30 has been considered ideal.	(Zhang et al., 2014)
Retention Times	SRT: Methanogen activity has been seen to be higher after 10 days. HRT: depending on feed, longer HRTs at around 8-10 days may be ideal for highest VFA yield	(Miron et al., 2000; Lim et al., 2008)

Biological VFA production routes

From mixed culture acidogenic fermentation, it is a mixed VFA stream that is produced. One use of the mixed VFA stream is for the production of polyhydroxyalkanoates (PHA), which is a biodegradable polymer and is moving towards a commercially produced environmentally friendly plastic. One study studied the optimal conditions of PHA production using VFAs produced from paper waste fermentation and determined optimal conditions of 10 g/L, 5:1:4 of acetic, propionic, and butyric acids, respectively (Al Battashi et al., 2020). This roughly corresponds to 83 mM, 13.5 mM, and 45 mM for acetic, propionic, and butyric acids. The industrial process for producing PHA is 5-10 times higher than conventional plastics however, the use of mixed VFA streams can significantly cut the costs by using the VFAs as a carbon source for the bacteria required for the PHA production process (Agnihotri et al., 2022). If mixed VFA streams are produced onsite of a wastewater treatment facility, it can be highly advantageous to utilize some of this stream as a carbon source in their biological nutrient removal processes. For nitrogen removal in wastewater, there is a COD requirement for the microbes carrying out the process of 5-10 mg COD/mg N for nitrification and denitrification (Henze 1991). Additionally, for phosphorus removal, 7.5-10.7 mg COD/mg P is commonly required to carry out the processes (Grady et al., 2011). Another developing use of mixed VFA stream is producing bioenergy. A microbial fuel cell is a system capable of harnessing energy using microbes, with the use of organic substrates (Lee et al., 2014). From these microbial electrochemical systems, we may also be able to obtain additional products for energy production such as biogas and hydrogen.

Waste to Energy Recovery Technologies

Wastewater is a valuable resource when considering capturing energy as a resource during treatment. Wastewater treatment has a high potential for energy capture because of the high organic matter content or also commonly known as COD. This is known to be captured as chemical energy that is contained in the wastewater and is considered to have a yield of 14.7 kJ/gCOD, which has been previously determined through analysis using a bomb calorimeter (Heidrich et al., 2010). In the United States, people produce about 60-120 gCOD/day which gives a ton of potential for recovering energy with wastewater streams (Mara, 2013). There are current and novel technologies that can utilize this available chemical energy and metabolic processes to the advantage of retrieving energy as a recovered resource. One way to estimate chemical energy that is available in wastewater is through measuring the COD. This estimates the total amount of carbohydrates, proteins, and lipids present for methane conversion in an anaerobic digestor. Domestic wastewater has been noted as barriers within implementing anaerobic digestion because of the possibility of low organic concentrations present, especially during summer (McCarty et al., 2011). This has led to finding new waste streams that require treatment and are high in organics for energy production and implementing new technology platforms that anerobic digestion has paved the way to discover

Currently, the most common way energy is recovered from wastewater treatment is from methane produced via anaerobic digestion and using this biogas and converting it into mechanical energy. The most common technology for large scale conversion of biogas to energy is the use of gas turbines (Kabeyi and Olanrewaju, 2022) as well as combined heat and power (CHP) production. From the wastewater plant, the biogas must be upgraded to remove contaminants such as such as hydrogen sulfide and siloxanes, prior to being supplied to gas turbines for energy production (EIA, 2022). An emerging option in North America is for upgrading biogas to pipeline quality natural gas for injection to the line and distribution. In smaller scale and research and development (R&D), fuel cells fueled with biogas have offered a promising option for direct electricity production (Scholz and Ellner, 2011). When fuel cells were initially being developed and researched, they were primarily designed for hydrogen to be the fuel which was oxidized at an anode and electrons would flow to a cathode for oxygen reduction, hence a conversion of biogas to hydrogen was required prior if biogas was being considered for use (Scholz and Ellner, 2011). Implementing fuel cells into wastewater treatment has also provided the opportunity to incorporate microbial processes with the technology for energy production. Microbial fuel cells (MFCs) can utilize the ability of microorganisms to channel electrons for electricity, methane, and hydrogen production (Lee et al., 2008). MFCs have become very attractive to implement in wastewater treatment for energy production because of the microbe's ability to convert organic matter in wastewater to electricity.

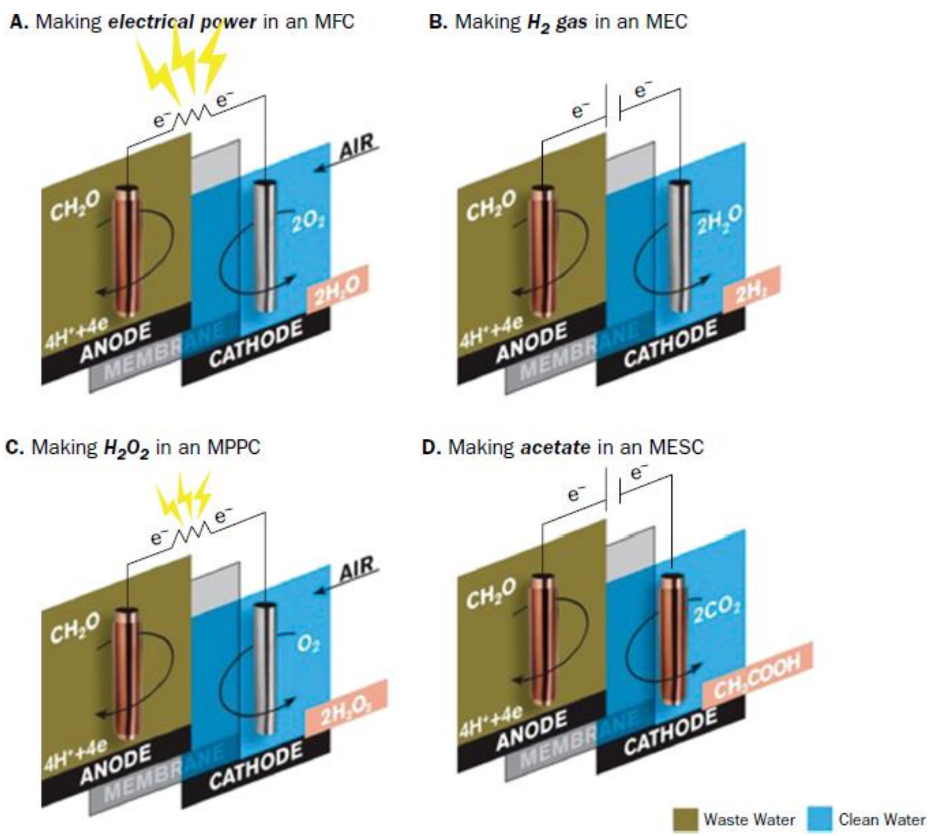


Figure 3: (a) MFC (b) MEC (c) MPPC (d) MESC Concepts (Rittman and McCarty, 2020)

Seen in Figure 3, there are several different configurations of fuel cells incorporated with microbial processes and are commonly classified as microbial electrochemical cells (MxCs), which almost all share the one common principle of containing an anode and there are microorganisms that utilize biodegradable substrates to generate electrical current (Wang and Ren, 2013). MFCs utilize microorganisms' ability to oxidize organic compounds to release electrons and protons, which will flow to the cathode and produce reactions for compound reductions (Cao et al., 2019). Some common MxCs besides MFCs are microbial electrolysis cells (MECs), microbial peroxide-producing cells (MPPCs), and microbial electro-synthesis cells (MESCs). These all provide different valuable outputs although the MEC provides electrical current along with hydrogen gas production which has an energy content of 120 MJ/kg while methane energy content is around 50 MJ/kg, making hydrogen a very valuable option for biofuel production (DOE, 2017; WNA 2016). For anaerobic treatment, MECs seem most beneficial for end products and do not require an input of oxygen which would add additional costs. As previously mentioned, during anaerobic treatment simpler compounds are resulted from hydrolysis such as sugars and fatty acids which are types of organic matter that can be oxidized in an MEC to release electrons that move to the anode, and in this process hydrogen ions move through a membrane to the cathode to be reduced to hydrogen gas (Rittman and McCarty, 2020).

The MFC was first developed in 2005 and discovered that the byproducts from fermentation could be used as substrates by bacteria, to use the anode as an electron acceptor, and transport electrons from the fermentative byproducts (Liu et al., 2005). Bacteria in the genera of *Geobacter*, *Shewanell*, and *Pseudomonas* became recognizably beneficial in this technology for having this ability to transfer electrons to an electrode directly, or through the production of mediators, such as phenazines (Lie et al., 2005; Bond and Lovely, 2003). *Shewanella* and *Geobacter* are two electrochemical bacteria that are known to be capable of direct electron transfer to the anode, where many other bacteria capable of electron transfer perform an indirect transfer causing using mediators (Cao et al., 2019). Indirect transfer of electrons can result in higher inefficiencies because the process requires the mediators to enter the bacteria cells and extract electrons to be supplied to the anode (Debabov, 2008). Overall, *Geobacter* has been noted to be one of the most important electrogens because of its ability to utilize several organic substrates (Zhao et al., 2016).

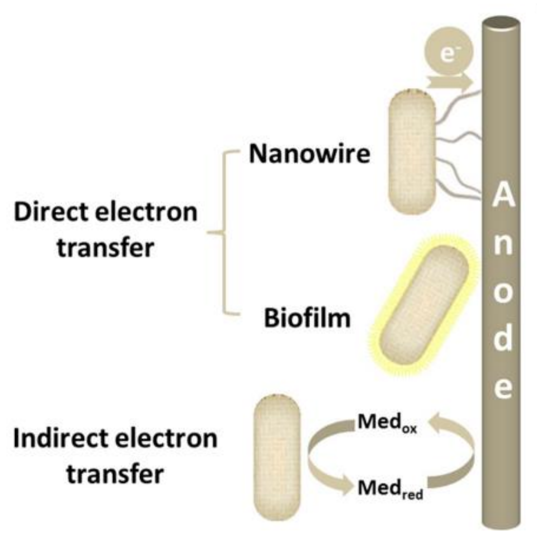


Figure 4: Direct Versus Indirect Electron Transfer to an Anode in MxCs (Cao et al., 2019)

MECs have gained popularity due to the lack of oxygen required within the system, and the voltage level required to produce hydrogen is much lower than the water electrolysis process and it provides a pathway for green biohydrogen generation (Zhang and Angelidaki, 2014). One study explored the effects of differing applied voltages ranging from 0.6V-1.2V in a waste activated sludge fed MEC. They determined 0.8 V to be the most ideal having the highest energy recovery efficiency and noted that the microbes preferably consumed VFAs in the order of acetate>propionate>butyrate>valerate in a single chamber MEC (Linji et al., 2013). To promote direct electron transfer shown in Figure 4, one study implemented a carbon-felt anode in two MECs with one having an applied voltage at 0.6V, where electro-assisted anerobic digestion was performed to analyze biogas production and current density. The carbon-felt anode increased methane production by 12.9% and increased to a current density of 0.1 A/m^2 (Zhao et al., 2016). Instead of anaerobic digestion in an MEC, one study coupled batch mode fermentation within the system to avoid the loss of VFAs to methane so they can be utilized as substrates in the system. The fermentation inoculum used was aerobic sludge to prevent methanogenic activity and was fed with food waste, carried out at pH 5, 6, 7, and 8 in batch mode and then inserted into a two-chamber reactor with an electroactive anode (Magdalena et al., 2023). They found that at pH 7, acetate was the predominant VFA which also resulted in the highest hydrogen gas yield (Magdalena et al., 2023). The goal of this study was to maximize the coulombic efficiency and found pH 7 reactors also reached the highest VFA removal at an average of around 90% at the end of operation and coulombic efficiency of an average 85%.

Integrating microbial electrochemical cells with anaerobic digestion to create clean energy can be a sustainable method for hydrogen production and cause a net positive energy output for treatment processes. There are several enhancements and studies needing to be made on MECs including pure cultures and enhancing the process for current production without a great extent of additional costs. The scale-up of MECs is also an important development for moving forward to commercial use.

Chapter 3 - Conventional and Electro-Selective Wastewater Fermentation

Introduction

Agricultural waste streams are a source growing in recognition of the potential for resource recovery. These agricultural waste streams can be high in nutrients and organic matter such as carbohydrates and proteins (Li et al., 2020). Agricultural waste streams may include animal manure, food processing wastewater, meat packing/slaughterhouse wastes, dairy waste, etc. When these waste streams enter the environment, this releases excess nutrients and pathogens that harm water sources and increase human health risks. One agriculture waste stream that is high in organic matter is swine wastewater. With global populations rising, animal products will continue to rise to ensure food security. Pork is one of the most common proteins for consumption, averaging 45 lb of pork meat per person, per year (USDA, 2023). This causes the growth of concentrated farming of pigs and hogs for a higher production at a cheaper cost, often termed as Confined Animal Feeding Operations (CAFOs). From these farms, large volumes of manure are produced, averaging about 5 L of wastewater per day per pig (Nagarajan et al., 2019). In recent decades, concentrated swine farming has greatly increased in the US with the number of hogs and pigs reaching 75.0 million head on US farms (USDA, 2023). This causes a significant amount of waste that needs to be properly processed instead of being released into the environment, causing environmental implications of excess nutrients being released like nitrogen and phosphorus. Excess nutrients can cause algal blooms in surface water sources, contaminate natural drinking water sources in rural regions, and increase the release of harmful greenhouse gases (EPA, 2012). On farms, swine waste is typically washed out and enters lagoons where the waste sits to promote removal of nutrients and settle out solids for a low cost and maintenance (Kuzniewski, 2023). While this is a low energy way to remove some solids and nutrients, there is no value added back in and all possible products formed are discharged. Agricultural manure management has begun to increase over the past few decades through implementing anaerobic lagoons for methane production, however this is typically only utilized for about 1-3% of the waste demand (AgStar, 2018). If this waste were to be utilized with resource recovery platforms, the high organic matter has potential for product production and utilization.

Anaerobic treatment is metabolically the most favorable to produce products that can be recovered from waste streams. Anaerobic treatment is advantageous for capturing the energy value of organic matter found in wastewater (Rittman and McCarty, 2020). Methane (CH_4) is a gas produced during anaerobic treatment that can be utilized towards energy production. During bacterial fermentation, hydrogen gas (H_2) and acetate are formed which feed methanogens and can be converted into methane. Although, under differing physical conditions, processes may be inhibited to allow for the methanogens to not produce methane and can build up from fermentation byproducts which obtains high value carbons. These carbon sources are volatile fatty acids (VFAs) and produced from the buildup of the acids if methanogens do not have optimal conditions.

Fermentation of swine wastewater

Fermentation of swine wastewater is a way to valorize the organic carbon available in the swine manure, rather than releasing it to the environment, by producing VFAs. Currently, most VFAs are derived from petrochemical processes, but there is high potential producing VFAs from waste streams. VFAs are a valuable carbon source produced as the final products of acidogenic fermentation in anaerobic digestion and are useful in biorefinery, food, and pharmaceutical industries (Sukphun et al., 2021). The VFAs being most commonly used are acetate, propionate, isobutyrate, butyrate, isovalerate, valerate, and caproate ranging in 2-6 carbons and are also known as short chain fatty acids (SCFA) (Wainaina et al., 2019). During anaerobic digestion, methanogenesis occurs by converting sugar, protein and lipids into methane and carbon dioxide. Methanogenesis consists of hydrolysis, acidogenesis, acetogenesis, and methanogenesis. Soluble organic molecules are formed as a result of hydrolysis and acidogenesis process begins where acidogens and acetogens, such as *Butyribacterium* and *Clostridium* metabolize the organic molecules using enzymes, resulting in VFA production (Cheah et al., 2019). After the VFAs have been produced, acetate will be present resulting in acetoclastic methanogens degrading acetate forming methane while simultaneously, carbon dioxide and hydrogen will be formed and utilized to reduce carbon dioxide to methane (Karakashev et al., 2006). If methanogens are inhibited, the concentrations of 2-6 carbon acids will continue to build. A common specific methanogen inhibitor is 2-bromoethanesulfonate (BES), which mimics the methyl coenzyme -A step of methanogenesis; however continued use of this is cost prohibitive for full scale application. To reduce costs, other environmental factors can be used for inhibition including pH as methanogens optimal pH occurs around 6.5-7.5, ammonia toxication as this is present in swine waste, and thermal pretreatments such as heat shock (Ishak et al., 2022). Additionally, other environmental factors contribute to the VFA profiles such as pH, temperature, inoculum, hydraulic retention time (HRT) and organic loading rate (OLR) (Sukphun et al., 2021).

Studies have shown promising results regarding using swine manure as a feedstock for fermentation. Studies have also tested the biogas production of swine waste which may give an estimate of the viability of product production as VFAs are an intermediate process to biogas.

One study performed alkaline fermentation (pH 10), for SCFA production feeding swine manure to a reactor containing anaerobic digested sludge as an inoculum. The VFA concentrations averaged 16,000, 5000, 6000, and 3000 mg COD/L (250, 43, 37, and 14 mM) of acetate, propionate, butyrate, and valerate, respectively (Zhang et al., 2020). Another study tested the VFA production of a swine manure fed reactor at pH 10 compared to uncontrolled and found pH 10 producing about 10,000 mg COD/L for total VFAs with the predominant VFAs being acetate, propionate, and butyrate (62, 49, and 48 mM) during a 24-day batch experiment (Lin et al., 2015).

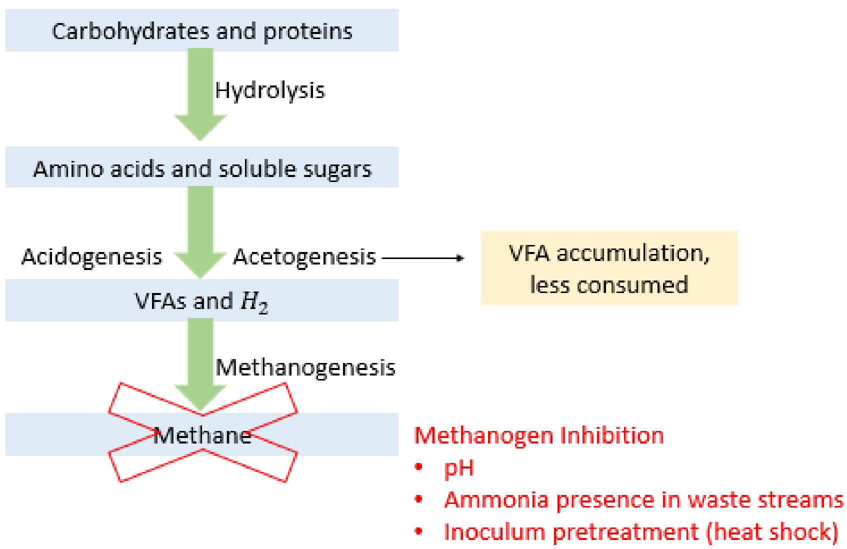


Figure 5: Methanogenesis Inhibition for VFA Accumulation

Separating VFAs from fermentative mixed liquors is possible through membranes, anion exchange resin, and distillation but still requires research and advancements. However, mixed-culture fermentation liquors can hold value to be used as external carbon sources to support wastewater treatment plant processes such as biological nitrogen, phosphorus, and sulphate removal, along with other biotechnologies such as bioplastics production, chain elongation, and electricity production via microbial electrolysis cells (MECs) (Perez-Esteban et al., 2022).

Process control can maximize the production of VFAs through pH, inoculum, HRT, SRT, and temperatures (Lee et al., 2014). Controlling the microbial process allows for enhancement of economically valuable products which helps with societal adaptation, while maintaining low environmental impacts.

Selective Fermentation *via* Microbial Electrolysis Cell (MEC)

MXCs are an emerging anaerobic environmental biotechnology that utilizes microorganisms to harvest chemical energy from organic matter within wastewater. MXCs come in differing configurations such as the MFCs requiring oxygen in the cathode and primarily producing electrical current. In the MEC, it is a highly beneficial technology for the production of hydrogen and higher carbon VFAs such as caproate. In the United States, municipal wastewater treatment consumes about 30.2 billion kWh every year, however the chemical energy harvested in the wastewater is about 2-4 times the energy used for treatment (McCarty et al., 2011). The microbes present in sludge and wastewater can be utilized for new technologies to use this energy that is available, such as MXCs. The energy harvested from MXCs is utilizing an anode and cathode to implement an interaction between living microbes and the surface of electrodes as electron donors and acceptors, respectively (Hassan et al., 2021). The microbes that are capable of consuming organic matter for electricity or hydrogen conversion in this technology are anode-respiring bacteria (ARB). The ARBs have restricted range of electron donors and are not effective at oxidizing complex substrates, which makes them require fermentative microbes to create byproducts that are simple substrates for the ARBs to utilize as electron donors (Rittman and McCarty, 2020; Parameswaran et al., 2009). The most favorable byproduct that the ARBs can utilize from the fermentative bacteria is acetate, however other acceptable donors that will be utilized are ethanol, formate, propionate, glucose, and other sugars (Rittman and McCarty, 2020).

MECs are commonly analyzed in terms of the current production normalized to the electrode surface area (A/m^2) and hydrogen production rates. This is because the biodegradable substrates within wastewater into electrical current and releases hydrogen protons (H^+) which get reduced to hydrogen gas in the cathode (Lu and Ren, 2016). However, this reaction will not occur spontaneously and a power source supplying a small voltage between the electrodes generally applied at 0.2-0.8V (Kadier et al., 2016).

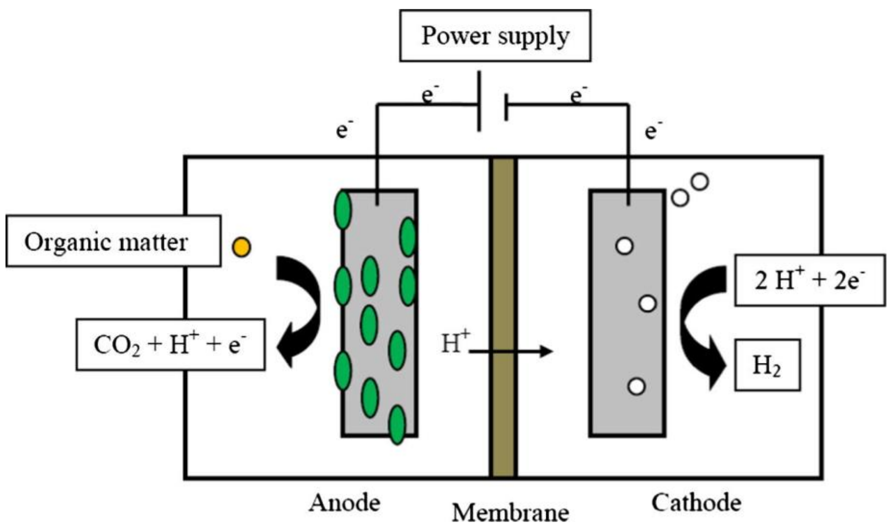


Figure 6: H-Type MEC Configuration (Kadier et al., 2016)

In this chapter, we investigate a microbial electrolysis cell (MEC) which is when oxygen (O_2) is absent in the cathode which allows for water (H_2O) to be electrolyzed to hydrogen gas (H_2) (Rittman and McCarty, 2020). Single chamber MECs may be less beneficial due to hydrogen loss to methanogens. A way to avoid this may be implementing a dual chamber MECs which separates the anode and cathode chambers by implementing an anion exchange membrane between the two chambers, with a simple configuration represented in Figure 6 (Kadier et al., 2016). A simple configuration is H-type used for fundamental studies while a flat plate configuration is a candidate for scale-up applications. In an H-type MEC, the anode and cathode can be put to optimal conditions for desired results. One parameter that will affect hydrogen production is the pH of the anode and cathode compartments. In the anode, the ARBs prefer neutral pH conditions and are affected by lower pH conditions. A phosphate buffer is commonly used in the anode to control the pH from dropping outside of optimal conditions (Sun et al., 2017). Syntrophic relationships have been studied to occur in MECs and this makes the process of hydrogen production from wastewater possible in this technology. Fermentative microbes have the ability to convert complex organic compounds into simpler compounds like SCFAs such as acetate and propionate, which can then be utilized by ARBs as electron donors to produce electrical current (Parameswaran et al., 2009).

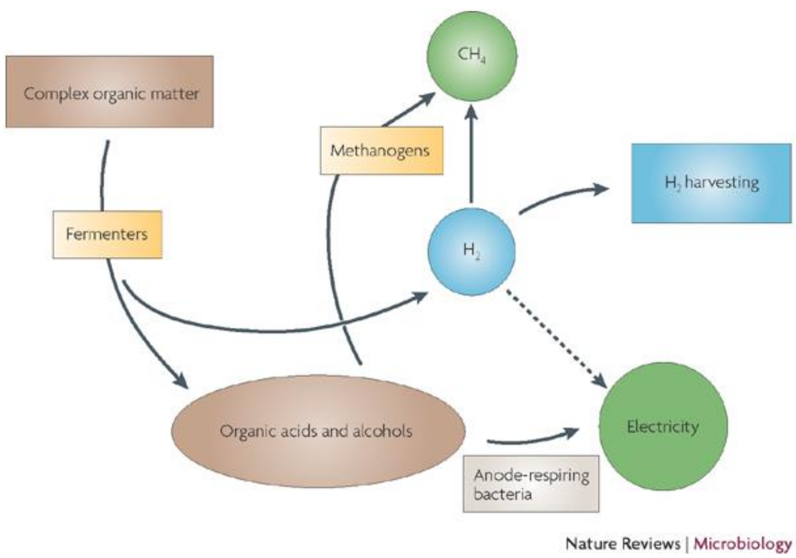


Figure 7: Fermentative Microbes and ARBs Syntropy in an MEC (Rittmann et al., 2008)

During fermentation, selective fermentation is the process of stimulating microbes to selectively ferment carbohydrates and proteins for VFA production. As previously stated, pH inoculum, temperature, and the feed can have influence on the fermentation process and the distribution of the products (Lusk et al., 2018). In an MEC, electro-selective fermentation is particularly valuable due to the conversion of carbohydrates and proteins to SCFAs (primarily acetate), which are electron donors to ARBs, and this can produce hydrogen and electric current. However, due to the ARBs nature of low carbon VFAs, the MEC can also accumulate high value longer chain VFAs such as valerate and caproate. In streams containing lipids in an influent feed, this can be beneficial because lipids degrade much slower than carbohydrates and proteins in anaerobic fermentation Liu et al., 2019). This can allow for lipids to be left behind and extracted for further use like biohydrogenation which long-chain fatty acids (LCFAs) are converted to saturated fatty acids and useful for producing transportation fuel (Liu et al., 2019).

Materials and Methods

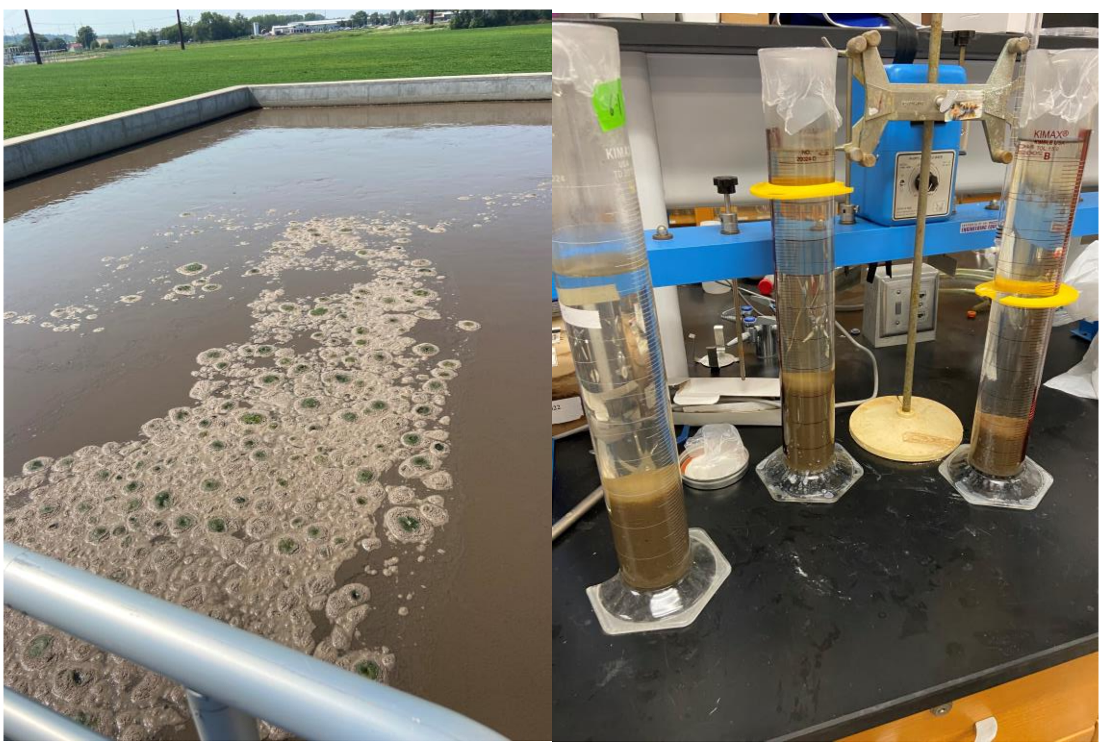
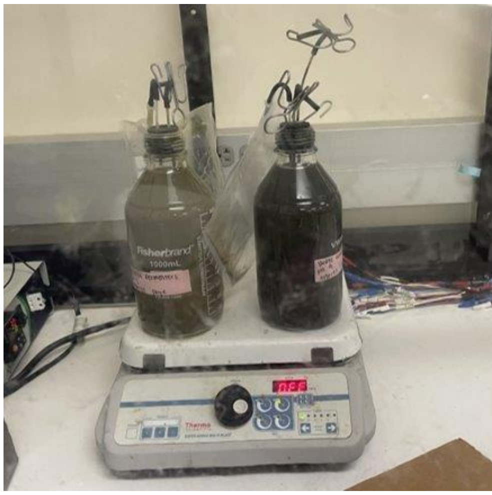


Figure 8: Anoxic Sludge Basin and Settled Sludge

Inoculum Acclimation

Fermenters



(25) (b)

Figure 9: (a): Trial 1 Fermentation Reactors in 1 L Bottles **(b):** Trial 2 Fermentation Reactors in 2 L Bottles

Activated sludge was collected in a 50 L LDPE Nalgene Carboy from the anoxic selector basin at the wastewater treatment plant located in Manhattan, Kansas. The activated sludge was allowed to be settled for 24 hours and 3L of the thickened solids were aliquoted equally to two 2-L reactors to serve as the inoculum. Each fermenter got 1500 ml of settled sludge in a 2 L bottle. Solids characterization (total and volatile suspended solids) of the inoculum was carried out, according to Standard Methods. An HPLC sample was initially taken for analysis of VFA concentrations. Initial total chemical oxygen demand (TCOD) and SCOD sludge analysis was performed using HACH TNT kits at 1:500 and 1:250 dilutions, respectively.

MEC

Figure 10: H-type MEC Fermentation Experiments Reactor

A H-type MEC contains two chambers, one containing an anode and one with a cathode both having a volume roughly of 650 ml. The anode compartment had a rectangular carbon felt anode with a surface area of 81.5 cm^2 and a cathode compartment contained a carbon cathode with a surface area of 16 cm^2. The anode was previously constructed and contained an acclimated biofilm containing ARBs that was acetate fed and grew in a media containing phosphate buffer and 1 mL of trace mineral media added to the phosphate buffer, which filled both the anode and cathode compartments. The phosphate buffer contained (per liter of deionized water): 12.04 g Na_2HPO_4, 2.06 g KH_2PO_4, and 0.41 g NH_4Cl. The trace mineral media contained (per liter of deionized water): 0.5 g of EDTA, 0.082 g $CoCl_2 \cdot 6\ H_2O$, 0.114 g $CaCl_2 \cdot 2\ H_2O$, 0.01 g H_3CO_3, 0.02 g $Na_2MoO_4 \cdot 2\ H_2O$, 0.001 g Na_2SeO_3 , 0.01 g $Na_2WO_4 \cdot 2\ H_2O$, 0.02 g $NiCl_2 \cdot 6\ H_2O$, 1.16 g $MgCl_2$, 0.59 g $MnCl_2 \cdot 4\ H_2O$, 0.05 g $ZnCl_2$, 0.01 g $CuSO_4 \cdot 5\ H_2O$, and 0.01 $KAL(SO_4)_2 \cdot 12\ H_2O$ (Parameswaran et al., 2009). Additionally, 1 mL of 4 g/L $FeCl_2$ and 0.5 mL of 37.2g/L $Na_2S \cdot 9\ H_2O$ solutions were added to the 1 L of phosphate buffer (Parameswaran et al., 2009).

In the anode compartment, the media was then replaced with 550 mL of settled anoxic sludge and 100 mL of the media containing phosphate buffer, trace mineral media, and stock solutions for pH buffering purposes. An initial glucose spike was then added at a concentration of 20 mM to initiate fermentation through simple sugars being used by fermentative microbes present in the inoculum and the pH was adjusted to 7 using 1 M NaOH. Two additional glucose spikes occurred while the rectors sat in bath mode to continue fermentation initiation before wastewater was added to begin HRT operations. The cathode compartment contained 650 mL of phosphate buffer with stock solutions and was adjusted to pH 11.5 with 1 M NaOH.

Inoculum Characteristics

After sludge analysis, casein, and cellulose, representative proteins, and carbohydrate substrates, were dosed to the fermentation reactors at 3.93 g/L, each. The protein and carbohydrate doses were chosen according to previous research (NSF final report, CBET Award: 1335884) and did not include lipids in the form of phosphatidylcholine, due to swine manure having an insignificant fat quantity (Chastain et al., 2003). After the addition of casein and cellulose, TCOD and SCOD were monitored periodically during batch operation along with VFAs. Once an increase followed by a stabilization of the SCOD and VFAs was noted, a second dose of casein and cellulose was added again at 3.93 g/L, 10 days after the first dose. Analysis of TCOD/SCOD and VFA concentrations were measured in same frequency until an additional increase in VFA and stabilization occurred. Table 3 shows initial characteristics of the inoculum after analysis. The initial VFA analysis of the inoculum is also shown in Figure 11.

Table 3: Anoxic Sludge Initial Characteristics

TCOD (mg/L)	19,900
SCOD (mg/L)	6,350
TS (mg/L)	12,812.5
VS (mg/L)	10,125
VS/TS	0.79

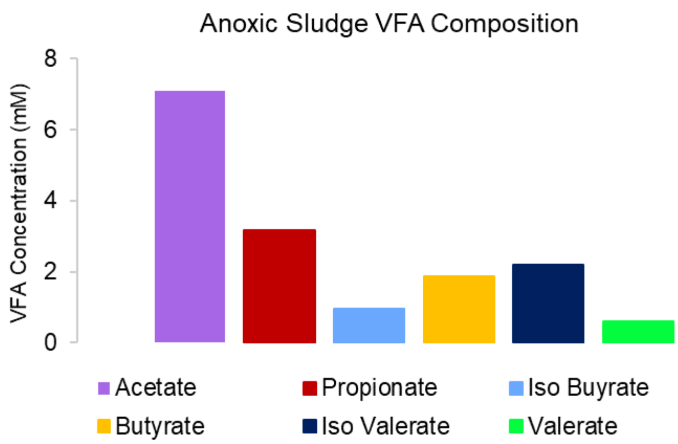


Figure 11: Initial VFA Composition of Anoxic Sludge Inoculum

Semi-Continuous Fermentation Operation

Following batch acclimatization, 500 ml of swine wastewater was added to maintain each fermenter at 2L operating volume. After wastewater addition, pH was adjusted to 5 and 9 in the two separate reactors using 4 M HCl and 5 M NaOH, respectively. Reactors operated at continuous HRTs moving forward at 10, 5, and 2 days in triplicate. Appropriate reactor effluent and influent feed volumes are shown in Table 1. Effluent volume was centrifuged daily, and the separated solids were resuspended with the influent wastewater to retain solids in the reactor. As a result, the SRT in the reactors were never removed and remained in the reactors throughout the experiments. Reactors were kept continuously stirred at 350 rpm on a Multi-Position Digital Stirrer at 30 ^{o}C (Thermo Fisher Scientific, USA).

Table 4: HRT Influent and Effluent Volumes

	10-day HRT Flowrate (mL/day)	5-day HRT Flowrate (mL/day)	2-day HRT Flowrate (mL/day)
Fermenters (Trial 1)	100	200	500
Fermenters (Trial 2)	200	400	1000
MEC	65	130	325

Analytical Methods

Wastewater and inoculum characterization

The HPLC (Shimadzu LC-20AT, USA) used an Aminex HPX-87H column (300 mm × 7.8 mm, Bio Rad Corp., USA) equipped with two detectors: photodiode array and refractive index detectors. For VFA quantification, the HPLC method was used at 0.65 mL/min pump flow rate and 50 ℃ oven temperature with 2.5 mM sulfuric acid as eluent. The initial TCOD/SCOD sludge analysis used HACH 822 kits, ranging from 20-1500 mg/L concentration. HACH vials were measured using a HACH DR 3900 spectrophotometer. The pH was measured and readjusted daily to pH 5, 7, and 9 using Oakton pH 550 benchtop pH meter. Centrifugation of the effluent was done at 15054 rcf with Eppendorf 5920 R (Eppendorf, Hamburg, Germany). Gas analysis was done measuring hydrogen and methane using Gas-Chromatography (GC) with a thermal conductivity detector (TCD). Argon was the carrier gas at a flow rate of 8.8 mL/min and pressure of 88.8 kPa. Temperatures of injection, column, and detector were 200, 80, and 150

°C, respectively. For MEC experiments, a VMP3 multichannel digital potentiostat was used for current density measurement and reference electrode maintenance (Bio-Logic USA, Knoxville, TN). The TS and VS of the reactors were analyzed according to the Standard Methods for Examination of Water and Wastewater 2540B and 2540E.

DNA Extraction and High Throughput Sequencing

DNA was extracted at the end of each 10-, 5-, and 2-day HRT experiment along with initial anoxic sludge samples used for inoculation of the reactors. A 50 ml sample was centrifuged from the reactors for concentrated solids that are suspended in the liquid. To extract the DNA, we used a E.Z.N.A.® Water DNA Kit. With the extracted DNA, we quantified the quality of the DNA with a nanodrop spectrophotometer to yield the 260/280 absorbance ratio. The DNA was sent for further analysis to the University of Kansas and once received was quantified using the Qubit dsDNA HS Assay Kit and a Qubit 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA). PCR was performed to generate 16S amplicons using a 1:100 dilution of each DNA sample as template. Amplicons were generated using primers Bakt_341F and Bakt_805R (Herlemann et al., 2011) that targeted the V3-V4 hypervariable region of the 16S rRNA gene. Dual indexed libraries were prepared from the generated amplicons using Nextera XT V2 indices *(Illumina, San Diego, CA)*. The libraries were quantified using the Qubit dsDNA HS Assay Kit and a Qubit 4 Fluorometer. The size distribution of the libraries was confirmed using the Agilent 4150 TapeStation System *(Agilent, Santa Clara, CA)* and the Agilent D1000 ScreenTape Assay *(Agilent, Santa Clara, CA)*. The prepped libraries were pooled and sequenced on a 2 x 300 Illumina MiSeq *(Illumina, San Diego, CA)* sequence run. The raw sequences resulting from the MiSeq run was processed using the Quantitative Insights Into Microbial Ecology2

(QIIME2, ver Amplicon 2024.2) (Bolyen et al., 2019) pipeline, and further processed as shown in Fig 12.

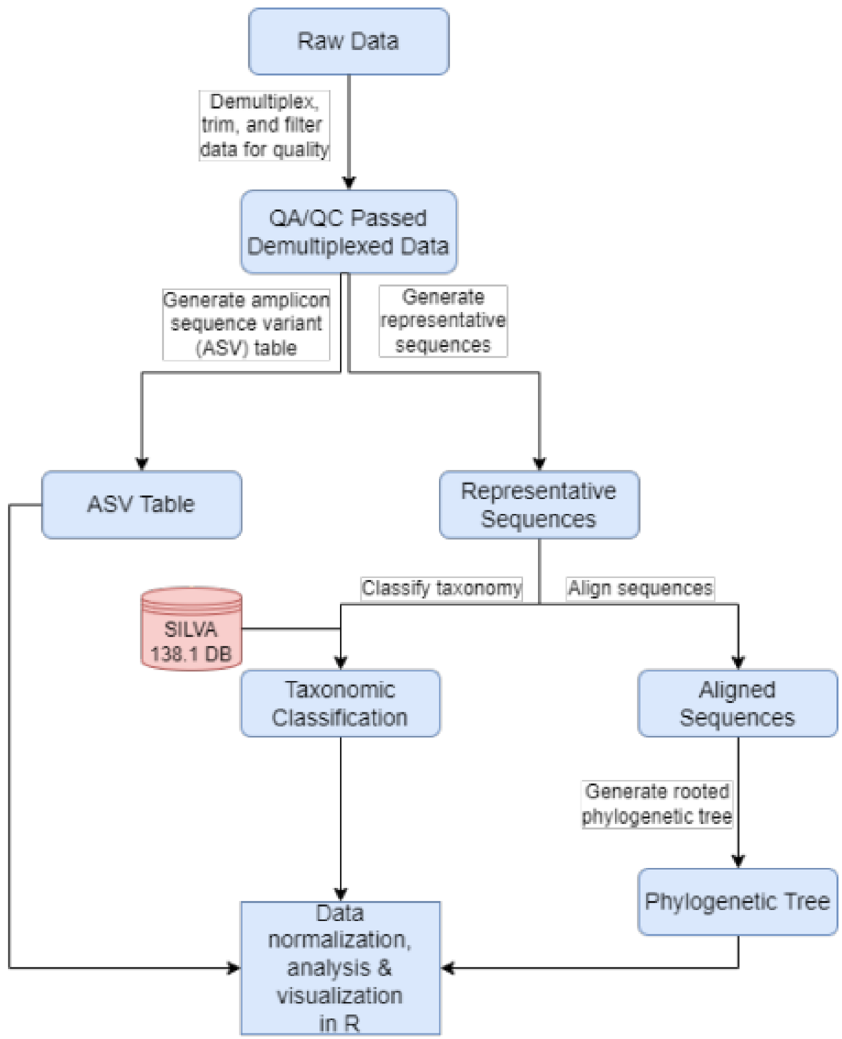


Figure 12: Raw Data Processing for DNA Analysis

VFA Analysis

VFA data from the HPLC is converted to COD from mM for an analysis of the efficiency of COD to VFA during fermentation. This is done through an electron balance and a COD conversion factor. The COD conversion factor shown represents a standard of 8 g COD is equivalent for one electron.

Acetate:

$$CO_2 + HCO_3^- + 8\ H^+ + 8\ e^- \rightarrow CH_3COO^- + 3\ H_2O$$

$$\left(\frac{mmol\ \boldsymbol{acetate}}{L}\right)\left(\frac{8\ meq}{mmol}\right)\left(\frac{8\ mg\ COD}{meq}\right) = \frac{mg\ COD}{L}$$

Lactate:

$$2\ CO_2 + HCO_3^- + 12\ H^+ + 12\ e^- \rightarrow CH_3CHOHCOO^- + 4\ H_2O$$

$$\left(\frac{mmol\ \boldsymbol{lactate}}{L}\right)\left(\frac{12\ meq}{mmol}\right)\left(\frac{8\ mg\ COD}{meq}\right) = \frac{mg\ COD}{L}$$

Propionate:

$$2\ CO_2 + HCO_3^- + 14\ H^+ + 14\ e^- \rightarrow CH_3CHOHCOO^- + 5\ H_2O$$

$$\left(\frac{mmol\ \boldsymbol{propionate}}{L}\right)\left(\frac{14\ meq}{mmol}\right)\left(\frac{8\ mg\ COD}{meq}\right) = \frac{mg\ COD}{L}$$

Iso-Butyrate and Butyrate:

$$4\ CO_2 + HCO_3^- + 20\ H^+ + 20\ e^- \rightarrow CH_3(CH_2)_2COO^- + 6\ H_2O$$

$$\left(\frac{mmol\ (\boldsymbol{iso}\ \boldsymbol{-})\boldsymbol{butyrate}}{L}\right)\left(\frac{20\ meq}{mmol}\right)\left(\frac{8\ mg\ COD}{meq}\right) = \frac{mg\ COD}{L}$$

Iso-Valerate and Valerate:

$$5\ CO_2 + HCO_3^- + 26\ H^+ + 26\ e^- \rightarrow CH_3(CH_2)_3COO^- + 8\ H_2O$$

$$\left(\frac{mmol\ (\boldsymbol{iso}\ \boldsymbol{-})\boldsymbol{valerate}}{L}\right)\left(\frac{26\ meq}{mmol}\right)\left(\frac{8\ mg\ COD}{meq}\right) = \frac{mg\ COD}{L}$$

Caproate:

$$6\ CO_2 + HCO_3^- + 32\ H^+ + 32\ e^- \rightarrow CH_3(CH_2)_3COO^- + 10\ H_2O$$

$$\left(\frac{mmol\ \boldsymbol{caproate}}{L}\right)\left(\frac{32\ meq}{mmol}\right)\left(\frac{8\ mg\ COD}{meq}\right)=\frac{mg\ COD}{L}$$

Fermentation Efficiency

Fermentation efficiency is the ratio of VFA accumulation as COD to the influent COD. This shows the COD in the wastewater that microbes are utilizing to convert to VFAs.

$$Fermentation\ Efficiency=\frac{\sum VFAs\ as\ COD}{Influent\ COD}*100\%$$

Coulombic Recovery

The coulombic recovery was calculated to determine the recovery of COD to current generation in the MEC. This was also utilized to determine the total COD recovery in the MEC operation. This is because the VFAs that accumulate are calculated within the fermentation efficiency. However, the COD that is being converted to current density is not considered hence the need for coulombic recovery being added.

$$Coulombic\ Recovery=\left[\frac{Current\ density\left(\frac{A}{m^2}\right)\times Anode\ area(m^2)\times\frac{1\ Coul}{A-sec}\times\frac{1\ e^{-}eq}{96{,}485\ Coul}\times\frac{86{,}400\ sec}{d}}{\left\{\left(\frac{g\ COD\ influent}{L}\right)+\left(\frac{g\ COD\ in\ reactor}{L}\right)\right\}\times\left(\frac{e^{-}\ eq}{8\ g\ COD}\right)}\right]$$

$$Total\ COD\ Recovery\ in\ MEC=(Coulombic\ Recovery)+(Fermentation\ Efficiency)$$

Results and Discussion

Once the batch fermentation experiments were completed with the addition of casein and cellulose to begin fermentation, swine wastewater was added as a substrate to the two pH 5 and 9 reactors. Swine wastewater is high in biodegradable organic resulting in the process of hydrolysis to occur to break down the carbohydrates and proteins for VFA accumulation. The swine waste was collected from a swine farm located in Manhattan, KS and the COD and VFA values would be measured upon collection. The batch operation VFA production can be seen in Figure 13, with the VFAs increasing as the casein and cellulose continue to go through hydrolysis and be converted to SCFAs. This occurred at a greater productivity at pH 9 as alkaline conditions typically enhance the hydrolysis process.

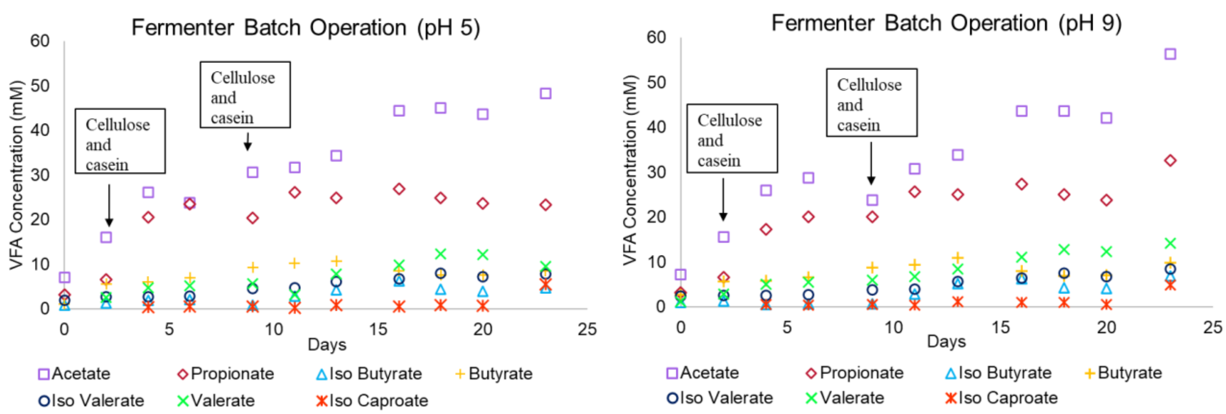
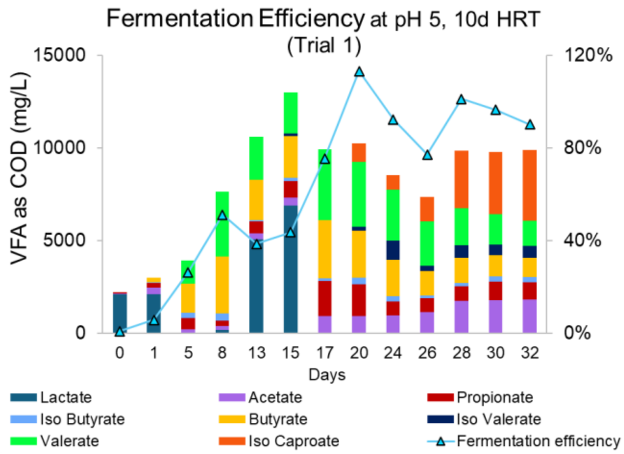
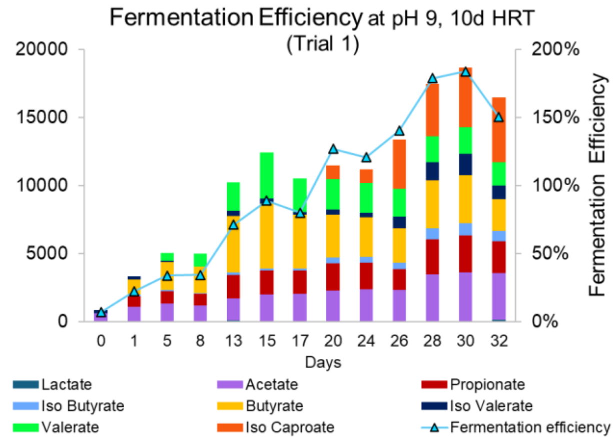


Figure 13: Batch Operation of Anoxic Sludge VFA Composition

10-Day HRT Operation



(a)



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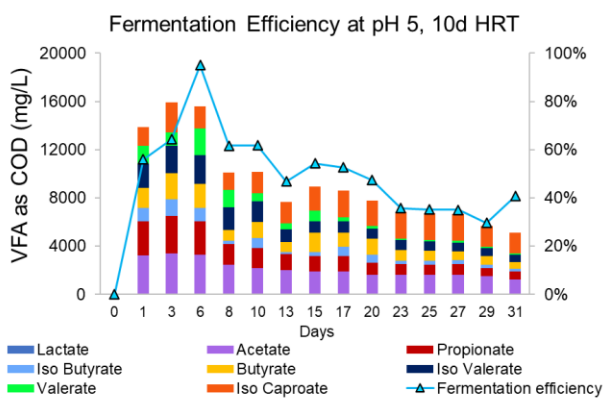

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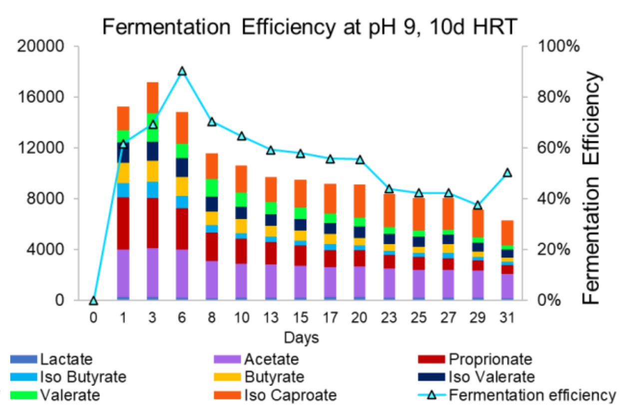


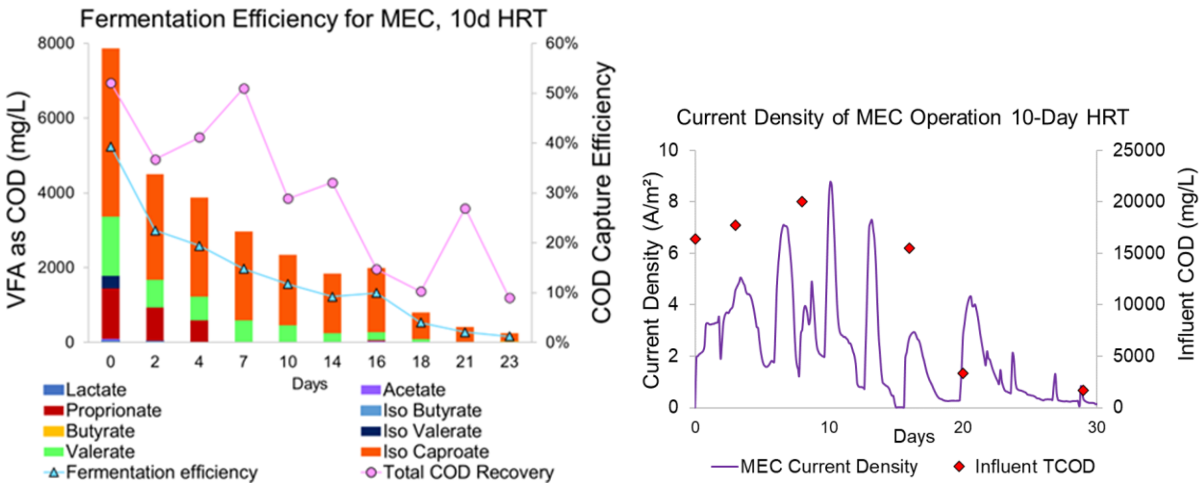
(d)

Figure 14: (a): Trial 1:10-Day HRT Fermentation efficiency for pH 5 reactor **(b):** Trial 1:10-Day HRT Fermentation efficiency for pH 9 reactor **(c):** Trial 2: 10-Day HRT Fermentation efficiency for pH 5 reactor **(d):** Trial 2: 10-Day HRT Fermentation efficiency for pH 9 reactor

Figure 14 shows the VFA profiles along with the efficiency of converting influent COD present in the wastewater to VFAs over a 10-day HRT timespan for pH 5 and 9 reactors during trials 1 and 2. The 10-day HRT was done in triplicate studies back-to-back. In Figure 14 (a), the first trial using glucose as an initial substrate, we saw lactate and acetate as the leading VFAs in pH 5 and transitioned to acetate, butyrate, and propionate being predominant at the end of the 10-

day operation. Figure 14 (b) shows the 10-day operation at pH 9 during trial 1 with leading VFAs of acetate and propionate at the beginning and transitioning to butyrate and isocaproate. The fermentation efficiencies for pH 5 and 9 during trial 1 were 62.6% and 95.4%, respectively. During trial 1, some fermentation efficiencies exceed 100% as the COD in the influent drops after being high (>10,000 mg/L), there is an increase in VFAs even when the influent may decrease for the next feed. During trial 1, the higher carbon VFAs likely accumulated later in the operation due to glucose being used to stimulate the fermentative microbes rather than casein and cellulose like trial 2 was.

In Figure 14 (c) and (d) show trial 2 fermentation efficiencies over the 10-day operation were measured. The leading VFAs were acetate and propionate produced in both of the reactors and began shifting to longer chain acids such as butyrate and isocaproate as the 15th day approached. The isocaproate showed early in the operation compared to trial, likely due to more complex substrates being utilized to initiate fermentation and was capable of production once the swine waste was added. While the concentrations were low, the lactate remained constant throughout the pH 9 studies and never appeared in the pH 5 reactor. Overall, the average fermentation efficiency during the 10-day HRT was higher in pH 9 at around 58% while pH 5 was at 52%.

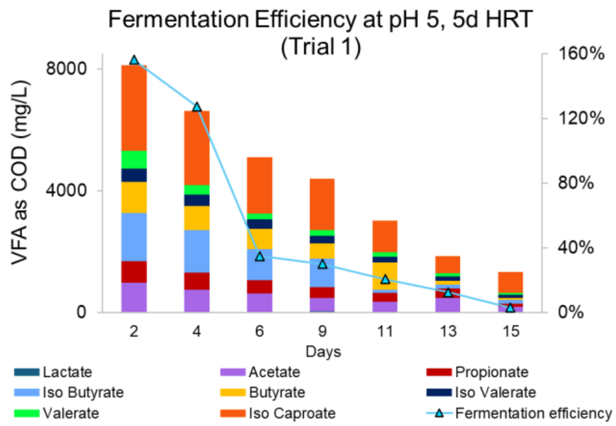


(a) (b)

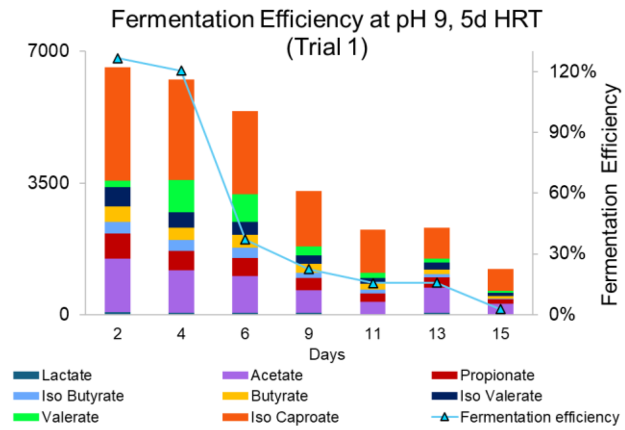
Figure 15: (a): 10-Day HRT COD Recovery for MEC **(b):** Current density and influent COD during 10-day HRT operation

Figure 15 (a) shows the fermentation efficiency over a 10-day HRT in the MEC reactor. The efficiency throughout the operation remained low because of the ARBs utilization of the lower carbon VFAs as they are more readily available as electron donors for the microbes. Throughout the operation, the higher carbon VFAs accumulate, primarily being valerate and isocaproate because of the ARBs preference for utilization of shorter chain acids. Due to the SCFAs being consumed, the total COD recovery was considered which accounts for the current density production from VFAs and COD. The total average COD recovery during the operation was 30%. The total VFAs continued to decrease as the influent COD decreased and this reflects the current density decreasing in Figure 15 (c). During high COD in the influent, the current density correspondingly increased to 8.7 A/m^2.

5-Day HRT Operation



(a)



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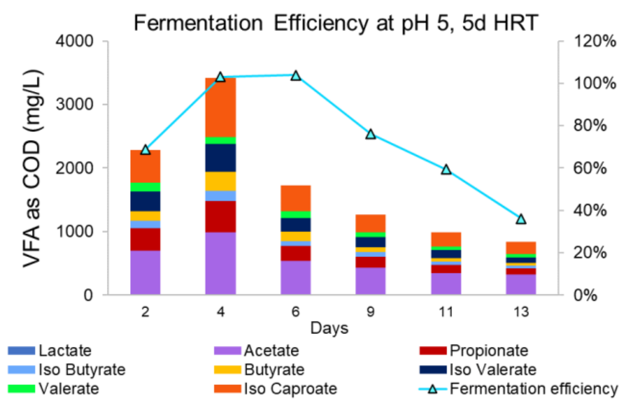
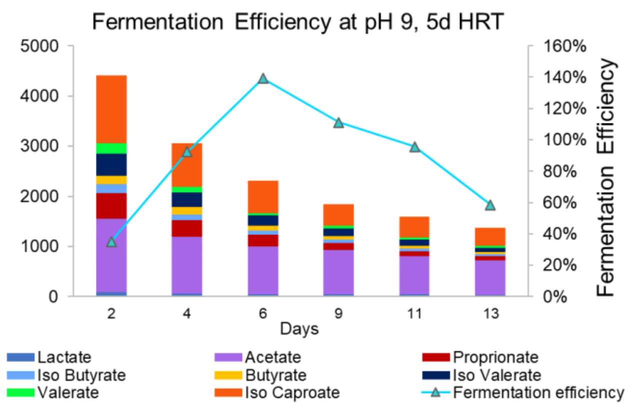


(c)

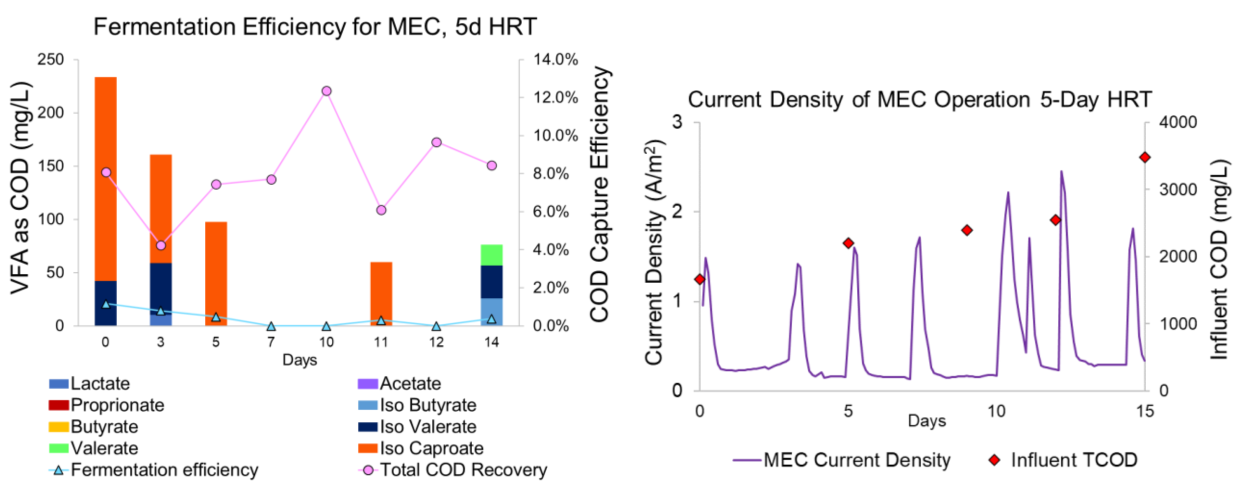


(d)

Figure 16: (a): Trial 1:5-Day HRT Fermentation efficiency for pH 5 reactor **(b):** Trial 1:5-Day HRT Fermentation efficiency for pH 9 reactor **(c):** Trial 2: 5-Day HRT Fermentation efficiency for pH 5 reactor **(d):** Trial 2: 5-Day HRT Fermentation efficiency for pH 9 reactor

Figure 16 shows the fermentation efficiency at pHs 5 and 9 under a 5-day HRT condition for trials 1 and 2. Figure 16 (a) and (b) show trial 1 fermentation products. During trial 1, the high carbon VFAs persist although overall fermentation efficiency decreases due to a decreased organic loading coming in. The more dilute influent stream continued to produce VFAs although decreased in concentrations. The leading VFAs persisted to be acetate and propionate with now shifting towards isocaproate. The average fermentation efficiencies during trial 1 for pH 5 and 9 reactors were 52% and 48.7%, respectively.

Figure 16 (c) and (d) show the fermentation efficiency and VFA production during trial 1 5-day HRT. The fermentation efficiencies for the pH 5 and 9 fermenters during this operation were 69.8% and 83.2%, respectively. Acetate became the leading VFA during this operation following isocaproate. The fermentation efficiency begins to drop after day 6 and this is because the influent COD drastically decreased. This drop in COD results in VFA concentrations decreasing as well on the first day of round two of the 5-day HRT. With the influent COD being around 1600 mg/L, the fermenters experienced higher washout also with the higher volume of influent and effluent added and removed. During this period of decreased influent COD, the fermentation efficiency remained high and above 100% because of the previous influent COD being very high (20,000 mg/L) and this resulted in the data showing more use of COD from the microbes than what was actually being utilized in the wastewater feed.



(a) (b)

Figure 17: (a): 5-Day HRT COD Recovery for MEC **(b):** Current density and influent COD during 5-day HRT operation

Figure 17 (a) shows the COD recovery and VFA accumulation for the MEC at a 5-day HRT operation. The efficiency continued to drop after the 10-day HRT operation which was due to a low COD in the influent wastewater which caused washout of VFAs. Regardless, the leading VFA continued to be the higher carbon VFAs of isocapraote until the final day of the 5-day HRT operation. This is likely from following full washout of detectable VFAs and the influent COD increased back to around 3500 mg/L, allowing the fermentative bacteria to produce longer chain SCFAs such as isobutyrate, isovalerate, and valerate. While washout occurred, the COD recovery persisted at around 12% on day 10 due to the current density production from the SCFA consumption. The average total COD recovery during this operation was 8%. This influent COD decrease also resulted in a decreased current density production seen in Figure 17 (c). While the COD was between 2,000-3,500 mg/L, the current density still responded to being 1.5-2.5 A/m^2 when fed with the wastewater.

2-Day HRT Operation

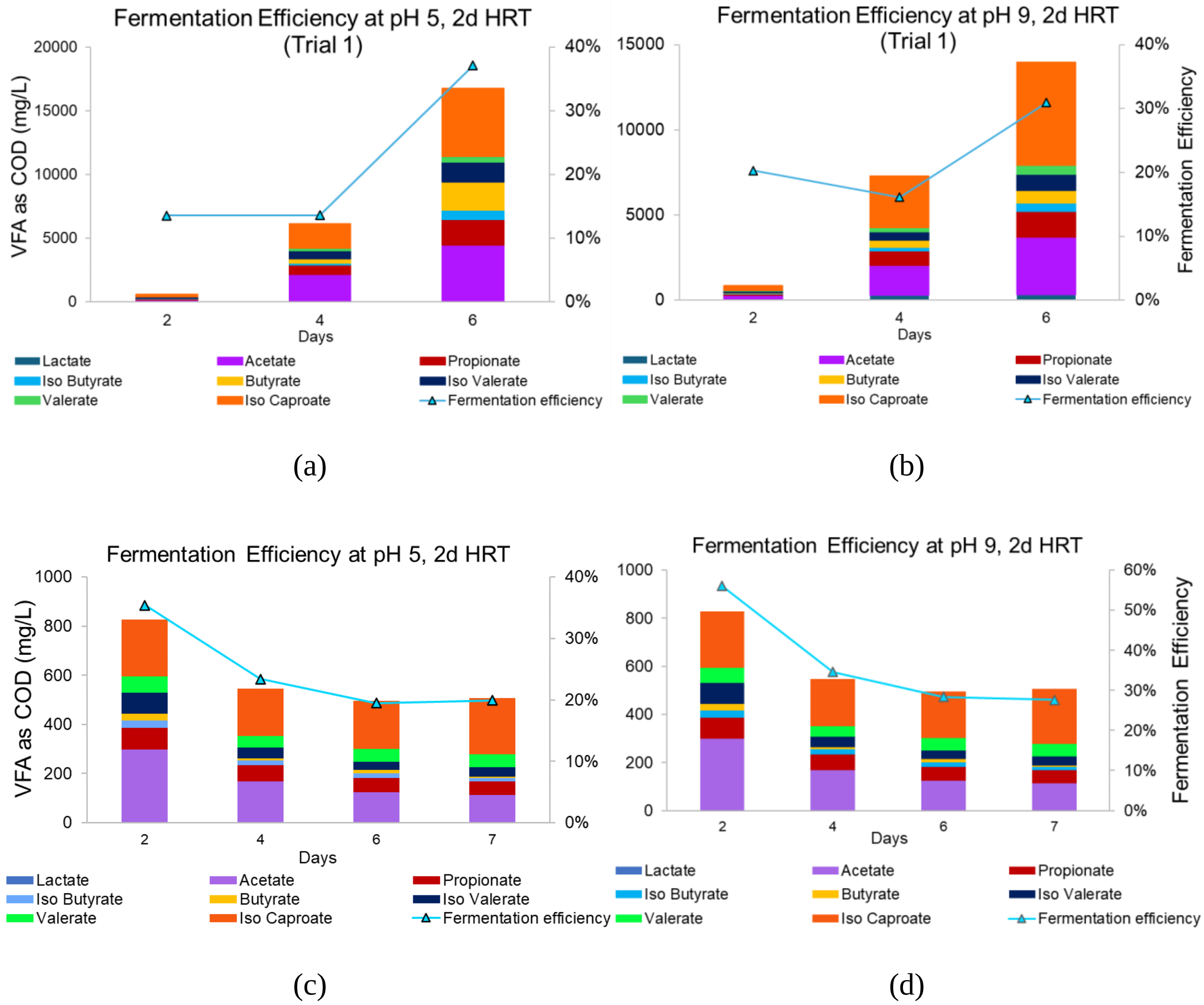
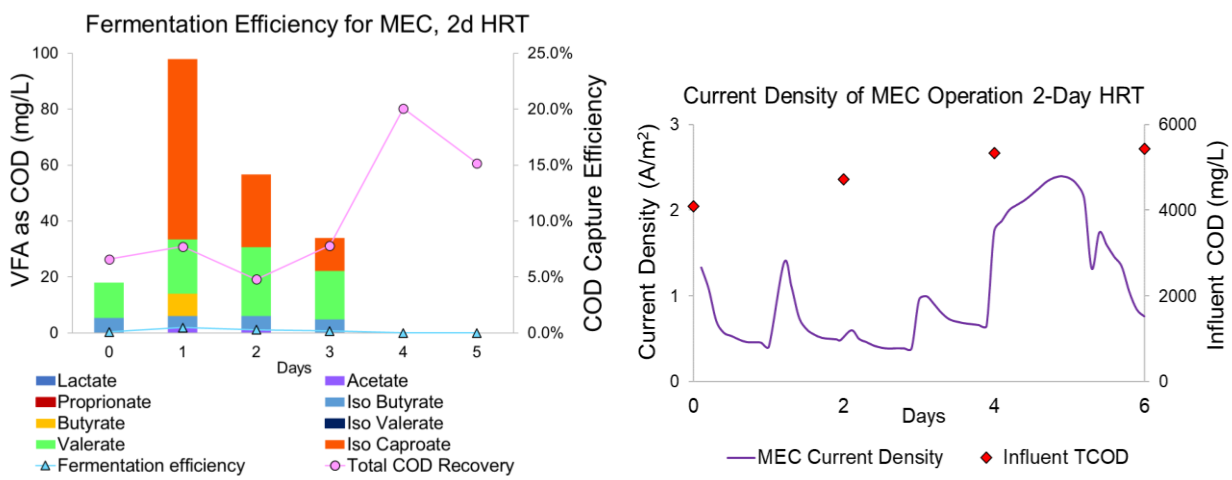


Figure 18: (a): Trial 1:2-Day HRT Fermentation efficiency for pH 5 reactor **(b):** Trial 1:2-Day HRT Fermentation efficiency for pH 9 reactor **(c):** Trial 2: 2-Day HRT Fermentation efficiency for pH 5 reactor **(d):** Trial 2: 2-Day HRT Fermentation efficiency for pH 9 reactor

Figure 18 shows the VFA profiles along with the efficiency of converting influent COD present in the wastewater to VFAs over a 2-day HRT timespan for pH 5 and 9 reactors during trials 1 and 2. During trial 1 shown in Figure (a) and (b), the influent COD increased significantly reflecting the increase in VFA concentrations during the operation. The VFA profiles remained consistent and similar in the fermentation efficiencies for pH 5 and 9 was 21.4% and 22.5%, respectively.

Trial 2 efficiencies and fermentation product production during 2-day HRT is shown in Figure 18 (c) and (d). The 2-day HRT continued to have a low influent COD and also a high volume of influent and effluent causing washout within the reactors. However, the VFA concentrations still showed acetate and isocaproate to be the leading VFAs and most VFAs were still detectable. The average fermentation efficiencies in pH 5 and 9 reactors were 24.6% and 36.6%, respectively. The average fermentation efficiencies for pH 5 and 9 over the 10-, 5-, and 2-day HRTs were 52.6% and 61.7%, respectively

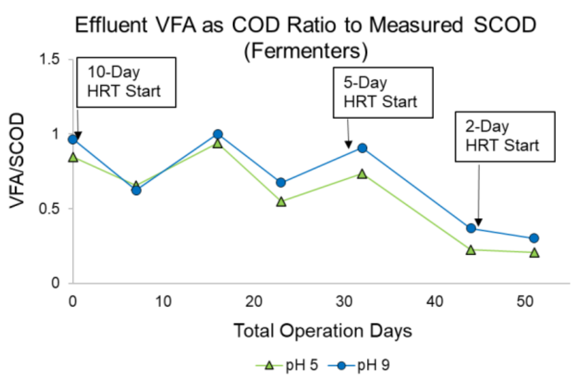


(a) (b)

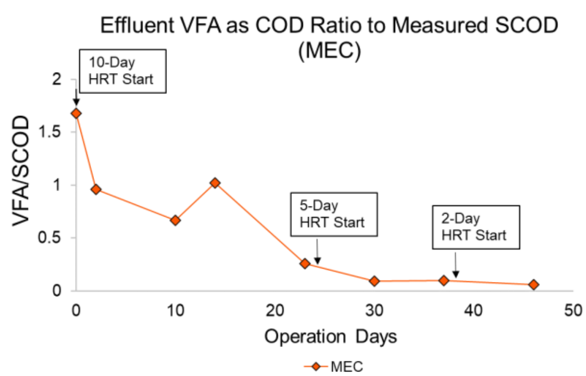
Figure 19: (a): 2-Day HRT COD Recovery for MEC **(b):** Current density and influent COD during 2-day HRT operation

In Figure 19 (a) the isocaproate began to build up again as the influent COD increased to about 5,000 mg/L during day 2. However, the fermentation efficiency continued to decrease as washout of higher carbon SCFAs occurred and the lower carbon SCFAs were consumed by ARBs to produce current density during by the third set of the 2-day operation. This is shown in the total COD recovery being solely the coulombic recovery at 15-20% during days 5 and 6. In Figure 19 (b), these lower carbon SCFAs are likely contributing to the higher current density production as the ARBs are utilizing the VFAs as electron donors before building up in the MEC. When the influent organic loading began to increase to nearly 6,000 mg/L, the current density reflected jumping to 2.4 A/m^2.

COD Balance



(a)

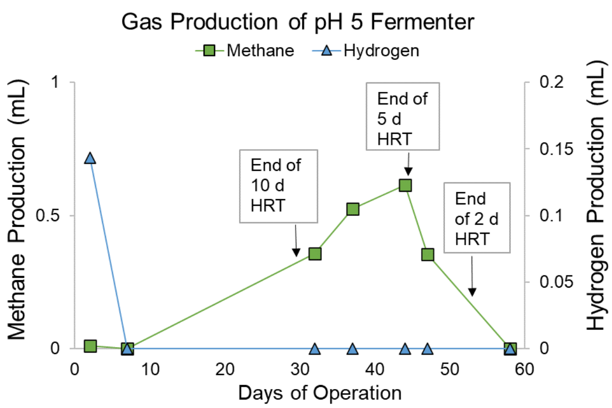


(b)

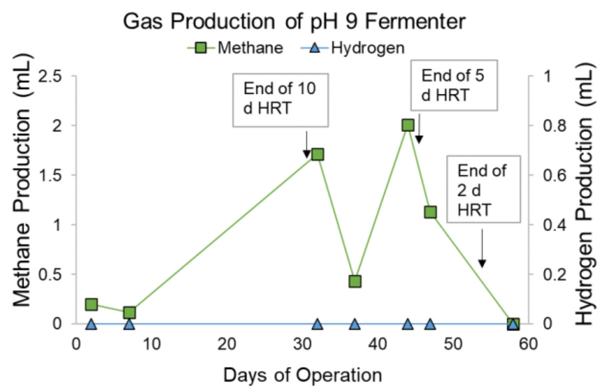
Figure 20: (a) Compared VFA to COD Calculation to Measured SCOD for pH 5 and 9 Fermenters **(b)** Compared VFA to COD Calculation to Measured SCOD for MEC

Figure 20 is the comparison of the calculated VFA as COD values previously used for fermentation efficiency and compared to the measured SCOD value using COD HACH TNT kits. The VFAs contribute to the SCOD fraction as they are organic acids present in the bioreactors and theoretically make up the majority of the SCOD content. As seen in Figure 20 (a), during the 10-day HRT operation, the VFAs likely make up the majority of the SCOD present in both fermenters. However, this changes as we approach 5- and 2-day HRT operations which is likely due to the COD being in the form of carbohydrates and proteins, and not yet converted to simpler soluble materials. The short HRT may not allow the microbes to break down the complex organic matter for VFA conversion in that time frame. This is even more prominent in the MEC operation in Figure 20 (b), during the 5- and 2- day HRT operations.

Gas Data



(a)



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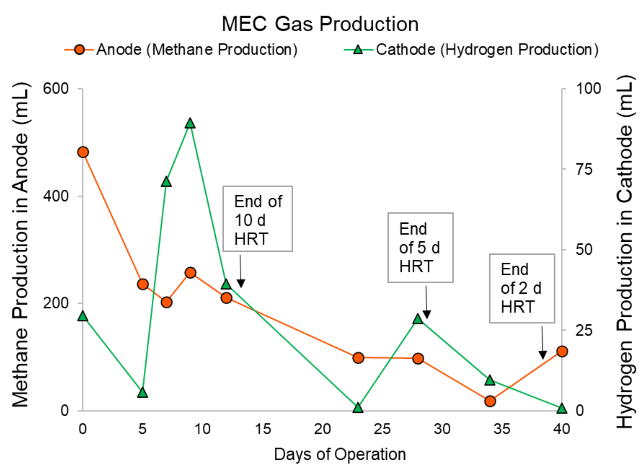


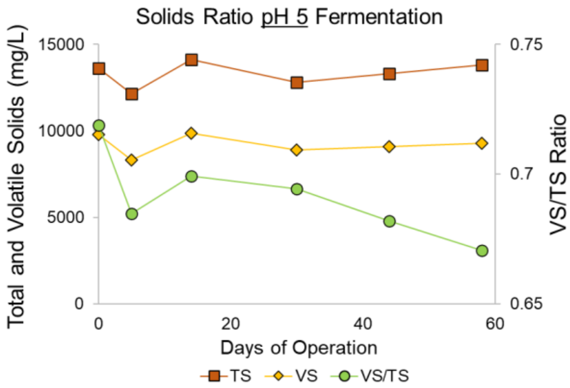
(c)

Figure 21: (a): Gas Production during HRT Operations for pH 5 reactor **(b):** Gas Production during HRT Operations for pH 9 reactor **(c):** Gas Production during HRT Operations for MEC

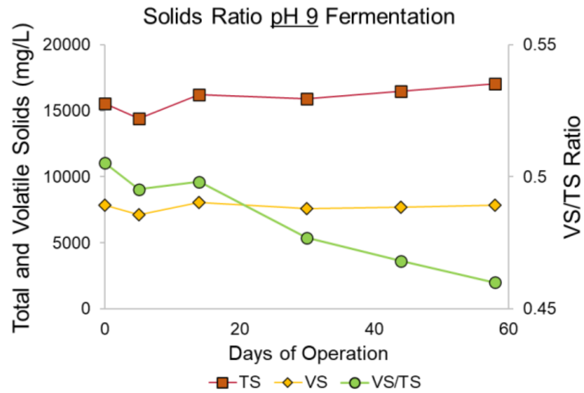
Figure 21 (a) and (b) are the gas production of hydrogen and methane throughout the HRT operations in pH 5 and 9 bioreactors. Both gas productions were significantly low as the reactors were opened daily for wastewater feeding and sparged to maintain anaerobic conditions. Methane production showed a gradual increase up to the end of the 5-day HRT operation which then came to steep decline during the 2-day HRT operation likely due to washout of some gas producing microbes as was seen from the fermentation efficiencies during the same operation. Overall, gas production may be more ideal in shorter HRT operations as longer HRTs tend to produce more methane. The methane production decreased in the 2-day operations for both pH 5 and 9 fermentation.

Figure 21 (c) shows the gas production of methane and hydrogen in the anode and cathode, respectively. In an H-type MEC, the anode will show significantly more methane production as the neutral pH conditions are more favorable for the present methanogens. However, similar to the fermentation reactors, the short HRT may be more beneficial if methane is not the desired production output as the methane production continued to decrease in short HRTs. The cathode will only produce hydrogen as the protons become reduced to hydrogen gas, making this not directly biological hydrogen production. During operation, the hydrogen gas averaged 30 mL per day during the entire operation, which began to decline as the influent COD significantly decreased leading to less VFAs produced or washed out which is less electron donors for the ARBs to produce hydrogen. This is also reflected in the decrease of methane throughout the operation. The beneficial component of the H-type is two valuable gases can be captured in this scenario, where hydrogen can be used as a valuable biofuel without energy intensive technologies such as gasification which requires high temperatures and oxygen to produce hydrogen gas from biomass (DOE, 2021). The methane could continue to be used for electricity production through implementation of a gas-turbine.

Solids Analysis



(a)



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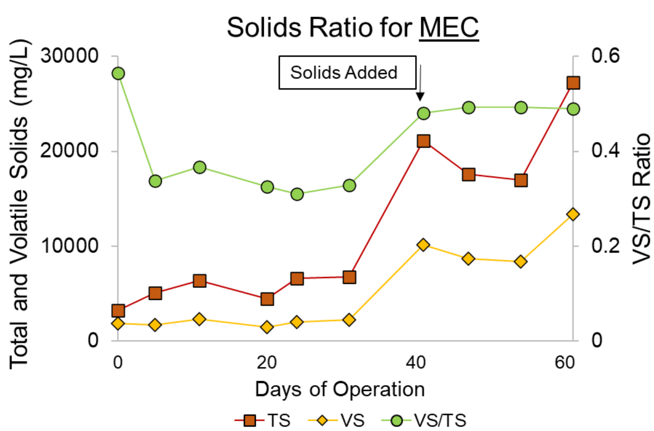


(c)

Figure 22: (a) TS and VS over a second HRT operation under pH 5 conditions **(b)** TS and VS over a second HRT operation under pH 9 conditions **(c)** TS and VS over a second HRT operation for the MEC system

Figure 22 represents the total solids (TS), volatile solids (VS), and the ratio VS/TS throughout all HRT operations in pH 5, 9, and MEC reactors from a second trial operation. The VS/TS ratio is a common indicator for anaerobic digestors to tell you how healthy the system is. The VS portion of the reactors represent the organic matter content with TS containing organic and inorganic material (Wang et al., 2017). A common healthy range for VS/TS is somewhere in the 0.5-1.0 range. In Figure 22 (b), pH 9 has consistently lower VS/TS ratio although the solids in the reactors are higher than pH 5 conditions overall. This may be caused by the increased nature at pH 9 causing more precipitate formation, signifying a lower VS overall. In Figure 22 (c), the MEC overall until additional solids were added which was more settled anoxic sludge. This performance of the MEC was not impacted due to the anode having significant biological growth of ARBs, however the VFA accumulation is affected as that is in the suspension.

pH Adjustment Requirements

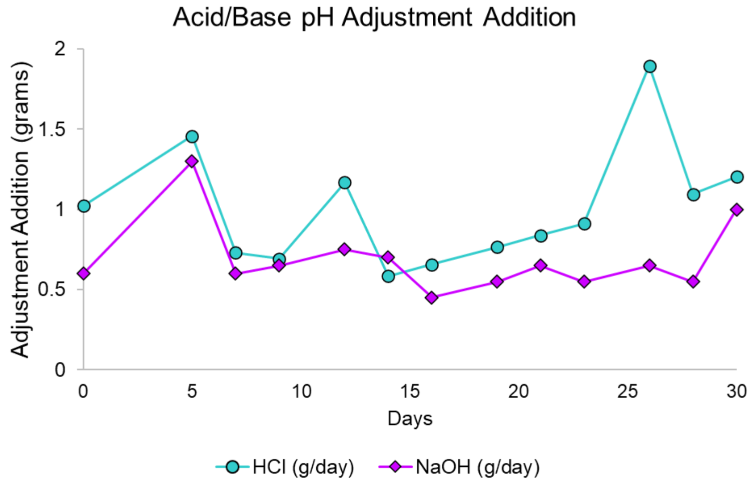


Figure 23: Acid and Base Requirement for Fermentation Reactors

To maintain the fermentation reactors at pH 5 and 9, daily pH adjustments were done using HCl and NaOH. The pH 5 reactor would increase in pH requiring only the HCl and the pH 9 reactor would decrease in pH only requiring the NaOH for adjustments. Overall, the pH 9 reactor required less grams of chemical per day for pH adjustment making this more economically feasible when scaled up. This data is only shown during a 10-day HRT operation meaning this is likely lower than what would be required at 5- and 2-day HRT operations because of the greater amount of water requiring adjustment entering.

DNA Analysis

Genus	Relative Abundance										
	T0 Anoxic Sludge (Fermenters)	pH 5 10-day HRT	pH 5 5-day HRT	pH 5 2-day HRT	pH 9 10-day HRT	pH 9 5-day HRT	pH 9 2-day HRT	T0 Anoxic Sludge (MEC)	MEC 10-day HRT	MEC 5-day HRT	MEC 2-day HRT
Clostridium_sensu_stricto_1	0.00	15.43	20.78	15.53	10.49	12.73	9.81	0.00	7.29	8.58	13.30
uncultured	10.37	10.54	3.88	3.54	25.08	13.10	13.36	7.26	20.13	20.97	11.54
Fastidiosipila	0.03	5.00	0.83	0.36	12.95	14.06	10.58	0.00	3.29	10.65	16.16
U29-B03	0.00	4.82	17.53	14.37	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Unclassified	11.21	3.50	5.22	8.61	5.30	4.27	8.76	19.32	6.44	5.38	4.69
Acinetobacter	1.03	2.68	2.14	3.31	0.96	6.55	9.72	0.47	0.33	0.00	0.00
Terrisporobacter	0.00	4.45	5.23	3.67	1.53	2.05	1.78	0.00	1.94	2.65	3.14
Parabacteroides	0.00	3.59	6.78	6.25	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Pseudomonas	0.00	1.09	1.64	3.26	2.47	3.40	2.04	0.07	0.16	1.74	2.02
Sarcina	0.06	3.03	2.91	2.25	1.84	1.94	1.67	0.00	0.57	0.96	1.87
Proteiniphilum	0.00	6.19	1.76	1.30	1.70	0.94	1.17	0.00	0.90	0.91	1.14
Turicibacter	0.06	1.76	3.31	1.53	1.47	2.41	1.67	0.00	2.48	4.15	9.46
Romboutsia	0.00	1.41	2.20	1.35	0.81	1.35	0.85	0.13	0.89	1.55	2.35
Christensenellaceae_R-7	0.00	1.33	0.37	0.50	2.43	2.12	1.46	0.00	5.19	5.71	4.10
Bacteroidetes_vadinHA17	0.00	0.58	0.03	0.18	0.26	0.40	0.62	0.00	4.58	5.71	4.10
Candidatus_Cloacimonas	0.05	0.85	0.18	0.12	0.82	3.81	1.57	0.00	0.92	0.29	0.33
W5053	0.00	0.63	0.12	0.13	1.92	2.32	1.70	0.00	0.70	1.00	0.85
UCG-004	0.00	1.05	2.64	2.24	0.00	0.00	0.00	0.00	0.03	0.00	0.00
Tissierella	0.00	0.49	0.00	0.00	2.45	1.37	0.60	0.00	0.00	0.00	0.00
DMER64	0.00	1.13	0.35	0.83	0.46	0.21	1.58	0.00	3.90	1.29	0.28
Fermentimonas	0.00	0.94	0.24	0.29	0.51	0.97	1.14	0.00	0.00	0.00	0.00
Anaerovorax	0.00	0.15	0.20	0.17	0.91	0.34	0.66	0.29	1.80	0.44	0.48
NK4A214_group	0.00	2.05	1.23	0.69	0.00	0.00	0.00	0.00	0.05	0.00	0.00
WCHB1-41	0.40	0.00	0.29	1.03	0.49	0.53	1.06	0.23	0.42	0.54	0.61
Alkaliphilus	0.00	0.33	0.00	0.08	0.29	0.30	0.17	0.00	0.00	1.46	1.67
Mariniphaga	0.00	0.21	0.04	0.05	0.61	1.67	0.94	0.00	0.30	0.98	1.21
Smithella	0.00	0.15	0.15	0.12	0.00	0.00	0.18	0.00	2.20	0.80	0.45
MBA03	0.00	0.37	0.00	0.00	1.16	0.29	0.12	0.00	0.00	0.00	0.00
mle1-27	6.15	0.00	0.00	0.00	0.00	0.00	0.00	1.01	0.00	0.00	0.00
Oscillibacter	0.00	0.95	0.76	0.36	0.00	0.00	0.00	0.57	0.00	0.00	0.00
Haliangium	9.50	0.00	0.00	0.00	0.00	0.00	0.00	2.48	0.00	0.00	0.00
Aquabacterium	0.12	0.00	0.11	0.53	0.00	0.47	1.32	0.00	0.37	0.06	0.00
Sutterella	0.00	1.18	0.50	0.20	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Muribaculaceae	0.00	1.00	0.35	0.25	0.00	0.00	0.00	0.16	0.00	0.00	0.00

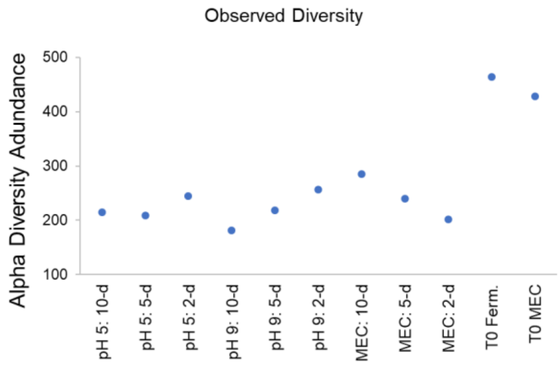
Figure 24: Relative Abundance from the high throughput sequencing analysis for all HRTs.

Figure 24 shows the relative abundance of the different bacterial genus present in the fermenters and MEC. There was a strong correlation of pH preference to the enriched microbes between the pH 5 and 9 reactors. The highest relative abundance in both pH 5 and second highest for pH 9 conditions in all HRT operations was *Clostridium sensu stricto 1* at 17.3% and 11.0%, respectively. Bacteria in the *Clostridium sensu stricto* genus are facultative microbes meaning they are capable of fermenting both carbohydrates and proteins with a preference in the production of butyrate, making them versatile and dominant fermentative microbes (Deng et al., 2022). Studies have shown a more acidic preference for microbes in this genus with their results showing the highest relative abundance at pH 5.5 (Liu et al., 2023). In all HRT operations, pH 5 remained slightly higher in butyrate and iso valerate which may contribute to the presence of U29-B03, which did not show a relative abundance in the inoculum and was likely enriched from the swine waste influent. The U29-B03 genus favors the deposition of short and medium chain fatty acids which may have contributed to the slight increase of the higher carbon fatty acids in pH 5 (Si et al., 2023). The third highest abundance genus was *Parabacteroides* which is a fermentative microbe that favors the deposition of acetate and propionate through carbs and protein fermentation (Lei et al., 2021). The U29-B03 and *Parabacteroides* microbes showed a strong acidic preference as these bacteria did not show a relative abundance in pH 9.

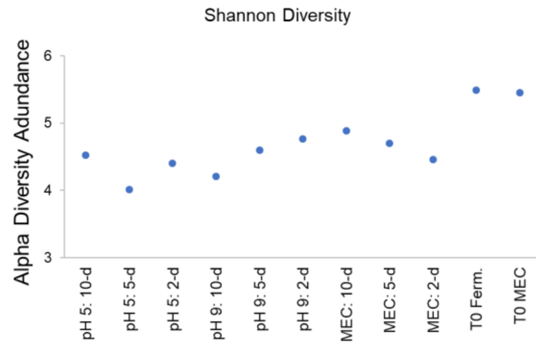
The highest relative abundance in pH 9 reactors in all HRT operations was *Fastidiosipila*. Bacteria from the genus *Fastidiosipila* are strictly anaerobic and have been shown in previous studies to convert proteins and carbohydrates primarily to acetic and butyric acids (Koo et al., 2019; Moestedt et al., 2020). In the pH 9 fermentation reactor, lactate had continually appeared throughout all HRT operations although continued to decrease as washout occurred during lower HRTs. In Figure 17, bacteria in the genus *Tissierella* maintained presence in the pH 9 reactor as it decreased in the pH 5 to being undetectable. *Tissierella* produces lactate and adapts to alkaline conditions which is likely the source of lactate throughout the pH 9 operations (Guan et al., 2021; Duan et al., 2019).

In Figure 24, the genus *Alkaliphilus* was enhanced likely through the inoculum containing a low relative abundance (<0.01%) as the fermenters contained low percentages of this genus as well. Bacteria in this genus are considered to be exoelectrogenic bacteria and in MECs, can live on the anode surface to utilize simple substrates to transfer electrons (Xu et al., 2022; An et al., 2023). Bacteria from the genus *Pseudomonas* are another important electroactive bacterium that became enhanced within the MEC and carry out significant electron transfer with an electrode present (Obata et al., 2020). Members of *Pseudomonas* produce compounds such as phenazines, which are involved in promoting electron transfer in MECs (Rhodes et al., 2021). The overall highest relative abundance present in the MEC throughout the operations was *Fastidiosipila* and became enhanced up to 16.1% in the 2-day HRT operation. The abundance of *Fastidiosipila* has been shown to be contributed to VFA production which services as electron donors for the ARBs (Zhu et al., 2023). The highest relative abundance difference with the pH 5 and 9 fermenters within the MEC is *Bacteroidetes vadinHA17* and *Christensenellaceae R-7*, which are commonly found in anaerobic fermentation methanogenic systems, which is expected to see more of these bacteria as neutral pH is ideal for methanogens (Wang et al., 2019; Saheb-Alam et al., 2019).

Alpha diversity



(a)



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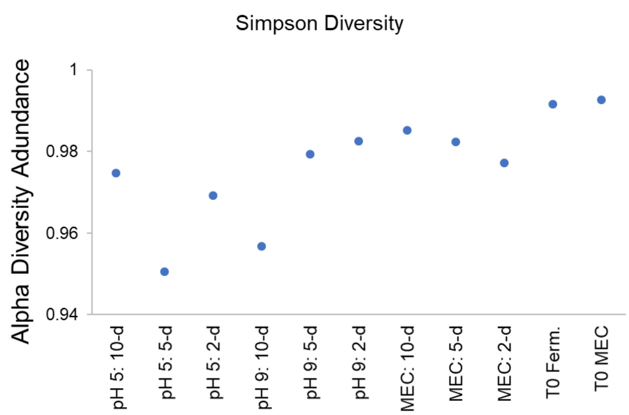

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Figure 25: (a) Total Amplicon Sequence Vs Observed in Each Sample (b) Shannon's Diversity Index (c) Simpson's Diversity Index

Figure 25 shows the alpha diversity of pH 5 and 9 HRT operations, MEC HRT operations, and the initial sludge samples used for inoculation. The alpha diversity of a system is commonly known as the species richness or the number of species present in an ecosystem (Andermann et al., 2022). Figure 25 (a) is the observed diversity which is the total amplicon sequence variants (ASV), which refers to sequences of the microbes present that differ from each other by a single nucleotide (Knecht 2021). Figure 25 (b) and (c) represent the alpha diversity using Shannon's and Simpson's diversity indices. Shannon's index measures uncertainty about identifying species in the sample and Simpson's method measures a probability that two individuals drawn from the sample will be different species (Roswell et al., 2021). The Shannon index also specifically stresses the number of different species while Simpson's method more so emphasizes the count of individuals of each species in a sample (Nagendra, 2002). Shown in Figure 25 in all diversity measurements, the diversity in the sludge inoculum is significantly higher than the diversity of the samples from the fermentation and MEC experiments. This shows the enrichment of similar species during the fermentation experiments, which are fermentative microbes.

Conclusions

This study demonstrated the VFA accumulation that can occur when methanogens are inhibited using pH and HRT as optimizable parameters for swine wastewater fed and anoxic sludge inoculum bioreactors. Implementing a fermentative environment within a MEC which included a bioanode, showed the syntrophic relationship that exists between fermentative microbes and ARBs. The overall fermentation efficiencies between pH 5 and 9 were 52.6% and 61.7%, respectively. While the overall fermentation efficiency remained low for the MEC at around 6 % because the VFAs were being consumed by the ARBs and being converted into current density. The average current density from 10-, 5-, and 2-day HRT operations were 2.2, 0.51, and 1.1 A/m^2, respectively. Fermentation efficiency and current densities dropped from low influent COD and higher HRTs which made washout occur in all reactors. From DNA analysis, we saw differing microbial communities showing the enhancement of bacteria through pH and a bioanode presence. In pH 5 *Clostridium sensu stricto 1* was at the highest relative abundance in all operations (17.3%) and second highest for pH 9 (11.0%). Bacteria from the genus U29-B03 was only present in pH 5 at the second highest average relative abundance of 12.2%. In the MEC there was a mix of bacteria capable of electron transfer, fermentative and methanogenic microbes given the neutral pH conditions. The two genus the became enhanced that are capable of electron transfer were *Alkaliphilus* and *Pseudomonas*, getting enhanced from <0.01% to 1.67% and 0.16% to 2.02%, respectively. The highest overall relative abundance in the MEC was *Fastidiosipila* at 10.0%.

Chapter 4 - Future Work and Conclusions

Future Work

Geoalkalibacter Species for Enhanced Microbial Electrolysis Cell

As previously mentioned, Geobacter is known to be one of the main bacteria capable of electron transfer in an MEC and to perform direct electron transfer from organic substrates to an anodes surface. To maximize current density and hydrogen gas yields, we can perform pure culture studies in an MEC that shows maximum recoveries and reduces losses to methanogens (Call et al., 2009). Different species have begun growing popularity for their abilities to produce high current densities in alkaline conditions. One of these species being *Geoalkalibacter ferrihydriticus*, a soda lake isolate that has been studied for its metal-reducing capabilities (Badalamenti et al., 2013). One study compared the hydrogen production of a pure culture MEC containing *Geobacter sulfurreducens* to a mixed culture MEC and found the two produced similar current densities, although the pure culture MEC showed less hydrogen losses to methane (Call et al., 2009).

Further studies on the enrichment of a pure culture bioanode may be beneficial for producing high current densities and higher hydrogen gas production. This MEC would start out as a pure culture MEC where the *Geoalkalibacter* would be grown on an anode similar to many studies that grow biofilms on an anode initially for the ARB to be present. The alkaline conditions would remain as this is preferred by the *Geoalkalibacter* microbes and the 10-, 5-, and 2-day HRT operations can be compared in current density, hydrogen production, and methane production to this study that was done at pH 7 and purely mixed cultures. This may enhance the utilization of VFAs as alkaline conditions promote hydrolysis, prevent losses of hydrogen/VFAs to methane, and gain electricity produced.

Syntrophic Relationships of Chain Elongating and Fermentative Microbes

The focus of fermentation experiments was to study ways to enhance the production of short-chain VFAs from swine wastewater. The main products produced were acetic acid, propionic acid, and butyric acid which are the main intermediate products during fermentation. While these are valuable separated and in a mixed stream, medium-chain fatty acids (MCFAs) have higher economic value and energy density and can be produced from chain elongation (Wang et al., 2020). MCFAs consist of fatty acids containing 6-12 carbons with an unrefined value of $1000/ton and refined value of $2000-$3000/ton (Wang et al., 2020). In a chain elongation process, ethanol is primarily used by chain elongating microorganisms (e.g., *Clostridium kluyveri*) (Roghair et al., 2018). However, several other biochemical processes compete resulting in consumption of ethanol to produce acetate (Roghair et al., 2018). There are a few ways this may be remediated. One study studied the effects of CO_2 as it effects the chain elongating microbes and the ethanol oxidizers, and when CO_2 is low enough this may produce more hydrogen and inhibit ethanol oxidation and to tunnel the ethanol to chain elongating microbes and not lose it to acetate. They found high CO_2 rates produced significantly increased amounts of caproic acid (Roghair et al., 2018). Another implementation of chain elongation with fermentation has studied syngas (H_2/CO) incorporation which can support ethanol production, direct production of MCFAs by species *Clostridium carboxidivorans* or *Eubacterium limosum,* and produce acetate which can be an electron acceptor for chain elongation (Baleeiro et al., 2021). In this study they found the presence of hydrogen increased butyrate and caproate production rates.

Future VFA production experiments should better understand how to enhance the production of MCFAs, which would further valorize the carbon present in wastewater. The use of hydrogen and ethanol in fermentative studies can enhance our understanding of incorporating chain elongating and fermentative microbes together. As these biological processes have several pathways, more studies are required for the understanding of possibly syntrophic relationships between the two.

Conclusions

Overall, this study discovered unique VFA profiles within a swine wastewater fermentation study at HRTs of 10-, 5-, and 2-days. This study also had unique findings due to the extended SRT and the lack of solid wasting. In pH 5 *Clostridium sensu stricto 1* was at the highest relative abundance in all operations (17.3%) and second highest for pH 9 (11.0%). The highest relative abundance in pH 9 was an enriched genus that has a preference to alkaline conditions (*Fastidiosipila)*, with an average relative abundance of 12.5%. The most optimal conditions for VFA production in this study were under pH 9 conditions and an HRT likely around 5 days as conditions began to decrease due to non-converted organics to VFAs being increased during the shorter operations however, methane production increased with longer HRTs. A possible remediation for this may be higher biomass concentrations if a lower HRT is preferrable.

For MEC operations, the 10-day HRT left the highest current density at 2.2 A/m^2. Two known bacteria capable of electron transfer (*Pseudomonas* and *Sarcina*), had shown significant enhancement of relative abundance of 1153% and 804%, respectively from the 10-day to the 2-day operations. The mixed culture MEC had shown promising data for electricity production and hydrogen production compared to other mixed culture MECs and further studies should include further enhancements of alkaline conditions and scale-up feasibility and integrating membrane separation technology such as an AnMBR for in-situ and continuous VFA separations.

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