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Breaking the Lignin Barrier with Termite TAV5 Treatment Technology (T4): Biopower and Biofuel from Agricultural Waste

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Breaking the Lignin Barrier with Termite TAV5 Treatment Technology (T^4): Biopower and Biofuel from Agricultural Waste

By

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Abstract

Breaking the Lignin Barrier with Termite TAV5 Treatment Technology (T^4): Biopower and Biofuel from Agricultural Waste

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The University of Texas at Arlington, 2023

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Bioenergy obtained from anaerobic digestion of lignocellulose biomass is one of the promising candidates to help move towards a fossil fuel free future. Agriculture waste has up to 90% lignocellulose content, and the eight leading US crops produce over 500 million tons of residue each year. Unfortunately, lignin is resistant to microbial attack under low oxygen conditions which normally occur in anaerobic digesters, and it often shields lignocellulose components (cellulose and hemicellulose) from decomposition as well. Traditional physical/chemical methods for destroying lignin are typically costly due to energy and chemical requirements and can create toxic intermediates (Rahimi et al., 2020).

Termites are commonly known for their ability to degrade wood, which contains high levels of lignin; microorganisms present in the hindgut of termites are responsible decomposition of lignin in wood. TAV5 (*Termite Associated Verrucomicrobia*) is the fastest-growing microorganism isolated from the hindgut of the *Reticulitermes flavipes termite*, the most widespread subterranean termite in North America. Its genome contains genes associated with methylotrophic competency which code for enzymes that structurally modify lignin (Kotak et al., 2015). TAV5 has been found

to degrade lignin in anaerobic conditions, which is quite unique since most of the pathways for biologically degrading lignin are aerobic. The overall goal of our research is to use TAV5 to destroy lignin and boost methane production from agriculture waste. Specific objectives are:

1. To determine optimal conditions for efficiently growing TAV5 in large volumes for field seeding of waste.

2. To determine the optimal addition of TAV5 to enhance methane production from agricultural wastes.

3. To conduct life-cycle environmental and economic assessments of biogas produced from agricultural waste using T^4 Technology, compared to baseline technologies (biogas produced without T^4 Technology and fossil fuels).

To accomplish the first objective, lab tests were conducted to find growth of TAV5 at different temperatures. Afterwards TAV5 was grown aerobically and anaerobically, and growth curves were developed and verified by using the cell count procedure. To accomplish the second objective, batch-scale reactors (125 mL) with four kinds of agricultural waste (rice straw, corn stover, rice husk and wheat straw) were used to determine the optimal ratios of TAV5 to anaerobic digester (AD) microorganisms to decompose waste and produce methane. Based on the results from batch-scale tests, life cycle analyses were conducted. POWER Tool was used to calculate costs and emissions associated with all the phases of life cycle. Estimates were based on a 20-year life of digester and 2% interest rate was used for cost analysis.

It was found that TAV5 shows limited growth over 37^{o}C and grows more quickly in aerobic conditions, which are less costly. Batch reactors showed that the optimal ratio between TAV5 and

WRRF sludge is 0.3 for corn stover and rice straw, 0.2 for wheat straw and 0.6 for rice husk. A higher ratio of TAV5 to WRRF sludge was needed for rice husk, which could be because it has a higher lignin content compared to other wastes used in this research, or possibly because rice husk was not ground, whereas the other waste streams were ground to have smaller particle size. No correlation was found between the lignin values of the other wastes and the TAV5 ratio, nor between the lignin values and the increase in methane production. Life cycle showed that the revenue generated by extra electricity production more than offsets the cost of growing TAV5. Rice straw showed the highest avoided cost due to higher increase in methane generation, whereas wheat straw showed the lowest increase in methane generation, thus lower avoided cost. Seeding digesters with TAV5 reduced emissions of carbon dioxide and criteria pollutant (NOx, SO_2) emissions, since the increased biogas production could be used to produce cleaner electricity than the average electricity from the power grid. However, VOC emissions increased because biogas itself has a high VOC content (around 50% methane). Rice straw showed the highest reduction in NOx, PM, SO_2 and CO_2 emissions because it generates the highest amount of methane, which in turn produces more clean electricity. Overall, the results show that seeding existing farm digesters with TAV5 will result in higher revenues and lower emissions, making the agriculture sector more sustainable.

Chapter 1: Introduction

1.1. Background: Agricultural Waste

Agricultural wastes can be defined as the residues obtained from production and processing of agricultural products such as crops, fruits, vegetables, meat, poultry, and dairy products (Obi et al., 2016). Generally, agricultural wastes can be divided into four categories i.e., crop residues, industrial processing wastes, livestock wastes, and fruit & vegetable wastes (Pattanaik et al., 2019), as shown in **Error! Reference source not found.**.

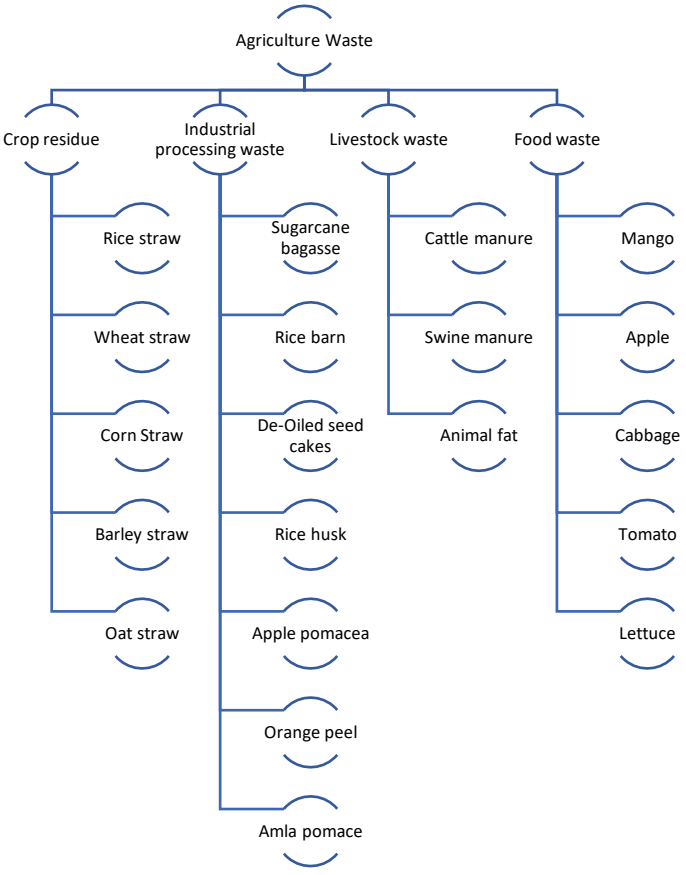

Figure 1-1: Agriculture waste categories defined by (Pattanaik et al., 2019)

Crop residues like straws, leaves, stovers, etc., are mostly generated from direct agricultural production at the field level. These crop residues are usually considered as the most abundant and cheapest source of organic waste and are easily transformable into many value-added products (Pattanaik et al., 2019).

In the past few years, sustainable development concerns in the US and around the world have prompted the increased interest in using crop residues as renewable energy source (Edenhofer et al., 2011; Pervaiz & Hummel, 2022; Ullah & Ali, 2021). It is evident from the fact that the amount of crop residue produced in the world is estimated at 3758×10^6 Mg/year, whereas for the US this number stands at around 488×10^6 Mg/year (Lal, 2005).

Crop residues as a renewable energy source can be used to substitute fossil fuels and contribute towards climate change mitigation. A portion of this residue should be left on the fields to prevent soil erosion, maintain soil organic matter, and enhance soil productivity; however, the U.S. Department of Energy estimates that 104 million tons could be sustainably harvested each year to provide a renewable domestic source of energy (Environmental and Energy Study Institute, 2017). If this energy is used on-farm for electricity, heat, or vehicle fuel (e.g., compressed natural gas or electric tractors or trucks for transport), then the agriculture/food production system becomes more sustainable and efficient, environmentally, and economically — and dependence on fossil fuel, with associated air pollution, is reduced.

1.2. Technologies for energy production from crop residue

Agro-industrial and crop residues are the non-woody biomass which can be used to produce energy. Various technologies can be used to convert agriculture waste biomass for energy generation, as shown in Figure 1-2 (Prasad et al., 2020).

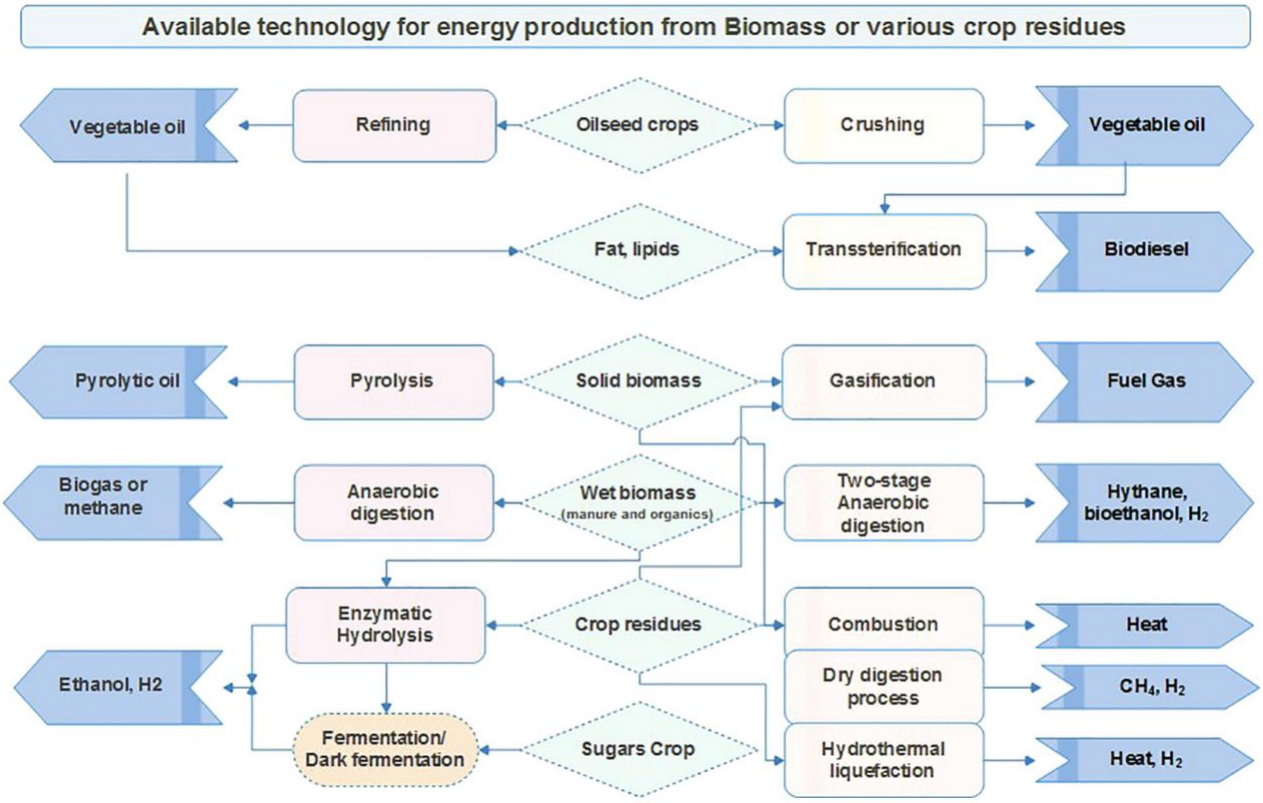

Figure 1-2: Technologies for energy production from agriculture waste (Prasad et al., 2020)

1.3. Termite TAV5 Treatment Technology (T^4)

Three major components of plant biomass include cellulose, hemicellulose, and lignin, as shown in Figure 1-3. Agriculture waste can have lignocellulose content of up to 90% (Saini et al., 2015). Even though lignocellulose biomass is one of the most abundant renewable energy resources, some studies have found that crop residue biofuels are not a viable option, energetically or economically (Andrews, 2006). A significant barrier to converting many agricultural residues to biogas is lignin. Major degradable constituents of lignocellulose — cellulose and hemicellulose — are often shielded by lignin. Most known biological pathways for lignin degradation are, unfortunately,

aerobic, and are not useful under the anaerobic conditions necessary for producing biogas. Traditional physical/chemical methods for destroying lignin are typically costly due to energy and chemical requirements and can create toxic intermediates (Bayer et al., 2007; Bugg et al., 2011; Jang et al., 2013; Kuthi et al., 2016; Lim & Wang, 2013; Millati et al., 2011; Mills et al., 2009; Wen et al., 2015; Zuroff & Curtis, 2012).

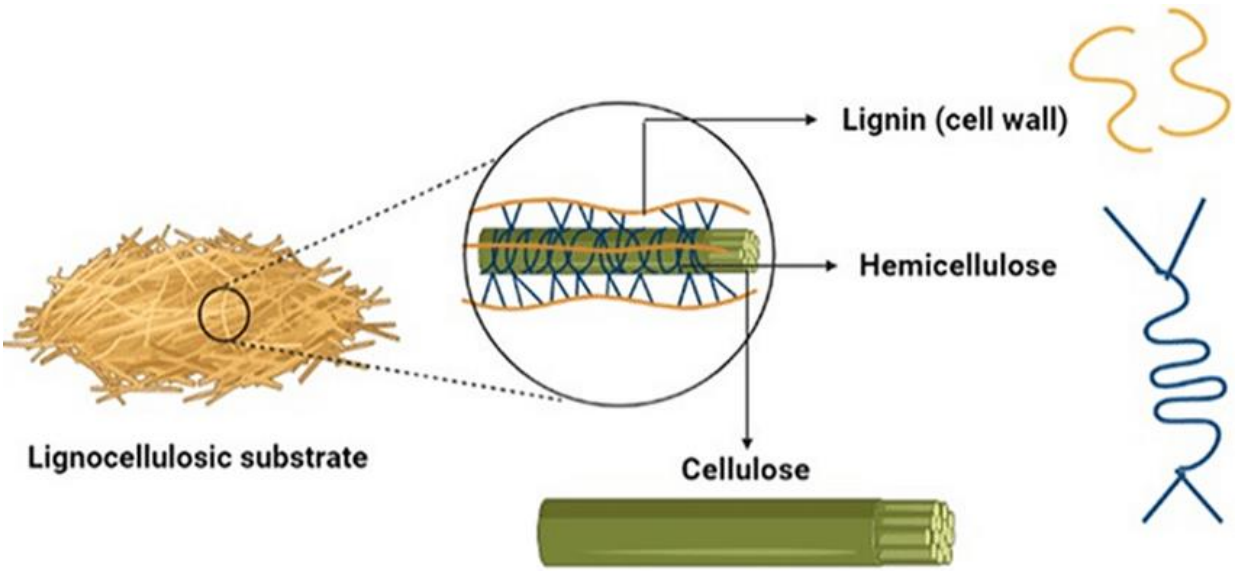

Figure 1-3: Components of biomass (Kuthi et al., 2016)

Although some studies have found that crop residue biofuels are not a viable option, energetically or economically (Andrews, 2006) all biofuels are not created equal. In particular, biogas generated via anaerobic digestion (AD) has advantages from a resource-efficiency perspective compared to liquid biofuels. Biogas yields 2-5 times more fuel per acre than liquid biofuels (Warren Weisman, 2011). The net energy ratio (the ratio of the net energy content of the biofuel to the total fossil fuel input to produce it) has been found to be 8:1 for corn to biogas to electricity, and 15-20:1 for corn to biogas to liquefied natural gas, but only 1-2:1 for corn to ethanol (Bauer, 2015). Anaerobic digestion of sustainably harvested crop residues (104 million tons), without treatment to increase

lignin degradability, would yield an estimated 22 billion m^3 of biogas, with energy content of 480 trillion BTUs. In 2014, the agricultural sector consumed 1,714 trillion BTUs of energy. Thus, without treatment to increase lignin degradability, sustainably harvested crop residues could provide 28% of the energy needs of the agricultural sector. However, this number can be significantly increased by using termite gut microorganisms.

1.4. Research Goal and Objectives

This work proposes a novel approach to break the lignin barrier for agricultural wastes, to produce renewable biogas: seeding with TAV5 (*Termite Associated Verrucomicrobia*), the fastest-growing microorganism isolated from the hindgut of Re*ticulitermes flavipes termite*, the most widespread subterranean termite in North America. TAV5 can grow in low levels of oxygen present in anaerobic digesters (AD). With use of TAV5, sustainably harvested crop residues (104 million tons per year) could provide 18% to 276% (based on type of agriculture waste) more of the energy needs of the agricultural sector. This assumes an increase of 18% to 276% in methane production due to TAV5, based on lab tests conducted for corn stover, rice straw, wheat straw and rice husk.

Our Termite TAV5 Treatment Technology (T^4) is innovative in using microorganisms themselves for lignin degradation in an anaerobic reactor, rather than a separate biological aerobic pre-treatment for lignin, or enzyme addition to the anaerobic reactor. Unlike other microorganisms able to degrade lignin (including the popular white rot fungi), TAV5 can grow in low levels of oxygen present in anaerobic digesters. This eliminates the need for a separate aerobic pre-treatment step, which would increase costs of processing waste and would convert part of the waste (which could potentially have been converted to methane, (CH_4) to carbon dioxide (CO_2) (Saini et al.,

2015).

The overall goal of this research is to increase the biogas production from agriculture waste without using any pretreatment. Specific objectives are:

1. To determine optimal conditions for efficiently growing TAV5 in large volumes for field seeding of waste.
2. To Determine the optimal addition of TAV5 to enhance methane production from agricultural wastes.
3. To conduct a life-cycle environmental assessment of energy products (heat, electricity, and vehicle fuel) generated from biogas produced from agricultural waste using T^4 Technology, compared to baseline technologies (biogas produced without T^4 Technology and fossil fuels).

1.5. Organization of Dissertation

This dissertation is outlined in the following manner:

- The second chapter provides the literature review for agriculture sector in the US, factors affecting anaerobic digestion process, and methods used to breakdown lignin.
- The third chapter describes the methodologies, experimental setup and laboratory procedures to address research objectives.
- The fourth chapter presents and analyzes the results.
- The fifth chapter summarizes the main conclusions of this study and also includes the recommendations for future work.

Chapter 2: Literature Review

2.1 Introduction

This chapter includes background information on agriculture waste generation and disposal in the US and explores the processes of converting agricultural waste into biogas through anaerobic digestion. It examines the benefits and drawbacks of different technologies currently used for lignocellulose degradation. Moreover, the chapter explores the potential of using TAV5 microorganisms from termite gut to enhance the efficiency of anaerobic digestion and the production of biogas.

2.2 Agricultural waste

The US is a major player in global agriculture, with a large selection of crops such as corn, wheat, and rice. In 2019, there were 2,023,400 farms across the United States, covering 897,400,000 acres of land with an average farm size of 444 acres. Of this land area, 339,900,000 acres were used for crop production. Agriculture and food sector contributed over $1.2 trillion to US gross domestic product (GDP) in 2021 and farms alone provided $164 billion, which is 0.7% of the total US GDP. Agriculture and the food sector also provided 21.1 million jobs, out of which 2.6 million were on-farm jobs alone, and more than 44% of land in the US is used for agriculture. Thanks to favorable growing conditions, US farmers planted 91.7 million acres of corn in 2019, according to the USDA National Agricultural Statistics Service (NASS) (Kassel et al., 2023; USDA, 2019).

The large production of crops in the United States creates a significant amount of crop residue or agricultural waste, which can be used as a feedstock to produce biogas. The eight leading US crops produce over 500 million tons of residue each year (Andrews, 2006).

As of 2022, 331 anaerobic digesters were already operational on livestock farms for converting manure to energy (US EPA, 2022). Agricultural residues could be added to these existing digesters to boost gas production. In addition, the nutrient-rich solid or liquid remaining after AD, called digestate, can be recycled on-farm as a high-quality fertilizer and soil amendment. This reduces the need to buy synthetic fertilizers and creates an even more sustainable agricultural food production system.

2.3 Anaerobic digesters

Anaerobic digestion is a series of complex biological processes, during which microorganisms break down organic materials in the absence of oxygen. During the process of anaerobic digestion, biogas is produced, which mostly contains methane (50-52%) and carbon dioxide (45-47%), along with traces of hydrogen sulfide, nitrogen, carbon monoxide, water vapor, etc. (Hernández & Martín, 2016). To produce and capture biogas, anaerobic digestion process is carried out in a sealed vessel called anaerobic digestor (AD), which can be designed based on organic matter and size requirements. AD can be single-stage batch reactors or continuous flow reactors. In a single-stage batch rector, all the feedstocks are loaded simultaneously into the AD and the complete process of digestion is allowed to occur. After digestion is completed, the AD is then emptied and reloaded with new feedstock. However, in a continuous flow reactor, feedstock is constantly added into the AD and digestate is constantly removed.

After anaerobic digestion process is complete the organic solids that did not degrade are separated from the water. This process of removing suspended solids and other materials from wastewater is called solid separation. The separated organic matter can be used as a fertilizer or soil amendment. This process is typically done by a combination of mechanical, physical, and chemical processes.

In the mechanical process, solids are separated from water by sedimentation, flotation, and filtration. The physical process involves gravity separation, which uses the differences in density and size of particles to separate solids from water. The chemical process involves the use of coagulants and flocculants that bind particles together and allow for their easy removal from the wastewater via gravity separation.

Currently 429 farm digesters are operating in the US and most of them treat dairy, swine and cattle manure with codigestion of mostly food waste and fats, oils and greases (FOG). Out of 429 only 4 use crop residues for codigestion due to presence of high lignin content in this type of waste (US EPA, 2022).

Two types of anaerobic digesters can be found around the US: Wet anaerobic digesters and dry anaerobic digesters. Dry digesters usually have a total solids content of 20 to 40%, whereas wet digesters have total solids content of 10 to 20%. Both types of ADs have pros and cons; however, many studies have found wet digesters to have better economic performance and a more advantageous energy balance. Nonetheless, dry AD offers multiple benefits like shorter retention times and lower water consumption.

These two types of anaerobic digesters are discussed in the following sections.

2.3.1 Wet anaerobic digester

Wet anaerobic digestion (WAD) is the most popular type of AD in the US. It involves submerging organic materials in a liquid solution such as water and then allowing microorganisms to break down the organic material. This process takes place in enclosed tanks, where temperatures can be carefully regulated to optimize the digestion process. Wet anaerobic digesters can handle a wide

variety of organic materials, including animal manure, food waste, and agricultural waste. According to a study by (Angelonidi & Smith, 2015) wet anaerobic digesters have improved energy balance, higher economic performance and are highly efficient at breaking down organic matter. WAD produces higher biogas yield when compared with other anaerobic digestion systems. WAD also produces a nutrient-rich digestate that can be used as a fertilizer. However, WAD requires the addition of extra water, it requires more energy to run and has a lower total solids content than dry anaerobic digestion. It also has a longer retention time, meaning it takes longer for the materials to break down completely.

2.3.2 Dry anaerobic digesters

Dry anaerobic digesters (DAD) involve exposing the organic matter to air in order to dry it out before it is fed into the digester. This type of system is typically used for materials that have a low moisture content, such as straw, wood chips, and sawdust. According to studies by (Kothari et al., 2014; Wang et al., 2023), DAD are particularly well-suited for the treatment of lignocellulosic materials, such as straw, as they are able to break down the complex sugars in these materials more effectively than wet anaerobic digesters, for reasons that are unclear. DAD also has several advantages over wet digesters like lower water content requirement, lower energy requirement for heating and mixing, lower reactor volumes, and relatively easier handling of digestate and higher organic loading rate (OLR). However, DAD are extremely sensitive to operational parameters like inoculum ratio, operating temperature, total solids and premixing. DAD carries a high risk of digester failure therefore proper parameters are needed for this process. Only a few lab scale and pilot scale studies have been conducted for DAD; therefore, only a limited information and data is currently available (Momayez et al., 2019). Research by (Angelonidi & Smith, 2015; Rapport et

al., 2012) found that dry anaerobic digestion systems have a higher capital cost than wet anaerobic digestion systems, but they also have a higher biogas yield per unit of feedstock. However, dry anaerobic digesters are more sensitive to variations in the quality of the feedstock and are less tolerant of impurities than wet anaerobic digesters.

2.4 Biodegradation of agricultural waste inside anaerobic digesters

Four stages of anaerobic digestion process are: hydrolysis, acidogenesis, acetogenesis and methanogenesis, as shown in **Error! Reference source not found.**. All of these stages are explained in the following section.

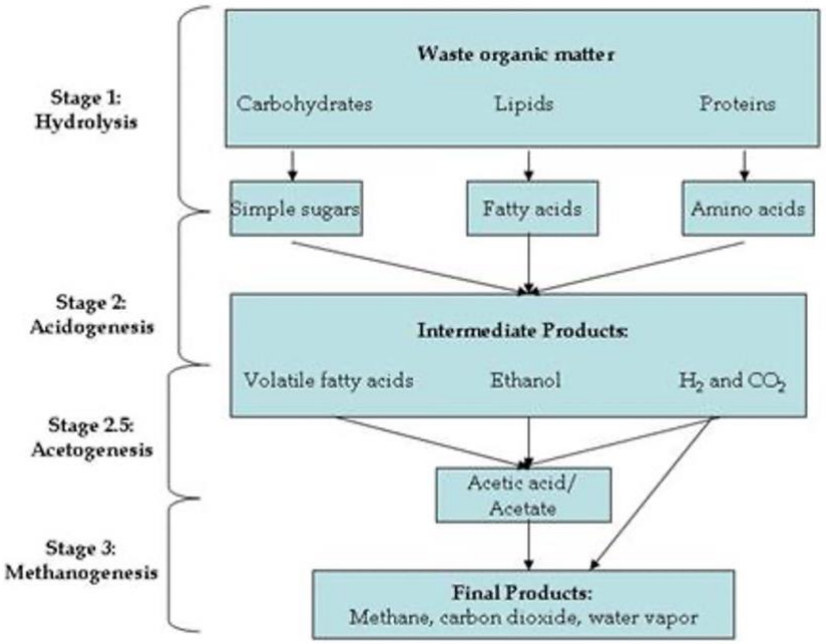

Figure 2-1: Four stages of anaerobic digestion adapted from (Khanal, 2008)

2.4.1 Hydrolysis

The first step of the anaerobic biodegradation process in an AD is hydrolysis, which is the process of breaking down complex molecules into simpler molecules by adding water. During this process, hydrolytic bacteria secrete hydrolases, which convert polymeric organic matter into sugars, ammino acids and long chain fatty acids (LCFA). These facultative bacteria can survive in both aerobic and anaerobic conditions.

Hydrolysis is a rate-limiting step and can slow the rate of overall anaerobic digestion process (Meegoda et al., 2018). The main reason for the slow rate is the overall structure of the feedstock and unavailability of free accessible surface area of particles. Hydrolysis is a key step in anaerobic digestion and is necessary for the subsequent acidogenesis, acetogenesis, and methanogenesis steps. For hydrolysis to be effective, a suitable environment must be provided, as it can be affected by temperature, pH and bacterial density in the inoculum used. Equation 1 shows an example of a hydrolysis reaction:

$$C_6H_{10}O_4 + 2H_2O \rightarrow C_6H_{12}O_6 + 2H_2 \quad (1)$$

2.4.2 Acidogenesis

Acidogenesis is the second stage of anaerobic digestion, during which organic acids and alcohols are produced. It is considered the fastest stage of the overall anaerobic digestion process. The primary products of this step include acetate, propionate, butyrate, valeric, formic, lactic, hydrogen, carbon dioxide and ammonia. Hydrogen, carbon dioxide and acetic acid produced during this phase will then be utilized by methanogens in the final stage of anaerobic digestion to produce methane Volatile fatty acids (VFA) production creates direct precursors for the final stage

of anaerobic digestion, since VFA acidification is a quite common cause for AD failure. Equations 2, 3 and 4 show examples of the steps involved in acidogenesis process for one particular organic (Bajpai, 2017):

$$C_6H_{12}O_6 \leftrightarrow 2CH_3CH_2OH + 2CO_2 \quad (2)$$

$$C_6H_{12}O_6 + 2H_2 \leftrightarrow 2CH_3CH_2COOH + 2H_2O \quad (3)$$

$$C_6H_{12}O_6 \rightarrow 3CH_3COOH \quad (4)$$

2.4.3 Acetogenesis

Acetogenesis, also known as accelerated methane production phase, is the third stage of the anaerobic digestion process. During this step, organic acids such as acetic acid, propionic acid, and butyric acid are converted into acetic acid, which is then used as a substrate for the methanogenesis step. During this process, pH is neutralized and both methanogenic bacteria and acid-producing bacteria develop synergy. Equations 5, 6 and 7 show examples of the reactions occurring during acetogenesis phase (Bajpai, 2017):

$$CH_3CH_2COO^- + 3H_2O \leftrightarrow CH_3COO^- + H^+ + HCO_3^- + 3H_2 \quad (5)$$

$$C_6H_{12}O_6 + 2H_2O \leftrightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (6)$$

$$CH_3CH_2OH + 2H_2O \leftrightarrow CH_3COO^- + 3H_2 + H^+ \quad (7)$$

2.4.4 Methanogenesis

Methanogenesis is the final step in anaerobic digestion in which methane and carbon dioxide are produced. This process is carried out by methanogens, which are obligate anaerobes convert

degradable organic matter into biogas. There are two groups of methanogens that perform this conversion: acetoclastic methanogens and hydrogenotrophic methanogens. Acetoclastic methanogens (70%) convert acetate into methane and carbon dioxide, whereas hydrogenotrophic methanogens (30%) use hydrogen and carbon dioxide to produce methane (Meegoda et al., 2018). Equations 8 and 9 show the reactions for methane production (Lohani & Havukainen, 2018).

$$CH_3COOH \rightarrow CH_4 + CO_2 \quad (8)$$

$$4CO_2 + H_2 \rightarrow CH_4 + 2H_2O \quad (9)$$

2.5 Factors affecting biodegradation in AD

The efficiency of the anaerobic digestion process can be severely affected even by the slightest change in factors and conditions required by microbes to perform various activities. Of these factors, the major ones are discussed below.

2.5.1 Temperature

Temperature highly affects methane production in AD. Based on the temperature, bacteria can be divided into three groups: psychrophilic (below 20 °C), mesophilic (20–45 °C), thermophilic (55–70 °C) (Lohani & Havukainen, 2018). Mesophilic ADs are most commonly used, since most of the methanogens exist in that range. However, it has been found that optimum temperature for hydrolysis is thermophilic, whereas for methanogenesis it is mesophilic. Hence, two-stage anaerobic digestors have been witnessed to produce more biogas.

2.5.2 pH

One of the most important factors that controls the bacterial activity in an AD is pH. Acidogens exist at pH 5.5-6.5, whereas methanogens prevail at 7.8-8.2. Therefore, a pH of 6.8 to 7.4 is considered an optimum range for anaerobic digestion (Lohani & Havukainen, 2018; Meegoda et al., 2018; Wang et al., 2023). pH levels outside of this range can inhibit the growth of microorganisms and slow down the biodegradation process. Over- acidification of AD can be controlled by using additives like enzymes, trace elements, alkali reagents, minerals, inorganic waste, carbon-based conductive materials, etc.

2.5.3 Organic loading rate

The organic loading rate (OLR) is the amount of organic matter added to the digester per unit of reactor volume per day. A high OLR can lead to overloading of the digester and inhibit biodegradation due to accumulation of VFA and other inhibitory compounds, while a low OLR can reduce the overall efficiency of the process. Optimum OLR depends on many factors like retention time, temperature, type of digestion and substrate composition. Therefore, no solid consensus can be found in literature for optimal OLR (Nkuna et al., 2022; Rocamora et al., 2020). **Error! Reference source not found.** shows different OLRs used in literature.

Table 2-1: Organic loading rates for corn stover and wheat straw

Waste type	**Type of digestion**	**OLR (kg VS/m^3/d)**	**Methane yield (m^3/kg VS_{fed})**	**References**
Corn stover	Dry	106.1	0.13	(Rocamora et al., 2020)
Corn stover	Wet	30	0.2	
Wheat straw	Dry	106.1	0.12	
Wheat straw	Dry	14.5	0.11	

2.5.4 Nutrient availability

The availability of nutrients, such as nitrogen and phosphorus, is critical for the growth of the microorganisms responsible for biodegradation. A deficiency in these nutrients can inhibit the growth of the microorganisms and slow down the biodegradation process. (NNFCC, 2023) found that at least 2.3 - 4.2 kg/ton of nitrogen and 0.2 - 1.5 kg/ton of phosphorous is required. However, a wide range of values are available in literature based on different types of substrates.

2.5.5 Microbial community

The microbial community present in the anaerobic digester can also affect biodegradation. A diverse microbial community is generally more effective at biodegrading a wide range of organic matter, while a less diverse community may have a lower biodegradation rate.

2.5.6 Inhibitors

Certain compounds present in the feedstock such as heavy metals, toxic compounds, and antibiotics can inhibit the growth of microorganisms and slow down the biodegradation process. Table 2-2 shows the maximum recommended concentrations for various toxic elements in ADs.

Table 2-2: Maximum recommended concentrations of toxic substances in anaerobic slurries adapted from (OLGPB, 1978)

Constituent	**Maximum Recommended Concentration**
Ammonia (NH_3)	1500-3000 mg/L
Calcium (Ca)	2500-4500 mg/L
Chromium (Cr)	200 mg/L
Copper (Cu)	100 mg/L
Cyanide (CN^{--})	<25 mg/L
Magnesium (Mg)	1000-1500 mg/L
Nickel (Ni)	200-500 mg/L
Potassium (K)	2500-4500 mg/L
Sodium (Na)	3500-5500 mg/L
Sodium chloride (NaCl)	40,000 ppm
Sulfate (SO_4^{2-})	5000 ppm

2.5.7 Trace elements

Microbial growth in ADs also depends on the availability of trace elements like cobalt (Co), Zinc (Zn), iron (Fe) and nickel (Ni). It has been found that Fe reduced the amount of hydrogen sulfide by 65% and also reduces the accumulation of VFAs. High concentrations of VFAs have been found to be toxic for AD microorganisms(Choong et al., 2016; Zhao et al., 2022). Cobalt deprivation can result in lower methanogenic activity and Ni is required for the growth of methanogens.

2.5.8 C/N ratio

An optimized carbon to nitrogen (C/N) ratio is crucial for enhanced biogas production in ADs. A C/N ratio between 15:1 to 30:1 is the most balanced to best run the AD. A lower C/N ratio results in ammonia accumulation, resulting in significant reduction in biogas production. A high C/N ratio means insufficient supply of nitrogen for proper call functioning, resulting in limited microbial growth, causing lower biogas production. C/N ratio can be adjusted using nutrients like ammonium sulfate, crop residues, etc.

2.5.9 Mixing

Mixing is an important factor, as it homogenizes the incoming feedstock with digester microorganisms and also keeps the solids suspended. Many studies have been done to find out the optimum mixing speed, intensity, and technology, as listed in **Error! Reference source not found.**-3. No optimal mixing speed can be found in the literature; however, many studies have found that increasing mixing speed does not increase biogas production. An average mixing speed

of 50 to 200 rpm was found to have positive impact on biogas production. It has also been found that intermittent mixing can provide increase in biogas production; however, (Lebranchu et al., 2017) found that reducing intermittent mixing to 15 min/hr can reduce biogas production.

Table 2-3: Impact of different shaking speeds and techniques on methane production

Shaking speed	**Time**	**Summary**	**Reference**
50 to 1500 rpm	Continuous	Noticed no difference in gas production from varying mixing intensity from 50 to 1500 rpm (impeller)	(Hoffmann et al., 2008)
100–200 rpm	Continuous, intermittent	No difference in gas production between 100 to 200 rpm	(Ong et al., 2002)
120 rpm for impeller 50-200 rpm (shaking table)	NA	The best results of mixing were obtained at 120 rpm (impeller) and 50–200 rpm (shaking table). Methane production decreases when the agitation rate increases.	(Hamdi, 1991)
NA	Intermittent	Results show that gas production was the same for (1) mixing for 2 h and non-mixing for 1 h; or (2) mixing for 7 h and non-mixing for 1 hour. Similarly, gas	(Lindmark et al., 2014)

		production was the same with (1) mixing for 10 min per 4 hours; and (2) mixing for 10 min/h.	
0, 60, 90 and 120 rpm	Continuous	Results showed that mixing at 90 and 120 rpm was favorable for hydrolysis and the acidification phase because an abundance of Proteobacteria, Chloroflexi, Firmicutes, Actinobacteria and Bacteroidetes was found.	(Ma et al., 2019)
NA	Intermittent	Results show that methane production was higher in case of intermittent mixing. Methane production rate decreased when the duration of mixing was reduced from 45 min/h to 15 min/h.	(Lebranchu et al., 2017)
140 rpm to 1000 rpm	Intermittent	The effect of mixing intensity on biogas production was analyzed at low speed (140 rpm) and high speed (1000 rpm) in primary sewage sludge.	(Stafford, 1982)

		It was observed that a low mixing speed of nearly 150 rpm maximized biogas production, whereas at a higher speed, i.e., above 700 rpm, gas production was reduced.	

2.6 Composition of biogas

Biogas composition consists primarily of methane and carbon dioxide, but the mixture of gases also includes a series of other substances and sometimes traces of volatile organic compounds (VOCs) and heavy metals (HMs). The primary component of biogas is methane (CH_4), with a typical concentration of 50-75%. Carbon dioxide (CO_2) is usually present in 30-50% concentration, with small amounts of hydrogen (H_2), hydrogen sulfide (H_2S), water vapor, and other trace gases (Li et al., 2019).

Methane is the primary component of biogas and is the most valuable component, as it can be used for energy production in the form of electricity and heat through combustion in a gas engine or gas turbine. Carbon dioxide, on the other hand, is a byproduct of the anaerobic digestion process, but it can be removed from the biogas to increase its methane content and make it more suitable for use as a fuel.

2.6.1 Methane

Methane (CH_4) is a hydrocarbon gas that is composed of one carbon atom and four hydrogen atoms. It is the primary component of natural gas and is also the primary component of biogas produced from anaerobic digestion of organic matter. Methane is a colorless, odorless, and flammable gas that is non-toxic and non-corrosive.

2.6.2 Carbon dioxide

Carbon dioxide (CO_2) is a colorless, odorless, and non-flammable gas that is composed of one carbon atom and two oxygen atoms. In anaerobic digestion, CO_2 is produced as a byproduct of the biodegradation of organic matter by microorganisms.

2.6.3 Hydrogen

Hydrogen (H_2) is a colorless, odorless, and flammable gas that is composed of two hydrogen atoms. Hydrogen can also be produced in anaerobic digesters through the hydrolysis of complex organic compounds by hydrolytic bacteria, which results in the production of simple sugars and hydrogen.

2.7 Major components of agricultural biomass

Agricultural waste generated during harvesting and processing is a lignocellulose rich material. This crop residue biomass waste is inexpensive and can be used as a renewable energy source. Major cultivated crops produce up to 5300 million tons of dry biomass around the globe. This biomass waste can be used to produce bioethanol, biodiesel, biogas, etc. First generation biofuels are produced from food crops like sugarcane and corn, whereas second generation biofuels are

produced using energy crops, agricultural waste, etc. Due to the abundance of agricultural residue, many countries across the world are developing technologies and policies to utilize this waste as a renewable energy source. Plant cell walls present in lignocellulose biomass provide strength and resilience to plants.

Cellulose, hemicellulose, and lignin are three major components of plant biomass. Cellulose is the main structural component of plant cell walls and is composed of glucose units connected by glycosidic bonds. Hemicellulose is the second main structural component of the cell wall and can be broken down into a variety of shorter chain polysaccharides. Lignin is a complex aromatic polymer that acts as an adhesive and provides strength and rigidity to the cell wall. It is composed of phenylpropane units and is cross-linked with other molecules (Collard & Blin, 2014; Houfani et al., 2020; Tarasov et al., 2018). These three components are important for the anaerobic digestion process, as they can be hydrolyzed and converted into biogas. The hydrolysis of cellulose and hemicellulose releases fermentable sugars, which can then be used by anaerobic bacteria to produce biogas. Lignin acts as a binding agent to cellulose and hemicellulose, which helps prevent them from being broken down too quickly and reduces the loss of organic dry matter during the anaerobic digestion process (Börcsök & Pásztory, 2021; Xia et al., 2021). Additionally, lignin can also act as a carbon source for microorganisms during anaerobic digestion (Chufo et al., 2015).

2.7.1 Cellulose

The most prevalent biopolymer found in nature is cellulose. It is the main component of lignocellulose biomass and consists of D-glucose monomers. Cellulose makes up 40 to 55% of total wood dry weight.

2.7.2 Hemicellulose

Hemicelluloses are also sugar polymers and are found next to cellulose. They are the second most important component of plant cell wall and make up 20 to 40% of total wood dry weight. Hemicellulose is most responsible for bio and thermal degradation of the fiber.

2.7.3 Lignin

Lignin is the second most abundant biopolymer and is composed of phenylpropanoid subunits. It is an abundant source of naturally generated aromatics found in nature. Typically, lignin is derived from guaiacyl, syringyl and hydroxyphenyl lignin subunits. Due to the presence of ether linkages, phenylpropanoid and a series of functional groups like benzyl alcohol, ether, methoxy etc., lignin becomes highly resistant to chemical and biochemical depolymerization. It enhances hydrophobicity, strength, rigidity and provides resistance against microbial attack on the plant cell walls. Lignin has higher a potential commercial value as well as higher energy density compared to polysaccharide polymers. However, efficient utilization of lignin is problematic due to its complexity.

Lignin is a vital part of secondary cell walls of plants and makes up 10 to 25% of plant biomass. It is connected to cellulose and hemicellulose via covalent and hydrogenic linkages and protects them from biological degradation as shown in Figure 2-2. Lignin is the only polymer present in plant cell walls that is composed of carbohydrates.

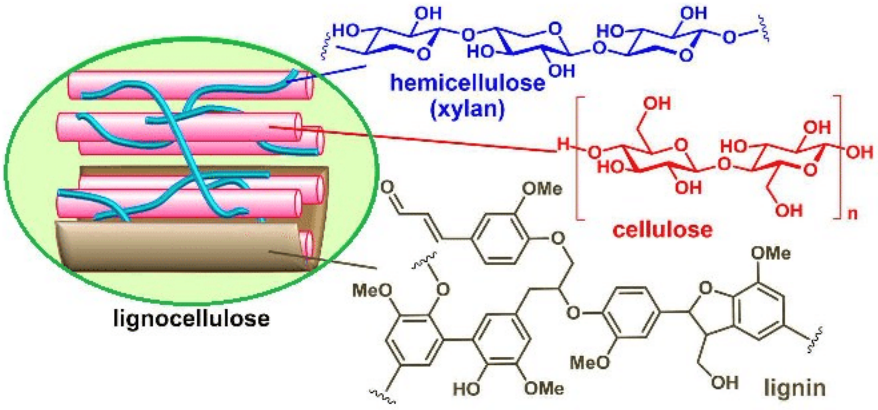

Figure 2-2: Lignocellulose composition (Tang, 2021)

2.8 Characteristics of agricultural waste

The quantity of cellulose, hemicellulose, and lignin in agricultural waste can vary depending on the type of crop and the growing conditions. However, on average, cellulose typically makes up around 35-50% of the total composition of agricultural waste, while hemicellulose and lignin typically make up around 25-30% each. Many studies have been done to characterize agricultural waste by determining the moisture content, volatile solids, cellulose, hemicellulose, and lignin. **Error! Reference source not found.** summarizes values found in agricultural waste based on several studies.

Table 2-4: Characteristics of agriculture waste

Waste	Cellu-lose	Hemi-cellulose	Lignin	Moisture content	Volatile solids	References
Rice husk	35-40%	15-25%	20-23%	4.6%-6.1%	74-82%	(Contreras et al., 2012; Q. Fang et al., 2019; Fathurahman & Surjosatyo, 2022; Ghatak & Mahanata, 2018; Singh, 2018)
Rice straw	35-38%	18-25%	12-24%	6.6-6.9%	75-84%	(Contreras et al., 2012; Dai et al., 2018; Du et al., 2019; Gou et al., 2018; Nguyen Van Hung et al., 2019)
Wheat straw	32-50%	2-45%	11-26%	5-7.8%	87-96%	(Baitha & Kaushal, 2019; Elsayed et al., 2016; Moset et al., 2018; L. Zhang et al., 2022)
Corn stover	37-41%	20-22%	7-18%	6.4%-13.6%	90-99%	(Hu & Yu, 2005; Khan et al., 2021; MAJOR, 2012; Morissette et al., 2013; Zhong et al., 2021)

2.9 Limitations in degrading lignocellulose waste

A limitation in using most microorganisms themselves to degrade lignin is the need for aerobic conditions. The popular white rot fungi, as well as most bacteria that degrade lignin, (*actinomycetes, α-* and *γ- proteobacteria, Rhodococcusjostii sp., Sphingobium sp., Bacillus sp*), do so aerobically, which requires a separate aerobic pre-treatment step (Ali et al., 2017; X. Fang et al., 2018; Muñoz et al., 2014; Seidl, 2009; Wen et al., 2015; Yuan et al., 2014). This separate step increases waste processing costs. An aerobic pre-treatment step also converts part of the waste directly to carbon dioxide, which has no energy value, rather than methane, thus limiting the energy return on investment (Andrews, 2006; Bugg et al., 2011; Zuroff & Curtis, 2012). Various recent studies have also investigated microaerobic pre-treatment of lignin. These studies of various substrates (sisal pulp, starch, brown water, food waste, and corn straw) have found that introducing limited air into an anaerobic digester as a pre-treatment step can increase methane production (Botheju et al., 2010; Charles et al., 2009; Lim & Wang, 2013; Mshandete et al., 2005; Ramos & Fdz-Polanco, 2013). The methane increases from these studies of however, ranged from 10-55%.

One previously used strategy for increasing lignin decomposition anaerobically is addition of enzymes (Botheju et al., 2010; M & A., 2013; Moraïs et al., 2012; R et al., 2011). (Jayasinghe et al., 2011) found manganese peroxidase (MnP) to be the most effective enzyme for increasing methane yield from mixed solid waste. However, MnP is expensive ($6500/g), and enzyme approaches to degrading lignin typically require fresh enzyme addition with each new batch of substrate (Jayasinghe et al., 2011; Rahman, 2018). Enzyme recycling processes are generally yet to be demonstrated under process relevant conditions and have not been scaled up to be economically feasible at an industrial scale (Weiss et al., 2013). In one study that did example

process-relevant conditions, (Weiss et al., 2013) found that hydrolysis yield for cellulose in corn stover could be maintained with 30% enzyme recycling. Addition of 70% of the original enzyme dosage with each new batch of waste, however, would still be costly.

2.10 Lignocellulose waste degradation with termite gut bacteria

Microorganisms found in the gut of termites and ruminants can digest lignocellulose. Many studies have found increased lignin removal and enhanced biogas production using termite gut bacteria. Table 2-5 shows recent studies done on different waste types using termite gut bacteria themselves. However, most of the studies only provided results based on a short period of time (all studies were less than 40 days), do not quantify the increase in methane production, just looked at one type of waste, or used a temperature not representative of AD temperatures.

Table 2-5: Literature review of studies using termite gut bacteria to destroy lignin

Reference	Waste used	Termite bacteria used	Description	Results	Limitations
(Kavitha et al., 2014)	Poultry waste	*Methanosarcina thermophile*	This 30-day study investigated enhancing biogas production using termite gut bacteria.	Study reports high methane concentration of 74.3%, compared to 48% from control.	• Study only lasted 30 days.
(Ben Guerrero et al., 2015)	Sugarcane bagasse, Napier grass	*Nasutitermes aquilinus, Cortaritermes fulviceps*	Study employed native termites to degrade sugarcane bagasse and napier grass for bioethanol production.	Growth of both strains of termite bacteria was found, meaning microorganisms were expressing the enzymes required to breakdown cellulosic substrates.	• Study did not quantify the percentage increase in bioethanol production.

(Auer et al., 2017)	Wheat straw	*M. parvus, T. hospes, N. ephratae and N. lujae*	Four gut microbiomes were used to test the potential for lignin degradation of wheat straw in an anaerobic bioreactor at 35^{o}C. Gas chromatograph was used to monitor composition of gas.	Wheat straw degradation varied from 26% to 49% after 20 days. Highest degradation was achieved by *N. ephratae*.	• Study only lasted for 20 days.
(Ngumah et al., 2017)	Gastroint-estinal contents of beef	*Archachatina maginata, Coptotermes formosanus*	Two strains of bacteria are used to enhance bio methanation of slaughtered beef	Four cases were used in the study: no bacteria, *Archachatina maginata, Coptotermes formosanus* and both bacteria combined. It was found that *Coptotermes formosanus* has the highest production of	• Study was carried out at room temperature; however, AD temperature can range between 30 to 40^{o}C. • Data was recorded for only 60

				biomethane.	days.
(Tsegaye et al., 2018)	Wheat straw	*Ochrobact-rum oryzae and Bacillus sp.*	Wheat straw is biodelignified by *Ochrobactrum oryzae*, which is further hydrolyzed by *Bacillus sp*. Study also compares separate and simultaneous bio delignification and hydrolysis system.	Biodelignification by *Ochrobactrum oryzae* resulted in 44.5% of lignin degradation within 16 days.	• Study did not quantify the increase in biofuel production
(Lazuka et al., 2018)	Wheat straw	Inoculum derived from *N. emphratae*	*N. emphratae* was used to transform lignocellulose into carboxylates under anaerobic conditions.	Sterile and non-sterile wheat straw samples were used in anaerobic conditions, and it was found that degradation of sterile wheat straw was greater.	• Study was only done for 11 days. • Does not quantify methane gas production

(Anukam et al., 2020)	Rice husk	*Morganella morganni*	Identification of lignin-degrading bacteria and potential for lignin, cellulose and hemicellulose degradation	Study was carried out for 30 days and lignin percentage was recorded on day 0, day 15 and day 30, which were 17.4%, 11.3% and 7.3%, respectively.	• Readings were only taken for 30 days • Study does not mention the temperature for optimal results.
(SIMOL et al., 2021)	Four ligninolytic indicator dyes	*Genus Bacillus*	Twenty-seven microbial isolates from *Coptotermes curvignathus* were used to identify presence of ligninolytic activity.	Research found three strains that showed great potential for lignin degradation.	• No waste was used • Study does not quantify actual potential of microbes to reduce lignin.
(Q. Zhang	Corn	*Enterobacter*	Three strains were used for	Out of three bacterial strains	• Study only

et al., 2021)	straw	*hormaechei (KA3)*	lignin degradation in corn straw.	used, *KA3* was found to be the most efficient one. It was found to increase the bio-gas production by 20% and methane production by 31%.	lasted 35 days • Only one kind of waste was used.
(Dumond et al., 2021)	Wheat straw	*N. ephratae, N. lujae, M. parvus, T. hospes*	Study employs 4 termite gut bacteria to degrade lignin under anaerobic conditions for 20 days.	Lignin removal of up to 37% is achieved. Study also proposes that gut microbes partially modified lignin polymer.	• Study was not long-term. • Did not look at effect of gut microbes on methane production
(Danso et al., 2022)	Wheat straw	*Streptomyces*	Study used bacterial species from *Microcerotermes* to find best strain for wheat straw degradation to produce	Out of 21 bacterial strains, *streptomyces* produced highest xylanase and cellulase production. It was	• Optimization of ethanol production was not included • Did not

			bioethanol	found that *MS-S2* can utilize wheat straw as a sole carbon source.	measure the biogas production
(Show et al., 2022)	Rice straw	*Morganella morganii*	Bacteria isolated from wood feeding termite was used to pretreat rice straw for 30 days to reduce the lignin content.	The microorganism was able to reduce the lignin content of rice straw by 53.27%.	• Did not measure the biogas production

2.11 TAV5 (*Termite Associated Verrucomicrobia*)

TAV5 (*Termite Associated Verrucomicrobia*) bacteria have great potential and advantage over fungi in the biotreatment of lignocellulose biomass because of their faster growth rate. It is the fastest-growing microorganism isolated from the hindgut of *Reticulitermes flavipes* termite and it is the most widespread subterranean termite in North America. TAV5 can grow in low levels of oxygen and is capable of degrading lignocellulose waste and converting it into useful products. TAV5 bacteria possess a unique suite of enzymes such as lignin peroxidase, laccase, and manganese peroxidase, which are responsible for lignin degradation. They also have multiple glycoside hydrolases which can hydrolyze different types of polysaccharides such as cellulose and hemicellulose. This makes them ideal for the biotreatment of lignocellulose waste. TAV5 bacteria also offer a sustainable alternative to surmount the drawbacks linked with conventional chemical catalysts (Saini et al., 2015).

(Rahimi et al., 2020) demonstrated that the addition of TAV5 resulted in removal of 46%, 34%, and 20% more acid soluble lignin from paper, yard, and wood waste, respectively, compared to reactors seeded with AD microorganisms only. It is significant that TAV5 was able to increase methane production when mixed with AD microorganisms: a pure culture did not have to be maintained (as has been done in some previous studies), which would be difficult if not impossible under field conditions involving waste (He et al., 2017; Xu et al., 2018). (Rahimi et al., 2020) study did not, however, test agricultural wastes.

2.12 Summary of previous studies and research objectives

Lignin is a major constituent of agriculture waste which does not degrade easily in anaerobic condition found in ADs. It severely limits biogas production, which in turns results in lower

production of vehicle fuel or electricity. Currently available methodologies to destroy lignin, i.e., mechanical, thermal, and acid/ base treatments, are generally expensive, energy- intensive, and can create toxic compounds that reduce biogas production. Biological treatment strategies generally fall into 2 categories: use of microorganisms themselves, and use of enzymes (Zabed et al., 2019).

Enzymes require addition with each new batch of waste, but termite gut bacteria have been found to regrow on their own. Recently many studies have investigated the use of different termite hind gut bacteria, but no study has looked into finding the optimal ratio of inoculum bacteria and TAV5 microorganisms for agricultural wastes like corn stover, rice straw, wheat straw and rice husk.

The overall goal of this study is to increase the biogas production from agricultural waste. Specific objectives are:

1. Determine optimal conditions for efficiently growing TAV5 in large volumes for field seeding of waste.
2. Determine the optimal addition of TAV5 to enhance methane production from agricultural wastes (corn stover, rice straw, wheat straw and rice husk).
3. Conduct a life-cycle environmental assessment of energy products generated from biogas produced from agricultural waste, compared to baseline technologies (biogas produced without TAV5 seeding from ADs).

Chapter 3: Methodology

3.1 Overview

Error! Reference source not found. provides an overview of the methodology. The following sections discuss the methods used to accomplish each objective in more detail.

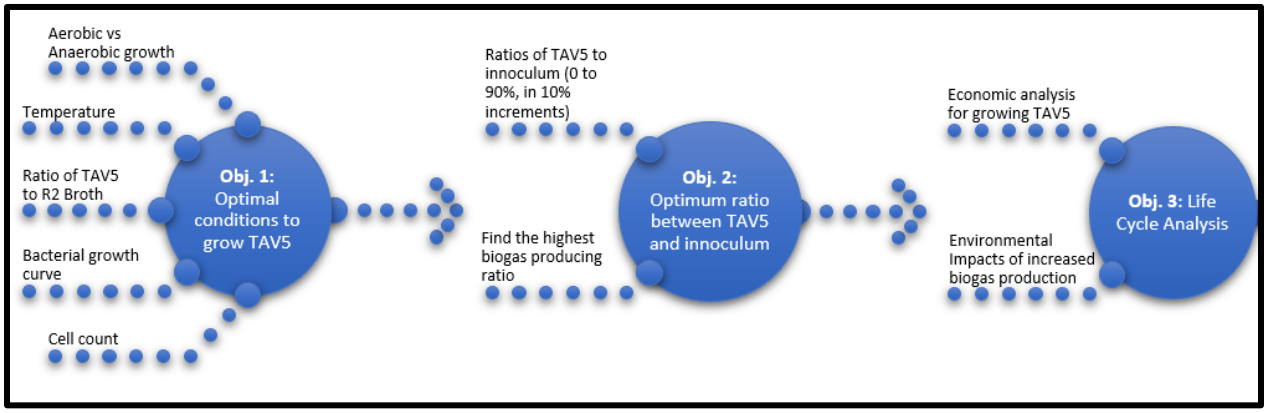

Figure 3-1: Summary of methodology

3.2 Methods to Address Obj. 1: Determine the optimal conditions to grow TAV5 in large volumes

3.2.1 Culturing TAV5

For culturing TAV5, R2A agar plates were prepared by mixing 18.2 g of R2A agar powder per 1 L of deionized water and autoclaved at 121°C. After sterilizing the mixture, it was poured onto petri dishes and was given an hour to solidify. Figures 3-2 and 3-3 show the autoclave and clean bench used to prepare culture plates.

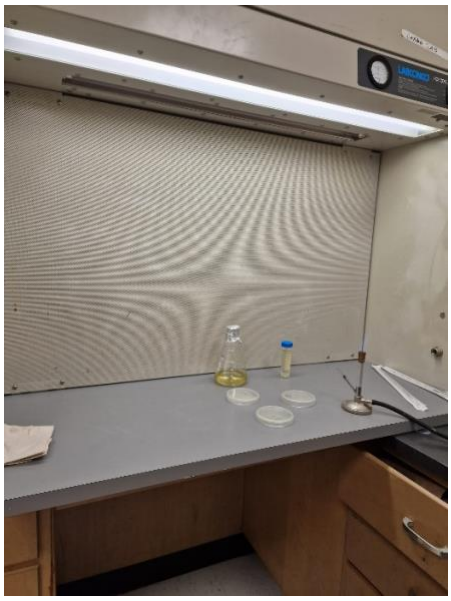

Figure 3-2: LABCONCO clean bench used to streak TAV5

Figure 3-3: Autoclaving R2A Agar

Petri dishes were then wrapped with parafilm and stored in the refrigerator. TAV5 obtained from Dr. Jorge Rodrigues UC Davis was streaked onto the petri dishes and was grown aerobically at

room temperature (around 24°C). Almost a week was given for TAV5 cells to grow on the petri dishes. After growing TAV5 on petri dishes aerobically, it was then again cultured in an anaerobic chamber (2% oxygen) because TAV5 is adapted to low-oxygen conditions of the termite gut (2-4% O_2 around the gut wall) at 30°C to check if it could grow anaerobically. The growth on the plates was observed after a week.

After growing TAV5 on petri dishes it was then shifted into sterilized plastic vials containing R2B Broth as shown in Figure 3-4. R2B broth was prepared by mixing R2B broth powder with deionized water at a ratio of 3.2 g per 1 liter of water and then autoclaving at 121°C.

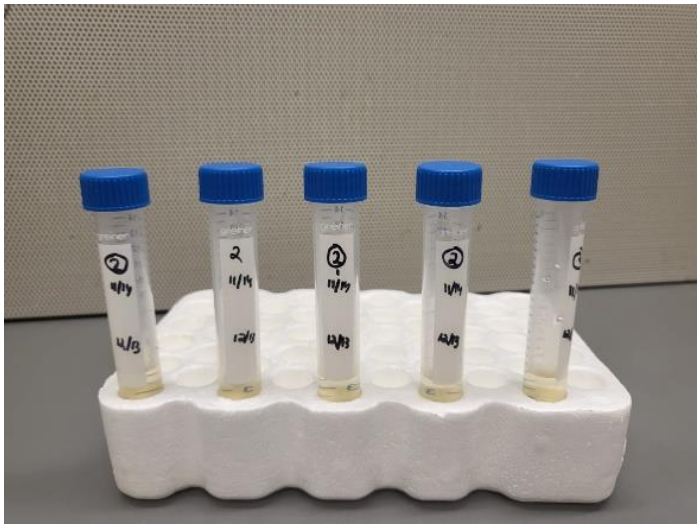

Figure 3-4: TAV5 in sterilized plastic vials

After moving TAV5 into R2B broth solution, the cultures were placed inside an incubator shaker at 30°C and a speed of 150 rpm. Initially the growth was measured by observing turbidity vs. a high contrast background, as shown in Figure 3-5.

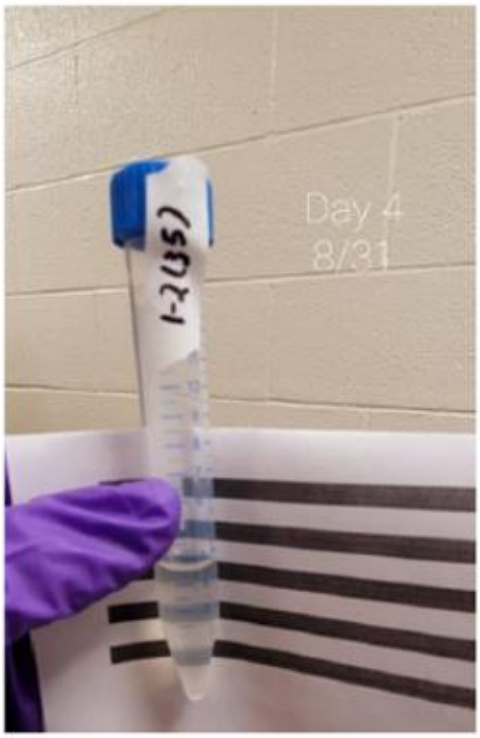

Figure 3-5: Initial growth assessment by turbidity vs. a high contrast background test

3.2.2 Comparing rates for aerobic vs. anaerobic growth

After growing TAV5 aerobically on petri dishes, its growth rate was compared in anaerobic conditions. For this purpose, TAV5 was grown on petri dishes inside an anaerobic chamber (Sheldon Bactron IV microaerophilic/environmental chamber) for one week and the growth was analyzed visually by counting the number of colonies.

To measure the growth rate of TAV5 in R2B broth, glass tubes were used. To simulate anaerobic conditions, Hungate anaerobic glass tubes (Avantor, Catalog no. 89167-170) were used, as shown in Figure 3-6. The rubber septa used in these glass tubes are sealed by plastic caps which keep these tubes anaerobic.

Figure 3-6: Hungate anaerobic glass tubes

Growth curves were measured every 8 hours until 120 hours at an optical density of 600. TAV5 cells (0.5 ml) stored in the refrigerator at 4^{o}C were added to a 3.5 ml of broth solution (1:4) in sterilized glass vials. Hach DR 2800 spectrophotometer shown in Figure 3-7 was used to find the optical density of the culture.

Figure 3-7: Using spectrophotometer to measure optical density of TAV5 culture

The growth curve was obtained to find the lag phase, log phase, stationary phase and death phase, as shown in Figure 3-8.

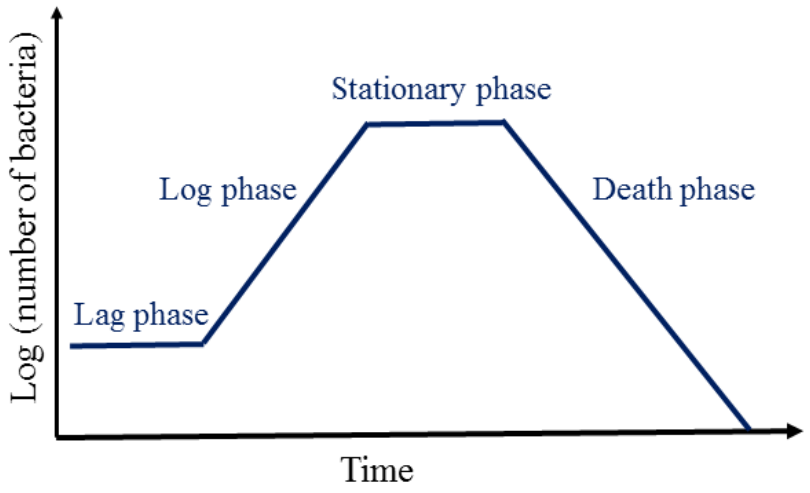

Figure 3-8: Microbial growth curve (Komorniczak, 2009)

3.2.3 Determining the impact of temperature on growth

After growing TAV5 at room temperature (24°C) in petri dishes, the temperature was increased to 40°C. However, no growth was observed after a week of incubation. Therefore, the temperature was gradually increased from room temperature to 40°C (at temperatures of 32, 34 and 37°C) in a constant temperature room. However, growth was observed only up to 37°C, with very slim to no growth observed at 40°C. The highest number of colonies after a week of growth was observed at 30°C.

3.2.4 Determining optimum ratio of TAV5 to R2 broth

The growth rate of TAV5 cells depends on how many cells of TAV5 are added in R2B broth. By adding more cells, the growth rate can be increased; however, to add more TAV5 cells, we need to grow more TAV5 cells. Therefore, to grow TAV5 cells optimally at large scales, different TAV5 to

R2B broth ratios were tested. For this purpose, 2-liter Erlenmeyer flasks were used. Each flask was filled with 1 liter of R2B broth and different ratios of TAV5 were tried varying from (1:33, 1:50 and 1:100). There was little to no growth for 1:100 in four days and very little growth in 1:50 flasks. However, at 1:33 growth was observed with cultures ready to be harvested (0.35 optical density) within 80 hours.

3.2.5 Cell count test

Batch reactors were seeded with TAV5 once it reached around 0.35 optical density. TAV5 cells were grown for three days in R2B broth, and each day the optical density of cultures was measured, and cells were streaked on the petri dishes as well. Since during log phase the cell growth increases exponentially, viable cell count procedure was used to make sure TAV5 cells are alive and growing exponentially at 0.35 optical density. TAV5 cells were mixed thoroughly before starting the procedure, which lasted three days. For this purpose, 1.7 ml microcentrifuge tubes were used as shown in Figure 3-9.

Figure 3-9: Microcentrifuge tube used for cell count

Each tube was labeled as tube 1 through tube 6. Each tube was filled with 0.9 ml of R2B broth and 0.1 ml of TAV5 was added to tube 1 and was mixed. Then 0.1 ml from tube 1 was added to tube 2 and same process was repeated for tube 3 to 6. 0.1 ml of culture was discarded from tube 6 to maintain homogeneity. Afterwards 0.1 ml of culture from each tube was streaked on R2A Agar plates. These plates were incubated at 30°C for about a week. After incubation, each plate was observed with naked eye and visible colonies were marked and counted. Using the number of colonies, colony-forming unit per milliliter (cfu/ml) was calculated. Figure 3-10 shows the petri dish with 10^{-6} dilution plated from a culture with optical density of 0.33.

Figure 3-10: TAV5 on R2A Agar plate at a dilution of 10^{-6}

3.2.6 Storing TAV5 in -80°C freezer

TAV5 cells were stored at -80°C to be used later. For this purpose, 50% glycerol solution was prepared. Micro tubes were used to store TAV5 in a 25% glycerol solution. Figure 3-11 shows the cultures stored in a -80°C freezer.

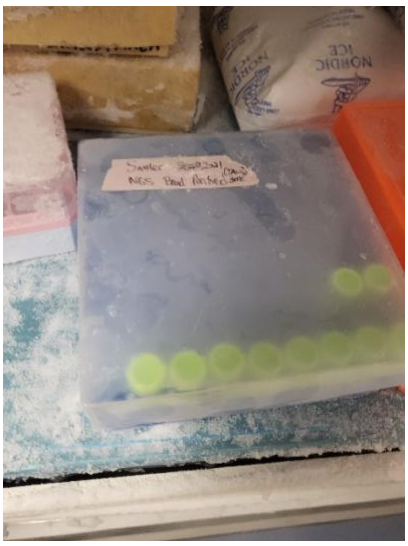

Figure 3-11: TAV5 cells stored in a -80^{o}C freezer

3.3 Methods to Address Obj. 2: Find the Optimal Ratio of TAV5 to Digester Microorganisms

3.3.1 Waste procurement

Agricultural wastes were obtained from the following sources: Rice husk from Golden Ridge Rice Mill, Wynne, AR; wheat straw from Russel Fields, Fort Worth; corn stover from a farm south of Waxahachie, TX; and rice straw from Hollister, CA. The wastes were sun dried to obtain a moisture content in the range provided by the literature.

Figure 3-12: Agricultural waste

3.3.2 Inoculum

This research uses WRRF (wastewater resource recovery facility) AD sludge as inoculum. Fresh sludge was obtained before starting the batch reactors from a continuously stirred AD at Village Creek Wastewater Treatment Plant located in Dallas-FortWorth area.

Figure 3-13: Collecting sludge from Village Creek WRRF

3.3.3 Batch reactors

Wheaton Serum bottles (125 mL) with rubber septa and aluminum crimp seals were used as batch reactors. Ratios of TAV5 to AD microorganisms were varied from 0% to 90% by weight (assuming they have density of water), in 10% increments. Each bottle was filled with 5 grams of waste. Wheat straw, corn stover and rice straw were ground using a coffee grinder and passed through HUMBOLDT Sieve no. 30 to obtain uniform particle size of 0.6 mm. The total number of bottles was 84 (10 microorganism increments x 4 wastes x 2 duplicates of each bottle + 4 controls – seed by itself). Each batch reactor was inoculated with 10 ml of WRRF sludge at a ratio of 1:2 (waste to sludge by weight). It was considered that TAV5 cultures and WRRF inoculum have a density of 1 g/ml. TAV5 was stored in a refrigerator (4^{o}C) for a maximum of one day before setting up the batch reactors. To prepare each batch reactor, deionized water, inoculum and TAV5 were mixed in a sterilized plastic vial and rotated 20 times by hand to achieve a homogeneous mix. This

mixture was then added to waste in the serum bottles to achieve a moisture content of 90% (accounting for the moisture content of the waste). Both sludge and TAV5 were stirred continuously using a magnetic stirrer at 50 and 150 rpm, respectively, to keep the cells floating. All the bottles were sealed with an aluminum seal and septa. Figure 3-14 shows the process of adding water, inoculum and TAV5 into the batch reactors.

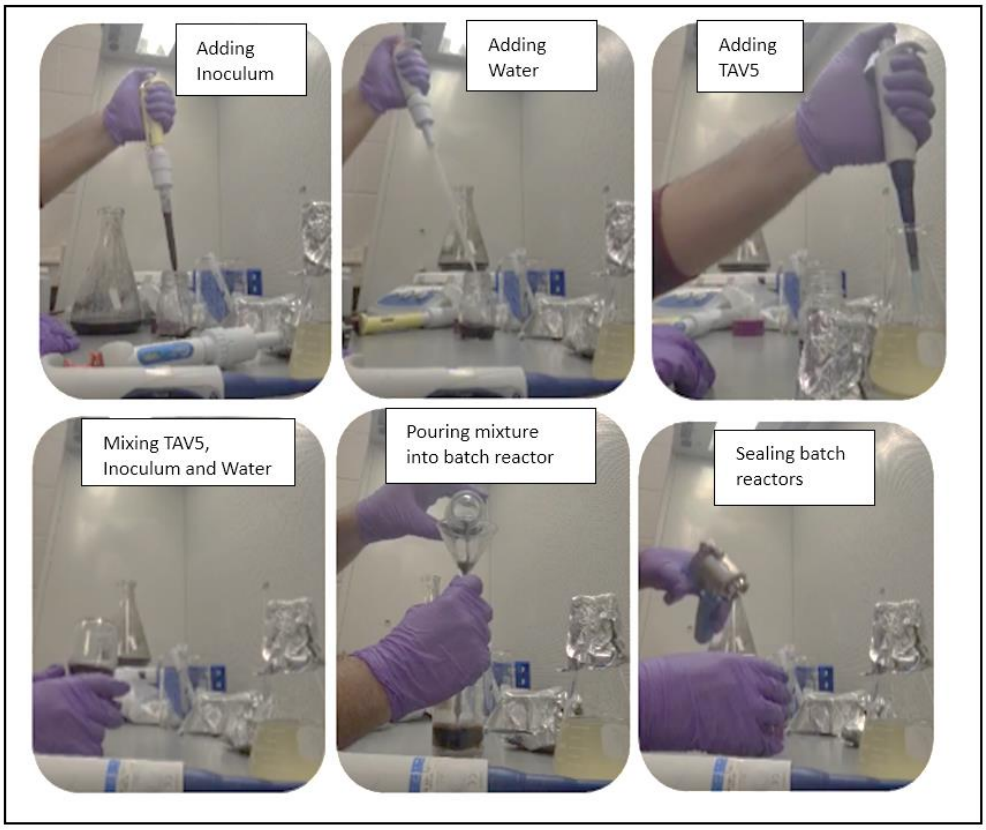

Figure 3-14: Steps for adding inoculum, water and TAV5 into batch reactors

Figure 3-15: Preparing batch reactors

Afterwards each bottle was flushed with nitrogen using a compressed nitrogen cylinder from Matheson Tri Gas as shown in Figure 3-16. One tube was used to inject nitrogen into the batch reactors at 5 psi and the other tube was used to induce vacuum. Each bottle was flushed for 5 minutes.

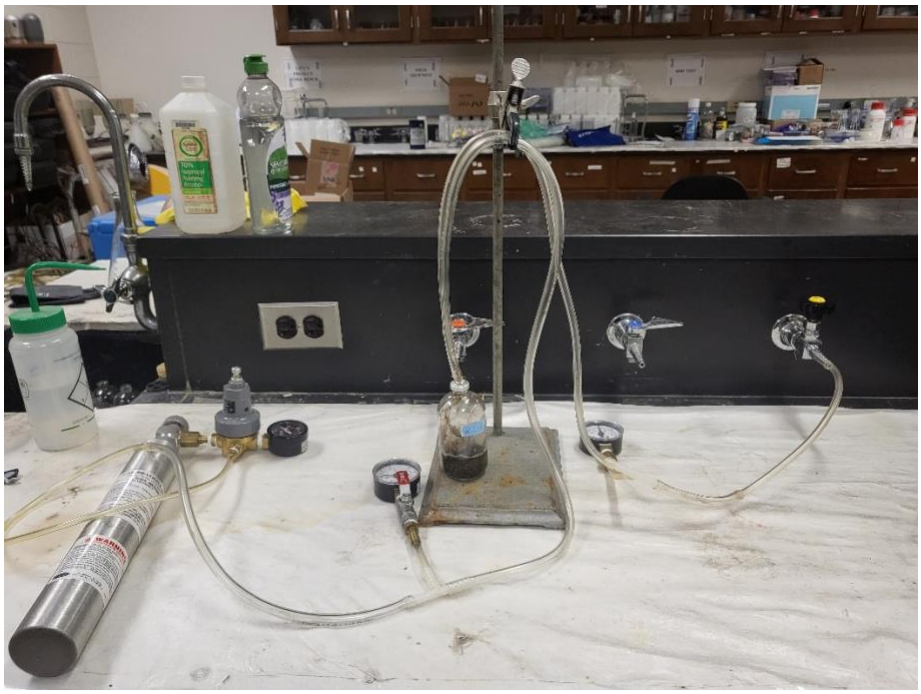

Figure 3-16: Batch reactor flushing station

After flushing, all the bottles were placed at 30^{o}C in a temperature-controlled room. Figure 3-17 shows the batch reactors after nitrogen flushing. Each bottle started to produce biogas within two days of setting up.

Figure 3-17: Batch reactors with rice straw

Figure 3-18: Temperature controlled room for batch reactors

3.3.4 Measuring biogas production

Gas volume was measured using 30 ml and 60 ml syringes. Gas composition was measured to find the concentration of methane in the biogas using SRI 8610c gas chromatograph equipped with Flame Ionization Detector (FID). Ultra-pure nitrogen gas from Matheson Tri Gas is used as carrier gas and hydrogen gas is used in FID detector (hydrocarbons ionize in the hydrogen gas flame). Carrier gas is set at 20 psi and hydrogen gas at 50 psi. Detector heat was set at 300^{o}C, column oven temperature was set at 101^{o}C and ECD detector was set at 350^{o}C. A gas calibration curve was prepared by injecting pure methane gas into the GC and noting down the peak values at 0.1 to 0.5 ml with an increment of 0.1ml. Biogas content was measured multiple times a week initially and then shifted to once a week, depending upon gas production.

pH was also checked multiple times a day for the first few days and then daily until pH stabilized over 6.5. To measure pH 5 ml syringes were used. A small drop was taken out as a sample and placed on the pH paper to obtain an estimate of the pH. If the pH was less than 6, potassium hydroxide was used as base to raise it to 6.5-7.0.

Figure 3-19: SRI Gas Chromatograph

3.3.5 Measurement methods for waste parameters

Waste parameters like lignin content, moisture content, organic carbon and volatile solids were measured. One-gram samples of all four waste streams were sent to Intertek labs to analyze for Carbon (C), Hydrogen (H), Nitrogen (N), Oxygen (O) and Sulphur (S) CHNO/S. Standard procedures were used to measure each parameter as shown in Table 3-1.

Table 3-1: Procedure to calculate waste parameters

Parameter	**Procedure**
Lignin	Klason lignin methodology was used to measure Acid Insoluble Lignin (Schwanninger et. al, 2002). Acid soluble lignin was measured via UV spectrophotometer using following formula: $$\%\text{ASL} = \frac{\text{UVabs} * \text{Volume}_{\text{filtrate}} * \text{Dilution}}{\varepsilon * \text{ODW} * \text{Pathlength}} * 100$$ UVabs = average UV-Vis absorbance for the sample at appropriate wavelength ε = Absorptivity of biomass at specific wavelength ODWsample = weight of sample in milligrams Pathlength = pathlength of UV-Vis cell in cm
Moisture content	Standard Methods APHA 2540B (wet weight basis)
Organic carbon	CHNO/S analyzer
Volatile solids	Standard Method APHA 2540-E

3.3.6 16S rRNA gene sequence analysis for bacterial identification confirmation

To verify that the bacteria used to seed batch reactors is TAV5, 16S rRNA gene sequencing was performed by the Life Sciences Core Facility (Department of Biology, UT Arlington, Arlington, TX) (Martin et al., 2018; Santos et al., 2017, 2018). Primers used for Polymerase chain reaction (PCR) amplification were 327F and 936R which cover variable regions V3-V6 (Sison-Mangus et al., 2015). GenBank sequence database (National Center for Biotechnology Information) was used to confirm the sequence data to be TAV5.

3.4 Methods to Achieve Obj. 3: Life Cycle Environmental and Economic Assessment of Growing TAV5

A life cycle analysis was done to compare economic and environmental impacts of adding TAV5 to existing farm digesters. Life cycle emissions or cost per m^3 of methane was estimated, considering the following life cycle phases:

- Raw material acquisition
- Manufacturing/construction
- Transportation
- Use/reuse
- End of life

It was assumed that existing farm digesters with an intake of 500 ton per year of crop residue will be used to add TAV5 with a lifetime of 20 years, which represents a reasonable estimate of the lifespan of an AD based on POWER Tool (Sattler et al., 2022). There is no initial raw material acquisition cost associated with TAV5 since it was grown using an already stored culture at UTA.

The manufacturing stage includes using autoclave to prepare broth, agar plates and sterlizing equipment, growing TAV5 on plate, then transferring it to broth cultures and then expanding it using onsite incubators. For use phase, it was assumed that once TAV5 is introduced into the digester, it will last for the lifetime i.e, 20 years for AD. POWER Tool was used to calculate the cost for electricity needed to grow TAV5 and estimated emissions due to the use of extra electricity and higher biogas conversion.

3.4.1 Overview of POWERTOOL

The POWER Tool is based on Microsoft Excel spreadsheet, estimates the following for the anaerobic digestion process shown in Figure 3-20: costs/benefits; pollutant emissions; and electricity, vehicle renewable natural gas (RNG), or pipeline RNG production. POWER Tool is based on data collected from over 200 peer reviewed articles, interviews were conducted with personnel from WRRF, fleet services, and solid waste collection services from several cities and meetings were held with a multi-disciplinary advisory group. Information sources are referenced in the POWER Tool itself.

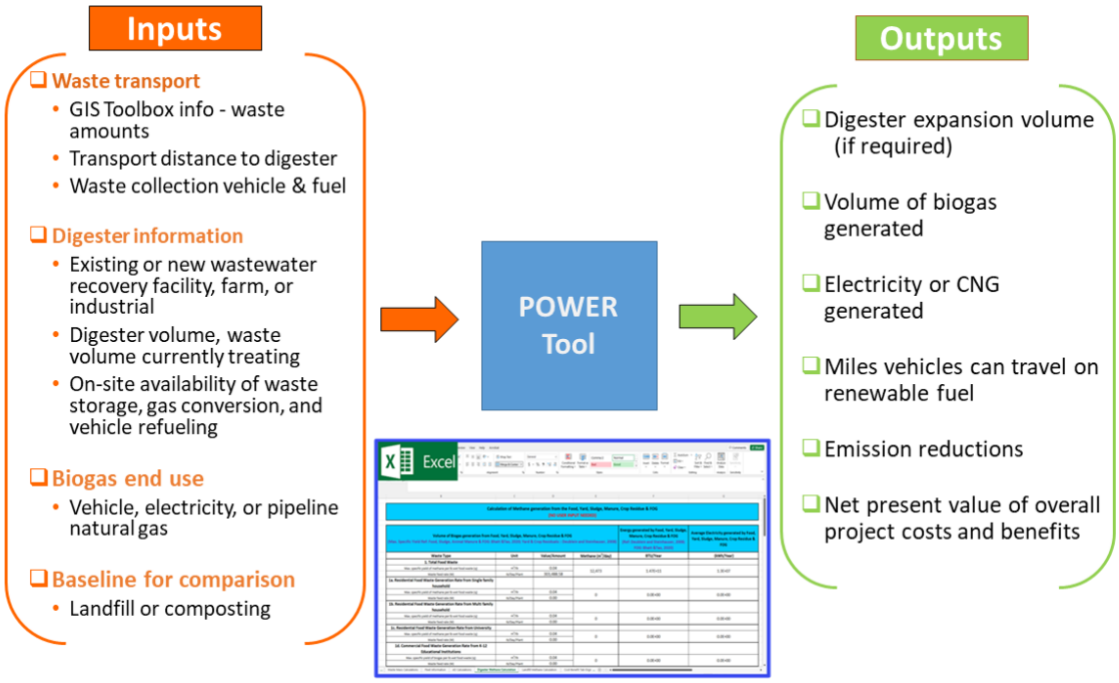

Figure 3-20: Overview of POWER Tool

Chapter 4: Results

4.1 Overview

This chapter provides and analyzes results for the three objectives defined in this study:

1. Determine optimal conditions for efficiently growing TAV5 in large volumes for field seeding of waste.

2. Determine the optimal addition of TAV5 to enhance methane production from agricultural wastes (corn stover, rice straw, wheat straw and rice husk).

3. Conduct a life-cycle environmental assessment of energy products generated from biogas produced from agricultural waste, compared to baseline technologies (biogas produced without TAV5 seeding from ADs).

4.2 Results for Objective 1: Determine optimal conditions for efficiently growing TAV5 in large volumes for field seeding of waste

4.2.1 Density/Day to harvest cells

Growth curves were obtained by measuring optical density using a spectrophotometer, as shown in Figure 4-1, for 1:4 TAV5 to broth ratio. Triplicates were used from two cultures named (1-1) and (1-2) grown from two different plates with TAV5. All the cultures were grown in aerobic conditions. Results for growth curve for all six samples are closely tracked, which indicates precision. Maximum growth occurs between hours 48 and 64. The TAV5 cells need to be harvested before the start of stationary phase, which begins right after the peak growth is achieved. Since the peak growth occurs between hours 48 and 64, TAV5 cells should be harvested at 0.35 optical density. The sudden spike for sample culture (1-1)-3 is because of contamination.

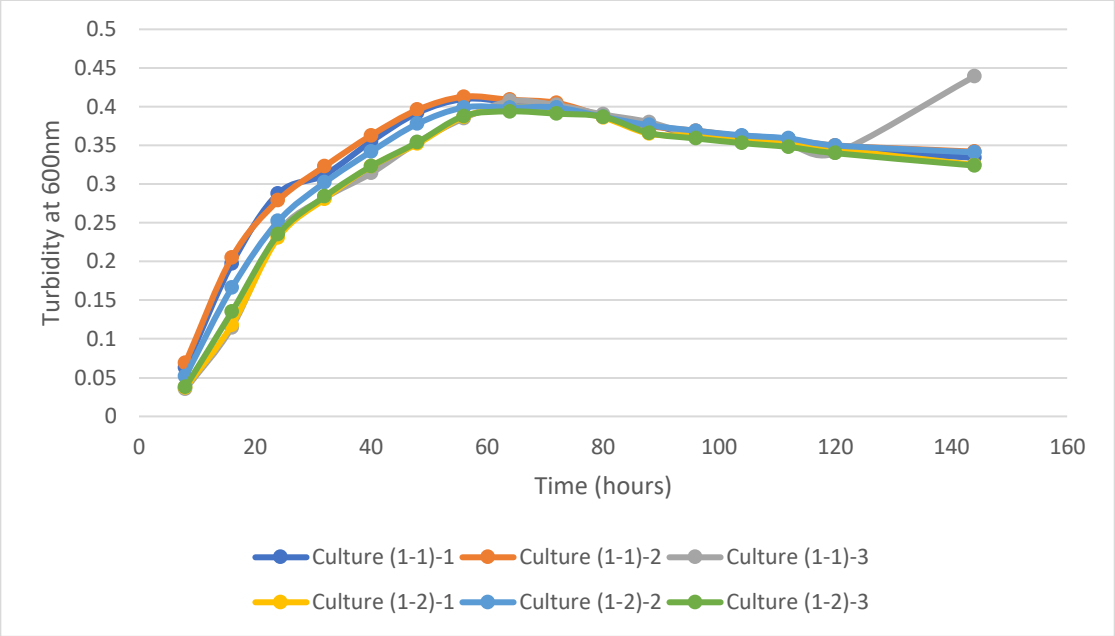

Figure 4-1: Growth curve of TAV5 for 1:4 TAV5 to broth ratio

4.2.2 Rates of aerobic vs. anaerobic growth

To compare growth of TAV5 in anaerobic conditions, airtight glass vials were used. Cultures were grown at 1:4 TAV5:broth solution. Figure 4-2 compares growth curves in aerobic and anaerobic conditions. Duplicates were grown aerobically, whereas triplicates were grown anaerobically from the same culture. TAV5 can grow both aerobically and anaerobically; however, higher growth was observed in aerobic conditions. This is fortunate, since aerobic conditions do not require a special anaerobic chamber, and thus are cheaper. Since it can also live in conditions of 2% oxygen, it can be used in ADs, making it an ideal candidate to destroy lignin in ADs without the need of any pre-treatment.

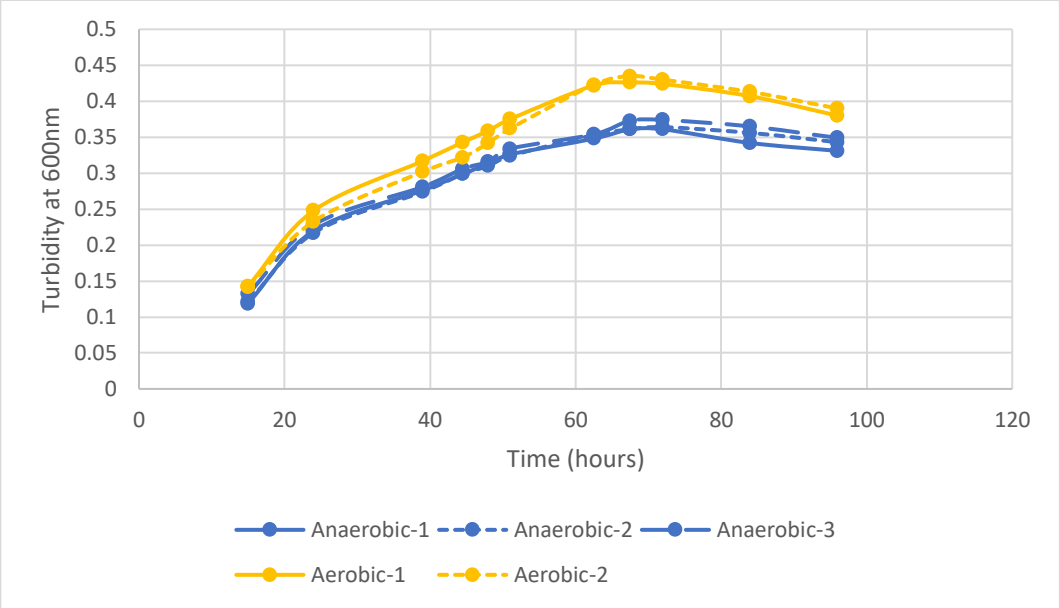

Figure 4-2: Aerobic vs Anaerobic growth curve for TAV5

4.2.3 Impact of temperature on growth

Originally TAV5 was streaked aerobically from the petri dish at room temperature, 30°C and 40°C. Good growth was observed at both room temperature and 30°C but no growth was observed at 40°C. The plates grown at 30°C were then streaked at 33°C, 35°C and 37°C and good growth was observed at all these temperatures, with the fastest growth observed at 30°C. After growing TAV5 at 37°C, multiple tries were done to jump from 37°C to 40°C but only very little to no growth was observed. Then same thing was tried with 39°C, which had the same result as 40°C. Thus, it was concluded that TAV5 growth over 37°C is very limited.

The plates incubated at 30°C were then used for broth cultures. Two batches were started initially, one at room temperature and other at 30°C. Both cultures showed growth, which was measured by observing turbidity of the culture vs. a high contrast background as shown in Figure 4-3. Cultures at room temperature were then identified to be TAV5 by 16s rRNA sequencing and stored at -80°C.

Afterwards broth cultures were grown at 32°C and 35°C and growth was observed. These cultures were also stored at -80°C after they were identified as TAV5.

Figure 4-3: Testing Turbidity of TAV5 vs high contrast background

4.2.4 Optimum ratio of TAV5 to R2B broth

Growth rate of TAV5 in R2B broth depends on the ratio of TAV5 cells and broth. If very low number of TAV5 cells are added in a very large R2B broth solution, then it will take a very long

time for the cultures to reach their peak. On the other hand, to add a higher number of TAV5 cell in broth solution, we need to grow more TAV5 cells first. Therefore, we need to determine the optimal ratio of TAV5 to R2B broth solution to grow large volumes of TAV5 efficiently.

The growth curves in Section 4.2.1 were developed using a 1:4 TAV5:broth ratio. TAV5 can be ready for harvesting within 48 hours for ratio of 1:4. For 1:4, however, a very large amount of initial TAV5 stock is required. Thus, in the experiments reported on in this section, growth curves were noted for 1:33, 1:50 and 1:100 TAV5:broth ratios, with TAV5 cells grown in 2000 ml, 1000 ml and 500 ml Erlenmeyer flasks. All the cultures were grown aerobically, and all the duplicates were grown together in same conditions. Almost no growth was observed for 1:100 in first 4 days and 1:50 showed very little growth. However, 1:33 showed good growth, with TAV5 cells gaining required optical density with 80 hours. Figure *4-4* shows the growth curves for 1:33 cultures. Since the amount of initial TAV5 required is lower, for commercial purposes it is more optimal to use 1:33 rather than 1:4, even though the time to harvest is longer. TAV5 cells should be harvested at 0.35 optical density, which can take 80 hours for 1:33

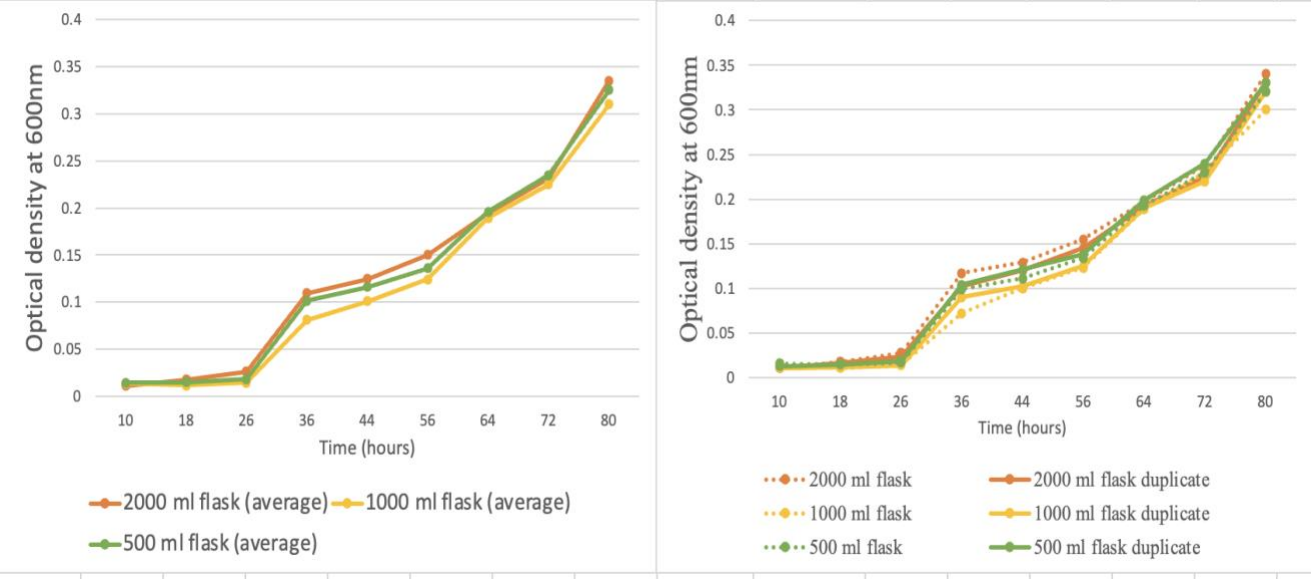

Figure 4-4: Optical density at 1:33 (TAV5 cells to R2B Broth solution)

4.2.5 Cell count – check of accuracy of growth curves

A cell count procedure was done to verify and ensure the accuracy of the growth curve by checking if TAV5 cells are alive and growing during the log phase (0.35 optical density) of the growth curve. During the log phase, the number of bacterial cells increases exponentially and shows uniform metabolic activity. However, as the number of cells increases, the nutrients are used up and waste products starts to accumulate. These unfavorable conditions stop the growth of bacteria and the number of cells alive reaches a plateau. This phase is called stationary phase, as the rate of cell growth and death becomes equal. Therefore, for research and industrial applications, cells are cultured at log phase. Table 4-1 shows the cfu/ml for TAV5. More detailed explanation is provided in appendix.

Table 4-1: Results for cell count

Day	Optical Density	No. of colonies	Dilution factor	CFU/ml
1	0.12	150	10^5	1.5 x 10^8
2	0.27	270	10^5	1.5 x 10^8
3	0.33	113	10^6	1.06 x 10^9

4.3 Results for Objective 2: Determine optimal TAV5 addition

TAV5 was added into batch reactors containing wheat straw, corn stover, rice straw and rice husk. Methane generated from each batch reactor was measured and results were compared to find the optimum ratio between TAV5 and WRRF sludge-based inoculum.

4.3.1 Waste characterization

Table 4-2 summarizes characteristics for each type of waste. All the values measured for moisture content and lignin content were found to be within the range of values found in the literature. Wheat straw and corn stover showed higher volatile solids compared to rice husk and rice straw. This might also be the reason for lower methane production for rice husk and rice straw. Rice husk

had the highest size since it was not ground because the particle size was already homogeneous, whereas the rest of the wastes were ground to achieve a uniform particle size.

Table 4-2: Waste characterization for agricultural waste

Waste	Volatile solids		Moisture content		Lignin Content		Particle size (mm)	Organic carbon test (%) (CHNO/S analyzer)			
	Measured	Range from literature	Measured	Range from literature	Measured	Range from literature		C	H	N	S
Rice husk	76%	74 - 81.6%	5.49%	4.6 - 6.1%	20%	20%-23%	3	34.7	4.04	0.08	<0.1
Rice straw	74%	74.9 - 84.4%	6.7%	6.6 - 6.9%	13%	12.3%-24%	0.6	38.2	4.9	0.28	<0.1

Wheat straw	95%	86.8 - 95.6%	6.36%	5 - 7.8%	17.3%	15%-20.82%	0.6	44.9	5.5	0.36	<0.1
Corn straw	96%	90.1 - 98.5%	10.14%	6.4 - 13.6%	15.2%	7%-18%	0.6	43.6	4.9	0.24	<0.1

4.3.2 Optimal TAV5 addition for corn stover

Methane produced from corn stover based batch reactors is plotted against time as shown in Figure 4-5. Different ratios of TAV5 were added to all batch reactors with each ratio having a duplicate as well. Reactors were incubated at 30^{o}C and started producing small amounts of methane within two days of setup. Methane production stayed low for the first 20-40 days but started to increase significantly once a stabilized pH was achieved. Generally, asymptotic values were reached by the reactors around day 85, which indicates that methane production had ceased.

The batch reactor with 30% ratio showed the most methane production. Figure 4-5 shows that batch reactors with 20%, 10% and 40% showed the second, third and fourth highest methane production, respectively. However, there is a difference in the production of biogas for the duplicates. This might be due to differing numbers of alive TAV5 cells added in each duplicate, since the duplicates of batch reactors without any TAV5 show similar results. In addition, moisture content could have been non-uniform in the bottles since constant mixing was not done during incubation. Some trial runs were done with constant mixing using a shaker but no significant increase in biogas production was observed. Therefore, for batch reactors constant mixing was not done. Also, the pH had to be measured using pH paper; a probe could not be inserted, or it would disturb the anaerobic conditions. Small liquid samples were taken out of the batch reactors using a syringe and a drop of sample was placed on the pH paper to estimate the pH. pH measurement with paper is not as accurate as with a probe; thus, there could be some difference in the pH maintained for each batch reactor. Some of the duplicates perform worse than batch reactors without any TAV5, which might be due to pH level staying low for too long, killing most of the methanogens. Initially, the pH dropped every 2 to 3 hours, making it very difficult to keep pH around 6.8. However, after the first few days, pH was stabilizing and would only drop every 12 to

24 hours. However, since it was not possible to constantly check the pH, sometimes pH dropped substantially between the intervals it was checked. This continuous change in pH could have negative impacts on methanogens inside batch reactors, resulting in varying methane production for duplicates.

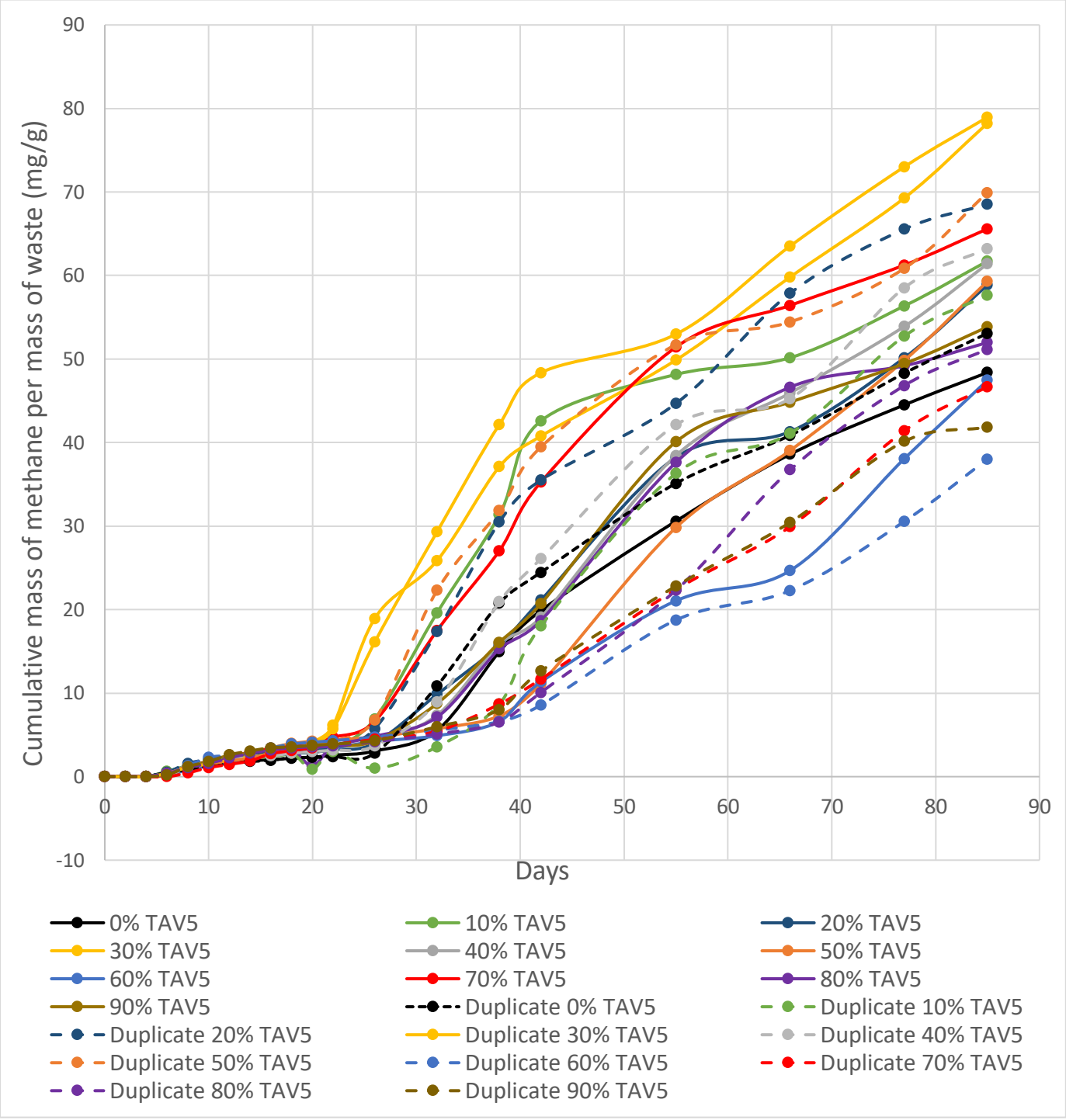

Figure 4-5: Cumulative methane generation for corn stover for varying ratios of TAV5

To calculate the range of increase in methane generation, an average of cumulative methane generated by batch reactors without TAV5 was taken and then compared with cumulative methane produced by both duplicates seeded with 30% TAV5. Batch reactors with 30% TAV5 produced 54% and 56% higher methane when compared to batch reactors without TAV5.

4.3.3 Optimal TAV5 Addition for Rice Straw

Figure 4-6 shows the cumulative methane generated for different ratios of TAV5 containing rice straw incubated at 30^{o}C. All the batch reactors took around 2 to 3 days to produce small amounts of methane. Methane production stayed low for the 20-40 days but started to increase significantly once a stabilized pH was achieved. Asymptotic methane values were achieved by day 85 and methane readings were stopped at that point.

Batch reactors with 30% TAV5 ratio produced 60% to 582% more methane when compared with batch reactors with no TAV5. Batch reactors with 90% TAV5 showed second best methane production, which is not a consistent trend compared to corn stover, where the top four best performing ratios ranged from 10% to 40%.

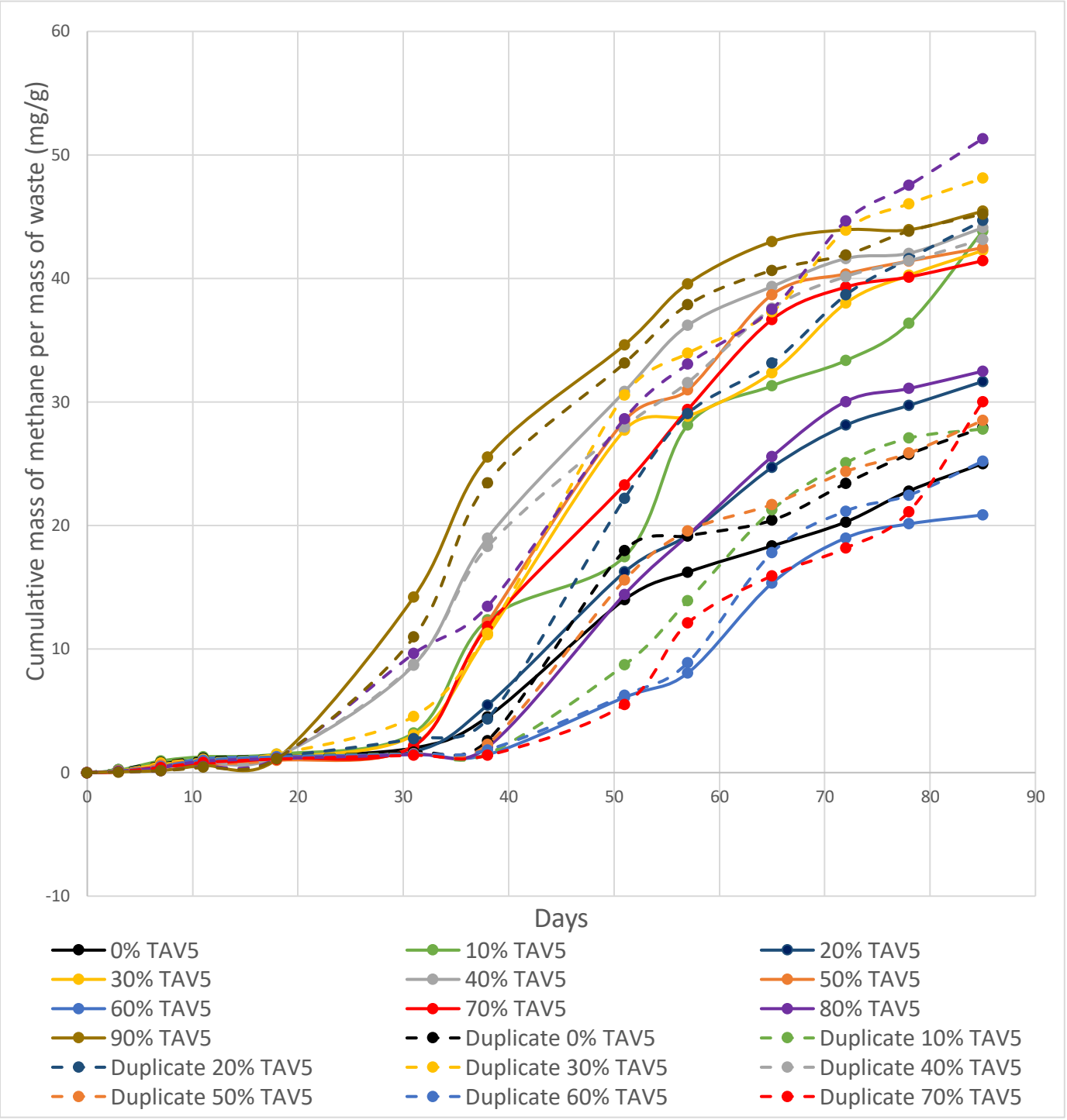

Figure 4-6: Cumulative methane generation for rice straw for varying ratios of TAV5

4.3.4 Optimal TAV5 Addition for Wheat Straw

Total cumulative methane produced by batch reactors based on wheat straw seeded with multiple ratios of TAV5 is shown in Figure 4-7. All the reactors were incubated at 30^{o}C for 85 days and it took around 2 days for the batch reactors to produce a small amount of methane. Methane production stayed low for the 20 days but started to increase significantly once a stabilized pH was achieved. Peak methane production occurred around day 32 and reactors reached asymptotic values around day 85.

The highest amount of methane was produced by "Duplicate 60% TAV5". However, the results for 60% TAV5 ratio are not consistent since the batch reactor "60% TAV5" generated 64% less methane. Therefore, we have selected 30% TAV5, which has more consistent results for duplicates, as the most optimal ratio between TAV5 and WRRF sludge microorganisms, as the duplicates are more consistent and produce more methane when compared to batch reactors with 0% TAV5. The 30% TAV5 reactors showed an increase of 34% and 59% in methane production.

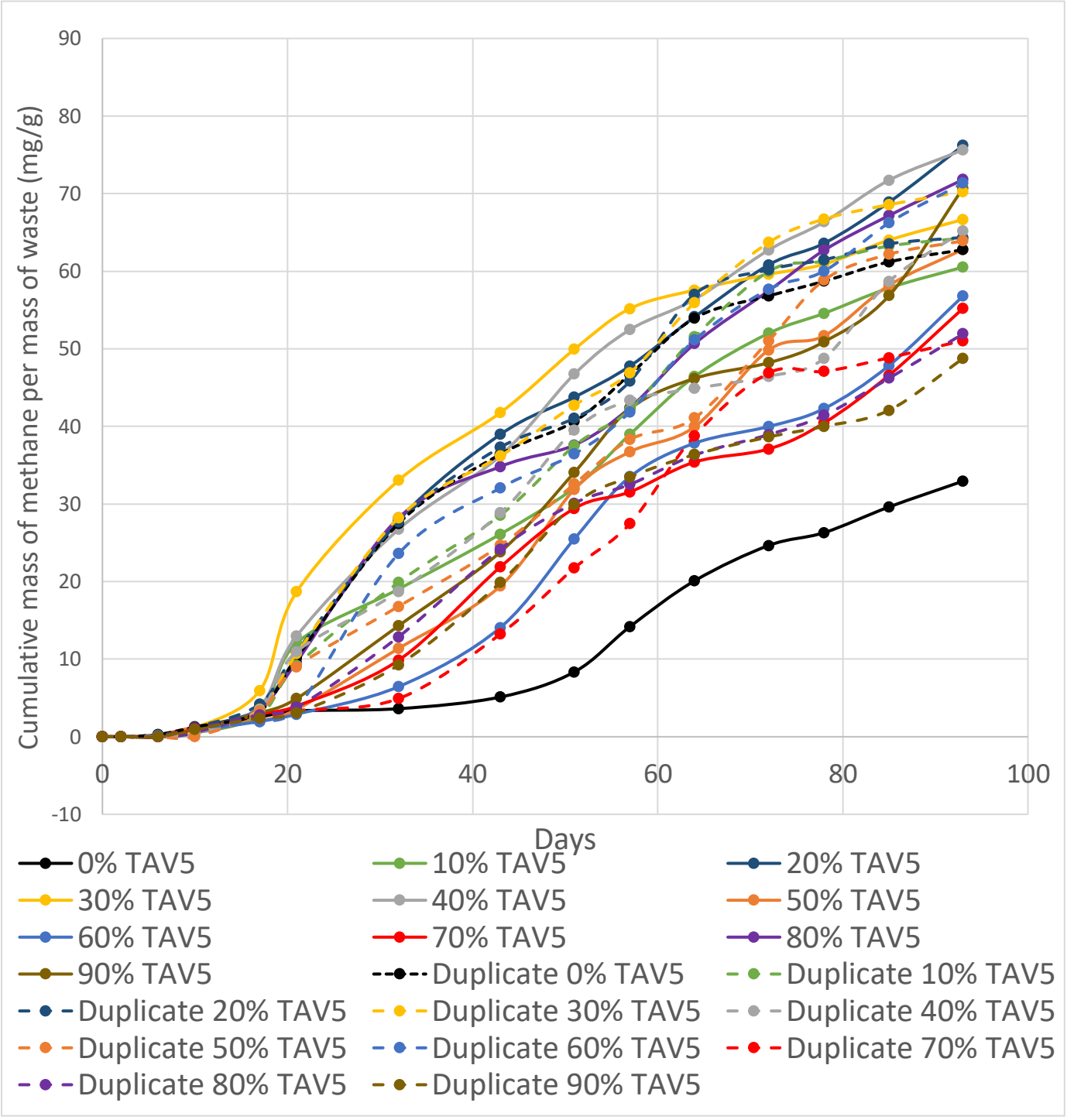

Figure 4-7: Cumulative methane generation for wheat straw for varying ratios of TAV5

4.3.5 Optimal TAV5 Addition for Rice husk

Figure 4-8 shows the methane production for rice husk-based batch reactors with different TAV5 ratios incubated at 30^{o}C for 68 days. An initial batch reactor test was done and the top 4 methane producing ratios (30%, 50%, 60% and 90%) were selected for a rerun. The rerun was done because the cumulative methane generation values for the duplicates did not match. The lag time for rice husk was less than a week, which is very little when compared to other agricultural wastes used in this research. Consistent pH of about 6.8 to 7 could be the main reason for this. Asymptotic values were achieved by 68th day.

In the re-run, the batch reactor with 50% TAV5 ratio produced the highest amount of methane but results from the duplicate are not consistent. Therefore, the optimum ratio selected is 60% TAV5, since both methane production from duplicates is consistent and higher than the batch reactors which are not seeded with TAV 5. 60% TAV5 increased methane production by 19% to 34%.

A higher ratio of TAV5 is needed in the case of rice husk when compared to the other three waste types. This is likely because of lower surface area to volume ratio since all other waste types were crushed to achieve consistent size. Rice husk was not crushed since it already has consistent size.

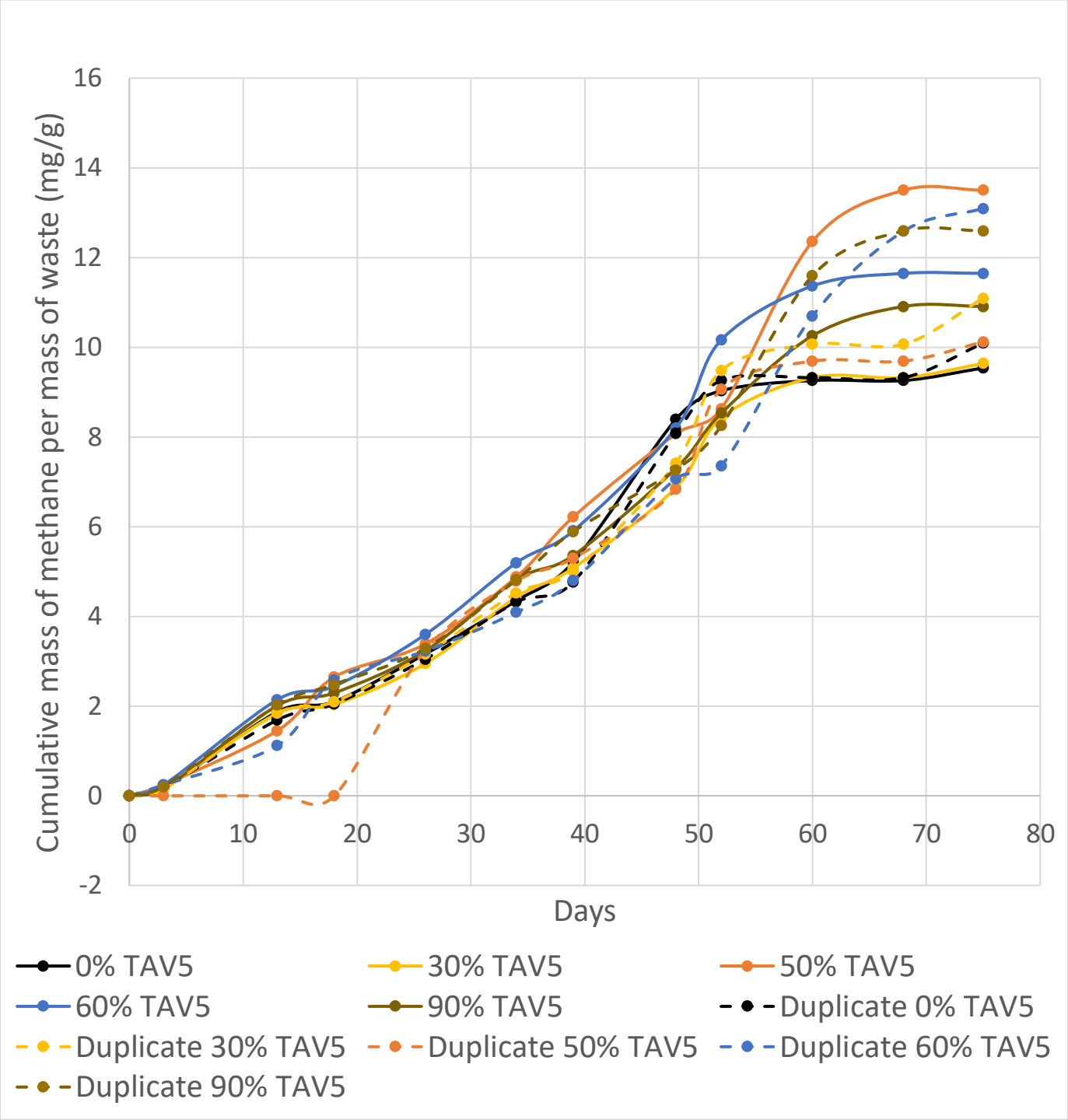

Figure 4-8: Cumulative methane generation for rice husk for varying ratios of TAV5

4.3.6 Comparison of lignin content, optimal TAV5 ratio and methane production increase for all agricultural wastes

Addition of TAV5 cells in certain ratios increased methane generation for all four agricultural waste types. It was found that TAV5 to inoculum ratios of 30%, 30% 20% and 60% produced the optimum results for corn stover, rice straw, wheat straw and rice husk, respectively.

Figure 4-9 shows the amount of methane produced for each waste. The time lag for rice straw to generate significant amount of methane might be due to pH since batch reactors for all the wastes started to produce higher amounts of methane once the pH stabilized. Methane production for rice straw, corn stover and wheat straw is not far off; however, rice husk produced very low amount of methane, which might be due to lower surface area available to microorganisms since it was not crushed.

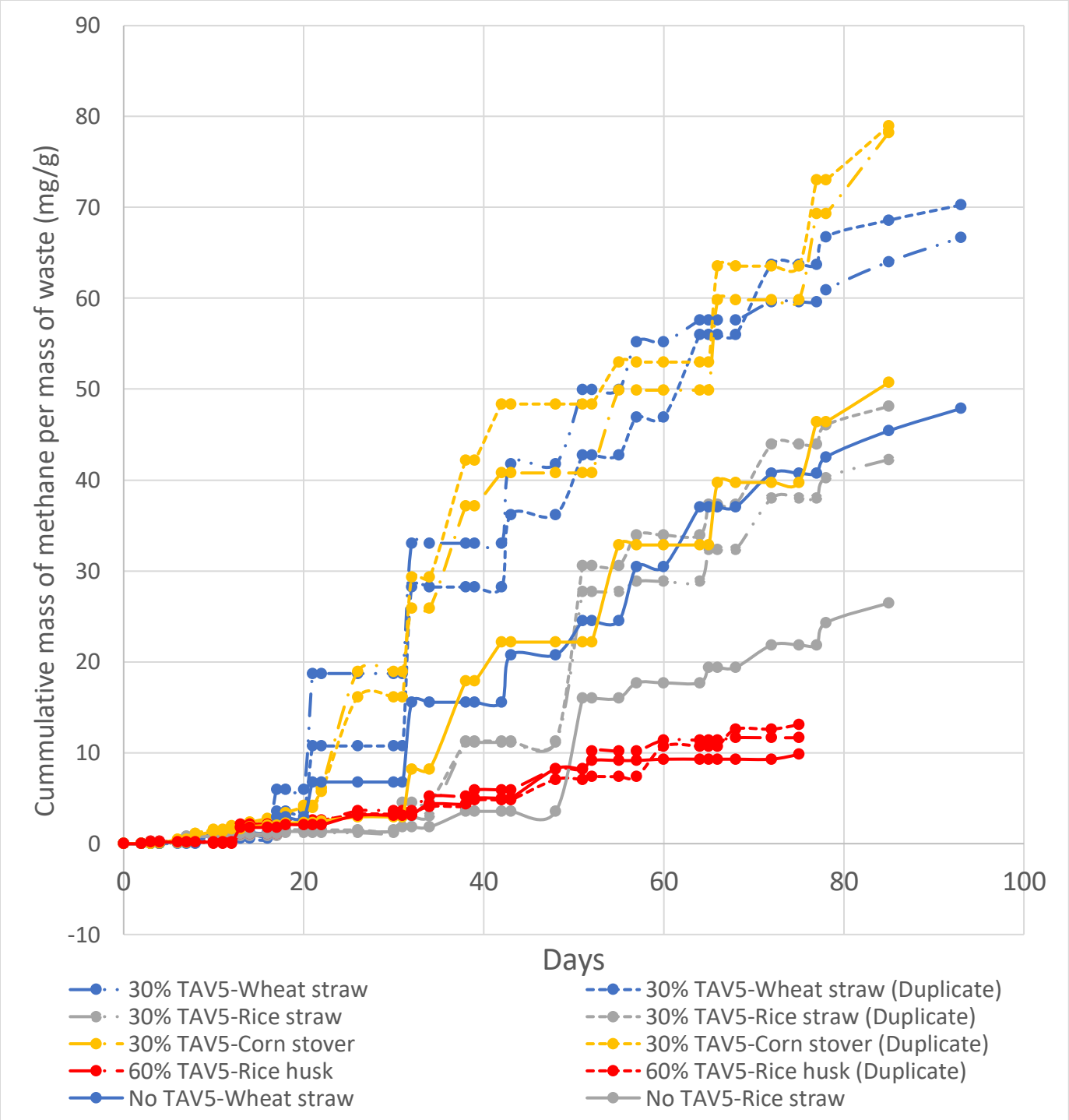

Figure 4-9: Comparison of methane production for different agricultural wastes

Table *4-3* summarizes the optimal TAV5/(water resource recovery facility digester microorganism) ratio for each of the 4 wastes, along with lignin content and percent increase in methane production

due to TAV5 addition at the optimal ratio. The measured lignin content for rice husk was the highest (20%), which may be one reason that it required a higher ratio of TAV5 to WRRF sludge. As mentioned previously, it also had a lower surface area-to-volume ratio, which gave microorganisms less access to break down the waste. There is not a correlation between the lignin values of the other wastes and the TAV5 ratio, nor between the lignin values and the increase in methane production.

Table 4-3: Comparison of lignin content, optimal TAV5 ratio, and increase in methane production due to optimal TAV5 addition, for all agricultural wastes tested

Waste	**Lignin content Measured**	**Optimal TAV5/WRRF ratio**	**Average increase in methane production (range)**
Corn stover	15.2%	30%	96% (77-115%)
Rice straw	13%	30%	182% (87-276%)
Wheat straw	17.3%	30%	26% (18-33%)
Rice husk	20%	60%	53% (28-77%)

4.4 Results for Objective 3: Economic and Environmental analysis for adding TAV5

The cost of adding TAV5 to farm digesters and subsequent environmental impacts from increased biogas were compared to the baseline case of no TAV5 addition, assuming a farm digester taking 500 tons of crop residue every year. The capacity of the farm digester is based on the equipment availability to grow TAV5 in the lab at UTA. Although this is a smaller amount of TAV5 production than industrial scale, we more accurately know the costs. Since economies of scale typically decrease costs, the cost of growing TAV5 in larger volumes would be expected to be lower.

The functional unit selected is cost and emissions from adding TAV5 for the lifetime of AD. It was also assumed that all the equipment needed to grow TAV5 i.e., clean bench, incubators, shakers etc. is already available in the lab at UTA and their capital cost and environmental impacts are not included. The POWER Tool was used to calculate the cost and estimate the emissions from farm digesters. All the stages of life cycle from material acquisition to end of life were considered. The analysis was based on the optimal ratio of TAV5 required for each waste (30% for corn stover, rice straw, wheat straw; 60% for rice husk).

4.4.1 Cost analysis

4.4.1.1 Material acquisition

The cost of acquiring agricultural waste residue and inoculum was considered zero since we are assuming that the existing farm digesters are already running, and they are located at a farm with waste available.

4.4.1.2 Manufacturing

For the manufacturing stage, the cost for growing TAV5 was considered. TAV5 was sourced from Dr. Jorge Rodriques at the University of California, Davis, on a petri dish and its cost was assumed to be zero. For rice straw, wheat straw and corn stover, 0.6ml of TAV5 is needed per gram of waste and 1.8ml per gram for rice husk. The total capacity of growing TAV5 in our lab at UTA is 60L at one time. Costs for equipment (Table 4-4) needed to grow TAV5 aerobically was not included since it was already available in our lab; it was also assumed that the lab that will grow TAV5 for the farm digesters (if not our lab) will have all the equipment available to grow TAV5.

Table 4-4: Equipment used to grow TAV5

Equipment	**Quantity**
MAX Q6000	2
Thermo Fisher Incubator: Model no. 490	1
Clean bench	1
Petri dishes	NA
-80ºC refrigerator	1
Refrigerator	1
Bunsen Burner	1
Glass tubes	NA
Plastic tubes	NA
Pipette	3
Pipette tubes	NA

Electricity used to grow a kg of TAV5 was calculated as shown below. Two types of incubator shakers were used, i.e., Max Q6000 and Brunswick Model 490. It was assumed that a TAV5 batch would be ready every two days (using TAV5 to broth solution ratio of 1:4), with a total capacity of producing 60L at a time.

Voltage for incubator shaker (Max Q6000): 120V

Ampere per incubator shaker (Max Q6000): 7.87A

Total wattage = (120*7.87) = 945 W

Voltage for incubator shaker (Brunswick Model 490): 120V

Ampere per incubator shaker (Brunswick Model 490): 9A

Total wattage = (120*9) = 1080 W

Energy for Max Q6000 Incubator: (2* 945 W* 24 hr/day)/1000 = 45.4 kWh/day

Energy for New Brunswick Incubator: (1080 W* 24 hr/day)/1000 = 25.9 kWh/day

Total Energy = 45.4 kWh + 25.9 kWh = 71.3 kWh/day

Electricity price = 0.12 kWh (US national average according to (ElectricRate, 2023))

Electricity cost per day for incubators = 0.12 kWh* 71.3 kWh/day = $8.5/day

$8.5/day * 2 days to grow TAV5/(60L grown at a time) = $0.283/L TAV5

Density of TAV5 is assumed to be the same as water: 1 g/mL or 1 kg/L

Electricity to grow TAV5 = $0.283/kg TAV5

It was assumed that no electricity cost is associated with the clean bench, and no additional costs associated with the refrigerators, since they are already in use. Bunsen burner use was minimal, so the cost is neglected. R2B broth is also needed to grow TAV5. To produce 1 kg of TAV5, 3.2 grams of R2B broth powder are needed, with costs as indicated below.

R2B broth price = $174 per kg

R2B broth needed per kg of TAV5 = 3.2 grams

Cost of R2B broth per kg of TAV5 = $174/kg * 0.0032kg = $0.55/kg TAV5

Total cost to grow TAV5 = Cost of R2B broth per kg + Cost of electricity per kg

Total cost to grow TAV5 = $0.283/kg TAV5 + $0.55/kg TAV5 = $0.833/kg TAV5

TAV5 will only need to be added once, at the beginning of digester operation. The cells are assumed to be maintained at log growth phase, so that cells that die are replaced by new cells at steady state. It was assumed that the digester size accommodates 500 tons per year (tpy), as explained above, and the residence time was assumed to be 17 days (Sattler et al., 2022). The batch size then was calculated to be (500 tons of waste/day) * (1 year/365 days) * 17 days = 23.3 tons

of waste/batch. WRRF inoculum was added to the lab-scale reactors at a ratio of 2:1 by weight, which would be 2*23.3 tons = 46.6 tons of inoculum for the field-scale digester accommodating 500 tpy. The amount of TAV5 to be added was then calculated as follows, using corn stover as an example.

TAV5 to be added = (Optimal ratio of TAV5 per weight of inoculum) * (weight of inoculum in the digester)

Optimal ratio of TAV5 for corn stover = 0.3

TAV5 to be added for corn stover = 0.3 * 46.6 tons of inoculum = 14.0 tons

To calculate the labor cost, it was assumed that the lab technician will spend 30 minutes each day to start new cultures and store the cultures that are ready. An hourly rate of $20 was assumed for this purpose.

Days needed to grow TAV5 for corn stover = 434

Total labor cost = 434 days * $20 per hour * 1 hour per day = $9,320

Table *4-5* shows the mass of TAV5 to be added for each waste, based on a 500 tpy digester. The cost for growing this amount of TAV5 can be estimated as follows, using corn stover as an example:

Cost of TAV5 for corn stover = $0.833/kg TAV5 * 14 tons of TAV5 * 1000 kg/ton = $11,662

Table 4-5 shows the costs of growing TAV5 for a 500 tpy digester for the other wastes. It is assumed that the original TAV5 stays in the digester for 20 years (assumed digester lifetime) with microorganisms that die being replaced by new microorganisms.

During that time, the amount of waste processed is 500 tons/year * 20 years = 10,000 tons.

So cost of TAV5 per ton of waste processed can be estimated as follows:

Cost of TAV5 for corn stover = $20,982

Waste processed over 20 years = 10,000

Cost of TAV5 for corn stover = $20,982/10,000 tons of waste processed = $2.09

Table 4-5 shows the cost of TAV5 per ton of waste processed for the other wastes. Rice husk has the highest cost since it needs the highest ratio of TAV5 and subsequently wheat straw has the lowest cost.

Table 4-5: Mass and cost of TAV5 for a digester processing 500 tons/year of agricultural waste

Parameter	**Corn Stover**	**Rice Straw**	**Wheat Straw**	**Rice Husk**
TAV5 percent	30%	30%	30%	60%
Mass of TAV5 to be added (tons)	14	14	14	28
Cost of electricity & broth	$11,662	$11,662	$11,662	$23,324
Labor cost	$9,320	$9,320	$9,320	$18,680
Total cost	$20,982	$20,982	$20,982	$41,964
Cost of TAV5 per ton of waste processed	$2.09	$2.09	$2.09	$4.19

4.4.1.3 Transportation

The transportation cost to deliver TAV5 from lab to the digester is also assumed to be zero because it was assumed that the lab which grows TAV5 is close enough to farm digester and does not need a heavy vehicle to deliver TAV5.

4.4.1.4 Usage

The increase in biogas production from using TAV5 results in higher electricity generation, meaning additional cost is required to convert biogas to electricity. Table 4-6 summarizes costs for biogas conversion to electricity using a microturbine, based on the POWER Tool.

Table 4-6: Cost for biogas conversion to electricity using microturbine

Waste type	**Additional capacity needed (kW)**	**Cost for biogas conversion (for 20 years)**
Corn stover	16	$167,431
Rice straw	21	$181,670
Wheat straw	14	$159,866
Rice husk	8	$142,067

The increase in electricity generation also means that the farm does not have to purchase this electricity from the grid. To calculate the avoided cost of purchasing electricity, due to the use of TAV5, the average increase in methane generation from the lab-scale reactors was used. Electricity produced from this methane was estimated according to the following formula.

Energy generated by waste (BTUs/year) = (Annual methane production, m^3/lb) * (Methane heating value, BTUs/ft^3) * (1 ft/0.3047 m)^3

Energy generated by corn stover (high estimate) (BTUs/year) = (147,265 m^3/lb * 911) * (1 ft/0.3047 m)^3 = 4.7x10^9 BTUs/year

Electricity Generated (kWh/year) = Energy generated (BTUs/year) * (Average conversion efficiency) /(3412 BTUs/kWh)

Electricity Generated by corn stover (high estimate) (kWh/year) = 4.7x10^9 BTUs/year * 0.2925 / (3412 BTUs/kWh) = 406,550 kWh/year

Electricity Generated by corn stover (low estimate) (kWh/year) = 401,338 kWh/year

Average Electricity generated by corn stover = 403,944 kWh/year

Electricity generated without TAV5 = 260,609 kWh/year

Increase in electricity for corn stover (high estimate) = 403,944 kWh /year - 260,609 kWh/year = 143,335 kWh

Electricity cost was assumed to be $0.12 per kWh (ElectricRate, 2023). Table 4-7 shows the electricity cost avoided for each type of waste. Annual cost was converted to present value assuming 2% interest rate (average value in the US for the 10-year period prior to the pandemic, Macrotrends) using a period 20 years. 20 years was the assumed digester lifetime, based on the economic life of AD, typically taken as 15-20 years (Urban et al., 2009). The negative sign in the table represents the costs avoided by adding TAV5 (negative costs, or benefits).

Table 4-7: Increase in electricity production and cost avoided by using TAV5

Estimate	**Corn stover**	**Rice straw**	**Wheat straw**	**Rice husk**
Increase in Electricity Production (kWh per year)	143,335	185,033	121,183	69,061
Cost avoided per year	-$17,200	-$22,204	-$14,542	-$8,287
Cost avoided for lifetime of digester (20 years)	-$344,000	-$444,080	-$290,840	-$165,740

The cost to add TAV5 to the AD was assumed to be zero since TAV5 needs to be added only once and it is a very inexpensive task. To calculate the overall cost for the use phase, we added additional cost for biogas conversion and cost avoided by increase in electricity production. Table 4-8 shows the overall cost associated with the use phase for the lifetime of the digester.

Table 4-8: Summary of total cost for use phase

	Corn Stover	**Rice straw**	**Wheat straw**	**Rice husk**

Cost for additional biogas conversion to electricity	$167,431	$181,679	$159,866	$142,067
Cost avoided by increase in electricity production	-$344,000	-$444,080	-$290,840	-$165,740
Total cost	-$176,569	-$262,401	-$130,974	-$23,673

4.4.1.5 End of Life

There is no cost associated with end of life since once TAV5 is grown, it can continue to be used as long as AD is running.

4.4.1.6 Summary of costs

Table 4-9 summarizes costs associated with adding TAV5 to a farm digester, over all phases of the 20-year life cycle. The negative sign indicates the avoided cost, which outweighs the manufacturing cost of TAV5. This means that using TAV5 increases revenue from selling electricity generated by ADs. Rice straw shows the highest avoided cost since it had the highest

increase in methane production. Only rice husk has a positive cost, meaning it is not financially viable to use rice husk for TAV5 seeding.

Table 4-9: Summary of costs associated with adding TAV5 to a farm digester, by life cycle phase

Life cycle phase	**Corn stover**	**Rice straw**	**Wheat straw**	**Rice husk**
Material acquisition	NA	NA	NA	NA
Manufacturing	$20,982	$20,982	$20,982	$41,964
Transport	NA	NA	NA	NA
Use	-$176,569	-$262,401	-$130,974	-$23,673
End of life	NA	NA	NA	NA
Total	-$155,587	-$241,419	-$109,992	$18,291

4.4.2 Environmental analysis

4.4.2.1 Material acquisition

Since agricultural waste is already present at the site, it is assumed that there are no emissions associated with acquiring it.

4.4.2.2 Manufacturing

TAV5 was grown inside incubator shakers. The amount of electricity needed to grow the TAV5 required for each waste (assuming a digester treating 500 tons/year of waste) was estimated in the previous section on costs. Emissions resulting from electricity usage were estimated using values from SimaPro, as shown in Table 4-10.

Table 4-10: Emission factors for regular US power mix

Emissions during electricity generation from regular power mix (lb/MWh) (Simapro)				
NOx	**SO_2**	**VOC**	**PM**	**CO_2**
0.3476	1.573	0.093	0.025	16.7

Table 4-11 summarizes electricity needed to grow TAV5 for each type of waste and resulting emissions.

Table 4-11: Emissions from electricity use to grow TAV5

Waste	TAV5 required (tons)	Electricity needed to grow TAV5 (kWh)	Emissions, kg for lifetime of AD				
			NOx	SO_2	VOC	PM	CO_2
Corn stover	14	33,297	5.3	23.8	1.4	0.4	252.8
Rice straw	14	33,297	5.3	23.8	1.4	0.4	252.8
Wheat straw	14	33,297	5.3	23.8	1.4	0.4	252.8
Rice husk	28	66,523	10.5	47.6	2.8	0.8	505

4.4.2.3 Transportation

Emissions from transportation were assumed to be zero since all the equipment needed to grow TAV5 is assumed to be available. Also, no emissions were included for the transport of TAV5 from lab to the digester because a small vehicle can be used for this purpose which is assumed not have significant emissions compared to the other phases.

4.4.2.4 Usage

The POWER TOOL was used to estimate the emissions resulting from biogas conversion to electricity using microturbines. Direct emissions of criteria pollutants depend on the power generation capacity, according to EPA equations for microturbines of 30-330 kW capacity, shown in Figure 4-10, 4-11, 4-12, 4-13 and 4-14 (EPA, 2016). Indirect emissions due to electricity consumption for biogas conversion were calculated using emission factors shown in Table 4-12 and 4-13. Emissions were calculated based on low and high methane increase estimates for all four wastes. Since electricity produced using biogas replaces electricity produced from general mix, emissions from the general mix were subtracted. The emission calculations assumed a farm digester with 500 ton per year waste input capacity.

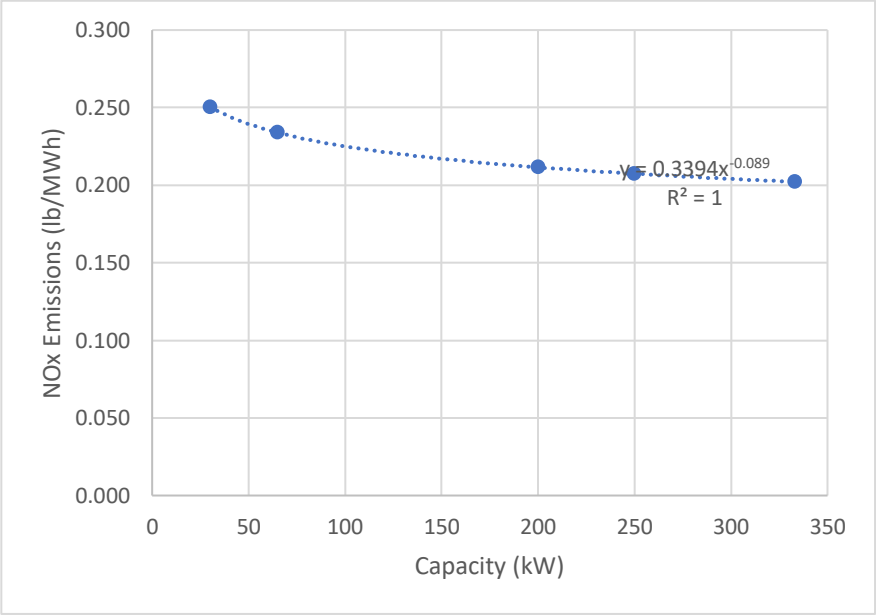

Figure 4-10: Regression curve for NOx emissions from biogas conversion using a microturbine (EPA, 2016)

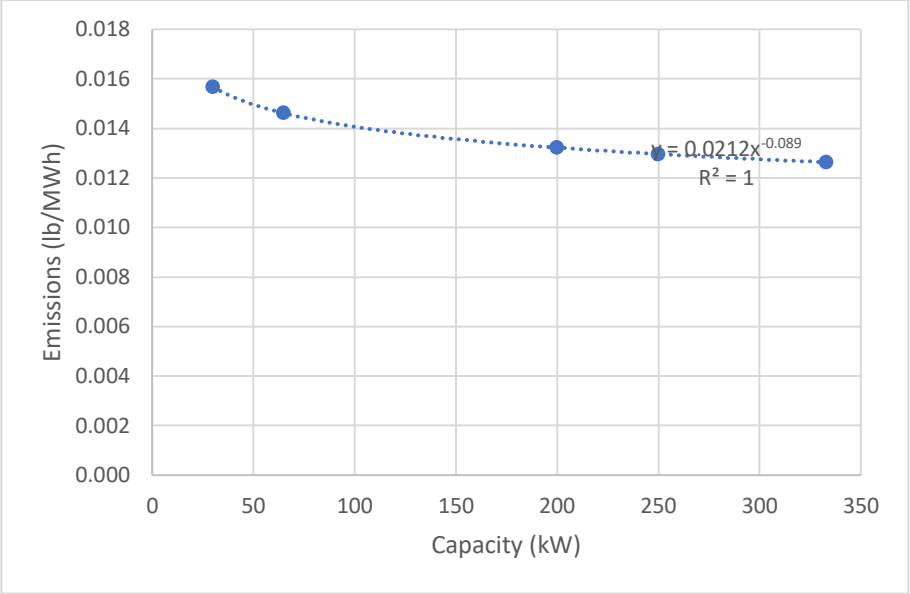

Figure 4-11: Regression curve for PM emissions from biogas conversion using a microturbine (EPA, 2016)

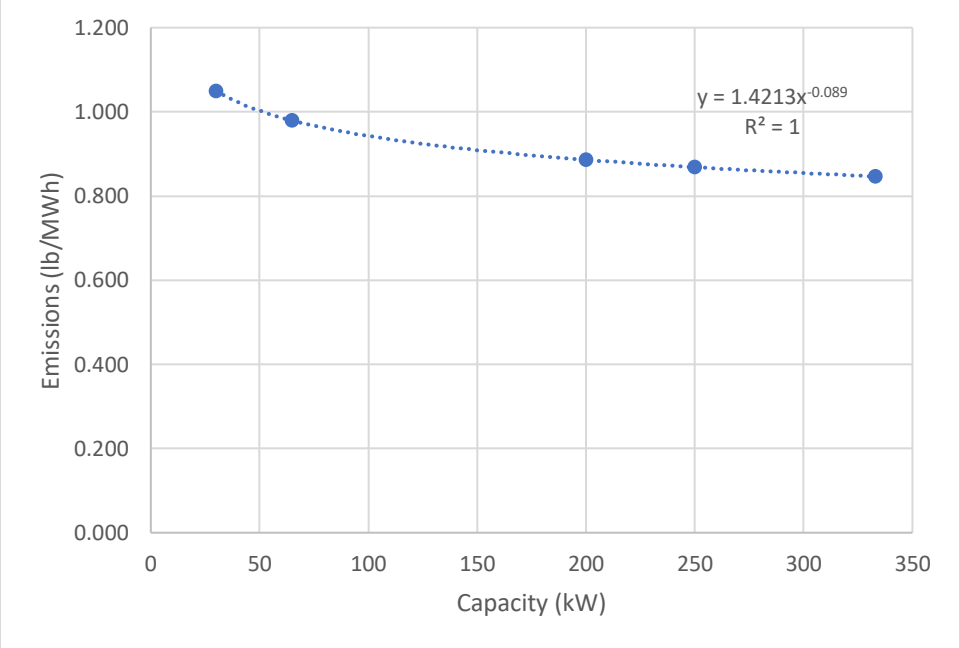

Figure 4-12: Regression curve for SO_2 emissions from biogas conversion using a microturbine (EPA, 2016)

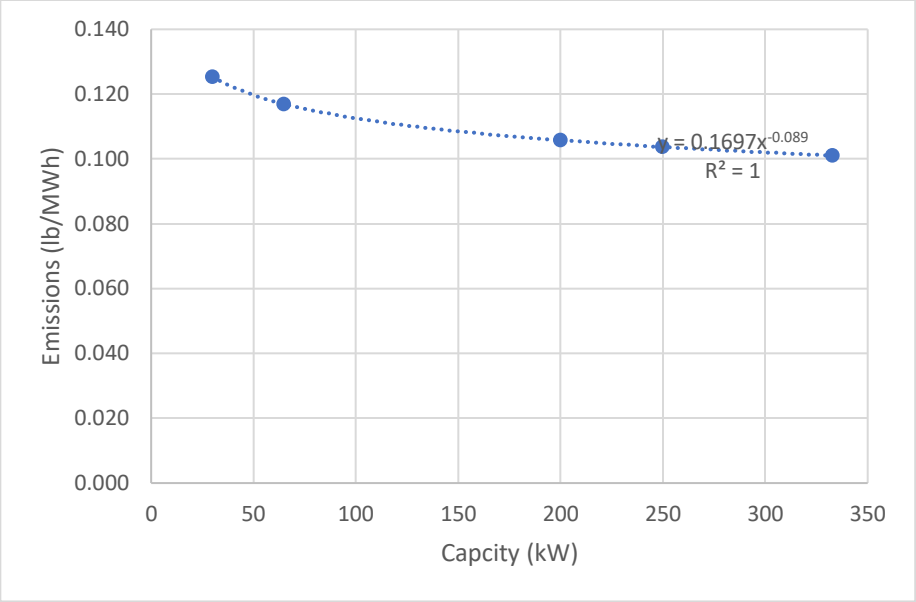

Figure 4-13: Regression curve for VOC emissions from biogas conversion using a microturbine (EPA, 2016)

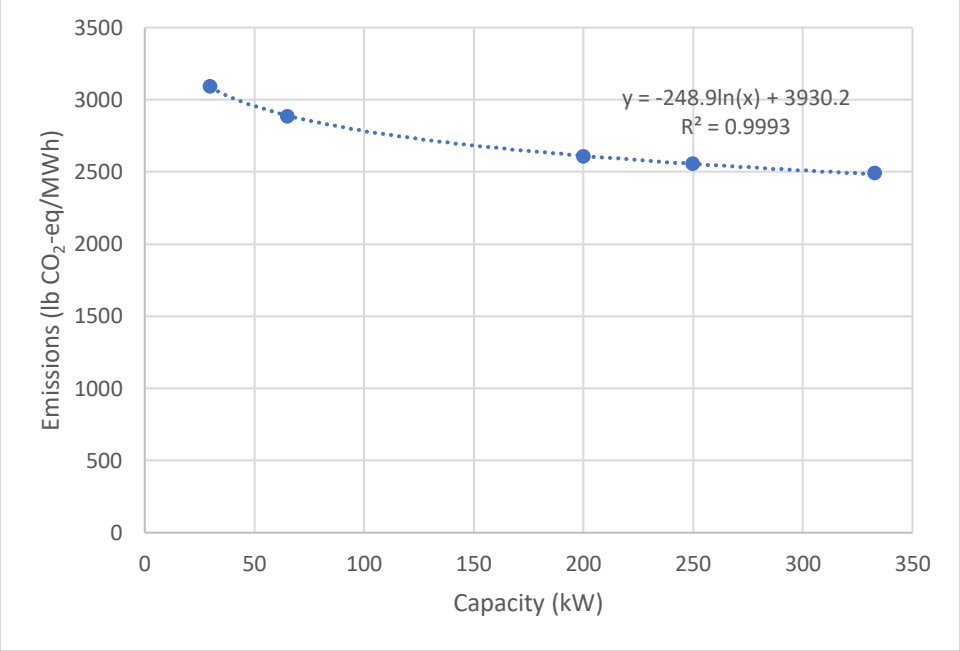

Figure 4-14: Regression curve for CO_2 equivalent emissions from biogas conversion using a microturbine (EPA, 2016)

Table 4-12: Emission factors for indirect emissions of criteria pollutants associated with biogas conversion

Indirect Emissions (lb/MWh) Or (lb/MMBTU) Ref: Simapro			
NOx	**PM**	**VOC**	**SO_2**
0.35	0.02	0.09	2
Emission for US electricity, low voltage (lb/Mwh) Ref: Simapro			
0.35	0.02	0.09	2

Table 4-13: Emission factors for indirect emissions of CO_2 associated with biogas conversion

Emission factors for CO_2	
Direct GHG Emissions (lb CO_2-e/MWh output) & (lb CO_2-e/MMBTU output)	80
Indirect GHG Emissions (lb CO_2-e/MWh output) & (lb CO_2-e/MMBTU output)	850
Emission for US electricity, (lb CO_2-e/MWh output) low voltage	850

According to Table 4-14, it was found that due to biogas increase by using TAV5, emissions from criteria pollutants SO_2, NOx and PM10 can be reduced. Additionally, reduction in GHG emissions was also calculated. This reduction in emissions is due to the cleaner electricity production from the increased biogas production, which replaces electricity largely generated by fossil fuels. However, there is an increase in VOC emissions because biogas itself contains methane, which may leak or slip out without being burned.

Table 4-14: Net emissions from biogas conversion to electricity (use phase)

Waste	Methane Production Scenario	Emissions, kg for lifetime of AD				CO_2 (metric tons for lifetime of AD)
		NOx	PM	VOC	SO_2	
All	Baseline - No TAV5	-211	-20	82	-1155	-1776
Corn Stover	Low	-359	-33	108	-1924	-2735
	High	-365	-33	109	-1953	-2770
	Avg.	-362	-33	108.5	-1938.5	-2753
	Increase over baseline	-128%	-65%	32%	-68%	-55%
Rice Straw	Low	-376	-34	111	-2012	-2841
	High	-440	-40	120	-2338	-3232
	Avg.	-408	-37	115.5	-2175	-3037
	Increase over baseline	-93%	-85%	41%	-88%	-71%

Wheat Straw	Low	-303	-28	99	-1634	-2380
	High	-374	-34	111	-1997	-2824
	Avg.	-338.5	-31	105	-1815.5	-2602
	Increase over baseline	-60%	-55%	28%	-57%	-47%
Rice Husk	Low	-262	-24	92	-1420	-2113
	High	-303	-28	99	-1634	-2380
	Avg.	-282.5	-26	95.5	-1527	-2247
	Increase over baseline	-34%	-30%	16%	-32%	-53%

4.4.2.5 End of life

There are no emissions associated with end of life because TAV5 will continue to work until the digester is under use.

4.4.2.6 Summary of emissions

Table 4-15 summarizes emissions for all life cycle phases of growing and use of TAV5. Rice straw shows the highest reduction in NOx, PM, SO_2 and CO_2 emissions whereas rice straw also resulted in highest VOC emissions. The reason for the highest reduction in emissions for rice straw is that it produced the highest amount of methane, which can be used to produce cleaner electricity, resulting in lower emissions. Due to increase in methane emissions, there is a higher chance of methane leakage, which results in higher VOC emissions.

Table 4-15: Summary of emissions

Corn stover					
Life cycle phases	**NOx**	**PM**	**VOC**	**SO_2**	**CO_2 (metric tons for lifetime of AD)**
	kg for lifetime of AD				
Material acquisition	NA	NA	NA	NA	NA
Manufacturing	5.3	0.4	1.4	23.8	0.25
Transport	NA	NA	NA	NA	NA
Use	-362	-33	108.5	-1938.5	-2753
End of life	NA	NA	NA	NA	NA
Total	**-357**	**-33**	**110**	**-1915**	**-2753**
Rice straw					

Material acquisition	NA	NA	NA	NA	NA
Manufacturing	5.3	0.4	1.4	23.8	0.25
Transport	NA	NA	NA	NA	NA
Use	-408	-37	115.5	-2175	-3037
End of life	NA	NA	NA	NA	NA
Total	**-403**	**-37**	**117**	**-2151**	**-3037**
Wheat straw					
Material acquisition	NA	NA	NA	NA	NA
Manufacturing	3.4	0.2	0.9	15.3	0.16
Transport	NA	NA	NA	NA	NA
Use	-338.5	-31	105	-1815.5	-2602

End of life	NA	NA	NA	NA	NA
Total	**-335.1**	**-31**	**106**	**-1800**	**-2602**
Rice husk					
Material acquisition	NA	NA	NA	NA	NA
Manufacturing	10.5	0.8	2.8	47.6	0.5
Transport	NA	NA	NA	NA	NA
Use	-282.5	-26	95.5	-1527	-2247
End of life	NA	NA	NA	NA	NA
Total	**-272**	**-25**	**98**	**-1479**	**-2247**

Chapter 5: Conclusion and Recommendations

5.1 Conclusions

5.1.1 Growth of TAV5

- A growth curve was developed for TAV5 and confirmed via cell counts. TAV5 can be ready for harvesting within 48 hours for ratio of 1:4 (TAV5 to broth solution) and can take 80 hours for 1:33. For 1:4, a very large amount of initial TAV5 stock is required; thus, for commercial purposes it is more optimal to use 1:33, even though the time to harvest is longer. TAV5 cells should be harvested at 0.35 optical density.
- TAV5 can grow both aerobically and anaerobically; however, higher growth was observed in aerobic conditions. This is fortunate, since aerobic conditions do not require a special anaerobic chamber, and thus are cheaper.
- TAV5 growth over 37^{o}C is very limited. The fastest growth was observed at 30^{o}C.

5.1.2 Optimal TAV5 ratio and gas production

The optimum ratios of TAV5 to WRRF sludge-based inoculum are shown in Table 5-1, along with observed percent increases in methane. A higher ratio of TAV5 to WRRF sludge was needed for rice husk which could be because it has a higher lignin content compared to other wastes used in this research or it might also be because rice husk was not grounded, whereas the other waste streams were ground to have smaller particle size. However, no correlation was found between the lignin values of the other wastes and the TAV5 ratio, nor between the lignin values and the increase in methane production.

Table 5-1: Optimal TAV5 ratios and increased gas production

Waste	Lignin measured	Optimal TAV5/WRRF ratio	Average increase in methane production (range)
Corn stover	15.2%	0.3	55% (54-56%)
Rice straw	13%	0.3	71% (60-82%)
Wheat straw	17.3%	0.2	47% (34-59%)
Rice husk	20%	0.6	27% (19-34%)

5.1.3 Economic and environmental analysis

Table 5-2 shows total costs associated with adding TAV5 to ADs. Including TAV5 in the ADs resulted in net negative costs for corn stover, rice straw and wheat straw but rice husk resulted in net positive cost. The revenue generated by extra electricity production more than offsets the cost of growing TAV5 for each waste type except rice husk. Rice straw shows the highest avoided cost due to higher increase in methane generation.

Table 5-2: Total cost per ton of waste processed for adding TAV5 for lifetime of digester

Waste type	**Cost for lifetime (20 yr) of digester**
Corn stover	-$155,587
Rice straw	-$241,419
Wheat straw	-$109,992
Rice husk	$18,291

Table 5-3 summarizes emissions for each waste type. Seeding AD with TAV5 reduced emissions of carbon dioxide and criteria pollutants (NOx, SO_2) emissions, since the increased biogas production could be used to produce cleaner electricity than the average electricity from the power grid. However, VOC emissions increased because biogas itself has a high VOC content (around 50% methane). Rice straw shows the highest reduction in NOx, PM, SO_2 and CO_2 emissions because it generates the highest amount of methane, which in turn produces more clean electricity.

Table 5-3: Summary of net emissions for each waste type

	Emissions (kg for lifetime of AD)				CO_2 (metric tons for lifetime of AD)
Waste type	**NOx**	**PM**	**VOCs**	**SO_2**	
Corn stover	-357	-33	110	-1915	-2753
Rice straw	-403	-37	117	-2151	-3037
Wheat straw	-335.1	-31	106	-1800	-2602
Rice husk.	-272	-25	98	-1479	-2247

5.2 Recommendations for future studies

Based on the results attained in this study, the following recommendations can be made for future research.

- Batch reactors were incubated at 30°C; however, AD temperature can range from 30 to 37°C (upper temperature limit for TAV5 growth). Future work can incubate reactors at different temperatures to examine how TAV5 increases methane production as a function of temperature.
- Test additional agricultural wastes as well as mixtures of waste.

- Measure methane production from crushed rice husk, to determine whether the low production was due to large particle size or high lignin content.
- Analyze bioreactor sludge to measure before and after bacterial compositions.
- Test the effect of varying pH values on TAV5 growth.
- Conduct a field scale testing of TAV5 seeding with the optimum ratios identified in this study.
- Test TAV5 in a real farm digester.

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Appendix

Cell count detailed results

Figure A1, A2 and A3 show the results from cell count procedure. TAV5 cells were grown in R2B Broth solution and each day a sample was taken out of those cultures to count cells. Serial dilutions ranging from 10^{-1} to 10^{-6} were done for every sample each day to reduce the number of colonies on the petri dishes. It would be impossible to count colonies without any dilution since there would be overwhelming growth which will result in merged colonies.

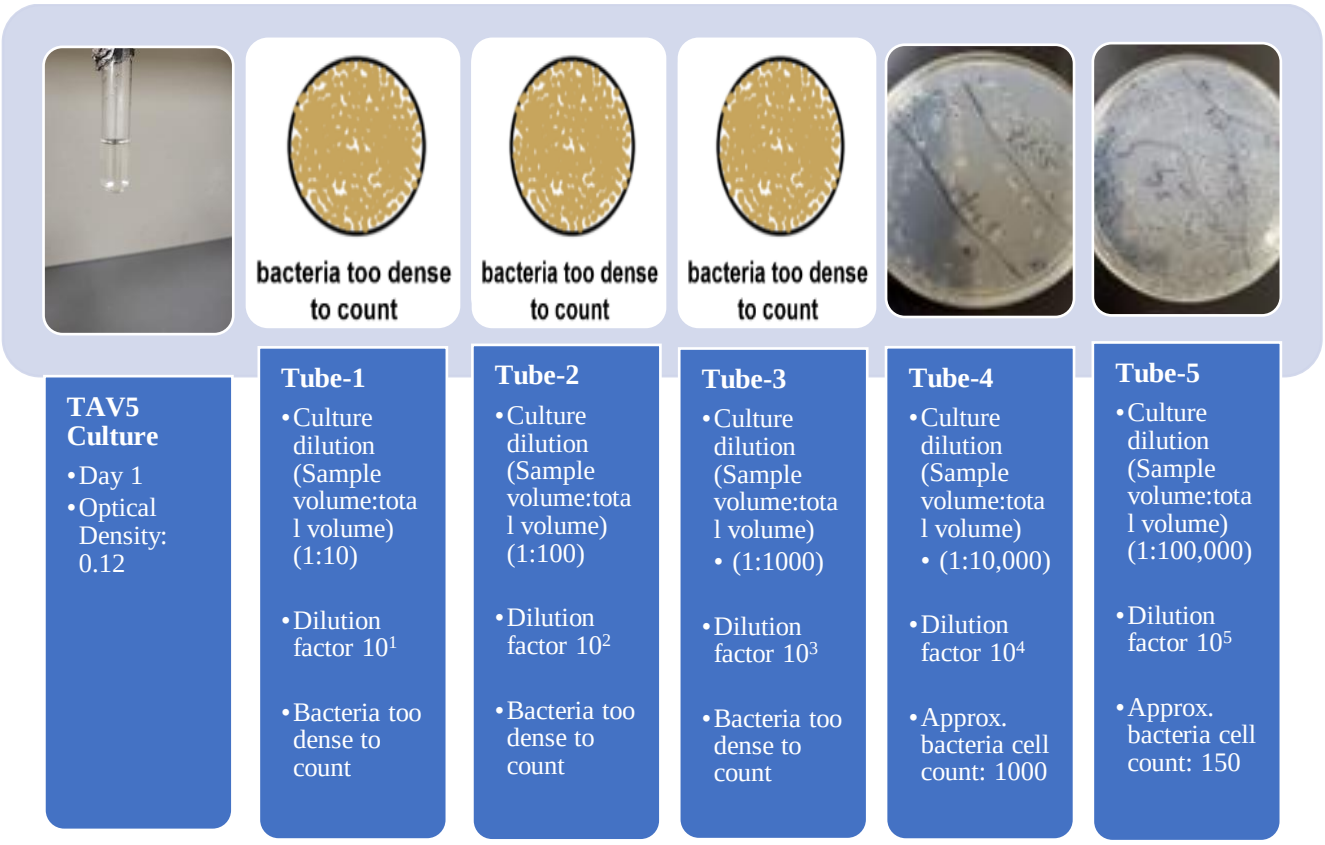

Figure A-1: Day 1 of Cell Count

Figure A-2: Day 2 of Cell Count

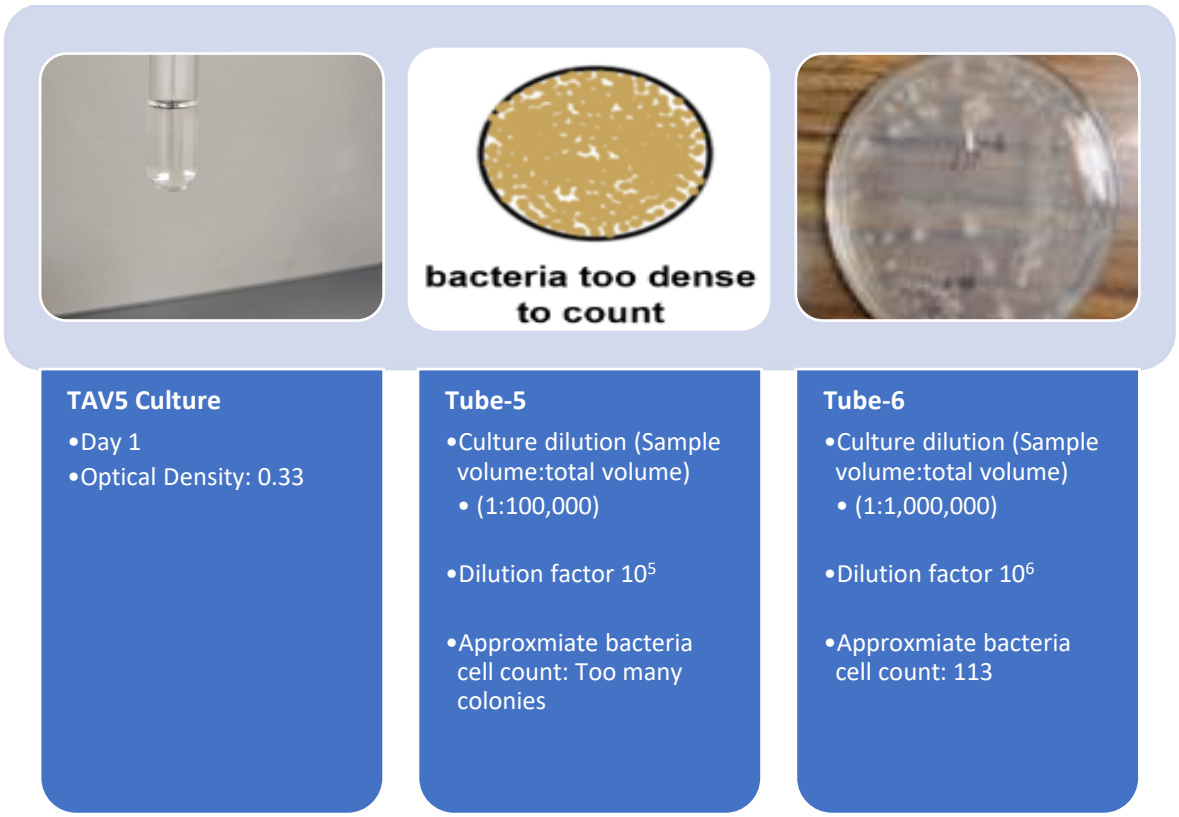

Figure A-3: Day 3 of cell count

On day 1 TAV5 cells reached an optical density of 0.12, with the number of cells around 150 at dilution of 10^{-5}. The number of cells for 10^{-4} dilution was over 1000 (too many to count); thus, this dilution was discarded. On the second day, the optical density of bacterial culture reached 0.27 and the number of bacterial colonies also increased to 270 for 10^{-5} dilution, showing growth. On third day the TAV5 culture reached optical density of 0.33 and the number of bacterial colonies reached 113 at dilution of 10^{-6}, which again shows high growth of bacteria. The number of cells increased exponentially from day 1 to day 3, showing that the cells are alive and growing. According to growth curves shown in the previous section, during log phase optical density of 0.35 is achieved. The results of cell count show exponential growth by third day at optical density of 0.33, which verifies our growth curves.

Biographical Information

Hussain Ali graduated from Department of Electrical Engineering, Government College University Lahore in July 2016 with a Bachelor of Science degree in Electrical Engineering. After graduation, he started his master's from US-Pakistan Center for Advanced Studies in Energy, National University of Sciences and Technology, Islamabad and got his degree in Electrical Engineering (Power) in 2019. His research focused on development of sustainable energy infrastructure and policies in Pakistan. He started his Ph.D. program under the supervision of Dr. Melanie Sattler in spring 2020 and received his Ph.D. degree in Civil Engineering from University of Texas at Arlington in summer 2023.