ANAEROBIC CO-DIGESTIONOF ABATTOIR AND WINERY SOLID WASTE FOR ENHANCED BIOGAS PRODUCTION

by

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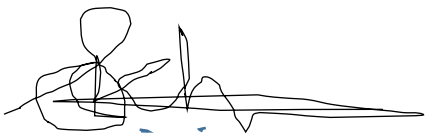
Declaration

I, **Sandile Charles Khumalo**, declare that the content of this thesis is my own unaided work, and that this thesis has not previously been submitted for any degree or examination in any other academic institution. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology or the National Research Foundation of South Africa.

All scientific concepts, theories, methodologies and material derivations and model developments used in this thesis and published in various scientific journals (except those of which the candidate is not the first author) were derived solely by the candidate and first author of the published manuscripts. Where appropriate, intellectual property of different scholars was acknowledged by using appropriate references; and the contribution(s) of co-authors to conference papers and published manuscripts and supervisory capacity (Dr VI Okudoh) and co-supervisory capacity (Prof O Oyekola) were as well recognized.



---------- 10/28/2020 ----------

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Abstract

The thesis investigated the potential application of abattoir and winery solid waste as co-substrates for enhanced biogas generation as well as stabilizing the AD process by using standard laboratory biochemical methane potential (BMP) techniques. Various input parameters on biodegradability, overall AD efficiency and bioenergetic kinetics were evaluated. The inoculum used was locally synthesized from zebra dung and ruminal content. Abattoir waste is rich in fats and proteins which makes it highly sought to produce good quality biogas with a high methane content. However, several challenges arise from sole processing of this type of wastes due to its low carbon-to-nitrogen (C/N) ratio. Thus, a supplemental substrate was used to mitigate issues associated with mono-digestion, namely winery solid wastes.

BMP tests were conducted in batches under mesophilic temperature (38±0.5 ºC) conditions, utilizing abattoir solid (A_s), cow blood (C_b) and winery solid (W_s) wastes in mono- and co-digestion modes for a period of 30 days. The parameters evaluated were substrate-ratio (1:1:21, 1:1 and 2:3), food-to-microorganism ratio (F/M) (0.5-2) and volatile solids (VS) concentration (5-20 gVS/L). For anaerobic co-digestion (AcoD) experiments binary blends (1:1 and 2:3) of A_sW_s and C_bW_s and a ternary blend (1:1:2) of $A_sC_bW_s$ were used to determine the effects of the simultaneous processing on specific methane production (SMP) and the overall digestion efficiency. Bioenergetic kinetics and parameter estimation were conducted using curve-fitting and least squares nonlinear regression techniques of experimental data points to the first order, and Gompertz, Logistic and Richard's model(s). The optimization of biogas production was also evaluated in two-lock steps. Firstly, a screening ABCD mixture design was developed to determine the optimal mixture blend composition, and to assess individual, synergistic and or antagonistic effects of each substrate within the mixture. The last step was evaluation of optimal conditions for methane production using identified optimal mixture blends from the previous step employing Response surface methodology (RSM) to a Central composite rotatable design (CCRD). Herein, the effects of organic load, food-to-microorganisms (F/M) ratio and initial reactor pH were investigated with SMP and maximum specific methane production rate (R_{max}) as the response(s) variables. After optimization, biogas production from the anaerobic co-digestion of five mixture blends consisting of A_sC_b (1:1), A_sW_s (2:3), C_bW_s (2:3), and $A_sC_bW_s$ (1:1:1 and 1:4:1), was studied using up-scaled two 5L acrylic custom built laboratory digesters equipped with a pH-control, gas-scrubbing and metering units and an automated data logging/control system for pH and temperature control. The digesters were run under mesophilic conditions to compare the operational efficiency of batch, single-stage semi-continuous and two-stage semi-continuous mode(s). For batch experiments, short retention periods of 5-15 days and semi-continuous experiments were run with varying hydraulic retention time (HRT) of (19 - 450 days), organic load (0.3-1.4 gVS/Ld^{-1}). The results from the BMP studies determined the highest SMP from mono-D of A_s and C_b to be 192 and 110 $NmLCH_4/gVS_{added}$. They also revealed that AcoD of 2:3 binary mixture blends of A_sW_s and C_bW_s yielded the highest SMP's of 370 and 354 $NmLCH_4/gVS_{added}$, while ternary blends yielded poorest results where a SMP of 22 $NmLCH_4/gVS_{added}$ was recorded. All kinetic models sufficiently simulated SMP with coefficient of determination (R^2) values above 90%. Furthermore, an increase in F/M and VS concentration negatively impacted the overall

digestion performance. This led to the conclusion that various factors play a significant role in the efficiency of the overall AD process, and that most particularly mixture compositions, organic load, F/M ratio and pH were identified as more relevant in the AD process. The optimal mixture blend was determined to be of (1:4:1) $A_sC_bW_s$ and A_sC_b (1:1) with a SMP of 112 and 104 $NmLCH_4/g\ VS_{added}$ and maximum SMP rate (R_{max}) of 11 and 14 $CH_4/gVS\ day^{-1}$, respectively. Overall, 21.3 % and 29.3 % improvements in SMPs were respectively recorded from ternary and binary blends, as the results of synergistic effects prompted by mixture blending. The antagonistic effects were only recorded for mixture of A_sW_s. The RSM optimization results showed organic load of 1.59 g VS/L, F/M of 0.25 gVS/gVS and initial pH of 6.5 as optimal values. A maximum SMP of 309 $NmLCH_4/gVS_{added}$ was predicted by the special cubic model for the $A_sC_bW_s$ mixture blend.

The experimental data was a close fit with SMP of 316 $NmLCH_4/gVS_{added}$ and maximum SMP rate (R_{max}) of 18 $CH_4/gVSday^{-1}$, respectively which was slightly higher than the predicted values as indicated by the coefficient of determination (R^2) of 96.7 %. The RSM was therefore successfully implemented in the optimization of SMP for AcoD of abattoir and winery wastes. Primary emphasis was given to $A_sC_bW_s$ (1:4:1) mixtures which resulted in the highest methane efficiency and stable operation. The highest methane yield of above 500 NmL was obtained in batch mode. Abattoir and winery waste can be successfully co-digested to improve biomethane production by optimizing AD process input parameters.

Dedication

"Seek simplicity and distrust it" Alfred North Whitehead (1861-1947)

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I would like to thank:

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(Opinions expressed in this thesis and the conclusions arrived at, are those of the author, and are not necessarily to be attributed to the National Research Foundation.)

Research Output

The following research outputs represent the contributions of the author to the scientific knowledge and development during his master's candidacy (2017-2020):

1. Khumalo, S.C Oyekola O.O and V.I Okudoh. (2021). Evaluating input parameter effects on the overall anaerobic co-digestion performance of abattoir and winery solid wastes. Article published in Elsevier journal: *Bioresources Technology Reports*. Volume 13, February 2021, 10063. https://doi.org/10.1016/j.biteb.2021.100635.
2. Khumalo, S.C Oyekola O.O and V.I Okudoh. Anaerobic co-digestion of environmentally recalcitrant abattoir and winery solid wastes in Proceedings of the 8th *International Conference on Engineering for Waste and Biomass Valorization,* May 31 - June 4, 2021, Guelph-Canada (Accepted conference paper).
3. Khumalo, S.C Oyekola O.O and V.I Okudoh. Anaerobic co-digestion of environmentally recalcitrant abattoir and winery solid wastes in Proceedings of the 8th *International Conference on Engineering for Waste and Biomass Valorization,* May 31 - June 4, 2021, Guelph-Canada (Accepted oral presentation).

Layout of Thesis

This research aimed at evaluating the potential application of abattoir and winery solid waste in an anaerobic co-digester to produce more biogas. The references are listed at the end as a separate chapter in accordance with the Harvard method of referencing.

The thesis is subdivided into the following chapters:

- **Chapter 1** namely, the introduction provides an insight into anaerobic digestion and applications in waste management. It also presents the statement of the problem, the justification and significance of this study, the hypothesis assigned to this project, the research objectives and delineation of the study.
- **Chapter 2** is about the literature review.
- **Chapter 3** presents the research methodology employed by providing a detailed description of the materials and methods used in this study.
- **Chapter 4** discusses and evaluates the biodegradability and biomethane potential of the tested substrates by focusing on mono- and co-digestion.
- **Chapter 5** is about a mixture blending for enhanced **AD** process efficiency using mixture interactions (i.e. either synergistic or antagonistic) to determine optimal mixture blends.
- **Chapter 6** is about evaluation of optimal conditions for processing above determined mixture blends using Response Surface Methodology (**RSM**).
- **Chapter 7** presents the findings on optimal mixture blends at their respective environmental processing conditions determined from **BMP** experiments, where these conditions were tested in upscaled five-liter laboratory digesters simulating full-scale plant operations.
- **Chapter 8** gives an overview of the research findings and provides the recommendations for future research. The answers to the research questions posed in chapter 1 are also analyzed and answered in this chapter.

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List of Symbols

Nomenclature

Symbol	Description	Units
P	Atomic phosphorous	
OH^-	hydroxyl ion	
O	oxygen	
Ni^{2+}	Nickel ion	
Na^+	Sodium ion	
N	Atomic Nitrogen	
K^+	Potassium ion	
H_2S	Hydrogen sulphide	
H_2	Hydrogen gas	
Co^{2+}	Cobalt ion	
CO_2	Carbon dioxide	
CH_4	Methane gas	
Ca^{2+}	Calcium ion	
S	Atomic sulphur	

Greek Symbols

Symbol	Description	Units
μ	Specific Growth Rate	day^{-1}
μ_{max}	Maximum Specific Growth Rate	day^{-1}
λ	Lag phase	day
v	Richard's Model shape factor	dimensionless
Δ	Variation of a Function	dimensionless
α		dimensionless
∞	Greater than Countable Numbers	dimensionless
β		dimensionless

Mathematical, Chemistry, Physical and Biological Functions

^oC	Degrees Celsius	
$EXP/\ e^{-1}$	Exponent	dimensionless
ΔG	Gibb's Free Energy Function	KJ mol^{-1}
ΔH	Reaction Enthalpy	KJ mol^{-1}
k	Kinetic Constant	day^{-1}
K_s	Half Velocity Constant	g $liter^{-1}$
log	Logarithm	dimensionless
ln	Natural Logarithm	dimensionless
R	Ideal Gas Constant	
R_{max}	Specific methane Production Rate	NmL g^{-1} VS day^{-1}
S	Concentration of Limiting Substrate	g $liter^{-1}$
t	Time	day
X	Biomass Concentration	g $liter^{-1}$
$Y_{X/S}$	Growth Yield	

Superscripts and subscripts

max	maximum
min	minimum
-1	per
x/s	Biomass/Substrate
s	Substrate

Glossary

Abbreviation	Full description
AD	Anaerobic digestion
AcoD	Anaerobic Co-digestion
BMP	Bio-methane Potential
BOD	Biological Oxygen Demand
CHP	Combined Heat and Power
CPI	Co-digestion Performance Index
GHG	Green House Gas
HRT	Hydraulic Retention Time
Mono-D	Mono Anaerobic Digestion
OLR	Organic Loading Rate
P_s	Standard Gas Pressure
SCOD	Soluble Chemical Oxygen Demand
TA_L	Total Alkalinity
T_s	Standard Gas Temperature
VFA	Volatile Fatty Acids
VOC	Volatile organic Compounds
VS	Volatile Solids
V_s	Standard Gas Volume

Clarification of terms

Acidogens	Acidogenic bacteria that convert simple and soluble compounds into CO_2, H_2, simple alcohols, and VFA's;
Acetogens	Acetogenic bacteria producing acetate/acetic acid, and H_2 from acidogenic bacteria synthesis by-products;
Anaerobic Digestion	Microbiological transformation (i.e. degradation) of organic waste (i.e. substrate) in the absence of oxygen (anaerobic) to its most reduced carbon state, and produces gaseous waste biogas;
Anaerobic Co-digestion	Simultaneous digestion of substrates;
Anaerobic Digester	Air-tight man-made vessel used for biogas production;
Biogas	A methane-rich renewable gaseous fuel produced via AD
Bio-energic	Thermodynamic description of biological active environments.
Biomethane Potential	Specific methane and extent of biodegradation of a given substrate at set process conditions.
Biotic Potential	Maximum growth rate for a given population at set specific environmental conditions
Carrying Capacity	The maximum number of species that can be supported by a system set at specific environmental conditions
Inoculum	An active source of microbiological transformers cultured for seeding the digesters.

CHAPTER 1

INTRODUCTION

The world's rapid urbanization, consumption of natural resources and increased organic waste generation coupled with the rising energy demand, have resulted in the increased usage of fossil fuels, leading to greenhouse gas (GHG) emission that negatively impacts the environment by causing the climate change. Thus, the priority was put on the protection of the environment across the globe, particularly in heavily industrialized and developing countries as depicted in **Figure 1-1**. Therefore, the protection of the environment across the globe made the introduction of a new alternative sustainable energy-source(s) imperative. For example in South Africa, the Department of Energy (DoE) has set goals beyond 2020 to contract about 20 GW of renewable energy, of which about 25% of the projected 10 GW electricity supply is to be generated from renewable biomass; and to achieve a zero-waste-to-landfills vision within 2030 (Waste Act No. 59 of 2008) (Presidency, 2012). This will assist not only in sustainable waste management practices, but also in tackling pollution and reducing health-related risks for residents and the environment at large. The biogas produced using by-product organic wastes from the agricultural and food-production industries, can serve as a sustainable and renewable energy source towards building a future green-energy economy sector.

Abattoirs produce large quantities of liquid and solid organic waste during the course of their operation with a high potential for methane generation, hence, making them a highly sourced feedstock for biogas production for increasing profits on initial capital investments for a sustainable biogas-economy (Cirne *et al.*, 2007; Palatsi *et al.*, 2010; Karthikeyan and Visvanathan, 2013; Y. M. Yoon *et al.*, 2014; Ortner *et al.*, 2015). However, processing high strong wastes, that are concentrated in lipids and proteins, on large-scale has been associated with various operational problems, such as scum formation, accumulation of intermediate products (e.g. long chain volatile fatty acids, ammonia etc.) and the potential inhibition of the microbiological processes controlling biogas generation (Palatsi *et al.*, 2010; Pagés-Díaz *et al.*, 2015).

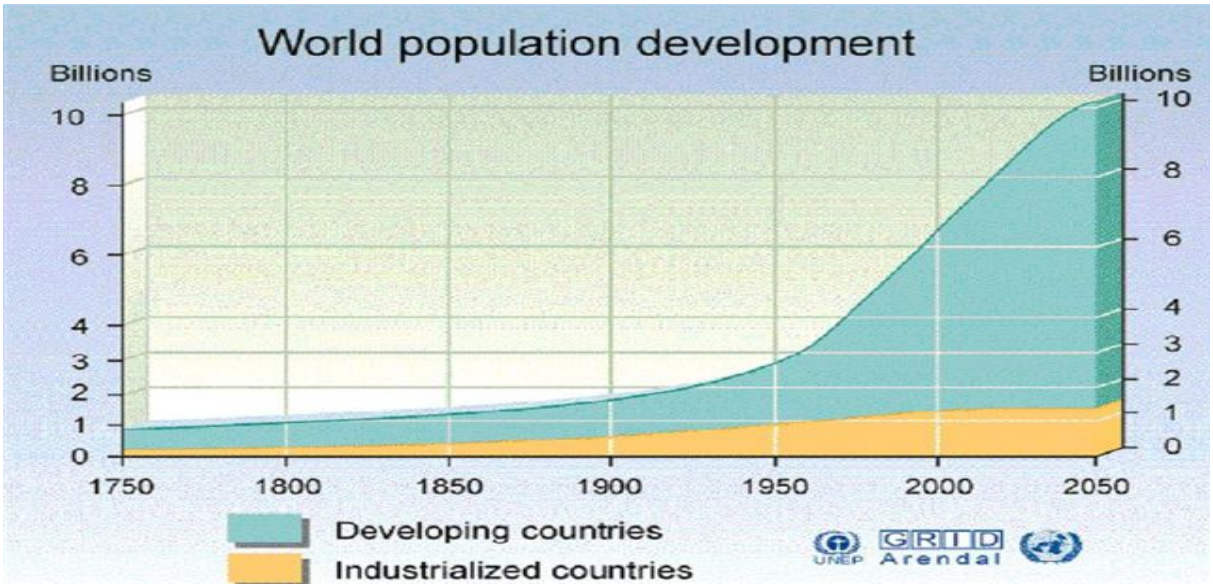


Figure 1-1: Projected world population growth (United Nations, 2017)

Dealing with the above anaerobic co-digestion (AcoD) of abattoir waste with residues/waste from winery industries has a beneficial advantage of balancing the nutrient requirements of the system, of diluting toxic material; and moreover, efficiently utilizing biogas-plant capacity. Although there is a large volume of research dealing with AcoD of abattoir waste with agricultural residue waste reported in literature, there was no research that was specifically conducted with the focus being on the use of winery waste as a co-substrate.

This thesis was therefore, focused on the evaluation of mono- and co-digestion of abattoir waste with solid winery waste in enhancing biogas production efficiency.

1.1 Background

The first records of AD date back to the 10th century BC where biogas was used for heating bath water in Assyria. In ancient China, solid anaerobic digesters were utilized as well in the mid-19th century. The construction of solid bio-digesters was also recorded at a leper colony in Bombay, India. Sludge digesters were also built in Exeter, UK to fuel street lamps in the 1890s (Bond and Templeton, 2011; Kigozi, Aboyade and Muzenda, 2014; Laks, 2017)

The rise in oil prices during the 1970s and energy security uncertainty motivated alternative energy research and allowed the spread of AD technology across different developing countries in the world. During the middle 1980s, AD eventually spread in industrial and urban waste management operations (Bond and Templeton, 2011).

In Asia, countries like China during the 1970s promoted the use of small-scale household digesters producing biogas for cooking and lighting purposes through government policy funds for every rural house-hold and facilitated the construction of over seven million digesters. In the year 2007, the number of biogas plants in China, was reported to be over 26 million. In India, the government also has a national biogas program that has been running since the 1980s, and during the late 1990s over 3 million digesters have been constructed (Bond and Templeton, 2011).

In developed countries, AD technology advanced over the past two decades, and more complex large-scale biogas plants have been constructed. In Germany alone, more than 3700 agricultural biogas plants were reported to be in operation as of 2007 (Seadi *et al.*, 2008). The world's largest commercial digester was built in South Africa in 1957. Currently, about 400-700 digesters, most of which are located in the Wastewater Management sector, have been constructed in South Africa between the 1970s and 1980s (Laks, 2017).

1.2 Problem Statement

Global industrial activity together with economic and population growth continue to be the significant green-house gas (GHG) emission drivers from fossil fuel combustion. Approximately 70 % of this combustion comes from cities alone as they consume more than half of the world's fuel and energy supply (IPCC, 2014). The high population density across global cities like Cape Town (SA) is driven by search for a better life e.g. social wellness, work, healthcare, etc., (see **Figure 1-2**). This presents a resource allocation challenge to municipalities as well as the huge amount of wastes generated (see **Figure 1-3**). The disposal of these wastes is always a big challenge, especially in SA. This is because of the prohibition of indiscriminate dumping in landfills coupled with rising costs of waste treatment, as well as local and global environmental health challenges associated with dumping (e.g. foul odors, leachate, etc.). In the Western Cape the by-product waste streams generated by abattoirs and the wine making industries have become more prevalent, as small business operators constantly fail to adhere to increasingly stringent government regulations. Thus, these wastes can be potentially applied in anaerobic digestion systems, reducing the hazardous risk to the environment while producing green energy and potentially adding a second revenue stream to these companies.

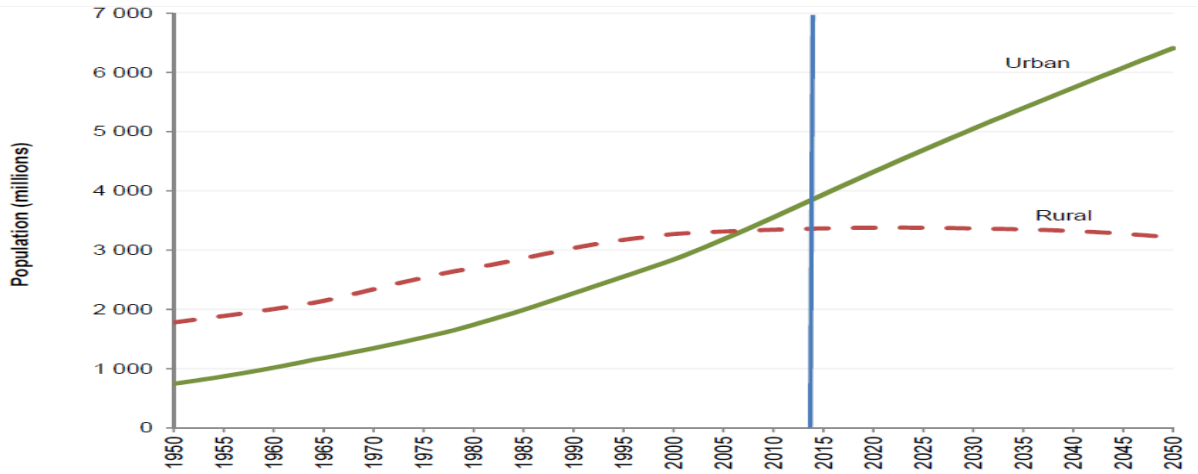


Figure 1-2: Projected growth in urban population from 1950 to 2050

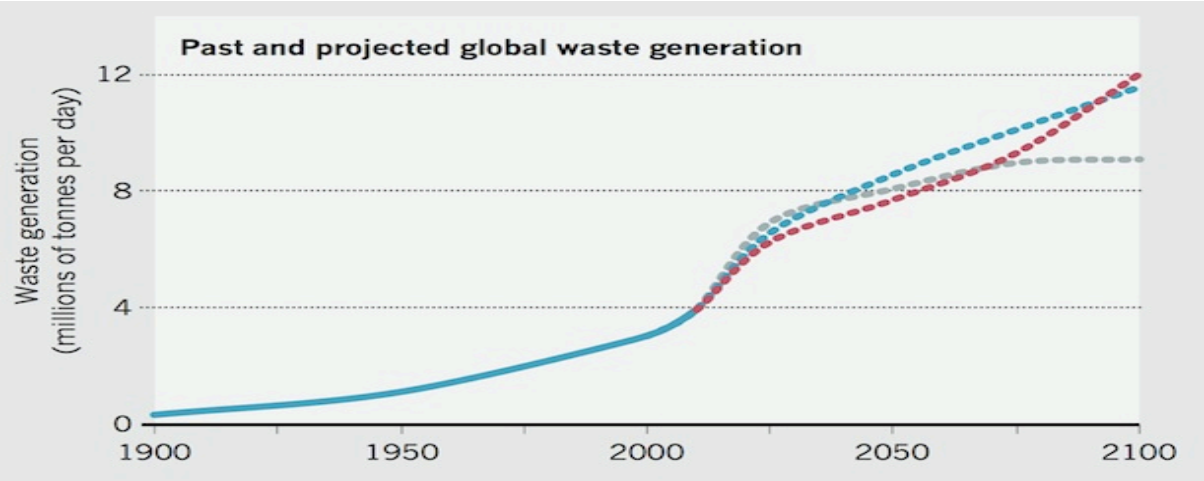


Figure 1-3: Projected global waste generation (source: https://www.smithsonianmag.com)

1.2.1 Justification

A sustainable and affordable supply of energy is a prerequisite for sustainable economic growth in developing countries. Its requirements are solely met through fossil-fuel based energy sources whose prices during the past years have increased and are continuously rising (Johansson *et al.*, 1992; EIA, 2017). For this reason, there is an urgent need to find and develop alternative renewable sources of energy such as biogas obtainable from organic waste which is readily available.

In South Africa there is no clear legislation guiding the use of abattoir waste as feedstock in AD with the industry in its infancy, except legislation governing the disposal off- and environmental protection acts, such as Meat Safety Act (Act No. 40, 2000); National Environmental Management: Air Quality Act (Act No. 39, 2008); and the National Environmental Management: Waste Act (Act No. 59, 2008) used interchangeably to regulate

the disposal of this waste. Thus, due to uncertainty regarding the disposal of abattoir waste, some illegal or unacceptable activities may occur with the resulting negative effects (Roux and Lasher-scheepers, 2016).

However, in developed countries where the AD technology has been integrated into the national energy grid, clear legislation pertaining to circumstances where use of abattoir waste is permissible has been drafted. Examples of those regulating legislations include the European Regulation (EC 1774/2002) on the treatment and further use of abattoir waste which is classified into three categories (H. Bouallagui *et al.*, 2009; Budiyono *et al.*, 2011; Valta *et al.*, 2015):

I. High risk material (i.e. containing animal diseases), cannot be used in AD under any circumstances;

II. High risk animal by-products (perished animals and/or animals slaughtered but not intended for human consumption), must be sterilised to 133 ºC under 3 bars for 20 mins.

III. Low risk material (meat containing wastes from food industry and slaughterhouse waste of animals fit for human consumption), must be treated to a minimum of 70 ºC for 60 minutes.

The diversion of organic waste and alternative treatment thereof, for biogas production via AD significantly reduces dependence on landfilling waste, and with an added benefit of clean and renewable energy production.

1.3 Hypotheses, Aims and Objectives

1.3.1 Hypotheses

The following hypotheses for this study were formulated:

I. Co-digestion provides a balance in nutrient content and dilutes toxins thereby improving system performance and biogas yield.

II. The composition and ratio of the co-substrates in the feed determine the quantity and quality of the biogas produced.

1.3.2 Aims and objectives

The main goal of this study was to enhance biogas production via anaerobic co-digestion of abattoir and winery solid wastes.

Objectives include:

1. To determine the biomethane potential (BMP) of abattoir and winery solid wastes, by comparing the amount of biogas generated substrates, in mono- and co-digestion;
2. To evaluate the mixture blend(s) for enhanced biomethane generation and associated synergistic/antagonistic effects;
3. To determine the optimum environmental input factors that would enhance the biological conversion of abattoir and winery solid waste; and
4. Evaluate optimized input variable in up-scaled AD experiments.

1.4 Significance of the research

Biogas produced from AD can be used as a green energy source, while the digestate is rich in agriculturally useful nutrients and can be used as a fertilizer. The use of biogas as a substitute to fossil fuels will also help combat climate change, by reducing the amount of greenhouse gas (GHG) released into the atmosphere.

To the best of our knowledge laboratory scale AD of abattoir and winery waste has never been studied before. Hence, this study will contribute to the scientific body of knowledge.
The targeting of the above-mentioned waste streams (i.e. abattoir and winery waste) is of importance for the South African economy, more particularly in the Western Cape, where the reduction of winery waste pollution and as well as abattoir waste will positively impact the quality of life. More research directed at addressing these issues are urgently needed.

1.5 Delineations

- The boundaries of this research are limited only to improving the production of biogas by co-digestion and will overlook the microbial community interactions, and only focus on process conditions that impact on the biogas production;
- The performance on pilot-scale continuous digestion operation of the investigated substrate(s) will be reserved for future study;
- The digestate will not be analyzed for potential use as a bio fertilizer.
- Economic benefits of redirecting waste streams to AD will not be evaluated.

CHAPTER 2

2 Literature Review

2.1 Abattoir waste

Abattoir waste (ABW) constitutes the uneatable parts of animals produced during the slaughtering process such as blood and other animal by-products (see **Figure 2-1**). The high moisture content of this waste makes it suitable for AD with a biogas production potential of 120-160 m^3 per ton of waste (Zafar, 2015). The high lipid concentration in ABW makes it attractive to AD processing, as they are easily degradable with a 72% theoretical CH_4 concentration (Ortner *et al.*, 2014). However, given its high fat and protein content, and low C/N, ABW inhibits microbial activity and creates process instability due to VFAs, ammonia and hydrogen sulfide build-up during fat and protein digestion. Hence, the need for some form of pretreatment prior to digestion in order to overcome these challenges (Hassib Bouallagui *et al.*, 2009; Battimelli *et al.*, 2010; Budiyono *et al.*, 2011; Affes *et al.*, 2013; Pagés-Díaz *et al.*, 2017; Salminen *et al.*, 2018).

Figure 2-1: Photograph of solid abattoir waste (source: http://shredding-machine.com/index.php/application/slaughterhouse-waste/)

According to DEA, (2017) the red meat processing industry consumes approximately 5.8 cubic meters of water, and at least 84 % is discharged to municipal sewers at pH = 5.7, COD = 2380-8942 mg/L, TSS = 189-3330 mg/L, TDS =595-2805 mg/L and TKN = 0.71-24 mg/L

contributes largely to the organic load of raw sewage. And with stringent municipal by-laws (COD = 3000-5000 mg/L and TSS = 500 mg/L) most abattoirs discharge after some form of pretreatment such as solids separation by screening, fat/oil flotation, primary settling, protein recovery (blood separation), pH correction, has been applied (see **Figure 2-2**). The solid waste fraction is usually processed further by rendering condemned waste (autoclaved at 130 ºC and 2 atm) as either animal feed or fertilizer (e.g. bone meal, pelletized pet food, non-grazed pasture, etc.); while condemned meat, according to the meat safety act (Act No. 40, 2000) is to be disposed-off by either first denaturing and subjecting to lime treatment prior to landfilling or by incineration which are energy intensive operations thus increasing the cost of operation subsequently. The major concerns of these treatments include but are not limited to control of air emissions during incineration, especially dioxins, nitrogen oxides and sulfur oxides; while rendering of condemned waste(s) can lead to the spread of animal diseases (e.g. transmissible spongiform encephalopathy) posing a health risk for both animals and human-beings.

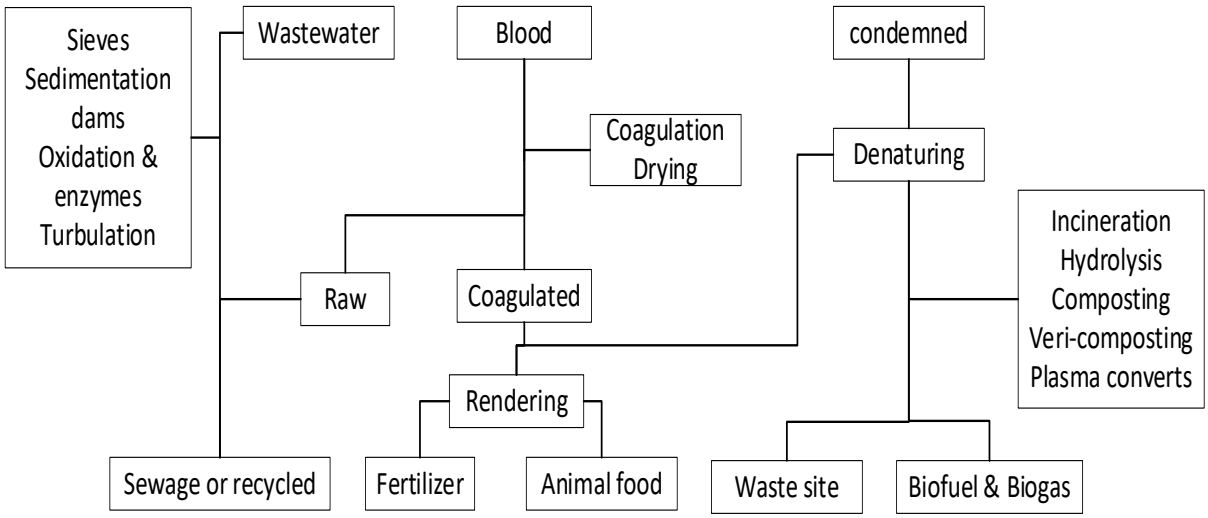


Figure 2-2: Current initiatives by Red meat abattoir for alternative solutions

On the other hand, small operators often have difficulties complying with municipal regulations due to financial reasons and resort to illegal dumping. Thus, anaerobic digestion can successfully be applied for energy recovery that can be recycled back to their operations, further for reducing costs of operations, and for stabilizing the by-product wastewater to comply with municipal by-laws regulations for discharge into municipal waste management facilities. The solid waste residues remaining are rich in nutrients and can be sold on the market as bio-fertilizers to further recover the capital investments of AD plants.

2.2 Winery solid waste

Winery solid waste (W_s) is a by-product solid residue left over from the crushing and screening operations of the beverage and wine producing industries, which contains residual organic acids, soluble proteins, soluble carbohydrates, macro-nutrients, nitrogen and phosphorous and trace elements that supports bacterial activity (Melamane, Strong and Burgess, 2007; Domínguez *et al.*, 2014) making it a feedstock for AD and biogas production (see **Table 2-1**).

However, this type of waste can be hazardous to the environment if it is not properly treated prior to discharges leading to surface and ground water pollution, foul odors, flies and pest infection (Makadia *et al.*, 2016). The wine making industry alone generates more than 20% of this waste, which consists of scores of crude grape pomace that can expedite considerable environmental hazards. A picture of winery solid waste dumped next to the vinyard and left to decompost is shown in **Figure 2-3**.

Figure 2-3: Photograph of fresh grape pomace (source: https:// u.osu.edu/wine/recycle-process/)

The W_S produced in South Africa is mainly composed of grape-marc which is a fibrous material consisting of approximately 8% seeds, 10% stems, 25% skins and 57% pulp, and filter waste generated from the crushing, draining and pressing of the wine production process (Dillon, 2011). Lo and Liao, (1986) successfully demonstrated the AcoD of winery solid waste with secondary dairy manure, where they reported an improvement in the methane production biodegradation efficiency. Similarly, AcoD of W_S with waste activated sludge resulted in improved biogas production efficiency was attributed to the high soluble chemical oxygen and polyphenolic compounds present in winery waste (Da Ros *et al.*, 2014).

The results obtained by the above-mentioned authors further motivated our research, that seeks to improve biogas yield by co-digesting abattoir and winery solid waste. The properties of both abattoir and winery solid waste could demonstrate synergistic effects of bacterial microbes in AD, which would allow for the stable operation and biogas production.

Table 2-1: Physical and biochemical characteristics of abattoir and winery waste

Indicator	Abattoir waste	Solid winery waste
pH	6.8 – 7.0 [a]	3.8 – 6.1 [b]
COD (mg L^{-1})	5200 – 11400 [a]	284000 [b]
TS (mg L^{-1})	570 –1690 [a]	65000 [b]
TKN (mg L^{-1})	19 – 74 [a]	526 [b]
Protein (mg L^{-1})	3250 – 7860 [a]	
K (g Kg^{-1})	-	11.8 – 37.9 [c]
Ca (g Kg^{-1})	-	5.4 – 20.6 [c]
P (g Kg^{-1})	-	0.22 – 1.72 [c]
Mg (g Kg^{-1})	-	1.3 – 4.3 [c]
Zn (mg Kg^{-1})	-	14 – 35 [c]
Mn (mg Kg^{-1})	-	0.2 – 100 [c]
Fe (mg Kg^{-1})	-	54 – 279 [c]
Cu (mg Kg^{-1})	-	5 – 279 [c]

[a] (Zafar, 2015); [b] (Eleutheria et al., 2016); [c] (Bustamante et al., 2008)

2.3 Biogas constituents and beneficial application

2.3.1 What is biogas?

Biogas is an odorless and tasteless secondary energy carrier gas rich in methane, and a renewable energy source. Biogas evolution from decaying organic matter occurs naturally in the absence of oxygen (i.e. anaerobic). It can also be voluntarily produced under anaerobic environmentally controlled conditions in air-tight vessels called digesters (Gerardi, 2003).

2.3.2 Biogas composition

Biogas is composed of methane, and of carbon dioxide, and it may contain small amounts of hydrogen sulfide saturated with water vapor depending on feedstock and processing conditions. The list of contributing gaseous compounds and the byproduct-impurities contained within a typical biogas sample is presented in **Table 2-2**. A generalized equation for biogas generation can be described by the Buswell equation (2-1), which was later modified by Boruff and Boyle for substrates containing nitrogen and sulfur described by of equations (2-2) and (2-3), respectively.

Table 2-2: Typical gases and trace compounds found in biogas (Schunurer and Jarvis, 2009)

Component	Formula	Content (v/v %)
Methane	CH_4	50 (%) – 75 (%)
Carbon dioxide	CO_2	25 (%) – 45(%)
Water vapor	H_2O	2 (%) -7 (%)
Ammonia	NH_3	Trace
Hydrogen sulphide	H_2S	Trace
Hydrogen	H_2	Trace
Oxygen	O_2	Trace
Nitrogen	N_2	Trace
Dust particles		Trace

$$C_aH_bO_c + \left(a - \frac{b}{4} - \frac{c}{2}\right) H_2O \rightarrow \left(\frac{a}{2} + \frac{b}{8} - \frac{c}{4}\right) CH_4 + \left(\frac{a}{2} - \frac{b}{8} + \frac{c}{4}\right) CO_2 \quad (2\text{-}1)$$

$$C_aH_bO_cN_d + \left(a - \frac{b}{4} - \frac{c}{2} + \frac{3d}{4}\right) H_2O \rightarrow \left(\frac{a}{2} + \frac{b}{8} - \frac{c}{4} - \frac{3d}{8}\right) CH_4 + \left(\frac{a}{2} - \frac{b}{8} + \frac{c}{4} + \frac{3d}{8}\right) CO_2 + dNH_3 \quad (2\text{-}2)$$

$$C_aH_bO_cN_dS_e + \left(a - \frac{b}{4} - \frac{c}{2} + \frac{3d}{4} + \frac{e}{2}\right) H_2O \rightarrow \left(\frac{a}{2} + \frac{b}{8} - \frac{c}{4} - \frac{3d}{8} - \frac{e}{4}\right) CH_4 + \left(\frac{a}{2} - \frac{b}{8} + \frac{c}{4} + \frac{3d}{8} + \frac{e}{4}\right) CO_2 + dNH_3 + eH_2S \quad (2\text{-}3)$$

2.3.3 Applications of biogas

Biogas obtained via anaerobic digestion can be used for:

- Direct combustion for heat production and cooking.
- Combustion in a combined heat and power plant (CHP) to produce electricity and heat.
- Upgraded biogas (e.g. Removal of impurities) to natural gas quality.

2.3.4 Benefits of biogas

The environmental impacts of AD are dependent on the management system that the digester amends or replaces as well as the actual use of the biogas produced. Typically, the anaerobic digestion followed by flaring of biogas, combustion of biogas for electricity, or production and use of bio-methane as fuel can provide a number of direct environmental benefits (Krich *et al.*, 2005) such as:

- Social and economic development
- Reduced greenhouse gasses (GHG) emissions
- Potential reduction of volatile organic compounds (VOC) emissions

- Fuel supply diversity
- Pathogen and weed seed control

The main benefit of biogas is that like most renewable sources it produces very little GHGs making it highly suitable for the environment (Johansson *et al.*, 1992; Rowse, 2011; REN21, 2015). With electricity being the major source of energy in SA, its production from non-renewables negatively impacts the earth's natural systems, and thus, an energy mix that includes biogas will significantly reduce GHG and VOC emissions.

2.4 The microbial dynamics of anaerobic digestion

The synthesis of biogas via AD is a complex process governed by enzymatic actions of a consortium of bacteria all working symbiotically between hydrogen producing (acetogens) and hydrogen consuming (homoacetogens, hydrogenotrophic, methanogens, etc.) bacteria.

In general, AD is the conversion of large and complex organic molecules to methane and carbon dioxide in the absence of oxygen. The process can be simply described as shown in equation (2-4).

$$\text{Complex Organic Molecule} \xrightarrow{\text{yields}} CH_4 + CO_2 + NH_3 + H_2 + H_2S \qquad (2\text{-}4)$$

The process involves a sequence of four microbiological transformation stages, which are hydrolysis, fermentation (acidogenesis), acetogenesis, and methanogenesis. A schematic representation of the processes involved is presented in **Figure 2-4** (Salminen and Rintala, 2002). The active bacterial community in AD form an anaerobic food chain where large and complex insoluble organic compounds are digested into simple compounds as they move along the chain. Within the food chain, symbiotic relationships, such as those between hydrogen-producing and hydrogen-consuming bacteria, exist (Gerardi, 2003; Henze *et al.*, 2015; Eleutheria *et al.*, 2016).

The anaerobes involved in AD are inactive in the presence of free molecular oxygen and can be divided into two groups (i.e. oxygen-tolerant and oxygen-intolerant). Some are strong acid producers (acetogens) whereas others reduce sulphate to hydrogen sulphide. The acid-forming bacteria are associated with methane-formers (methanogens) that include facultative anaerobes with the ability to ferment simple and soluble organic compounds, and to strict anaerobes that ferment complex proteins; carbohydrates and lipids (Gerardi, 2003).

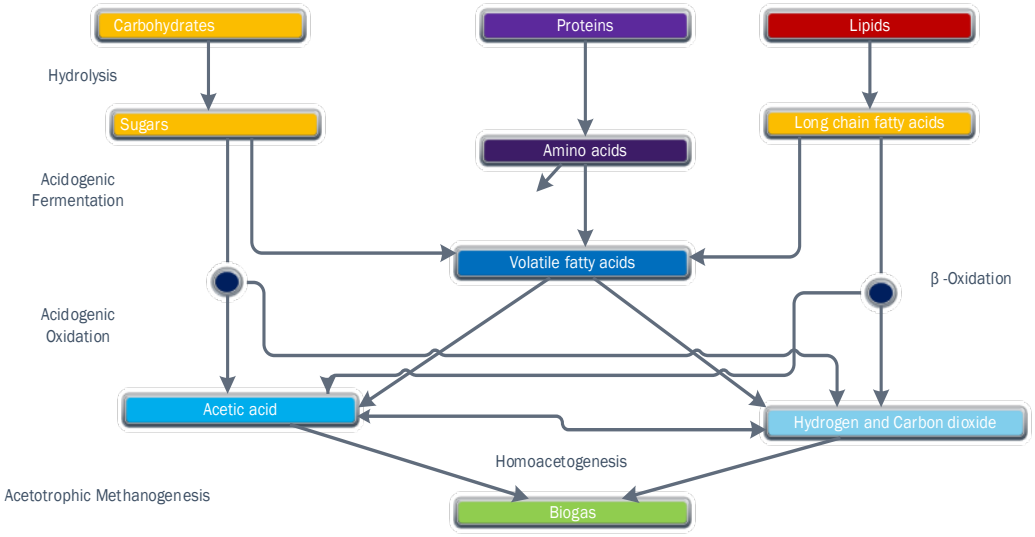


Figure 2-4: Anaerobic digestion food-chain reproduced from (Salminen and Rintala, 2002)

2.4.1 Hydrolysis

The first stage in AD is hydrolysis, where complex organic polymers (e.g. polysaccharides, fat/oil, etc.) are reduced into their simple monomers (e.g. monosaccharaides, peptides, amino acids, etc.). The decomposition of organic matter is characterized by the activity of the enzymes (e.g. cellulose, amylase, protease, and lipase) excreted by the hydrolytic bacteria (Kigozi, Aboyade and Muzenda, 2014). The bacteria can hydrolyze substrate using enzymes (i.e. endoenzymes and exoenzymes):

I. Endoenzymes: are produced inside the bacterial cell and can degrade water soluble substrate within the cell wall
II. Exoenzymes: are produced inside the cell and excreted as slime to the insoluble substrate which facilitates solubilization. Once in the soluble form, further hydrolysis can be performed within the cell. The production of exoenzymes and the solubilization of substrates are quite long and can take several hours or a few days to be achieved. Examples of exoenzymes enzymes and their associated substrates and products are listed in **Table 2-3**

All bacteria can produce endoenzymes but not all can produce exoenzymes, and no single form of bacteria can produce all the necessary exoenzymes required to hydrolyze a variety of substrates present in an anaerobic digester. Therefore, a large and complex culture of the bacterial community is required to ensure the optimal functioning of an anaerobic digester (Gerardi, 2003).

Table 2-3: Commonly occurring hydrolytic enzymes in AD (Gerardi, 2003)

Substrate	Exoenzyme	Example	Product
Polysaccharides	Saccharolytic	*Cellulomonas*	Simple Sugar
Proteins	Proteolytic	*Bacillus*	Amino Acid
Lipids	Lipase	*Mycobacterium*	Fatty Acids

2.4.2 Acidogenesis

The second stage in AD is the acidogenic fermentation of products from the hydrolysis stage by acid forming bacteria producing volatile organic acids such as acetic; propionic; butyric acid, alongside ethanol; carbon dioxide and hydrogen.

The dominant species in anaerobic digesters are bacteria (i.e. obligate; and facultative anaerobes) alongside a small population of protozoa, fungi and yeast.

2.4.3 Acetogenesis

Acetogenesis is the third AD stage described as the oxidation of fermentation of by-products into a soluble substrate utilized by methanogenic bacteria such as formate, acetate, H_2, and CO_2 (see **Table 2-4**). There are two paths in which acetogenesis may occur namely homoacetogenesis, and syntrophic acetogenesis.

2.4.3.1 Homoacetogenesis

Homoacetogenesis is the production of acetate as a sole product from CO_2 and H_2:

$$4H_2 + 2HCO_3^- + 2H^+ \rightarrow CH_3COO^- + 4H_2O + H^+ \qquad \Delta G^o = -104.6 \frac{KJ}{mol} \quad (2\text{-}5)$$

2.4.3.2 Syntrophic acetogenesis

Syntrophic acetogenesis is the production of acetate from the oxidation of VFA's from fermentation stage equation (2-6) and (2-7).

$$CH_3CH_2COO^- + H^+ + 3H_2O \rightarrow CH_3COO^- + HCO_3^- + 2H^+ + 3H_2 \qquad \Delta G^o = +76 \frac{KJ}{mol} \quad (2\text{-}6)$$

$$CH_3(CH_2)_2CH_2COO^- + 2H_2O + H^+ \rightarrow 2CH_3COO^- + 2H^+ + 3H_2 \qquad \Delta G^o = +48 \frac{KJ}{mol} \quad (2\text{-}7)$$

Syntrophic acetogens are obligate H_2 producers that grow symbiotically with the methanogens, and only survive at a low H_2 partial pressure with significant changes resulting in the loss of activity. Commonly found acetogenic species are listed in **Table 2-4** alongside with their respective preferred substrate and product(s).

Table 2-4: Acetogenic bacteria found in mesophilic anaerobic digesters

Substrate	Product	Species	T (°C)
Butyrate	Acetate	*Syntrophobacter wolinii.*	35-40
	H_2/CO_2, and Formate	*S. fumaroxidans.*	35-40
Propionate	H_2/CO_2, and Formate	*Syntrophomonas wolfei.*	35-40
H_2/CO_2	Acetate	*Clostridium aceticum.*	30-37

Acetogenic bacteria have a very slow reproduction/regeneration rate of approximately three days (Gerardi, 2003).

2.4.3.3 Beta-oxidation

This is the degradation of long chain fatty acids to acetate and propionate and is a cyclic process where the release of one acetate unit per cycle is obtained. The end products are acetate & propionate depending upon whether the LVFAs are even or odd carbonic acids.

2.4.4 Methanogenesis

The final stage in AD is methanogenesis where low molecular weight organic compounds from the previous stages are converted into biogas (i.e. CH_4 and CO_2), by archaebacterial methane-formers (methanogens) which are among the oldest species on the earth, and perhaps the most strictly anaerobic (Zeikus, 1977).

$$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O \qquad \Delta G^o = -135.5\frac{KJ}{mol} \qquad (2\text{-}8)$$

$$CH_3COO^- + H_2O + H^+ \rightarrow CH_4 + HCO_3^- + H^+ \qquad \Delta G^o = -32.3\frac{KJ}{mol} \qquad (2\text{-}9)$$

$$4CH_3OH \rightarrow 3CH_4 + HCO_3^- + H^+ + H_2O \qquad \Delta G^o - 79.9\frac{KJ}{mol} \qquad (2\text{-}10)$$

$$4HCOO^- + 4H^+ \rightarrow CH_4 + 3CO_2O + H_2O + \qquad \Delta G^o = -36.1\frac{KJ}{mol} \qquad (2\text{-}11)$$

Methanogens are predominantly terrestrial, and aquatic in nature. They commonly occur in decomposing organic matter where oxygen is removed via microbiological activity. For example, these methanogens can be localized in deep-sea volcanic vents; deep sediments; geothermal springs; and in digestive tracts of ruminant animals which symbiotically obtain their carbon and energy from the decomposition of cellulose and polysaccharides from plants (Miller and Wolin, 1974; Zeikus, 1977; Gerardi, 2003). They have a very slow reproduction rate compared to acidifiers (see **Table 2-5**).

A list of commonly found methanogenic archaea in AD, with alongside their preferred substrates, synthesis product(s), and optimum range of environmental conditions for highest bacterial activity, is presented in **Table 2-6**.

Table 2-5: Average kinetic parameters of acidifiers and methanogens (Henze et al., 2015)

Process	Conversion rate (g COD g^{-1} VSS day^{-1})	Yield (g VSS g^{-1} COD)	K_S (mg COD/L)	μ_{max} (day^{-1})
Acidogenesis	13	0.15	200	2
Methanogenesis	3	0.03	30	0.12
Overall	2	0.03-0.18		0.12

K_S: Substrate saturation constant; and μ_{max}: Maximum specific cell growth rate

Methanogenesis is said to be the rate-limiting step during methane production phase; and considers pH neutrality for their optimal performance which can easily shift to acidic in poorly buffered systems, that can be caused by the accumulation of intermediate products from the hydrolysis/acidification phase.

Table 2-6: Methanogenic archaea found in mesophilic AD (Gerardi, 2003)

Substrate	Product	Species	pH	T (°C)
Acetate	CH_4/CO_2	*Methanothrix soengenii.*	7.4-7.8	35-40
		Methanosaeta concilli.	7.1-7.5	35-40
		Methanosarcina acetivorans.	6.5-7.5	35-40
H_2/CO_2	CH_4/CO_2	*Methanobacterium bryantii.*	6.9-7.2	37-39
		Methanobrevibacter arboriphilus.	7.8-8.0	30-37
		Methanolacinia paynteri.	6.6-7.2	40
Formate	CH_4/CO_2	*Methanobrevibacter smithii.*	7.0	37-39
		M. ruminantium.	7.0	37-39
H_2/CO_2		*Methanococcus voltae.*	6.5-8.0	35-40
		M. maripaludis.	6.5-8.0	35-40
		M. tatii.	7.0	37-40
		Methanocorpusculum aggregans.	6.4-7.2	35-37

There are two major pathways of methanogenesis namely, aceticlastic cleavage of acetate (Acetotrophic methanogenesis) and carbon dioxide (CO_2) reduction with hydrogen (H_2) (Hydrogen-trophic methanogenesis) (Gerardi, 2003; Seadi *et al.*, 2008; Schunurer and Jarvis, 2009). The relative kinetic and thermodynamic characteristics of the above types of methanogenesis are presented in **Table 2-7** where about 70% of methane produced during AD is derived from acetotrophic methanogenesis, while the residual 30% is synthesized by reduction of CO_2 with H_2 (Seadi et al., 2008).

Table 2-7: Methanogenic reactions, and their associated kinetic and thermodynamic parameters (Henze *et al.*, 2015)

Reaction	ΔG^0 (KJ/mol)	T_d (day)	K_S (mg COD/L)	μ_{max} (day^{-1})	Equation
Acetotrophic methanogenesis	-32.3	5.8[a]	30[a]	0.12[a]	
					(2-9)
		1.0[b]	300[b]	0.71[b]	
Hydrogenotrophic methanogenesis	-135.5	0.2	0.06	2.85	
					(2-8)

[a] *Methanosarcina spec*. and [b] *Methanosaeta spec.*

2.4.5 Sulphate-reducing bacteria

The sulphate-reducing bacteria are also found in anaerobic digesters and the presence of sulphur compounds in the feed promotes their rapid growth. The sulphate-reducing bacteria also utilize hydrogen and acetate as carbon sources (see equations (2-12 to (2-16), the same substrates required by methanogens resulting in a competition for substrate between the two bacterial groups. At a substrate-to-sulphate that is greater-than 2; the sulphate-reducing bacteria outcompete the methanogens for acetate, and at the values between 2 and 3 the competition becomes intensified and at a value greater than 3 methanogenic bacteria are favored (Gerardi, 2003)

$$4H_2O + SO_4^{2-} + H^+ \rightarrow HS^- + 4H_2O \qquad \Delta G^o = -152.9\frac{KJ}{mol} \quad (2\text{-}12)$$

$$CH_3COO^- + SO_4^{2-} \rightarrow 2HCO_3^- + HS^- \qquad \Delta G^o = -47.6\frac{KJ}{mol} \quad (2\text{-}13)$$

$$2CH_3(CH_2)_2CH_2COO^- + SO_4^{2-} \rightarrow 4CH_3COO^- + HS^- + H^+ \qquad \Delta G^o = -27.8\frac{KJ}{mol} \quad (2\text{-}14)$$

$$2CH_3CHOHCOO^- + SO_4^{2-} \rightarrow 2CH_3COO^- + 2HCO_3^- + HS^- + H^+ \qquad \Delta G^o - 80.0\frac{KJ}{mol} \quad (2\text{-}15)$$

$$2CH_3CH_2OH + SO_4^{2-} \rightarrow 2CH_3COO^- + HS^- + H^+ + H_2O \qquad \Delta G^o = -66.4\frac{KJ}{mol} \quad (2\text{-}16)$$

2.5 Anaerobic co-digestion

When AD is applied to a homogenous mixture of two or more organic substrates the process is termed “anaerobic co-digestion” which is currently the most prevalent form of biogas synthesis to-date (Seadi *et al.*, 2008).

Anaerobic co-digestion (AcoD) is rapidly being adopted for treating various organic waste streams, with several benefits, such as dilution of toxins, balance nutrition, high methane content, and increased digestion rates resulting from synergistic effects amongst different microbial groups involved in AD (Khalid *et al.*, 2011; Mata-Alvarez *et al.*, 2014).

A study on the AcoD of cattle manure with organic kitchen waste conducted by Aragaw, Andargie and Gessesse (2013), demonstrated an increase of 47% in biogas yield when compared to the mono digestion experiments. Similarly, the AcoD of municipal bio-waste with sewage sludge increased biogas yield 4-fold when compared to mono-digestion of sewage sludge. The authors attributed their findings to an increase from 8.9 to 12.6 in the C/N ratio that stabilized AD (Kuglarz and Mrowiec, 2007).

The batch AcoD of manure, solid slaughterhouse waste, and fruit and vegetable waste was studied by Alvarez, et al., (2006), and the improved biogas yield of 0.29 m^3/kg VS for manure and slaughterhouse waste mixture was recorded. However, the authors noted that batch AcoD of fruit and vegetable waste with either manure or slaughterhouse waste was not possible, as it resulted in the accumulation of VFAs and subsequent digester collapse.

However, in studies conducted by Bouallagui, et al. (2009), a 52% increase in biogas yield was achieved, when fruit and vegetable waste AcoD with abattoir waste in an anaerobic sequencing batch reactor while the results were associated with the synergistic effects on the microbial population, with increased biodegradability and efficiently buffered AD system.

In the research conducted by Zhang and Banks (2012) to study or evaluate the AcoD of mechanically recovered organic fractions of municipal solid waste with pig intestines (PI) and/or floatation fat (FF), and pig blood in a continuously stirred tank reactor, the finding revealed that AcoD was only feasible at low OLR of 2 $kgVSm^{-3}d^{-1}$ for both PI/FF and pig blood as co-substrate. When the OLR was increased to 3 and 4 $kgVSm^{-3}d^{-1}$ an inhibitory behavior was noted resulting in reduced biogas production rates was noted (Zhang and Banks, 2012).

Da Ros, et al. (2014), successfully demonstrated the AcoD of wine lees and waste activated sludge, which produced 0.38 m^3/kg_{COD} biogas with a 65% CH_4 content; and have attributed their findings to the high content of soluble COD and phenols present in wine lees. Similarly, the AcoD of winery waste and cow manure anaerobic digestion in a fixed film reactor also demonstrated positive effects on methane production and process stability at mesophilic conditions (Lo and Liao, 1986). A summary of various anaerobic AcoD of substrates is provided in **Table 2-8**.

Table 2-8: Proportionate biogas production rates and methane yield from co-digestion of various organic wastes

Substrate [a]	Mixing proportions	Reactor configuration	T ^{0}C	OLR	Biogas		Effect [b]	References
				Kg/m^{3}d	m^3/kg d	CH_4 (%)	(%)	
CM: SW	24:1	Fed-Batch	35	2.9	0.29	-	76 (VS)	(Alvarez and Lidén, 2008)
		Batch	38	-	0.238	-	47 (biogas)	(Aragaw, Andargie and Gessesse, 2013)
RS: OKW: PM	1:0.4:1.6	Batch	37	54	383.9 [f]	56.9	55.8 (VS)	(Ye et al., 2013)
FOG: WAS	-	2-CSTRs	55	1.83	25.1 [d]	70	65 (COD)	(Li, Champagne and Anderson, 2015)
FVW: MPR	3:1	Fed-Batch	30	2.7	0.45	63	78 (VS)	(Garcia-Peña et al., 2011)
FVW: FW	1:1	CSTR	35	3	490 [f]	64	74.9 (VS)	(Lin et al., 2011)
FVW: AW	9:1	ASBR	35	2.5	0.61	64	85 (VS)	(H. Bouallagui *et al.*, 2009)
SW: SS	1:1	Semi CSTR	35	3.1	608.6 [f]	68	62 (VS)	(Borowski and Kubacki, 2015)
OFMSW: SHW	4:1	CSTR	37	4			-	(Zhang and Banks, 2012)
PS: PSW: RG	35:47:1	Semi CSTR	37	3.2	0.38	71	55 (COD)	(Rodríguez-Abalde, Flotats and Fernández, 2017)
AW: STS	1:1	Fed-batch	37	-	-	-	90	(Ahmad et al., 2014)
WSW: SDM	1:1	Fixed-film reactor	35	7.78	2.77 [e]	72	70 (COD)	(Lo and Liao, 1986)
WSW: WAS	22:68		37	2.8	0.38	64.8	70 (COD)	(Da Ros et al., 2014)
ABP: SS	1:7	Batch	35	-	400 [f]	63		(Luste and Luostarinen, 2010)

[a] FVW: Fruit and Vegetable waste, SW: Slaughterhouse Waste, FOG: Fat, Oil and Grease, OKW: Organic Kitchen Waste, CW: Cattle Manure, WAS: Waste Activated Sludge, MPR: Meat Processing Residues, FW: Food Waste, AW: Abattoir Waste Water, OFMSW: Organic Fraction of Municipal Solid Waste, PS: Pig Slurry, PSW: Pasteurized Slaughterhouse Waste, RG: Recycled Glycerin, Sewage Sludge, WSW: Winery Solid Waste, SDM: Screened Dairy Manure

[b] Performance measured in either volatile solids (VS) destruction and/or chemical oxygen demand (COD) removal [c] m^3/m^3 day, [d] L/ day, [e] L CH_4/ L day, [f] L CH_4/ Kg VS $_{added}$

2.6 Biomethane potential and toxicity tests

The BMP assay is a useful experimental research tool for evaluating AD suitability of an anaerobically incubated substrate sample seeded with an active microbiological culture (inoculum) for a given period of time until total degradation of organic matter is achieved (Angelidaki and Sanders, 2004; Hansen *et al.*, 2004). A control sample is also included in the assay to measure background methane generation by the inoculum, and the biodegradability of a substrate is determined by monitoring cumulative methane production per unit VS or COD added. An anaerobic environment is induced by flushing the headspace of the assay vessels with a mixture carbon dioxide and hydrogen (80/20 v/v%) or pure hydrogen gas, incubating at optimal temperature and monitoring substrate-conversion (biodegradability) and/or gas production rate over the given period. BMP assays are also useful in determining substrate-inhibitory concentrations and toxic effects. It should also be noted that BMP assays are just approximate indicators of actual biodegradability as some of the organic matter is used for extracellular activity by the microorganisms involved in AD.

2.7 Determination of biological and kinetic parameters

The widely used kinetic curve-fitting models available in the literature share one common feature which is that they all consist of at least two biologically relevant parameters. As Fitzhugh (2018) points out, a selection of a model is relative to analytical objectivity and the nature of the data set.

The primary objective of curve-fitting is both:

- Descriptive- in the sense that information contained within a data set is consolidated to a few relative parameters; and
- Predictive-in the sense that future behavior and/or data can be estimated (e.g. growth rates, lag-phase, responses. etc.) for designing and efficiently running of biogas plants

To better understand the dynamics of biogas production via AD for specific substrate utilization in the design and operation of anaerobic digesters, kinetic modelling is a useful evaluation tool given that it allows the determination of biodegradability rates based on the occurrence of microscopic biochemical reactions and the input environmental factors impacting the process.

2.7.1 Population growth kinetics

There are two types of population growth patterns that have been observed and which are dependent on specific environmental conditions (Yin *et al.*, 2003), namely, Exponential growth

(Reverse L-shaped or J-shaped) and Sigmoidal growth (**S**-shaped) (see **Figure 2-5**). The factors that have an impact on population growth are either density dependent (i.e. influenced by the size, limited resources and presence of predators) or density-independent (PAW) which is short for **P**henomena (e.g. disaster, etc.), **A**biotic (e.g. nutrient concentrations, etc.) and **W**eather (temperature) (Amagu Echiegu, 2015).

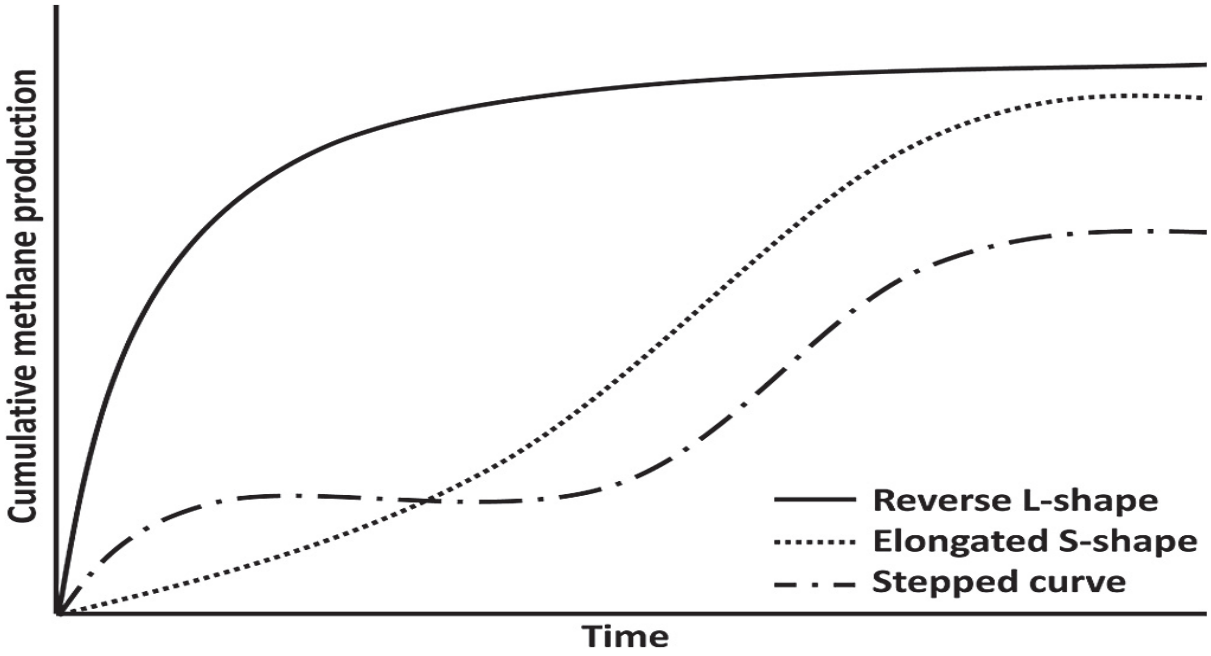


Figure 2-5: Typical examples of curve fitted population growth data (Ware and Power, 2017)

i. **Exponential growth**

Exponential growth (J-curve): occurs under ideal environmental conditions with unlimited resources thus, improving geometric growth rates. Initially population growth, is slow due to the small number of reproducers that might be homogeneously dispersed across the system. And as population increases similarly the growth rates increases yielding an exponential fit of the observable response as the system approaches carrying the capacity. The maximum growth rate for any given population is referred to as its biotic potential. This type of growth pattern is commonly found in bacteria.

ii. **Sigmoidal growth**

Sigmoidal growth (S-curve) typically occurs in four-stages within a fixed geographic environment containing a stable population. The four stages include the lag phase, the exponential growth phase, the transition growth phase and plateau phase.

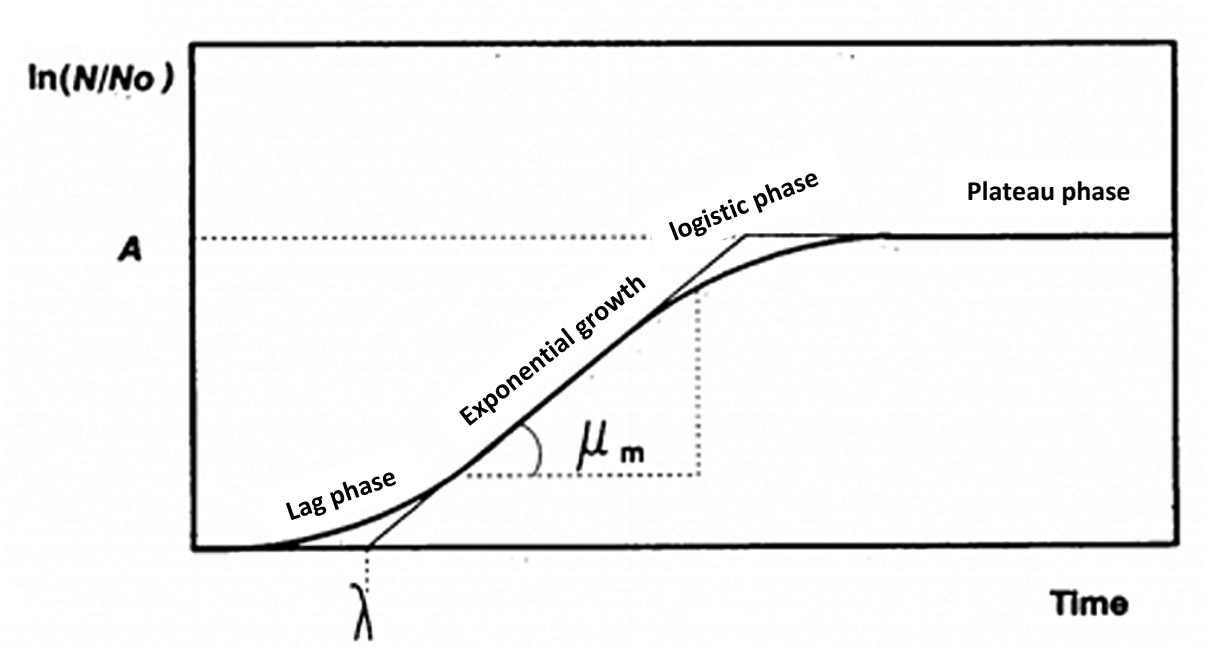


Figure 2-6: Typical bacterial growth curve (Ware and Power, 2017)

- **Lag phase**

In this phase population growth is initially very slow as microorganisms acclimatize to the new environmental conditions before multiplying, hence the term lag-phase.

- **Exponential growth phase**

This phase is characterized by rapid increases in population as number of species accumulates. As a result, the rate of natality tremendously exceeds the one of mortality as there is an abundance of resources and minimal environmental resistance.

- **Transition (logistic) growth phase**

Logistic growth occurs as population size slowly approaches the infinite carrying capacity of the system at set environmental conditions. During this phase, some environmental resistance is notable in the slowing growth rate response as population continues to grow, and availability of resources becomes a limiting factor as competition for survival amongst species rises. The natality rate declines as mortality rates increases, and this eventually slows the entire population growth. This type of growth eventually occurs in any system containing a stable population.

- **Plateau (decay) phases**

During this phase, the population growth ca either increase at a slow pace or not increasing at all. In here, the system remains at stable levels as the rate of natality reaches an equilibrium with rate of mortality.

2.7.2 Kinetic models

Several models used to describe AD, ranging from simple mathematical expressions to more complex functions with modifications to describe the biological transformation process. The most commonly used kinetic models in literature focused on describing the specific growth rate of microorganisms and rate of substrate consumption. However, specific methane generation rate is a useful AD performance indicator since it is easy to measure and can directly be correlated to substrate conversion rate.

2.7.2.1 First-order exponential function

The first order kinetic model has been widely used to evaluate and define simple process parameters such as hydrolysis rates and constants (Deepanraj, Sivasubramanian and Jayaraj, 2017; Hagos *et al.*, 2017; Nguyen *et al.*, 2019).

The first-order kinetic function (k) evaluates AD performance of different substrates based on their limiting organic loading concentrations, and on their specific biodegradability rate and/or biogas generation.

$$\mathrm{y(t)} = \mathrm{y_0}(1 - \mathrm{e}^{-k\mathrm{t}}) \tag{2-17}$$

However, defining a system where microorganisms utilize complex substrates biodegradation occurs via several biological transformation stages, yielding various intermediate products from multiple step reactions. Therefore, other models that factor in the above mentioned are used to simulate the microbial growth kinetics ("bio-energetic").

2.7.2.2 Gompertz function

The Gompertz model was derived by Benjamin Gompertz (1832) to estimate human mortality. This function was further adopted to describe the growth of microorganisms by assuming growth rate of microbes within a given environment declining in size, such that the rate of change (i.e. measured variable defining growth) (e.g. size, weight, etc.) is described by the following mathematical expression:

$$\frac{d\log x}{dt} = \mathrm{k}(\log Y_\infty - \log x) \tag{2-18}$$

Integrating the above function yields the following expression:

$$x(t) = Y_{\infty} e^{e^{-k(t-I)}} \quad (2\text{-}19)$$

$$x(t) = \mathrm{A} + \mathrm{B}e^{-k(t-I)} \quad (2\text{-}20)$$

Where x, k, and Y∞, are the sizes, growth rate and the asymptotic values are at zero mortality, respectively; A and B are the lower and upper asymptotes, respectively, and t represents the age of the population at the point of inflection. According to equation (2-20) the point of inflection is always found to be around 36.8% of the asymptote. However, this may not be the case in all population growth systems. Thus, it is only considered for growth processes in which the point of inflection is approximately one third of the maximum growth.

2.7.2.3 Verhulst function

The Verhulst (logistic) growth model was derived by Pierre Verhulst (1838) and is amongst the simplest S-shaped growth models. This model assumes that the rate of growth of organisms declines with population size defined by the following expression:

$$\frac{dx}{dt} = x(k - \log x) \quad (2\text{-}21)$$

$$\mathrm{x(t)} = \frac{x(\infty)}{1+EXP(-k(t-x))} \quad (2\text{-}22)$$

2.7.3 Model selection and comparison

The comparison of models based on the coefficient of determination (R^2), the statistical residual sum of squares (RSS) and Root of the Mean Squares of the errors (RMSE) alone do not reveal sufficient information, as each model differs in the type and number of biological and kinetic parameters used for predictive modelling (Ware and Power, 2017). In **Figure 2-7** the relationship between specific growth rate of microorganisms to the saturation constant is presented, which depicts a system following the Monod kinetic model (1965) that relates microbial growth rates in an aqueous environment to the concentration of limiting nutrient.

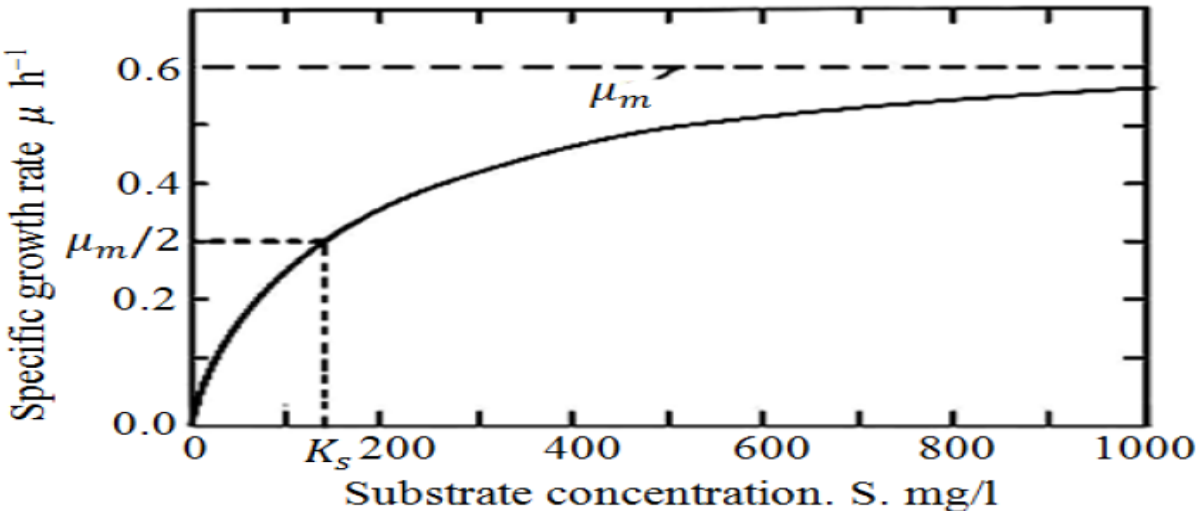


Figure 2-7: Relationship between specific growth rate and substrate saturation constant (Amagu Echiegu, 2015)

2.8 Biogas digester technology

A digester is an engineered chamber constructed to occlude air and stimulate the growth and multiplication of methanogens. A digester should be a simple tank, a roofed lagoon, or an intricate design with internal baffles, or packed with coarse material (e.g. Plastic rings, rocks, etc.) for attached bacterial growth (Krich *et al.*, 2005). A great number of anaerobic digesters, each performing AD in subtly different ways, is categorized as either industrial or domestic.

2.8.1 Industrial digester

Industrial anaerobic digesters are predominantly used in developed countries to reduce pressure on landfill sites, and the biogas by-product is considered as a renewable energy source. These systems can either be classified as low rate systems, in which long hydraulic retention time (HRT) is applied; or as high rate systems, in which HRT is short (de Mes *et al.*, 2003).

- Low rate systems are predominantly used for waste streams such as slurries and solid waste. Common examples include complete mixed digester, plug flow digester, and anaerobic sequencing batch digester.
- High rate systems are common in wastewater works plants (e.g. Contact process, anaerobic filter, fluidized bed, upflow anaerobic sludge bed and induced sludge bed).

A schematic representation of the AD systems commonly applied in industrial applications is presented in **Figure 2-8**.

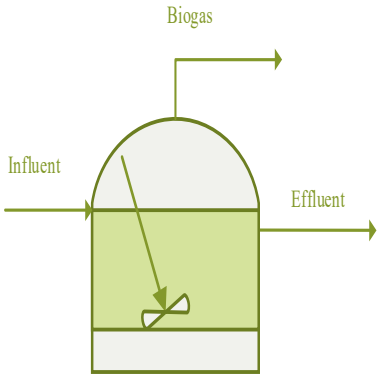
2.8.1.1 Anaerobic digestion of solid waste and slurries

The anaerobic digesters used for solid waste treatment are categorized according to the total solids (TS) percentage in their waste streams (de Mes *et al.*, 2003).

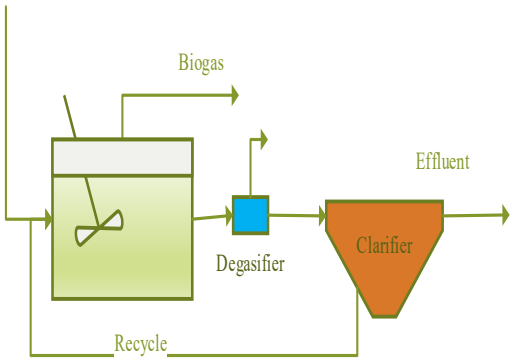
- 15-25% low solids anaerobic digestion: wet fermentation
- > 30% high solids anaerobic digestion: dry fermentation

All wet digesters are operated as continuous processes with complete mixed digester as the most common type (see **Figure 2-8**). In these systems mixing is either mechanical, hydraulic or pneumatic to achieve uniformity and facilitate the release of gas bubbles from the slurry (Weiland, 2010). They also require that biological sludge and organic waste undergo a series of pre-treatment steps including the separation of inorganic matter; liquefaction; and removal of toxic substances prior to AD (de Mes *et al.*, 2003).

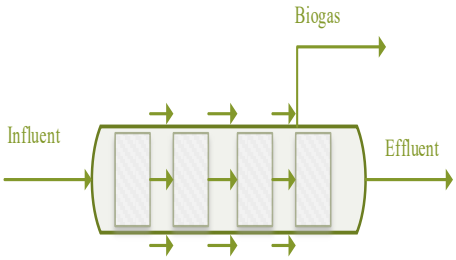
Dry digesters are used alternatively for digestion of high organic solid waste (e.g. Crop residues, municipal solid waste, etc.). It is performed in batches in which digested waste is blended with fresh feed and/or the waste is moderately inoculated by circulating process fluid (Schunurer and Jarvis, 2009).



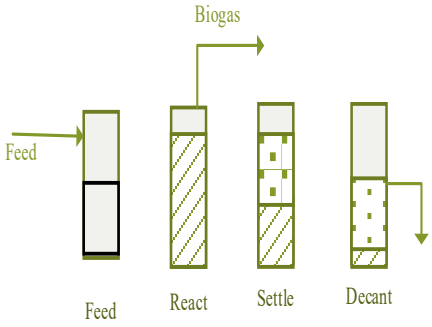
(a) Schematic Drawing of a Complete Mixed Digester



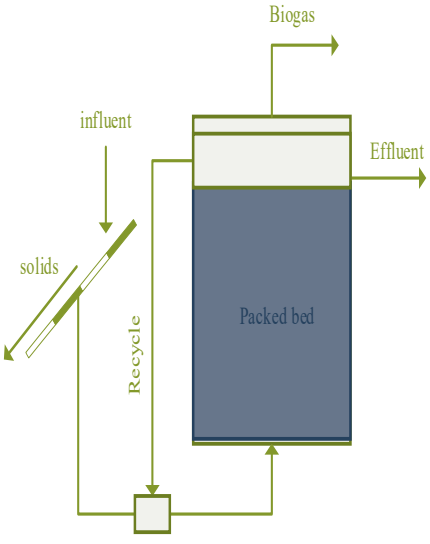
(b) Schematic Drawing of a Contact Stabilization digester



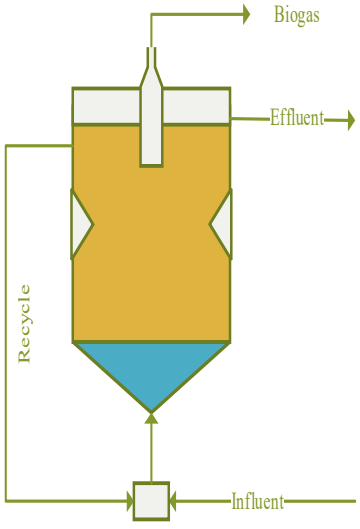
(C) Schematic Drawing of a Plug Flow digester



(d) four phases of an ASBR Cycle



(e) Schematic Drawing of a Fixed Film Digestion System



(f) Schematic Drawing of an Upflow Anaerobic Sludge Blanket (UASB) Digester

Figure 2-8: Simplified graphical representation of industrial biogas digesters

2.8.1.2 Stage separation

To achieve an efficient digestion of organic matter, stage separation in two or more digestion vessels is done to improve the selective growth and enrichment of microbes (Khalid *et al.*, 2011). The acidification stage (i.e. Hydrolysis and acidogenesis) and methane formation stage (i.e. acetogenesis and methanogenesis) are performed at their optimal environmental conditions. The advantages of a two-stage system over a single-stage reactor include an increase of stability with optimum pH control, a higher loading rate, an increase of specific activity of methanogens resulting in a higher methane yield, an increase of **VS** reduction efficiencies, and a high potential for pathogen control (Hagos *et al.*, 2017).

An overview of the advantages and disadvantages of the different types of AD technologies is described in **Table 2-9**.

Table 2-9: An overview of the advantages and disadvantages of AD technological applications

Digester	Disadvantages	Advantages
One-stage	High retention times, Form and scum	Simple design with less technical failure
Two-stage	Complex and expensive to construct and maintain, and requires solids removal prior to 2nd-stage feeding	Increased overall digestion due to recirculation
Dry-digester	Complex and expensive transport and handling of waste, only structured material can be used, and material handling and mixing is difficult	High biomass retention, controlled feeding special niches, and pre-treatment is much simple
Wet-digestion	Scum formation during, high water and energy demand, short-circuiting, sensitivity to shock loading	Dilution of inhibitor with fresh water
Batch	Channeling and clogging, Lager volume requirements, lower biogas yield	No mixing, stirring and pumping are required, low input in terms of the process and mechanical demand, low capital cost
Continuous	Rapid acidification and lager VFA production	Simplicity in design and operation, and low capital cost
High-rate	Loner start-up time, channeling at low feeding rates	Higher biomass retention, controlled feeding, lower investment costs, and no supporting material required

2.8.2 Domestic digesters

Domestic biogas digesters are most popular in developing countries for biogas production in households for cooking and heating purposes, thus reducing the dependence on fossil fuels. Examples of domestic digesters include the Floating drum, Balloon digester, and Fixed Dome digester (see **Figure 2-9**).

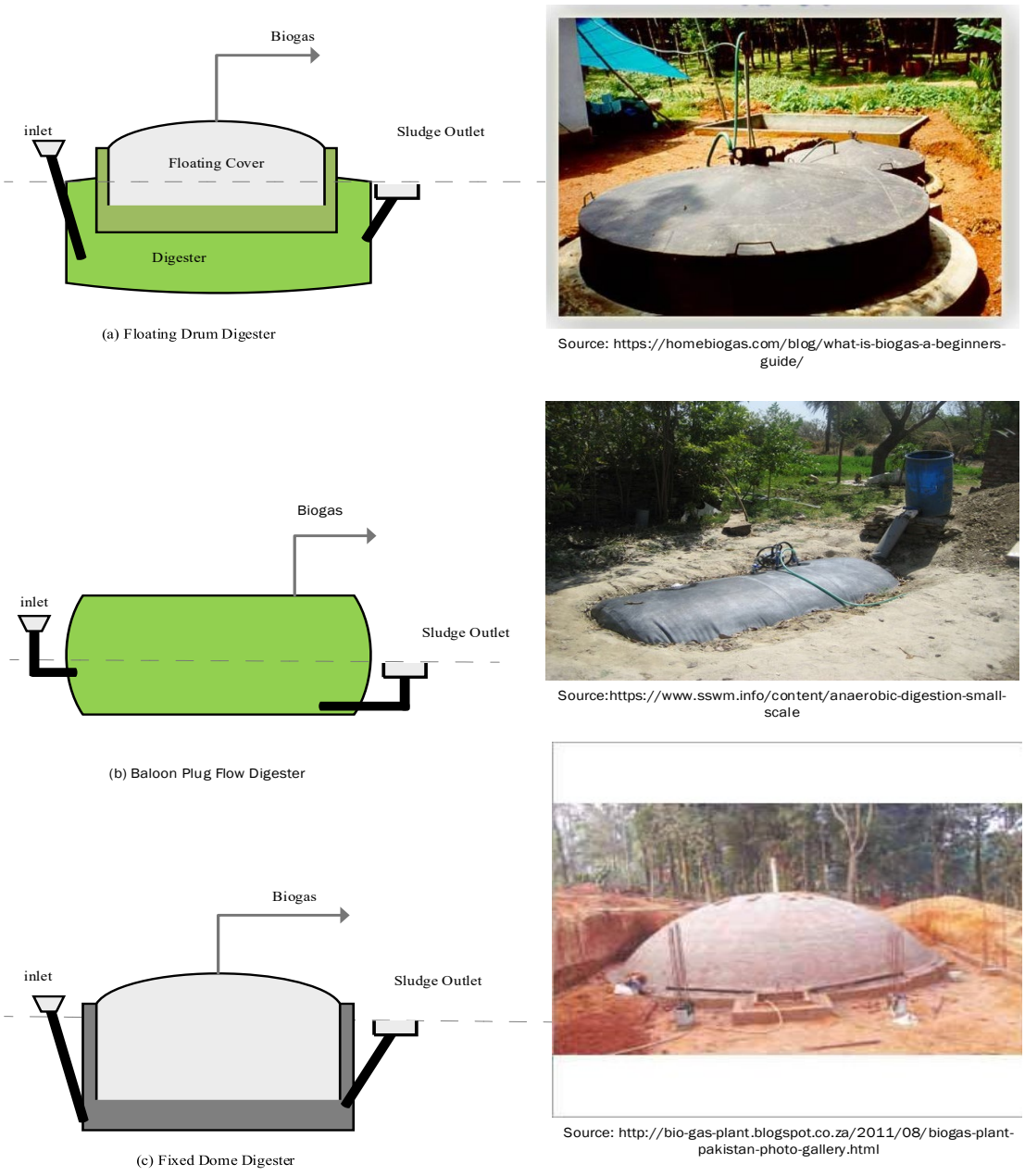


Figure 2-9: Simplified graphical representation and images of domestic digesters

2.9 Operational parameters impacting on AD

The performance of AD, like most biological processes, is closely linked to environmental conditions (i.e. Temperature; pH; nutrients balance; C/N ratio; organic loading rate (OLR); Hydraulic Retention Time (HRT); moisture content; the presence of inhibitors or toxins etc.); and the successful operation of anaerobic digester rests primarily on maintaining the environmental conditions to optimal comfort of the bacteria involved in AD (Gerardi, 2003; Seadi *et al.*, 2008; Kigozi, Aboyade and Muzenda, 2014).

2.9.1 Temperature

There are three temperature ranges in which AD is known to occur, namely Psychrophilic [10-20 ºC]; Mesophilic [20-40 ºC]; and Thermophilic [40-60 ºC]. However, it is worth noting that methanogens are inactive in extremely low and high temperature; and that the optimum biogas production from AD occurs under mesophilic, and thermophilic conditions (see **Figure 2-10**) (Kigozi, Aboyade and Muzenda, 2014).

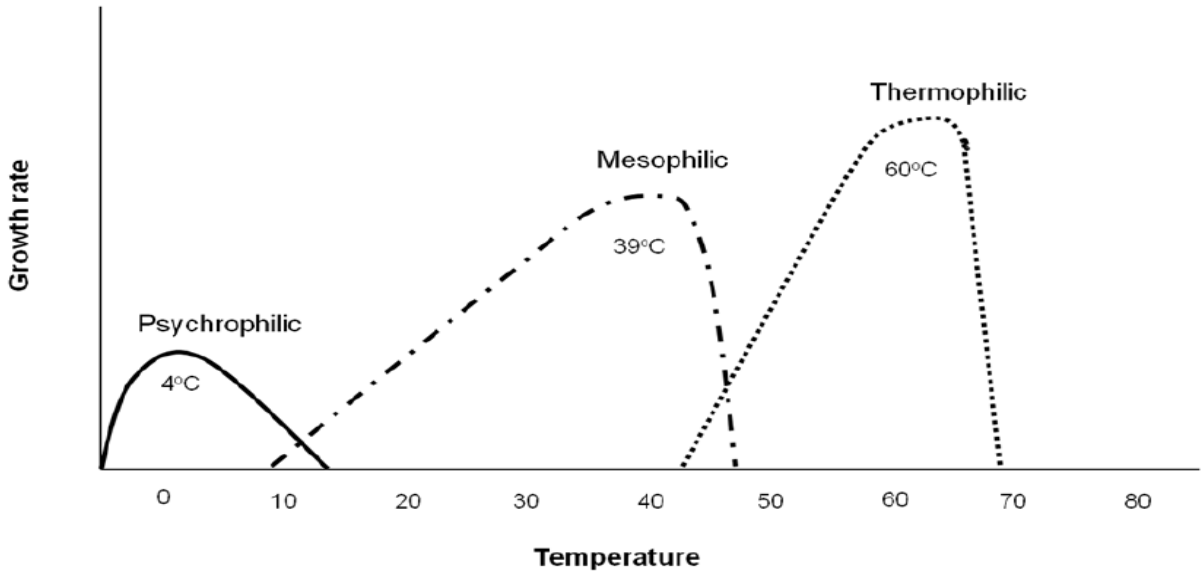


Figure 2-10: Temperature effects on the bacterial growth activity

The effects of temperature on the overall microbiological activity, and biogas production during AD can be modelled using the van't Hoff's equation (2-23):

$$r_T = r_0(\theta)^{(T-T_0)} \quad (2\text{-}23)$$

Thermophilic digesters are highly attractive as they allow higher organic loading rates (OLR) and are characterized by higher methane yield; substrates digestion; and pathogens removal. At organic solids and bacterial sludge is much shorter as they facilitate faster reaction rates during hydrolysis resulting in a reduced digester volume. However, the thermophilic microbes are extremely sensitive to incremental changes in their environment, high energy maintenance; and the long reproduction time (<30 days) makes them less attractive in commercial applications (Ray, et al., 2013).

Mesophilic digesters on the other hand are more tolerant to changes in the environment; stable and easier to maintain with minimal energy requirements. However, they require long retention times with a low methane yield hence the need of large reactor volumes for efficient conversion of organic waste to biogas. (Ray, et al., 2013).

2.9.2 pH and alkalinity

There exist two groups of bacteria in terms of the optimal AD performance namely acidogens and methanogens. The former thrives in the pH range of 5.5-6.5; and the later in the of pH range 7.8-8.2. The optimal pH range for combined culture of bacteria is close to neutral since methanogenesis is considered the rate limiting stage (Kigozi, Aboyade and Muzenda, 2014).

In the study conducted by Latif *et al.*, (2017) to assess the influence of low pH on the continuous anaerobic digestion of waste activated sludge the results revealed that there was a 50% reduction in the methane production efficiency at pH 5.5, which was as well associated with the observed increase in VFAs that led to a shift in the microbiological properties towards acidogens and 88% reduction in the methanogenic archaea population.

2.9.3 Mixing

The mixing of digester contents is necessary to achieve the optimal contact between substrate and microorganism for enhanced anaerobic digestion (Kigozi, Aboyade and Muzenda, 2014). Maintaining a uniform distribution of temperature, microbes and substrate throughout the slurry minimizes the settling of particulate matter and the formation of grit and scum, thus improving biogas production efficiency (Lindmark *et al.*, 2014). However, contradictory results were obtained by Karim *et al.*, (2005) where they noted higher biogas production in the unmixed digesters.

The mixing in digesters can be achieved using either mechanical stirring of the digester content or via gas or sludge recirculation. The mechanical mixing is considered the most effective method, but however mechanically agitated digester often experiences clogging and fouling of the mixer impeller.

2.9.4 Retention time

In anaerobic digestion there exists two important retention time parameters, which are Sludge Retention Time (SRT); and Hydraulic Retention Time:

2.9.4.1 Sludge retention time

SRT is the average time spent by the bacterial sludge inside the digester and is the most important retention time in anaerobic digestion, since if too short the reactor would quickly acidify and affect the performance of methanogenic bacteria. At least 15 days are necessary to ensure both sufficient methanogenic activity and the hydrolysis and acidification of lipids at 35 ^{o}C. Short values of this parameter results in the washout of the important bacterial media, and is thus considered the most important retention time in AD (Gerardi, 2003).

2.9.4.2 Hydraulic retention time

HRT is the average time spent by the input slurry in the digester and a function of the volatile solids conversion to biogas in anaerobic digestion, as its values are known to affect the rate and amount of methane production. The HRT is considered as the most important operational and design parameter in AD (Gerardi, 2003). A linear relationship between HRT and digester temperature is known to exist up to 35 ºC (Kigozi, Aboyade and Muzenda, 2014)

The retention times can be calculated using the following equation:

$$\mathrm{HRT} = {}^{V_R}/_{Q} \tag{2-24}$$

2.9.5 Organic loading rate

Organic loading rate (OLR) can be described as the volume amount of organic materials fed to the digester per day. The production of gas is highly dependent on the OLR, especially methane, and increases with increasing OLR to the digester until methane-forming bacteria are no longer capable of degrading the VFAs. A shift in the microbial community structure induced by changes in OLR, and process dynamics reducing biogas productivity are associated with decrease in both bacterial and archaeal biomass (Ferguson, Coulon and Villa, 2016).
The volume, rate, and composition of the biogas produced can be taken as digester performance indicators (Gerardi, 2003).

The OLR can be calculated according to the following equation:

$$\mathrm{OLR} = \frac{Q * C_S}{V_R} \tag{2-25}$$

Where Q (m^3m^{-3} day^{-1}) is the substrate volumetric loading rate; C_S is the substrate concentration (Kg VS or Kg COD); and V_R is the digester working volume (m^3).

2.9.6 Food to microorganism ratio

Food-to-microorganism (F/M) ratio is defined as the ratio of VS in the substrate to the VS in the inoculum initially present during batch AD. According to Feng *et al.* (2013) F/M ratio can be considered one of the most important operational parameters in AD, with each substrate having different optimum F/M ratios due to its physical and chemical characteristics and the system's VFAs buffering capacity. However, a high F/M ratio may lead to inhibition and toxicity; while low F/M ratio hinders the synthesis of enzymes that promote AD directly impacting lag time for CH_4-generation (Feng *et al.*, 2013).

2.9.7 Nutrients

All microbiological transformation processes including AD in addition to carbon; oxygen; and hydrogen require certain macro (N; P; and S) and micro (trace metals such as Ca; K; N; Co; etc.) nutrients in sufficient concentrations capable of enabling the microbes to perform their normal metabolic activities (Grerardi, 2003). The nutritional balance is dependent on the COD strength of the waste to be digested, ideally, the most suitable COD: N: P of 1000:7:1 and 350:7:1 for high strength waste and low loading rates, respectively. Note that these values are approximately at the optimum C/N ratio of 25:1.

The active bacteria in AD use nitrogen to meet their protein requirements, and in the case of low C/N, the nitrogen is rapidly consumed during AD, with none left over to react with the remaining carbon, hence the effects of this process on the gas production. In cases where nitrogen is excessively in high concentrations it produces more ammonia, which is a strong base that raise the pH above the desired levels, and inhibit the methane-forming bacteria and ultimately gas production (Kigozi, Aboyade and Muzenda, 2014).

2.9.8 Volatile fatty acids

VFAs are produced as intermediate products during AD and are required in sufficient concentrations to maintain a balance across the food-chain. However, high concentrations exceeding 13000 mg L^{-1} can inhibit the AD process and accumulation thereof reducing system pH and subsequently decreasing the methanogenic archaeal population (Gerardi, 2003).

2.9.9 Toxicity

The biogas production in AD can be adversely affected by various inorganic and organic substance that are either present in the substrate feed or produced as intermediates. The most widely reported types of toxicity are ammonia; hydrogen sulphide; heavy metals; and many common household detergents; antibiotics and other organic solvents (Kigozi, Aboyade and Muzenda, 2014). A list of commonly occurring nutrients in anaerobic digestion systems and their known toxic/inhibitory levels to microbial activity is presented in **Table 2-10**.

The methane-forming bacteria can however be acclimatized to a certain extent to toxic compounds, and the values of toxicity can be assessed according to the following criteria:

- The ability of the microbes to adapt to a constant concentration of toxic waste
- The absence or presence of other toxic waste
- Changes in operational parameter

Table 2-10: Toxic and inhibitory concentration for commonly found nutrients and trace elements in AD

Component	Concentration
Na^+	30 g L^{-1}
K^+	3 g L^{-1}
Ca_2^+	2.8 g L^{-1}
Mg_2^+	2.4 g L^{-1}
NH_4^+	pH-related (80-150ppm)
Sulfide	pH-related (< 1000ppm)
CO_2	> 1 bar and < 0.2 bar
Nitrate / nitrite	> 50 ppm
Free **VFA**s	pH-related

There also exists two types of toxicity in AD (i.e. acute and chronic toxicity) briefly articulated below (Gerardi, 2003):

2.9.9.1 Acute toxicity

This type of toxicity is a result of rapid exposure of an un-acclimatized bacterial culture to relatively high concentrations of toxic waste.

2.9.9.2 Chronic toxicity

The chronic toxicity is a result of the gradual and relatively long-term exposure of an un-acclimatized bacterial culture to toxic waste. During this type of toxicity, the microbial culture can be acclimatized to the toxic waste in two ways:

I. The microbes can either repair their broken enzymes or adjust and degrade the toxic compounds;
II. They can grow a large and diverse microbial culture that will be able to develop the enzyme system that has the capability to degrade the toxins present in the digester.

2.9.10 Ammonia

Ammonia is one of the crucial nutrients for the growth of microorganisms which is utilized as a protein source for by the microbes (Chiba, 2014). However, during the digestion of nitrogen-containing organic compounds such as urea and proteins, ammonia is largely released in the ionized form (NH_4^+). The released ammonia is more toxic in the unionized form (NH_3) form which can lead to an increase in the pH value of the digester as the acceptable pKa value limit

is 9.3 (Ma, et al., 2016). The pKa value can be calculated according to the following equations **(2-26**) and **(**2-27) (Diamantis, et al., 2007):

$$\frac{NH_3}{[NH3]*[NH_4^+]} = \frac{1}{1+10^{pKa-pH}} \quad (2\text{-}26)$$

$$pKa = 4 * 10^{-8} * T^3 + 9 * 10^{-5} * T^2 - 0.0356 * T + 10.072 \quad (2\text{-}27)$$

Ammonia is more toxic to the methanogens in the unionized (NH_3) form than in the ionized form (NH_4^+) as it can readily diffuse through the cell membrane, resulting in a proton in balance and potassium (K^+) deficiency, while in its ionized form higher concentrations will directly inhibit the enzymatic synthesis of methane (Gerardi, 2003). However, the ionized form of ammonia can provide a buffering capacity when it reacts with the carbon dioxide and produces bicarbonate that balances the pH of the digester when the rate of acetogenesis exceeds methanogenesis.

2.9.11 Hydrogen sulphide

The inhibitory effects of hydrogen sulphide are of two kinds: (i) the competition for the vital acetate substrate between sulphate reducing bacteria and methanogens; (ii) and the direct inhibition of methane production by the presence of sulphide ions in the system (Gerardi, 2003).

2.10 Pre-treatment for enhancing AD efficiency

The pre-treatment of organic waste makes a substrate more biodegradable (i.e. Extent and kinetics), and enhances biogas production (Mata-Alvarez *et al.*, 2014). The pre-treatment techniques employed in AD can either be physical, chemical, and/or biological (Kondusamy and Kalamdhad, 2014).

2.10.1 Physical pre-treatment

Physical pre-treatments methods are employed to reduce particle size of substrates which increases the surface area accessible to hydrolytic bacteria; and are classified into three categories, namely, mechanical, thermal and ultrasonic (microwave).

2.10.1.1 Mechanical pre-treatment

Mechanical pre-treatment disrupts weak physical bonds and is performed by chopping and grinding substrate to reduce particle size increasing the substrate(s) surface area enabling enough substrate-bacteria contact.

2.10.1.2 Thermal pre-treatment

This pre-treatment technique utilises temperature to pre-treat the substrate with the intention of increasing its solubilisation. It also facilitates pathogen removal, improves dewatering performance and reduces viscosity.

2.10.1.3 Microwave pre-treatment

Ultrasonic pre-treatment technique uses waves to disrupt the substrate's cells to promote cavitation inside the cell.

2.10.2 Chemical pre-treatment

Chemical treatment with acidic, alkaline and oxidative compounds breaks the chemical bonds of long chain polymers to simple forms, thereby increasing solubility and access by microorganisms.

2.10.3 Biological pre-treatment

In a study by Cirne, et al. (2007), assessing the effects of lipid concentration (5%-47% COD% w/w) in anaerobic digestion, the authors observed an improved hydrolysis rate with addition of lipase enzyme, and concluded that high concentrations of lipids and enzyme were inhibitory for the methane production phase. However, the process recovered after a long time, in the end, a methane recovery of above 93% for all the tests performed.

2.10.4 Combined pre-treatment strategies

The pre-treatment strategies combined for high efficiency of the process resulting in highly soluble substrate easily accessible to the microorganisms, provide those economic benefits of combined pre-treatments outweighing initial investments.

A study conducted on various pre-treatment methods for waste activated sludge (Kim et al., 2003) demonstrated that a combination of thermochemical treatment improved biogas production, COD removal, and VS destruction. Thermal treatment at 120 ^{0}C followed by chemical addition 7g/l NaOH provided the best results such as biogas production of 3367 l/m^{3}, SCOD removal (61.4%), and VS removal (46.1%).

In an experiment conducted by Cavaleiro, et al. (2013), on the physical, chemical and enzymatic pre-treatment of meat-processing waste it was revealed that alkaline and enzymatic pre-treatment steps produced higher concentrations of soluble/colloidal COD and free LCFA during the hydrolysis phase.

Similarly, a study on the saponification of slaughterhouse waste indicated enhanced biodegradability for waste that was treated with sodium hydroxide at 120 ^{O}C for 30 minutes, and the authors attributed these results to the increase in bioavailability of the fatty wastes (Battimelli *et al.*, 2010).

A summary of work done by various researchers on the effects of different substrate pretreatment methods is presented in **Table 2-11**. In anaerobic co-digestion, pre-treatment is applied to a single substrate, and the one with a poorest biodegradability profile is usually considered.

Table 2-11: The effects of different types of AD feedstock(s) pretreatment methods and their overall impact on the digestion process

Pretreatment, Substrate [a], Reactor configuration, Mesophilic (35-37 °C)		**Improvements**		**References**
		Organic [b]	**Biogas [c]**	
Thermal	70 °C for 30 min, MR, Batch	0.3-0.8 g/g VS	nr	(Cavaleiro et al., 2013)
	121 °C for 30 min, WAS, Batch	36.7% (COD)	32.4 % (70%)	(KIM et al., 2003)
Ultrasound	35000 KJ/Kg TS, 20KHz, 750 W, WAS, Batch	26% (COD)	31.4%	(Lizama et al., 2017)
	42 kHz for 120 min, WAS, Batch	38.9 % (VS)	20.7% (70%)	(KIM et al., 2003)
Chemical	7 g/L NaOH, WAS, Batch	29.8% (VS)	13.4 % (70%)	(KIM et al., 2003)
	0.3 g NaOH g^{-1} TS for 24 h, MR, Batch	0.3-0.8 g/g (VS)	nr	(Cavaleiro et al., 2013)
	5.0% NaOH (w/w) for 24h, CS, Batch	44.4%	37% (60%)	(Zhu, Wan and Li, 2010)
Thermal	0.04 mole NaOH g^{-1} COD 70 °C for 60 min, SW, Fed-Batch	97% (VS)	52.7% (75)	(Affes et al., 2013)
+	0.156 g NaOH g^{-1} VS 120 °C for 3 h, SW, Fed-Batch	1–5 g/L (VS)	nr	(Battimelli et al., 2010)
Chemical	0.04 mole NaOH g^{-1} COD 60 °C for 30 min, SW, Fed-Batch	0.1–0.2 g/g VS	[d]	(Battimelli, Carrère and Delgenès, 2009)
	0.3 g NaOH g^{-1} TS 55-121 °C for 20 min, MR, Batch	0.3-0.8 g/g VS	nr	(Cavaleiro et al., 2013)
	7 g/L NaOH 121 °C for 30 min, WAS, Batch	67.8%, 46.1%	37.8% (70%)	(KIM et al., 2003)
Biological	Lipase, 3.6 IU g^{-1}, LPW, Batch	nr	[e]	(Cirne et al., 2007)
	10 IU g^{-1} Lipase, MR, Batch	0.3-0.8 g/g VS	nr	(Cavaleiro et al., 2013)

[a] SW: Slaughterhouse, FPW: Lipid-Rich, WAS: Waste Activated Sludge, MR: Meat Process Residues, Corn Stover waste; [b] Organic matter degradation efficiency expressed as either **COD** and/ or VS in brackets; [c] Biogas production increase in percentage and methane content in brackets; [d] Biogas produced 90% of theoretical maximum; [e] Biogas produced 93% of theoretical maximum; nr, not reported.

CHAPTER 3

3 Materials and Methods

This chapter presents a detailed description of the materials used in this study. The sample collection and preparation, digester set-up, and experimental protocols followed are also presented in this chapter together with analytical methods deduced for data analysis. The justified use of materials and techniques is also discussed in this chapter.

A logical series of steps followed in determining substrate(s) and co-substrates suitable for enhanced biomethane production carried out as follows:

- A proximate and ultimate analysis of the substrates were conducted under reproduceable conditions;
- The theoretical methane potential (TMP) of the substrate(s) and co-substrates were obtained by applying (CHNS) ultimate analysis results to the Buswell and Boyle equation.
- A series of conventional BMP assays based on mono- and co-digestion of substrate(s) are performed in triplicate under reproduceable conditions,
- The results of the BMP assay were used to obtain relevant kinetic data for enhancing biogas production, and the biodegradability index by comparing BMP to the TMP yield (Chapter 4).
- Mixture blend designed experiment based on BMP results for optimizing biogas production, and enhancing synergistic effects of the mixture (Chapter 5),
- Optimization experiments around environmental factors effect(s) to enhanced biogas production designed experiments (Chapter 6),
- Lab-scale batch, and semi-continuous AD experiments were conducted to estimate a desirable HRT, OLR and mode of operation for the chosen mixture (Chapter 7).

3.1 Feedstocks collection, preparation and storage

3.1.1 Reagent solution description

All the steps used in preparation of reagent solutions are detailed below

3.1.1.1 Sodium hydroxide stock solution

A 10 N sodium hydroxide (NaOH) stock solution used to prepare various dilutions was prepared with lab grade pellets (NaOH, CAS-No. 1310-73-2, B & M Scientific.) and de-ionized water flushed with nitrogen gas.

3.1.1.2 Hydrochloric acid stock solution

A 5 N hydrochloric acid (HCl) stock solution was prepared by diluting lab grade concentrated solution (HCl: 36.46, 32 % CP-grade, Batch-No. 15477W0, B & M Scientific.). The 10 N HCl stock solution was further diluted to 1 N HCl and used as pH-control/adjustment solution and used to prepare an acidified-saline solution.

3.1.1.3 Acidified-saline solution

An acidified-saline solution used as discharge fluid in the gasometer(s) was prepared with normal table salt. The salt was dissolved in warm water (30 ±5 ºC) while constantly stirring until the saturation point was reached, after which the pH was lowered to 2 using HCl solution.

The acidified-saline solution decreases the solubility and diffusion of gasses, in particular the CO_2 which is approximately 25 times soluble in water at 25 ºC than CH_4 (Strömberg, Nistor and Liu, 2014)

3.1.1.4 Acetic acid and glucose solution

Laboratory grade glacial acetic acid and dextrose powder were used to prepare dilute concentrations (1 – 7 g L^{-1}) that were used in specific activity test assays, and during inoculum synthesis.

3.1.2 Substrate description

3.1.2.1 Abattoir solid and cow blood

The primary substrate used in this study is abattoir waste which consisted of condemned meat waste (A_s) (**Figure 3-1A)**, cow blood (C_b) (**Figure 3-1C**) and ruminal contents (RC), that were obtained from OSDAM abattoirs (Paarl, Western Cape, SA) on the 8th June 2018 in sterile plastic containers. According to recommendations from previous studies (Salminen and Rintala, 2002; Escudero *et al.*, 2014; Valta *et al.*, 2015; Ware and Power, 2016) abattoir wastes in this category must be sterilized by heating for 20 minutes at 133 °C under 2 bars in a pressurized vessel prior to their application as feedstock in AD.

3.1.2.2 Winery waste

Fresh winery solid waste W_s (**Figure 3-1B**) which was obtained from Agricultural Research Council (Stellenbosch, Western Cape, SA) was made dry in the sun followed by size reduction step using a hammer-mill (RSA Scientific Sr-no. 401).

3.1.2.3 Zebra dung

Fresh zebra dung (ZD) collected from Vreidenheim Farm (Stellenbosch, Western Cape, SA) in sterile plastic bags was placed and kept in a cooler-box at 0 ºC for an hour during transit to the Waste-2-Energy Lab at CPUT, Bellville, Western Cape, SA). The ZD samples were then flushed with anaerobic gas (N_2) for 5 minutes prior to storage in laboratory-coolers set at -4 ºC, and further synthesized into inoculum.

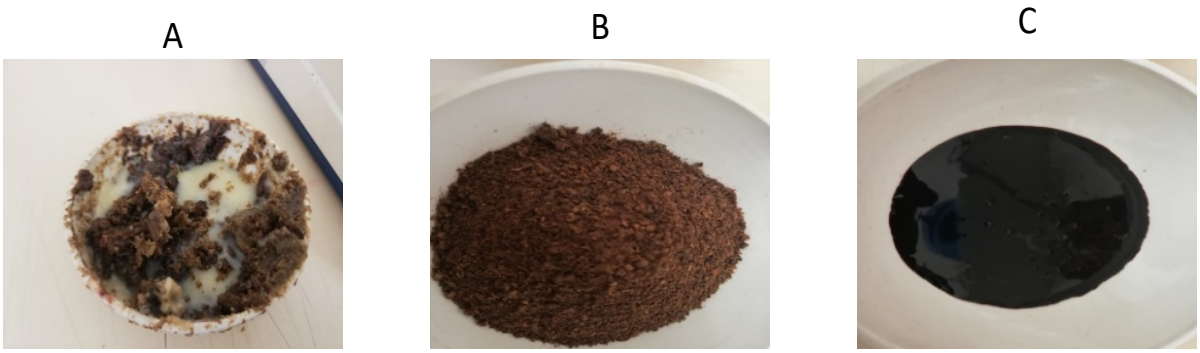


Figure 3-1: Photographic image of the three substrates evaluated A: sterilized and microwave pretreated abattoir solid; B: winery solid; and C: abattoir liquid (cow blood) wastes

3.1.3 Abattoir-winery waste co-substrate mixture blends

The co-substrate mixture blends from abattoir wastes (i.e. solids and cow blood) were blended with winery solid waste in varying proportions (see **Figure 3-2**), which were based on all the experimentally designed aims and objectives set to investigate the hypotheses made in **Section 1.3**.

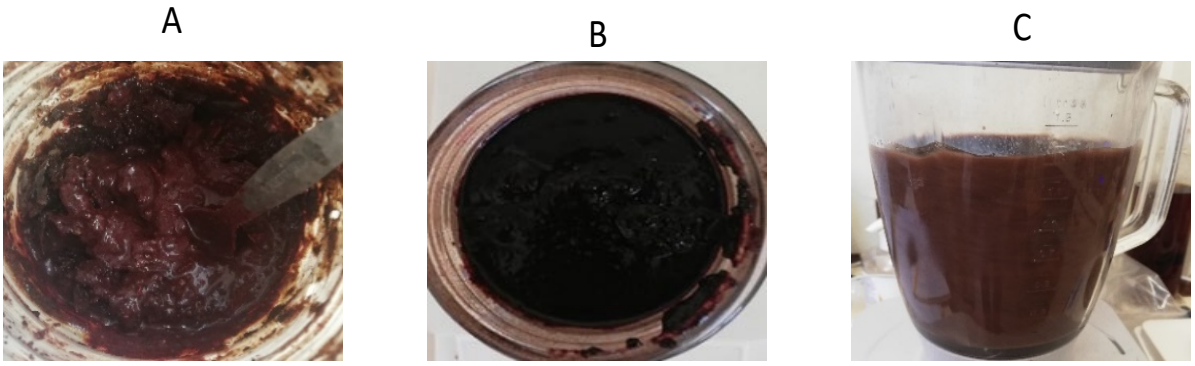


Figure 3-2: Photographic images of co-substrate mixture blends

3.1.4 Inoculum Preparation

The inoculum used throughout this study was carefully prepared in three stages described below. A laboratory-induced anaerobic environment was maintained in all three stages of the preparation process.

Stage 1: **Mixing**

To synthesize the inoculum used in all anaerobic digestion experiments, 206 grams of ZD and 208 grams of RC were carefully transferred into sterilized food blender (Kenwood Multi-Pro, FDM780BA, 1000 W) filled with 0.5 L de-ionized water and blended for 10 minutes to prepare the inoculum-seed. After this process, the blended product was then transferred to a deflated 3 L re-usable plastic container, which was thereafter sealed and kept at room temperature for a period of one month. After that period, the container was filled with the biogas containing a determined concentration of 29.3 and 38.9 % for CH_4 and CO_2, respectively.

Stage 2: **Inoculum acclimatization (Anaerobic synthesis)**

In this stage, the produced inoculum-seed was then transferred into 3 L of de-ionized water contained in a 5 L continuously stirred (150 rpm) batch digester at 38 ±5 ^oC. The mixture was slowly acclimatized to the new substrates (i.e. A_s, C_b and W_s) by feeding it per week with 1-2 g of raw substrate with acetic acid and glucose solutions which were frequently added to the maintain activity of the anaerobic culture. After two months, the headspace gas (i.e. wet gas composition) quality was recorded at 59 % CH_4 and 9.2 % CO_2.

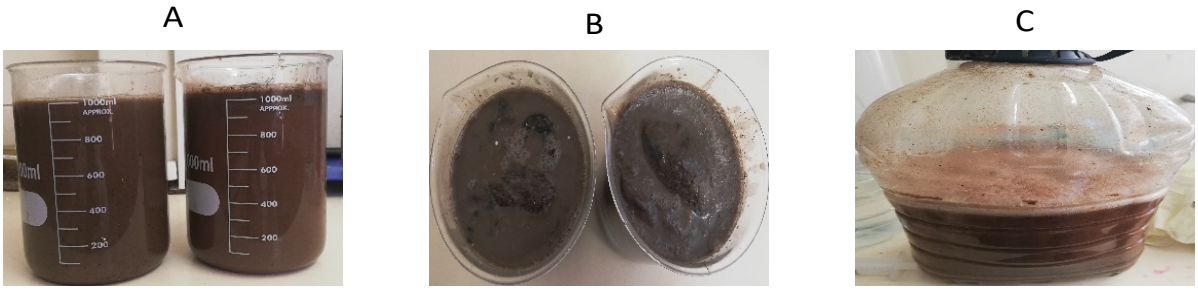


Figure 3-3: Inoculum preparation

Step 3: **Screening and characterization of inoculum**

The inoculum was then sieved to remove large inert and recalcitrant organic matter (> 2mm) and characterized. After characterization, the inoculum was stored in three-liter re-usable plastic containers (see **Figure 3-3**) at - 4 ±1 ºC for later use in all AD experiments. Prior to AD experiments the inoculum was degassed for 7-14 days at 38 ±5 ºC to reduce residual CH_4 emission.

3.2 Analytical methods

3.2.1 Scientifically Standardized Methods/Protocols and Assumptions Applied

The International Union of Pure and Applied Chemistry (IUPAC) standard temperature and pressure conditions were applied assuming perfect mixing and ideal gas behavior:

- Standard gas volume (V_s) = 22.4 m^3
- Standard Temperature (T_s) = 0 ºC (273.15 K)
- Standard Pressure (P_s) = 101.325 KPa (1 atm)

The following assumptions were used to simplify the complex nature of anaerobic digestion:

- Ideal microbiological conditions, meaning complete digestion
- Perfect mixing yielding homogeneity inside the digester;
- Input substrate consists of C, H, O, N and S;
- The products of reaction include only CH_4, CO_2, NH_3; and H_2S; and
- Negligible ash accumulation inside the digestion vessel.

3.2.2 Sample pretreatment

3.2.2.1 Sterilization

A conventional pressure cooker (Russell Hobbs, RHEP7, 70 KPa, 6.0 L; 50/60 Hz) was used to sterilize abattoir solid waste (excluding ruminal content) to reduce bacterial activity and

destroy pathogens, thus reducing risk of exposure. The time of exposure to treatment consisted of the initial five minutes leading the samples from atmospheric conditions to 133 °C and 2 bars followed by 20 minutes of heating at constant conditions.

3.2.2.2 Microwave

A commercial domestic microwave oven (DEFY, DMO 367, 2450 MHz frequency, 700 W) was used to irradiate the abattoir (solid) samples. The microwave pre-treatment was conducted in batches using 100 g of sample placed in a covered microwave dish to avoid losses caused by hot spot formation during the treatment for a period of five minutes.

3.2.3 Sample characterization

3.2.3.1 Proximate analysis

The analysis for TS, VS and TOC were performed following the standard methods described in Sluiter *et al.* (2008). For TS determination, samples with known volume/weight were placed in crucibles and dried in a laboratory convective oven performed at 90 ±5 ºC rather than 105 ±5 ºC to a constant weight as suggested to avoid the volatilization of VFA's, due to the acid nature of the substrates tested (Angelidaki and Sanders, 2004). After cooling the samples in a desiccator, these samples were then analyzed for TS by weighing them. After that, the samples were oxidized by incineration in a muffle furnace at 575 ±25 ºC for a period of four hours with the intention of determining the VS concentration determination.

3.2.3.2 Ultimate analysis

The elemental composition of carbon, hydrogen, nitrogen and sulphur excluded moisture and ash content of the substrates. This analysis which consisted of making the substrates dry and ash-free took place at Central Analytical Facilities (CAF) in Stellenbosch by means of combustion using CHNS element analyzer with oxygen determined as the difference, while the induced coupled plasma mass spectrometry (ICP-MS) was used to analyze the metal concentrations of each substrate. The ultimate analysis results were used to estimate the empirical formula, theoretical methane potential (TMP), theoretical oxygen demand (TOD), and carbon-to-nitrogen (C/N) ratio.

3.3 Anaerobic batch digestion equipment set-up and procedure

3.3.1 Biomethane potential (BMP) assay

A conventional BMP protocol based on the principles described by Owen et al. (1979) and revised by other scholars or scientist (Hansen *et al.*, 2004; Angelidaki *et al.*, 2009; Strömberg,

Nistor and Liu, 2014) with minor modifications as depicted in **Figure 3-4** for directly measuring methane yield, was followed throughout this study.

All BMP assays were carried-out at 38 ±5 ºC which is within the mesophilic temperature range of 20 to 40 ºC (Kigozi, Aboyade and Muzenda, 2014). Anaerobically controlled environmental conditions were induced by flushing the headspace of assay vessels for five minutes with N_2-gas (Nitrogen Baseline 5.0, UN No. 1066, Afrox gas, Epping, South Africa), prior to inoculum and sample adding and just before sealing and incubating.

The BMP assay equipment was designed and set-up into three processing units as depicted in **Figure 3-5** and **Figure 3-6**. **I→II→III**

I. Anaerobic Digestion Section:

- Two temperature-controlled water baths were fitted with water circulating immersion thermostats (FMH electronics); 500- and 1000-mL digesters (Duran Schott Bottles); A gas production line was also fitted with a plastic clamp.

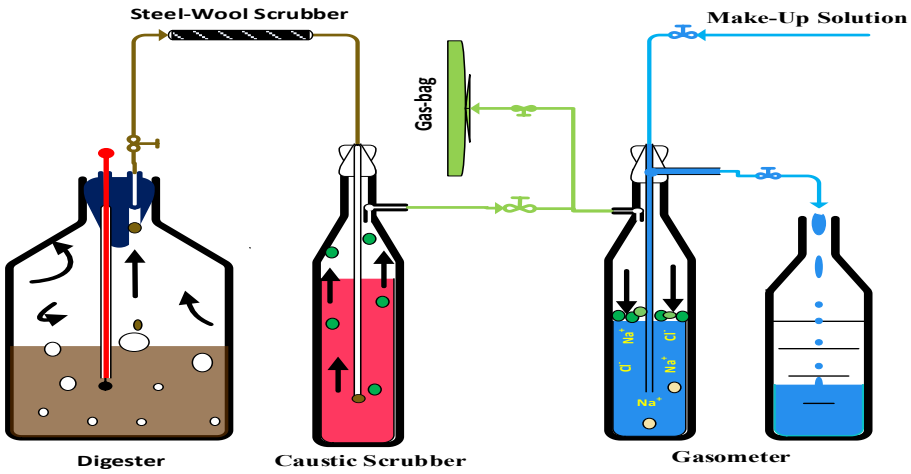


Figure 3-4: A schematic representation of the modified BMP assay procedure

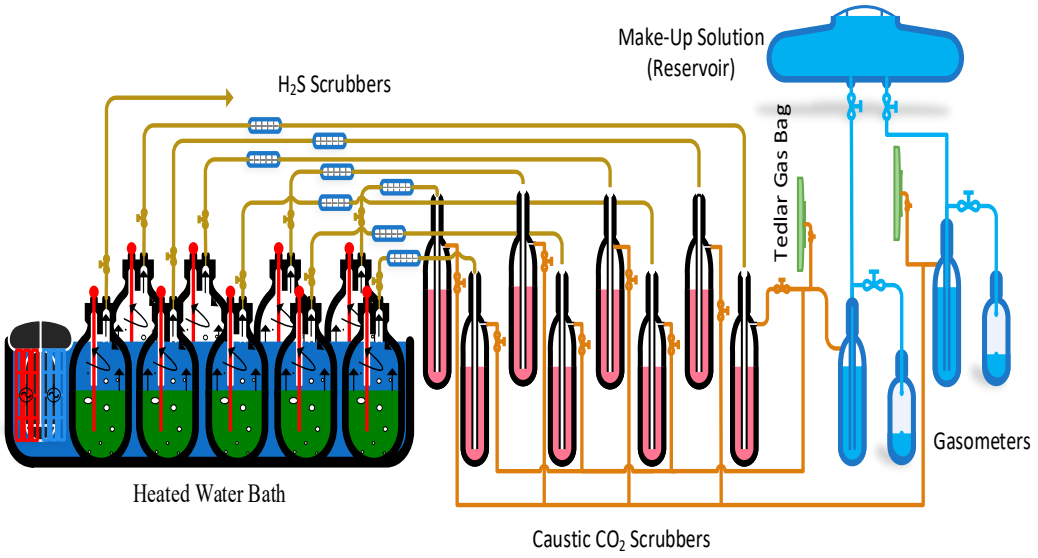


Figure 3-5: Schematic view of BMP assay experimental set-up

II. Gas Scrubbing Section:

- 60 mL polystyrene tubes were packed with steel wool, for H_2S trapping; and 500- and 1000-mL gas washing bottles were filled with 3 N NaOH solution for CO_2, and NH_3 trapping. A pH indicator phenolphthalein powder was also added to the solution to aid in determining necessary scrubber saturation points.

III. Gas measuring unit section (Gasometer):

- 500- and 1000-mL simple, accurate, and easy to calibrate lab-scale gasometer(s) made with re-usable plastic bottles filled with acidified-saline solution. A 1000-mL Tedlar gas bag(s) (Sigma-Aldrich) used for long term gas storage were attached to the gasometer(s).

Figure 3-6: Photographic image of BMP assay experimental set-up

3.4 Biomethane potential and biodegradability experiments

3.4.1 Substrate and inoculum

Three different substrates were used in this study, namely abattoir solid (A_s) and liquid (cow blood) (C_b), and winery solid (W_s) waste. A detailed description of the substrate collection, preparation and preservation can be found in **section 3.1.**

3.4.2 Experimental procedure

The study into the anaerobic biodegradability, biomethane potential, and toxicity of abattoir and winery solid waste was led by three series of experiments (**Table 3-1**):

3.4.2.1 Natural biodegradability and toxicity assays

The first experiment sought the determination of the natural biodegradability extent of A_s, C_b and W_s wastes in mono- and co-digestion experiments with high organic load (OL) and low F/M ratio of 20 gVS/L and 2 g $VS_{sub}g^{-1}VS_{inoc}$, respectively. The volume of inoculum was maintained constant at 400 mL in all the assays for consistency, while the measurement of tested substrates was done on VS basis. Moreover, to ensure natural conditions the headspace of the assays was not flushed with anaerobic gas, and no form of pH-control/adjustments agents

was added to the digestion vessel. In these experiments freshy prepared inoculum (see **section 3.1.4**) was source of active anaerobes.

3.4.2.2 Mono-digestion experiments

In the second set of experiments the performance of the now acclimatized inoculum synthesized in a five-liter fed batch laboratory digesters (see **section 3.1.4**), was conducted for the mono-digestion of A_s and C_b, respectively. These experiments were conducted under controlled laboratory experiments with an OL and F/M ratio of 10 gVS L^{-1} and 1 $gVS_{sub}g^{-1}VS_{inoc}$, respectively.

3.4.2.3 Specific methanogenic activity tests

Specific methanogenic activity (SMA) assays were also conducted before starting the last series of experiments to assess the quality of the inoculum following the method described by Soto, Méndez and Lema (1993). In these experiments conducted for three to five days, acetic acid was used as substrate, and the substrate organic concentrations selected was 2 gVS L^{-1}. The SMA assays were conducted with an inoculum size concentration of 0.81 gVSS L^{-1}, utilizing the 500- and 1000-mL anaerobic digestion vessels for the first and second SMA assays, respectively.

3.4.2.4 Biomethane potential from mono- and co-digestion experiments

The last series of experiments was aimed at determining the optimum F/M ratio, organic loading, and compare mono- and co-digestion performance. More specifically, three F/M were applied: 0.5, 1 and 2 $gVS_{sub}g^{-1}VS_{inoc}$, and organic concentrations of 5, 10 and 20 gVS L^{-1}, respectively. The co-digestion effects were tested on dual and triple co-substrate mixtures of A_s, C_b and W_s, the ratio applied in the triple substrate co-mixture 1:1:2 ($A_sC_bW_s$), and two ratios for the dual co-mixtures (i.e. 1:1 and 2:3 of A_sW_s and C_bW_s) g VS_{sub} $g^{-1}VS_{sub}$, respectively.

Table 3-1: Experimental design for anaerobic mono- and co-digestion

Run	Substrate	Mix	Pre-treatment	V_R	Sample mass	F/M	Temp	Incubation
				mL	gVS L^{-1}		±5 °C	day
Anaerobic Biodegradability and Toxicity Assays								
1	A	-	Yes [a]	450	20	2	38	34
2	B	-	No	450	20	2	38	34
3	W	-	Yes [b]	450	20	2	38	34
4	AB*W	1:1	Yes [c]	450	20	2	38	34
5	AB+ W	1:1	No	450	20	2	38	34
6	Inoculum	-	-	450	-	-	38	34

Anaerobic Mono-Digestion Assay								
1	A	-	Yes [a]	900	10	1	38	30
2	B	-	No	900	10	1	38	30
3	Inoculum	-	-	900	-	-	38	30
Specific Methanogenic Activity Assays								
1	AA	-	-	400	7	-	38	3
2	Inoculum	-	-	400	-	-	38	3
3	AA	-	-	900	1	-	38	5
4	Inoculum	-	-	900	-	-	38	5
Anaerobic Mono- and Co-Digestion Assays								
1	A	n/a		900	20	2	38	30
2	B	n/a		900	20	2	38	30
3	W	n/a		900	20	2	38	30
4	A + B + W	1: 1: 2		900	20	2	38	30
5	Inoculum	-		900	-	-	38	30
6	A + W	1:1		400	10	1	38	30
7	B + W	1:1		400	10	1	38	30
8	Inoculum	n/a		400	-	-	38	30
9	A + W	2:3		900	5	0.5	38	32
10	B + W	2:3		900	5	0.5	38	32
11	Inoculum	-		900	-	-	38	32

[a] Microwave thermal pre-treatment; [b] Mechanical size reduction pre-treatment; * pre-treated substrate; **n/a** - not applicable.

A is the solid fractions of abattoir waste; **B** is the liquid fraction (cow blood) of abattoir waste; **W** is the winery solid waste;

3.5 Optimization for biomethane production efficiency

The idea that co-digestion at the theoretical C/N range alone will enhance the biomethane is unfounded, considering different phenomena such as the complex AcoD process dynamics of abattoir and winery wastes, the microbiological symbiotic interactions, and environmental input factors. Thus, enhanced performance, stability, and biomethane generation can only be achieved by setting and controlling these input factors at their optimum levels.

Therefore, two key studies were conducted to reveal more information about:

(i) mixture blending to address **(Objective 2)**; and

(ii) input factor(s) effects on overall AD performance and their optimal levels (**Objective 3)**.

3.5.1 Screening mixture design

3.5.1.1 Substrates

Three substrates, abattoir solid (A_s), cow blood (C_b), and winery solid (W_s) wastes were investigated in anaerobic co-digestion experiments. (See section **3.1** for detailed methods).

3.5.1.2 Experimental design

A screening ABCD mixture design seen in **Table 3-2** for factor screening was created using JMP software (JMP® Pro® SAS 13.2.1, 64-bit, Institute Inc., USA), which generated 10 mixtures that were used to study the effects of various mixture blends on the overall AD performance, with the assumption that important components in the mixture would have pronounced linear effect(s).

However, as noted by Snee and Marquardt (2016) special cases can exists nullifying the above statement and, therefore, to determine if significant curvature/nonlinear blending (NBL) effects exist, center points were added to the design and with only A_s, C_b, and W_s factors given that the design was reduced to a three factor-five level simplex centroid design.

A full cubic model accommodating substantial curvature in the response surface over the design space was used to fit nonlinear ternary and binary blends using equation **(3-1**, with the last three model terms representing binary blends that are cubic in nature.

$$Y = \beta_A A + \beta_B B + \beta_C C + \beta_{AB} AB + \beta_{BC} BC + \beta_{ABC} ABC + \delta AB(A - B) + \delta AC(A - C) + \delta BC(B - C) \quad (3\text{-}1)$$

Sufficient levels of each factor in the mixture were also needed to fit experimental data to the special quadratic model described in equation (3-2), which is useful in prediction of higher order curvature for non-linear blending (NLB) in the response surface throughout the design space interior.

$$Y = \beta_A A + \beta_B B + \beta_C C + \beta_{AB} AB + \beta_{BC} BC + \beta_{ABC} ABC + \beta_{AABC} A^2 BC + \beta_{ABBC} AB^2 C + \beta_{ABCC} ABC^2 \quad (3\text{-}2)$$

Table 3-2: Measured mixture composition following **ABCD** screening mixture design

Mixture	Substrate (g)	Mixing Ratio					
		B (%VS)		**W** (%VS)		**A** (%VS)	
		Design	**M**easured	**D**esign	**M**easured	**D**esign	**M**easured
$M_{1\ Avg.}$	1.49 ± 0.05	0	0	0.5	0.46	0.5	0.54
$M_{2\ Avg.}$	2.34 ± 0.02	0.33	0.34	0.33	0.32	0.33	0.34
$M_{3\ Avg.}$	2.64 ± 0.08	0.5	0.51	0.5	0.49	0	0
$M_{4\ Avg.}$	3.31 ± 0.08	0.5	0.51	0	0	0.5	0.49
$M_{5\ Avg.}$	2.14 ± 0.02	0.17	0.16	0.17	0.18	0.66	0.66
$M_{6\ Avg.}$	4.53 ± 0.05	0	0	0	0	1	1
$M_{7\ Avg.}$	3.51 ± 0.05	0.14	0.17	0.66	0.66	0.17	0.17
$M_{8\ Avg.}$	1.71 ± 0.02	0	0	1	1	0	0
$M_{9\ Avg.}$	7.24 ± 0.06	0.66	0.67	0.17	0.17	0.17	0.16
$M_{10\ Avg.}$	9.12 ± 0.04	1	1	0	0	0	0

3.5.1.3 Biomethane Potential Assay

All BMP assays were conducted with standard methods and protocols observed (see **3.3.1**) for detailed description of the method.

3.5.1.4 Co-digestion performance index

The co-digestion performance index (CPI) was evaluated for the binary and ternary mixture blends based on linear mixtures using the method described in section **3.8.6**.

3.5.1.5 Kinetic study

To better understand the AD system towards efficiency in biodegradation kinetics and optimization of biogas production, an evaluation of the "bio-energetic" parameters was conducted using nonlinear regression techniques (see section **3.8.7**).

3.5.2 Optimization via Central Composite rotatable design (CCRD)

3.5.2.1 Substrates

The optimal mixture blend "M_i" identified from the design in **section 3.5.1** was used in the optimization experiments.

3.5.2.2 Experimental design

A five-level-three factor Central Composite Rotatable Design (CCRD) was chosen for optimizing environmental input factors affecting biogas production efficiency, with factors and levels investigated presented in **Table 3-3.** Organic load/concentration (OL) X_1, food-microorganism (F/M) ratio X_2, and initial substrate pH (pH) X_3 were selected according to their

level of significance based on literature or scholar sources and pre-experimental studies on the studied substrate.

Table 3-3: Selected optimization variables and associated coded factors and levels

Factors and Response Variables	Symbol	Factor Levels				
		- α	**-**	**0**	**+**	**α**
Organic Loading (g VS L^{-1})	X_1	0.78	1.5	3.25	5	5.72
F/M ratio (g $VS_{substrate}$ g^{-1} $VS_{inoculum}$)	X_2	0.09	0.25	0.625	1	1.16
pH	X_3	6.19	6.50	7.25	8	8.31
SMP (mL CH_4 g^{-1} VS_{added})	Y_1	.	.	.	.	.
R_{max} (mL CH_4 g^{-1} VS day^{-1})	Y_2	.	.	.	.	.

The optimal pH range for anaerobic digestion is reported to be around 6.5-8.2 (Budiyono, Syaichurrozi and Sumardiono, 2013), while Sibiya, Muzenda and Tesfagiorgis, (2014) reported the highest methane production from a thermophilic digester to be maintained at a pH of 6.5. According to Kalloum *et al.* (2014) optimization of the AD process can be achieved by carefully selecting inoculum sources acclimatized to handle shock organic loading at set F/M ratios; while various F/M ratios ranging from 0.1 to 5 have been reported in the literature. However, all the authors concede that the principle is determined by the nature of substrate(s), the microbiological nature of the system, and the optimum operating conditions.

Thus, given the above submissions various factors and their levels for optimization studies were therefore chosen to deduce the optimal conditions for efficient AcoD of abattoir and winery solid waste. By so doing, an experimental design was created using JMP software (JMP® Pro® SAS 13.2.1, 64-bit, Institute Inc., USA) which generated 20 runs with 8 corner points, 6 axial (star) points, and 6 center points which were also added to measure the quadratic effects as shown in **Table 3-4**. In addition, the experimental runs were conducted in randomized run-order to mitigate noise interference in the response. The axial points were calculated to be 1.6818 and -1.6818 for the higher and lower design space boundaries, respectively.

Table 3-4: Central Composite Rotatable Design random run-order with chosen factors and their levels

Run	Replicates	X_1	X_2	X_3	Temperature	V_R
1	3	**-**	+	+	38 ºC	400
20	3	+	+	+	38 ºC	900
15	3	0	0	0	38 ºC	400
3	3	**-**	+	**-**	38 ºC	400
8	3	0	0	0	38 ºC	900
17	3	0	0	0	38 ºC	400
11	3	-	-	-	38 ºC	900
18	3	-	-	+	38 ºC	900
16	3	**-α**	0	0	38 ºC	400
2	3	+	-	-	38 ºC	400
9	3	0	0	0	38 ºC	900
5	3	0	0	0	38 ºC	400
12	3	+	-	+	38 ºC	400
10	3	0	0	**-α**	38 ºC	900
7	3	**α**	0	0	38 ºC	900
13	3	0	0	0	38 ºC	400
4	3	0	**-α**	0	38 ºC	400
19	3	0	0	**α**	38 ºC	900
14	3	0	**α**	0	38 ºC	400
6	3	+	+	**-**	38 ºC	400
Control 1	3	*	*	*	38 ºC	900
Control 2	3	*	*	*	38 ºC	900

3.5.2.3 Biomethane potential assay

All BMP assays were conducted with standard methods and protocols observed (see **section 3.3.1**) for a detailed description of the method.

3.6 Lab-scale AD equipment

The lab-scale digestion instrumentation was designed and set-up into four processing units and/or sections as depicted in **Figure 3-7 and Figure 3-8**.

I. Anaerobic digestion section:

- 5 L (x2) custom designed (Glass Chem; Stellenbosch; SA) acrylic round-shaped 5000 mL bottom digester(s) with 5 openings on the detachable lid fitted with an automated stirrer, in-line Hanna instruments pH-probe (HI1280 with 0 -13 pH; and 0-80 ºC) and temperature-probe;

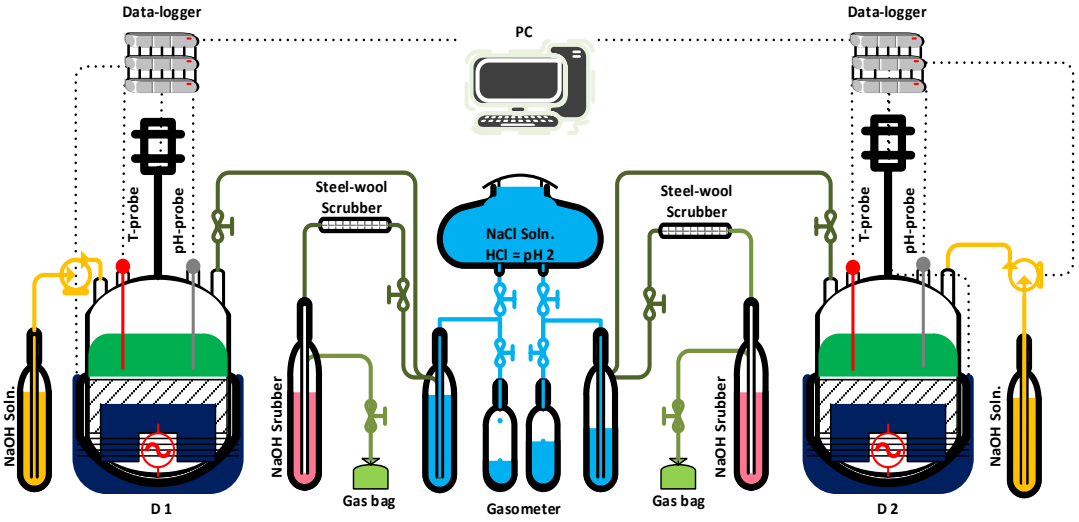


Figure 3-7: Schematic represetation of the 5L lab scale **AD** set up

Figure 3-8: Photographic image of the 5L laboratory-scale experimental **AD** set-up

- An automated pH-control unit consisting of a small peristaltic pump, and a 1000 mL reusable plastic container filled with 1N NaOH solution; and
- An automated heating-mantle (Glass-Chem Laboratory Equipment Pvt. Ltd.) to maintain AD temperature constant.

II. Gas scrubbing section:

- 1000 mL caustic scrubbers (**see section 3.3.1.)**

III. Gasometer section:

- 1000 mL gasometer (**see section 3.3.1**.)

IV. Automated control-loop and data logging section:

A computer with a custom installed Glass Chem Data logging (HID terminal user interface) software, was connected to monitor and record process temperature, pH and stirrer-speed output.

3.6.1 Biogas composition analysis equipment

A portable biogas analyzer BIOGAS 5000 (Geotech, UK) (see **Figure 3-9**) was used to analyze the contents of the produced gas.

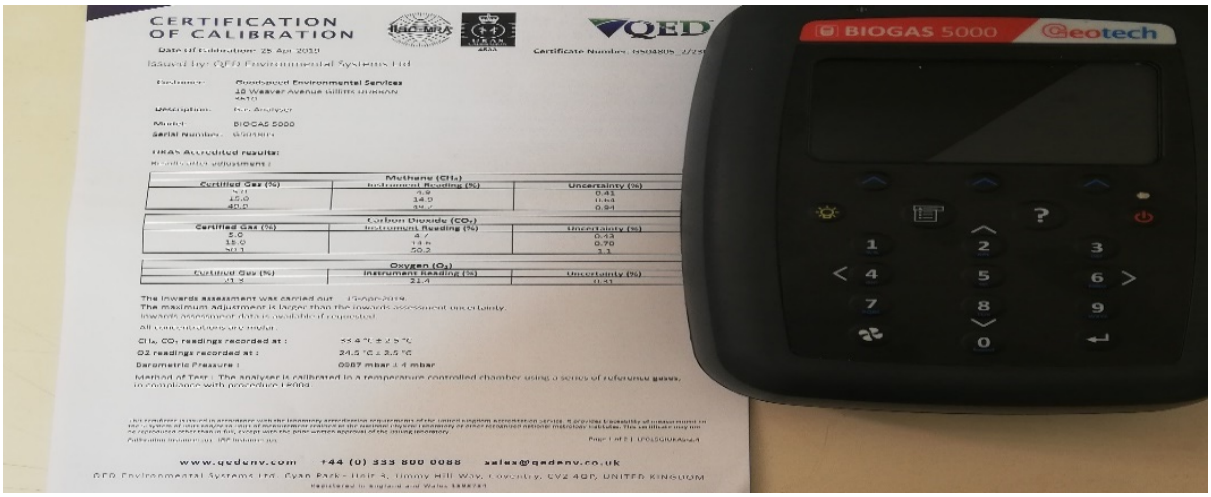


Figure 3-9: Portable biogas analyzer (BIOGAS 5000)

The BIOGAS 5000 is a simple, easy to use and calibrate gas analyzer that provides accurate online gas analysis with standard gas cells for methane, carbon dioxide and oxygen detection with a range of 0 – 100%. Two optional gas cells were also fitted for hydrogen (H_2) and hydrogen sulphide (H_2S) detectable within the range of 0 – 1000 ppm.

3.7 Laboratory scale anaerobic digestion experiments

The laboratory scale AcoD experiments were conducted after the conclusion of each major BMP study, to further evaluate the performance of optimally determined mixture compositions at set environmental conditions.

3.7.1 Substrates

Five mixture compositions namely, $R_{3\text{-}1}$, $R_{3\text{-}2}$, M_4, M_7, and M_9 determined from BMP and optimization studies as potential AcoD mixture blends, were used as substrate in various laboratory scale studies. The mix ratio (i.e. $A_sC_bW_s$) used for $R_{3\text{-}1}$, $R_{3\text{-}2}$, M_4, M_7, and M_9 was

(0:60:40), (40:60:0), (50:0:50), (34:33:33) and (17:17:66), respectively determined on a VS-basis.

3.7.2 Experimental procedure

The lab-scale AcoD experiments were conducted utilizing three modes of operation (i.e. Digester configuration), namely batch, single-stage semi-continuous, and two-stage semi-continuous configurations.

3.7.2.1 Batch mode anaerobic co-digestion experiments

The first set of experiments were conducted in batch with organic load of 5 gVS L^{-1} for $R_{3\text{-}1}$, $R_{3\text{-}2}$, which ran simultaneously in digesters one and two (i.e. D_1 and D_2) for 15 days. In the second set of batch experiments, an organic load of 1.5 gVS L^{-1} was applied for M_4 and M_7 running concurrently in D_1 and D_2, respectively. The third and last set of batch lab-scale experiments were performed on M_9 mixture blends with organic loading concentrations of 1.5 gVS L^{-1} for a period of 15 days. All batch experiments were conducted with conditions automatically set at mesophilic (38 ±5 ºC), pH of 7 ±0.2 and mechanically stirred at 300 rpm (**see section 3.6** for more details).

3.7.2.2 Specific methanogenic activity tests

To assess D_1 and D_2 performance following the first set of batch experiments and prior to switching to semi-continuous mode, specific methanogenic activity (SMA) tests were conducted using acetic acid solution with conditions. For the first SMA assays an organic loading concentration of 1 gVS L^{-1} was applied to both D_1 and D_2

3.7.2.3 Single-stage semi-continuous anaerobic co-digestion experiments

Semi-continuous AcoD experiments with M_9 mixture blends were conducted in parallel utilizing D_1 and D_2 configured for single-stage operation. Both digesters were previously running in batch mode. Thus, to facilitate the switch to semi-continuous operation, firstly D_1 and D_2 were operated as fed-batch digesters with HRT and OLR of 19 days and 0.64 g VS L^{-1} day^{-1}, respectively, and secondly the working volume (V_R) was increases from 4.5 to 4.75 liters. The systems were permitted to stabilize for two days, then 0.3 liters of digestate was removed while 0.05 liters of feed was being added, which consequently resulted in the start of single stage semi-continuous operation with the V_R maintained at 4.5 liters. The OLR was slowly increased from 0.28 to 0.35 and finally to 0.55 gVS L^{-1} day^{-1} with HRT of 450; 360 and 450 days, respectively.

3.7.2.4 Two-stage semi-continuous anaerobic co-digestion experiments

For the two-stage semi-continuous configuration, the digesters (D_1 and D_2) were operated in series, with output digestate from 'hydrolysis/acidification-stage' (D_1) used as feed to the 'methanogenic-stage' (D_2). The pH in D_1 ranged from 5.50 to 6.50 (±0.05) which was regulated naturally using OLR and HRT, and in D_2 the automatic control unit deployed kept pH within the 6.70-7.00 (±0.01) range.

3.8 Data analysis

3.8.1 Biomethane yield evaluation

3.8.1.1 Total solids, volatile solids and fixed carbon determination

The various samples studied were evaluated for Ts, FS and FC using equations (3-3) to (3-5).

$$\mathrm{TS}(\%) = \frac{\mathrm{A}-\mathrm{B}}{\mathrm{C}-\mathrm{B}} * 100 \quad (3\text{-}3)$$

$$\mathrm{VS}(\%) = \frac{\mathrm{D}-\mathrm{B}}{\mathrm{A}-\mathrm{B}} * 100 \quad (3\text{-}4)$$

$$\mathrm{FC} = {}^{VS(\%)}/_{1.8} \quad (3\text{-}5)$$

Where A is the weight of dried sample plus crucible; B weight of crucible; C weight of wet sample and crucible; and D is the weight of ash plus crucible in grams.

3.8.1.2 Theoretical methane potential, oxygen demand and C/N ratio

The theoretical methane potential (TMP), and theoretical oxygen demand (TOD) based on the Buswell-Boyle equation (2-3), was determined stoichiometrically using equation (3-6) and (3-7), respectively and the and C/N ratio for each co-substrate mixture on a VS basis was calculated with equation (3-8).

$$\mathrm{TMP}\left[\mathrm{NmL}\frac{\mathrm{CH_4}}{\mathrm{g\,VS}}\right] = 22.4 * \left[\frac{\left(\frac{a}{2}+\frac{b}{8}-\frac{c}{4}-\frac{3d}{8}-\frac{e}{4}\right)}{12a+b+16c+14d+32e}\right] * 1000 \quad (3\text{-}6)$$

As Korsak and Moreno (2006) highlights, the estimation of TMP and the organic matter available for AD transformation based on VS has one fault as it includes microorganisms, and FC (e.g. exopolymers, and organic matter absorbed in locks or biofilms.

$$\mathrm{TOD}\left[\mathrm{mg}\frac{\mathrm{O_2}}{\mathrm{g\,VS}}\right] = 22.4 * \left[\frac{\left(2a+\frac{b}{2}-c-\frac{3d}{2}\right)}{12a+b+16c+14d}\right] * 100 \quad (3\text{-}7)$$

$$\mathrm{C}/_{\mathrm{N}} = \frac{W_1C_1W_1+W_2C_2+W_3C_3}{W_1N_1W_2+N_2W_1+W_3N_3} \quad (3\text{-}8)$$

Where W_i is the volatile solids weight of the corresponding substrate in the mixture, C_i is the carbon content in g kg^{-1} VS, and N_i is the nitrogen content in gkg^{-1} VS.

3.8.1.3 Pretreatment specific energy demand

The specific energy demand was calculated according to Pecorini *et al.*, (2016), considering the power requirements for microwave/pressure-cooker pre-treatment, the exposure time applied for each treatment, and mass of treated mash (kg VS). the SPED (kJ kg^{-1} VS) was calculated according to equation (3-9).

$$\mathrm{SPED} = \frac{P*t}{m} \quad (3\text{-}9)$$

Where SPED is short for "specific energy demand"; P is the power output of the microwave/pressure cooker (kW); t is the treatment exposure time (s); and m is the mass of treated mash (kg VS).

3.8.1.4 Biogas production measurement and correction

The biogas produced is assumed to be saturated in water vapor which can lead to overestimation of biogas yield and, subsequently, to poor estimation of substrate BMP (Strömberg, Nistor and Liu, 2014). Therefore, to provide an accurate estimate of biogas produced and mitigate associated errors, water vapor pressure at ambient temperature and pressure conditions was accounted for in all gas measurements according to equation (3-10), which is obtained using the modified Arden-Buck (1961) equation (3-11).

$$P_{dry} = P_{wet} - P_{vapor} \quad (3\text{-}10)$$

$$P_{vapor} = 6.1121\mathrm{EXP}\left|\left(18.678 - \frac{T_c}{234.5}\right)\left(\frac{T_c}{257.14+T_c}\right)\right| \quad (3\text{-}11)$$

where T_c is the temperature of the gas at measurement (°C), and P_{vapor} is the water vapor pressure measured in horse-power (where 1hp = 0.1 KPa).

3.8.2 Gas composition analysis

A sample of the gas produced was analyzed for CH_4 and CO_2 once or twice a week using the portable Biogas 5000 (Geo. Tech, UK) analyzer. The methane content was then corrected using equation (3-12) as proposed in the German standard procedure VDI 4630 (Kafle and Kim, 2012).

$$\mathrm{CH_{4\,corrected}} = \left|\frac{\mathrm{CH_4(\%)}}{\mathrm{CH_4(\%)+CO_2(\%)}}\right| \cdot 100 \tag{3-12}$$

3.8.3 Net methane yield calculation

The produced gas was corrected by subtracting CH4 produced by the blank assay from the active assay at the same time of sampling using equation (3-13) and reported as net CH4 volume produced.

$$\mathrm{Net\ V_{CH_4\,Production}(mL)} = \mathrm{V_{CH_4\,Sample}} - \mathrm{V_{CH_4\,Blank}} \tag{3-13}$$

Assuming ideal gas behavior the combined gas law equation (3-14) was applied to standardize and report produced gas volumes (@ STP, IUPAC).

$$\mathrm{V_{STP}(NmL)} = \mathrm{V} * \left[\frac{\mathrm{T_S}}{\mathrm{T}} * \frac{\mathrm{P_{dry-biogas}}}{\mathrm{P_S}}\right] \tag{3-14}$$

The BMP of each sample was determined by normalizing the generated CH_4 volume against the initial VS added to obtain the SMP (P_0) using equation (3-15).

$$\mathrm{SMP}\left[\frac{\mathrm{NmL}}{\mathrm{gVS}}\right] = \frac{\mathrm{Net\ V_{STP}(NmL)}}{\mathrm{g\ VS_{added}}} \tag{3-15}$$

3.8.4 Biodegradability efficiency

The biodegradability efficiency or extent of degradation (f_d) is defined as the ratio of the experimentally determined specific methane potential (SMP_{time}) to the theoretically methane potential (TMP).

$$f_d = \frac{\mathrm{SMP_{time}}}{\mathrm{TMP}} \tag{3-16}$$

3.8.5 Inhibition or toxic effects

The inhibitory/toxic effects were evaluated using the following equation

$$I = \left|1 - \frac{\mathrm{CH_{4\ Experiment}}}{\mathrm{CH_{4\ Control}}}\right| * 100\ \% \quad (3\text{-}17)$$

3.8.6 Co-digestion performance index

As previously discovered in **Chapter 2**, AcoD of substrates can potentially improve or reduce specific methane yield due to either the synergistic and/or antagonistic effects. Therefore, a co-digestion performance index (CPI) was determined following the steps outlined by Ebner *et al.* (2016), which compare the biomethane potential of the co-digestion experiments to the weighted sum of the mono-D biomethane potentials as an indicator of synergistic or antagonistic interactions. The CPI was calculated as the ratio of the biomethane potential from the co-digestion experiments ($BMP_{AcoD\ i,\ n}$) to the weighted average ($BMP_{mono\ i,\ n}$) considering the VS content (%VS) of each substrate's biomethane potential ($BMP_{mono\ i,\ n}$) using equation (3-18).

$$\mathrm{CPI} = \frac{\mathrm{BMP_{Experiment}}}{\mathrm{BMP_{Theoretical}}} = \frac{\mathrm{BMP_{AcoD,}}_i}{\sum_i^n \%VS * \mathrm{BMP_{mono,}}_i} \quad (3\text{-}18)$$

where *i* is substrate component; and n is the number of replicates; and %VS is the percentage volatile solids of each substrate contained by the mixture and a sum of 100. Thus, a CPI greater or less than one is an indicator of synergistic or antagonistic interactions, respectively. If the CPI is equal to one the substrate work independently from the mixture.

3.8.7 Kinetic parameter(s) and evaluation model selection

The production of methane was simulated through non-linear regression techniques with SPSS software (IBM® SPSS® Statistics 25, IBM Corp., USA), by fitting experimental data using the first-order, modified Gompertz, Verhulst (logistic), and Richards models, equation (3-19); (3-20) ;(3-21); and (3-22), respectively, and "Bio-energetic" parameters were evaluated in all AD experiments.

$$\mathrm{P(t)} = \mathrm{P_0}\left(1 - \mathrm{e^{-kt}}\right) \quad (3\text{-}19)$$

$$\mathrm{P(t)} = \mathrm{P_0 EXP}\left|-\mathrm{EXP}\left|\frac{\mathrm{R_{max} * e}}{\mathrm{P_0}}(\lambda - \mathrm{t}) + 1\right|\right| \quad (3\text{-}20)$$

$$\mathrm{P(t)} = \frac{\mathrm{P_0}}{1 + \mathrm{EXP}\left|4\frac{\mathrm{R_{max}}}{\mathrm{P_0}}(\lambda - \mathrm{t}) + 2\right|} \quad (3\text{-}21)$$

$$\mathrm{P(t)} = \mathrm{P_0}\left[1 + \mathrm{vEXP}|1 + \mathrm{v}| * \mathrm{EXP}\left|\frac{\mathrm{R_{max}}}{\mathrm{P_0}}(1 + \mathrm{v})\left(1 + \frac{1}{\mathrm{v}}\right)(\lambda - \mathrm{t})\right]\right|^{(-1/\mathrm{v})} \quad (3\text{-}22)$$

The Schnute model equation (3-23) with only mathematical relevant symbols representing the parameters found in all the models was also used as an additional model selection criterion as suggested by Zwietering *et al.* (1990) and Ware and Power (2017). The criteria for model selection are presented in **Table 3-5**.

$$P(t) = \left[P_0 * \frac{1-b}{a}\right]\left[\frac{1-b*\mathbf{EXP}|a\lambda+1-b-at|}{1-b}\right]^{\frac{1}{b}} \quad (3\text{-}23)$$

Where P_t (mL CH_4 g^{-1} VS_{added}) is the cumulative CH_4 yield recorded at time t (day); P_0 (mL CH_4 $g^{-1}VS_{added}$) is the potential CH_4 yield; R_{max} (mL CH_4 $g^{-1}VS_{added}day^{-1}$) is the maximum CH_4 production rate; λ indicates lag phase (day) duration; e is the natural exponent (exp (1) = 2.7183); and k (day^{-1}) is the first order rate**.**

Table 3-5: Model selection criteria (Zwietering et al., 1990; Ware and Power, 2017)

Model	Parameters	Selection criterion
Gompertz	3	a > 0; b = 0
Richards	4	a > 0; b < 0
Logistic	3	a > 0; b = -1
Linear	2	a = 0; b = 1
Quadratic	2	a = 0; b = 0.5
Power law	2	a = 0; b = 0
Exponential	3	a < 0; b = 0

The statistical determined regression parameters from the Schnute model and four-parameter and three-parameter kinetic model(s) considered were also used to determine if the use of a fourth parameter is economical using equation (3-24).

$$f = \frac{(RSS_2 - RSS_1)/(df_1 - df_1)}{RSS_1/df_1} \text{ tested abgainst } F_{DF_1}^{DF_2 - DF_1} \quad (3\text{-}24)$$

Where RSS_1 (Schnute model); RSS_2 (three-parameter model); df_2 (Schnute model); and df_1 (three-parameter model),

3.8.8 Statistical Evaluation

3.8.8.1 One-way analysis of variance

Average values for all measurements were statistically evaluated using one-way Analysis of Variance (ANOVA). The calculated F value and tabulated F value were then compared to assess whether differences between two values is significant or not. Thus, if the F-ratio is

greater than the F-critical or the calculated p-value is greater than the level of significance (α), there is at least a significant difference between two means. In this case, the least significant difference (LSD) was calculated at α=0.05 using equation (3-25).

$$LSD_{\alpha} = t_{\alpha} * SQR\left[2\frac{MS}{n}\right] \quad (3\text{-}25)$$

Where t_{α} is the tabulated value identified from the degree of freedom for error (d*f*) and α, S^2 is the mean square for error (MS); and n is the number of replications on which the means were computed. MS and d*f* were also calculated using the one-way ANOVA in SPSS software (IBM® SPSS® Statistics 25, IBM Corp., USA).

3.8.8.2 Goodness of fit

The production of methane was simulated by fitting experimental data into four kinetic models through the nonlinear regression curve fitting package in SPSS software (IBM® SPSS® 25, IBM Corp., USA) to solve for the "bio-energic" parameters, which were further evaluated for their mathematical soundness and biological appreciation.

The goodness of the fit was determined by considering both the Residual Sum of Squares (RSS) and the coefficient of determination R-Square (R^2). Furthermore, a fifth model that only considers mathematical symbols was used to compare and select a model that would closely simulate the production of methane from the three substrates.

CHAPTER 4

4 Biomethane Potential from Co-Digestion of Environmentally Recalcitrant Abattoir and Winery Waste

4.1 Abstract

Biomethane potential (**BMP**) assays are efficient tools for estimating recoverable methane from organic matter through anaerobic digestion (**AD**) and evaluating economic feasibility and/or improving efficiency of existing plants. This study focused on **BMP** from environmentally recalcitrant abattoir solids ($\mathbf{A_s}$), cow-blood ($\mathbf{C_b}$) and winery solid ($\mathbf{W_s}$) wastes, to evaluate the effects of various input parameters on biodegradability and overall **AD** performance. For this purpose, a series of **BMP** assays were conducted following standard protocols at mesophilic conditions (38±5 ^{o}C) for $\mathbf{A_s}$, $\mathbf{C_b}$, $\mathbf{W_s}$ individually; and then in combination of $\mathbf{A_sC_bW_s}$, $\mathbf{A_sW_s}$ and $\mathbf{C_bW_s}$. The parameters evaluated were substrate-ratio (1:1:2, 1:1 and 2:3), food-to-microorganism ratio (F/M) (0.5-2) and volatile solids (VS) concentration (5-20 gVS/L). The inoculum used as active source of anaerobic microorganisms was synthesized from zebra dung and ruminal content. The production of methane was also simulated by curve-fitting experimental data using first order, modified Gompertz, logistic and Richards kinetic models via least square nonlinear regression analysis techniques of experimental data. The highest methane potentials of 369.56, 354.13, 192.28, and 110.24 NmL CH_4/gVS_{added} were obtained for $\mathbf{A_sW_s}$, $\mathbf{C_bW_s}$, $\mathbf{A_s}$ and $\mathbf{C_b}$ respectively. All models sufficiently simulated methane production profiles with over 90% accuracy, with an exception to results in inhibited systems. Furthermore, an increase in F/M and VS concentration negatively impacted the overall digestion performance. Abattoir and winery waste can be successfully co-digested to improve biomethanation, by optimizing **AD** process input parameters. Thus, further research including mixture design, parameter optimization and pilot scale experiments are required, to better understand performance on full scale **AD** plants.

Keywords: Biomethane potential, anaerobic co-digestion, abattoir, winery solid waste

4.2 Introduction

Over the last decades, there has been an increase in emission of green-house gases (GHG) resulting in global warming and consequent climate change. This change has great negative impact on the environment which has been traced to the world's rapid urbanization, increased energy-demand, organic waste pollution and increased dependence on fossil fuels. Anaerobic digestion (AD) is an alternative solution simultaneously addressing organic waste management issues and providing clean renewable energy in the form of biogas as a by-product. AD is the sequential conversion of large and complex organic molecules to methane (CH_4) and carbon dioxide (CO_2) in the absence of oxygen. AD is carried-out by a consortium of bacterial and archaeal populations working symbiotically in a four-step process (i.e. hydrolysis, acidogenesis, acetogenesis, and methanogenesis) (Gerardi, 2003; Henze *et al.*, 2015; Eleutheria *et al.*, 2016). When substrates are simultaneously subjected to AD the process is known as anaerobic co-digestion (AcoD) (Mata-Alvarez *et al.*, 2014). There is wide variety of materials used in AcoD specifically for biogas production such as abattoir waste, wastewater, municipal waste and animal manures (Laks, 2017).

Abattoir wastes have particularly gained wider attention owing to increased legal restrictions on disposal, treatment costs and environmental consciousness. However, treatment of abattoir waste residues comes with its own challenges, as this type of waste is rich in fat and protein content (Salminen and Rintala, 2002; Battimelli *et al.*, 2010; Palatsi *et al.*, 2011; Valta *et al.*, 2015; Affes *et al.*, 2017) which is a plus on the theoretical aspect for CH_4 gas generation but still with associated operational issues (e.g. scum formation, concentration of long chain volatile fatty acids and ammonia, etc.) thereby reducing system performance (Luste and Luostarinen, 2010; Manuel and Canas, 2010; Ortner *et al.*, 2015; Labatut, 2017). Hence, pre-treatment including AcoD with other substrates is a viable option, and has demonstrated to have synergistic effects on biogas yield and quality by balancing C/N ratio, energy and nutritional requirements for sustainable growth of microorganisms involved in AD (Mata-Alvarez *et al.*, 2014; Divya, Gopinath and Merlin Christy, 2015; Hagos *et al.*, 2017).

Winery solid waste (Ws) is an attractable carbonaceous co-substrate that improves biomethane production efficiency when co-digested with abattoir residues, as they contain relevant nutrients in sufficiently large concentration (Da Ros *et al.*, 2014; Okudoh *et al.*, 2017; Zacharof, 2017) and a relatively high C/N ratio resulting in balanced AD. With the lack of information from literature on the use of abattoir and winery solid wastes as feedstock in AcoD for biogas production, further research is required to fill the gap by determining relevant parameters

including optimum mixture proportions, operating conditions (e.g. temperature, pH, organic loading, retention time, etc.), kinetic profiles and mathematical models to be adopted for an efficiently running AD process.

The determination of recoverable CH_4 from a given substrate via AD is a useful economic evaluation tool, considering the energy value of the produced gas as an accounting- and engineering design factor for investments into anaerobic digestion plants. A simple and efficient method used in the evaluation of a feedstock's suitability for AD and production of CH_4 is biomethane potential (BMP) assay (Angelidaki and Sanders, 2004; Hansen *et al.*, 2004; Angelidaki *et al.*, 2009). BMP assays provide crucial parameter analysis including CH_4 yield, biodegradability extent, and reaction kinetic rates (Strömberg, Nistor and Liu, 2014) hence, their vital importance in the design and construction of new and optimized AD plants.

To the best of our knowledge and survey of literature, no previous work has been conducted evaluating the application of abattoir and winery waste in AcoD for biomethane production. Thus, this study aimed at determining the physiochemical characteristics for anaerobic biodegradation of abattoir solid ($\mathbf{A_s}$) and cow-blood ($\mathbf{C_b}$) and winery solid ($\mathbf{W_s}$) wastes and their biomethanation potentials in mono- and co-digestion systems. Further, effects of F/M ratio and organic load (OL) toxic/inhibitory effects on quality and quantity of biogas were evaluated.

4.3 Aim and Objectives

The aim was to determine the biomethane potentials of ($\mathbf{A_s}$, $\mathbf{C_b}$ and $\mathbf{W_s}$) in mono- and co-digestion systems.

The objectives for this phase of the project were:

- To evaluate inhibitory toxic effects of abattoir ($\mathbf{A_s}$ & $\mathbf{C_b}$) and winery solid waste ($\mathbf{W_s}$);
- To determine the anaerobic biodegradability and biomethane potential from the mono- and co-digestion of abattoir (**A** & **B**) and winery solid waste ($\mathbf{W_s}$);
- To evaluate the synergistic or antagonistic effects from co-digestion experiments of abattoir ($\mathbf{A_s}$ & $\mathbf{C_b}$) and winery solid wastes ($\mathbf{W_s}$);
- To evaluate food-to-microorganism (F/M) ratio effects for the substrates; and
- To evaluate the effects of organic loading concentrations.

4.4 Materials and Methods

Standard methods and protocols described in detail in **CHAPTER 3** were followed throughout this study.

4.4.1 Substrate and inoculant

Three different substrates were used in this study, namely abattoir solid ($\mathbf{A_s}$) and liquid (cow blood) ($\mathbf{C_b}$), and winery solid ($\mathbf{W_s}$) waste. A detailed description of the substrate collection, preparation and preservation can be found in **section 3.1**

4.4.2 Experimental set up and procedure

All details about experimental set-up and procedure are provided in **CHAPTER 3 section 3.2** to **section 3.7**

4.5 Data Analysis

All details on data analysis are provided in **CHAPTER 3 section 3.8.1** to **section 3.8.8**

4.6 Results and Discussions

4.6.1 Substrate physical and chemical characteristics

The physical and chemical characteristics of the three substrates and inoculum are presented in **Table 4-1**. All substrates had an appreciably high VS/TS ratio found to be above 90 % considering the volatile matter content of dried solids desirable for AD. For both $\mathbf{A_s}$ & $\mathbf{C_b}$ pH was found to be slightly alkaline while $\mathbf{W_s}$ was in the acidic region. The C/N ratio for $\mathbf{W_s}$ was within the recommended range (20-30) required for stable anaerobic digestion (Gerardi, 2003), while the one for $\mathbf{A_s}$ was slightly below the recommended range. Substrate $\mathbf{C_b}$ had the poorest C/N ratio similar to that obtained for sheep, pig and chicken waste blood (Zhang and Banks, 2012; Ortner *et al.*, 2014; Y. M. Yoon *et al.*, 2014). Substrate $\mathbf{A_s}$ had the highest TMP (816 NmL/gVS) compared to TMP of both to $\mathbf{C_b}$ and $\mathbf{W_s}$ which were similarly lower (483 and 444 NmL/gVS, respectively).

Table 4-1: Substrates and inoculum physiochemical characteristics

	A_s	W_s	C_b	Inoculum
TS (%) (wet-basis)	32.13	87.93	15.01	0.73
VS (%) (wet-basis)	30.19	80.05	14.95	0.728
VS/TS (wet-basis)	0.940	0.910	0.996	0.997
pH (1:5 dilution for A_s and W_s)	7.68	4.53	7.8	6.78
Ca (mg/kg)	640.5 ± 107.5	-	180.5 ± 4.5	98
Cu (mg/kg)	20.62 ± 0.003	-	3.39 ± 0.25	0.44
Co (mg/kg)	0.0375 ± 0.0055	-	-	-
Fe (mg/kg)	136.56 ± 25.38	-	2478.17 ± 78.94	24.26
K (mg/kg)	1912.5 ± 384.5	-	1660.5 ± 99.5	125
Mg (mg/kg)	155.5 ± 24.5	-	77.5 ± 3.5	20
Na (mg/kg)	2758 ± 550	1191.90 [a]	8539 ± 48	768
Ni (mg/kg)	0.1785 ± 0.0215	-	0.063 ± 0.008	0.029
Zn (mg/kg)	36.08 ± 6.75	-	15.733 ± 0.02	2.36
P (mg/kg)	10.65 ± 0.05	0.16 (%) [a]	0.43	
C (%)	65.60 ± 0.3	50.40 [a]	48.8 ± 1.1	
N (%)	4.15 ± 0.45	1.76 [a]	13.93± 0.23	
H (%)	6.03	8.96 [a]	7.35 ± 0.15	
S (%)	0.35 ± 0.05	0	0.6	
C/N Ratio	18.41	28.63 [a]	4.05	
Empirical Formula	$C_{474}H_{920}O_{104}N_{26}S$	$C_{28}H_{47}O_{21}N$	$C_{222}H_{423}O_{99}N_{55}S$	
TOD (mg O2/g VS)	2.33	1.27	1.38	
TMP (STP mL/g VS)	816.27	443.89	482.84	

[a] Results determined from previous analysis study of grape pomace obtained from **ARC**; **TMP** is the Theoretical methane potential. **TOD** is the Theoretical oxygen demand; **TOD** Total organic carbon; **C/N** is Carbon-Nitrogen Ratio

Thus, co-digestion of these three substrates is expected to be efficient when compared to mono-AD. Apart from the VS content and C/N ratio the macro- and micronutrient concentration of the substrate play critical roles in the biotic environment and are required at balanced concentrations. Thus, Induced Coupled Plasma (ICP) spectroscopy analysis of the three substrates was conducted considering that various trace (e.g. iron, nickel, cobalt, copper, zinc, etc.) elements are required for growth and extracellular activity, where optimal concentrations are determined by enzymes and proteins required for a particular task (Ünal *et al.*, 2012). A detailed result with all determined physiochemical characteristics can be found in **Table C-1**.

4.6.2 Natural biodegradability and toxicity assay

The natural biodegradability and toxicity results are summarized in **Table 4-2** while **Figure 4-1** presents the **AD**-profiles expressed as cumulative methane yield (NmL) over time (day). The highest methane production was recorded for $\mathbf{C_b}$ assay (236.90 NmL), followed by the pre-treated combination ($\mathbf{A_sC_bW_s}$)* (112.60 NmL).

Table 4-2: BMP results from anaerobic mono- and co-digestion of abattoir and winery solid waste natural without induced anaerobic conditions

Substrate	Replicates	V_R (L)	C/N	F/M	Incubation (day)	Net CH_4 (mL)	t_{80} (day)	SMP	f_d (%)
A_s	3	0.45	18.41	1:1	34	21.48	27	2.38	0.43
C_b	3	0.45	4.05	1:1	34	236.90	24	26.26	4.81
W_s	3	0.45	28.36	1:1	34	10.40	28	1.15	0.21
$(A_sC_bW_s)$*	3	0.45	19.74	1:1	34	112.60	23	12.54	2.29
$A_sC_bW_s$	3	0.45	19.76	1:1	34	7.89	33	0.87	0.16
Inoculum	3	0.45	-	1:1	34	37.21	23	-	-

C/N, Carbon/Nitrogen ratio; F/M, Food-to-microorganism ratio; SMP, Specific methane potential; V_R, reactor working volume; Net CH_4, net methane volume produced; t80, time taken to produce 80% methane volume; f_d, extent of biodegradation

Mono-AD of $\mathbf{C_b}$ produced biomethane from Day 1 and proceeded exponentially towards Day 15 and then remained constant from Day 21 after which a second exponential phase was noted towards the last day. A similar trend was noted for ($\mathbf{A_sC_bW_s}$)*. However, the amount of biomethane was doubled for the mono-AD of $\mathbf{C_b}$ at each measurement interval. The above results are illustrated in **Figure 4-1 a-c.**

The absence of lag-phase in these assays was attributed to high concentration of proteins supplied by the $\mathbf{C_b}$ substrate; and the addition of microwave pre-treated $\mathbf{A_s}$ with highly soluble fats/lipids content and $\mathbf{W_s}$ resulted in preferential biodegradation during co-digestion which resulted as well in a more stable AD transformation. The combined substrates ($\mathbf{A_sC_bW_s}$) CH_4-production began after 2-weeks with significant inhibition noted from Day 15 to Day 21. This was determined to be around 43 % of the total biogas produced and lasted until Day 28 as the system started to recover with CH_4-production inhibition reduced to only 0.06 % from the 31st day till the last day of experiments.

The mono-AD of $\mathbf{A_s}$ and $\mathbf{W_s}$ was significantly inhibited from Day 1 to Day 20 and only showed signs of recovery from Day 21 until Day 28. This inhibition was more pronounced on $\mathbf{A_s}$ assays where inhibition was around 72%, which was associated with the production of LCFAs which

are known to result in reduction of methanogenic activity (Cavaleiro, Pereira and Alves, 2008; Battimelli *et al.*, 2010; Affes *et al.*, 2013). The long delay/lag-phase is consistent with the observations by Palatsi *et al.* (2011) on abattoir wastes in sequential batch test with a third pulse at 15 g COD L^{-1}. For $\mathbf{W_s}$ the inhibition was most likely due to poor biogas quality (i.e. CO_2 concentrated) considering the high activity observed in the gas scrubbers of these systems, as they had to frequently be replenished due to acidification.

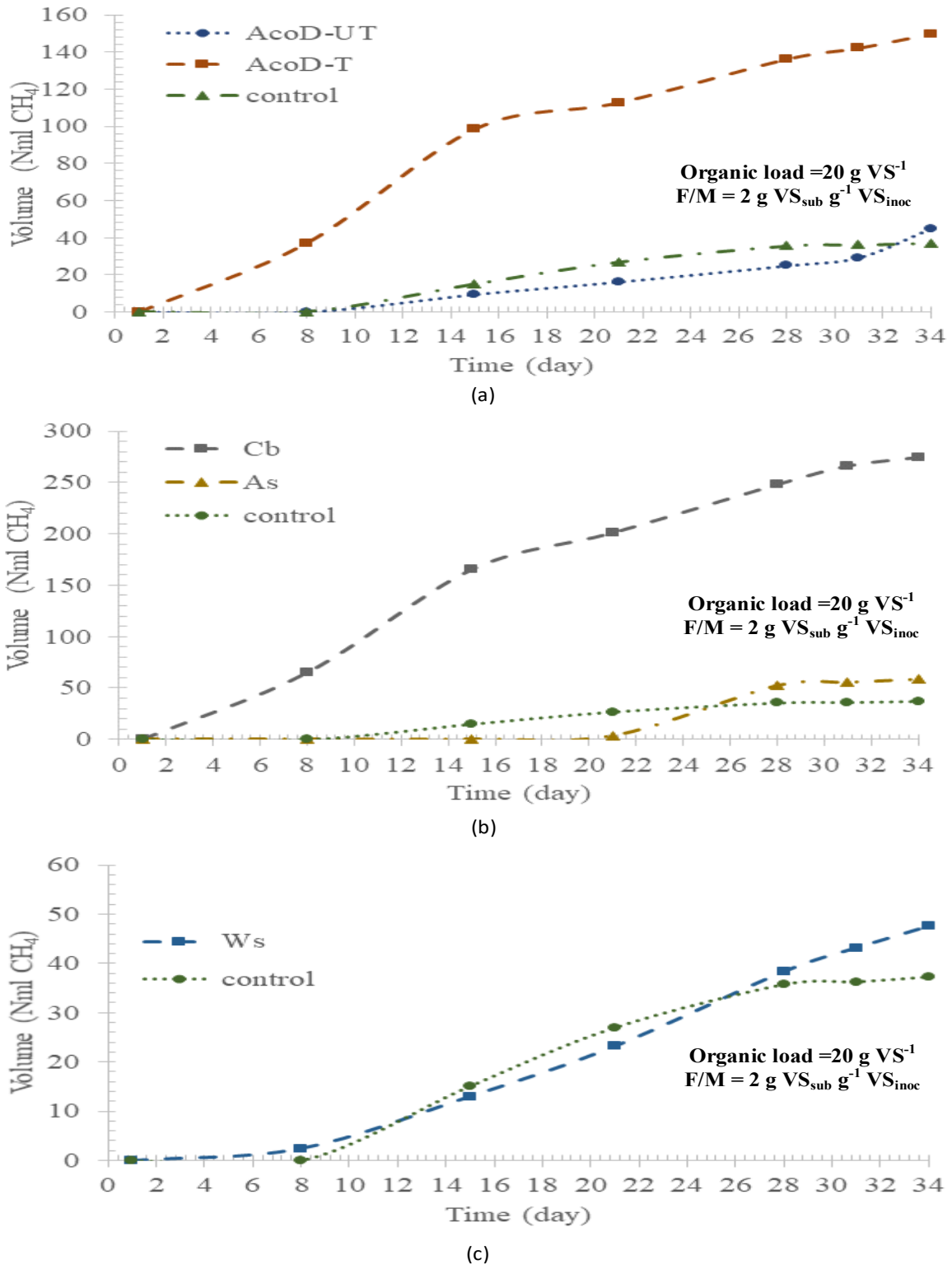


Figure 4-1: Cumulative methane yield profiles for (a) AcoD of microwave pre-treated combined substrates ($\mathbf{A_sC_bW_s}$)*and untreated ($\mathbf{A_sC_bW_s}$), (b) mono-AD of microwave pre-treated $\mathbf{A_s}$ and $\mathbf{C_b}$; and (c) mono-D of $\mathbf{W_s}$.

4.6.3 Biomethane potential from mono-AD of abattoir waste

Table 4-3 summarizes the results from mono-AD of abattoir wastes and the AD-profiles are presented in **Figure 4-2**. The **C_b** produced more biomethane compared to **A_s**, where an average of 1908.72 ±8.5 NmL CH_4 was obtained for the former and 1095.72 ±5.92 NmL CH_4 for the latter. The biodegradability (f_d) for both **C_b** and **A_s** was determined to be 39.8 % and 13.5 %, respectively.

Table 4-3: BMP results from the anaerobic mono-D of **A_s** and **C_b**

Substrate	C/N	V_R (L)	F/M	TMP (NmL g^{-1}VS)	Net V CH_4	SMP (NmL $g^{-1}VS_{added}$)	t_{80} (day)	f_d (%)
A_s	18.41	0.9	1/1	816.27	1095.72 ±5.92	110.24 ±0.60	20	13.5
C_b	4.05	0.9	1/1	482.84	1908.72 ±8.5	192.28 ±0.87	15	39.8
Inoculum		0.9	-	-	39.62 ±0.80	-	18	-

SMP: Specific methane potential; TMP: Theoretical methane potential; C/N: Carbon to Nitrogen ratio; F/M: Food to microorganism ratio; V_R: Reactor working volume; Net V CH_4: Net Volume methane produced; t80: time taken to produce 80% methane; and f_d: biodegradability

Methane production was immediate for **C_b** and proceeded exponentially until Day 15 and continued slowly in the upward trajectory towards the last day of experiments. At this point, the biodegradability (f_d) was appreciably high at 39.8%. For A_s, the production of CH_4 was delayed and only recorded a significant continuous increase from Day 15 to Day 21, resulting in the poorest f_d record of 13.5 % which may be due to a slow hydrolysable fat content.

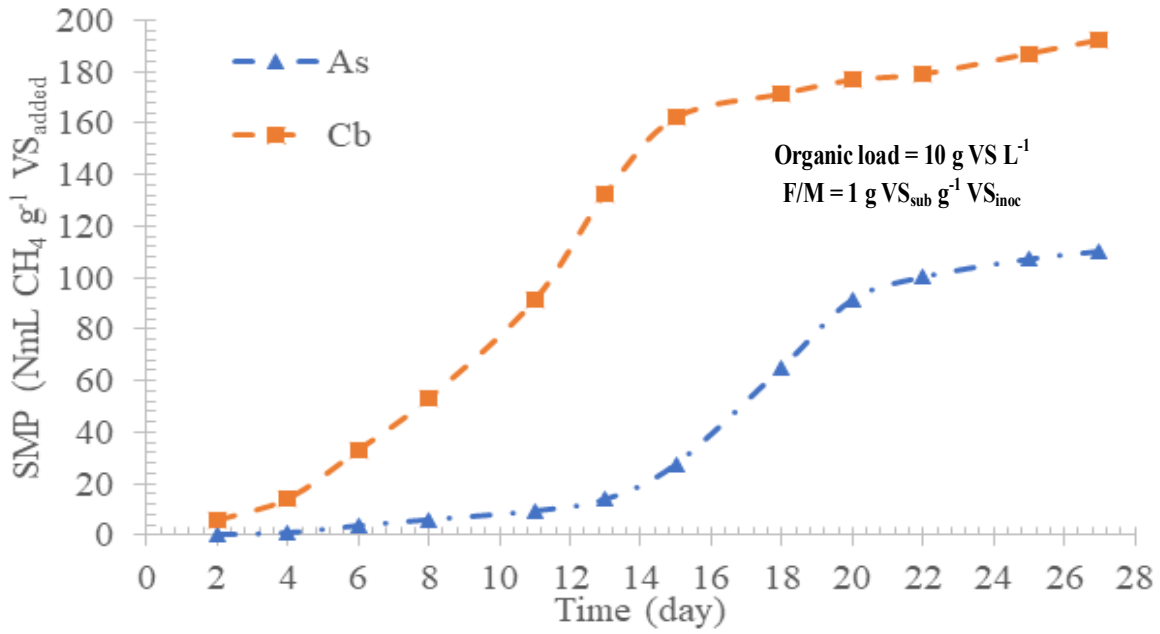


Figure 4-2: Cumulative methane production profiles for mesophilic mono-D of **A_s** and **C_b** at OLR and F/M of 10 g VS L-1, and 1 g VSsub g-1VSinoc, respectively.

4.6.4 Specific methanogenic activity (SMA)

The SMA assay results and experimental conditions are summarized in **Table 4-4**, where SMA for the aceticlastic archaea was determined using acetic acid as sole substrate. An average SMA of 2.6 g COD_{CH4} g^{-1}VSS d^{-1} was obtained for both tests comparable to the results obtained by Johanna *et al.* (2009), where a SMA of 2.39 g COD_{CH4} g^{-1}VSS d^{-1} on acetate was reported for anaerobic sludge treating urban solid waste.

Table 4-4: Specific Methanogenic Activity (**SMA**) assay results

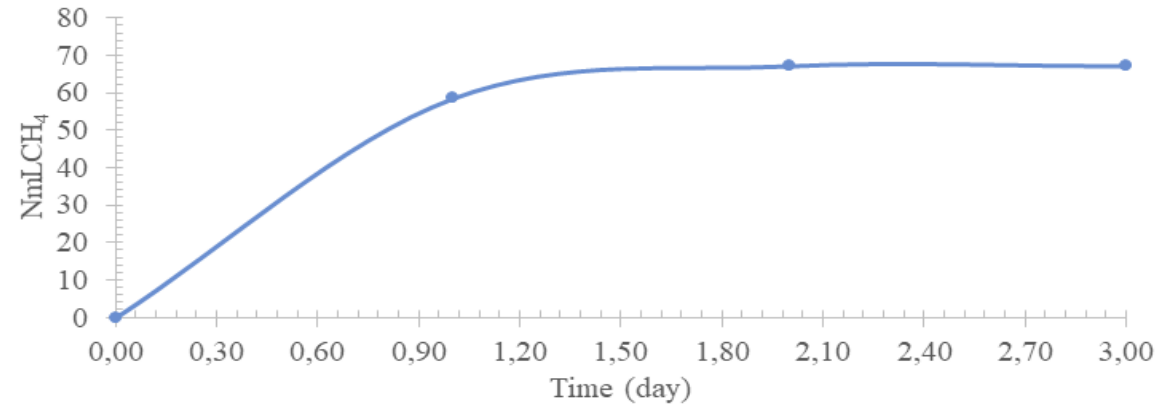
Assay	$V_{inoc.}$ (mL)	Mass (g)	X_0 ($g^{-1}L$)	V_R (mL)	T (° C)	pH	Time (day)	Net V (mL CH_4)	SMA ($gCOD_{CH4}$ g^{-1}VSS d^{-1})
SMA_1	200	3.24	8.1	0.4	38	7	3	67.2446	2.6635
Control	200	3.24	8.1	0.4	38	7	3	-	nr
SMA_2	450	7.29	8.1	0.9	38	7	3	149.5270	2.6324
Control	450	7.29	8.1	0.9	38	7	3		nr

X_0: Cell concentration; V_{inoc}: Volume inoculum; V_R: Reactor working volume; Net V CH4: Net Volume methane produced;

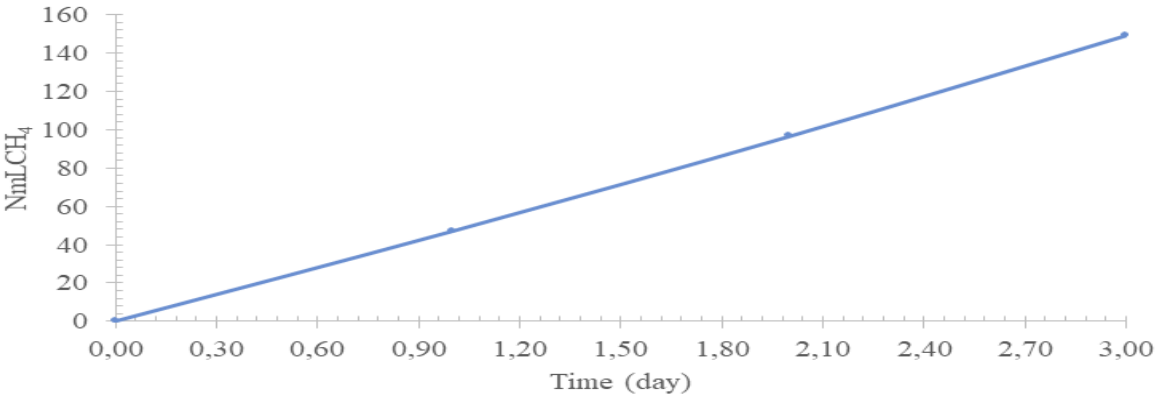
The SMA was thus, significantly above the recommended minimum activity for standardized BMP assays (Hansen *et al.*, 2004; Angelidaki *et al.*, 2009) indicating good health in the aceticlastic archaea population; and attributable to the meticulous inoculum synthesis and acclimation to high strength organic loads.

The average cumulative methane production profile for SMA I assays is presented in **Figure 4-3a**, where CH_4 production was noted immediately from the beginning of the assay which proceeded exponentially over 24-hours period, and started to plateau on the second day of measurement, before eventually reaching a constant value on the third and last day of the test.

For SMA II **Figure 4-3b** assays a linear cumulative methane production profile was observed, the straight-line fit of the cumulative methane yield observed was in conjunction with the tested organic load. An average SMA of 2.6 g COD_{CH4} g^{-1}VSS d^{-1} was obtained for both tests comparable to the results obtained by Johanna *et al.* (2009), where a SMA of 2.39 g COD_{CH4} g^{-1}VSS d^{-1} on acetate was reported for anaerobic sludge treating urban solid waste. Thus, SMA, significantly above the recommended minimum activity for standardized BMP assays (Hansen *et al.*, 2004; Angelidaki *et al.*, 2009) has indicated good-health conditions in the aceticlastic archaea population, which were as well attributed to the meticulously inoculum synthesis and acclimation to high strength organic loads.



(a) SMA 1



(b) SMA 2

Figure 4-3: Cumulative methane AD profiles for specific methanogenic activity assays a) SMA I; and b) SMA II

4.6.5 The effects of input parameters on biomethane potential of abattoir and winery solid waste

The results of this study are summarized in **Table 4-5**, and **AD** profiles are presented in **Figure 4-4** as cumulative methane over time. At high organic load and F/M ratio of 20 gVS L^{-1} and 2, respectively mono-AD of $\mathbf{C_b}$ and $\mathbf{W_s}$ produced 819.7 and 490.0 NmL CH_4, respectively followed by the AcoD of $(\mathbf{A_sC_bW_s})$* which produced 441.2 $NmLCH_4$. The worst performance was observed from the mono-AD of $\mathbf{A_s}$ assays where the lag phase was noted to be almost the entire incubation period producing a total average of 6 $NmLCH_4g^{-1}VS_{added}$, which was significantly smaller than that obtained from a previous experimental run with a half of the tested organic load. Biogas production was immediate for the three best performing assays (i.e. mono-AD of $\mathbf{C_b}$ and $\mathbf{W_s}$; and AcoD of $\mathbf{A_sC_bW_s}$*), with the highest activity noted for $\mathbf{W_s}$ assays.

Table 4-5: BMP results from the anaerobic mono- and AcoD of abattoir and winery waste at varied F/M ratios and organic load

Substrate	Mix	C/N	V_R (L)	F/M	TMP ($NmLg^{-1}VS$)	Net V CH_4 (mL)	SMP ($NmLg^{-1}VS_{added}$)	t_{80} (day)	f_d (%)
A_s	-	18.4	0.9	1.92	816.27	125.99	6.29	*	0.77
C_b	-	4.1	0.9	1.92	482.84	819.66	41.06	26	5.01
W_s	-	28.4	0.9	1.94	443.89	490.01	24.33	6	5.48
(ABW)*	1/1	19.9	0.9	1.96	477.29	441.19	21.59	8	4.52
Inoculum	-	nr	0.9	-	-	76.23	-	26	-
A_sW_s	1/1	23.3	0.4	0.96	634.20	289.31	10.75	14	1.70
C_bW_s	1/1	16.1	0.4	0.97	463.47	412.35	22.87	11	4.93
Inoculum	nr	nr	0.4	nr	nr	182.27	nr	17	nr
A_sW_s	2/3	23.4	0.9	0.48	628.15	1859.28	369.56	19	58.83
C_bW_s	2/3	16.2	0.9	0.49	463.32	1793.40	354.13	15	76.43
Inoculum	-	nr	0.9	nr	nr	167.63	-	21	-

* system significantly inhibited determination of t_{80} was inconclusive with various points noted; V_R: reactor working volume; t_{80}: time taken for 80% methane volume of methane to be produced; nr: data not reported

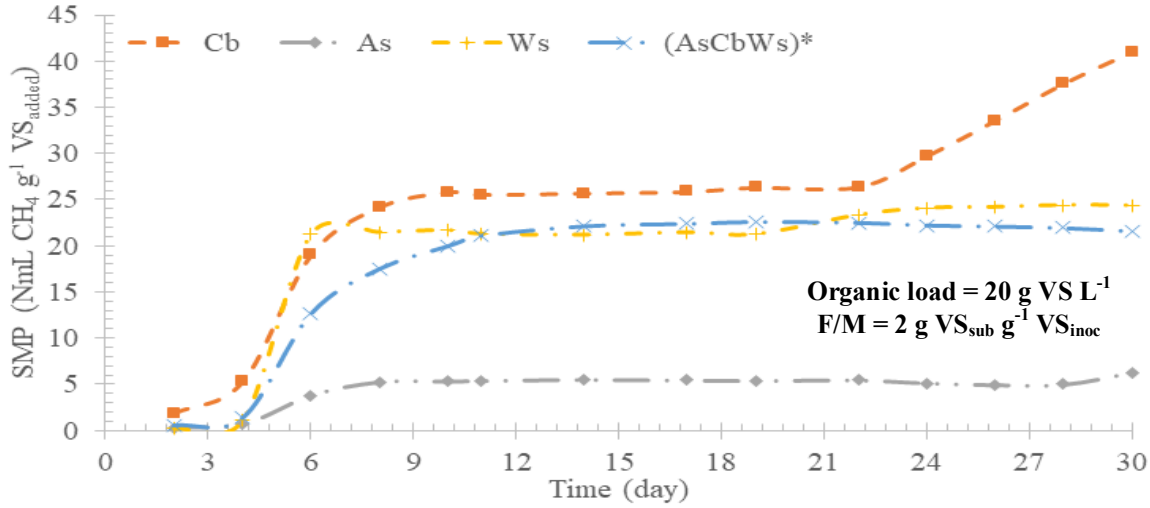
However, the quality of biogas was poor and highly concentrated in CO_2 which is observable in the quick acidification of scrubber liquor **(see Figure 4-5)**. Similarly, he **C_b** assays were subject to inhibition considering the low C/N ratio, likely due to high NH_3/NH_4^+ concentrations. The process eventually recovered from inhibition on the 22nd day as microbes acclimatized to the toxic environment, and a second exponential phase was recorded towards the last day of experiments.

The AcoD assays were more stable without inhibition even though the biogas produced over the digestion period was lower than that produced by mono-AD of **C_b** and **W_s**. The quality of biogas produced was good, and the overall performance indicated synergistic effects when compared to mono-AD of **A_s** which can be attributed to a balance in the C/N ratio and reduced toxicity generated by long chain volatile fatty acids (LCVFAs) (Alvarez and Lidén, 2008; Cavaleiro *et al.*, 2009; Palatsi *et al.*, 2011; Affes *et al.*, 2013; Xia *et al.*, 2015).

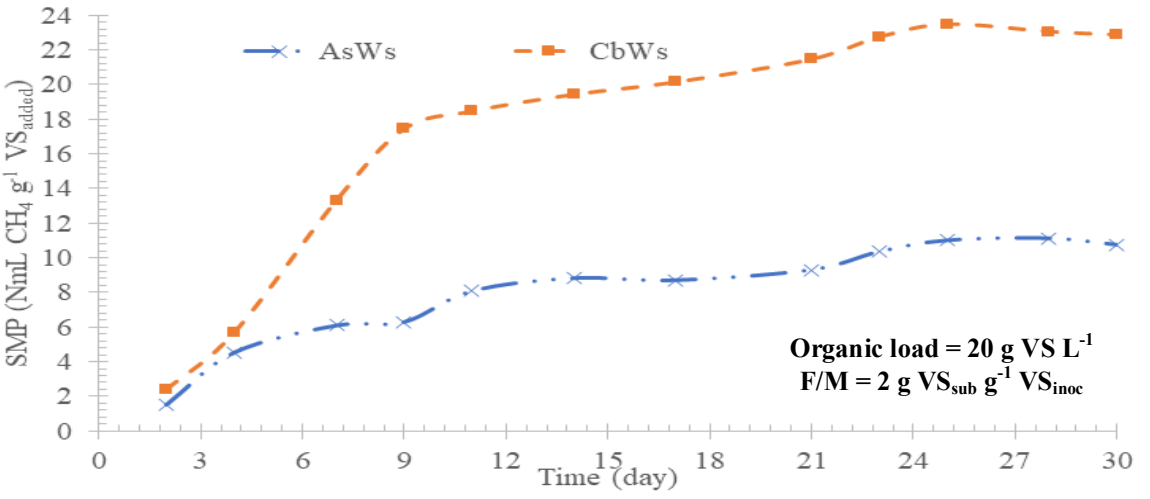
The mono-AD and AcoD experiments performed poorly than expected when compared to the results obtained by other researchers (Battimelli, Carrère and Delgenès, 2009; Palatsi *et al.*, 2011; Esposito *et al.*, 2012; Cavaleiro *et al.*, 2013; Xia *et al.*, 2015). The treatment of slaughterhouse wastes could not provide expected results possibly due to the chosen pre-treatment methods, inoculum and/or F/M ratio applied.

Another likely factor could be the high organic load chosen in the experimental design. With AcoD experiments, it became a necessity to separate $\mathbf{A_s}$ and $\mathbf{C_b}$ and then use them as co-substrates in binary mixtures with $\mathbf{W_s}$ to mitigate shock-loading and toxicity.

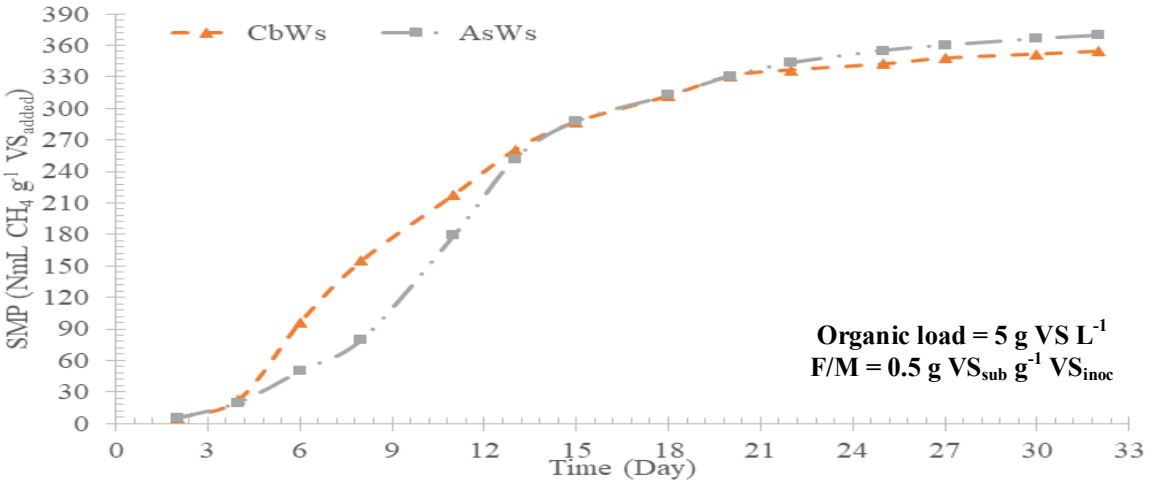
There were no improvements in biomethane yield(s) obtained for the 1:1 binary mixture of $\mathbf{C_bW_s}$ and $\mathbf{A_sW_s}$ with organic load and F/M ratio of 10 gVS L^{-1} and 1, respectively. The lack of improvement was due to the presence of high levels of phenolic compounds contributed by $\mathbf{W_s}$ (Mkruqulwa, Okudoh and Oyekola, 2019). However, the AD process proceeded un-inhibited without delay (lag) most likely due to the presence of highly soluble sugars from $\mathbf{W_s}$, rapid acclimatization of microorganisms and reduced toxic/inhibitory effects (Moukazis, Pellera and Gidarakos, 2017). Biomethane generation was recorded on Day 2 and proceeded exponentially and started to plateau on Day 9. It slowed down towards the last day where CH_4 yield from the control samples was slightly higher than the experimental value and finally reached values of 11 and 23 $NmLCH_4g^{-1}VS_{added}$ for $\mathbf{A_sW_s}$ and $\mathbf{C_bW_s}$, respectively.



(a)



(b)



(c)

Figure 4-4: Cumulative methane production profiles for (a) mono-AD and AcoD of $\mathbf{A_s}$, $\mathbf{C_b}$, $\mathbf{W_s}$ and $(\mathbf{A_sC_bW_s})$*, (b) AcoD of $\mathbf{A_sW_s}$ and $\mathbf{C_bW_s}$, and (c) AcoD of $\mathbf{A_sW_s}$ and $\mathbf{C_bW_s}$

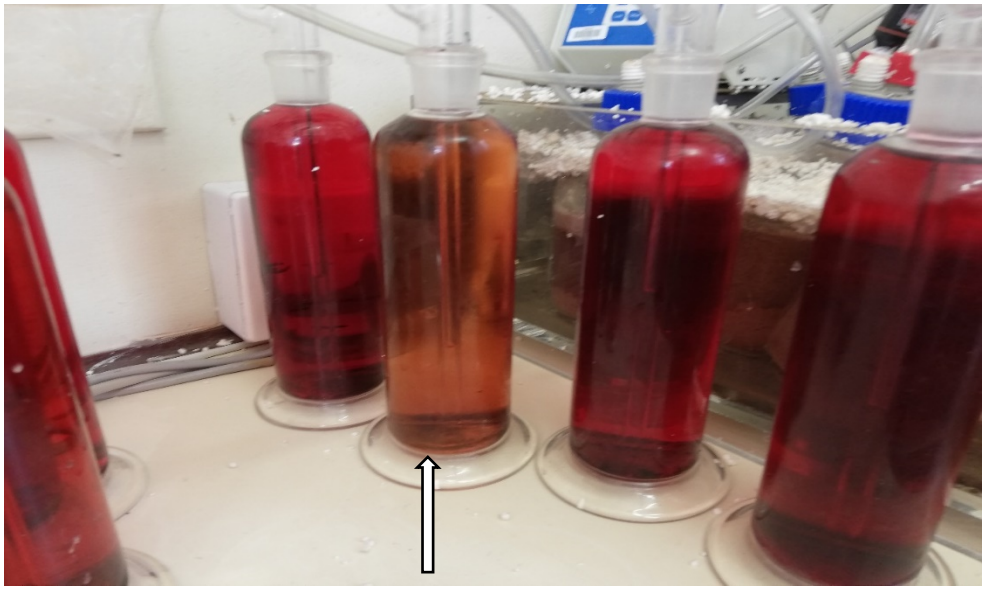


Figure 4-5: Photographic image of spent NaOH scrubbing liquor

When organic load and consequently the F/M was reduced to 5 gVS L^{-1} and 0.5, respectively for 2:3 mixtures of $\mathbf{A_sW_s}$ and $\mathbf{C_bW_s}$, CH_4-yield improved exponentially with recorded values reaching 370 and 354 $NmLCH_4g^{-1}VS_{added}$, respectively.

The SMP results for the co-substrates was similar to that obtained by Zhang and Banks (2012) for AcoD of mechanically recovered organic fraction of municipal solid waste (mr-OFMSW) with pig intestine/floatation fat (PI/FF) and sheep blood that yielded 357 and 358 $NmLCH_4\ g^{-1}VS_{added}$.

4.6.6 Kinetic study

The results of the kinetic study and bio-energic parameter estimation were only possible for $\mathbf{A_s}$ and $\mathbf{C_b}$ mono-AD (at organic loading of 10 gVS L^{-1} and F/M of 1) and the 2:3 binary mixtures of $\mathbf{A_sW_s}$ and $\mathbf{C_bW_s}$ AcoD (organic loading of 5 gVS L^{-1} and F/M of 0.5) which are summarized in **Table 4-6.** The nonlinear regression curve fitting of results with first order, Gompertz, Logistic and Richard's kinetic models of SMP over time for (a) mono-AD of **A**, (b) mono-AD of $\mathbf{C_b}$, (c) AcoD of $\mathbf{A_sW_s}$, and (d) AcoD of $\mathbf{C_bW_s}$. are shown in **Figure 4-6.** For mono-AD of $\mathbf{A_s}$ the Richard's model presented the smallest error (0.3%) between the measured and predicted SMP, followed by the logistic and Gompertz model (0.9% and 2.2%, respectively), while the largest margin of error was recorded for the first-order kinetic model.

The mono-AD of $\mathbf{C_b}$ was slightly different with the smallest error (0.4%) recorded for the logistic model then respectively followed by Richard's, Gompertz models (0.7% and 1.6%, respectively) and first-order kinetic model (11.2%). These results are further justified by the

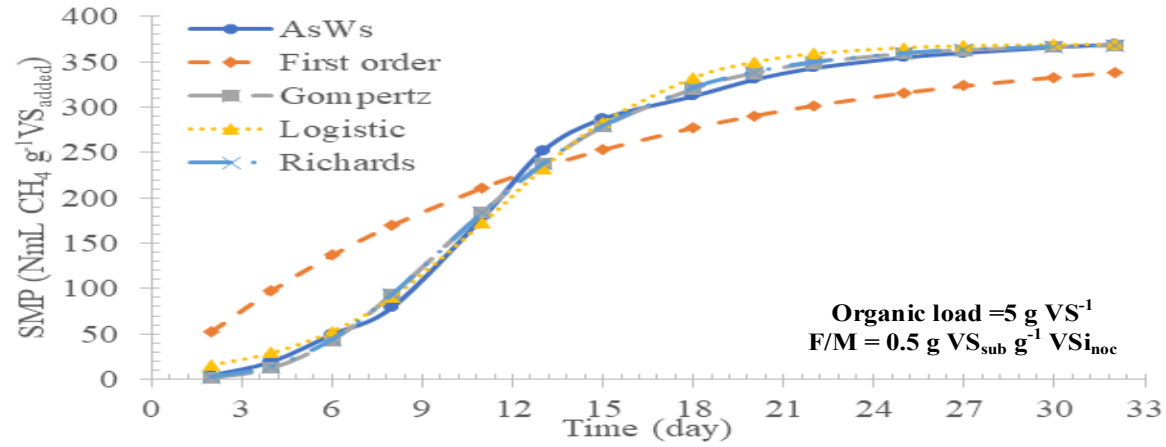
differences in the lag-phase determined for both substrates, where it was predicted to be longer for **A_s** (12.75-15.78 days) and shorter for **C_b** (4.47-4.98 days). For the AcoD of **A_sW_s** and **C_bW_s** the logistic model presented the smallest error (0.1%) between measured and predicted **SMP** was followed by Richard's model (0.5-0.7%), Gompertz model (0.5-0.6%) and the highest margin of error was from the first-order kinetic model (5.4-8.5%). The lag-phase for both **AcoD** mixtures was lower than the one for mono-AD, with the ranges of (4.95-5.37 days) and (2.96-3.18 days) for **A_sW_s** and **C_bW_s**, respectively. These results were theoretically attributed to the synergistic effects of the co-mixtures, the maturity of the inoculum (i.e. increased activity), reduced organic load and F/M ratio with a positive influence on the overall AD performance.

Table 4-6: Kinetic study results showing model selection and bio-energic parameter estimation

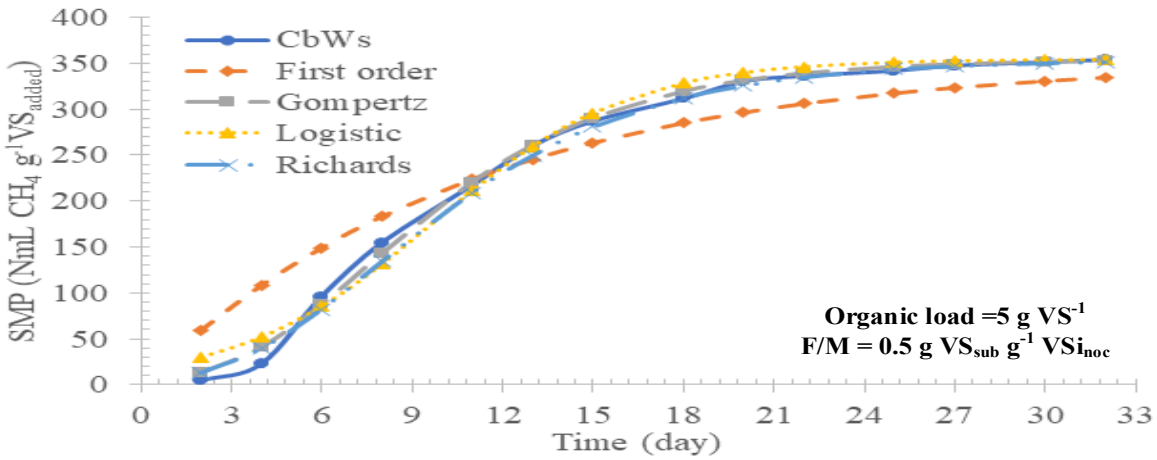
	Model	a	b	k	SMP		Error	μ_m	λ	ν	R^2
				day^{-1}	($NmLCH_4\ g^{-1}VS_{added}$)		(%)	($NmLCH_4\ g^{-1}VS\ day^{-1}$)	day		
					predicted	measured					
A_s	1st-order	-	-	0.045	77.53	110.24	-29.7	-	-	-	0.638
	Gompertz	-	-	nr	107.83	-	-2.2	13.69	12.75	-	0.991
	Logistic	-	-	nr	109.26	-	-0.9	12.91	13.13	-	0.997
	Richards	-	-		109.91	-	-0.3	13.28	15.78	1.9	0.999
C_b	1st-order	-	-	0.081	170.70	192.28	-11.2	-	-	-	0.849
	Gompertz	-	-	-	189.20	-	-1.6	16.09	4.47	-	0.993
	Logistic	-	-	-	191.53	-	-0.4	16.22	4.98	-	0.995
	Richards	-	-	-	190.84	-	-0.7	13.28	4.74	0.62	0.995
A_sW_s	1st-order	-	-	0.077	338.12	369.56	-8.5	-	-	-	0.858
	Gompertz	-	-	-	367.29	-	-0.6	30.61	4.95	-	0.997
	Logistic	-	-	-	369.17	-	-0.1	30.64	5.37	-	0.992
	Richards	-	-	-	367.58	-	-0.5	5.39	4.96	0.72	0.997
C_bW_s	1st-order	-	-	0.091	334.88	354.13	-5.4	-	-	-	0.905
	Gompertz	-	-	-	352.39	-	-0.5	28.30	2.96	-	0.996
	Logistic	-	-	-	353.75	-	-0.1	27.14	3.18	-	0.986
	Richards	-	-	-	351.61	-	-0.7	0.002	2.96	**	0.996

a and b: Schnute kinetic model parameters; k: First order kinetic constant; μ_m: Maximum specific methane generation rate; λ: Lag phase; ν: Richards model shape factor; and nr: Data not reported; **

The coefficient of determination (R^2) lowest value was obtained with first-order kinetic modelling of mono-AD (0.638-0.849) and increased with AcoD (0.858-0.905). The R^2 values were highest (0.999) with Richard's model for mono-AD of **A_s** with a shape factor (ν) of (1.9); while mono-AD of **C_b**, AcoD **C_bW_s** and **A_sW_s** were sufficiently defined by the logistic (R^2 = 0.995) and Gompertz (R^2 = 0.996-0.997) models, respectively.



(a) AcoD abattoir solid + winery solid (A_sW_s)



(b) AcoD cow blood + winery solid (C_bW_s)

Figure 4-6: Cumulative specific methane potential kinetic profiles for anaerobic mono- and co-digestion of **A_s**, **C_b**, **A_sW_s**, and **C_bW_s** wastes

The highest specific methane generation rate (μ_m) was measured in the AcoD mixtures, with values 2-fold than measured by mono-AD. A visual assessment of the three sigmoidal fitted kinetic models (i.e. Gompertz, logistic and Richard's) and R^2-values alone was not enough for choosing the most appropriate model defining AD of the tested substrates. Thus, a more rigorous statistical approach using the students-t and F-ratio tests was employed and summary of the results is presented in **Table 4-7**.

For mono-AD of **A_s** and **C_b**, the student-t test with ($a > 0$; $b < 0$ at 95% confidence intervals) indicating acceptance of the Richard's model and was confirmed with F-test for **A_s**. Both the Gompertz and logistic models ($f = 47.052$ and 8.618, respectively) were not F distributed ($F = 3.072$). Therefore, an additional parameter (i.e. v) was required to accurately simulate biodegradation patterns and predict methane production profiles. However, for **C_b** closer

inspection using the RSS and the F-test suggested the logistic model (f = 3.096), which was more accurate.

Table 4-7: Statistical analytical data for kinetic model selection

	a min [a, b]	b min [a, b]	b max	f [d]		F	RSS		
				Gompertz	Logistic	table	Gompertz	Logistic [e]	Richards
A_s	0.425	**-3.647**	-0.899	47.052	8.618	3.072	209.293	**63.178**	35.048
C_b	0.200	**-2.463**	0.062	6.879	**3.096**	3.072	417.776	**311.367**	280.883
A_sW_s	0.183	-0.803	0.347	**1.370**	17.489	2.753	801.322	1937.25	790.573
C_bW_s	0.133	0.546	0.937	49.036	225.159	2.753	715.862	2851.486	715.908

[a] a and b are Schnute model parameters; [b] min and max are 95% confidence limits [c] Bold face data indicates acceptance of logistic model with t test; [d] Bold face data indicates acceptance of given model with F tests; [e] Bold face data indicates that the RSS with Gompertz model is greater than RSS with logistic model

For AcoD of **A_sW_s** the student-t test with (a > 0; b < 0 at 95% confidence intervals) the F-test (F = 2.753) found Gompertz model (f = 1.370) to be F-distributed indicating acceptance. However, for **C_bW_s** even though the f-value was not F-distributed with either Gompertz or logistic models (f = 49.036 and 225.159) an additional parameter obscured the results. This was more evident in the RSS values obtained and in the error between measured and predicted SMP. Therefore, the Gompertz model was the more accurate model for simulating the results.

4.7 Conclusion and recommendations

The BMP from AcoD of 2:3 abattoir and winery solid wastes (i.e. **A_sW_s** and **C_bW_s**) with respective F/M and organic loads of 0.5 and 5 g VS L^{-1}, resulted in the highest methane yields and in most stable and efficient digestion performance. An increase in F/M ratio and organic loading, reduced methane yield and affected system performance an indication of inhibitory/toxic effects. Kinetic modelling was only possible for the uninhibited systems, where all models were able to define and simulate AD profiles with over 90% of accuracy. Thus, considering the results of this study further research should be conducted including mixture blending, optimization of process input parameters and mode of operation (i.e. either batch or continuous) to better understand the effects of AcoD of abattoir and winery solid waste on large scale operations.

CHAPTER 5

5. Screening Mixture Designed Experiments: Evaluation of Optimal Blends and Synergistic/Antagonistic Effects during Co-Digestion of Abattoir and Winery Solid Wastes

5.1 Abstract

Various mixture proportions consisting of abattoir solid wastes ($\mathbf{A_s}$), cow blood ($\mathbf{C_b}$), and winery solid wastes ($\mathbf{W_s}$) were blended to enhance biogas production. The primary objective was the screening of optimal mixtures by assessing synergistic effects prompted by proportional mixing. Thus, mesophilic batch anaerobic co-digestion (**AcoD**) assays were conducted, utilizing the **ABCD** mixture design of experiments for a three-factor mixture design with specific methane production (**SMP**) and maximum specific methane production (μ_m) as response variables of interest. Kinetic modelling was used to estimate μ_m and the Gompertz model was found more suitable for simulating experimental results with $\mathbf{R^2}$ values above 90%**.** The highest **SMP** of 112 $NmLCH_4$/g VS was obtained for a ternary mixture consisting of 66 % of $\mathbf{C_b}$, 17 % of $\mathbf{W_s}$, and 17 % $\mathbf{A_s}$; and second highest of 104 $NmLCH_4$/g VS was obtained from the binary mixtures consisting of equal parts $\mathbf{C_b}$ and $\mathbf{A_s}$, which also had the highest recorded μ_m of 14 $NmLCH_4$/g VS day. The experimental data was fitted using mixture design models to determine if there are synergistic and/or antagonistic interactions within the mixture blends. The ternary and binary mixtures resulted in an average 21.3 % and 29.3 %, respectively increase in **SMP**s compared to **mono-AD**, with synergistic effects attributed to a balancing of nutrient requirements for stable and enhanced **AcoD** performance by each contributing component within the mixture. The only significant antagonistic effects were observed for the binary mixtures of $\mathbf{A_s}$ and $\mathbf{W_s}$. The optimal mixture blend for SMP was determined to be 46 % $\mathbf{C_b}$, 39% $\mathbf{A_s}$ and 15 % $\mathbf{W_s}$, with a total yield of 106 $NmLCH_4$/g VS_{added} corresponding to μ_m of 7.7 $NmLCH_4$/g VS per day.

Keywords: Anaerobic co-digestion, Winery solid wastes, Abattoir solid wastes, Cow blood, Mixture experimental design

5.2 Introduction

Anaerobic digestion (AD) is an efficient bio-engineering tool used in waste management practices that converts organic matter into renewable green-energy in the form of biogas, with a by-product nutrient-rich digestate applicable to agricultural operations (Gerardi, 2003; Seadi *et al.*, 2008). With intensified researches for its application in treatment of various organic waste stream (e.g. agricultural, industrial, food wastes, etc.), it has been sought as means of simultaneously dealing with the runaway environmental pollution and energy-crisis.

Abattoir waste is rich in fats and proteins with a high biomethane potential (BMP) and thus, a highly suited AD feedstock for biomethanation (Pitk, Kaparaju and Vilu, 2012; Wang, Jena and Das, 2018). However, the mono-anaerobic digestion (mono-AD) of organic waste residues from abattoir operation is prone to failure (Schwede *et al.*, 2013; Ortner *et al.*, 2014, 2015). As the biodegradation of this waste produces long chain fatty acids (LCFA), which are known to be rate limiting in the hydrolysis stage, and whose accumulation is known to be toxic to the acetogenic bacteria and methanogenic archaea (Cirne *et al.*, 2007; Cavaleiro, Pereira and Alves, 2008; Battimelli *et al.*, 2010; Palatsi *et al.*, 2010; Chen *et al.*, 2014). Furthermore, their low C/N ratio produces high concentrations of ammonia which are detrimental to the performance of methanogenic archaea (Luna del Risco, 2011; Chen *et al.*, 2014).

Anaerobic co-digestion (AcoD) of abattoir wastes with carbon-rich substrates such as agricultural waste residues is a recommended solution for mitigating the problems associated with mono-AD of this type of waste (Weiland, 2010; Mata-Alvarez *et al.*, 2014; Divya, Gopinath and Merlin Christy, 2015; Das and Mondal, 2016). The **AcoD** of substrates provides a balance in C/N ratio and other macro- and micro-nutrients essential for **AD** thereby, reducing the probability of ammonia and lipid inhibition and improving the overall **AD** performance (Salminen and Rintala, 2002; Cirne *et al.*, 2007; Palatsi *et al.*, 2011; Kovács *et al.*, 2013; Chen *et al.*, 2014; Ortner *et al.*, 2015).

The enhancement of biodegradability and improved biomethane yields from co-digestion of substrates with varying characteristics has previously been demonstrated by various researchers (Sosnowski, Wieczorek and Ledakowicz, 2003; Kuglarz and Mrowiec, 2007; Li, Champagne and Anderson, 2015; Neshat *et al.*, 2017; Wannapokin *et al.*, 2018).

Winery solid waste (grape marc/pomace) is generated in large amounts by the wine producing industries with an estimated annual amount of 5 tons per hector across the globe (Zacharof, 2017). The production and consumption of wine were originally dominant in European countries, and recently over 67 countries, such as Australia, New Zealand, Argentina, Chile and South Africa, across the globe are nowadays competing in this market (Zacharof, 2017). Grape pomace, if not properly treated can be hazardous to the environment leading to air, surface and ground water pollution (Begalli, Codurri and Gaeta, no date; Dillon, 2011; Conradie, Sigge and Cloete, 2014; Domínguez *et al.*, 2014). On the other hand, it is a carbonaceous substrate concentrated with nutrients (e.g. soluble sugars, phosphates, nitrates, etc.) (Bustamante *et al.*, 2008; Conradie, Sigge and Cloete, 2014; Makadia *et al.*, 2016). Which is what informed the decision for AcoD of abattoir with winery solid waste to improve the C/N ratio and balancing nutrients supply thereby, improving biomethanation efficiency.

The AcoD of abattoir waste (mainly poultry, fish, pig and animal manure) has successfully been experimented with a variety of wastes such as sewage sludge (Luste and Luostarinen, 2010; Borowski and Kubacki, 2015), organic fractions of municipal solid waste (Zhang and Banks, 2012), kitchen waste (Aragaw, Andargie and Gessesse, 2013), fruit and vegetable waste (H. Bouallagui *et al.*, 2009; Pagés-Díaz *et al.*, 2017), and rendering plant waste (Bayr *et al.*, 2012). The **AcoD** of winery solid wastes was as well experimented with waste activated sludge (Da Ros *et al.*, 2014), screened dairy manure (Lo and Liao, 1986), winery wastewater, and cassava biomass (Mkruqulwa, Okudoh and Oyekola, 2019). For most of these studies mentioned, the randomly selected co-substrate combinations resulted in synergistic effects, and at times antagonistic effects were determined in the biogas yields. The use of experimental mixture designs has been previously demonstrated by (Pagés-Díaz *et al.*, 2014) for the evaluation of component interactions within mixtures, and optimization of contributing fractions for improved digestion efficiency. Besides the mixture blending, optimization of the environmental biological process parameters (e.g. Temperature, pH, organic load) is also essential for optimal running of **AD** systems (Mata-Alvarez *et al.*, 2014; Divya, Gopinath and Merlin Christy, 2015; Dahunsi *et al.*, 2016).

Nevertheless, to the best of our knowledge no study has been conducted utilizing both abattoir and winery solid wastes as co-substrates. Thus, aim of this study was to apply a three-factor mixture design for abattoir (i.e. solid fractions and cow blood) and winery solid wastes in **AcoD**

evaluating quadratic and cubic synergistic/antagonistic effects of various mixture blends, and evaluate their biomethane potentials.

5.3 Aims and Objective

The aim and objective of this study were to improve the anaerobic co-digestion of abattoir and winery waste for enhanced biomethanation using:

- Mixture design principles to evaluate and screen for mixture factor interactions; and
- To determine optimal mixture blends for further evaluation.

5.4 Materials and Methods

Standard methods and protocols described in detail in **CHAPTER 3** were followed throughout this study.

5.4.1 Substrate and inoculant

Three different substrates were used in this study, namely abattoir solid ($\mathbf{A_s}$) and liquid (cow blood) ($\mathbf{C_b}$), and winery solid ($\mathbf{W_s}$) waste. A detailed description of the substrate collection, preparation and preservation can be found in **section 3.1**

5.4.2 Experimental set up and procedure

All details about experimental set-up and procedure are provided in **CHAPTER 3 section 3.2** to **section 3.7**

5.4.3 Data Analysis

All details on data analysis are provided in **CHAPTER 3 section 3.8.1** to **section 3.8.8**

5.5 Results and discussion

5.5.1 Mixture Characteristics

The physical and chemical characteristics of the mixture blends used in the designed experiments is presented in **Table 5-1**. The abattoir solid ($\mathbf{A_s}$), cow blood ($\mathbf{C_b}$) and winery solid ($\mathbf{W_s}$) wastes are substrates concentrated in organic content with varying characteristics, making them suitable for mixture blending. The highest C/N ratio and total solids (28 and 87.93%) were found in $\mathbf{M_8}$ ($\mathbf{W_s}$ pure blend) amongst the tested mixtures, which were within the appreciable levels for stable biomethanation (Gerardi, 2003; Seadi *et al.*, 2008).

The other pure blends tested (i.e. $\mathbf{M_6}$ and $\mathbf{M_{10}}$) had the highest theoretical methane potentials of 816.27 and 482.84 $NmLCH_4/gVS$, respectively, and a relatively lower C/N ratios of 18 and 4.05, respectively, due to the presence of lipids and proteins which may inhibit biomethanation as previously indicated by other researchers (Banks and Wang, 2006; Cirne *et al.*, 2007; Palatsi *et al.*, 2011; Zhang and Banks, 2012; Escudero *et al.*, 2014). The presence of lipids results in slow hydrolysis rate and fats cover the cell membrane reducing activity (Gerardi, 2003), while digestion of proteins produces ammonia which is toxic to methanogenic archaea (Bayr *et al.*, 2012; Kovács *et al.*, 2013). Thus, are not suitable as **mono-AD** substrates and optimal biomethanation. Therefore, mixture blending of these substrates will complement each other, balancing C/N ratio for efficient **AcoD**.

5.5.2 Biomethane potential

Data analysis results from the biomethane potential assays of mixture blends $\mathbf{M_1}$ to $\mathbf{M_{10}}$ are presented through **Figure 5-8** to **Figure 5-10** with **SMP** ($N\ mLCH_4\ /g\ VS_{added}$) as the response variable and independent time (t) variable in days of incubation. The highest **SMP**s of 85.54, 104.09, 80.24 and 111.87 $NmLg^{-1}VS_{added}$ were recorded for mixture blends $\mathbf{M_2}$, $\mathbf{M_4}$, $\mathbf{M_7}$, and $\mathbf{M_9}$, respectively. A two- to three-fold reduction in **SMP** was observed in mixture blends $\mathbf{M_3}$, $\mathbf{M_5}$, and $\mathbf{M_6}$ with recorded values of 40.37, 35.84 and 39.00 $NmLg^{-1}VS_{added}$. The lowest **SMP**s recorded were 7.12, 2.50 and 17.24 $NmLg^{-1}VS_{added}$ from the mixture blends of $\mathbf{M_1}$, $\mathbf{M_8}$ and $\mathbf{M_{10}}$, respectively.

Table 5-1: Physical and chemical characteristics of the mixture blends

Mixture	**M_1**	**M_2**	**M_3**	**M_4**	**M_5**
pH	7.25 ± 0.05	7.66 ± 0.02	7.65 ± 0.01	7.82 ± 0.01	7.44 ± 0.03
Mix ratio (**C_b: W_s: A_s**)	0:46:54	34:32:34	51:49:0	51:0:49	16:18:66
TS (%)	45.66	27.91	25.92	20.55	30.37
VS (%)	92.60	94.77	95.24	96.74	94.29
C/N	23	17	16	11	18
TMP (N mL /g VS)	622.71	567.51	440.01	646.77	688.23
TOC	0.555	0.528	0.462	0.570	0.590
TOD (mg O_2/g VS	1.78	1.62	1.26	1.85	1.97
Organic load (g VS/L)	1.6	1.6	1.6	1.7	1.5
Mixture	**M_6**	**M_7**	**M_8**	**M_9**	**M_{10}**
pH	7.74 ± 0.01	7.65 ± 0.01	7.53 ± 0.09	7.61 ± 0.01	7.76 ± 0.01
Mix ratio (**C_b: W_s: A_s**)	0:0:100	17:66:17	0:100:0	66:17:17	100:0:0
TS (%)	32.13	42.80	87.93	19.80	15.01
VS (%)	93.97	92.87	91.04	97.06	99.57
C/N	18	23	28	11	4.05
TMP (N mL /g VS)	816.27	479.90	394.57	521.54	482.84
TOC	0.656	0.481	0.436	0.505	0.486
TOD (mg O_2/g VS	2.33	1.37	1.13	1.49	1.38
Organic load (g VS/L)	1.5	1.5	1.5	1.5	1.5

TS: Total solids; **VS**: Volatile solids; **C/N** Carbon to nitrogen ratio; **TMP**: Theoretical methane potential; **TOC**: Total organic carbon; and **TOD**: Theoretical oxygen demand

5.5.3 Kinetic model selection and parameter evaluation

A summary of the "Bio-energetic" parameter(s) estimates that were used to simulate anaerobic digestion profiles of mixture blends tested is presented in **Table 5-2.** The Gompertz function received an exceptional "goodness of fit" evaluation when used to simulate AD profiles from observed experimental data fitted with coefficient of determination (**R^2**) greater than 0.992. However, for mixture blends of **M_{10}** and **M_1** the Richards model was used to simulate AD with an estimation of parameters with **R^2** values of 0.991 and 0.975, respectively.

Table 5-2: Kinetic model selection and associated bio-energic parameter estimates

	Model	a	b	μ_m	λ	v	R^2	RSS	df
M_{10}	Richards	--	--	1.213	15.868	382.002	0.991	3.475	11
M_9	Gompertz	--	--	7.114	0.606	--	0.994	97.074	12
	Logistic	--	--	6.645	0.609	--	0.999	21.132	12
	Richards	--	--	6.869	0.639	1.131	0.999	20.475	11
	Schnute	0.248	-1.146	--	--	--		20.464	10
M_8	Gompertz	--	--	2.285	1.293	--	--	5.138	12
	Logistic	--	--	2.89	1.294	--	--	5.138	12
	Schnute	14.529	-62.875	0.636	0.119	--	0.467	115.649	10
M_7	Gompertz	--	--	4.914	0	--	0.992	21.393	12
M_6	Gompertz	--	--	8.662	3.329	--	0.998	4.848	12
M_5	Gompertz	--	--	3.325	0.703	--	0.995	4.673	12
M_4	Gompertz	--	--	14.893	0.599	--	0.995	39.000	12
M_3	Gompertz	--	--	8.884	3.040	--	0.995	11.194	12
M_2	Gompertz	--	--	4.348	0	--	0.955	270.481	12
M_1	Richards	--	--	0.948	1.688	1.688	0.975	1.214	11

a and **b:** Schnute kinetic model parameters; **k:** first order kinetic constant; μ_m: maximum specific methane generation rate; **λ:** lag phase; **v:** Richards model shape factor; **RSS**: Residual sum of squares; **df**: degrees of freedom and --: data not reported

For $\mathbf{M_9}$ mixture blends, the Gompertz, Logistic and Richards model(s) simulated the AD profiles with relatively high $\mathbf{R^2}$ values of 0.994, 0.999 and 0.999, respectively. The Logistic function was determined to have the best fit based on **F**-ratio and student t-test(s) analysis of $\mathbf{M_9}$ results. All models failed in simulating methane generation of the $\mathbf{M_8}$ mixture blends due to observed inhibitory and toxic effects, and the systems did not recover for the duration of this experimental study. Therefore, only observed methane generation data from $\mathbf{M_8}$ were considered. The $\mathbf{M_9}$ mixture blends was the most efficient AcoD process observed with lag phase (**λ**) and maximum specific methane production rate ($\boldsymbol{\mu_m}$) determined to be 0.61 days and 6.65 $NmLg^{-1}VS\,d^{-1}$. The exponential growth phase was long and lasted for a period of 20 days as the system reached its carrying capacity (see **Figure 5-1**), which was a good indication of stability and of efficiency of the AD process with minimal environmental resistance for microorganism growth.

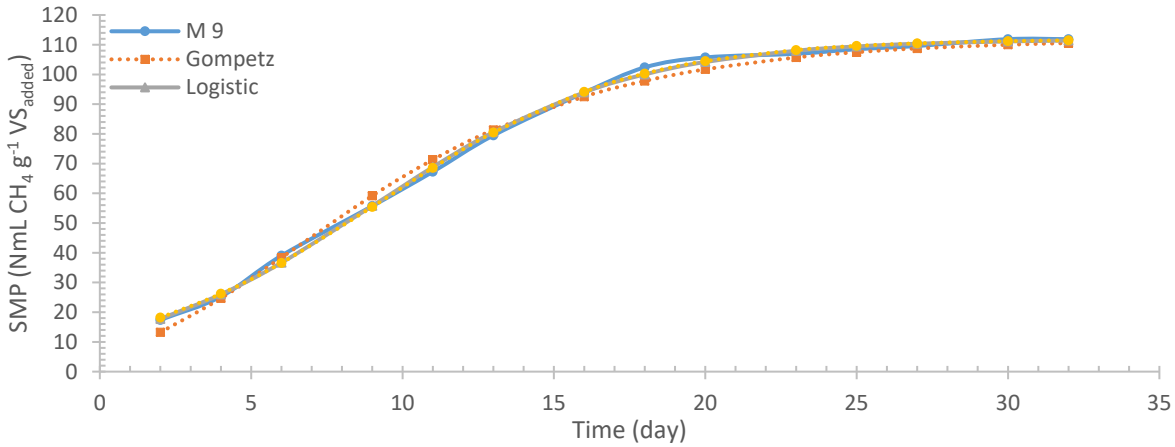


Figure 5-1: M_9 AcoD kinetic model curve-fitting profiles

The second most efficient AcoD experiment was noted for **M_4** mixture blends which had a similar lag phase response (0.60 days) as the one of **M_9** mixture blends. However, the rate of methane generation from **M_4** was twice the rate observed in **M_9** mixtures with a recorded μ_m of 14.87 $NmLg^{-1}VS$ day. The exponential growth phase was approximately 10 days as specific methane yield reached its asymptotic value (see **Figure 5-2**). The anaerobic digestion profiles of **M_2** and **M_7** had similar characteristics (see **Figure 5-3** and **Figure 5-4**) with no delay in methane generation (i.e. $\lambda = 0$) and specific methane generation rates were estimated to be 4.35 and 4.91 $NmLg^{-1}VS$ day, respectively.

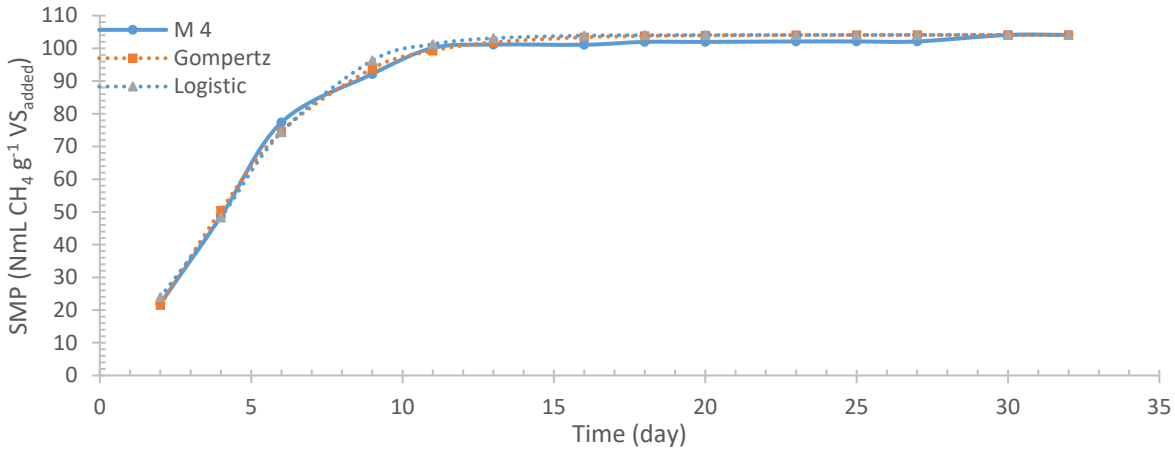


Figure 5-2: M_4 AcoD kinetic model curve-fitting profiles

Even though the specific methane yields obtained from **M_2** were slightly higher, AD of **M_7** mixture blends slightly demonstrated higher **μ_m**, which could be associated with C/N ratio of the two mixture blends and synergistic effects provided by the **W_s** fraction of **M_7**.

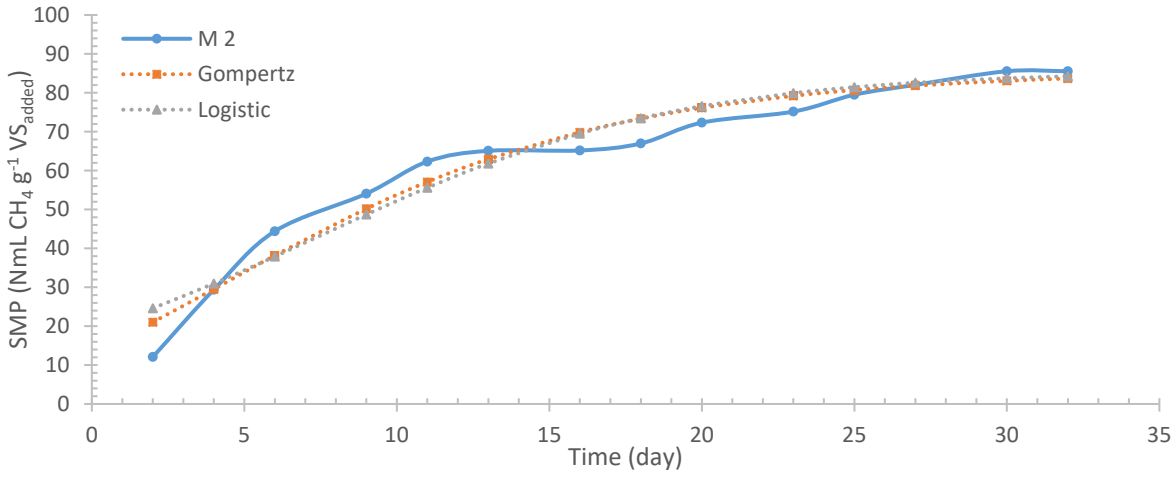


Figure 5-3: M_2 AcoD kinetic model curve-fitting profiles

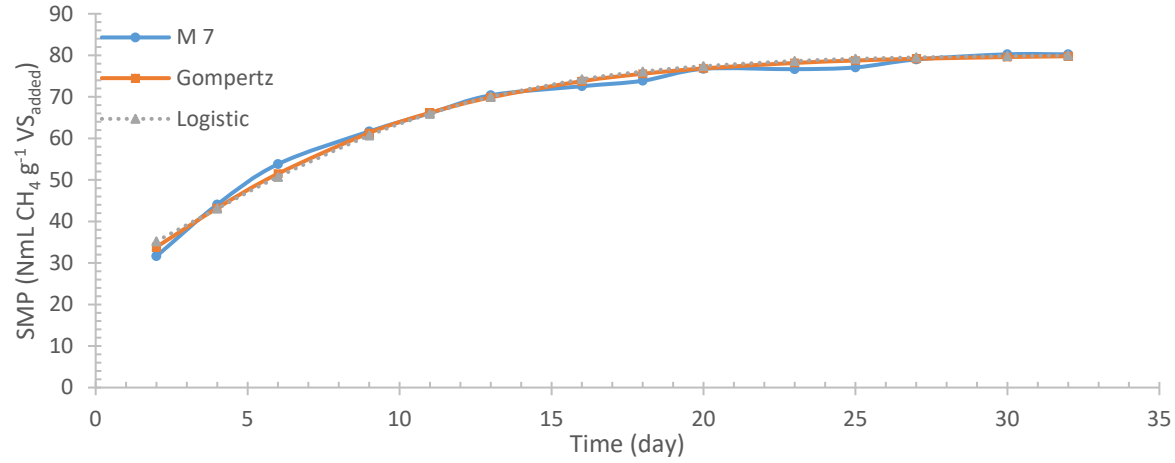


Figure 5-4: M_7 AcoD kinetic model curve-fitting profiles

For **M_3**, **M_5** and **M_6** mixture blends **AD** kinetic profiles agreed well with the Gompertz model with R^2-value of 0.995 and 0.998, respectively, and this demonstrates systems operating with minimal and/or no hinderance on biomethane production. The recorded **μ_m** for the **M_3** mixture blend was 8.88 NmL g^{-1}VS day with a **λ** delay in CH_4-generation of 3.04 days. At this rate, the SMP was expected to reach the 100 NmL CH_4 $g^{-1}VS_{added}$ mark, and unfortunately CH_4-generation from these blends were quick to approach their asymptotic values (see **Figure 5-5**) as the carrying capacity of the system was reached on the 13th day.

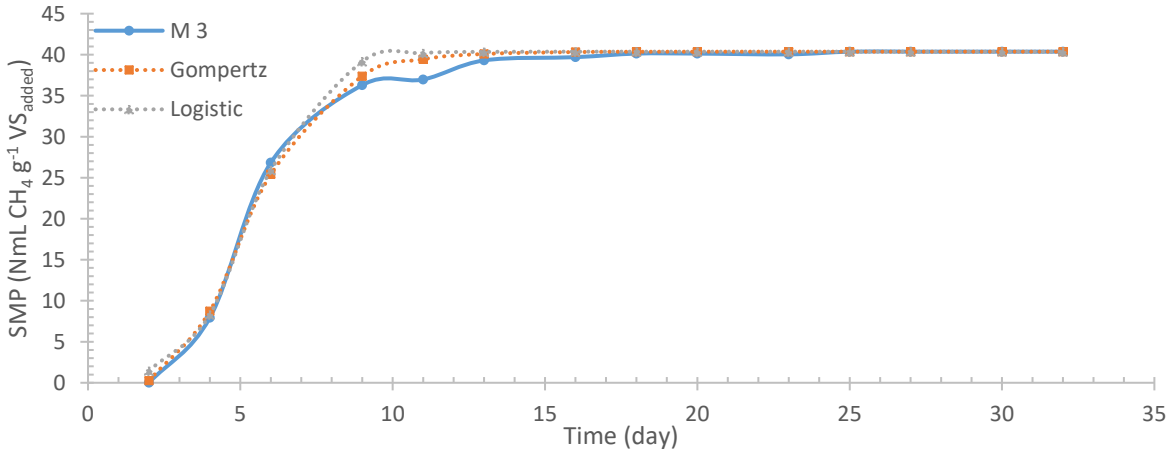


Figure 5-5: **M_3** AcoD kinetic model curve-fitting profiles

In **Figure 5-6** the AcoD kinetic profile for **M_5** mixture blends is presented, with the lowest **μ_m** of 3.33 NmL g^{-1}VS day which occurred in the shortest **λ** of 0.7 days observed from simulation of AD experimental data. These mixture blends contained the smallest amount of **W_s** and **C_b** compared to **A_s** with reduced lag periods in CH_4-generation possibly due to the initial balance in quick- and slow biodegradable organic matter with evidently flat AD profiles.

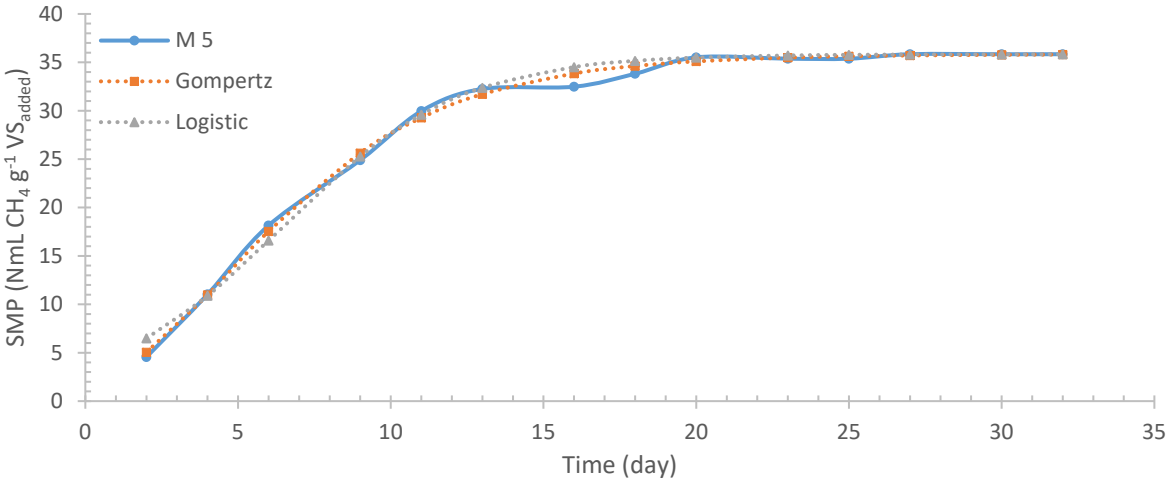


Figure 5-6: **M_5** AcoD kinetic model curve-fitting profiles

For **M_6** and **M_3** mixture blends similarities in the profiles were noted (see **Figure 5-7**), the **μ_m** and the **λ** observed were 8.66 NmL g^{-1}VS and 3.33 days, respectively. This observation was not expected, considering that **M_6**, a pure blend of **A_s**, and **M_3** a binary blend in equal proportion of **C_b** and **W_s**.

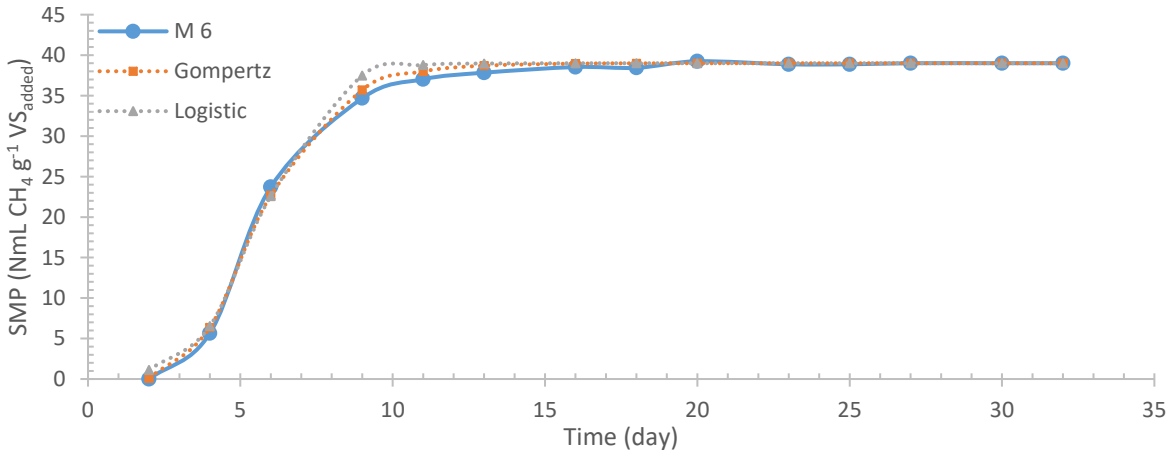


Figure 5-7: $\mathbf{M_6}$ AD kinetic model curve-fitting profiles

The simulation of AD profiles and estimation of "bio-energetic" parameters for the pure blends of $\mathbf{M_8}$ and $\mathbf{M_{10}}$, and the binary mixture blends of $\mathbf{M_1}$ were marred by the toxic and inhibitory behavior observed throughout this study.

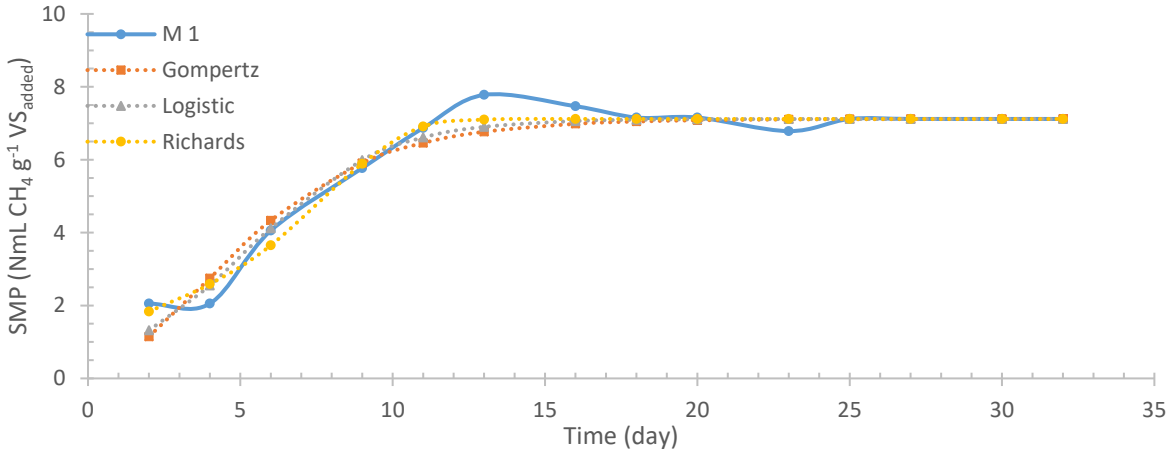


Figure 5-8: $\mathbf{M_1}$ AcoD kinetic model curve-fitting profiles

For the $\mathbf{M_1}$ mixture blends the Richards model provided the best fit for experimentally observed data (see **Figure 5-8**) with $\mathbf{R^2}$-value of 0.975, the $\boldsymbol{\mu_m}$ of these mixtures was estimated to be 0.95 NmL g^{-1}VS and the $\boldsymbol{\lambda}$ for CH_4-generation was 1.7 days. For the $\mathbf{M_8}$ pure blend, all sample attempts to simulate the AD process using either the Gompertz, Logistic and/or Richards functions have failed (see **Figure 5-9**), as it was noted from the observable experimental data that these systems were irreversibly inhibited. The estimation of relevant parameters conducted using the Schnute model with $\mathbf{R^2}$-value of 0.467, the $\boldsymbol{\mu_m}$ and the $\boldsymbol{\lambda}$ were determined to be 0.64 N mL g^{-1}VS and 0.1 days, respectively.

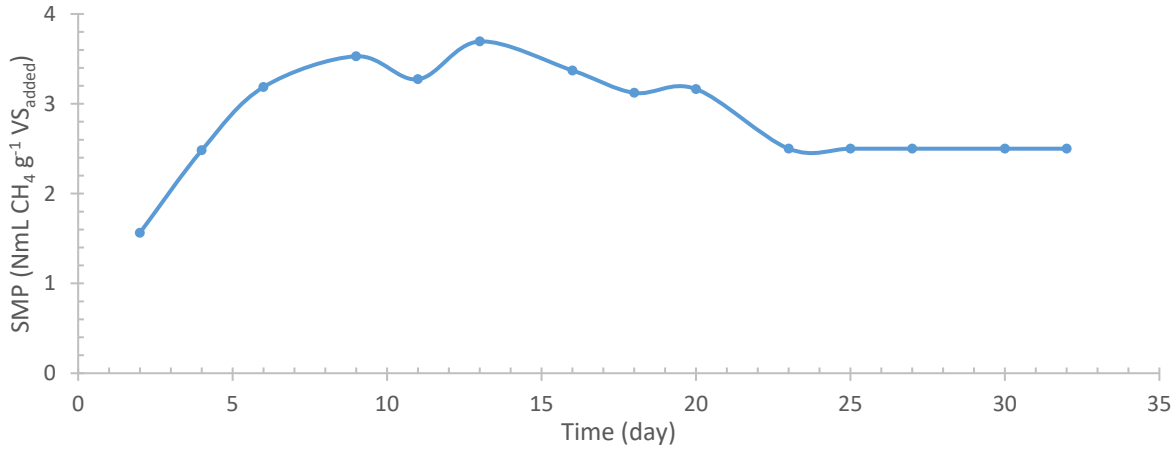


Figure 5-9: $\mathbf{M_8}$ AD kinetic model curve-fitting profiles

The $\mathbf{M_{10}}$ pure blend samples were expected to be the worst performers with significant inhibitory behavior with flat AD profiles considering the low C/N ratio of these samples. However, amongst the worst performing systems $\mathbf{M_{10}}$ was the quickest to recover from the inhibition (see **Figure 5-10**) and towards the end of digestion experiments the system had fully recovered. The Richards model best described the AD profiles with $\mathbf{R^2}$-value of 0.991, and the $\boldsymbol{\mu_m}$ and the $\boldsymbol{\lambda}$ were estimated at 1.21 N mL g^{-1}VS and 15.7 days, respectively.

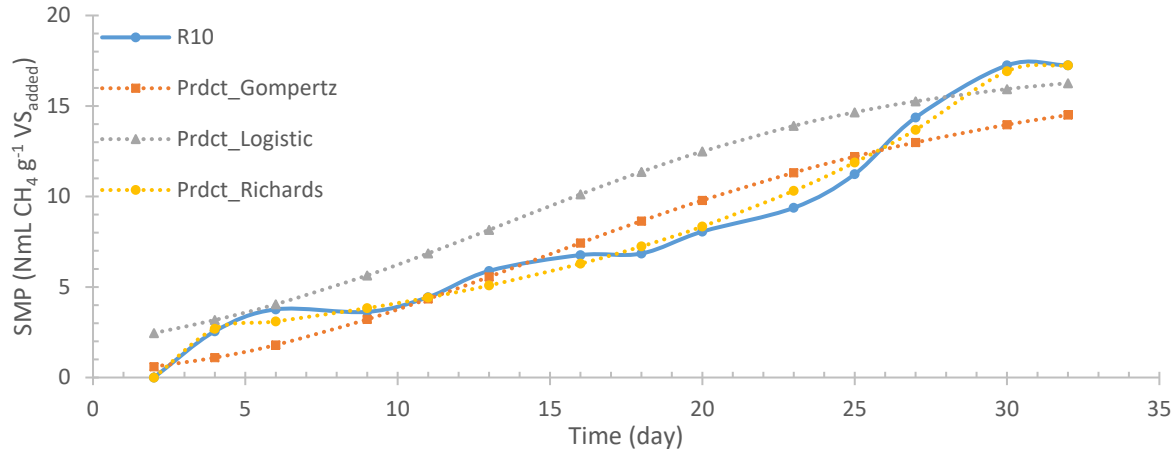


Figure 5-10: $\mathbf{M_{10}}$ AD kinetic model curve-fitting profiles

5.5.4 Model fitting and regression analysis

The linear, quadratic and special cubic model predicted the response using the least square fits at α set at 0.05. The special cubic model was found more suitable for predicting both SMP and methane generation rate. The p-values for SMP and methane production rate were 0.3641 and 0.1104, respectively which is higher than the significance test value set at 0.05 for the summary

of fits for this model. **Table 5-3** presents the test of significance and analysis of variance (ANOVA) as the sum of squares and $\mathbf{R^2}$ of the models employed and error.

Table 5-3: Test of significance and analysis of variance (ANOVA) regression report for the linear, quadratic and special cubic model

Response variable	Sum of Squares	RMSE	R^2 (%)	F	p-Value
SMP					
Linear	2102	42	15	0.5956	0.5769
Quadratic	10358	32	72	2.0221	0.2574
Special cubic	11102	33	77	1.6551	0.3641
Rate					
Linear	39	4	22	0.9854	0.4197
Quadratic	139	3	77	2.7269	0.1763
Special cubic	163	2	91	4.8884	0.1104

The significance of regression coefficients is summarized in **Table 5-5**, where the only significant effects were determined on the microscopic level wherein the presence of $\mathbf{C_b}$ and $\mathbf{W_s}$ was found to have an influence on the $\boldsymbol{\mu_m}$ with p-values of 0.04218 and 0.04571, respectively. The SMP and $\boldsymbol{\mu_m}$ response variables modelling was simulated according to equation(s) (5-1 and 5-2), respectively as shown below:

$$24.45\mathbf{C_b} + 11.24\mathbf{W_s} + 26.52\mathbf{A_s} + 153.88\mathbf{C_bW_s} + 293.33\mathbf{C_bA_s} - 62.01\mathbf{W_sA_s} + 875.08\mathbf{C_bW_sA_s} \quad (5\text{-}1)$$

$$1.13\mathbf{C_b} + 1.45\mathbf{W_s} + 7.77\mathbf{A_s} + 33.30\mathbf{C_bW_s} + 37.87\mathbf{C_bA_s} - 14.96\mathbf{W_sA_s} - 157.44\mathbf{C_bW_sA_s} \quad (5\text{-}2)$$

The response variables (i.e. experimental and model predicted) of individual, binary and ternary mixtures of $\mathbf{A_s}$, $\mathbf{C_b}$ and $\mathbf{W_s}$ subjected to AD as is detailed above (through **Figure 5-1** to **Figure 5-10**) are summarized in **Table 5-4**. Furthermore, mixture profiles, represented as a function of the mixture proportions of the three substrates, are depicted in **Figure 5-11**; the graph provides a plot of the predicted responses as a function of the mixture experimental design. The contour grids represent the predicted response values, which were plotted over the range of the chosen experimental conditions. These plots were utilized in determining the most desirable response values and optimal mixture proportions. The mixture blends containing only

individual fraction of $\mathbf{A_s}$ ($\mathbf{M_6}$), as expected yielded more methane in comparison to those of $\mathbf{M_8}$ and $\mathbf{M_{10}}$ containing only **Ws** and $\mathbf{C_b}$, respectively.

The graph of SMP (**Figure 5-11**) revealed that the highest methane yield zones were located towards the right-hand side of the triangle concentrated in $\mathbf{C_b}$ and $\mathbf{A_s}$, which were as expected, from the theoretical evaluation to have a positive response on total methane yield.

Table 5-4: Experimentally observed and predicted response variable obtained from the AD of various mixture blends

Mix	SMP	SMP*	μ_m	μ_m *	t_{80}	f_d	pH
	($NmLCH_4\ g^{-1}$ VS)		($NmLCH_4\ g^{-1}$ VS day^{-1})		(day)	(%)	
$\mathbf{M_1}$	7,1181	3.3774	0.948	0.8716	9	1.1	6.60
$\mathbf{M_2}$	85,5413	95.9483	4.348	3.8645	19	15.1	6.52
$\mathbf{M_3}$	40,3677	56.31693	8.884	9.6156	8	9.2	6.76
$\mathbf{M_4}$	104,0905	98.8198	14.893	13.9154	7	16.1	6.73
$\mathbf{M_5}$	35,8392	69.8111	3.325	6.1646	10	5.2	6.03
$\mathbf{M_6}$	39,0012	26.5209	8.662	7.7692	8	4.8	7.03
$\mathbf{M_7}$	80,2377	50.5498	4.914	2.6258	10	16.7	6.73
$\mathbf{M_8}$	2,5001	11.2398	0.636	1.4525	3	0.6	11.29
$\mathbf{M_9}$	111,8666	86.7686	6.450	6.8656	15	21.4	6.73
$\mathbf{M_{10}}$	17,2443	24.4539	1.213	1.1282	27	3.6	7.28

SMP* model predicted specific methane potential; μ_m * model predicted specific methane generation rate

On the other hand, $\mathbf{W_s}$ was the individual fraction that contributed the least to methane yield. However, its presence in the binary and ternary blends cannot be left unsaid, because it provided a balancing effect on the C/N ratio by adding the required macro- and micronutrients to the AcoD system, and by buffering the entire digestion system from the toxic by-products associated with mono-AD of abattoir wastes (e.g. ammonia, sulphates, etc.), an effect which resulted in a stable and efficient digestion process. This was more evident in the AcoD trials containing all three substrates concentrated in $\mathbf{C_b}$ ($\mathbf{M_9}$), which produced 111.9 $NmLCH_4$/g VS and those of $\mathbf{M_2}$ and $\mathbf{M_7}$ with equal proportions; and those concentrated in $\mathbf{W_s}$ yielded similar results 85.5 and 80.2, respectively, (**Table 5-4**). What was most interesting to note was the performance of the binary mixture blends containing equal proportions of $\mathbf{C_b}$ and $\mathbf{A_s}$ ($\mathbf{M_4}$), which produced 104,1 $NmLCH_4$/g VS, and which was attributed to the presence of readily biodegradable protein within the mixture supplied by $\mathbf{C_b}$.

Table 5-5: Regression coefficients, standard error and test of significance for estimates based on the data fitted through the special cubic model

Coefficient	Effects on SMP			Effects on Rate		
		Standard Error	p-value		Standard Error	p-value
C_b	24.4539	32.3225	0.50430	1.1282	2.2790	0.04218
W_s	11.2398	32.3225	0.75101	1.4525	2.2790	0.04571
A_s	26.5209	32.3225	0.47205	7.7691	2.2790	0.06236
C_bW_s	153.8804	162.7047	0.41404	33.3013	11.4719	0.12879
C_bA_s	293.3295	162.7047	0.16920	37.8668	11.4719	0.28334
W_sA_s	-62.0117	162.7047	0.72850	-14.9567	11.4719	0.56922
$A_sC_bW_s$	875.0779	1072.28	0.47436	-157.4407	75.6307	0.65456

In this study, AcoD of various mixture blends resulted in SMP, ranging from 2.5 to 112 $NmLCH_4/g$ VS. which was significantly lower than the one reported in the previous studies by Alvarez and Lidén (2008); Hassib Bouallagui *et al.* (2009); Luste and Luostarinen (2010); Palatsi *et al.* (2011); Kovács *et al.* (2013); and Pagés-Díaz *et al.* (2014). But considering the differences in the chosen operating parameters (e.g. retention times, mix ratios, etc.), the methane yields from similar studies ranged from 300 to 800 $NmLCH_4/g$ VS. Another response variable considered was maximum specific methane generation rate (μ_m), the mixture profile plot (see **Figure 5-12**) determined the maximum response situated also on the right-hand side of the triangle, which was concentrated in C_b and **As** and coinciding with the binary mixtures of M_4.

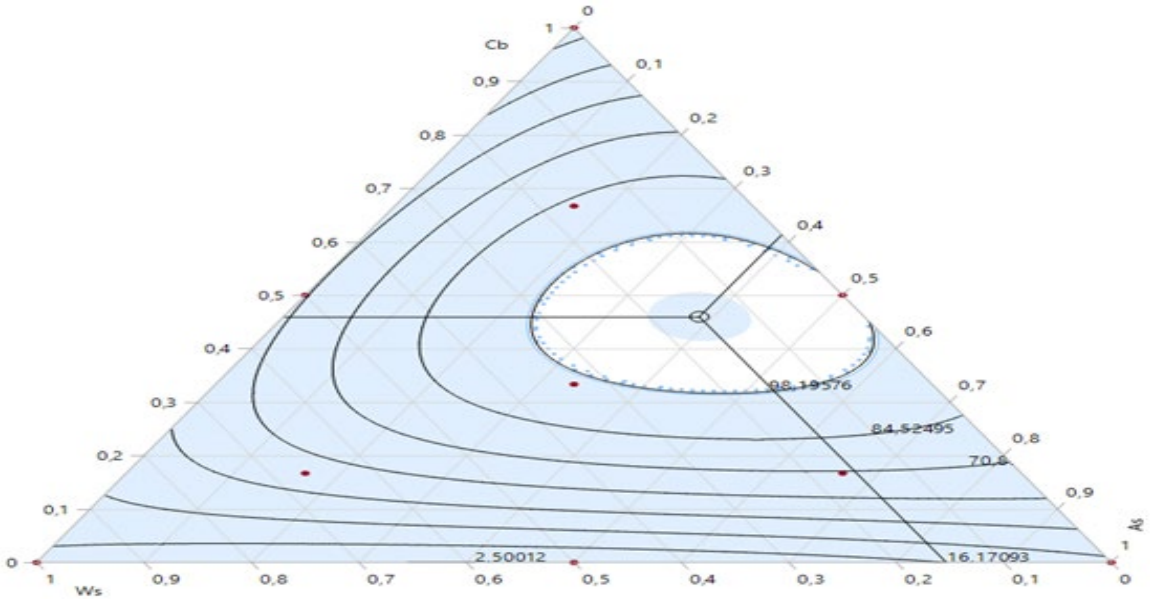


Figure 5-11: Mixture profile at maximized desirability levels for SMP response

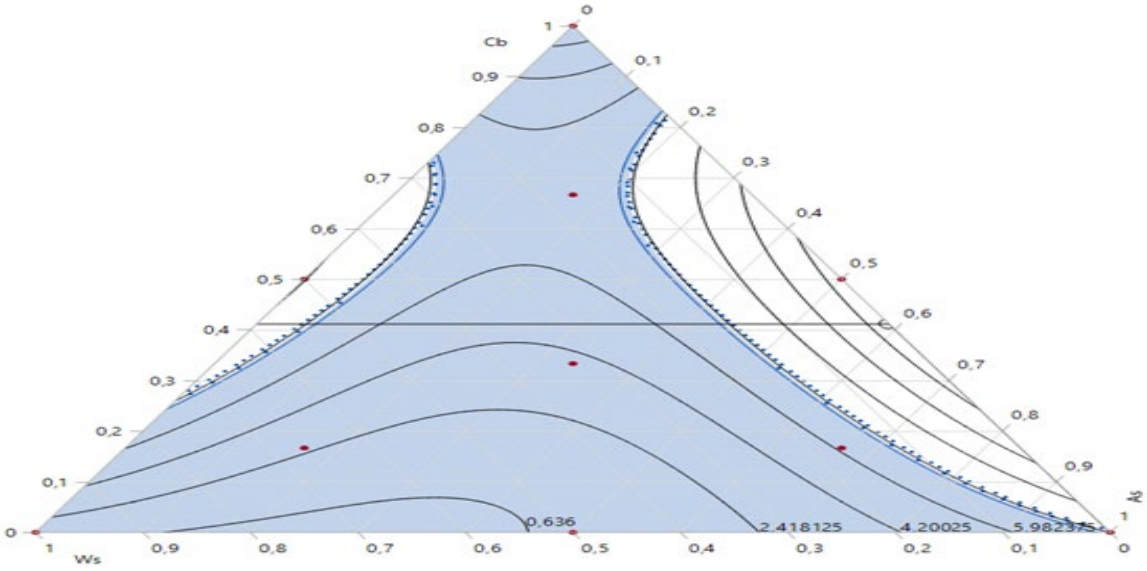


Figure 5-12: Mixture profile at maximized desirability levels for μ_m response

5.5.5 Synergistic and antagonistic interactions

In this study, a system adopted by Pagés-Díaz *et al.*, (2014) to simulate methane production with the first terms of the fitted special cubic model was used, with the assumption that there are no synergistic interactions existing within the mixture blends. By so doing, the study applied the full model which includes quadratic and cubic terms to determine if synergistic and/or antagonistic interactions exist. The fitted models were used to determine the contributions of individual fractions within the mixture. The highest coefficient for SMP was noted for both $\mathbf{C_b}$ and $\mathbf{A_s}$, but $\mathbf{C_b}$ had the lowest for $\boldsymbol{\mu_m}$. Although the binary mixture blend of $\mathbf{M_3}$ and $\mathbf{M_4}$ was assumed to be synergistic on both SMP and $\boldsymbol{\mu_m}$, it displayed no significant interactions with a p-value > 0.05. Similarly, the findings of ternary mixture blends of $\mathbf{M_2}$, $\mathbf{M_5}$, $\mathbf{M_7}$ and $\mathbf{M_9}$, which were also assumed to be synergistic towards **SMP**, revealed that the mixture blends had antagonistic effects towards $\boldsymbol{\mu_m}$ by exhibiting a negative beta coefficient of -157.44 though not reaching significance (p-value > 0.05) of 0.645. For $\mathbf{M_1}$ binary mixture blends antagonistic interactions were noted for both response variables, although they did not reach significant levels. This could be explained by the presence of soluble and highly biodegradable VS contents supplied by $\mathbf{W_s}$ and high fat content of $\mathbf{A_s}$ that generated high concentrations of volatile fatty acids leading to rapid acidification of the digestion medium.

The highest increase in SMP was 45.4 %, which was obtained from ternary mixture blends of $\mathbf{M_2}$ followed by binary mixtures of $\mathbf{M_9}$, $\mathbf{M_7}$ and $\mathbf{M_5}$ that reached a SMP enhancement of 25.3 %, 23.8 %, and 22.8 %, respectively. The average increment in SMP response from the ternary

mixture blends was 29.3 %. Other improvements in SMP were from the binary mixture blends of $\mathbf{M_4}$ where the second highest increment in SMP reached was 32.9 %. $\mathbf{M_3}$ mixture blends resulted in the smallest increment of about 9.6 % in SMP. While $\mathbf{M_1}$ mixture blends resulted in a 10.9 % reduction of the expected SMP. When $\boldsymbol{\mu_m}$ was considered, a converse was painted, where the binary mixture blends of $\mathbf{M_3}$ and $\mathbf{M_4}$ recorded higher increments of 57.6 % and 45.1 %, respectively. While only the $\mathbf{M_9}$ mixture blends resulted in the slightest increase in $\boldsymbol{\mu_m}$ of 10, 6 %. The other ternary blends resulted in significant reduction $\boldsymbol{\mu_m}$ as high as 40.7 % for $\mathbf{M_2}$ followed by $\mathbf{M_7}$ and $\mathbf{M_5}$ with reductions of 28.1 % and 10.1 %, respectively.

And expectedly $\mathbf{M_1}$ mixture blends were the worst performing in terms of $\boldsymbol{\mu_m}$ where reductions as high as 444.2% were recorded.

Based on the above results, one can conclude that AcoD of all three substrates is a viable option treating abattoir and winery wastes. The appropriate choice of mixture proportions is emphasized to bring about synergy. Thus, statistical evaluation is considered a valuable instrument in the efforts of optimizing biological transformation processes such as anaerobic digestion.

5.5.6 Optimization of response variables

The purpose mixture blending is to optimize the desired response variable(s) **Y(s)**, by testing various combinations of components and their effects on the overall mixture. The optimization process is used to select the optimal mixture proportions from a determined range that would improve AcoD; and to increase methane yield and the maximum specific methane generation rate.

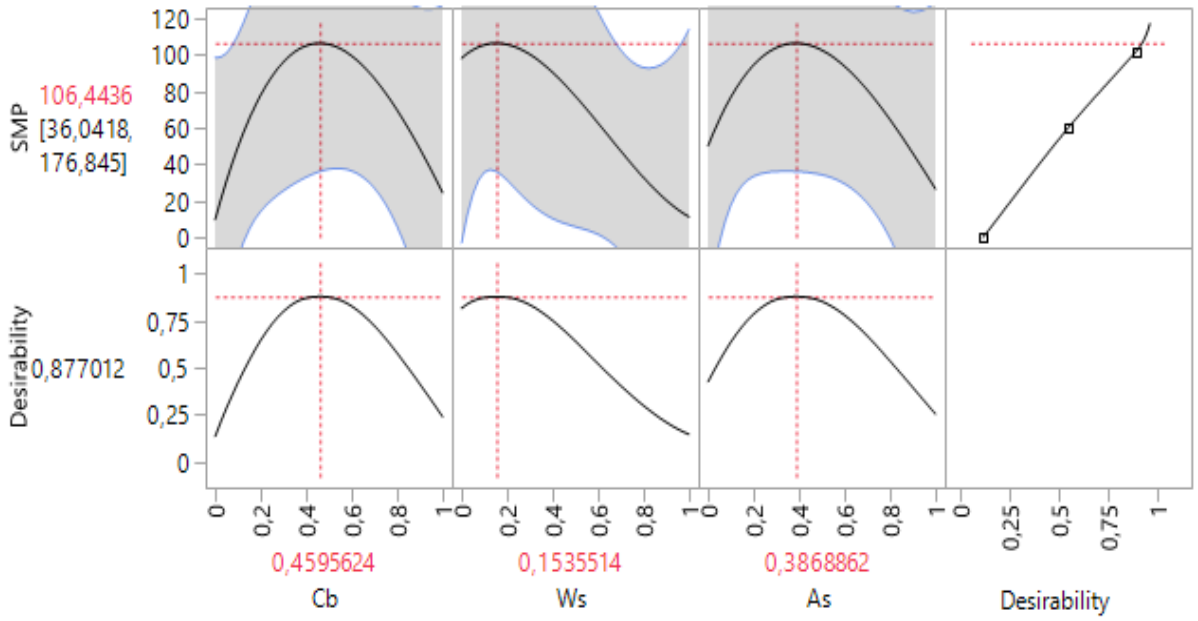


Figure 5-13: Mixture model prediction profiler

The two response variables had different optimal mixture combinations, and SMP was prioritised as a response variable of interest. The Optimum mixture blend was determined to be 46 % of $\mathbf{C_b}$, 15 % of $\mathbf{W_s}$ and 39 % of $\mathbf{A_s}$ on the VS basis that yielded 106 $NmLCH_4/g$ VS, with a $\boldsymbol{\mu_m}$ of 7.74 $NmLCH_4/g$ VS day (**Figure 5-13**). The desirability was found to be 87 %, which was a good indicator for optimization.

5.6 Conclusion and recommendations

In this present work, the AcoD of abattoir solid waste, cow blood and winery solid wastes was studied employing **ABCD** screening mixture design of experiments. These designs were used to determine the existence of synergistic and/or antagonistic interactions using statistical tools to assess the overall **AcoD** performance. The SMP improvements recorded a 29.3 % increase in comparison to individual performance of individual fractions obtained using linear models. Note that this increase was attributed to synergistic effects from some of the studied mixture blends. The overall results enabled the proper screening of candidate mixture blends for optimization of AcoD performance using statistical tools. The highest SMP was obtained from $\mathbf{M_9}$ mixture blends (111 $NmLCH_4/gVS_{added}$) with $\boldsymbol{\mu_m}$ of 6.5 $NmLCH_4/gVS$ day; while the model predicted optimal conditions was that of a mixture blend consisting of 46 % $\mathbf{C_b}$, 39% $\mathbf{A_s}$ and 15 % $\mathbf{W_s}$, which would yield a slightly less SMP of 106 $NmLCH_4/g$ VSadded and corresponding $\boldsymbol{\mu_m}$ of 7.7 $NmLCH_4/gVS$ day. Thus, with the above results considered further optimization studies considering the various input parameters (e.g. F/M ratio, organic load, temperature, pH, etc.) that would result in maximal yields are needed.

CHAPTER 6

6 Optimization via Response Surface Methodology

6.3 Abstract

This study evaluated anaerobic co-digestion of abattoir solid wastes, cow blood and winery solid wastes and the effects of selected influential process parameters namely, organic load, food to microorganism's (F/M) ratio, and feed initial pH. Response surface methodology (**RSM**) with the central composite rotatable design (**CCRD**) design of experiments which determined the specific methane production (**SMP**) and maximum specific methane generation rate (μ_m) were found to be strongly influenced by organic load and F/M ratio while initial pH partially affected the response(s). The experiments were conducted under batch mesophilic temperature (38±5 ^oC) range. The anaerobic co-digestion (**AcoD**) was conducted with a retention time of 32 days. The **SMP** was measured using a tailor-made liquid displacement unit, and μ_m was determined via kinetic mathematical modelling. The Logistic and Richard's models were found most suitable for simulation of experimental data with coefficient of determination (R^2) of above 99%; while, the highest **SMP** was determined to be 316 $NmLCH_4/g\ VS_{added}$ with a μ_m of 18 NmL/gVS day. **RSM** optimization was successful with R^2 value of 96.9% with an adequate precision (signal-to-noise ratio) of 12.54. Thus, further usage of abattoir solid wastes, cow blood and winery solid wastes is encouraged for enhanced biomethane production.

Keywords: Anaerobic co-digestion, Specific Methane Production, Bio-energic, Winery solid wastes, Abattoir solid wastes, Cow blood, Response Surface Methodology, Central Composite Rotatable Design

6.4 Introduction

The optimization of bioprocess parameters is an important step for the success of anaerobic digestion (**AD**) process. There have been several studies in the literature (Lee, Lee and Park, 1999; Blonskaja, Menert and Vilu, 2003; Cirne *et al.*, 2007; Pecorini *et al.*, 2016; Affes *et al.*, 2017) that were conducted to improve the efficiency of **AD** process by including substrate pre-treatment and multi-stage processing. Although, these methods have been successfully implemented in laboratory scale, the economic feasibility during scale-up to commercialization has been proved to be difficult in certain instances (Ek *et al.*, 2011; Curry and Pillay, 2012).

Thus, there are several initiatives, including simultaneous substrate processing (anaerobic co-digestion) and parameter optimization, that were implemented instead of using pre-treatment and multi-stage processing. This chapter, therefore, aimed at evaluating and optimizing identified significant process environmental conditions for enhanced biogas production from anaerobic co-digestion (AcoD) of abattoir solid wastes (**A_s**), cow blood (**C_b**) and winery solid waste (**W_s**). Note that the optimization study was conducted in batch laboratory-scale digesters by applying experimental data to the response surface methodology (**RSM**) model. **RSM** is a set of mathematical and statistical techniques used in modelling analysis of experimental data, where the response variable of interest is affected by various input variables and optimization of the response is set as the primary objective (Lenth, 2015). In this study, the primary objective is determination of the influence of organic loading, F/M ratio and initial pH on the overall AcoD process using laboratory bench scale reactors in batch mode, and eventually optimization of specific methane production (SMP) and maximum specific methane generation rate (**μ_m**) using **RSM** with a Central Composite Rotatable Design (**CCRD**).

6.5 Aim and Objectives

The aim and objective of this study were to improve the **AcoD** of **A_s**, **C_b** and **W_s** for efficient and stable biogas production using **RSM** optimization for a **CCRD**, by:

- Optimizing the substrate organic loading (**OL**) concentrations;
- Substrate initial substrate **pH**; and
- Food-to-microorganisms (**F/M**) ratio.

6.6Materials and Methods

Standard methods and protocols described in detail in **CHAPTER 3** were followed throughout this study.

6.6.1 Substrate and inoculant

Three different substrates were used in this study, namely abattoir solid (**A_s**) and liquid cow blood (**C_b**), and winery solid (**W_s**) waste. A detailed description of the substrate collection, preparation and preservation can be found in **section 3.1**

6.6.2 Experimental set up and procedure

All details about experimental set-up and procedure are provided in **CHAPTER 3 section 3.2** to **section 3.7**

6.6.3 Data Analysis

All details on data analysis are provided in **CHAPTER 3 section 3.8.1** to **section 3.8.8**

6.7 Presentation and discussion of the research findings

6.7.1 Biomethane potentials, kinetic model selection and regression analysis

The results from BMP assays, AcoD performance and bio-energic parameters are presented in **Table 6-1**. The BMP test results were grouped into their respective experimental design categories, namely, corner-; alpha-; and center-points.

Table 6-1: Specific methane yield, AD kinetic model and bio-energic parameter estimates from the CCRD designed experiments

Run	Kinetic Model	SMP		Error	μ_m	λ	v	R^2
		$NmLg^{-1}VS_{added}$		%	$NmLg^{-1}VS\ day^{-1}$	day		
		Experimental	Model					
1	Logistic	229.63	226.06	1.56	12.313	11.327	--	0.990
20	Logistic	54.43	52.73	3.12	1.739	0	--	0.975
15	Richards	148.89	148.52	0.25	9.512	9.785	2.574	0.994
3	Logistic	273.81	271.83	0.72	18.311	14.132	--	0.996
8	Richards	142.31	141.42	0.63	7.947	8.783	2.559	0.994
17	Richards	133.63	133.37	0.19	8.374	9.173	2.911	0.994
11	Logistic	316.17	313.14	0.96	17.896	10.278	--	0.996
18	Richards	136.40	135.39	0.74	6.826	5.780	2.509	0.996
16	Logistic	116.62	113.30	2.85	5.473	10.545	--	0.992
2	Richards	119.26	117.16	1.76	6.665	11.462	10.180	0.997
9	Richards	148.71	147.76	0.64	8.279	8.829	2.572	0.994
5	Richards	148.18	144.71	2.34	8.137	8.835	2.391	0.994
12	Richards	83.42	82.81	0.73	4.835	10.428	2.502	0.996
10	Logistic	197.91	195.78	1.08	9.637	5.967	--	0.998
7	Richards	94.53	94.26	0.29	4.480	6.154	5.566	0.994
13	Richards	132.45	131.90	0.42	7.722	8.747	2.600	0.994
4	Logistic	208.74	207.31	0.69	11.401	8.063	--	0.993
19	Richards	61.59	61.31	0.45	3.366	7.908	3.022	0.994
14	Richards	139.80	138.98	0.59	8.081	9.003	2.318	0.994
6	Gompertz	28.15	28.14	0.04	2.023	0	--	0.938

μ_m: **M**aximum specific methane generation rate; **λ:** Lag phase; **v:** Richards model shape factor; and **nr**: Data not reported

The AcoD profiles of the design center space observed (see **Figure 6-1**) agreed well with previous observations made from the mixture design studies, in which the studied mixture (**M_9**) for optimization studies was chosen (see **CHAPTER 5**). The average SMP, which was estimated to be around 140.88 ±8.43 ($NmLg^{-1}VS_{added}$) was in a reasonable agreement with the

results obtained from **M**9 namely 111.87 ($NmLg^{-1}VS_{added}$), when considering organic loading and **F/M** ratio in the center point design that were applied in **M**9 blends.

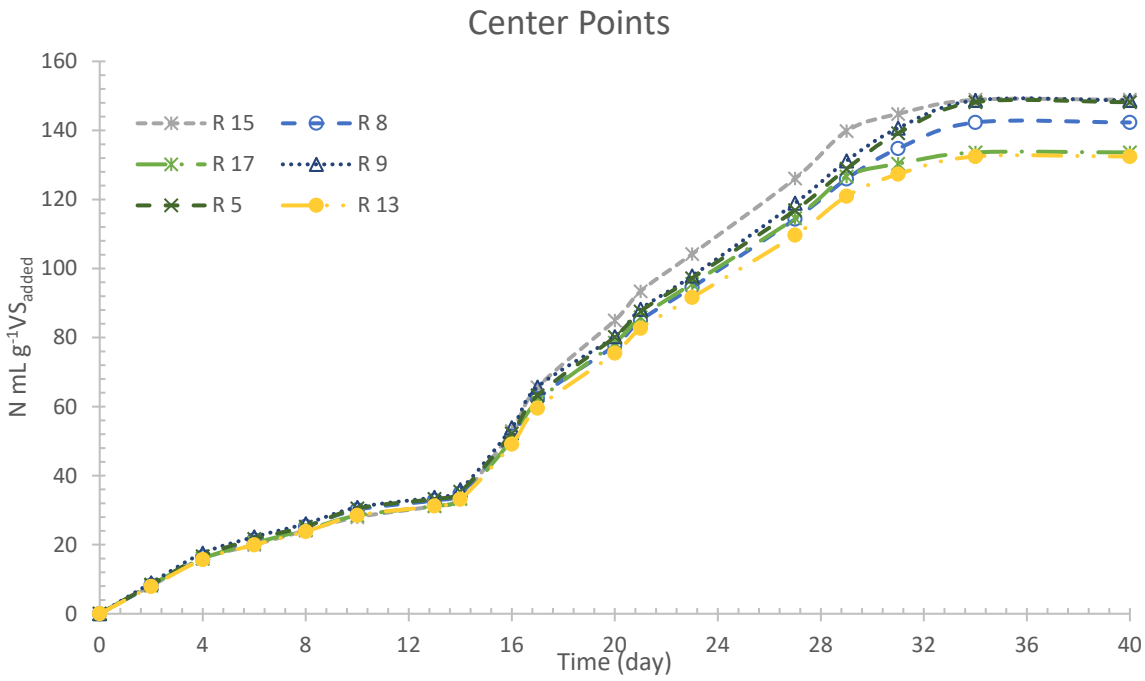


Figure 6-1: Cumulative specific methane yield for center points from the **RSM** study of abattoir and winery waste AcoD via **CCRD**

The kinetic modelling study evaluation of their bio-energic parameters had similar characteristics, where non-linear regression curve fitting of experimental data points that were used in the modified Richard's model with coefficient of determination ($\mathbf{R^2}$) of 0.994. The maximum specific methane generation rate ($\boldsymbol{\mu_m}$) and lag time ($\boldsymbol{\lambda}$) for biomethanation were estimated to be around 8.43 ±0.71 ($NmLg^{-1}VS\ day^{-1}$) and 8.99 ±0.23 days, respectively with a shape factor (**v**) of 2.601 ±0.21. The observations made from the design center space were a reasonable sufficient basis for the navigation of the entire design space, and an easy way of evaluation of digestion performance.

The highest overall observed SMP of 316.17 ($NmLg^{-1}VS_{added}$) was obtained within the design space from run eleven ($\mathbf{R}_{11}$) (see **Figure 6-2**) and experimental data points perfectly fitting the Logistic model with a $\mathbf{R^2}$ of 0.996, and $\boldsymbol{\mu_m}$ and $\boldsymbol{\lambda}$ of 17.90 ($NmL\ g^{-1}VS_{added}\ day^{-1}$) and 10.28 days, respectively, were determined from non-linear regression analysis of experimental observations using SPSS statistical software package.

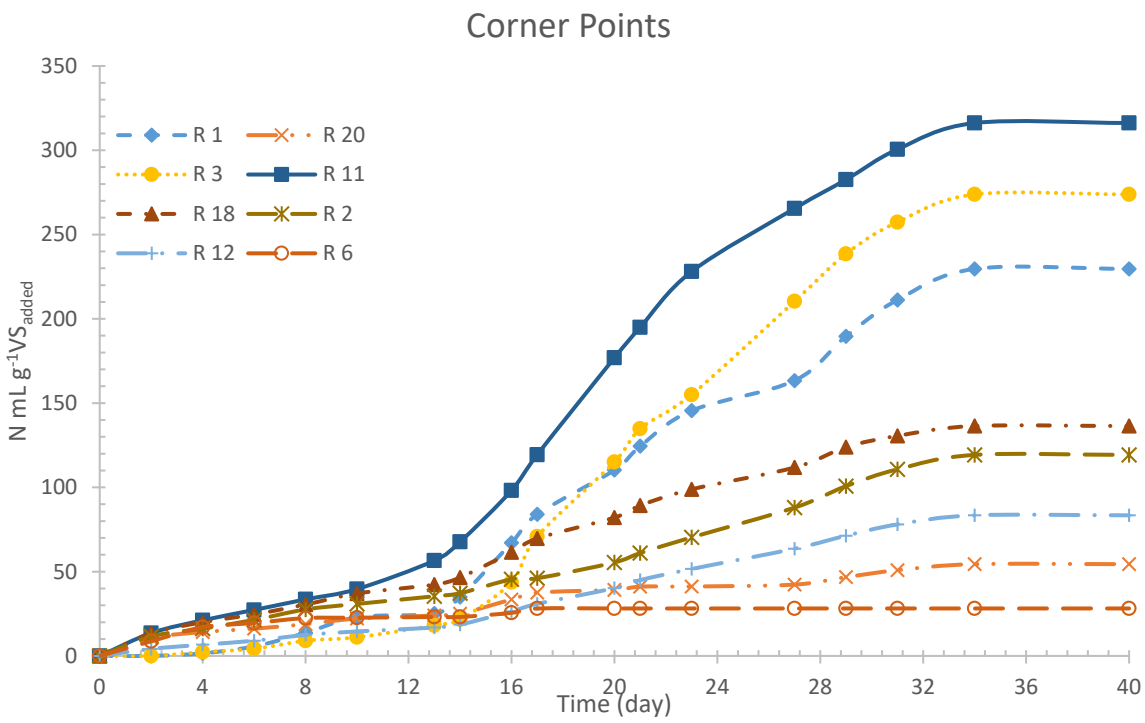


Figure 6-2: Cumulative specific methane yield for experimental (corner) points from the **RSM** study of abattoir and winery waste AcoD via **CCRD**

The second and third best observable methane production of 229.63 and 273.81 ($NmLg^{-1}VS_{added}$) were from $\mathbf{R}_1$ and $\mathbf{R}_3$, respectively where both **AcoD** profiles from the respective experimental runs followed the Logistic model and determined $\boldsymbol{\mu_m}$ and $\boldsymbol{\lambda}$ of 14.13 ($NmLg^{-1}VS_{added}$ day^{-1}); 18.31 days; 11.33 ($NmLg^{-1}VS_{added}$ day^{-1}) and 12.31 days.

For $\mathbf{R}_8$ and $\mathbf{R}_2$ AcoD, kinetic profiles had similar characteristics to the design center point experimental runs with slightly negative deviations from the average SMP**,** which were 136.40 and 119.26 ($NmLg^{-1}VS_{added}$) for the respective runs. For $\mathbf{R}_2$ the influence of factor levels was much stronger as SMP was significantly reduced to 83.42 ($NmLg^{-1}VS_{added}$) at these settings, however. However, similarities in AcoD kinetic profiles to the center were observed.

The worst AcoD performance was observed in $\mathbf{R}_{20}$ and $\mathbf{R}_6$ exhibiting significantly inhibited microbiological systems, with poor $\boldsymbol{\mu_m}$ of 1.74 and 2.02 ($NmLg^{-1}VS_{added}$), respectively even though methane generation was immediate and more especially $\mathbf{R}_{20}$ was able to produce a reasonable total methane volume around 240 (mL), but still with poor SMP considering the organic strength of the mixture.

The star/alpha design points were set to evaluate individual factor influence beyond the design space determined to be **-1.68** and **1.68** for the minimum and maximum limits, respectively.

Amongst these set of experimental runs two produced a positive response when compared to the center namely, $\mathbf{R}_4$ and $\mathbf{R}_{10}$ whose SMP were of 208.74 and 197.91 ($NmLg^{-1}VS_{added}$), respectively and which agreed well with the Logistic model with $\mathbf{R^2}$ above 0.990, $\mathbf{R}_4$ had the third highest overall estimated $\boldsymbol{\mu_m}$ of 11.401 ($NmLg^{-1}VS_{added}$) which was comparable with that obtained from $\mathbf{R}_1$ with a slightly shorter lag phase of 8.06 days for the former (see **Figure 6-3**). There was no significant deviation in the analysis of results from $\mathbf{R}_{14}$ and $\mathbf{R}_{16}$, while significant individual factor influence was observed for $\mathbf{R}_7$ and $\mathbf{R}_{19}$.

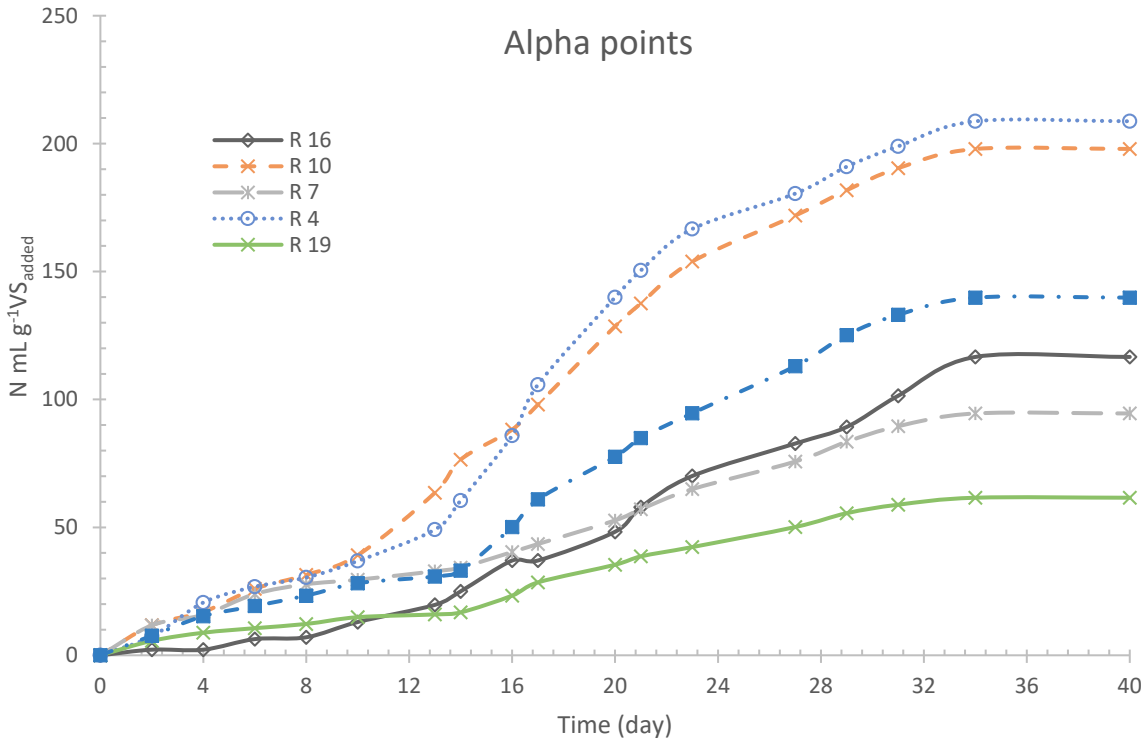


Figure 6-3: Cumulative specific methane yield for star (alpha) points from the **RSM** study of abattoir and winery waste AcoD via **CCRD**

6.7.2 RSM optimization and factor interactions analysis

The experimental runs determining and predicting biomethane yields from the **CCRD** for AcoD of abattoir and winery solid waste, and the regression model equation and coefficients are presented in **Table 6-2**. A second-order polynomial model failed to predict the response (**Y**) as there were significant variations in the observed $\mathbf{R}^2$-value and **adj.** $\mathbf{R}^2$-value which were used to validate the model-efficiency for practical applications.

Thus, a third-order polynomial was generated by adding cubic effects of $\mathbf{X_1}$ which was a more significant factor based on the **F**-value obtained from the second-order polynomial regression.

Table 6-2: Surface response study of BMP from AcoD of abattoir and solid winery waste

Run	Coded Values			Real Values			Actual	Predicted
	x_1	x_2	x_3	Organic load	F/M	pH		
1	-	+	+	1.5282	0.31	8.08	229.63	203.27
20	1	1	1	4.9781	1.00	8.00	54.43	46.43
15	0	0	0	3.2304	0.65	7.25	148.89	141.75
3	-	+	-	1.5197	0.30	6.51	273.81	275.21
8	0	0	0	3.2891	0.66	7.29	142.31	141.75
17	0	0	0	3.2799	0.66	7.24	133.63	141.75
11	-1	-1	-1	1.4926	0.30	6.61	316.17	309.01
18	-1	-1	1	1.5214	0.30	80.8	136.40	138.21
16	-α	0	0	0.7965	0.16	7.26	116.62	127.34
2	1	-1	-1	5.0042	1.00	6.62	119.26	130.47
9	0	0	0	3.2861	0.66	7.29	148.71	141.75
5	0	0	0	3.2935	0.66	7.29	148.18	141.75
12	1	-1	1	4.9889	1.00	8.02	83.42	66.87
10	0	0	-α	3.2444	0.65	6.21	197.91	197.45
7	α	0	0	5.7785	1.16	7.30	94.53	105.25
13	0	0	0	3.2816	0.66	7.25	132.45	141.75
4	0	-α	0	3.3344	0.67	7.25	208.74	207.79
19	0	0	α	3.2437	0.65	8.37	61.59	83.48
14	0	α	0	3.2594	0.65	7.27	139.80	162.18
6	1	1	-1	5.0093	1.00	6.52	28.15	11.18

$\mathbf{X_i}$ – factor; i = 1; 2; and 3 is organic load, **F/M**, and pH, respectively

All response surface model regression coefficients were statistically validated using the ANOVA tool in the JMP software package (JMP® Pro 13.0.0, SAS Institute Inc., USA), and the results are presented in **Table 6-3**.

The large **F**-ratio (variance) and low associated **p**-values (probability) from the statistical evaluation of the AcoD optimization results were good indicators of significance of coefficients used by the model, with a strong influence on SMP response with probabilities ($p < 0.05$). The coefficient of determination ($\mathbf{R}^2$) for the fitted third-order polynomial model was determined to be 0.9690 with an **adj.** $\mathbf{R}^2$ of 0.9345 which was as well considered significant. The Adequate Precision, also known as the signal to noise ratio, was 12.54, which indicated a passable signal for using the model to appraise the design space boundaries.

The Model **F**-ratio of 28.12 indicated the model's significance, with only a <0.01% probability of an **F**-ratio this large occurring as noise. All linear terms, quadratic- and cubic term(s) $(\mathbf{X_2})^2$, $(\mathbf{X_1})^2$ and $(\mathbf{X_1})^3$, respectively, were found to be the significant model contributors.

Table 6-3: Test of significance and ANOVA for all regression coefficients

Source	Sum of Squares	d*f*	Mean Squares	F-Ratio	p-Value
X_1	45179.738	1	45179.738	135.2064	< ,0001
X_2	2510,751	1	2510,751	7,5138	0,0228
X_3	15681,351	1	15681,351	46,9285	< ,0001
X_1*X_2	3653,843	1	3653,843	10,9346	0,0091
$X_1* X_3$	5745,384	1	5745,384	17,1938	0,0025
X_2*X_3	4886,156	1	4886,156	14,6225	0,0041
$(X_1)^2$	1167.486	1	1167.486	3.4939	0,0944
$(X_2)^2$	3367.672	1	3367.672	10.0782	0,0113
$(X_3)^2$	2,964	1	2,964	0,0089	0,9270
$(X_1)^{3*}$	36693,922	1	36693,922	59,2190	< ,0001
Model	93952,980	10	9395,30	28.12	< 0.0001
Error	3007,385	9	334,15		
Lack of Fit	2716,1253	4	679,031	11.66	0.0095
Pure Error	291,2593	5	58,252		
R-Squared	0.9690				
R-Squared Adj	0.9345				
RMSE	18.28				
Mean of Response	145.7315				
Adequate Precision	12.54				
Press	49226,70				

*__NB:__ higher-order polynomials appear to better fit the data but may result in overfitting of the modelling terms

While the quadratic effects of initial pH $(\mathbf{X_3})^2$ were found to be insignificant. The goodness of fit of the model was analyzed using the coefficient of determination ($\mathbf{R}^2$). The Lack of Fit **F**-ratio of 11.66 proved the Lack of Fit to be non-significantly relative to the pure error considering that there was only a 0.95% probability that an **F**-ratio this large could occur, which meant that the model could be used to closely simulate the specific methane yield from **AcoD** of abattoir and winery solid waste.

In **Figure 6-4** a plot of the **RSM** predicted against actual/measured SMP is presented. This was obtained through multiple regression analysis of experimental data fitting the special cubic polynomial model (equation 6-1**)** to simulate the SMP (**Y**) response to the three coded independent factors, namely, organic load ($\mathbf{X}_1$), **F/M** ratio ($\mathbf{X}_2$), and pH ($\mathbf{X}_3$) and interactions amongst factors.

$$\mathbf{Y}\left(\frac{NmL}{gVS}\right) = 141.75 - 126.11\mathbf{X_1} - 13.56\mathbf{X_2} - 33.89\mathbf{X_3} - 21.37\mathbf{X_1X_2} + 26.8\mathbf{X_1X_3} + 24.71\mathbf{X_2X_3} - 9.00(\mathbf{X_1})^2 + 15.29(\mathbf{X_2})^2 - 0.45(\mathbf{X_3})^2 + 42.26(\mathbf{X_1})^3 \quad (6\text{-}1)$$

The optimal conditions for the AcoD of abattoir and winery solid waste mixture (**M**$_9$) were obtained by solving the above polynomial expression in JMP statistical software package. The highest predicted SMP by the **RSM** model with a desirability of 86.89% was 309.01 with lower and upper limit of 273.89 and 344.14 ($NmLg^{-1}VS_{added}$), respectively for statistically determined optimum conditions set at $\mathbf{X}_1$= -1 (1.5 $gVSL^{-1}$), $\mathbf{X}_2$ = -1 (0.25 $gVS_{substrate}g^{-1}$ $VS_{inoculum}$) and $\mathbf{X}_3$ = -1 (6.5).

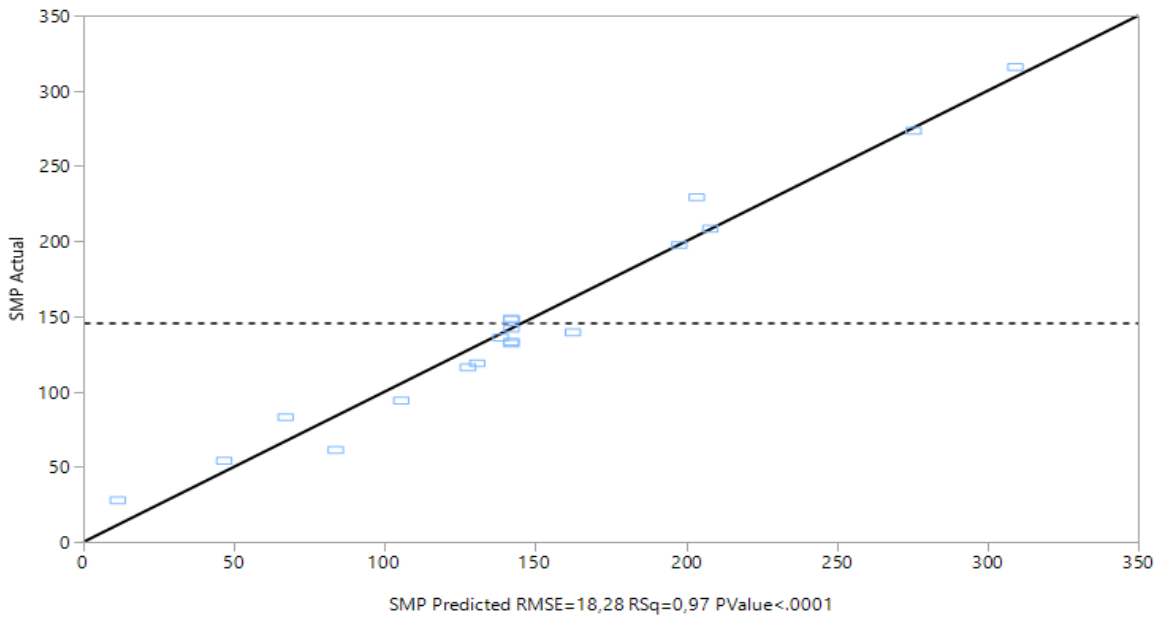


Figure 6-4: Graph of **RSM** predicted against actual **SMP** for **AcoD** of abattoir and winery solid waste

To validate the efficiency of the model for predicting the **SMP,** the determined conditions optimum were applied to three independent replicates where the average SMP observed was 316.17 ($NmLg^{-1}VS_{added}$), which was slightly higher than predicted although it fell within the 95% mean confidence interval range. The combined effects of organic load and F/M ratio are depicted in **Figure 6-5**. It was noted that an increase in organic load and F/M ratio beyond the centre point resulted in a reduction of SMP, while conversely reducing these two factors to a certain degree improved SMP. Feng *et al.*, (2013) found that a lower F/M ratio reduced the technical AD time, while improving methane production because of the reduction in accumulated VFA's concentration.

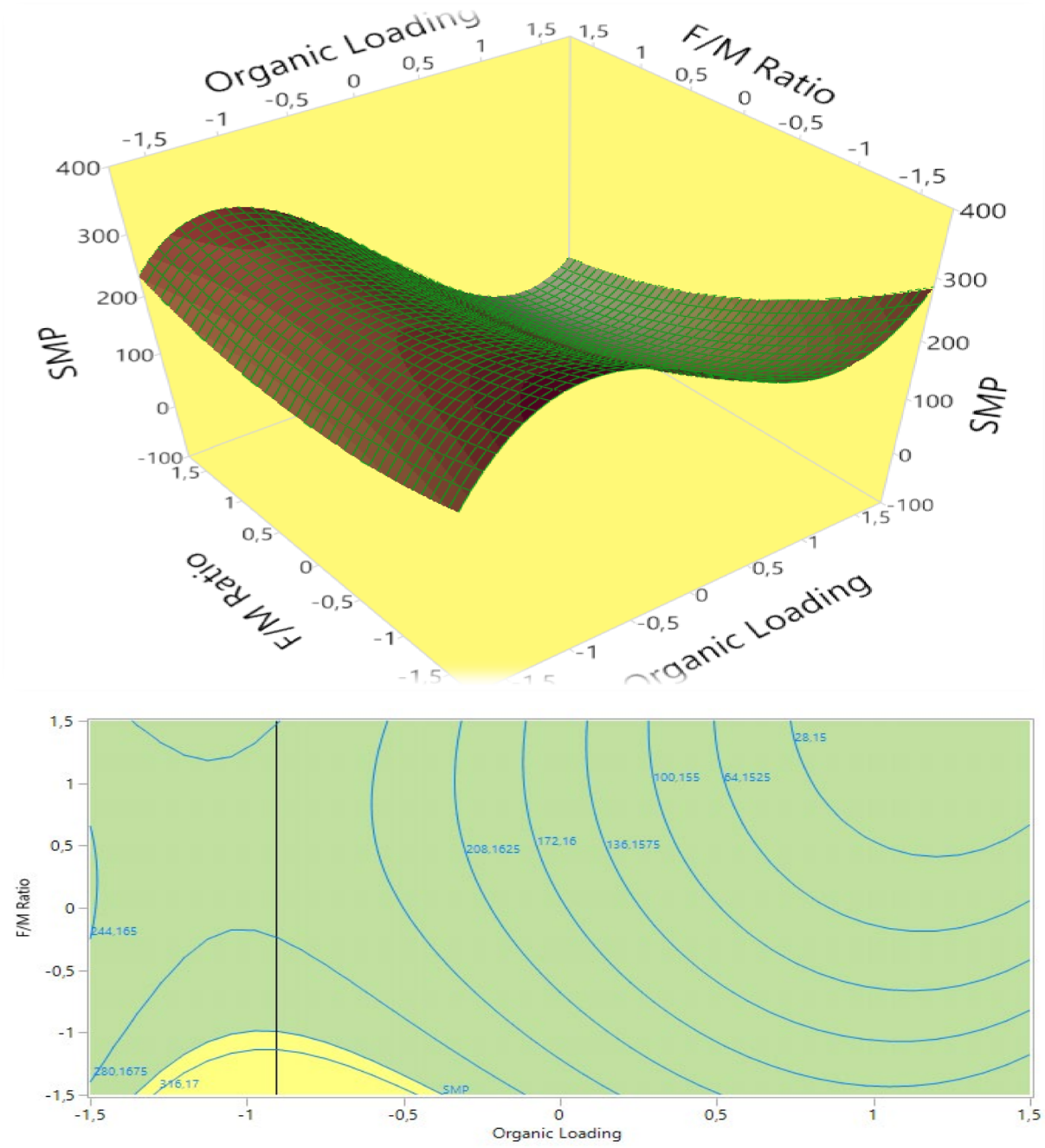


Figure 6-5: Response surface **3-D** curvature plot and contours for organic load and F/**M** ratio effects on the **AcoD** of abattoir and winery solid waste.

However, further decrease in F/M ratios could yield a negative results as it was noted by the study conducted by Y. Yoon *et al.* (2014) for piggery slaughterhouse waste, where an increase in F/M ratio from 0.1-1.5 resulted in increased methane yield for intestine residues, while that of blood increased with F/M ratio of 0.1-1 with further increase to 1.5 decreasing methane yield which was attributed to the high protein content of the blood waste that increased NH_3-NH_4^+ concentration inhibiting the **AD** process. Both individual and combined factor effects were

deemed to be significant ($p < 0.05$), quadratic effects of both organic load and F/M ratio were found to be found to be insignificant ($p > 0.05$), while the special use of cubic effects of organic load was found to be significant.

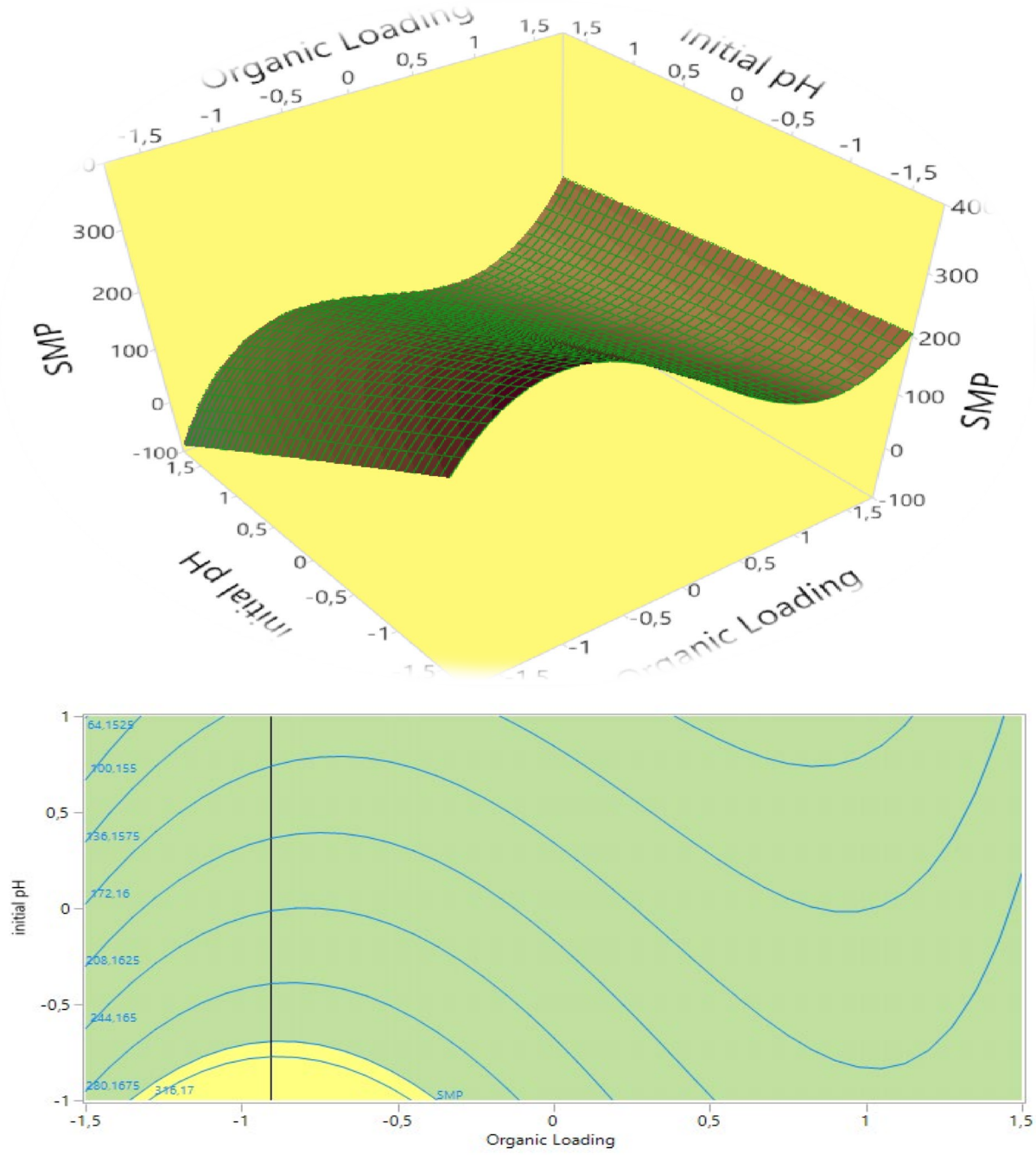


Figure 6-6: Response surface **3-D** curvature plot and contours for organic load and pH ratio effects on the **AcoD** of abattoir and winery solid waste

Similarly, increases in organic load and initial pH resulted in poor digester performance while the reductions improved performance significantly (see **Figure 6-6**). This result could be

largely attributed to the fact that various micro-organism existing within in **AD** systems have different optimal pH performance.

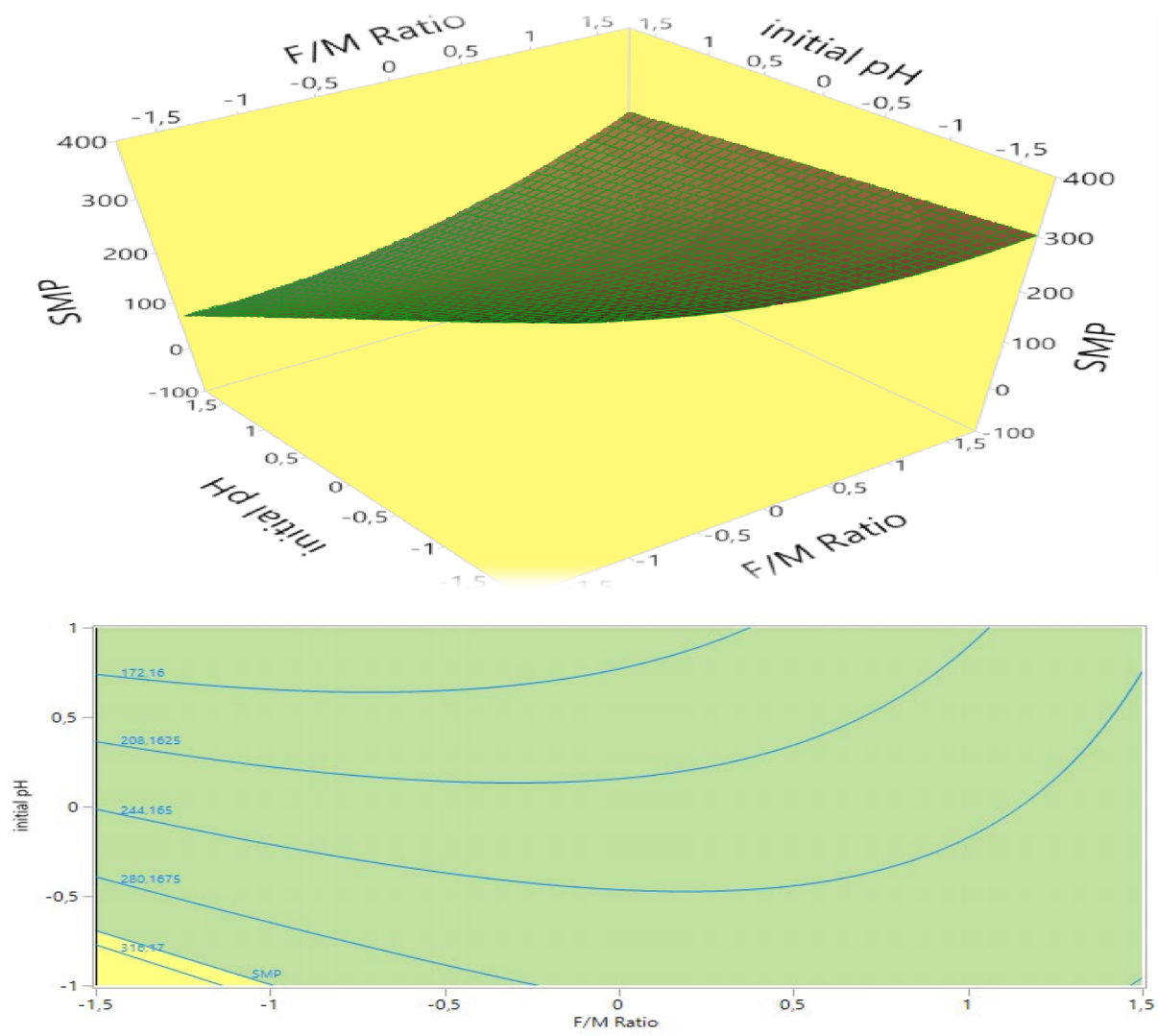


Figure 6-7: Response surface **3-D** curvature plot and contours for **F/M** ratio and pH effects on the AcoD of abattoir and winery solid waste

In this study's case lowering the pH during start-up phase improved hydrolysis rate enhancing the conversion of slowly biodegradable fraction of abattoir wastes. Even though methanogens are notably sensitive to extremely low pH at values around 6.5, the system performance was not affected (see **Figure 6-7**). Similarly, Sibiya, Muzenda and Tesfagiorgis, (2014) found a pH of 6.5 to be optimal for anaerobic digestion of grass silage, hence, the result being attributed to rapid growth of hydrolytic and acidogenic bacteria required for breakdown of organic matter. In the confirmation of this view, Latif, Mehta and Batstone (2017) further highlighted that a pH of 6.5 could yield added benefits in the access of material that is non-degradable at higher

pH values which can be proven useful in two-stage **AD** systems, where the first-stage is operated at lower pH. A cubic plot of the **RSM** study is presented in **Figure 6-8** which summarizes the detailed results of this study, where the highest **SMP** can be found the beyond the corner points.

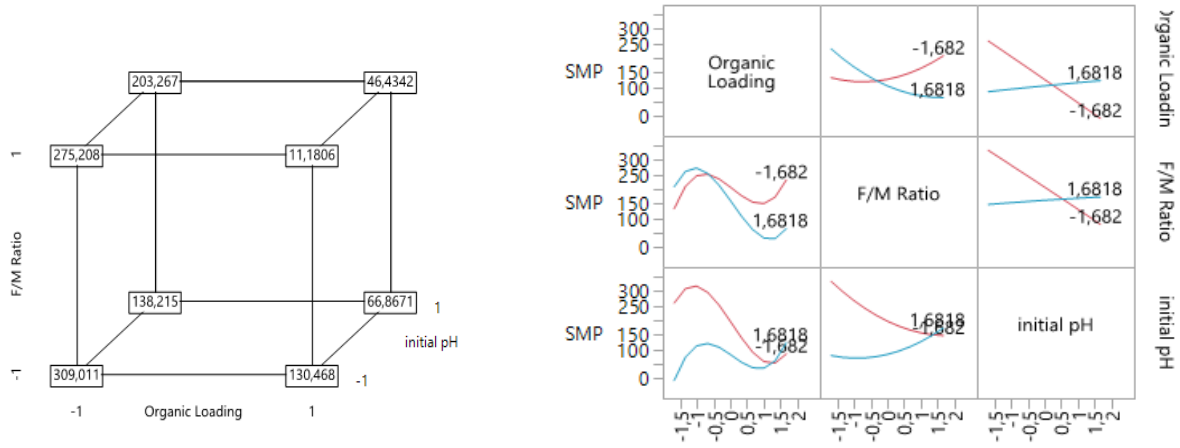


Figure 6-8: Cube plot and interaction profiles representation of the **RSM** model for the optimization of input factors

6.8 Conclusion and recommendations

The **AcoD** of abattoir solid wastes, cow blood and winery solid wastes was evaluated and found to enhance the specific methane yield. In this study the optimal conditions for enhanced methane production from a mixture blend of abattoir solids, cow blood and winery solid wastes were determined to be organic load, F/M ratio and initial pH of 1.59 gVS/L, 0.25 gVS/gVS and 6.5, respectively which would yield a predicted SMP of 309 $NmLg^{-1}VS_{added}$ with a maximum desirability of 86.9%. The closest run to the optimal was that of R_{11} which yielded a SMP of 316 $NmLg^{-1}VS_{added}$ with input factors of organic load, F/M ratio and pH of 1.5 g VS/L, 0.3 gVS/gVS and 6.6, respectively

CHAPTER 7

7 LAB-SCALE AcoD EXPERIMENTS

7.1 Abstract

This study compared the operational efficiency of batch, single- and two-stage semi-continuous anaerobic co-digestion of a mixture of abattoir solid ($\mathbf{A_s}$), cow blood ($\mathbf{C_b}$) and winery solid ($\mathbf{W_s}$) wastes. To this purpose five mixtures namely, $\mathbf{A_sC_b}$ (50:50), $\mathbf{A_sW_s}$ (40:60), $\mathbf{C_bW_s}$ (40:60), $\mathbf{A_sC_bW_s}$ (33:33:33) $\mathbf{A_sC_bW_s}$ (17:66:17) with **BMP** previously identified in the previous studies (i.e. **Chapters 4, 5** and **6**), were utilized. The tests were conducted on two custom built 5L acrylic laboratory-scale digesters equipped with a pH-control, gas-fixing, gas metering units and an automated data logging/control system for pH and temperature control. Batch experiments were conducted on all mixtures applying short retention periods of 5-15 days; and the primary emphasis was put on the $\mathbf{A_sC_bW_s}$ (17:66:17) mixtures which resulted in the highest methane efficiency and stable operation. The highest methane yield of above 500 NmL was obtained in batch mode.

Keywords: Anaerobic co-digestion; abattoir waste; winery waste; microwave pre-treatment; batch; semi-continuous, two-stage semi-continuous

7.2 Introduction

In this chapter, co-digestion of abattoir and winery waste was explored using two laboratory scale five-liter anaerobic digesters considering the optimum compositions and environmental applicable conditions that were determined in the previous chapters.

The rise in population, and the increased demand of food and energy have become popular more significantly in the meat production and beverage processing industries. Consequently, abattoirs and wineries have been producing large quantities of organic residual wastes, which were traditionally used after downstream processing as rich protein source for animal feeding. In the case of abattoir waste and winery solid waste that were sent to distilleries for recovery of ethanol and tartaric acid small portions of those wastes were directly disposed of by land spreading (Da Ros *et al.*, 2014). However, stringent legal restrictions, rising treatment costs and environmental consciousness, were implemented to treat organic residues and wastes particularly those from wastewater works treating high strength organic wastes such as those from the meat and wine processing industries. With the increasing awareness that was due to

animal diseases outbreaks such as transmissible encephalopathy (TSE) or scrapie, tighter process control and hygiene have restricted downstream animal by-products utilization (Pesta G., Mayer Pittroff, 2007; Ortner *et al.*, 2015). Furthermore, the disposal of organic residues and the landfilling of wastes has been legally constrained, with high disposal costs which led small operators to resorting to illegal dumping (Roux and Lasher-scheepers, 2016).

Thus, anaerobic digestion (**AD**) has become an attractive solid waste management tool. Apart from producing biogas, and being a green energy source, **AD** is a proven technology that destroys pathogens by yielding a stabilized material usable as fertilizer for land application for the farming industry (Schunurer and Jarvis, 2009).

However, relatively little and/or no research has been conducted on the co-treatment of solid abattoir and winery waste residues in **AD**, and with few **AD** plants built particularly for treating wastes from abattoirs, and some having diversified their operations for multiple feedstock intake (co-digestion) (Ek *et al.*, 2011). Abattoir wastes are known to be problematic feedstock in **AD** due to their high lipid and protein content (Salminen and Rintala, 2002; Battimelli *et al.*, 2010; Palatsi *et al.*, 2011; Valta *et al.*, 2015; Affes *et al.*, 2017). The degradation of proteins produces ammonia, of which the unionized form is detrimental methane producers found in the **AD**-microorganism consortium. While the lipids, are associated with scum formation, clogging of **AD** process equipment is also linked to the accumulation of long chain fatty acids (LCFAs) (Luste and Luostarinen, 2010; Manuel and Canas, 2010; Ortner *et al.*, 2015; Labatut, 2017).
A primary solution to issues associated with digestion of abattoirs waste consists of performing co-digestion experiments with other types of waste. Anaerobic co-digestion (**AcoD**) can be briefly described as simultaneous **AD** of two or multiple organic residues that differ in their physiochemical characteristics, and which can enhance microbial activity facilitating a stable system performance. Moreover, AcoD balances the macro and micronutrients availability, dilutes inhibitory and/or toxicity of the substrates and increases the allowable organic loading rates (**OLR**) (Mata-Alvarez *et al.*, 2014). The **AcoD** of abattoir with other organic waste residues was proved to be successful in wastes such as fruit and vegetables (Hassib Bouallagui *et al.*, 2009; Pagés-Díaz *et al.*, 2015), pig slurry and glycerin (Rodríguez-Abalde, Flotats and Fernández, 2017), municipal solid waste and various organic crops (Zhang and Banks, 2012), sewage sludge (Luste and Luostarinen, 2010; Borowski and Kubacki, 2015) and rendering plant waste (Bayr, 2014). Therefore, **AcoD** of abattoir waste with winery solid waste was regarded as a viable option considering the demonstrated feasibility of **AcoD** of abattoir waste

with fruit and vegetable wastes which are considered to be similar in physiochemical characteristics to winery waste by different researchers (Bouallagui *et al.*, 2004; Alvarez and Lidén, 2008; Pagés-Díaz *et al.*, 2017). However, to the best of our knowledge of existing literature, there is no information on the **AcoD** of abattoir and winery solid waste that is available, except the one that reports data focusing on the **mono-D** or either **AcoD** with wastes of similar physiochemical characteristic.

Thus, the following study was considered a novelty that required **BMP** analysis, optimization of mixture blends and organic loading concentrations for an efficient and sustainable operation that was conducted on a separate study utilizing standard **BMP** tests. For this reason, both laboratory-scale batch and semi-continuous experiments were performed on the optimal mixture blends to assess the viability of abattoir and winery waste **AcoD** for large-scale operations by examining operational parameters such as organic loading rate (**OLR**) and hydraulic retention time (**HRT**). A 2-stage semi-continuous operation was also tested to determine the effects on the overall digestion process.

7.3 Aims and Objectives

The overall aims and objectives of this study were to investigate the performance of anaerobic digestion (**AD**) of the optimal mixture blends from **BMP** studies in laboratory-scale digesters by testing the most efficient operating modes such as:

- Batch
- Single-Stage Semi-Continuous
- Two-Stage Semi-continuous

7.4 Materials and Methods

Standard methods and protocols described in detail in **CHAPTER 3** were followed throughout this study.

7.4.1 Substrate and inoculant

Three different substrates were used in this study, namely abattoir solid ($\mathbf{A_s}$) and liquid (cow blood) ($\mathbf{C_b}$), and winery solid ($\mathbf{W_s}$) waste. A detailed description of the substrate collection, preparation and preservation can be found in **section 3.1**

7.4.2 Experimental set up and procedure

All details about experimental set-up and procedure are provided in **CHAPTER 3 section 3.2** to **section 3.7**

7.4.3 Data Analysis

All details on data analysis are provided in **CHAPTER 3 section 3.8.1** to **section 3.8.8**

7.5 Results and Discussion

The anaerobic co-digestion (**AcoD**) of abattoir solid (**A_s**), cow blood (**C_b**) and winery solid (**W_s**) waste was performed through three different modes of operation namely, batch, single-stage semi-continuous and two-stage semi-continuous on a laboratory-scale at mesophilic condition (38±5 ºC)

7.5.1 Physiochemical Characteristics

To the best of our knowledge, there are no studies that comparatively explored the anaerobic co-digestion of abattoir waste together with winery solid waste. Thus, specific methane production from this study was compared with the results from BMP assay presented in the preceding chapters. The inoculum had a volatile solids-to-total solids ratio of 0.997 and a slightly neutral pH of 6.78.

Table 7-1: Physiochemical characteristics of the inoculum and co-substrates

Parameters	Units	A_sW_s	C_bW_s	A_sC_b	$A_sC_bW_s$		Inoculum
Mix ratio		(40:60)	(40:60)	(50:50)	(34:33:33)	(17:36:17)	-
pH		7.30	7.64	7.82	7.64	7.61	6.78
TS	(%)	47.64	26.53	20.55	27.91	19.80	
VS	(%)	44.05	25.23	19.88	26.45	19.22	
VS/TS		0.925	0.951	0.967	0.948	0.971	0.997
BMP_{30}	(NmL CH_4 g^{-1} VS)	369.56 [a]	354.13 [a]	104.09 [a]	85.51 [a]	111.87 [a]	-
TBMP	(NmL CH_4 g^{-1} VS)	628.15	463.32	646.77	479.90	521.54	-
TCOD	(mg O2 g^{-1} VS)	1.79	1.32	1.85	1.37	1.49	-
C/N	(mole/mole)	22.44	16.23	11.11	22.58	10.57	-

C/N is the carbon/nitrogen ratio; BMP_{30} is biomethane potential in 30 days; TBMP is theoretical biomethane potential; TCOD is theoretical chemical oxygen demand; TS is total solids; VS is volatile solids

7.5.2 Lab-scale anaerobic co-digestion batch experiments

The results from the lab-scale batch experiments are summarized in **Table 7-2**, and the daily and cumulative biomethane production (**Figure 7-1** and **Figure 7-2**) and respective gas compositions from AcoD of the five chosen mixtures under batch operation mode are illustrated. The experiments were conducted utilizing short retention periods. The first batch experiments conducted on **D_1** and **D_2** utilizing 40:60 mixtures of **A_sW_s** and **C_bW_s**, respectively showed strong signs of inhibited systems producing a fraction of the expected methane yield in comparison to the BMP (which was over 280 $NmLg^{-1}VS_{added}$) at the same organic loading

of 5 gVS L^{-1} and retention period. A poor CH_4 and high CO_2 content in the gas were measured for both $\mathbf{A_sW_s}$ and $\mathbf{C_bW_s}$, which was most likely due to significant levels of hydrogen sulphide and ammonia generated in the larger reaction volume.

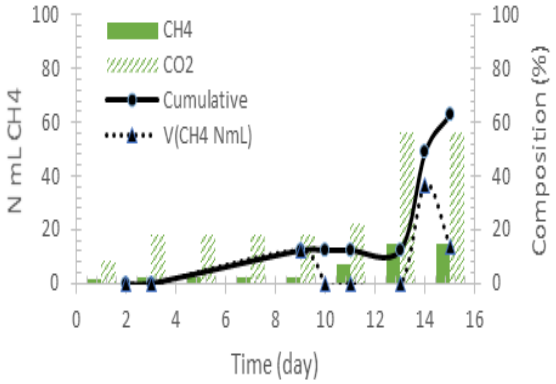
Table 7-2: Results from the five-liter laboratory-scale batch AcoD of abattoir and winery solid waste

Reactor	Substrate	Ratio	V_R (mL)	X_0 (g VS L^{-1})	F/M	Cum Yield (NmL)	CH_4	BMP [a]	T (±5 °C)	Incubation (day)
Batch: Anaerobic co-digestion with R_3 mixtures										
D_1	C_bW_s	2:3	4000	5	0.25	62.97			38	15
D_2	A_sW_s	2:3	4000	5	0.25	89.21			38	15
Batch: Anaerobic co-digestion with M_4 and M_7 mixtures										
D_1	C_bA_s	1:1	4500	1.5	0.25	151.85			38	6
D_2	$A_sC_bW_s$	17:17:66	4500	1.5	0.25	118.62			38	13
Batch: Anaerobic co-digestion with M_9 mixtures										
D_1	$A_sC_bW_s$	17:66:17	4500	1.5	0.25	486.96			38	15
D_2			4500	1.5	0.25	546.07			38	15

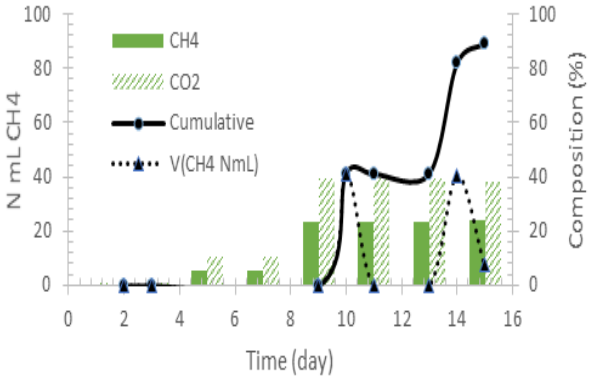
V_R: Reactor working volume; **X_0**: Organic load/concentration; **F/M**: Food to microorganism ratio; Cum CH_4: cumulative methane yield; **T**: Temperature; and Incubation: incubation period

For the second set of experiments conducted on $\mathbf{D_1}$ and $\mathbf{D_2}$, the mixtures used were $\mathbf{A_sC_b}$ and $\mathbf{A_sC_bW_s}$ (50:50 and 17:17:66 respectively), where a more stable AD operation was noted with similar biomethane production profiles and methane yields that are equivalent to the BMP results. More specifically both mixtures performed as expected and produce over 150 NmL CH_4 on the 6th day and 120 NmLCH_4 on the 13th day of operation, respectively. Similarly, BMPs (of 100 and 85 $NmLg^{-1}VS_{added}$, respectively) were obtained for the mixtures from the previous work.

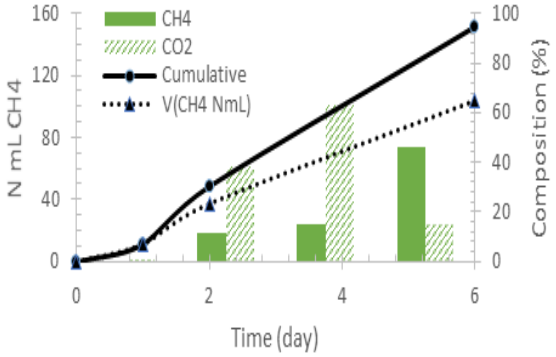
The last set of batch experiments conducted on $\mathbf{A_sC_bW_s}$ (17:66:17) mixtures recorded the highest methane yields of 490 and 550 NmLCH_4 on $\mathbf{D_1}$ and $\mathbf{D_2}$, respectively. Biomethane generation was noted on the 6th reaching and it peaked on the 15th day of operation, the total production was consistent with the BMP of the mixture (110 $NmLg^{-1}VS_{added}$) at relevant retention period. Note that the slight differences in CH_4 yields and composition from each digester can be attributed to variable changes in operating parameter (i.e. Temperature, pH, stirring, etc.).



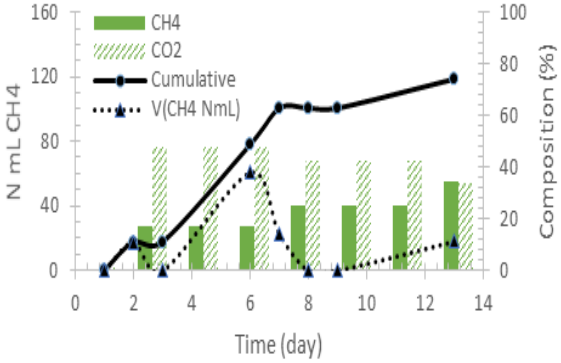
(a) AW [40:60] D1



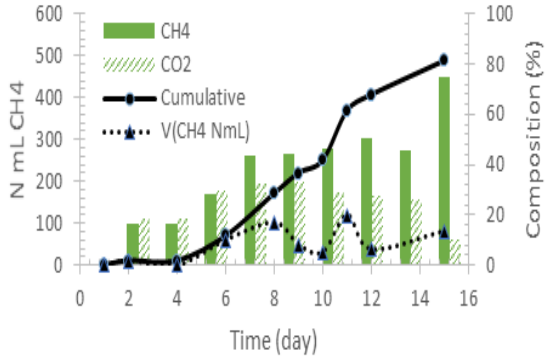
(b) BW [40:60] D2



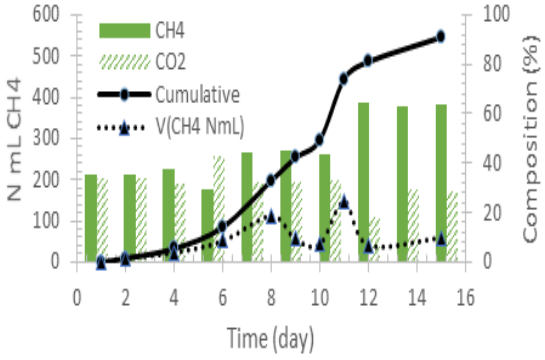
(c) AB [50:50] D1



(d) ABW [33:34:33] D2



(e) ABW [17:36:17] D1



(f) ABW [17:36:17] D2

Figure 7-1: Accumulated, daily methane and biogas composition profiles from lab-scale batch anaerobic co-digestion of abattoir and winery solid waste

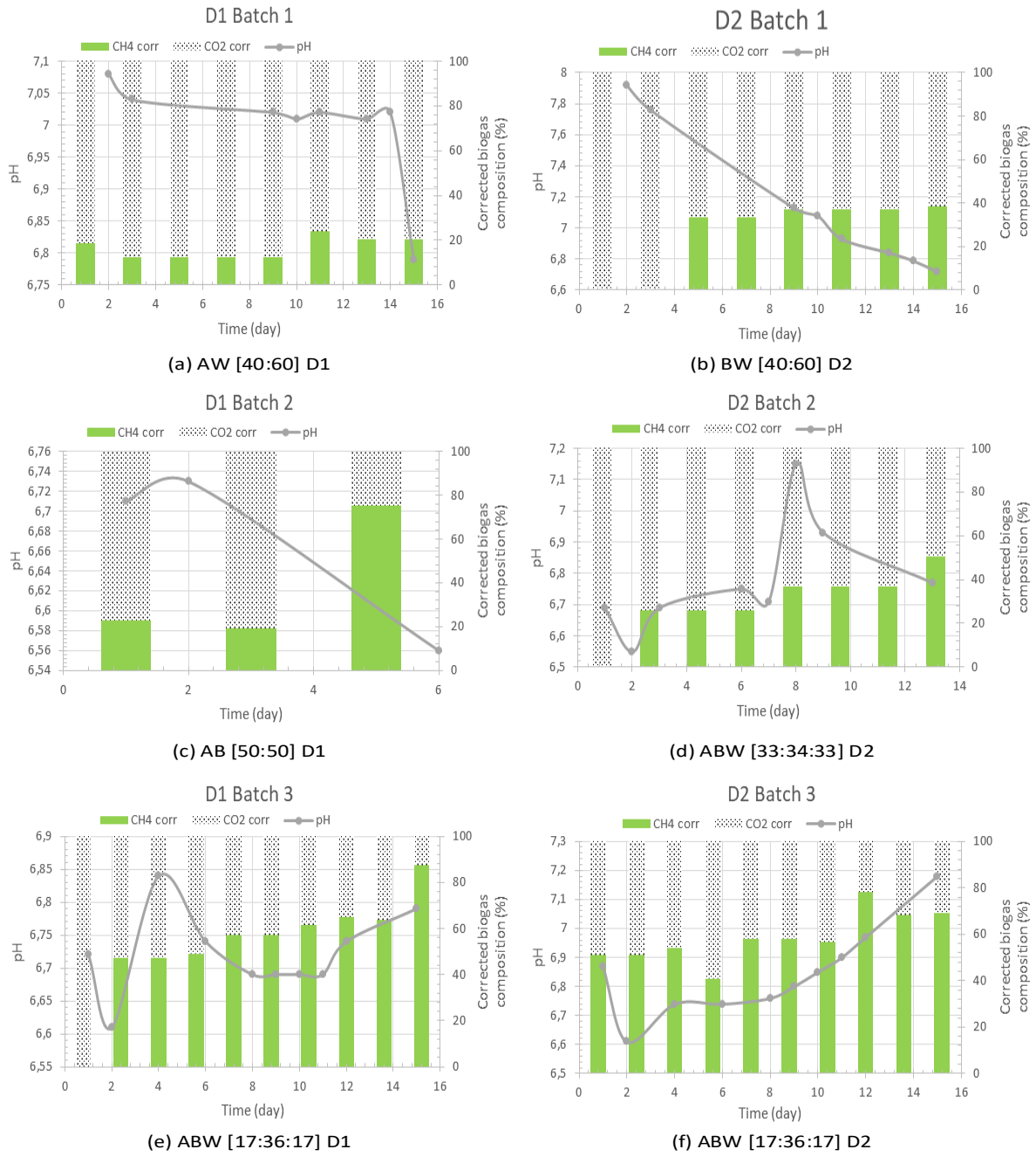


Figure 7-2: pH and corrected biogas composition profiles from the batch anaerobic co-digestion of abattoir and winery solid waste

7.5.3 Specific methanogenic activity tests

In **Table 7-3** the specific methanogenic activity (**SMA**) test results from both tests (i.e. I and II) with acetic acid as the sole substrate are presented. Specific methane generation was notably immediate for both tests with a linear trajectory towards the 2nd day of operation, after which a stationary phase occurred indicating substrate exhaustion (see **Figure 7-3**). For **SMA I** on **D_1**

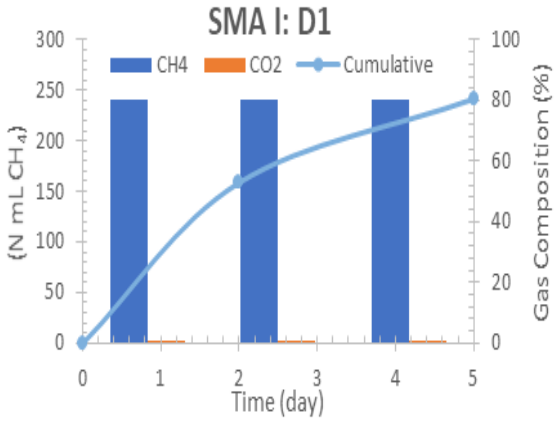
and **D_2** specific methane volumes were above 225 mL and 240 mL, respectively. The specific methane volumes were significantly higher for **SMA II** on **D_1** reaching values above 1180 mL.

Table 7-3: Specific Methanogenic Activity (**SMA**) results with acetic acid as substrate

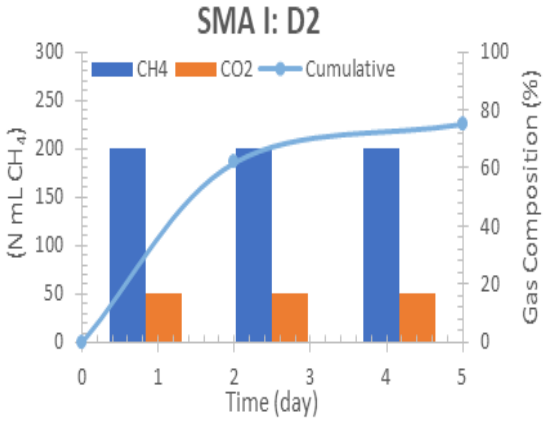
Assay	Reactor	$V_{inoc.}$ (mL)	Mass (g)	X_0 (g VS L^{-1})	V_R (mL)	T (°C)	pH	Time (day)	V CH_4 (mL)	Activity (CH_4) (g COD_{CH4} g^{-1}VSS d^{-1})
SMA_1	**D_1**	1000	16.2	3.6	4.5	38		3	225.4605	1.0716
SMA_1	**D_2**	1000	16.2	3.6	4.5	38		3	242.3135	1.1517
SMA_2	**D_1**	4000	64.8	14.4	4.5	38		3	1186.9312	2.3487

$V_{inoc.}$: Volume inoculum**; X_0: O**rganic load/concentration; **V_R**: reactor working volume; **T**: Temperature; and **V CH_4**: Volume methane produced

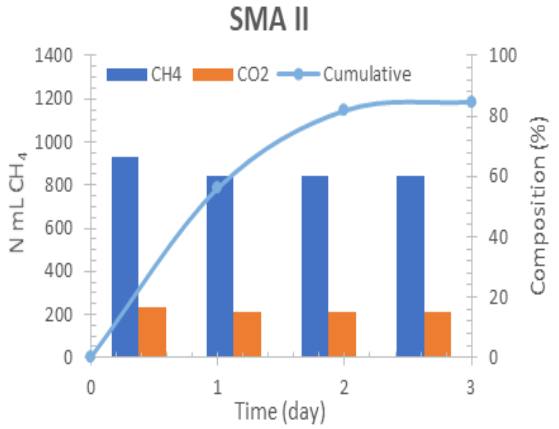
The results were significantly in agreement with the reasonable high **SMA** recorded, as for **SMA II** the methanogenic activity on acetic acid was 2.35 gCOD-CH_4 g^{-1}VSS day^{-1}; whereas for the **SMA I** on **D_1** and **D_2** an activity of 1.07 gCOD-CH_4 g^{-1}VSS day^{-1} and 1.15 gCOD-CH_4 g^{-1}VSS day^{-1}, respectively was observed. The **SMA** recorded for acetic acid were comparable with the results of 2.39 gCOD-CH_4 g^{-1}VSS day^{-1} using acetate as sole substrate obtained by Johanna *et al.*, (2009) while Soto, Méndez and Lema, (1993) reported values that were higher than 1.07 gCOD-CH_4 g^{-1}VSS day^{-1} on enriched pure cultures utilizing acetic acid as sole substrate. Therefore, recorded activities suggested good adaptation of the anaerobic microbial population of the inoculum used in the anaerobic co-digestion of abattoir and winery solid wastes.



(a) 1 g/L acetic acid



(b) 1 g/L acetic acid



(c) 7 g/L acetic acid

Figure 7-3: Specific methanogenic activity (**SMA**) biomethanation profiles on (a) **D_1** with 1 g/L acetic acid, (b) **D_2** with 1 g/L acetic acid, and (c) **D_2** with 7 g/L acetic acid

7.5.4 Lab-scale anaerobic co-digestion single- and two-stage semi-continuous experiments

The chosen operating parameters and performance results for the semi-continuous AcoD experiments are summarized in **Table 7-4**, while the **AcoD** profiles are presented in **Figure 7-4**. For the single-stage set-up the highest cumulative methane yield of 344.11 NmLCH_4 was obtained from **D_1** while **D_2** was slightly lower yielding a cumulative total of 266.60 NmL CH_4. Even though similar operating parameters were applied to both **D_1** and **D_2** the former operated on a slight acidic region approaching neutrality with a pH range of 6.6- 6.9; the insulation used maintained a constant temperature for the entire duration of the experiments; while **D_2** had a slight alkaline pH range of 7.21-7.25. The temperature control in the unit proved difficulty with the measured value which consistently fell below the set-point that resulted in a slight reduction of microbial activity and hence, the occurrence of lower methane yields in comparison to **D_1**.

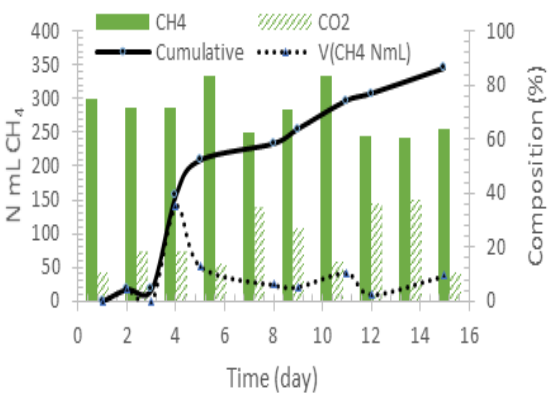
Table 7-4: Operating parameters and system performance from the single- and two-stage semi-continuous anaerobic co-digestion experiments

Parameter	Unit	Day 1-2	Day 3-5	Day 8-11	Day12-15
Single-stage D1					
HRT	(day)	38	450	360	450
OLR	(gVS L^{-1} day^{-1})	0.64	0.28	0.35	0.55
SMP	(NmLCH_4 g^{-1} VS_{added})	17.32	208.47	296.37	344.11
$CH_{4\ corrected}$	(%)	83.5	76.4	73.4	73.6
Single-stage D2					
HRT	(day)	38	450	360	450
OLR	(gVS L^{-1} day^{-1})	0.64	0.28	0.35	0.55
SMP	(NmLCH_4 g^{-1} VS_{added})	45.86	201.52	252.71	266.60
$CH_{4\ corrected}$	(%)	74.4	71.9	80.4	48.9
		Day 1-7	**Day 8-10**	**Day 11-14**	**Day15-17**
Two-stage D1					
HRT	(day)	450	90	360	180
OLR	(gVS L^{-1} day^{-1})	0.56	1.39	0.35	0.69
SMP	(NmLCH_4 g^{-1} VS_{added})	72.53	283.9	298.36	381.53
$CH_{4\ corrected}$	(%)	47.8	20.1	17.6	20.7
Two-stage D2					
HRT	(day)	450	90	360	180
OLR	(gVS L^{-1} day^{-1})	0.56	1.39	0.35	0.69
SMP	(NmLCH_4 g^{-1} VS_{added})	39.72	70.33	90.93	166.20
$CH_{4\ corrected}$	(%)	47.3	45.6	44.4	28.4

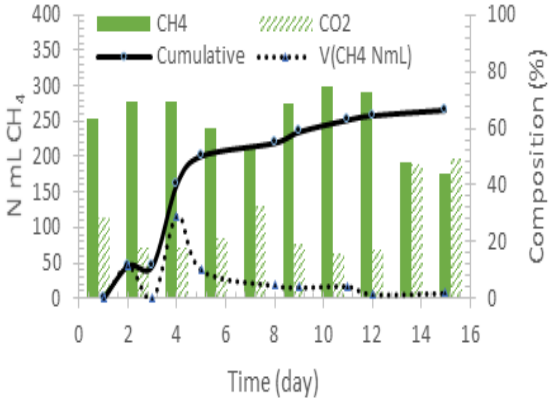
$CH_{4\ corrected}$ is corrected methane composition; **HRT** is hydraulic retention time; **OLR** is organic loading rate; **SMP** is specific methane potential

The two-stage semi-continuous AcoD experiment utilizing both **D_1** and **D_2** had interesting results, considering a combined methane yield accumulative total of 452.50 NmLCH_4 that was obtained from both digesters. Cases where **D_1** operated as hydrolysis/acidogenic stage without any form of pH control allowing the system to drop to values as low as pH 5.5, promoted a hydrolytic/acidogenic bacterial activity with an interesting significance which was that the obtained yield of 344.11 NmLCH_4 was similar to that recorded for single-stage semi-continuous operation mode of the same unit. In **D_2**, the pH was kept neutral by sodium hydroxide (NaOH) addition deploying the automated control feature of system and a pH range of 6.71-6.98 was recorded, promoting methanogenic archaea activity was recorded. However, methane yield of 108.39 NmL CH_4 from **D_2** was considerably lower than the one from **D_1** and about a half the one that was produced during single-stage operation mode.

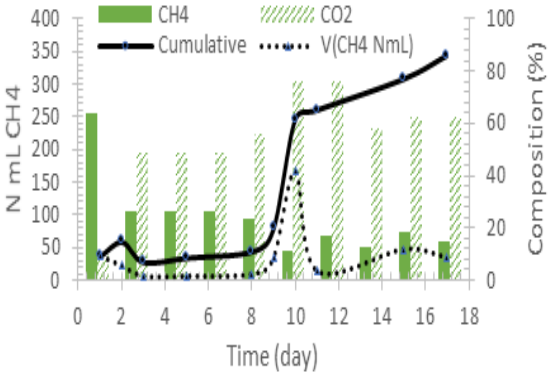
The production of methane during single-stage semi-continuous mode on both **D_1** and **D_2** was immediately noted after the 2nd day of feeding with **HRT** and **OLR** set at 19 days and 0.64 gVS L^{-1} day^{-1}, respectively. The **HRT** was then increased to 450 days and **OLR** reduced to 0.28 gVS L^{-1} day^{-1} between the 3rd and 5th day of operation resulting in a peak in methane yield and improved gas composition. In context where **D_1** recorded accumulative methane yield of 208.5 $NmLCH_4$, the biogas produced was composed of 62.4-83.2% CH_4. For **D_2** in the same period the methane yield was similar 201.5 $NmLCH_4$ and was produced under same conditions although the biogas composition was lowered at 54.3-68.9%.



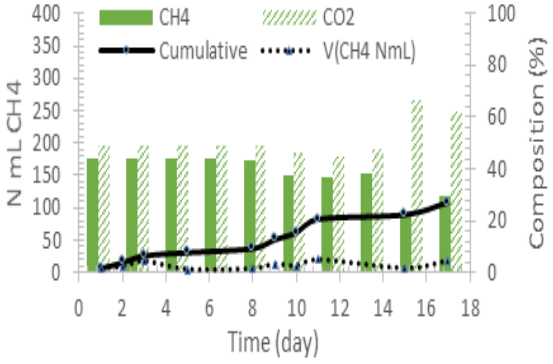
(a) Single-stage semi-continuous D1



(b) Single-stage semi-continuous D2



(c) Two-stage semi-continuous D1



(d) Two-stage semi-continuous D2

Figure 7-4: Accumulated, daily methane and biogas composition profiles from lab-scale semi-continuous operation (a) **D_1** single-stage, (b) **D_2** single-stage, (c) **D_1** two-stage and (d) **D_2** two-stage

The **HRT** was then reduced to 270 days and **OLR** increased to 0.35 gVS L^{-1} day^{-1} between the 8th and 11th day of operation without significant influences on either biogas composition or

methane yield, as the accumulative curve continued the upwards trajectory. But when the **HRT** was slightly reduced to 225 days and **OLR** increased to 0.55 gVS L^{-1} day^{-1} from the 12th towards the last day of the operation, biogas composition was affected more profoundly on **D_2** where values of 44.1-48.1% CH_4 were recorded.

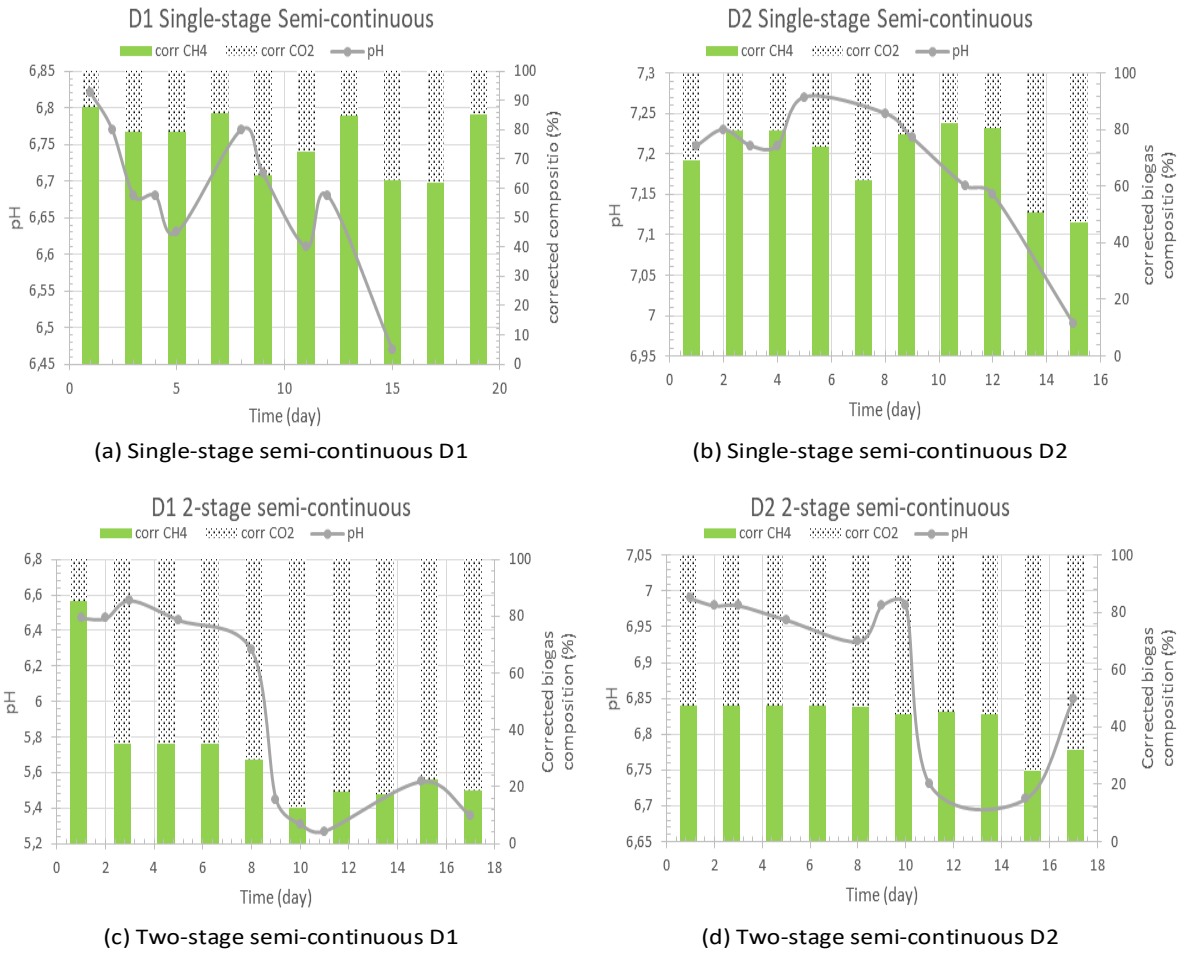


Figure 7-5: pH and corrected biogas composition profiles from the single- and two-stage semi-continuous anaerobic co-digestion of abattoir and winery solid waste.

For the two-stage operation, the **HRT** was set at 450 days and respective **OLR** at 0.55 gVS L^{-1} day^{-1}. Thus, between the 1st and 5th day of operation large volumes of biogas were produced with accumulated methane yield reaching only 35.11 $NmLCH_4$, where the pH in **D_1** dropped to a range of 6.46-6.57. Note that the impact of decreased pH was immediately noticed in the quality of the biogas produced which was highly concentrated in CO_2 with a composition as low as 26.4-63.9 % CH_4. While the pH in **D_2** remained fairly within the neutral range 6.93-6.99, and although the biogas produced was significantly lower than that obtained in the 1st stage, the quality of the gas improved in the 2nd stage where the composition was constant at 44.1% CH_4.

The **HRT** was then reduced to 90 days and **OLR** increased to 1.38 gVS L^{-1} day^{-1} from the 8th to the 10th day and the pH in **D_1** further dropped to 5.31 releasing more CO_2 from the reaction liquor while the accumulated methane yield reached an appreciable value of 246.48 $NmLCH_4$ during what was noticed as an exponential phase where the highest daily biogas yield was recorded. A similar trend was also observed on **D_2**, but gas composition remained constant while methane yield of 51.79 $NmLCH_4$ was significantly lower than it was in the 1st stage.

Thus, to try and improve the operational efficiency of the system(s) the **HRT** was increased to 360 days and **OLR** reduced to 0.35 gVS L^{-1} day^{-1} from the 11th to 14th day, which reduced the performance in the 1st stage as biogas production was 10-fold less than that obtained on the 10th day although on the contrarily a slight improvement on the 2nd stage was notable as biogas production was twice that of the previous day of operation.

The operating parameters were altered again between the 15th and last day of the operation, where the **HRT** and **OLR** were set at 180 days and 0.69 gVS L^{-1} day^{-1} which slightly recovered operational performance on both the 1st and 2nd stage. However, the biogas composition on the 2nd stage was impacted as more CO_2 was recorded on the produced gas with methane quality dropping to 29.6% CH_4.

7.6 Conclusion and recommendations

The results obtained in this study suggests that co-digestion of abattoir and winery solid waste at given mix ratios is a viable option for biogas and/or biomethane production optimization. Moreover, the selection of appropriate process equipment further enhances the recoverable energy from in the form of biogas from the sourced organic waste materials.

CHAPTER 8

8 Conclusion and Recommendations

8.1 Introduction

This chapter concludes the study undertaken and provides recommendations. Anaerobic co-digestion presents a multitude of benefits compared to mono-digestion. Simultaneous use of co-substrates yields efficiency of anaerobic digesters and subsequently enhances biogas and methane production. Moreover, the economic viability of the process becomes more feasible considering the tipping fees for waste management that can be obtained for industrial scale operations. However, for sustainable long-term operation **AcoD** requires proper implementation guided by the understanding of the effects of the various input parameters. Overall, the implementation of industrial scale **AcoD** processes could simultaneously address the issues of growing waste management and energy demand by providing a renewable source of energy in the form of biomethane. The aim of this study was to enhance biogas production via anaerobic co-digestion of abattoir and winery solid wastes. The experimentally determined biomethane potentials, optimal mixture blending and input parameters (i.e. organic load, F/M and pH) from **AcoD** of abattoir and winery solid wastes were then used to determine the mode of operation ranging from batch, single- and two-stage semi-continuous that could practically be applied in industrial scale operations for maximal efficiency. This chapter is concluded by presenting future research area suggestions and overall summary of this research.

8.2 Overview of findings

This section considers the key findings of this research and discusses each step undertaken to achieve the overall objectives of this study. The primary objective of this study was enhancing biogas production from the AcoD of abattoir and winery solid waste. The investigation was conducted by first determining SMP from mono-AD and by evaluating the impacts of microwave pre-treatment and AcoD on the overall digestion performance using BMP assays for a period of 30 days. All samples were first analyzed for TS, VS, their elemental composition and trace elements to determine their C/N ratio and nutritional value. The inoculum used in all

experiments was locally synthesized using zebra dung and cow ruminal contents as source of anaerobic flora. Thus, specific methanogenic activity tests were consistently carried out to verify the quality of the inoculum. The biogas produced was bubbled through sodium hydroxide solution and passed through steel-wool scrubbers to remove impurities and directly measure methane production. The quality of the produced gas was measured at least once a week with a portable gas analyzer. It was the microwave treated abattoir solids and AcoD samples that performed efficiently by producing good quality biogas to a certain extent.

Biomethane potential assays

The inoculum had significant high average SMA of 2.66 g COD_{CH4} g^{-1}VSS d^{-1}. The maximum efficiency was determined at F/M ratio of 0.5 g/g (VS) and organic load of 5 g VS L^{-1}. Note that higher organic loads and F/M ratio resulted in reduced digestion performance and consequently in methane yields due to a build-up of inhibitory/toxic components.

The experimental data were simulated using curve fitting and mathematical modelling method(s) through the Gompertz, logistic and Richard's kinetic models with R^2 ranging from 0.991-0.999. The first-order kinetic model was also useful in the calculation of hydrolysis rate constants of the variable samples, with values ranging from 0.045-0.091 day^{-1}. However, it is worth highlighting that kinetic modelling was only possible for the un-inhibited samples.

Optimization of methane yield

The secondary objective(s) was the optimization of response variable(s) (i.e. SMP and maximum specific methane production rate) firstly, by determining optimal mixture blends employing an ABCD Mixture Design of Experiments to evaluate the synergistic/antagonistic effects using statistical tools (**CHAPTER 5**) and secondly, a Response Surface Methodology was applied utilizing a Central Composite Rotatable Design to evaluate optimal input parameters (i.e. organic load, F/M ratio and initial pH) to promising mixture blends (**CHAPTER 6**).

Mixture design experiments

The highest improvement was obtained from $\mathbf{M_9}$ mixture blends where a 29.3% in SMP increase was recorded in comparison to the performance of individual fractions, which was obtained using linear models. This result could be largely attributed to synergistic effects that were promoted by correct balancing of nutritional requirements for an efficient AD system.

The overall results allowed for proper screening of optimal mixture blends for further optimization with the use of statistical data obtained throughout the course of this study. The optimization of process parameters for enhanced biogas production with high quality methane content has been and will continue to be one of the adopted strategies for anaerobic digestion systems from laboratory to industrial operations. In this study, the combination of some of the critical process parameters and their effects on the overall efficiency on anaerobic digestion were explored.

Response surface methodology design of experiments

The AcoD of abattoir solid wastes, cow blood and winery solid wastes was evaluated using the design of experiment tools which generated a total of 20 randomized experimental runs (i.e. R_1-R_{20}) that were replicated at least three-times for statistical validation purposes. The optimization using **RSM** models was efficiently used to predict and simulate optimal conditions for enhanced methane production, where a third-order polynomial equation was efficiently fitted with experimental data with $\mathbf{R^2}$ value of 0.97.

In this study, the optimal conditions for enhanced specific methane production from the optimal mixture blend consisting of (17:66:17) abattoir solids, cow blood and winery solid wastes were determined to be organic load, F/M ratio and initial pH of 1.6 g VS/L, 0.25 g VS/g VS and 6.5.

8.3 Recommendations

To advance the scientific knowledge input into the potential application of abattoir and winery waste as co-substrates for production of high-quality biogas the following recommendations in future studies have been noted:

- The biogas produced in the 1st stage of a two-stage semi-continuous digester operation should be recirculated and/or bubbled in the 2nd stage to allow maximum utilization of the hydrogenotrophic methanogenic archaeal population.

8.4 Suggestions for future research

There is a rise in research interests for alternative renewable energy sources due to rapid population growth that has led to increase in energy demand, and waste management issues across the globe. Although many initiatives were implemented, more emphasis should primarily be given to anaerobic digestion process because it can successfully be used to simultaneously address both issues of growing energy needs and waste management crisis by providing green renewable energy, in the form of biomethane from organic waste streams, that

can be directly used as fuel for heating, cooking, and/or be converted in CHP plants to produce electricity. Thus, there is a dire need for other researchers evaluate other feedstocks that can be utilized by AD processes for optimal efficiency.

Below is a set of key recommendations that future research should be primarily focused on:

- AcoD of other carbon rich feedstocks with abattoir waste for enhancing the AD process and maximizing biogas yield should be investigated.
- The input parameters, such as pH, temperature, F/M ratio, organic loading rated, retention times, mixing, that directly impact the overall AD process should be prioritized.
- Future research studies on the effects of various pretreatment methods of substrates and their economic feasibility for successful implementation of industrial scale operations should be undertaken.
- Studies focusing on the most efficient mode of digester operation for maximal utilization of feedstock(s) should be conducted.
- Future studies aimed at making AcoD much more economical for wide spread rollout to the poorest communities.
- The impact of AD processes on the mitigation of the runaway global warming, and the effect because of increased reliance on fossil fuels should be analyzed.

8.5 Evaluation of the study

This study primarily focused on enhancing biogas production from abattoir and winery solid wastes. The introduction in **CHAPTER 1** gave an overview of the background of AD processes which were the basis from which the hypothesis and objectives of this study were drawn. A comprehensive literature review was conducted in **CHAPTER 2** to develop a solid theoretical foundation for in-depth understanding of AD processes. This simplified the processes of developing a thorough methodology found in **CHAPTER 3** to complete the tasks that were required to address the set of objectives assigned to this research with the intention of verifying the hypothesis and assumptions made for this study.

The quality of the feedstock for biogas production was characterized using standard protocols, which were useful in determining the theoretical methane yields. BMP assays following standard methods were conducted for a period of 30 days to determine methane yields. The results that were obtained through **CHAPTER 4**, **CHAPTER 5** and **CHAPTER 6** were sufficient to conclude that enhancement biogas production from AcoD of abattoir and winery

solid wastes is possible, when provided optimal mixture blends, input parameters and operational digester modes are employed.

8.6 Conclusion

This study investigated the enhancement of biogas production by AcoD of abattoir and winery solid wastes. In determining the BMP from abattoir and winery solid wastes the effects of mixture blending, effects of various input parameters and scale-up procedures were evaluated. Thus, as a basis of the hypothesis and assumptions made it was possible to conclude that AcoD of abattoir and winery solid wastes enhances biogas production efficiency.

The significance of this study has to do with the contribution it makes to the science community towards sustainable production of renewable energy research. It highlighted the importance of various input factors directly impacting the overall AD process with the use of mathematical modelling and statistical tools, through which economic feasibility of the process on industrial scale can be established. This study can be used as a baseline for developing futuristic biogas plant designs for a maximal efficiency.

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Appendices

- **Appendix A**: Raw material characterization and sample calculations;
- **Appendix B**: Experimental raw data and sample calculations;
- **Appendix C**: Raw table,
- **Appendix D**: Raw graphs;

Appendix A: Sample and inoculum characterization raw data and sample calculations based on the stoichiometry of ultimate analysis results

A.1 **Total solids and volatile solids calculations based on abattoir sample I** (refer to **Table C-1:** Proximate substrate and inoculum analysis raw data)

$$\mathrm{TS}[\%] = \left[\frac{\mathrm{A}-\mathrm{B}}{\mathrm{C}-\mathrm{B}}\right] * 100$$

$$= \left[\frac{106.67 - 74.42}{174.80 - 74.42}\right] * 100$$

$$= 32.13\ \%$$

$$\mathrm{VS}[\%] = 100 - \left[\frac{\mathrm{D}-\mathrm{B}}{\mathrm{A}-\mathrm{B}}\right] * 100$$

$$= 100 - \left[\frac{76.20 - 74.42}{106.67 - 74.42}\right] * 100$$

$$= 94.48\ \%$$

The TS % and VS % estimate of the sample was then determined as the average value of the analyzed samples, namely I; II; IV and V

$$\mathrm{TS}[\%] = \frac{32.13 + 32.01 + 32.08 + 31.68}{4}$$

$$= 32.15\ \%$$

Moisture content was then determined as the difference as follows:

$$Moisture\ [\%] = 100 - 32.15$$

$$= 67.85\ \%$$

$$\mathrm{VS}[\%] = \frac{94.48 + 93.89 + 93.98 + 93.52}{4}$$

$$= 93.97\ \%\ on\ dry\ basis$$

Ash content based on volatile matter then determined as the difference as follows:

$$Ash[\%] = 100 - 93.97$$

$= 6.03\ \%\ on\ dry\ basis$

A.2: Determination of carbon/nitrogen **(C/N)** ratio, cow blood ($\mathbf{C_b}$) sample calculations (refer to **Table C-2**).

The sample calculation for **C/N** ratio was based on the cow blood ($\mathbf{C_b}$) mole ratio and applying equation **(3-8** as follows:

$$C/_N = \frac{W_1C_1 + W_2C_2 + W_3C_3}{W_1N_1 + W_2N_2 + W_3N_3} = \frac{W_1C_1 + 0 + 0}{W_1N_1 + 0 + 0} = \frac{a}{d} = \frac{221.5444}{54.6990}$$

$= 4.0502$

A.3: Determination of the theoretical organic carbon **(TOC)** ratio, for cow blood ($\mathbf{C_b}$) sample calculation (refer to **Table C-2**).

The **TOC** was estimated using the stoichiometric mole ratio for the $\mathbf{C_b}$ sample by applying equation **(3-5** as follows:

$$TOC = \frac{12.0107a}{12.0107a + 1.0079b + 15.999c + 14.0067d + 32.065e} = \frac{2660.9042}{5477.9776}$$

$= 0.4857$

A.4: Determination of the theoretical chemical oxygen demand **(TCOD)**, for cow blood ($\mathbf{C_b}$) sample calculation (refer to **Table C-2**).

The **TCOD** was also estimated stoichiometrically for the $\mathbf{C_b}$ sample applying equation Error! Reference source not found. as follows:

$$TCOD = \frac{8(4a + b + 2c - 3d - 2e)}{12.0107a + 1.0079b + 115.999c + 14.0067d + 32.065e}$$

$$= \frac{7553.8677}{5475.4669}$$

$= 1.3807\ mg\ O_2\ g^{-1}\ VS$

A.5: Determination of the theoretical methane potential **(TMP)**, for cow blood ($\mathbf{C_b}$) sample calculation (refer to **Table C-2**).

Similarly, the **TMP** was also estimated based on the mole ratio composition for the B sample by applying equation (**3-6**) and assuming ideal gas behavior, where one mole of gas occupies a volume of 22.4 liters at standard temperature and pressure conditions (**STP**), thus, the theoretical methane production was estimated as follows:

$$\mathrm{TMP}\left[\frac{N\ ml}{g\ VS}\right] = 22.4\left[\frac{\left[\frac{\mathrm{a}}{2}+\frac{\mathrm{b}}{8}-\frac{\mathrm{c}}{4}-3\frac{\mathrm{d}}{2}-\frac{\mathrm{e}}{4}\right]}{12.0107\mathrm{a}+1.0079\mathrm{b}+15.999\mathrm{c}+14.0067\mathrm{d}+32.065\mathrm{e}}\right]*1000$$

$$= 22.4\left[\frac{118.02918}{5475.6388}\right]*1000$$

$$\underline{= 482.8393\ \mathrm{N\ mL}g^{-1}\ VS}$$

Appendix B: AD experimental raw data and sample calculations

IUPAC standard conditions were applied in all calculations assuming ideal conditions and perfect mixing (refer to **3.2.1 Scientifically Standardized Methods/Protocols and Assumptions Applied**) for details. All calculations were based on $\mathbf{A_sW_s}$ sample on day-2 of **AcoD-**experiments.

B1. **Carbon/Nitrogen ratio sample calculation**

The sample calculation for **C/N** ratio was based on the (**AW**) mole ratio; **VS** composition of each substrate contributing to the mixture applying equation **(3-8** as follows:

$$C/_N = \frac{W_1C_1 + W_2C_2}{W_1N_1 + W_2N_2} = F_1\frac{C_1}{N_1} + F_2\frac{C_2}{N_2}$$

$$= (0.49 * 18.4083) + (0.51 * 28.3590)$$

$$\underline{= 23.4}$$

B2. **Theoretical Chemical Oxygen Demand sample calculation**

$$\mathrm{TCOD} = (0.49 * 2.3327) + (0.51 * 1.2680)$$

$$\underline{= 1.7948\ \mathrm{m}g\ O_2\ g^{-1}\ VS}$$

B3. Theoretical Methane Potential sample calculation

$$\mathrm{TMP}\left[\frac{N\ ml}{g\ VS}\right] = (0.49 * 816.2701) + (0.51 * 443.8946)$$

$$\underline{= 628.1484\ N\ mL\ g^{-1}VS}$$

B4. Water vapor pressure calculations

The water vapor pressure on day 2 was calculated as follows:

$$P_{vapor} = 6.1121\mathrm{EXP}\left|\left(18.678 - \frac{T_c}{234.5}\right)\left(\frac{T_c}{257.14 + T_c}\right)\right|$$

$$= 0.61121\mathrm{EXP}\left|\left(18.678 - \frac{26.2}{234.5}\right)\left(\frac{26.2}{257.14 + 26.2}\right)\right|$$

$$\underline{= 3.40239\ KPa}$$

B5. Biogas volume normalization calculations

$$P_{dry} = P_{wet} - P_{vapor}$$

Applying standard temperature and pressure condition results in the combined gas law equation:

$$V_{STP}[N\ mL] = V * \frac{P}{P_S} * \frac{T_S}{T} = V * \frac{P_{atm} - P_{vap}}{P_S} * \frac{T_S}{T}$$

$$= 35.5733 * \frac{101.325 - 3.40239}{101.325} * \frac{298.15}{299.35}$$

$$= 34.24097\ N\ mL\ biogas$$

B6. The percentage of methane produced was corrected following the VDI 4630 method:

$$CH_{4\ corrected} = \left| \frac{CH_4(\%)}{CH_4(\%) + CO_2(\%)} \right| = \frac{17.2}{17.2 + 0.7}$$

$$= 0.9609$$

B7. The net methane was determined to be the product of normalized clean biogas yield and the corrected methane content minus that of the blank/control assay

$$V_{STP}^{Sample} * CH_{4\ corrected} - V_{STP}^{Blank} * CH_{4\ corrected} = 34.2410 * 0.9609 - 9.6092$$

$$= 32.9022 - 9.6092$$

$$= 23.2930\ NmL\ CH_4$$

B8. The specific methane yield was calculated as follows:

$$SMP\left[\frac{NmL}{gVS}\right] = \frac{Net\ V_{STP}(NmL)}{g\ VS_{added}} = \frac{23.2930}{5.03}$$

$$= 4.6308\ NmL\ CH_4 g^{-1} VS_{added}$$

B9. The overall biodegradability of substrate at set lab-environmental conditions over given period of digestion observed, was determine as:

$$f_d = \frac{SMP_{time}}{TMP} = \frac{369.56}{628.15}$$

$$= 0.63$$

Appendix C: Raw Tables

Table C-1: Proximate substrate and inoculum analysis raw data

Description	Preparation		Sample grams	Crucible		A	B	C	D	TS [a] %	VS [b]
Abattoir solid waste	Sterilized + pasteurized (microwave)		100.38	74.42	**I**	106.67	74.42	174.80	76.20	32.13	94.48
			100.74	74.62	**II**	106.87	74.62	175.36	76.59	32.01	93.89
			98.38	73.71	**II**	105.96	73.71	172.09	75.65	32.08	93.98
			101.80	77.04	**IV**	109.29	77.04	178.84	79.13	31.68	93.52
Blood [c]	Raw		29.00	74.42	**I**	78.79	74.42	103.42	75.97	15.07	99.65
			29.13	74.62	**II**	78.98	74.62	104.75	76.35	14.97	99.60
			29.05	73.71	**II**	78.07	73.71	103.76	75.96	15.01	99.48
			29.08	77.04	**IV**	81.4	77.04	106.12	78.96	14.99	99.56
Winery waste	Sun dried		22.62	74.42	**I**	94.32	74.42	97.00	76.21	87.98	91.01
			19.09	74.62	**II**	91.41	74.62	93.70	76.13	97.95	91.01
			20.91	73.71	**II**	92.08	73.71	94.60	75.36	87.85	91.02
			16.65	77.04	**IV**	91.98	77.04	93.70	78.34	87.93	91.12
Inoculum [c]	Raw		24.62	74.42	**I**	74.58	74.42	99.00	74.43	0.65	99.94
			21.80	74.62	**II**	74.78	74.62	96.40	74.68	0.73	99.63
			22.00	73.71	**II**	73.87	73.71	95.70	73.74	0.73	99.81
			19.58	77.04	**IV**	77.2	77.04	96.6	77.08	0.82	99.75

[a] TS = (A-B)/(C-B) *100 (%); [b] VS = 100-((D-B)/(A-B) *100) *100) (%); [c] TS analysed at 90 ±5 °C to prevent volatilization

A is (dried sample + crucible); B is crucible; C is (wet sample + crucible); and D is (Ash + crucible) weights

Table C-2: Ultimate analysis raw data and

Substrate	Preparation	Sample size mg	Analysis	C %	H %	N %	S %	O %
A_s	Dried /milled	7.527	Elemental (CHNS)	65.3	10.6	4.6	0.4	-
		4.583		65.9	10.7	3.7	0.3	-
C_b	Dried /milled	5.653	Elemental (CHNS)	47.7	7.2	13.7	0.6	-
		4.909		49.9	7.5	14.5	0.6	-
				VS (%)				
A_s	Based on proximate analysis dried blood and abattoir solids have an organic around 93.97 and 99.57 %, respectively.			61.37	10.01	4.33	0.41	17.84
				61.95	10.08	3.51	0.28	18.14
C_b				44.84	6.79	12.87	0.55	28.92
				46.86	7.02	13.64	0.56	25.89
				Average **VS** mass (g)				
A_s	Assuming 100 grams of sample			61.6631	10.0407	3.9232	0.3472	17.9957
C_b				45.8527	7.3460	13.2592	0.5525	27.4026
W_s [a]				46.4900	6.4700	1.9200	0.000	45.1200
				Average **VS** moles (g)				
A_s				5.1340	9.9616	0.2789	0.0108	1.1248
C_b				3.8177	7.2881	0.9426	0.0172	1.7128
W_s				3.8707	6.4190	0.1365	0	2.8202
	Mole Ratio			**a**	**b**	**d**	**e**	**c**
A_s				474.1161	919.934	25.7556	1	103.8733
C_b				221.5444	422.9421	54.6990	1	99.3948
W_s				28.35904	47.0295	1	0	20.6622
Theoretical Methane Yield, Chemical Oxygen Demand, Total Organic Carbon and Carbon-Nitrogen Ratio								
	Empirical Formula [$C_a H_b O_c N_d S_e$]			**TMP** N mL g^{-1} VS		**TCOD** mg O_2 g^{-1} VS	**TOC**	**C/N**
A_s	$C_{474.1161} H_{919.934} O_{103.8733} N_{25.7556} S$			816.2701		2.3327	0.6563	18.4083
C_b	$C_{221.5444} H_{422.9421} O_{99.3948} N_{54.6990} S$			482.8393		1.3807	0.4857	4.0502
W_s	$C_{28.35904} H_{47.0295} O_{20.6622} N$			443.8946		1.2680	0.4650	28.3590

Table C-3: Micro- and macro-nutrients and trace elements of the substrates and inoculum

Element	A_s	W_s	C_b	Inoculum
	mg/kg	mg/kg	mg/kg	mg/kg
B	1.108 ± 0.144	-	1.001 ± 0.0315	0.512
Al	56.908 ± 3.182	-	8.254 ± 0.209	7.572
V	0.0825 ± 0.0145	-	0.0135 ± 1.5	0.010
Cr	0.432 ± 0.0015	-	0.131 ± 0.026	0.195
Mn	2.289 ± 0.426	-	0.2635 ± 0.0085	1.240
Fe	136.564 ± 25.377	-	2478.167 ± 78.938	24.259
Co	0.0375 ± 0.0055	-	-	-
Ni	0.1785 ± 0.0215	-	0.063 ± 0.008	0.029
Cu	20.617 ± 0.00334	-	3.390 ± 0.248	0.443
Zn	36.0805 ± 6.7505	-	15.733 ± 0.024	2.361
As	20E-3 ± 1E-3	-	0.014 ± 0.001	8 E-3
Se	0.375 ± 0.0825	-	1.0905 ± 0.0335	7 E-3
Sr	2.627 ± 0.414	-	0.372 ± 0.014	0.529
Mo	0.30 ± 0.045	-	-	-
Cd	0.0255 +/- 0.0035	-	2.65 E-3 ± 0.45 E-3	1.7 E-3
Sb	7 E-3 ± 1E-3	-	11.2 E-3 ± 5.8 E-3	10 E-3
Ba	2.080 ± 0.028	-	0.141	0.332
Hg	5.85 E-3 ± 0.45 E-3	-	3.3 E-3 ± 0.6 E-3	2.1 E-3
Pb	0.0985 ± 0.0155	-	88 E-3 ± 3 E-3	24 E-3
Ca	640.5 +/- 107.5	0.06%	180.5 +/- 4.5	98
K	1912.5 +/- 384.5	1.77%	1660.5 +/- 99.5	125
Mg	155.5 +/- 24.5	NR	77.5 +/- 3.5	20
Na	2758 +/- 550	1191.90	8539 +/- 484	768
Si	145 +/- 28	-	27 +/- 1	81
Cyanide	-	0.92	-	-

Table C-4: Raw data results for blank assays C_{b1} **Mono**- and **AcoD** experiments R_1

Date	Time	T room	C_{b1-1}	C_{b1-2}	CH_4	CO_2	H_2	H_2S	O_2	Bal
	day	°C	mL		%		ppm		%	
28/09/2018	Blank assay									
30/09/2018	2	23.2	3.36	3.01	0.2	0.1	0	1	1.1	98.6
02/10/2018	4	20.4	4.15	3.83	0.2	0.1	0	1	1.1	98.6
04/10/2018	6	24.8	13.64	18.06	0.2	0.1	0	1	1.1	98.6
06/10/2018	8	26.2	3.98	4.53	0.2	0.1	0	1	1.1	98.6
08/10/2018	10	27.3	3.10	3.92	34.6	0.1	84	1	0.9	64.4
09/10/2018	11	23.0	7.93	8.52	34.6	0.1	84	1	0.9	64.4
12/10/2018	14	23.4	4.31	3.86	34.6	0.1	84	1	0.9	64.4
15/10/2018	17	23.0	5.54	4.96	34.6	0.1	84	1	0.9	64.4
17/10/2018	19	24.1	3.12	4.43	34.6	0.1	84	1	0.9	64.4
20/10/2018	22	27.1	8.44	9.06	34.6	0.1	84	1	0.9	64.4
22/10/2018	24	25.9	8.06	9.82	34.6	0.1	84	1	0.9	64.4
24/10/2018	26	31.2	6.12	5.83	34.6	0.1	84	1	0.9	64.4
26/10/2018	28	28.9	4.12	5.08	34.6	0.1	84	1	0.9	64.4
28/10/2018	30	31.5	8.52	7.64	34.6	0.1	84	1	0.9	64.4
		pH								

Table C-5: Raw data results for C_{b1} **Mono-** and **AcoD** experiments R_1

Date	Time	T_{room}	C_{b1-1}	C_{b1-2}	CH_4	CO_2	H_2	H_2S	O_2	Bal
	day	°C	mL		%		ppm		%	
28/09/2018	F/M = 1.92; average **VS** for (**B**) = 19.96 g;									
30/09/2018	2	23.2	42.15	42.17	9.2	0.3	161	1	1.6	89.6
02/10/2018	4	20.4	73.9	73.76	9.2	0.3	161	1	1.6	89.6
04/10/2018	6	24.8	325.7	279.84	9.2	0.3	161	1	1.6	89.6
06/10/2018	8	26.2	112.19	115.26	9.2	0.3	161	1	1.6	89.6
08/10/2018	10	27.3	41.99	44.87	10.8	1.7	319	3	1.3	86.1
09/10/2018	11	23.0	2.84	3.26	10.8	1.7	319	3	1.3	86.1
12/10/2018	14	23.4	8.26	8.12	10.8	1.7	319	3	1.3	86.1
15/10/2018	17	23.0	10.19	9.84	10.8	1.7	319	3	1.3	86.1
17/10/2018	19	24.1	14.65	15.37	10.8	1.7	319	3	1.3	86.1
20/10/2018	22	27.1	10.52	10.36	27.0	0.9	98	4	0.9	71.2
22/10/2018	24	25.9	83.16	78.85	27.0	0.9	98	4	0.9	71.2
24/10/2018	26	31.2	89.52	92.36	27.0	0.9	98	4	0.9	71.2
26/10/2018	28	28.9	90.38	93.78	27.0	0.9	98	4	0.9	71.2
28/10/2018	30	31.5	82.72	85.74	27.0	0.9	98	4	0.9	71.2

Table C-6: Raw data results for $\mathbf{A_{s1}}$ **Mono-** and **AcoD** experiments $\mathbf{R_1}$

Date	Time	T_{room}	A_{s1-1}	A_{s1-2}	CH_4	CO_2	H_2	H_2S	O_2	Bal
	day	°C	mL		%		ppm		%	
28/109/2018	F/M = 1.92; average **VS** for (**A**) = 20.02 g;									
30/09/2018	2	23.2	10.14	7.06	1.7	0.2	1756	2	1.7	96.5
02/10/2018	4	20.4	10.14	10.96	1.7	0.2	1756	2	1.7	96.5
04/10/2018	6	24.8	87.63	83.02	1.7	0.2	1756	2	1.7	96.5
06/10/2018	8	26.2	36.22	38.75	1.7	0.2	1756	2	1.7	96.5
08/10/2018	10	27.3	5.84	4.96	1.7	0.2	1756	2	1.7	96.5
09/10/2018	11	23.0	9.76	9.98	22.2	0.7	42	1	0.6	76.5
12/10/2018	14	23.4	6.41	6.58	22.2	0.7	42	1	0.6	76.5
15/10/2018	17	23.0	3.28	4.86	22.2	0.7	42	1	0.6	76.5
17/10/2018	19	24.1	2.04	3.73	22.2	0.7	42	1	0.6	76.5
20/10/2018	22	27.1	9.56	10.84	22.2	0.7	42	1	0.6	76.5
22/10/2018	24	25.9	1.98	2.32	40.8	0.5	42	1	0.6	59.9
24/10/2018	26	31.2	1.44	2.46	40.8	0.5	42	1	0.6	59.9
26/10/2018	28	28.9	5.55	5.78	40.8	0.5	42	1	0.6	59.9
28/10/2018	30	31.5	35.42	37.57	40.8	0.5	42	1	0.6	59.9

Table C-7: Raw data results for $\mathbf{W_{s1}}$ **Mono**- and **AcoD** experiments $\mathbf{R_1}$

Date	Time	T_{room}	W_{s1-1}	W_{s1-2}	CH_4	CO_2	H_2	H_2S	O_2	Bal
	day	°C	mL		%		ppm		%	
28/109/2018	F/M = 1.94; average **VS** for (**W**) = 20.14 g;									
30/09/2018	2	23.2	7.38	7.06	33.9	0.6	1856	109	1.3	64.2
02/10/2018	4	20.4	23.64	18.06	33.9	0.6	1856	109	1.3	64.2
04/10/2018	6	24.8	430.74	438.61	25.3	0.4	26	1	0.9	73.4
06/10/2018	8	26.2	8.24	7.82	25.3	0.4	26	1	0.9	73.4
08/10/2018	10	27.3	7.77	8.08	17.8	0.8	94	1	0.9	82.1
09/10/2018	11	23.0	0	0.46	17.8	0.8	94	1	0.9	82.1
12/10/2018	14	23.4	2.73	1.83	17.8	0.8	94	1	0.9	82.1
15/10/2018	17	23.0	10.25	11.54	17.8	0.8	94	1	0.9	82.1
17/10/2018	19	24.1	0.08	0	17.8	0.8	94	1	0.9	82.1
20/10/2018	22	27.1	53.32	55.69	17.8	0.8	94	1	0.9	82.1
22/10/2018	24	25.9	24.85	26.21	17.8	0.8	94	1	0.9	82.1
24/10/2018	26	31.2	8.43	9.02	17.8	0.8	94	1	0.9	82.1
26/10/2018	28	28.9	9.56	9.84	17.8	0.8	94	1	0.9	82.1
28/10/2018	30	31.5	6.56	7.86	17.8	0.8	94	1	0.9	82.1
		pH								

Table C-8: Raw data results for $\mathbf{A_sC_bW_{s1}}$ **Mono**- and **AcoD** experiments $\mathbf{R_1}$

Date	Time	T_{room}	$A_sC_bW_{s1-1}$	$A_sC_bW_{s\ 1-2}$	CH_4	CO_2	H_2	H_2S	O_2	Bal
	day	°C	mL		%		ppm		%	
28/109/2018	F/M = 1.96; average **VS** for (**A**) = 5.02 g; (**B**) = 5.06 g; (**W**) = 10.36 g; (**ABW**) = 20.43 g;									
30/09/2018	2	23.2	17.3	17.0	48.6	8.6	1743	21	0.7	42.1
02/10/2018	4	20.4	22.63	23.96	48.6	8.6	1743	21	0.7	42.1
04/10/2018	6	24.8	292.48	295.15	48.6	8.6	1743	21	0.7	42.1
06/10/2018	8	26.2	129.94	125.15	48.6	8.6	1743	21	0.7	42.1
08/10/2018	10	27.3	67.96	65.48	48.6	8.6	1743	21	0.7	42.1
09/10/2018	11	23.0	35.62	35.28	25.4	3.1	28	2	0.9	70.6
12/10/2018	14	23.4	29.84	27.37	25.4	3.1	28	2	0.9	70.6
15/10/2018	17	23.0	12.06	11.76	25.4	3.1	28	2	0.9	70.6
17/10/2018	19	24.1	8.34	9.25	25.4	3.1	28	2	0.9	70.6
20/10/2018	22	27.1	7.34	7.62	25.4	3.1	28	2	0.9	70.6
22/10/2018	24	25.9	2.42	3.54	16.7	0.2	148	3	0.9	82.6
24/10/2018	26	31.2	2.76	4.08	16.7	0.2	148	3	0.9	82.6
26/10/2018	28	28.9	0.62	1.26	16.7	0.2	148	3	0.9	82.6
28/10/2018	30	31.5	0	0.83	16.7	0.2	148	3	0.9	82.6

Table C-9: Calculated results for **R_1 AcoD** experiments

Time	P_{vapor}	C_{b2}	C_{b1}	A_{s1}	W_{s1}	$A_sC_bW_{s1}$	B_{1cum}	$A_{s\,cum}$	$W_{s\,cum}$	$A_sC_bW_{scum}$
day	KPa	Daily gas (N mL); corrected CH_4 and CO_2 gas composition (%)					CH_4 (N mL g^{-1} VS)			
2	3.0025	2.0763	39.9237	7.5242	6.9372	14.1825	1.8960	0.2722	0.2414	0.5924
4	2.6604	2.6378	70.9011	9.3606	20.3162	19.4622	5.3157	0.6080	1.1193	1.4157
6	3.3426	10.2470	284.3394	74.0341	414.9656	240.6783	19.0464	3.7945	21.2176	12.6921
8	4.5465	2.7304	106.0088	32.2831	7.6090	101.4810	24.2202	5.2709	21.4599	17.5245
10	3.9843	3.3487	35.9023	4.6228	7.2565	53.756	25.8510	5.3345	21.6539	19.9912
11	3.0938	8.0277	2.5794	9.3658	0.2154	30.6705	25.5781	5.4014	21.2660	21.0993
14	2.4872	3.9789	6.9122	6.1506	2.1314	25.2060	25.7250	5.5099	21.1742	22.1380
17	2.6121	5.1241	8.4698	3.8621	10.2057	10.4531	25.8926	5.4468	21.4266	22.3988
19	3.4024	3.6636	12.6225	2.7222	0.0373	7.5448	26.3414	5.3998	21.2465	22.5887
22	2.6121	8.3570	9.6773	9.4714	49.9620	6.5650	26.4076	5.4554	23.3126	22.5010
24	2.7760	8.5942	75.5778	2.0477	23.5549	2.8853	29.7631	5.1284	24.0555	22.1117
26	3.5456	5.5745	82.3452	1.8025	7.8126	3.2406	33.6090	4.9400	24.1667	22.1075
28	2.9666	4.3495	84.5004	5.3069	8.8026	0.9050	37.6242	4.9878	24.3878	21.9389
30	3.5042	7.5249	76.1327	33.6735	6.4445	0.3937	41.0611	6.2941	24.3342	21.5899
C/N							4.05	18.41	28.36	19.90
TCOD (mg O_2 g^{-1} VS)							1.3811	2.3330	1.2680	1.5576
TMP (N mL g^{-1} VS)							482.84	816.27	443.89	545.02

Table C-10: Raw data results for blank assays C_{b2} **AcoD** experiments

Date	Time	T_{room}	C_{2-1}	C_{2-2}	CH_4	CO_2	H_2	H_2S	O_2	Bal
	day	°C	mL		%		ppm		%	
15/10/2018				Blank assay						
17/10/2018	2	24.1	17.96	150.5	4.4	0.9	1750	6	1.9	92.8
19/10/2018	4	22.1	23.80	24.18	4.4	0.9	1750	6	1.9	92.8
22/10/2018	7	25.9	43.24	43.56	28.2	0.5	42	2	1.9	69.4
24/10/2018	9	31.2	34.32	36.02	28.2	0.5	42	2	1.9	69.4
26/10/2018	11	28.9	14.96	14.42	33.0	0.6	51	2	1.6	64.8
29/10/2018	14	24.6	13.47	13.22	33.0	0.6	51	2	1.6	64.8
01/11/2018	17	21.8	17.36	16.72	33.0	0.6	51	2	1.6	64.8
05/11/2018	21	21.8	15.27	14.92	62.4	1	41	1	6.2	30.4
07/11/2018	23	22.8	1.93	3.46	62.4	1	41	1	6.2	30.4
09/11/2018	25	26.9	6.19	4.86	62.4	1	41	1	6.2	30.4
12/11/2018	28	23.9	7.04	8.25	62.4	1	41	1	6.2	30.4
14/11/2018	30	26.7	3.74	4.12	62.4	1	41	1	6.2	30.4
		pH								

Table C-11: Raw data results for co-digestion experiments C_bW_{s2} **AcoD** experiments

Date	Time	T_{room}	BW_{2-1}	BW_{2-2}	CH_4	CO_2	H_2	H_2S	O_2	Bal
	day	°C	mL		%		ppm		%	
15/10/2018	F/M = 0.97; average **VS** for (**B**) = 5.06 g; (**W**) =5.00 g; and totaling (**BW**) = 10.06 g									
17/10/2018	2	24.1	35.58	38.66	23.1	0	1688	4	1.7	75.2
19/10/2018	4	22.1	52.99	54.33	23.1	0	1688	4	1.7	75.2
22/10/2018	7	25.9	128.38	126.94	85.3	4.4	268	2	1.6	8.7
24/10/2018	9	31.2	82.86	83.37	85.3	4.4	268	2	1.6	8.7
26/10/2018	11	28.9	26.12	27.33	63.3	3.7	268	2	1.5	31.5
29/10/2018	14	24.6	25.36	23.56	63.3	3.7	268	2	1.5	31.5
01/11/2018	17	21.8	24.62	26.28	63.3	3.7	268	2	1.5	31.5
05/11/2018	21	21.8	28.41	30.28	53.2	1.9	170	2	1.2	43.7
07/11/2018	23	22.8	16.5	16.25	53.2	1.9	170	2	1.2	43.7
09/11/2018	25	26.9	14.94	12.36	53.2	1.9	170	2	1.2	43.7
12/11/2018	28	23.9	3.27	3.35	53.2	1.9	170	2	1.2	43.7
14/11/2018	30	26.7	1.73	2.16	53.2	1.9	170	2	1.2	43.7

Table C-12: Raw data results for co-digestion experiments $\mathbf{A_sW_{s2}}$ **AcoD** experiments

Date	Time	T_{room}	AW_{2-1}	AW_{2-2}	CH_4	CO_2	H_2	H_2S	O_2	Bal
	day	°C	mL		%		ppm		%	
15/10/2018	F/M = 0.96; average **VS** for ($\mathbf{A_s}$) = 5.09 g; ($\mathbf{W_s}$) =4.87 g; and totaling ($\mathbf{A_sW_s}$) = 9.96 g									
17/10/2018	2	24.1	28.79	29.71	22.0	0	1986	57	1.1	76.9
19/10/2018	4	22.1	50.66	50.24	22.0	0	1986	57	1.1	76.9
22/10/2018	7	25.9	59.46	58.88	77.2	1.3	285	2	1.2	20.3
24/10/2018	9	31.2	36.62	38.07	77.2	1.3	285	2	1.2	20.3
26/10/2018	11	28.9	35.2	33.89	68.0	2.8	220	2	1.4	27.8
29/10/2018	14	24.6	21.84	21.43	68.0	2.8	220	2	1.4	27.8
01/11/2018	17	21.8	17.92	14.39	68.0	2.8	220	2	1.4	27.8
05/11/2018	21	21.8	22.58	20.73	47.4	1.7	215	3	2.9	48.0
07/11/2018	23	22.8	14.04	13.56	47.4	1.7	215	3	2.9	48.0
09/11/2018	25	26.9	12.06	13.24	47.4	1.7	215	3	2.9	48.0
12/11/2018	28	23.9	8.60	9.48	47.4	1.7	215	3	2.9	48.0
14/11/2018	30	26.7	0	0	47.4	1.7	215	3	2.9	48.0
		pH								

Table C-13: Calculated results for **R_2 AcoD** experiments

Time	P_{vapor}	C_{b2}	CH_4	CO_2	C_bW_{s2}	CH_4	CO_2	A_sW_{s2}	CH_4	CO_2	$C_bW_{s2\ cum}$	$A_sW_{s\ 2cum}$
day	KPa	Daily gas (N mL); corrected CH_4 and CO_2 gas composition (%)									CH_4 (N mL g^{-1} VS)	
2	3.0025	13.3365			37.5891			28.4692			2.4104	1.5202
4	2.6604	19.5838			52.7643			49.6079			5.7082	4.5363
7	3.3426	40.6394			117.0399			56.1012			13.3016	6.0895
9	4.5465	32.3343			73.9537			34.3640			17.4381	6.2934
11	3.9843	13.6814			23.9431			31.4626			18.4579	8.0797
14	3.0938	12.7236			22.4337			20.1720			19.4230	8.8279
17	2.6121	16.4812			23.6788			15.2801			20.1384	8.7072
21	2.6121	14.6309			27.9022			20.5873			21.4574	9.3056
23	2.7760	2.5990			15.4915			13.0535			22.7388	10.3558
25	3.5456	5.2143			12.6376			11.7101			23.4766	11.0083
28	2.9666	7.3312			3.1138			8.5029			23.0574	11.1261
30	3.5042	3.7131			1.8027			0.000			22.8675	10.7531

C/N	16.1381	23.2735
TCOD (mg O_2 g^{-1} VS)	1.3249	1.8123
TMP (N mL g^{-1} VS)	463.4735	634.2031

Table C-14: Raw data results for blank assays C_{b3} **AcoD** experiments

Date	Time	T room	C_{b3-1}	C_{b3-2}	C_{b3-1}	CH_4	CO_2	H_2	H_2S	O_2	Bal
	day	°C		mL		%		ppm		%	
01/11/2018					Blank assay						
03/11/2018	2	26.2	10.28	11.36	11.92	0.9	0.1	188	3	1.5	97.5
05/11/2018	4	21.8	15.27	14.76	15.81	0.9	0.1	188	3	1.5	97.5
07/11/2018	6	22.8	33.99	30.86	32.72	9.3	4.5	187	3	1.8	84.5
09/11/2018	8	26.9	38.28	35.74	33.97	9.3	4.5	187	3	1.8	84.5
12/11/2018	11	23.9	19.74	19.36	20.35	10.5	6.7	198	1	1.2	81.6
14/11/2018	13	26.7	20.36	22.84	19.72	10.5	6.7	198	1	1.2	81.6
16/11/2018	15	26.1	18.36	20.28	19.54	10.5	6.7	198	1	1.2	81.6
19/11/2018	18	26.5	18.56	18.22	18.47	10.5	6.7	198	1	1.2	81.6
21/11/2018	20	23.2	16.12	15.35	14.77	10.5	6.7	198	1	1.2	81.6
23/11/2018	22	23.4	15.81	16.46	14.86	29.4	2	87	1	0.8	67.8
26/11/2018	25	25.6	15.35	17.64	16.22	29.4	2	87	1	0.8	67.8
28/11/2018	27	21.6	10.23	10.46	9.88	29.4	2	87	1	0.8	67.8
01/12/2018	30	25.2	4.12	2.36	3.83	29.4	2	87	1	0.8	67.8
03/12/2018	32	21.8	3.36	1.64	2.96	29.4	2	87	1	0.8	67.8
		pH	6.73	6.82	6.77						

Table C-15: Raw data results for co-digestion experiments C_bW_{s3} **AcoD** experiments

Date	Time	T room	$C_bW_{s\,3-1}$	$C_bW_{s\,3-2}$	$C_bW_{s\,3-1}$	CH_4	CO_2	H_2	H_2S	O_2	Bal
	day	°C		mL		%		ppm		%	
01/11/2018		F/M = 0.49; average **VS** for (C_b) = 2.53 g; (W_s) =2.54 g; and totaling (C_bW_s) = 5.07 g									
03/11/2018	2	26.2	39.02	36.76	37.94	18.1	2	1843	1	1.8	78.1
05/11/2018	4	21.8	116.83	118.04	120.71	18.1	2	618	1	1.8	78.1
07/11/2018	6	22.8	431.58	435.94	425.47	44.4	3.2	288	1	1.6	50.8
09/11/2018	8	26.9	355.24	366.17	350.86	44.4	3.2	288	1	1.6	50.8
12/11/2018	11	23.9	353.13	365.96	354.25	65.6	3.7	98	1	1.3	29.4
14/11/2018	13	26.7	250.62	257.98	248.42	65.6	3.7	98	1	1.3	29.4
16/11/2018	15	26.1	161.43	163.25	158.32	65.6	3.7	98	1	1.3	29.4
19/11/2018	18	26.5	151.78	142.96	152.61	65.6	3.7	98	1	1.3	29.4
21/11/2018	20	23.2	106.83	118.04	114.18	65.6	3.7	98	1	1.3	29.4
23/11/2018	22	23.4	45.22	44.64	46.71	46.4	0.8	54	1	0.6	52.2
26/11/2018	25	25.6	45.92	47.82	44.37	46.4	0.8	54	1	0.6	52.2
28/11/2018	27	21.6	38.65	38.25	38.42	46.4	0.8	54	1	0.6	52.2
01/12/2018	30	25.2	25.02	23.15	23.14	46.4	0.8	54	1	0.6	52.2
03/12/2018	32	21.8	15.89	17.38	15.88	46.4	0.8	54	1	0.6	52.2
		pH	6.81	6.73	6.74						

Table C-16: Raw data results for co-digestion experiments $\mathbf{A_sW_s}_{3}$ **AcoD** experiments

Date	Time	T_{room}	$A_sW_{s\ 3\text{-}1}$	$A_sW_{s\ 3\text{-}2}$	$A_sW_{s\ 3\text{-}1}$	CH_4	CO_2	H_2	H_2S	O_2	Bal
	day	°C		mL		%		ppm		%	
01/11/2018	F/M = 0.48; average **VS** for ($\mathbf{A_s}$) = 2.49 g; ($\mathbf{W_s}$) =2.54 g; and totaling ($\mathbf{A_sW_s}$) = 5.03 g										
03/11/2018	2	26.2	33.74	37.26	35.72	17.2	0.7	102	0	1.8	80.3
05/11/2018	4	21.8	97.38	95.76	93.42	17.2	0.7	102	0	1.8	80.3
07/11/2018	6	22.8	182.45	183.38	186.55	46.5	2.4	144	1	1.6	49.5
09/11/2018	8	26.9	190.36	195.47	186.55	46.5	2.4	144	1	1.6	49.5
12/11/2018	11	23.9	540.25	351.74	541.42	68.9	1.8	206	2	0.5	28.8
14/11/2018	13	26.7	406.82	410.36	403.69	68.9	1.8	206	2	0.5	28.8
16/11/2018	15	26.1	204.25	198.76	206.19	68.9	1.8	206	2	0.5	28.8
19/11/2018	18	26.5	142.72	150.37	140.64	68.9	1.8	206	2	0.5	28.8
21/11/2018	20	23.2	106.63	103.42	105.28	68.9	1.8	206	2	0.5	28.8
23/11/2018	22	23.4	81.24	78.32	84.26	80.3	0.9	161	1	0.6	18.2
26/11/2018	25	25.6	78.76	75.93	77.35	80.3	0.9	161	1	0.6	18.2
28/11/2018	27	21.6	35.16	34.98	36.93	80.3	0.9	161	1	0.6	18.2
01/12/2018	30	25.2	35.09	33.76	38.62	80.3	0.9	161	1	0.6	18.2
03/12/2018	32	21.8	20.24	18.72	19.98	80.3	0.9	161	1	0.6	18.2
		pH	6.72	6.77	6.85						

Table C-17: Calculated results for co-digestion experiments **R_3**

Time	P_{vapor}	C_3	CH_4	CO_2	$C_bW_{s\,3}$	CH_4	CO_2	$A_sW_{s\,3}$	CH_4	CO_2	$C_bW_{s\,3cum}$	$A_sW_{s\,3cum}$
day	KPa	Daily gas (N mL); corrected CH_4 and CO_2 gas composition (%)									CH_4 (N mL g^{-1} VS)	
2	3.4024	9.6092	90.0	10.0	23.1655	90.1	9.9	23.2110	96.1	3.9	4.5744	4.6136
4	2.6121	13.5428	90.0	10.0	91.5667	90.1	9.9	76.8457	96.1	3.9	22.6557	19.8880
6	2.7760	21.4759	67.4	32.6	372.4386	93.3	6.7	150.0828	95.1	4.9	96.1997	49.7196
8	3.5457	23.2615	67.4	32.6	296.4297	93.3	6.7	150.7104	95.1	4.9	154.7345	79.6758
11	2.9666	11.7867	61.1	38.9	318.1926	94.7	5.3	498.8634	97.4	2.6	217.5668	178.8335
13	3.5042	12.2906	61.1	38.9	217.0084	94.7	5.3	368.4186	97.4	2.6	260.4185	252.0630
15	3.3824	11.4017	61.1	38.9	135.3734	94.7	5.3	179.1858	97.4	2.6	287.1502	287.6793
18	3.4632	10.9997	61.1	38.9	124.6486	94.7	5.3	124.3993	97.4	2.6	311.7641	312.4058
20	2.8440	9.2067	61.1	38.9	95.4046	94.7	5.3	90.9568	97.4	2.6	330.6032	330.4850
22	2.8786	14.1765	93.6	6.4	29.5385	98.3	1.7	64.3340	98.9	1.1	336.4361	343.2725
25	3.2837	14.8310	93.6	6.4	28.8708	98.3	1.7	59.0310	98.9	1.1	342.1371	355.0059
27	2.5804	9.4052	93.6	6.4	27.8457	98.3	1.7	25.3871	98.9	1.1	347.6357	360.0521
30	3.2065	3.1139	93.6	6.4	19.4986	98.3	1.7	31.1683	98.9	1.1	351.4860	366.2473
32	2.6121	2.4465	93.6	6.4	13.4141	98.3	1.7	16.6868	98.9	1.1	354.1348	369.5641

C/N	16.2313	23.4
TCOD (mg O_2 g^{-1} VS)	1.3242	1.7948
TMP (N mL g^{-1} VS)	463.3243	628.1484

Appendix D: Kinetic modelling sample calculation

	Model	a	b	μ_m	λ	ν	R^2	RSS	df
M 10	I	--	--	0.627	4.132	--	0.898	38.155	12
	II	--	--	0.661	5.859	--	0.929	26.582	12
	III	--	--	1.213	15.868	--	0.991	3.475	11
M 9	II	--	--	7.114	0.606	--	0.994	97.074	12
	III	--	--	6.645	0.609	--	0.999	21.132	12
	III	--	--	6.869	0.639	1.131	0.999	20.475	11
	IV	0.248	-1.146	--	--	--	--	20.464	10
M 7	I	--	--	4.914	-4.890	--	0.992	21.393	12
	II	--	--	3.953	-6.901	--	0.986	41.020	12
M 6	I	--	--	8.662	3.329	--	0.998	4.848	12
	II	--	--	9.369	3.585	--	0.993	16.098	12
M 5	I	--	--	3.325	0.703	--	0.995	4.673	12
	II	--	--	3.035	0.561	--	0.991	12.624	12
M 4	I	--	--	14.893	0.599	--	0.995	39.000	12
	II	--	--	13.828	0.508	--	0.992	63.414	12
M 3	I	--	--	8.884	3.040	--	0.995	11.194	12
	II	--	--	9.761	3.328	--	0.989	23.484	12
M 2	I	--	--	4.348	-2.786	--	0.955	270.481	12
	II	--	--	3.622	-4.458	--	0.935	396.47	12
M 1	I	--	--	0.853	0.780	--	0.940	2.950	12
	II	--	--	0.800	0.847	--	0.960	1.956	12
	III	--	--	0.948	1.688	1.688	0.975	1.214	11

Appendix E: Statistical data evaluation

	Model	a	b	μ_m	λ	ν	R^2	RSS	df
				$\frac{\text{NmL } CH_4}{\text{g VS day}}$	day				
R 20	**I**	--	--	2.002	-1.807	--	0.953	103.467	12
	II	--	--	1.859	-2.006	--	0.969	66.459	12
	III	--	--	2.257	-1.038	3.264	0.979	46.342	11
	IV	0.326	-4.136	--	--	--	0.979	45.564	10
R 19	**II**	--	--	2.457	4.593	--	0.933	283.615	12
	III	--	--	2.549	5.750	--	0.970	126.125	12
	III	--	--	3.559	8.432	4.601	0.992	35.364	11
	IV	0.353	-3.647	2.860	7.952	--	0.992	35.364	10
R 18	**I**	--	--	5.366	3.218	--	0.953	948.030	12
	II	--	--	5.447	4.176	--	0.981	379.644	12
	III	--	--	7.227	6.257	3.688	0.995	106.772	11
	IV	0.241	-2.369	5.644	5.354	--	0.996	88.794	10
R 17	**I**	--	--	6.350	6.762	--	0.943	1388.850	12
	II	--	--	6.452	7.534	--	0.976	571.470	12
	III	--	--	8.786	9.585	3.818	0.992	198.835	11
	IV	0.344	-0.3031	7.043	9.143	--	0.992	190.313	10
R 16	**I**	--	--	5.554	9.515	--	0.977	329.539	12
	II	--	--	5.458	9.926	--	0.991	128.398	12
	III	--	--	6.855	10.944	2.307	0.994	83.986	11
	IV	0.206	-0.957	5.184	9.760	--	0.996	59.218	10
R 15	**I**	--	--	7.648	8.088	--	0.978	1598.024	12
	II	--	--	7.530	8.470	--	0.979	645.544	12
	III	--	--	10.070	10.196	3.456	0.992	254.607	11
	IV	0.338	-2.735	8.072	9.771	--	0.992	242.974	10
R 14	**I**	--	--	6.347	6.886	--	0.946	1276.923	12
	II	--	--	6.430	7.622	--	0.979	511.500	12
	III	--	--	8.670	9.531	3.596	0.992	184.354	11
	IV	0.326	-2.789	6.927	9.051	--	0.993	174.501	10
R 13	**I**	--	--	5.911	6.297	--	0.943	1239.354	12
	II	--	--	6.051	7.184	--	0.977	508.781	12
	III	--	--	8.260	9.308	3.868	0.992	169.456	11
	IV	0.336	-3.016	6.604	8.824	--	0.993	160.963	10
R 12	**I**	--	--	3.679	7.801	--	0.950	391.123	12
	II	--	--	3.701	8.457	--	0.981	149.089	12
	III	--	--	5.126	10.709	4.051	0.996	32.473	11
	IV	0.317	-2.698	3.989	9.959	--	0.997	26.854	10
R 11	**I**	--	--	20.016	10.488	--	0.972	4087.895	12
	II	--	--	18.669	10.382	--	0.991	1302.622	12
	III	--	--	24.445	11.587	2.960	0.998	272.115	11

	IV	0.440	-2.816	20.344	11.505	--	0.998	272.115	10
R 10	**I**	--	--	9.928	5.957	--	0.976	103.467	12
	II	--	--	9.882	6.540	--	0994	66.459	12
	III	--	--	12.130	7.439	2.238	0.998	46.342	11
	IV	0.241	-1.546	9.842	6.858	--	0.999	59.204	10
R 9	**I**	--	--	6.276	6.095	--	0.941	1492.304	12
	II	--	--	6.437	7.023	--	0.975	633.102	12
	III	--	--	8.842	9.303	4.081	0.991	216.830	11
	IV	0.320	-2.951	6.988	8.674	--	0.992	202.057	10
R 8	**I**	--	--	6.021	6.051	--	?	1363.672	12
	II	--	--	6.189	7.008	--	0.976	572.003	12
	III	--	--	8.521	9.305	4.089	0.992	184.863	11
	IV	0.329	-3.033	6.759	8.720	--	0.993	173.071	10
R 7	**I**	--	--	3.056	0.408	--	0.927	567.822	12
	II	--	--	3.055	1.297	--	0.957	331.594	12
	III	--	--	4.497	6.408	9.121	0.991	66.271	11
	IV	0.427	-6.349	3.674	5.570	--	0.992	63.410	10
R 6	**I**	--	--	2.023	-3.471	--	0.933	28.845	12
	II	--	--	1.583	-5.467	--	0.920	*34.337*	12
R 5	**I**	--	--	6.263	6.258	--	0.943	1413.396	12
	II	--	--	6.419	7.164	--	0.976	586.515	12
	III	--	--	8.791	9.346	3.945	0.992	192.418	11
	IV	0.335	-3.030	7.010	8.833	--	0.993	181.918	10
R 4	**I**	--	--	12.409	8.121	--	0.965	2271.394	12
	II	--	--	12.072	8.312	--	0.988	765.484	12
	III	--	--	15.609	9.485	2.864	0.996	280.619	11
	IV	0.446	-3.021	13.267	9.572	--	0.996	278.137	10
R 3	**I**	--	--	19.412	13.722	--	0.987	1223.150	12
	II	--	--	19.139	14.102	--	0.995	614.562	12
	III	--	--	20.263	14.215	1.191	0.995	603.049	11
	IV	0.211	-0.396	17.387	13.532	--	0.997	391.260	10
R 2	**I**	--	--	3.711	2.262	--	0.920	945.002	12
	II	--	--	3.825	3.539	--	0.995	536.559	12
R 1	**I**			13.602	11.132	--	0.986	934.417	12
	II			13.049	11.287	--	0.992	532.543	12
	III			13.059	11.289	1.002	0.992	532.542	11
	IV	0.823	-0.647	12.280	10.851	--	0.993	470.406	

Appendix D: Raw Graphs

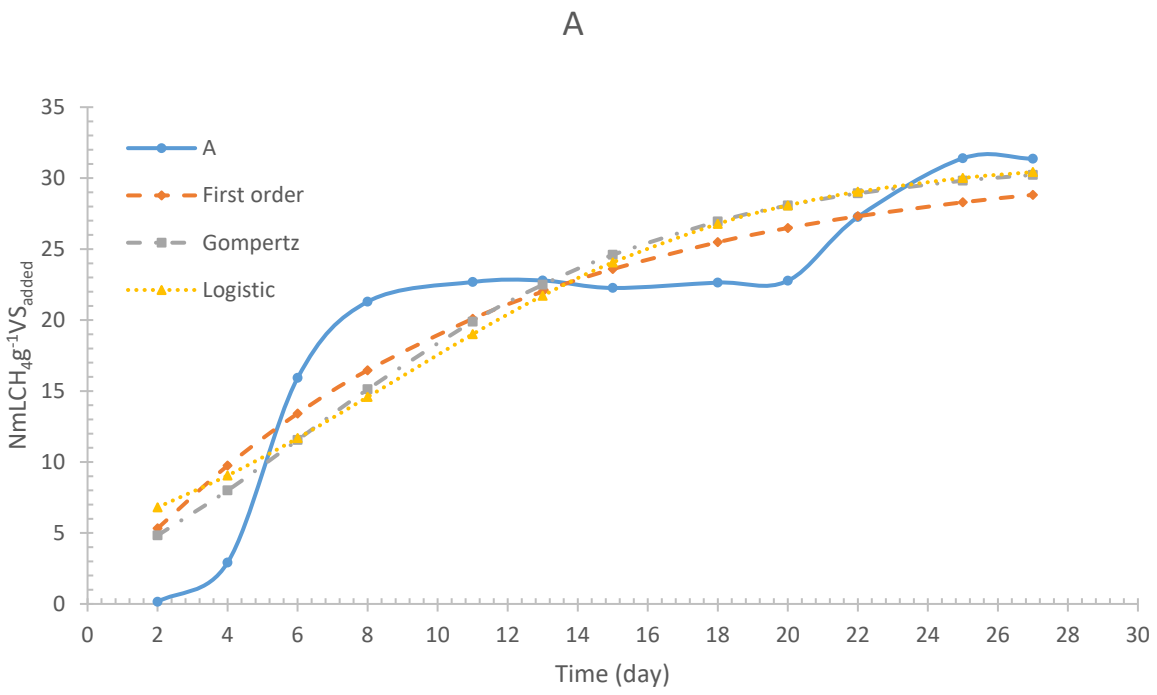

Figure: Cumulative **SMP** kinetic profiles for mesophilic **mono-D** of solid abattoir at OLR and F/M of 10 g VS L^{-1}, and 1, respectively.

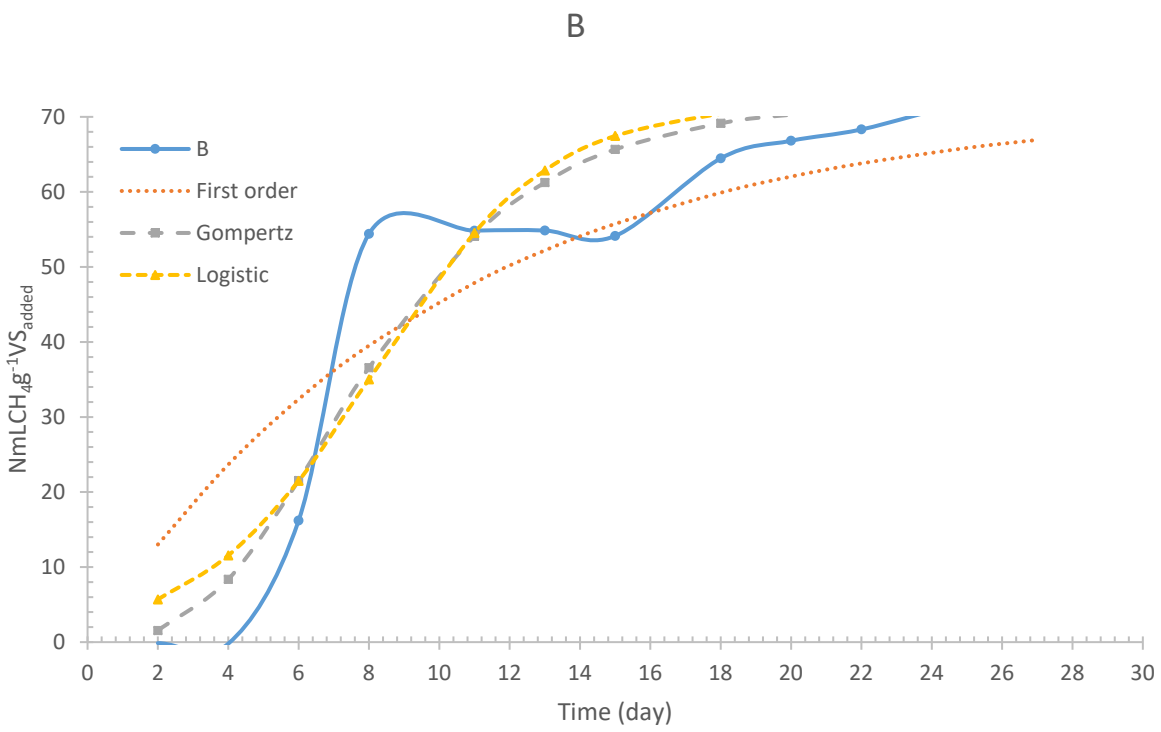

Figure: Cumulative **SMP** kinetic profiles for mesophilic **mono-D** of cow blood at OLR and F/M of 10 g VS L^{-1}, and 1, respectively.

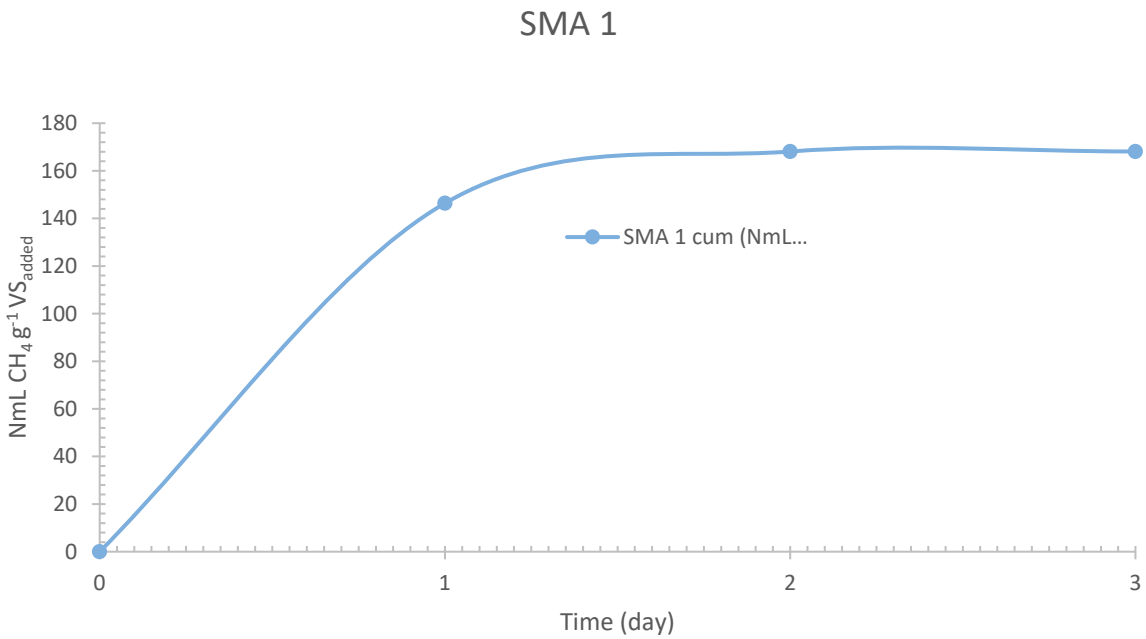

Figure: Cumulative methane **AD** profile from **SMA** test with organic loading of 0.25 g VS L^{-1} acetic acid

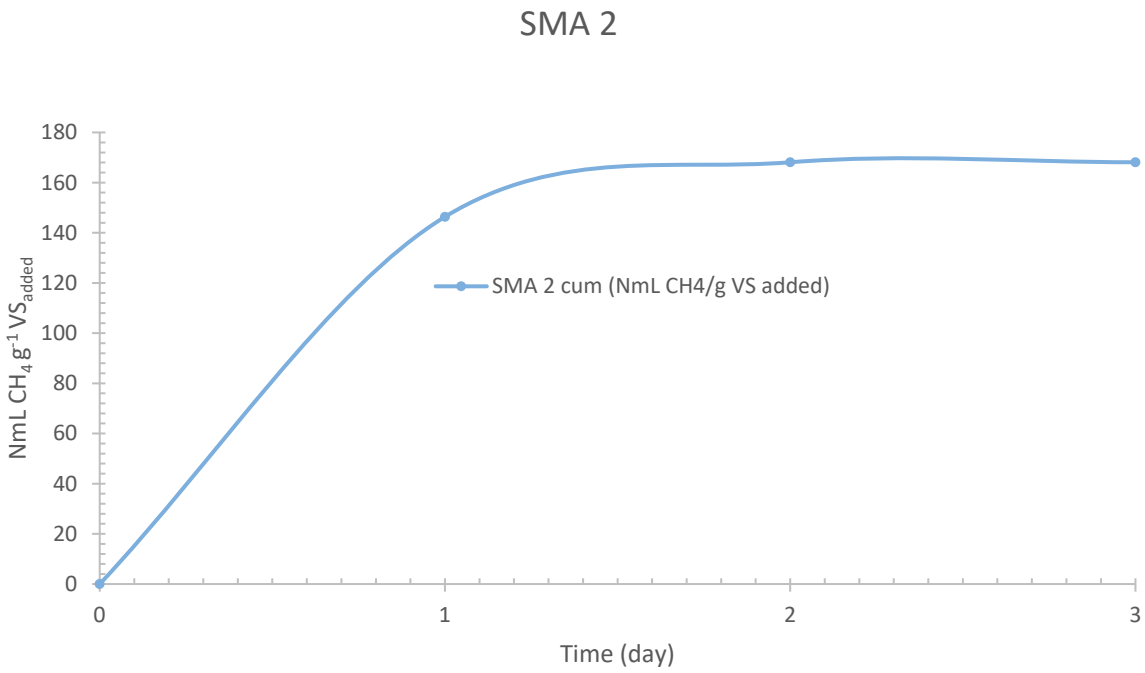

Figure: Cumulative methane **AD** profile from **SMA** test with organic loading of 0.9 g VS L^{-1} acetic acid

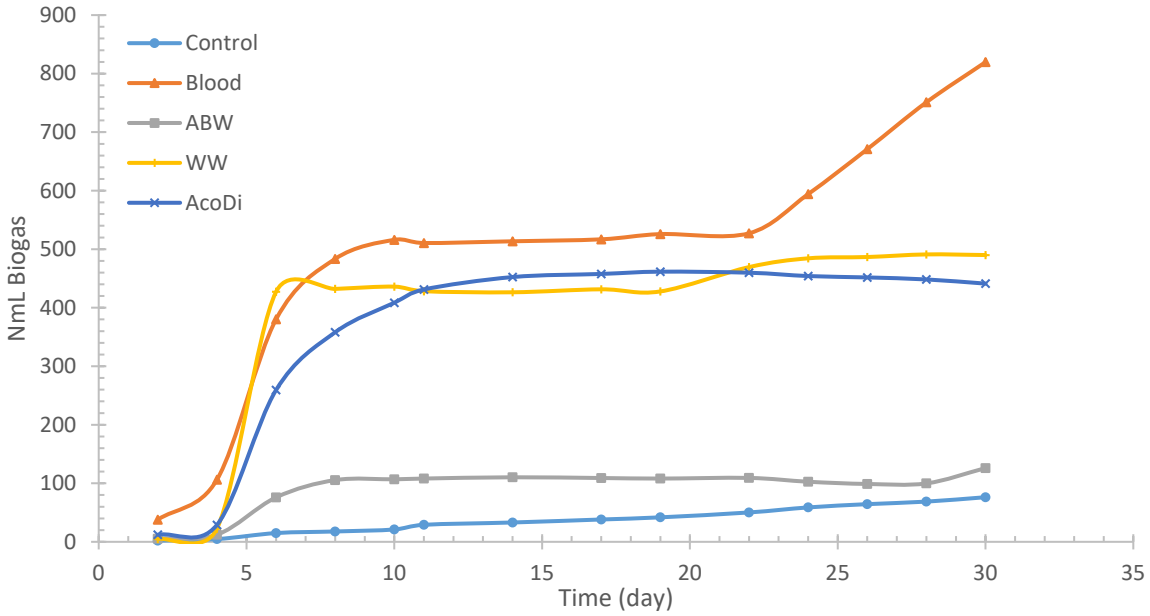

Figure: Cumulative methane production profiles for mesophilic mono- and co-digestion of abattoir and winery waste at OLR and F/M of 20 g VS L^{-1}, and 2, respectively.

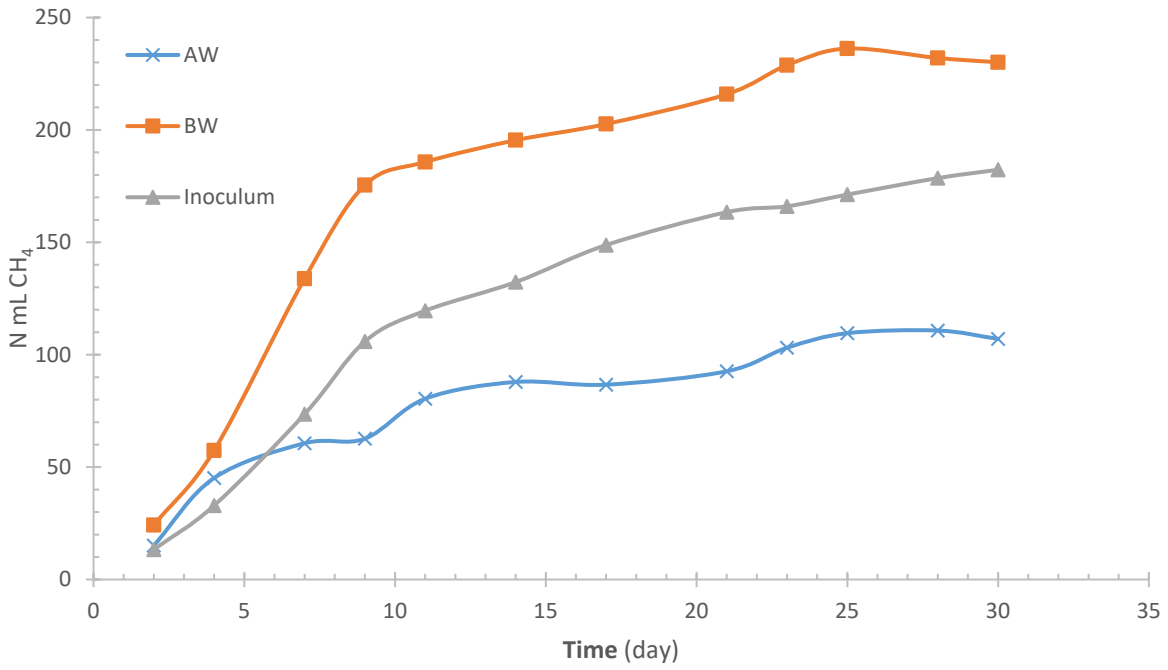

Figure: AcoD profiles for 50:50 mixture blends of **A**:W and **B**:W and control

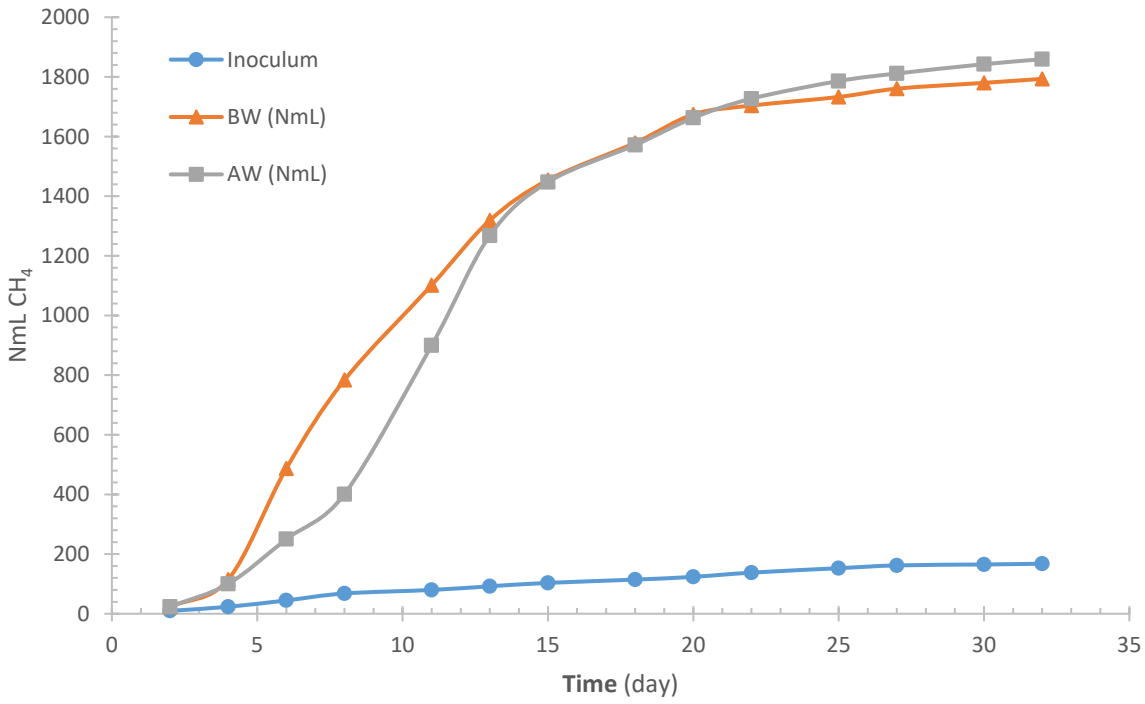

Figure: AcoD profiles for 40:60 mix ratios of **A**:**W** and **B**:**W** and control

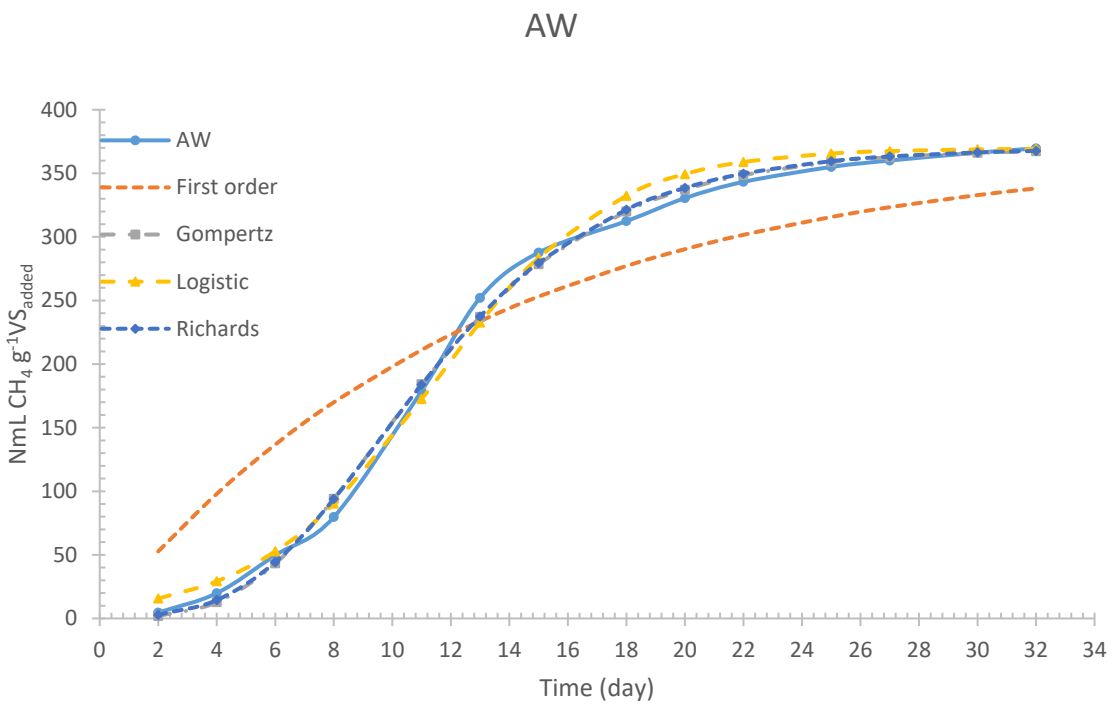

Figure: Cumulative specific methane production **AcoD** kinetic profile for 40:60 **A:W**

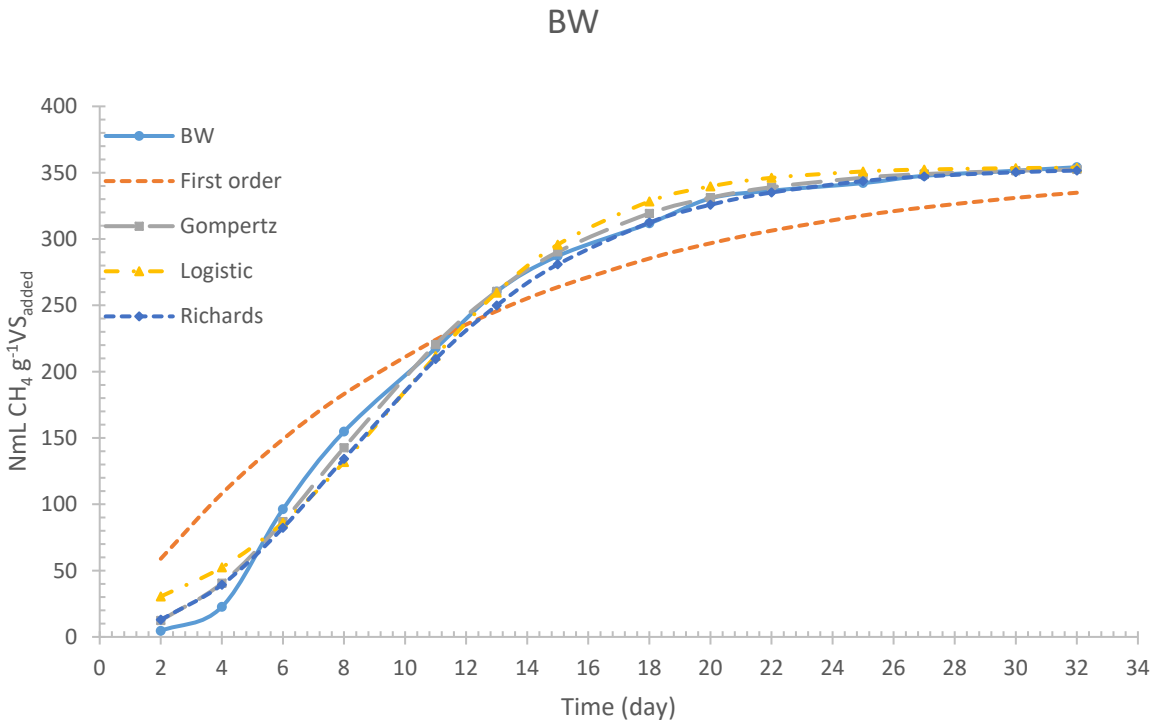

Figure: Cumulative specific methane production **AcoD** kinetic profile for 40:60 **B:W**

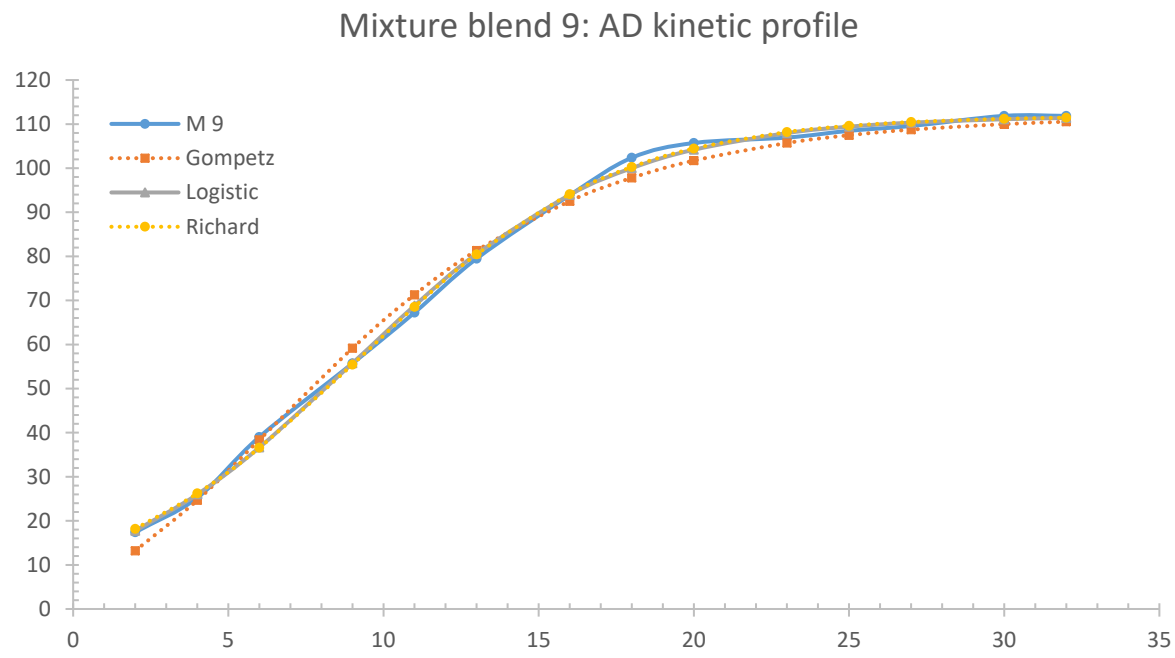

Figure: M_9 AD kinetic profiles

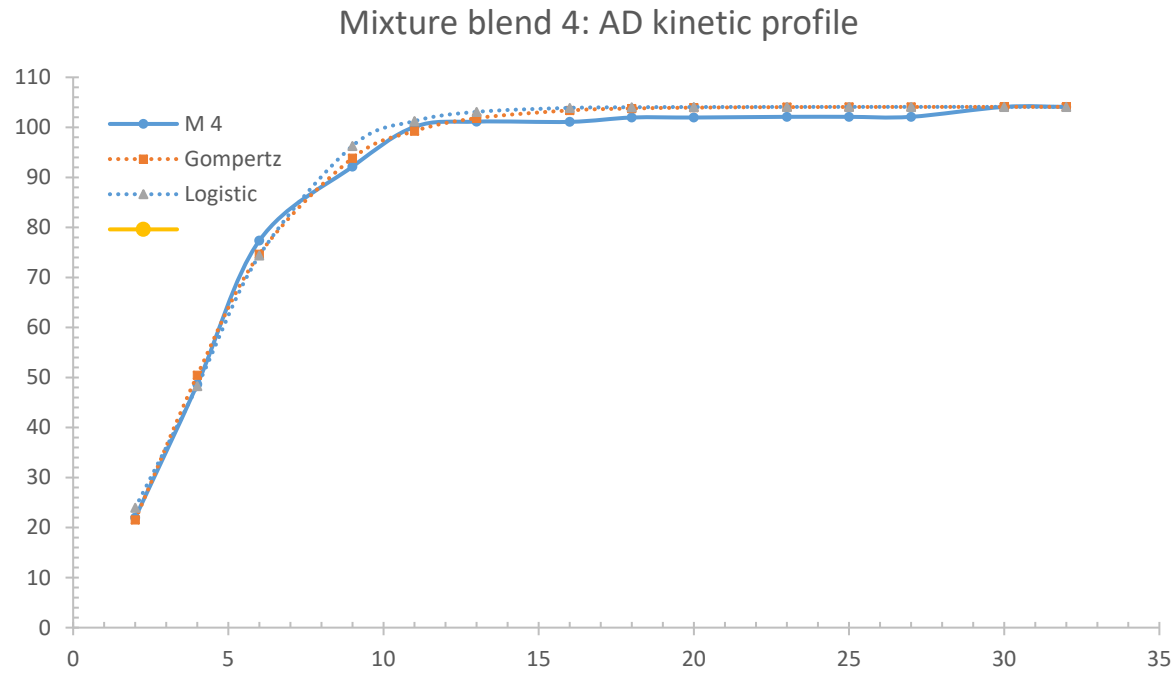

Figure: M_4 AD kinetic profiles

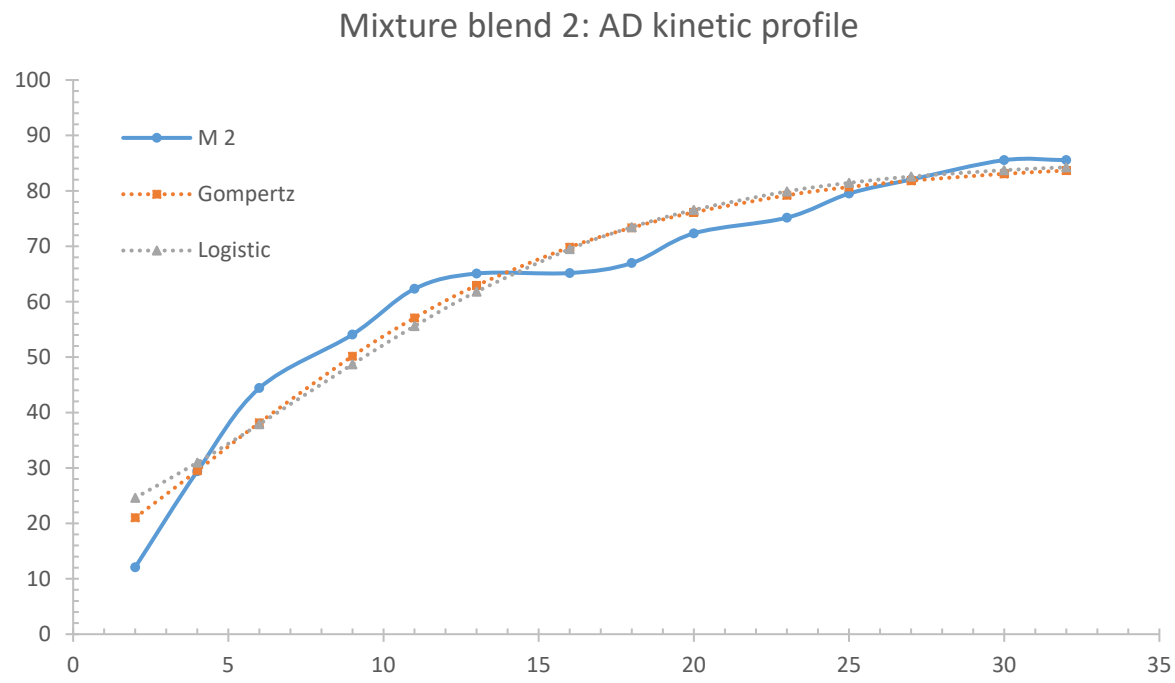

Figure: M_2 AD kinetic profiles

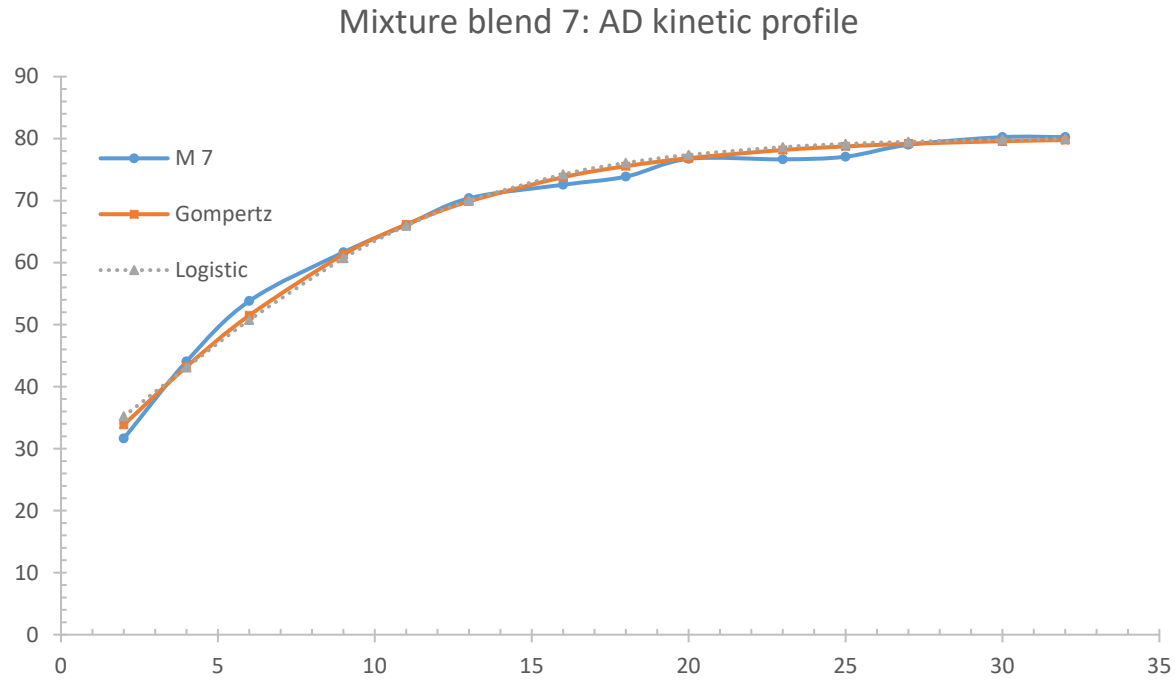

Figure: M_7 AD kinetic profiles

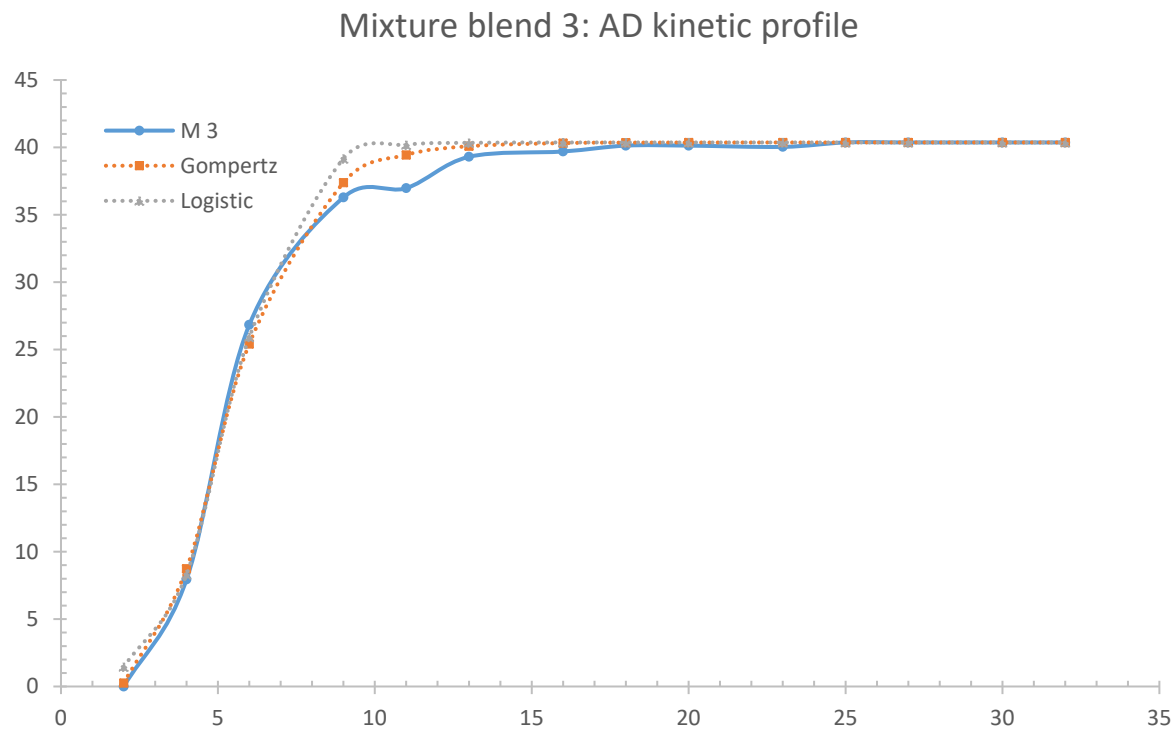

Figure: M_3 AD kinetic profiles

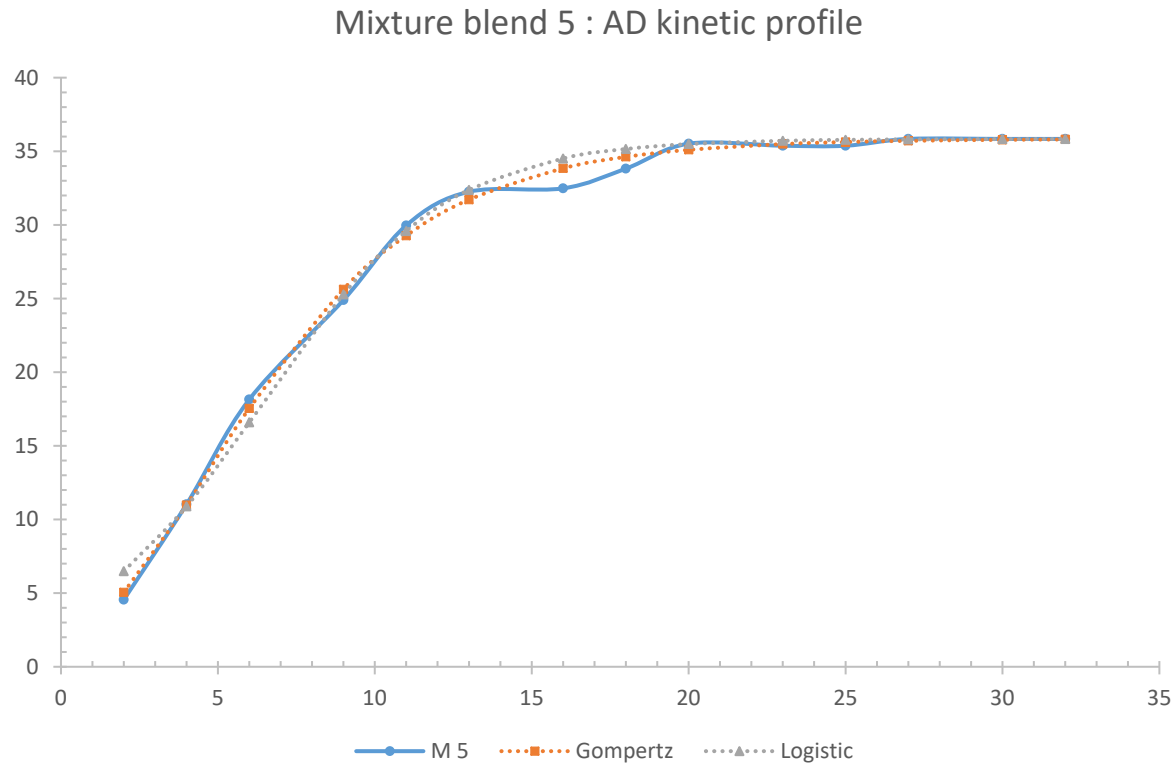

Figure: M_5 AD kinetic profiles

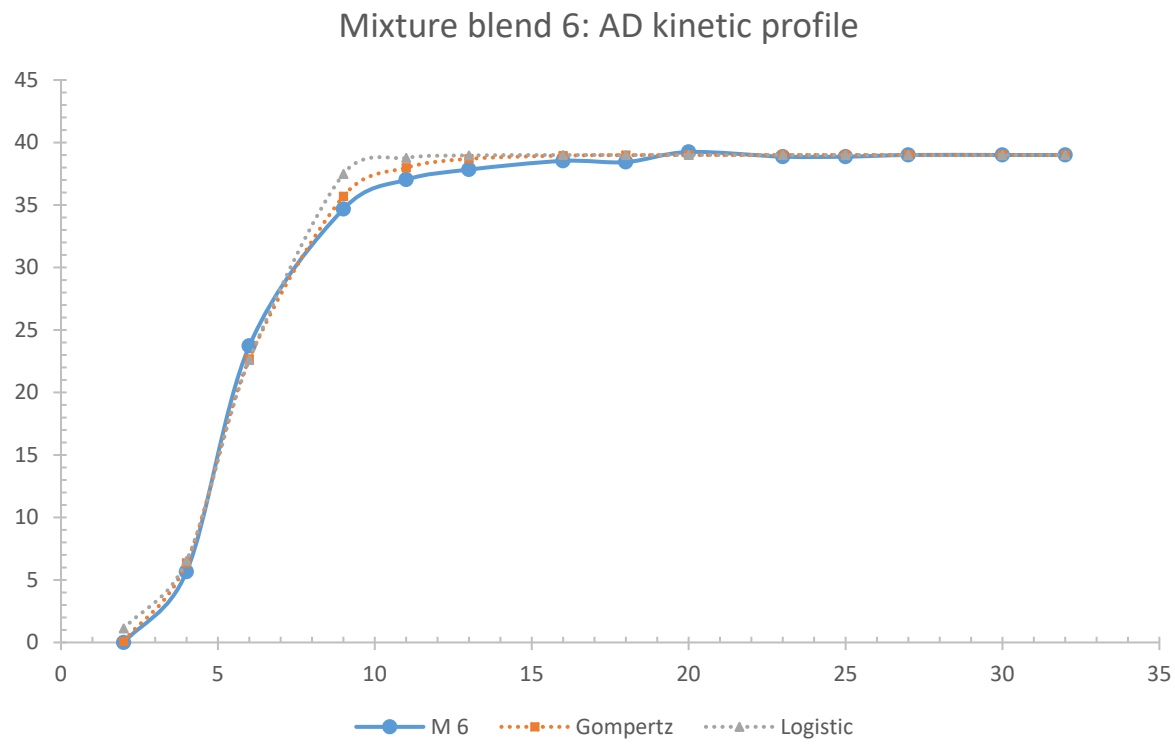

Figure: M_6 AD kinetic profiles

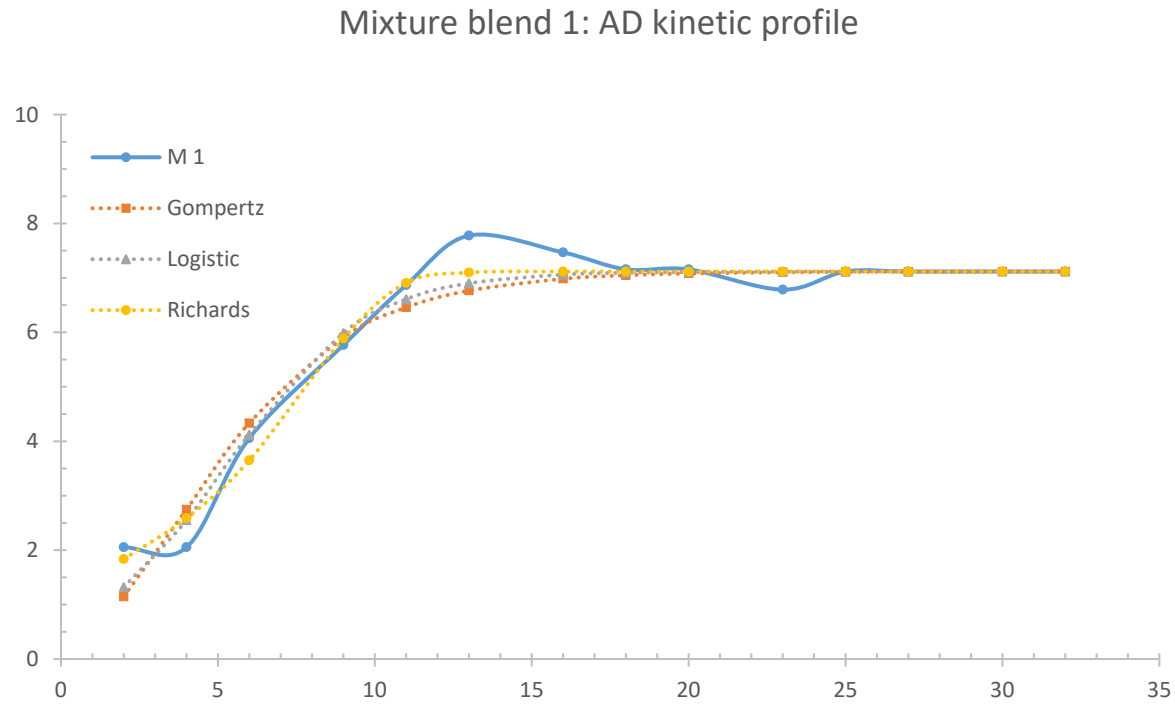

Figure: M_1 anaerobic digestion kinetic profiles

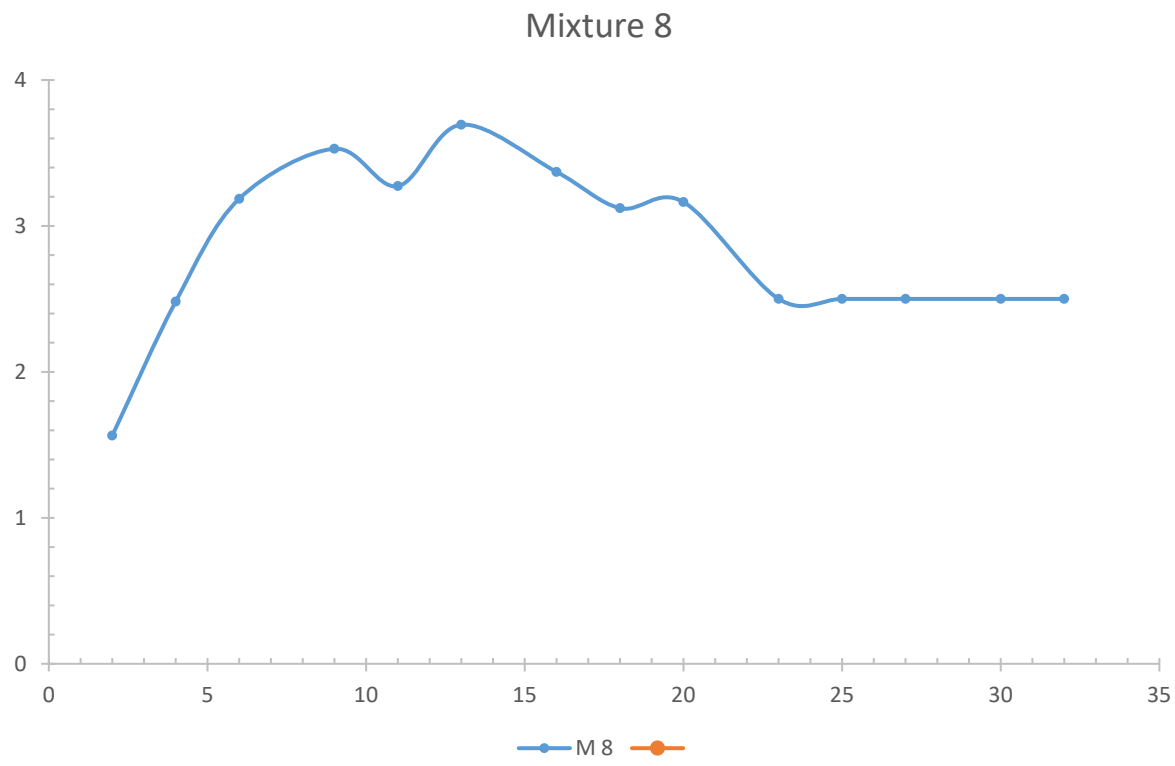

Figure 0-1

Figure: M_8 anaerobic digestion kinetic profiles

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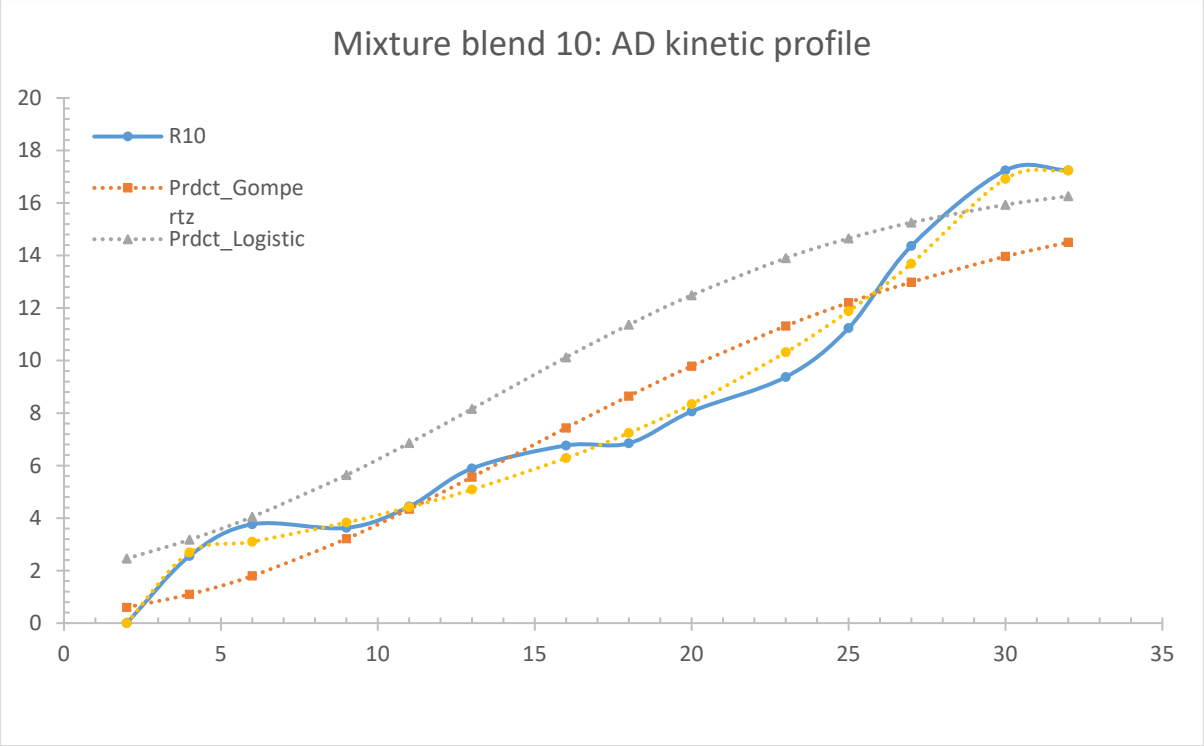

Figure: M_{10} anaerobic digestion kinetic profiles