Cape Peninsula University of Technology

EFFECTS OF TEMPERATURE, SUBSTRATE-TO-INOCULUM RATIO, NUTRIENT AUGMENTATION, AND INOCULUM ACCLIMATION ON THE ANAEROBIC DIGESTION OF PRIMARY WINERY WASTEWATER SLUDGE

by

Walusungu Kaira

217160085

Thesis submitted in fulfilment of the requirements for the degree of Master of Engineering: Chemical Engineering in the Faculty of Engineering

Supervisor: Dr Pamela Welz

Co-supervisor: Dr Debbie de Jager

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ABSTRACT

The wine industry generates substantial volumes of organic-rich primary winery wastewater sludge (PWWS), posing both economic and environmental challenges if not managed effectively. Currently, only 17% of wineries surveyed in the Western Cape province of South Africa employ anaerobic digestion (AD) for treatment of their PWWS, with others opting for composting and off-site disposal. With rising energy costs and increasingly stringent environmental regulations, circular waste management practices are essential for achieving long-term economic and environmental sustainability. This study addresses the need for a circular bio-economy approach by evaluating the feasibility of AD as a waste management and energy solution for wineries, aligned with South Africa's National Development Plan 2030 and UN Sustainable Development Goals (SDGs 7, 12, and 13).

This thesis investigated the variability of PWWS from six wineries (designated A-F) and the optimization of anaerobic digestion (AD) of the PWWS for methane (CH_4) production. The study evaluated the influence of key parameters such as inoculum acclimation, substrate composition, operational temperature, inoculum-to-substrate ratio (ISR), and micronutrient supplementation on CH_4 yields and process stability.

The variability of PWWS from winery A during the crush and post-crush seasons was examined. Results revealed significant intra-site variability ($p<0.05$), likely due to differences in winery operations during those periods. The total solids (TS), volatile solids (VS), and chemical oxygen demand (COD) content of post-crush PWWS were found to be 74%, 171%, and 132% higher, respectively, than those of crush-season PWWS. Inter-site variability was also assessed across PWWS from wineries B through E, as well as lees from winery F. Substantial variation was observed, with COD, total organic carbon (TOC), TS, and VS concentrations ranging between 33.0–181.7 g/L, 6.8–20.2 g/L, 32.8–153.1 g/L, and 26.0–123.5 g/L, respectively.

Theoretical biomethane yields at standard temperature and pressure (STP) were determined based on the COD:VS ratio of each sample. The estimated CH_4 yields were 567 $mLCH_4/gVS_{added}$ and 490 $mLCH_4/gVS_{added}$ for winery A crush and post-

crush season PWWS, respectively, 654 $mLCH_4/gVS_{added}$ for winery B PWWS, 251 $mLCH_4/gVS_{added}$ for winery C PWWS, 470 $mLCH_4/gVS_{added}$ for winery D PWWS, 544 $mLCH_4/gVS_{added}$ added for winery E PWWS, and 1 434 $mLCH_4/gVS_{added}$ for lees from winery F. The actual CH_4 yields achieved at 37°C without added nutrients were 204 $mLCH_4/gVS_{added}$, 65 $mLCH_4/gVS_{added}$, 43 $mLCH_4/gVS_{added}$, 53 $mLCH_4/gVS_{added}$, and 765 $mLCH_4/gVS_{added}$ for PWWS from wineries A-E and lees from winery F, respectively. With the addition of nutrients at 37°C, the CH_4 yields achieved were 154 $mLCH_4/gVS_{added}$, 28 $mLCH_4/gVS_{added}$, 114 $mLCH_4/gVS_{added}$, 62 $mLCH_4/gVS_{added}$, and 584 $mLCH_4/gVS_{added}$ for PWWS from wineries A-E and lees from winery F, respectively.

This study found that in comparison to ambient temperatures, when the temperature was elevated to 37°C, CH_4 production was enhanced and time required to reach maximum yield was reduced. However, heating is only justified when the increase in CH_4 production exceeds 30 mL CH_4/g VS compared to unheated conditions. For small-scale wineries, lower-cost strategies such as insulating digesters or painting them black to enhance solar heat absorption may be more appropriate and cost-effective than active heating.

The addition of a micronutrient blend containing 14 trace elements produced mixed results, likely due to substrate-specific nutrient imbalances and potential synergistic effects with temperature. These findings suggest that nutrient supplementation strategies must be tailored to the specific characteristics of the PWWS substrate and the operating conditions of the digester.

The study also highlighted the critical role of inoculum acclimation and substrate type in determining methanogenic pathways and overall process performance. When the inoculum was switched from a municipal waste-activated sludge-acclimated inoculum (MWASi) to a PWWS-acclimated inoculum (PWWSi), biomethane yields increased from 0 to 49 mL CH_4/g VS added. Moreover, analysis revealed that the dominant methanogenic populations in PWWSi were composed of both acetoclastic and hydrogenotrophic communities, indicating active methanogenesis through multiple metabolic routes.

Kinetic modelling showed that the Chen and Hashimoto and Cone models provided the best fit for predicting AD performance of PWWS from wineries B through E at 37°C. At ambient temperature, the Cone and Logistic models were more accurate. For the digestion of lees from winery F, the Logistic model consistently provided the best prediction regardless of temperature or nutrient supplementation.

These findings reveal significant variability in PWWS characteristics across wineries and seasons, underscoring the importance of a tailored approach to AD. Effective AD of PWWS requires optimization of process parameters based on specific substrate properties and the use of a well-acclimated inoculum. The study emphasizes the need for customized, site-specific strategies to enhance biogas production and improve the overall feasibility of AD in the wine industry.

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LIST OF ABBREVIATIONS

AD	Anaerobic digestion
ADg	Anaerobic digestate
AIC	Akaike information criterion
AM	Acetoclastic methanogens
AMHBI	Applied Microbial and Health Biotechnology Institute
BMP	Biochemical methane potential
C&H	Chen & Hashimoto
C:N	Carbon to nitrogen ratio
DOE	Design of Experiment
DWS	Department of Water and Sanitation
HM	Hydrogenotrophic methanogens
ICP-OES/AES	Inductively coupled plasma optical/atomic emission spectrometry
IPW	Integrated production of wine
ISR	Inoculum to substrate ratio
LCFA	Long chain fatty acids
MCC	Microcrystalline cellulose
MM	Methylotrophic methanogens
MWAS	Municipal waste activated sludge
MWASi	Municipal waste activated sludge fed inoculum

NWMS	National Waste Management Strategy
PWWS	Primary winery wastewater sludge
PWWSi	Primary winery wastewater sludge fed inoculum
RMSE	Root mean square error
SCFA	Short chain fatty acids
SOC	Soil organic carbon
SRB	Sulfate reducing bacteria
SWWS	Secondary winery wastewater sludge
VFA/VOA	Volatile fatty acids/volatile organic acids
WWTPs	Wastewater treatment plants
WWW	Winery wastewater
STWWS	Secondary tannery wastewater sludge

NOMENCLATURE

Alk	Alkalinity (mg$CaCO_3$/L)
BOD	Biological oxygen demand (mg/L)
COD	Chemical oxygen demand (g/L)
HRT	Hydraulic retention time (days)
NH_3-N	Ammonia as nitrogen (mg/L)
OLR	Organic loading rate (mgCOD/day)
PO_4^{2-}-P	Phosphate as phosphorus
SS	Suspended solids (g/L)
TAN	Total ammonia nitrogen (g/L)
TN	Total nitrogen (g/L)
TOC	Total organic carbon (g/L)
TP	Total phosphorus (mg/L)
TS	Total solids (g/L)
VOAt	Total volatile organic acids (mg/L)
VS	Volatile solids (g/L)

CHEMICAL FORMULAE

aluminium	Al
acetic acid/acetate	CH_3COOH/CH_3COO^-
ammonia/ammonium ion	NH_3/NH_4^+
ammonium chloride	NH_4Cl
butyric acid/butyrate	$CH_3CH_2CH_2COOH/CH_3CH_2CH_2COO^-$
calcium	Ca
calcium carbonate	$CaCO_3$
cadmium	Cd
calcium hydroxide	CaOH
carbon dioxide	CO_2
chromium	Cr
cobalt	Co
copper	Cu
formic (methanoic) acid	CH_2O_2
hydrochloric acid	HCl
hydrogen	H_2
hydrogen ion	H^+
hydrogen peroxide	H_2O_2
hydrogen sulfide	H_2S
hydroxylamine	NH_2OH

hydroxide ion	OH^-
hypochlorite ion	ClO^-
iron	Fe
lead	Pb
methane	CH_4
monochloramine	NH_2Cl
magnesium	Mg
manganese	Mn
nickel	Ni
nitrate	NO_3^{2-}
nitric acid	HNO_3
nitrogen	N
oxygen	O_2
peroxodisulfate	$S_2O_8^{2-}$
phosphorus	P
propionic acid/propionate	$CH_3CH_2COOH/CH_3CH_2COO^-$
potassium	K
potassium dichromate	$K_2Cr_2O_7$
sodium	Na
sodium hydroxide	NaOH
sulfate ion	SO_4^{2-}

sulfur	S
sulfuric acid	H_2SO_4
valeric acid/valerate	$CH_3(CH_2)_3COOH/CH_3(CH_2)_3COO^-$
Zinc	Zn

CHAPTER 1: INTRODUCTION AND BACKGROUND

1.1. Background

Global wine production is mainly concentrated in eight (8) countries which produce over 75% of the wine worldwide: Italy (19.3%), France (17.7%), Spain (13.8%), United States of America (8.7%), Australia (4.9%), Chile (4.8%), Argentina (4.4%), and South Africa (4.0%) (OIV, 2023). One of the by-products of making wine is winery wastewater (WWW) (Figure 1-1) (Welz et al., 2016). For each L of wine produced, 0.7 to 14 L per L of WWW is generated (Zahedi et al., 2020). In 2022, South Africa wineries produced 864.7 million L of wine from 523 wine cellars, which crushed over 1.37 million tonnes of grapes, amounting to over 1 billion L of WWW generated (SA Wine Industry Information & Systems, 2022).

The suspended solids (SS) in WWW are usually separated from the WWW by gravity in settling basins to form primary winery wastewater sludge (PWWS). Treating 1 L of WWW yields PWWS containing 1.6 g – 79.6 g of total solids (TS) that is either composted or sent to landfills (Welz et al., 2016; Mader et al., 2022; Vlotman et al., 2022). This practice is costly to wineries, due to increasing landfill fees and negative environmental consequences that can affect the rating of wineries via the Integrated Production of Wine (IPW) system.

It has been estimated that solid waste management is responsible for 1.6 $GtCO_{2eq}$ worldwide, accounting for 5% of global carbon (C) emissions (Dell'Orto & Trois, 2024). Organic waste, such as PWWS, is responsible for most of these emissions due to its high biodegradable fraction. There has also been a legislative push toward greening the South African economy, of which prime examples are the Carbon Tax Act (2019) and the National Waste Management Strategy (NWMS). This promotes waste prevention, reduction, reuse, recycling, and recovery to reduce landfilling of biodegradable solid waste (Hector & Lambrechts, 2024). These recent developments are forcing industries to divert their organic waste from landfills. Welz et al. (2023) found that only 17% of wineries surveyed in the Western Cape province of South Africa employ AD for treatment of their PWWS. Other common practices in

the region include composting, (33%), and out-sourcing of organic to waste management companies for off-site disposal (25%) (Welz et al., 2023).

Generation of organic winery wastes is highly seasonal and the effluent composition changes constantly. Wineries generally have an organic peak over the harvesting and crushing season which makes them potential candidates for energy recovery through anaerobic digestion (AD) (Howell et al., 2016). Anaerobic digestion offers a robust, cost effective solution for managing PWWS and similar organic wastes (Da Ros et al., 2017a). Anaerobic digestion (AD) convert organic into biogas and nutrient-rich anaerobic digestate (ADg), making it a better option than compared to composting (Lin et al., 2018). Additionally, AD offers lower greenhouse gas emissions by capturing methane (CH_4), which can be used for energy generation to reduce costs. This offers environmental benefits over both landfilling and composting (Appels et al., 2008; Muhlack et al., 2018).

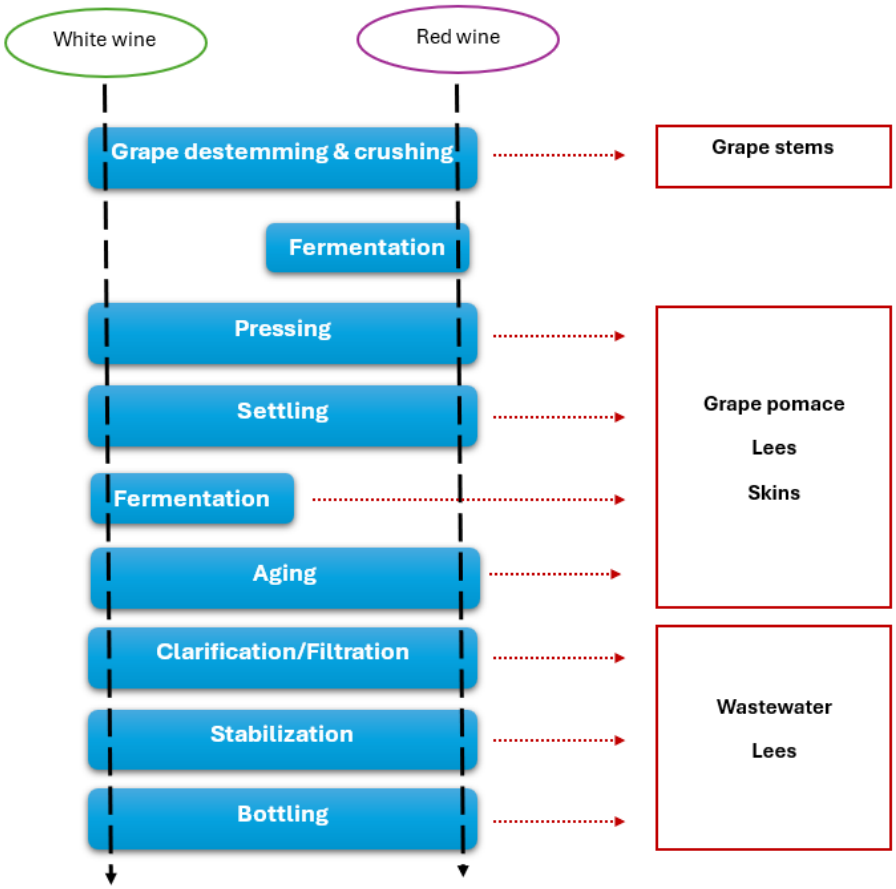

Figure 1-1: Winemaking process and by-products generated at each stage (Gomes et al., 2023)

1.2. Problem statement

The treatment and disposal of PWWS generated at winery wastewater treatment plants (WWWTPs) is a major challenge due to economic, environmental, and legislative factors. Surveys in the Western Cape found that 25% of small to medium wineries pay for off-site disposal and 33% use PWWS for composting. Considering the ongoing energy crisis, landfill shortages, increasing global carbon emissions, stringent environmental regulations, particularly the imminent organic waste to landfill ban and escalating costs of PWWS disposal, there is a need for cheaper and sustainable waste management approaches.

1.3. Research questions

i. What are the characteristics of the crushing season and off-crush season PWWS?
ii. What are the factors that affect biogas yield using AD?
iii. What are the optimal process conditions necessary for highest biogas yield?
iv. What are the characteristics (chemical composition) of PWWS, biogas and the ADg produced?
v. Is it possible to use the ADg as biofertilizer?
vi. Which kinetic models best describe the AD of PWWS?
vii. Does the source of PWWS have an impact on AD performance?
viii. Is AD of PWWS a robust, cost-effective and sustainable waste management strategy for the wine industry?

1.4. Research aim and objectives

The aim of the research is to reduce the volume of PWWS through AD and simultaneously generate biogas and ADg that can be potentially used as a biofertilizer.

The objectives of this research are:

i. To characterise the seasonal PWWS from the settling delta of a local winery.

ii. To investigate which factors (inoculum to substrate ratio (ISR), temperature and nutrient supplementation) have significant effects on the conversion of organic winery waste into biogas using AD.
iii. To optimise AD of PWWS for sludge volume reduction and biogas production, based on key factors identified using factorial design.
iv. To characterise PWWS, biogas and ADg produced.
v. To compare ADg to commercial biofertilizer.
vi. To evaluate and compare various kinetic models in order to identify the model that best describes the AD of PWWS.
vii. To assess the effect of inoculum acclimation on the start-up period, process stability, and CH_4 yield under optimum conditions.
viii. To make recommendations on AD as waste management strategy.

1.5. Scope of the thesis

Chapter 1: This chapter provides the introduction and background to the study. It outlines the research problem, highlighting on the economic and environmental challenges surrounding PWWS disposal. The chapter defines the research questions, aim, and objectives, as well as details the significance of the study.

Chapter 2: This chapter contains a literature review that provides an overview of the winery industry, generation of PWWS, treatment techniques, and waste management practices. The chapter also provides details about AD technology, including factors that affect AD and their effects on biogas yield.

Chapter 3: This chapter contains the research design and methodology. The materials and methods used to meet the set objectives of the study are described, including sampling, storage, analytical techniques, experimental setup and parametric optimisation.

Chapter 4: Results of biomethane potential (BMP) experiments on PWWS samples taken from the crush and non-crush seasons from winery A, including full characterisation of the ADg at varying ISR, temperature, and nutrient additions are presented and discussed in this chapter.

Chapter 5: This chapter covers the characterisation and optimisation of BMP for PWWS from 4 different wineries (sludge B-E), and lees (sludge F). The chapter includes a comparative analysis, evaluating the performance and fit of established kinetic models in the literature.

Chapter 6: This chapter investigates and reports on the effect of inoculum in the BMP of PWWS.

Chapter 7: This chapter reports on the conclusions of the whole study and provides recommendations.

1.6. Delineation of the research

This research primarily focused on the BMP and reduction of solids of PWWS using AD as a way of minimising waste generation. The research did not cover the following:

- Investigation on the inhibitory concentrations of specific contaminants contained in PWWS on CH_4 generation and/or substrate degradation.
- The extraction and/or recovery of other contaminants from the PWWS.
- Microbial metagenomics of the inoculum.

This research focused on establishing a baseline for biomethane yield of PWWS to address immediate industry needs for waste minimization and energy recovery. Therefore, investigations such as inhibitory kinetics and microbial metagenomics were excluded to focus on the technical feasibility of AD.

1.7. Significance and contributions of the research

The wine industry generates substantial volumes of organic-rich PWWS, posing economic and environmental challenges if not managed effectively. With increasing energy costs and stricter environmental regulations, circular waste management practices are crucial for economic and environmental sustainability.

The background and literature review highlight several critical factors that motivated this study. These motivations are derived from the existing body of knowledge and research gaps identified in the field of winery waste management, which are:

(i) There is a limited understanding of the optimal process parameters required for AD of PWSS. While the importance of factors such as organic loading rate (OLR), temperature, and nutrient supplementation in AD of other substrates has been established, there is a gap in understanding the optimal combination of these parameters for maximising biogas production and sludge volume reduction of PWWS.

(ii) Literature highlights the complex composition of WWW, including organic acids, nitrogen (N), phosphorus (P), sugars, and other compounds. However, there is a gap in comprehensive characterisation studies that link the chemical composition of WWW to PWWS and the chemical composition of PWWS to its biogas potential and ADg quality.

(iii) Data on the seasonal and/or source specific variation in the characteristics of PWWS and effect on AD performance and biogas production is lacking.

(iv) Data on the character of ADg from AD of PWWS is lacking.

(v) Data on the kinetics of AD of PWWS is limited.

Addressing these gaps in knowledge through this research contributes valuable insights to the field of waste management, resource recovery, and sustainable energy production in the wine industry.

This study aims to contribute to reducing the environmental impact of winery operations through valorisation of PWWS by exploring AD as a sustainable waste management strategy. The study advances the understanding of the factors influencing biogas yield and sludge volume reduction in AD of PWWS. Furthermore, by investigating the optimal process conditions and developing model equations to relate operational parameters to biogas production, this study may assist in decision making involving development and operations of AD treatment plants using PWWS as feed. Lastly, the characterisation studies provide insights into the composition of PWWS and ADg, which will be necessary to assess other possible options for reuse or disposal.

CHAPTER 2: LITERATURE REVIEW

2.1. Wineries and effluents

Winery wastes consist mainly of pomace (spent seeds and skins from crushing), lees (dead yeasts and solids from fermentation), and WWW from washing, cleansing and disinfection of bottles, equipment and cellar floors (Keyser et al., 2003; Bertran et al., 2004). Winery wastewater is a high strength organic waste that is low in nitrogen (N) (100-640 mg/L) and phosphorus (P) (240-657 mg/L) with sufficient trace elements for bacterial growth (Keyser et al., 2003; Vlotman et al., 2022). Other compounds include alcohols, hexose sugars (glucose and fructose), organic acids (acetic, lactic, citric, malic, succinic and tartaric), soluble proteins, peptides and tartrates (Keyser et al., 2003; Welz et al., 2016). Quantification of WWW from individual wineries is difficult because it depends on the amounts of grapes crushed, the cellar dimensions and the technologies applied (Mosse et al., 2011). Studies have shown that a wide range of WWW (0.7 to 14 L) is generated for each L of wine produced (Da Ros et al., 2017b). The composition of WWW depends on the winemaking technologies employed and seasonal cellar activities such as crushing and bottling. The organic content of WWW peaks during the crush season. On average, the chemical oxygen demand (COD) measurements range from 3500 to 7700 mg/L across all seasons (Mader et al., 2022). Much of the organic fraction is soluble and highly biodegradable due to the presence of ethanol, sugars, and organic acids (Mader et al., 2022). The removal of particulate organic material from WWW generates 0.21-0.28 kg of volatile solids (VS) per kg of COD removed (Da Ros et al., 2017b). To safely dispose/reuse WWW, some form of treatment is required to minimize environmental degradation and to comply with relevant legislation.

Table 2-1: Winery wastewater pollutants

	Unit	Min	Max	Source
COD	mg/L	340	296119	(Vlotman et al., 2022; Mader et al., 2022)
BOD	mg/L	6	130000	(Vlotman et al., 2022; Mader et al., 2022)
TS	mg/L	1602	79635	(Vlotman et al., 2022)
TVS	mg/L	130	54952	(Vlotman et al., 2022)
pH		2	13	(Vlotman et al., 2022; Mader et al., 2022)
TN	mg/L	0.7	415	(Vlotman et al., 2022; Mader et al., 2022)
P	mg/L	0.4	280	(Vlotman et al., 2022; Mader et al., 2022)
K	mg/L	1.3	1270	(Vlotman et al., 2022; Mader et al., 2022)
Na	mg/L	5.4	3060	(Vlotman et al., 2022; Mader et al., 2022)
SS	mg/L	60	30300	(Vlotman et al., 2022; Mader et al., 2022)
VOAt	mg/L	1	11254	(Mader et al., 2022)

COD = chemical oxygen demand, BOD = biological oxygen demand, TS = total solids, TVS = total volatile solids, TN = total nitrogen, P = phosphorous, K = potassium, Na = sodium, TSS = suspended solids, VOAt = total volatile organic acids

2.2. Winery wastewater treatment and sludge generation

There are many different methods used to treat WWW, ranging in complexity. One of the main goals of the treatment process is to reduce the organic load usually measured as biological oxygen demand (BOD) or chemical oxygen demand (COD) (Welz et al., 2016). Preliminary treatment will typically involve settling or screening of larger particles like grit, seeds, stems, and grapes (Mosse et al., 2011). Effluents are then allowed to settle in a sedimentation tank or basin to remove most of the SS as PWWS (Welz et al., 2016). Small wineries in South Africa generally do not treat their effluent further and reuse the water for “beneficial irrigation” after pH dosing in compliance with the Department of Water and Sanitation (DWS) general authorisations guidelines for the disposal of “biodegradable industrial wastewater” (DWS, 1998). Medium to larger wineries usually treat their WWW further using secondary (biological) or advanced physicochemical methods such as ozone-based advanced oxidation (Davididou & Frontistis, 2021), heterogeneous photo-Fenton, reverse osmosis (Ioannou et al., 2013), and photocatalytic advanced oxidation (Souza et al., 2013). Such systems require high capital and operating costs and often require skilled personnel to operate and are generally not economically feasible for small wineries (Holtman et al., 2018). Many wineries utilise waste stabilisation ponds or constructed wetlands which are

simple and cheap to operate. However, odour problems are associated with ponds, and long hydraulic retention times (HRTs) are required, which translates into large spatial footprints (Holtman et al., 2018). Primary winery wastewater sludge is generated in primary sedimentation before the WWW is treated further or used for irrigation (Da Ros et al., 2017b). It is estimated that PWWS represents 12% of the total organic solid waste produced by wineries (Da Ros et al., 2017b). In most instances, seasonal cleaning activities result in WWW and PWWS that is rich in organics but can also contain varying amounts of inorganics from cleaning and disinfection products (Bertran et al., 2004). The total solids (TS) of the sludge generally ranges from 129.0 to 194 g TS/kg while the ratio of volatile solids (VS) to TS reported in literature is very high (over 80%), probably due to the high biodegradability of the raw WWW, however outliers can exist due to technical differences (for example, conditioner doses and filter press settings) and season (Da Ros et al., 2017). Consequently, AD is the most appropriate approach to mitigate and valorise the high organic matter content in PWWS (Zahedi et al., 2020).

2.3. Sludge handling and disposal

Winemaking generates several types of solid waste, including lees, fining agents, filtration aids, and grape marc (also known as pomace) – the skins, seeds, and stems remaining after pressing (Welz et al., 2016). Winery wastewater generated from cleaning and disinfecting cellar equipment and floors contains a variable mix of solid grape residues, chemicals, and filter aids (Welz et al., 2016; Zahedi et al., 2020).

Recent advancements have seen industrial bio-refineries established to extract value-added products from grape pomace and lees, such as phenolics, dietary fibre, cellulose, grapeseed oil, and tartaric acid (Zhang et al., 2017a; Sirohi et al., 2020; Filippi et al., 2022). Some of the lower value solid wastes can be composted for beneficial use, this includes vermi-composting and co-composting (Bertran et al., 2004). However, some wineries resort to landfilling their solid waste, this approach represents a missed opportunity. Not only does it pose an economic burden on the industry and environmental burden on landfill sites, but it also ignores the potential for extracting or producing valuable products and/or recovering energy from this organic-rich waste.

2.4. Anaerobic treatment

Anaerobic digestion is a biological decomposition process that occurs in the absence of oxygen (O_2) (Lohani & Havukainen, 2018). Most of the processes are controlled directly by the microorganisms themselves. However, the operational conditions such as temperature, pH, concentrations of essential trace nutrients and toxicants can play major roles in modifying reaction rates of individual sub-processes (Lohani & Havukainen, 2018). The decomposition of organic matter is a multi-step process of a series and parallel reactions namely hydrolysis, acidogenesis, acetogenesis and methanogenesis (Lohani & Havukainen, 2018; Jamaldheen & Goyal, 2020).

2.4.1 Hydrolysis

Hydrolysis of the complex organic matter is an important first step in anaerobic biodegradation. During hydrolysis, hydrolytic bacteria transform the insoluble complex organic substrate (carbohydrates, proteins, lipids) into soluble monomers and polymers (Meegoda et al., 2018). This process is catalysed by microbial enzymes including proteases, lipases and cellulases that hydrolyse proteins to amino acids, lipids to long-chain fatty acids (LCFA) and polysaccharides to simple sugars (Lohani & Havukainen, 2018; Meegoda et al., 2018). Hydrolysis is usually the rate-limiting process for the overall digestion of substrates with high SS/COD ratios (Meegoda et al., 2018; Lohani & Havukainen, 2018).

Equation 1 shows an example of hydrolysis reaction where organic waste is broken down into a simple sugar (in this case glucose).

$$C_6H_{10}O_4 + 2H_2O \rightarrow C_6H_{12}O_6 + 2H_2 \quad \text{…equation 1}$$

2.4.2 Acidogenesis

The second step, acidogenesis, is generally believed to proceed at a faster rate than all other stages of the AD process (Meegoda et al., 2018). In this stage, the hydrolysis products (amino acids, LCFA and simple sugars) which are relatively soluble compounds, are converted into variety of small organic compounds, mainly volatile fatty acids (VFAs), including acetic acid (CH_3COOH), propionic acid (CH_3CH_2COOH), butyric acid ($CH_3CH_2CH_2COOH$), valeric acid ($CH_3CH_2CH_2CH_2COOH$) and formic acid (HCOOH) (Lohani & Havukainen, 2018; Jamaldheen & Goyal, 2020). Other organic acids such as lactic acid ($C_3H_6O_3$) as well as hydrogen (H_2), carbon dioxide

(CO_2) and ammonia (NH_3) are also formed via acidogenesis (Lohani & Havukainen, 2018; Jamaldheen & Goyal, 2020). Hydrogen, CO_2 and CH_3COOH can be used directly by methanogens (Lohani & Havukainen, 2018).

Acidogenesis is mainly performed by fermentative microorganisms, and the end products depend on the conditions of the reactor medium. For instance, acetate (CH_3COO^-) is the main end-product if H_2 is effectively removed by H_2-scavenging organisms such as hydrogenotrophic methanogens (HM) (Lohani & Havukainen, 2018). However, if methanogenesis is retarded and H_2 accumulates, more reduced products such as propionate ($CH_3CH_2COO^-$) and butyrate ($CH_3CH_2CH_2COO^-$) are likely to accumulate (Lohani & Havukainen, 2018). Therefore, effluents of overloaded or disturbed anaerobic reactors often contain these more reduced intermediate products and become acidic. Equations 2 and 3 represent typical acidogenic reactions where glucose is converted into CH_3COOH and CH_3CH_2COOH, respectively.

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad \text{…equation 2}$$

$$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2CO_2 + 4H_2 \quad \text{…equation 3}$$

2.4.3 Acetogenesis

In the third stage, the short-chain fatty acids (SCFA) that are produced during acidogenesis are further converted to CH_3COOH, CO_2 and H_2 by acetogenic bacteria (Lohani & Havukainen, 2018; Jamaldheen & Goyal, 2020). There are two types of acetogenic bacteria, namely hydrogen-producing acetogens and homoacetogens (Lohani & Havukainen, 2018). Equations 4 and 5 show the production of CH_3COOH from $CH_3CH_2CH_2COOH$ and CH_3CH_2COOH, respectively, by hydrogen-producing acetogens.

$$2CH_3CH_2CH_2COOH + 4H_2O \rightarrow CH_3COOH + 2CO_2 + 6H_2 \quad \text{…equation 4}$$

$$2CH_3CH_2COOH + 2H_2O \rightarrow CH_3COOH + CO_2 + 3H_2 \quad \text{…equation 5}$$

2.4.4 Methanogenesis

The final stage of AD is called methanogenesis in which degradable organic material is finally converted to a biogas containing CH_4. Three types of methanogenic archaea are known to be responsible for AD, namely acetoclastic methanogens (AM), hydrogenotrophic methanogens (HM) and methylotrophic methanogens (MM) (Lohani & Havukainen, 2018; Karakashev et al., 2006; Oliveira et al., 2025). Acetoclastic

methanogens convert CH_3COOH into CH_4 and CO_2 via equation 6 (Meegoda et al., 2018). Hydrogenotrophic methanogenesis is the production of CH_4 from dissolved H_2 and CO_2 by HM (Lohani & Havukainen, 2018; Oliveira et al., 2025). These methanogens produce CH_4 by the reaction shown in equation 7 (Meegoda et al., 2018). Methylotrophic methanogens produce CH_4 from a variety of methyl compounds, or the methyl group of CH_3COOH (Costa & Leigh, 2014; Liang et al., 2024). The major constituents of biogas typically produced are CH_4 (50–80%), CO_2 (20–50%) and hydrogen sulfide H_2S (1%) (Jamaldheen & Goyal, 2020). In cases where sulfate (SO_4^{2-}) is present in sufficiently high concentrations, sulfate-reducing bacteria (SRB) may compete with the methanogens for substrate (Lohani & Havukainen, 2018). Methanogens suffer an acute sensitivity to O_2. For example, in a study by Meegoda et al (2018) 99% of the HM species *Methanococcus voltae* and *Methanococcus vannielli* were killed after exposure to O_2 for 10 hours.

$$CH_3COOH + 2H_2O \rightarrow CH_4 + CO_2 \quad \text{…equation 6}$$

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \quad \text{…equation 7}$$

2.5. Factors affecting anaerobic digestion

2.5.1 Temperature

Anaerobic processes can occur in a wide range of temperatures from psychrophilic (<20°C) to mesophilic (25–40°C) to thermophilic (45–60°C) (Meegoda et al., 2018; Lohani & Havukainen, 2018). The temperature of the AD reactor influences the physical and chemical properties of the substrate which in turn affects the thermodynamic and kinetic reactions of the biological processes (Lohani & Havukainen, 2018). Different behaviours are observed for psychrophilic, mesophilic and thermophilic conditions due to the temperature effects on the microbial populations. In general, anaerobic degradation occurs at higher temperatures with faster microbial growth rates and consequently higher gas production rates and shorter residence times as depicted in Figure 2-1 (Weinrich & Nelles, 2021).

There are several advantages with increasing temperatures. For instance, the hydrolytic rates increase, leading to higher availability of soluble products for downstream microbial reactions. The general increase in chemical and biological

reaction kinetics leads to higher solids reduction, shorter reaction times and lower HRTs (Da Ros et al., 2017b). Moreover, increased temperature also reduces the time required for pathogen and polyphenol destruction during AD (Lohani & Havukainen, 2018). Two main advantages of using thermophilic conditions are increased TS reduction and increased biogas yield as evidenced in a study by Da Ros et al. (2017) where the TS removal improved by 12% and biogas yield increased by 18% when the temperature was increased from 37˚C to 55˚C. However, high temperatures can have negative effects as well. Increasing temperature and hydrolysis rates increases the fraction of free NH_3 that is inhibitory to some microorganisms (Lohani & Havukainen, 2018). Thermophilic conditions may lead to higher costs (due to increase energy input requirements) and possibly lower net energy output if no significant increase in CH_4 yield is achieved.

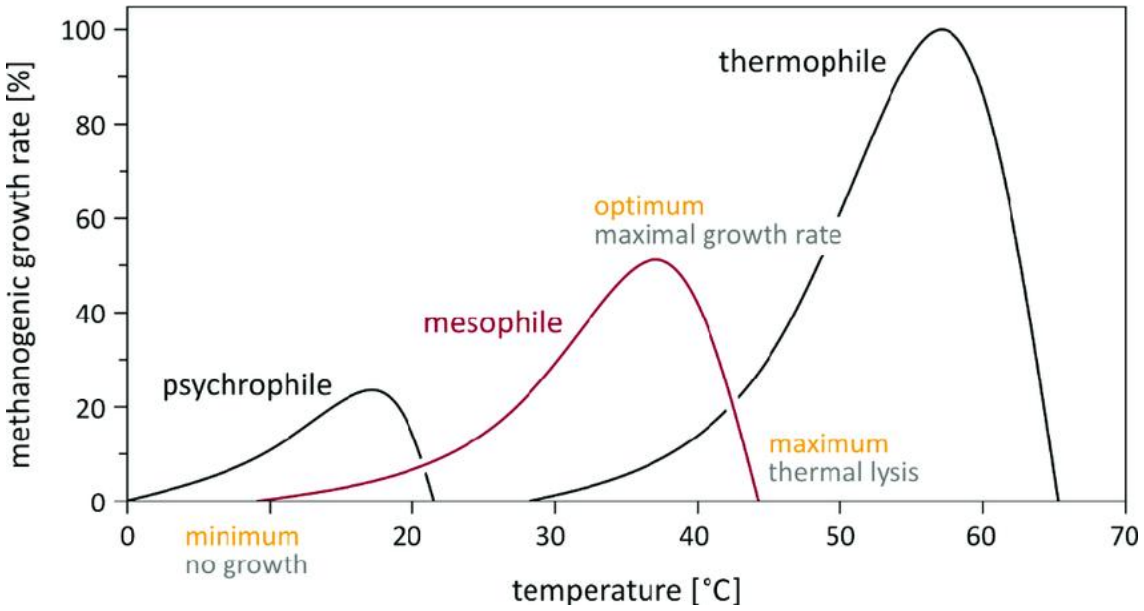

Figure 2-1: Effect of temperature on growth rate of methanogens (adapted from Weinrich & Nelles, 2021)

2.5.2 pH

The optimum range of pH for AD that has been reported in literature is 6.5 to 7.5 (Bamba et al., 2021; Lohani & Havukainen, 2018). At lower pH values, the growth of methanogenic archaea reduces, leading to lower CH_4 yields. The greatest risk for

digester failure is a result of acid accumulation which can occur with high VS loading (Lohani & Havukainen, 2018) At reduced pH, acidogens become more active, and the increased amounts of organic acids can lower the pH to below 5.0, which is lethal to methanogens (Lohani & Havukainen, 2018). While fermentative bacteria function in the pH range 4.0 to 8.5 (optimal 5.0 to 6.0), methanogens function from pH 5.5 to 8.5 (optimal 6.5–8.0) (Lohani & Havukainen, 2018). Acetoclastic methanogens are particularly vulnerable to pH levels < 6.5 (Lohani & Havukainen, 2018).

Zhai et al. (2015) emphasized the significance of controlling the pH at the initial stage of AD to improve CH_4 production. The study reported an enhancement in reactor performance when the pH was controlled during the first 4 days of the digestion period using sodium hydroxide (NaOH) and hydrochloric acid (HCl), and resulted in 1.9-10 times more biogas production from AD. However, with some substrates, for example, sugar beet, the highest CH_4 yield (73%) was achieved when the pH was adjusted to 8, which illustrates that microbes can also have different pH sensitivity and responses in different environments (Bamba et al., 2021).

Low pH values in digesters contents could be due to the lack of buffering capacity in the substrate and/or excessive accumulation of VFAs by acidogenic bacteria (Cai et al., 2025). High pH could be due to prolific methanogenesis, resulting in a high concentration of NH_3 that may impede acidogenesis (Lohani & Havukainen, 2018). After the digestion process, the pH values are generally observed to increase, often beyond the optimal range, likely due to the conversion of organic N to ammonium ions (NH_4^+) (Bamba et al., 2021). Therefore, initial control of pH and NH_3 and NH_4^+ within the desired ranges can prevent excessive VFAs production until the reactor becomes stable (Yang et al., 2025). For instance, Sheng et al. (2013) used ammonium chloride (NH_4Cl) as a source of ammonia nitrogen (NH_3-N) during AD of food waste and found that CH_4 production was enhanced by 5.1% when they added 1.1 g/L of NH_3-N, compared with the reactors without additions of NH_3-N. Further addition of NH_3-N by 1.9 to 2.82 g/L resulted in decreased CH_4 yields by 8% and 18%, respectively.

2.5.3 Buffer capacity

During AD, buffer capacity is essential to relieve the acid stress during acidogenesis. Rapid acid accumulation is related to high amounts of easily biodegradable

carbohydrates in the substrate. High buffer capacity helps maintain a stable pH, ensuring that microbial communities can function effectively at optimal conditions. Research by Hu et al. (2024) and Singhal et al. (2023) showed that addition of biochar can enhance CH_4 production rates of the organic fraction of municipal solid waste by improving buffer capacity through the presence of alkali and alkaline earth metals.

A strong buffer contains a relatively high concentration of a weak acid and its conjugated base, so that the effect of acidic or alkaline substrate components and metabolic products is balanced by the reaction of free hydrogen ions (H^+) or hydroxide (OH^-) ions with the existing base or acid (Figure 2-2) (Weinrich & Nelles, 2021). There are several ways to enhance the buffer capacity of anaerobic digestors, including addition of alkaline materials such as calcium hydroxide (CaOH), co-digestion with other high alkalinity wastes or wastes with varied VFA production rates, and microbial inoculation and acclimatization. A wide range of organic acids (including tartaric, malic, lactic acids), and polyphenols are expected in PWWS. The wide range of polyphenols and organic acids present in PWWS (along with their conjugate bases) could assist in provide buffering capacity.

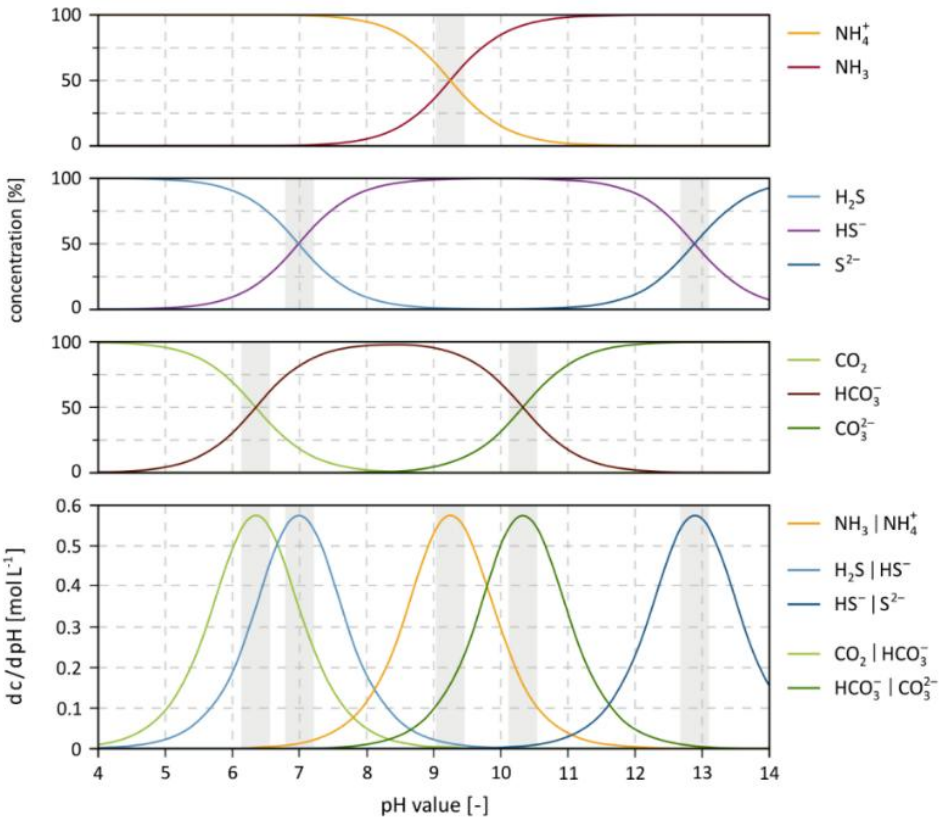

Figure 2-2: Speciation diagram as a function of pH (adapted from Weinrich & Nelles, 2021)

2.5.4 Hydraulic retention time

Hydraulic retention time refers to the mean residence time of the substrate within the reactor volume and is a key parameter that affects biochemical properties of organic materials (Meegoda et al., 2018; Lohani & Havukainen, 2018). The HRT plays an important role in AD, especially for methanogens at low operational temperatures (Lohani & Havukainen, 2018). The HRT should be long enough to provide sufficient methanogenic activity (Lohani & Havukainen, 2018). Generally, for stable operations, longer HRT corresponding to large digester sizes promote solids degradation and maximum biogas yield as compared to shorter HRTs that reduce the extent of hydrolysis (Dareioti et al., 2022; Appels et al., 2008; Lee et al., 2011; Zupančič & Jemec, 2010).

Methanogenesis starts at HRT between 5 and 15 days at 25°C and between 30 and 50 days at 15°C, depending on the characteristics of the substrates (Lohani & Havukainen, 2018).

The optimum HRT differs with substrate type, organic loading, solids concentration, temperature, and the microbial consortium that is present. Ideally, for perfectly mixed suspended growth continuous reactors, HRT is inversely proportional to the OLR, according to Equation 8 (Appels et al., 2008).

$$HRT = \frac{V_R}{OLR_{vol}} \quad \text{…equation 8}$$

HRT [days], reactor volume (V_R) in [m³], substrate volumetric feed rate OLR_{vol} in [m³/d]

2.5.5 Inoculum to substrate ratio

The inoculum to substrate ratio, namely the ratio of VS from the inoculum to VS from the substrate, is a key parameter of BMP tests. The amount of substrate available for growth and metabolism of microorganisms in biological systems is dependent on the ISR.

It is recommended that the portion of VS from the inoculum be greater than that from the substrate to minimize acidification or inhibition problems (Holliger et al., 2016). Holliger et al. (2016) further recommends that's ISRs should for most applications be

between 2 and 4. For easily degradable substrates where rapid accumulation of fermentation intermediates such as VFAs could lead to inhibition of AD, an ISR ≥ 4 should be applied.

At low ISR, rapid microbial growth takes place whereas at high ISR, there is insufficient microbial growth substrate (Lohani & Havukainen, 2018). If the applied ISR is too low, organic acids accumulate, causing an irreversible acidic state of the digester (Lohani and Havukainen, 2018). A study by Zahedi et al. (2020) found that decreasing the ISR of WWW resulted in a decrease in specific CH_4 generation, but an increase in total CH_4 production (Zahedi et al., 2020).

2.5.6 Toxic and inhibitory substances

The AD process may be inhibited by the presence of toxic substances causing an upset of biogas production, decomposition of organic matter or even failure of a digester (Seadi et al., 2008; Chen et al., 2008). These kinds of substances form part of the feed substrate or are by-products of the metabolic activities of the bacterial consortium in the digester. Some of these substances are essential in small concentrations for microbial growth but inhibit AD at higher concentrations (Seadi et al., 2008). There is a wide variety of inhibition/toxicity levels for most substances reported in literature. This is probably due to the varying bacteriological mechanisms such as acclimation, synergism and antagonism, on different digester conditions (Chen et al., 2008). Several substances have the potential to inhibit, or are toxic to AD:

-Ammonia

The extent of NH_3 inhibition depends on the NH_3 concentration in the feed substrate, pH and temperature (Appels et al., 2008). Bacterial and archaeal membranes are more permeable to NH_3 than NH_4^+, making the former more toxic to microbial function during AD. At mesophilic temperatures (35°C) and below pH values of 7, 99% is present as NH_4^+, while the amount of NH_3 increases significantly at higher pH values (Yenigün & Demirel, 2013a; Yang et al., 2025). At mesophilic temperatures and pH value of 7, the pKa of NH_4^+ is approximately 9.0, while at thermophilic temperatures (55°C), the pKa drops to approximately 8.4 (Yenigün & Demirel, 2013a; Yang et al., 2025). Working at thermophilic temperatures conditions increases the risk of NH_3 inhibition as compared to mesophilic temperatures due to release of free NH_3 (Seadi

et al., 2008). A reason for this is the increased degradation of nitrogen-rich organic matter, as well as the increased permeability of cell membranes at higher temperatures (Capson-Tojo et al., 2020). Particularly, methanogenic archaea are sensitive to NH_3, and 50% methanogenesis inhibition has been found at concentrations exceeding 600 mg/L of NH_3-N under thermophilic conditions and pH around 7.6 (Appels et al., 2008; Lindorfer et al., 2012; Zupančič et al., 2012). However, some studies have shown that methanogens can acclimate to higher NH_3 concentrations over time, resulting in reactor stability under thermophilic conditions (Yenigün & Demirel, 2013).

-Metals

Inorganic salts can contain significant concentrations of aluminium (Al), magnesium (Mg), sodium (Na), calcium (Ca), and potassium (K) that can affect AD (Chen et al., 2008). The optimal growth of HM occurs at concentrations of about 350 mg Na/L (Appels et al., 2008). The toxicity of salt is mainly associated with dehydration of the anaerobic bacterial or archaeal cells (Chen et al., 2008). It has been reported that methanogens can be inhibited by up to 50% by Na from 6 to 40 g/L, depending on the sludge history, synergistic and antagonistic effects and the test method used (Nayono, 2009). The toxic metals such as copper (Cu), zinc (Zn), iron (Fe), cobalt (Co), cadmium (Cd), and nickel (Ni) have been definitively identified as inhibitors of AD (Chen et al., 2008; Appels et al., 2008). Extensive studies performed on factors affecting AD and/or anaerobic reactor performances found metal toxicity to be one of the leading causes of AD upset or failure (Chen et al., 2008). However, metal toxicity depends on speciation under anaerobic conditions and digester operating conditions, especially the pH (Chen et al., 2014). Studies by Lindorfer et al. (2012) found that sulfur (S), Na and Ni inhibited AD of various energy crops at concentrations of 0.5 g/l, 5.0 g/l and 2.0 mg/l respectively.

2.5.7 Macronutrients

Microorganisms present during AD require essential elements for their growth. With the exception of organic C, the most important macronutrients are N and P (Li et al., 2023; Chen et al., 2008). Nitrogen is essential for the formation of amino acids and proteins (Hussain et al., 2015; Xi et al., 2023). Phosphorus is required for the synthesis

of nucleic acids (DNA and RNA), phospholipids for cell membranes, and for cellular energy transfer (Hussain et al., 2015; Xi et al., 2023). Xi et al (2023) reported that the optimal concentration of P is 100-500 mg/L. The carbon-to-nitrogen (C:N) and carbon-to-nitrogen-to-phosphorus (C:N:P) ratios are key indicators of AD performance (Bertran et al., 2004; Bamba et al., 2021). A high C:N ratio resulting from low N in PWWS can negatively impact the digestion process. It is reported that high CH_4 yields are obtained with a C:N:P ratio of 100:3:1 (Xi et al., 2023; Hussain et al., 2015). Large deviations from this ratio could result to insufficient nutrients for microbial growth and poor buffering capacity or (Lohani & Havukainen, 2018).

2.5.8 Micronutrients

Micronutrient supplementation can also be applied to further stimulate CH_4 production since some microelements such as Ni, Co and Fe may be vital for methanogenic growth and activity. Nutrient concentrations vary strongly between AD plants, therefore the application of mineral additives should be plant specific to avoid over-dosage and inhibition. Historically, the main substrates used in digestors were manure and mixed organic wastes, where the supply of macro- and micronutrients was quite good and deficiencies were rarely encountered or irrelevant (Lindorfer et al., 2012). Only in the application of AD to industrial wastewater treatment has the augmentation of trace elements gained popularity (Lindorfer et al., 2012). More recently, several authors have described an increase of CH_4 production after trace element addition in AD of energy crops (Paritosh et al., 2020; Mancini et al., 2018). Chan et al. (2019) found that during AD of food waste and domestic wastewater, CH_4 yield was optimal at 10 mg/L Cu. Khan et al. (2021) found that AD of molasses was optimal at Ni concentration of 2-4 mg/L while the optimal concentration of Co was determined by Li et al. (2023) at 2 mg/L for the same substrate.

Lindorfer et al. (2012), sampled over 1500 digesters and found that addition of micronutrients increased microbial biomass, restricted accumulation of VFAs and increased overall biogas yield by up to 22% in AD plants using energy crops. This suggests that higher OLR without acidification may be possible if micronutrients are added to substrates that are nutrient deficient. Additional studies by Da Ros et al. (2017) found that trace metal augmentation (Fe, Co and Ni) improved the stability and biogas yields during AD of waste activated sludge and lees, particularly under

thermophilic conditions. The study further noted that AD of wastes from single sources showed low concentrations of several trace elements, highlighting the benefits of co-digestion.

2.6. Theoretical methane yield

The BMP determines the fraction of organic carbon in each substrate that can be converted to CH_4. The theoretical CH_4 potential represents the CH_4 generation potential under ideal conditions. Numerous methods for calculating theoretical CH_4 potential exist and are based on COD, composition, and kinetic models (discussed in section 2.7) (Shitophyta et al., 2023). Deviation from the ideal CH_4 yield is due to the variable degree of matrix complexity among different types of biomass, or even among biomass of the same type, depending on its source and state (Lymperatou et al., 2023).

Buswell developed a model to estimate the biogas composition (CH_4, CO_2, H_2S and NH_3) from the chemical composition of organic substrates, such as C, H, N and S (Gallego Fernández et al., 2022). The Buswell equation (equation 9) is a commonly used method to determine the theoretical BMP of a biomass of known elemental composition (Lymperatou et al., 2023). The Buswell equation is a stoichiometric approach and assumes complete degradation of the substrate into CH_4, CO_2, NH_3 and H_2S.

$$C_xH_yO_zN_vS_u + \frac{4x-y-2z+2u}{4}H_2O \rightarrow \frac{4x+y-2z-3v-2u}{8}CH_4 + \frac{4x-y+2z+3v+2u}{8}CO_2 + vNH_3 + uH_2S \quad \text{…equation 9}$$

Another widely accepted method for estimating theoretical CH_4 yield involves using the COD to VS ratio (COD:VS) . This method relies on the stoichiometric relationship between CH_4 and its COD, where the complete oxidation of one mole of CH_4 requires two moles of O_2 (Yasim & Buyong, 2023; Mohamed et al., 2018). Therefore, 1 kg of COD can produce a maximum of 0.35 m^3 of CH_4 at standard temperature and pressure (STP) (equation 10). This method is useful for complex substrates where elemental composition is difficult to determine, making the Buswell equation unsuitable.

$$Theoretical\ CH_4 = COD{:}VS \times 0.35m^3 \quad \text{…equation 10}$$

2.7. Kinetic models used in anaerobic digestion

Kinetics models used in AD describe the rate of biogas production over time. These mathematical models use biological and operational parameters to predict and simulate digester performance. Studying these kinetics is important for predicting CH_4 yield and optimizing the design and operation of full-scale anaerobic digesters.

2.7.1 Modified Gompertz Model

The Modified Gompertz (MG) model (equation 11) is a widely used empirical model to describe the cumulative CH_4. It is popular due to its good fit to experimental data, especially for batch AD. The model assumes that biogas production is proportional to microbial activity (Nielfa et al., 2015). The equation predicts reactor behaviour based on the total potential CH_4 yield (P_{max}), the maximum CH_4 production rate (R_{max}), and the duration of the initial lag phase (λ) (Mohamed et al., 2018; Yasim & Buyong, 2023). This model accounts for the lag phase and offers insights into all phases of microbial growth (Llanos-Lizcano et al., 2024).

$$P(t) = P_{max} \times \exp\{-\exp\left[\frac{R_{max} \times e}{P_{max}}(\lambda - t) + 1\right]\} \quad \text{…equation 11}$$

2.7.2 Cone Model

The Cone model (equation 12) is another empirical model used to describe cumulative CH_4 production. It is often used for its simplicity and good fit to the exponential phase (Li et al., 2015). Similar to the Gompertz model, the cone model predicts CH_4 yield based on P_{max}.

$$P(t) = \frac{P_{max}}{1+(k_C t)^{Fs}} \quad \text{…equation 12}$$

2.7.3 Logistic Model

The Logistic model (equation 13) is another widely used empirical growth model (Mohamed et al., 2018). This model assumes that the rate of CH_4 production is proportional to amount of total CH_4 produced (Mohamed et al., 2018). The model captures sigmoidal growth patterns and explicitly accounts for a lag phase (Li et al., 2015). However, it may lack a direct account for underlying biological processes (Mohamed et al., 2018; Llanos-Lizcano et al., 2024).

$$P(t) = \frac{P_{max}}{1-\exp\left(\frac{4R_{max}(\lambda-t)}{P_{max}}\right)+2}$$ …equation 13

2.7.4 Chen and Hashimoto Model

The Chen and Hashimoto (C&H) model (equation 14) incorporates biological knowledge of AD as well as operating conditions. It focuses particularly on the relationship between CH_4 production, HRT, reactor volume, and temperature (Mohamed et al., 2018). This model is often used for sizing AD plants and can be adapted for batch and continuous systems (Mohamed et al., 2018).

$$P(t) = P_{max} \times (1 - \frac{K_{CH}}{HRT \times \mu_m + K_{CH} - 1})$$ …equation 14

2.7.5 First-Order Model

This simplified model assumes that the CH_4 production follows first order kinetics, where cumulative CH_4 production is simulated using an exponential rise to a maximum. The first-order kinetic model (equation 15) is a simple model that doesn't account for a lag phase and assumes that the rate of reaction is proportional to the concentration of a limiting reactant (Nielfa et al., 2015; Llanos-Lizcano et al., 2024). Despite being widely used, this model does not always accurately capture the S-shaped curve commonly observed in the cumulative CH_4 production of batch systems.

$$P(t) = P_{max} \times (1 - \exp(-ut))$$ …equation 15

The cumulative CH_4 potential (P(t)) in each model's equation is estimated by fitting the model to experimental cumulative CH_4 data. Various software packages, such as Excel Solver, R, and Python can be used to optimize model parameters for the best fit to the experimental data. The quality of the fit is assessed using statistical metrics such as the coefficient of determination (R^2), root mean square error (RMSE), and Akaike Information Criterion (AIC), which help evaluate the model's reliability and suitability.

2.8. Anaerobic digestate as fertilizer

The use of ADg as a fertilizer provides a cost-effective alternative to traditional chemical fertilizers while promoting a circular economy through recycling organic waste (Simioni et al., 2023). Compared to inorganic fertilizers, ADg reduces the risk of nutrient leaching and water contamination (Nikolaidou et al., 2024; Walsh et al., 2012).

Several studies have shown the agronomic benefits and environmental advantages of applying ADg as fertilizers or soil conditioners. Anaerobic digestate from chromium tanned leather shavings and tannery sludge has been reported to contain high levels essential macronutrients including as N, P, and K, as well as various micronutrients that contribute to soil health and plant growth (Simioni et al., 2023). Studies using ADg from agricultural and livestock waste show that application of these ADg produce crop yields as high as those from chemical fertilizers, particularly in grassland systems (Nikolaidou et al., 2024; Walsh et al., 2012). Mora-Salguero et al. (2025) found that traditional organic fertilizers such as biowaste compost and farmyard manure (rich in C with highly stable and recalcitrant organic matter and C:N ratios > 10) was improved when mixed with raw ADg from food wastes.

The use of ADg as fertilizer helps maintain favourable soil organic carbon (SOC) levels due to high C content (Mora-Salguero et al., 2025). Additionally, ADg can improve the soil N, P and K content, and enriches microbial diversity more than chemical fertilizers (Mora-Salguero et al., 2025). Digestate from agricultural and livestock waste has been found to improve soil structure and microbial activity (Nikolaidou et al., 2024; Song et al., 2021). Additionally, ADg from agricultural and livestock waste also contains various micronutrients, including Ca, Mg, P, Zn, Cu and B, which are essential for plant health (Brychkova et al., 2024; Walsh et al., 2012; Nikolaidou et al., 2024). Similar to chemical fertilizers, the N in ADg is predominantly in available in inorganic form, which is readily available for plant uptake (Simioni et al., 2023; Nikolaidou et al., 2024).

The use of ADg can promote healthy soil ecosystems such as nutrient cycling and C sequestration, which are key for sustainable agriculture (Karimi et al., 2022). Conversely, overuse of ADg can possibly lead to unfavourable microbial and chemical balances in the soil which negatively impact plant health (Garcia-Lopez et al., 2023).

In conclusion, the use of ADg with a favourable nutrient profiles can promote soil health. This practice reduces the reliance of the agriculture industry on chemical

fertilizers. The use of ADg as fertilizer or soil conditioner also supports a circular economy and reduces waste to landfill. Despite well documented studies on the use of ADg from tannery sludge, food waste, and municipal waste as fertilizer, there is little literature on the use of PWWS ADg as fertilizer. Therefore, the possible use of ADg from PWWS as a fertilizer requires investigation before adoption.

CHAPTER 3: MATERIALS AND METHODS

3.1. Introduction

This chapter provides a comprehensive description of the experimental set-up and procedures and materials used to meet the study objectives (refer to Section 1.4). The experimental procedure was divided into 3 stages (Figure 3-1): (i) BMP tests of crush and off-crush season from the first study site were conducted to optimise 3 operating parameters (temperature, ISR, nutrient augmentation) and establish baseline BMP, (ii) the optimised parameters obtained from the first were applied to BMP tests of 5 other solid waste streams from different wineries, and (iii) the effects of acclimation of the inoculum to the intended substrate was investigated by cross inoculation of PWWS, municipal waste activated sludge (MWAS) and secondary tannery wastewater sludge (STWWS) with inocula acclimated to PWWS and MWAS.

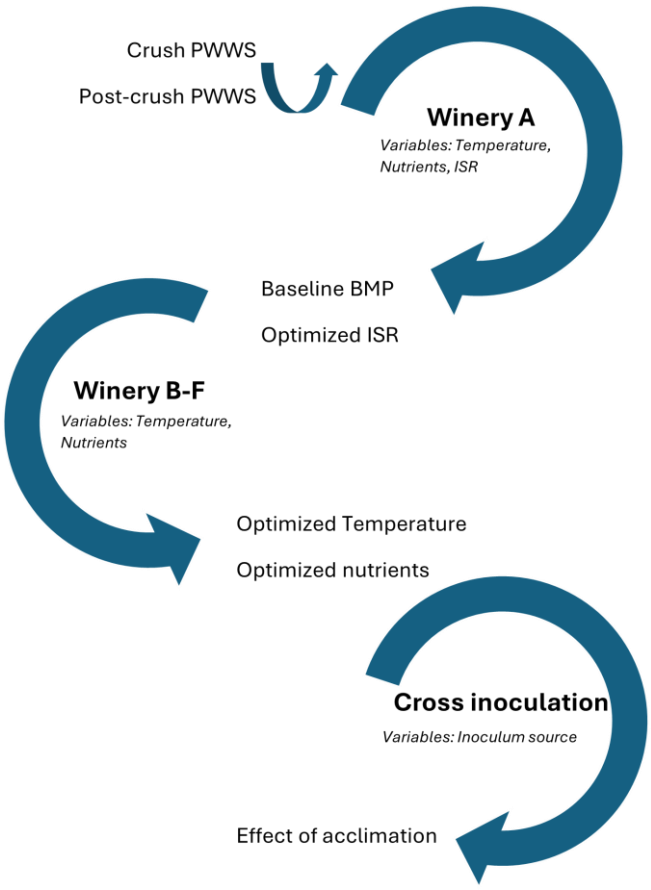

Figure 3-1: Experimental workflow of the sequential optimisation approach

3.2. Sampling of primary winery wastewater sludge

Primary winery wastewater sludge from the setting delta of a local winery (Winery A) in the Western Cape was manually and randomly sampled using the grab sampling technique into polyurethane airtight 20 L storage containers. Collection of samples was performed during the crush season (February to April) and off-crush season (July to October). The sampling events aimed to capture representative samples of the year's total PWWS produced divided into crush and off-crush wastes. The containers were filled to approximately 80% capacity to cater for any expansion caused by gas formation. Samples were immediately transported to the laboratory and were thoroughly homogenised using a food blender on arrival. Aliquots were taken for physical and chemical analysis (±500 mL), which were performed within 48 h of arrival. The remainder of the sludge was reserved for BMP experiments and was immediately frozen at -18°C until required. Thawing of frozen samples was performed at 4°C, increasing to ambient conditions before use in BMP tests.

An additional 4 samples of PWWS, and one sample of lees were collected from other wineries in the Western Cape (Wineries B to F). All samples were collected, prepared and as stored as described for the PWSS from Winery A.

3.3. Inoculum preparation

The inoculum was cultivated by incubating PWWS anaerobically in an airtight 20 L bucket fitted with a PVC tube and Hoffman clamp at room temperature for 60+ days. The biogas produced was collected into Tedlar Bags and measured regularly to monitor the microbial activity of the inoculum. The CH_4, CO_2, H_2S, and O_2 content of the biogas was analysed using a Geotech biogas 5000 analyser (Warwickshire, England). After stable digestion, if biogas production rate and quality stabilised and the CH_4 fraction produced from the inoculum dropped below 50% of the peak, small amounts (±100 mL) of PWWS were added. The inoculum was used for experimentation only once the CH_4 content of the biogas started declining after peaking above 40%, indicating that the substrate was depleting. This PWWS inoculum was kept active by regular feeding and bi-weekly biogas analysis and was

used for all experiments. The inoculum samples were collected into 50 ml polypropylene tubes before each experiment for physicochemical analyses.

3.4. Biogas analysis

Biogas was collected in 1L Tedlar bags (Sigma, Darmstadt, Germany cat no: 30237-U). The contents were qualitatively and quantitatively analysed when sufficient biogas was produced (> ~100 ml). The volume of biogas was determined using a graduated gas-tight syringe. Qualitative analyses consisted of determining the CH_4 (%vol), CO_2 (%vol), and O_2 (%vol), as well as the H_2S content (parts per million) of the gas using a Geotech biogas 5000 analyser (Warwickshire, England).

Figure 3-2: Geotech biogas 5000 analyser

3.5. Characterization of inoculum and substrate

All inocula and PWWS samples were characterised. The TS and VS concentrations were determined in triplicate following the loss on ignition standard methods using an oven at 105°C overnight and a furnace at 550°C for 2 h, respectively (Gerenfes et al., 2022).

The chemical analyses were performed in duplicate using a Merck Spectroquant Pharo® 100 instrument (Darmstadt, Germany) together with Merck cell tests or kits for: total COD (cat no: 14555), total organic carbon (TOC) (cat no: 14879), total nitrogen (TN) (cat no: 14537), total volatile organic acids (VOAt) as acetic acid

equivalent (cat no: 01763), for total nitrogen (TN) (cat no: 14537, total phosphate (TP) as phosphorus (PO_4^{2-}-P) (cat no: 14729), and total alkalinity (alk) as calcium carbonate ($CaCO_3$) (cat no: 101758), total ammonium nitrogen (TAN) as NH_3-N (cat no: 00683). All of the tests were conducted following the manufacturer's instructions. Merck's equivalent standard tests and method principles are shown in Table 3-1**.**

The concentrations of cations and CHNS (%wt.wt) were determined at the Central Analytical Facility at Stellenbosch University. The samples were dried at 100°C in an oven overnight and ground to a powder using a pestle and mortar followed by using acid-assisted microwave extraction of 0.5 g of the dried powder in 6:1 (vol.vol) nitric acid (HNO_3) and hydrogen peroxide (H_2O_2) in a MARS microwave digestion system (CEM corporation, Matthews, NC, USA) at 210°C (ramp time of 20 min, hold time of 10 min). Thereafter, metal concentrations (chromium (Cr), iron (Fe), lead (Pb), Al, Cd, Co, Cu, Fe, Ni, Pb, Zn) and other cations (Ca, K, Mg, Na) were quantified by inductively coupled plasma - atomic emission spectroscopy (ICP-AES) using a Thermo ICap 6200 ICP-AES instrument, while ultra-trace analyses were performed by ICP-mass spectrometry (ICP-MS) using an Agilent (Santa Clara, USA) 7900 ICP-MS instrument. Quantification of C, H, N and S (%wt.wt) were determined using an Elementar (Hamburg, Germany) Vario EL cube Elemental analyser.

A Eutech instrument (Ayer Rajar, Singapore) was used for the measurement of pH following the prescribed standard procedure of operation.

Table 3-1: Principles of Merck equivalent test methods

Test/ Cat no	Method principle
COD/14555	The sample is oxidised in the presence of known excess sulfuric acid (H_2SO_4) and potassium dichromate ($K_2Cr_2O_7$). The intensity of the greenish colour of the released Cr (III) is measured spectrophotometrically at 605 nm.
TOC/14879	Acidification of the sample prior to digestion eliminates dissolved CO_2 and anions of CO_2. Digestion with H_2SO_4 and peroxodisulfate ($S_2O_8^{2-}$) emits CO_2. The CO_2 reacts with a blue indicator solution producing a blueish-green colour that is measured spectrophotometrically at 605 nm.
VOAt /01763	VFAs in the sample react with dioles to form fatty acid esters that react with hydroxylamine (NH_2OH) to form hydroxamic acids that complex with Fe (III). The intensity of the resultant red complexes is measured spectrophotometrically at 500 nm .
TAN/00683	NH_3 reacts with hypochlorite ions (ClO^-) to form monochloramine (NH_2Cl) which in turn reacts with a substituted phenol to form a blue indophenol derivative measured spectrophotometrically at 690 nm.
TN/14537	Koroleff's method is based on the oxidation of organic and inorganic N species to nitrate (NO_3^{2-}). The NO_3^{2-} reacts with 2,6 dimethylphenol to 4-nitro-2,6-dimethylphenol to form a red nitro compound that is measured spectrophotometrically at 340 nm.
TP/14729	Ortho-P reacts with molybdate to form molybdophosphoric acid that is reduced to phosphomolybdenum by ascorbic acid forming a blue colour that is measured spectrophotometrically at 690 nm.
Alk ($CaCO_3$)/101758	The reaction of protonatable substances in water alters the pH, causing a corresponding change in the colour of an indicator. This colour change directly reflects the acid capacity of the solution. The resultant colour is measured spectrophotometrically at 605 nm .

COD = chemical oxygen demand, TOC = total organic carbon, VOAt = total volatile organic acids, TAN = total ammonia nitrogen, TN = total nitrogen, TP = total phosphorous, Alk = alkalinity

3.6. Biomethane potential tests

The BMP tests were carried out in gas tight borosilicate glass reactors (500 mL or 1000 mL) following the standard procedure described by (Holliger et al., 2016). This method provides a framework for conducting controlled BMP tests to assess the theoretical maximum amount of CH_4 that a specific substrate can produce through AD. For each experimental reactor, a constant volume of inoculum was added, to which different initial volumes of PWWS were added to achieve the desired ISR. The reactor contents were made up to working volumes (500 mL and 1 L in 500 mL and 1 L reactors, respectively) using deionised water. For each experiment, 4 controls were included: (i) inoculum only, (ii) substrate only, (iii) inoculum with acetate (substrate for AM), and (iv) inoculum with microcrystalline cellulose (substrate for hydrolytic taxa). The inoculum and substrate controls were used to identify endogenous CH_4 production, while the acetate and cellulose served as positive controls to validate the metabolic health of the inoculum. All experiments (including controls) were conducted in duplicate or triplicate.

The pH of each reactor was adjusted to pH 7±0.3 using HCl and/or NaOH. The borosilicate glass reactors were immediately flushed with N_2 gas for 2 min and sealed with screw caps retrofitted with flexible PVC tubing connected to a Tedlar bag (Figure 3-3). Reactors were then incubated in a heated water bath at 37°C (mesophilic) and ambient (indoor) temperature. The ambient temperature was logged for the duration of the experiments using a Extech® RHT35 USB Humidity/Temperature/Barometric Pressure Datalogger (cat no: 793950441350). Mixing was achieved by gently swirling each reactor vessel daily for the duration of the experiment (±21days). The biogas was analysed every 1-2 days throughout the duration of experiments (Section 3.4).

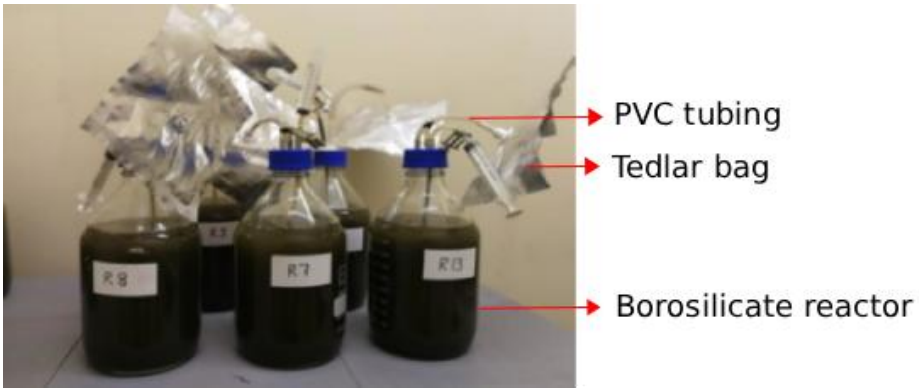

Figure 3-3: Biomethane potential reactors

3.7. Optimization of process parameters

3.7.1 Optimization of inoculum to substrate ratio, temperature, and nutrient augmentation for the anaerobic digestion of seasonal primary winery wastewater sludge from winery A (Experiment 1)

Scoping experiments (experiment 1a and 1b) were performed using a multi-level categoric design of experiments (DOE) methodology. The software package Design-Expert® Version 10 (Stat-Ease, Inc., Minneapolis, USA) was used to define the experimental design matrix.

For experiment 1a, the design matrix was designed to investigate 2 factors: ISR (ISR 2, ISR 3, ISR 4) and nutrients augmentation (no nutrients, with nutrients (+N)), measuring one response variable: CH_4 yield ($mLCH_4/gVS$) as shown in Table 3-1. The choice of factors for optimisation and levels was based on previous studies in literature, particularly Holliger et al. (2016). This matrix resulted in 12 experimental reactors/runs for the crush season (excluding controls) and 12 runs for the off-crush season (excluding controls). This BMP assay was carried out in 1 L reactors at ambient temperature as described in section 3.5. The nutrients added were 5 mg/L Cu, 20 mg/L Ni, and 20 mg/L Co based on deficiencies in the PWWS required for efficient AD (Chen et al., 2008; Schmidt et al., 2014).

Table 3-2: Factors and levels for design matrix (experiment 1a)

Factors	
ISR	Nutrients
Levels	
2	No nutrients
3	Nutrients added
4	-
Response	
mLCH4/gVSadded	

ISR = inoculum to substrate ratio, VS = volatile solids

In experiment 1b, the variable combination (ISR and with/no nutrients) resulting in the highest response in terms of specific biogas and CH_4 generation in experiment 1a

were repeated at ambient and mesophilic temperatures simultaneously for both the crush and post crush season PWWS to determine the effect of temperature on AD efficiency.

3.7.2 Optimization of temperature, nutrient augmentation, and substrate source for the anaerobic digestion of primary winery wastewater sludge and less from wineries A-E (experiment 2)

Experiment 2 was performed using a multi-level categoric DOE methodology in Design-Expert® Version 10 (Stat-Ease, Inc., Minneapolis, USA). Approximately 100 L of four batches of PWWS (PWWS A-D) and one batch of lees (E) were collected, homogenized and stored during the crush season by methods described in section 3.2. The same inoculum from experiment 1 was used after continual feeding between experimental periods with PWWS from winery A. Following the characterization of the PWWS, lees, and inoculum, BMP assays were carried out in 1L reactors at ISR of 4 as described in Section 3.6**.** The study investigated the effects of nutrient addition, and temperature (ambient versus 37°C) on biogas and specific CH_4 production (Table 3-3). Data outputs were analysed by fitting relevant kinetic models as described in section 3.8.

Table 3-3: Factors and levels used for design matrix (experiment 2)

Factors		
Temperature	Nutrients	Source
Levels		
Ambient	No nutrients	PWWS A
37ºC	Nutrients added	PWWS B
-	-	PWWS C
-	-	PWWS D
-	-	Lees E
Response		
mLCH4/gVSadded		

PWWS = primary winery wastewater sludge, VS = volatile solids

3.7.3 Comparison of anaerobic digestion of primary winery wastewater sludge, municipal waste activated sludge and secondary tannery wastewater sludge using different inocula (experiment 3)

Experiment 3 was designed to investigate the effect of inoculum acclimation on the biodegradability of different substrates. The study employed a multi-level categoric DOE methodology in Design-Expert® Version 10 (Stat-Ease, Inc., Minneapolis, USA) (Table 3-4). Approximately 20 L of PWWS, MWAS and STWWS were collected, homogenized and stored by methods described in section 3.2. Two inoculums, one PWWS fed inoculum (PWWSi) and another MWAS fed inoculum (MWASi), were cultivated by periodically feeding with the relevant substrate over a period of 90+ days. In experiment 3a, PWWSi was used as inoculum to determine the BMP of PWWS, MWAS and STWWS in a cross-inoculation design. Similarly, in experiment 3b MWASi was used to determine the BMP of PWWS, MWAS and STWWS. All assays were done in triplicate. The operational parameters used (ISR and temperature) were informed by optimal conditions determined during experiment 1 and 2.

Table 3-4: Factors and levels used for design matrix (experiment 3)

Factors	
Inoculum	Substrate source
Levels	
PWWSi	PWWS
MWASi	MWAS
-	STWWS
Response	
mLCH4/gVSadded	

PWWS = primary winery waster sludge, MWAS = municipal waste activated sludge, STWWS = secondary tannery wastewater sludge, PWWSi = PWWS fed inoculum, MWASi = MWAS fed inoculum

3.8 Determination of kinetic models

Kinetic models can be used to predict the BMP in AD. These models are helpful for describing the behaviour of complex feedstocks in the modelled system. It is therefore important to choose a suitable kinetic model for evaluating the BMP of PWWS. In this study, the Logistic, Cone, Modified Gompertz, First-Order, and Chen & Hashimoto

(C&H) models were adopted to fit and evaluate the BMP during AD of sludge A-E. Three statistical measures (correlation coefficient (R^2), AIC and RMSE were used to fit and validate the models in order to identify the most suitable model for the AD of the PWWS. The kinetic parameters were determined by nonlinear regression, via the solver function in Excel to minimize the sum of squares error.

3.9 Energy balances

Simple thermodynamic energy balances were conducted by subtracting the energy required for heating from the energy produced by the generated CH_4. The energy required to heat was calculated using equation 16, where m is the mass of reactor contents, C_p is the specific heat capacity, T is the temperature difference between the reactor and ambient temperature, U is the overall heat transfer coefficient, A is the reactor surface area, and t is the retention time. Assuming convective heat transfer coefficients of 500 W/m^2 and 10 W/m^2 for the liquid side (forced convection due to stirring) and air side (due to natural convention of air), respectively.

$$E_{loss} = m \cdot C_p \cdot \Delta T + U \cdot A \cdot \Delta T \cdot t \quad \text{…equation 16}$$

The energy gained from CH_4 is estimated by equation 17, where V_{CH4} is the volume of CH_4 collected at STP, and LHV is the lower heating value of CH_4 (35.8 MJ/m^3),

$$E_{gain} = V_{CH4} \cdot LHV \quad \text{…equation 17}$$

Assuming no further heat losses, and that both the heater and any energy conversion systems operated at 100% efficiency, the net energy is estimated using equation 18.

$$E_{net} = E_{gain} - E_{loss} \quad \text{…equation 18}$$

CHAPTER 4: CHARACTERISATION AND ANAEROBIC DIGESTION OF PRIMARY WINERY WASTEWATER SLUDGE

4.1. Introduction

The findings presented in this chapter provide fundamental information on the biodegradation of PWWS. The objectives were to: (i) characterise PWWS based on seasonality, understanding how the composition of the PWWS varies throughout the year and estimate its theoretical suitability for AD; (ii) determine an ideal ISR for the BMP of PWWS; (iii) evaluate the impact of nutrient augmentation on the BMP of PWWS; iv) investigate and compare the influence of mesophilic and ambient temperatures on the BMP of PWWS.

4.2. Characterisation of primary winery wastewater sludge

The seasonal variation of PWWS harvested during and post-crush is discussed in this section with reference to their applicability as feedstocks for AD. The physicochemical characteristics of PWWS from Winery A for the crush and post-crush seasons and the inocula used in the BMP experiments were determined according to the methods described in Section 3.5 and Appendix A. The results are presented in Table 4-1 and Table 4-3.

4.2.1 Organics, solids and macronutrients

The seasonal TS, VS and COD content of the post-crush PWWS were 74%, 171%, and 132% higher than the crush PWWS, respectively (Table 4-1). It was hypothesised that this was due to: (i) use of fining agents to remove suspended particles from wine during the crushing season (ii) residual grape organic solids from the crush season still present in the wastewater settling basin, (iii) the presence of lees and other solids from washing vats during the post-crush season. As expected, the C:N ratio of the post

crush season PWWS was lower than the crush season PWWS, likely due to the use of ammonia-based cleaning chemicals during the post crush season. The C:N ratio in the PWWS from both seasons was lower than the ideal ratio for AD of carbonaceous substrates, implying that AD may not be stable. The optimal C:N ratio for carbonaceous substrates such as PWWS has been widely reported as 20–30, while for proteinaceous substrates such as tannery sludge and food waste it has been reported as 6–9 (Table 4-2) (Berhe & Leta, 2019; Parra-Orobio et al., 2016). The higher C:N ratio in the crush PWWS (C:N 11.6) suggests that this may be better suited to AD than post-crush PWWS (C:N 7.1). The AD of substrates with low C:N ratios are susceptible to instability due to the potential accumulation of NH_3 and VOAs. At higher temperatures, higher C:N ratios are required to reduce the risk of inhibition by NH_3, suggesting greater NH_3 inhibition at mesophilic temperatures than ambient temperatures (Wang et al., 2014).

Substrates with higher VS typically have better potential for favourable conversion to CH_4 because VS represents the organic fraction, namely the microbial biomass and other organics (such as carbohydrates, lipids, and proteins). High VS typically translates to greater quantities of organic matter, which includes biodegradable substrate and microbial biomass, available for the key metabolic stages of AD (Mächtig et al., 2020). Similar to the C:N ratio, the VS:TS ratio indicates that the crush PWWS (VS:TS 0.82) is more suited to AD than the post-crush PWWS (VS:TS 0.52). This strengthens the previous observation based on the C:N ratio. The VS:TS of crush PWWS and all inocula used was below the optimal (0.8) (Rasmeni et al., 2022). However, it's important to understand that the VS:TS ratio by itself is not always a reliable measure of potential CH_4 yields due to the wide variety of microbes present during AD (Wang et al., 2020).

Table 4-1: Characteristics of primary winery wastewater sludge, inoculum, and winery wastewater for winery A (average and standard deviation from the mean, n =3)

Parameter (*n*=3)	Crush PWWS	Crush Inoculum 1	Crush Inoculum 2	Post-crush PWWS	Post-crush Inoculum 1	Post-crush Inoculum 2	Winery effluent
Elemental CHNS analysis (g/kg dry weight)							
C	29.4 ± 2.45	32.5 ± 2.2	25.2 ± 2.9	40.1 ± 1.3	26.5 ± 0.3	20.4 ± 3.9	ND
H	0.55 ± 0.05	4.86 ± 0.55	4.85 ± 0.42	6.61 ± 0.43	3.62 ± 0.52	2.28 ± 0.64	ND
N	2.54 ± 0.04	2.86 ± 0.16	1.89 ± 0.25	5.67 ± 0.14	2.25 ± 0.20	1.78 ± 0.39	ND
S	0.38 ± 0.04	0.89 ± 0.05	0.57 ± 0.15	0.34 ± 0.03	0.38 ± 0.07	0.38 ± 0.08	ND
C:N	11.6	11.6	13.3	7.1	11.8	11.5	ND
Characterisation (g/L wet weight homogenised sludge, g/L wastewater)							
COD	149 ± 5	35.5 ± 1.0	206 ± 14	346 ± 26	95.2 ± 8.8	71.7 ± 5.6	6.79 ± 1.20
TP	0.70 ± 0.01	0.17 ± 0.01	0.82 ± 0.20	1.65 ± 0.13	0.49 ± 0.09	0.44 ± 0.06	0.20 ± 0.07
TN	11.5 ± 0.5	2.3 ± 0.4	2.5 ± 1.07	14.3 ± 0.5	2.4 ± 0.8	4.5 ± 0.1	0.03 ± 0.02
VOA	28.5 ± 0.7	8.98 ± 0.35	44.6 ± 10.4	104 ± 5	26.1 ± 1.1	23.3 ± 2.36	1.37 ± 0.15
HS^-	0.08 ± 0.01	0.06 ± 0.01	0.08 ± 0.01	0.75 ± 0.02	0.11 ± 0.01	0.13 ± 0.02	ND
NH_4^+-N	1.89 ± 0.04	1.3 ± 0.17	5.39 ± 0.06	1.18 ± 0.35	1.02 ± 0.21	1.16 ± 0.13	ND
TS	173 ± 10	257 ± 7	62.3 ± 4.1	301 ± 14	145 ± 2	117 ± 1	ND
VS	91.9 ± 11	97.8 ± 8.7	32.8 ± 22	247 ± 9	67.9 ± 1.5	85.3 ± 1.4	ND
COD:VS	1.62	0.36	6.28	1.40	1.40	0.84	ND
VS:TS	0.53	0.38	0.53	0.82	0.49	0.72	ND
COD:TN:TP	213:16:1	209:14:1	251:3:1	210:9:1	194:5:1	163:10:1	34:0.2:1

PWWS = primary winery wastewater sludge; ND = not determined; COD = chemical oxygen demand; TP = total phosphate, TN = total nitrogen, VOA= volatile organic acids, TS=total solids; VS=volatile solids

Despite N being an essential macronutrient for microbial growth, high concentrations of NH_4^+-N are particularly toxic to methanogens (Czatzkowska et al., 2020). While the NH_4^+-N in the crush and post-crush PWWS was well below the reported inhibitory threshold of 3.4-5.77 g/L NH_4^+-N, crush inoculum 2 had a NH_4^+-N concentration of 5.39 g/L, indicating possible inhibition (Table 4-1) (Yang et al., 2025).

Phosphorus is an essential macronutrient required for the growth and metabolic function but has also been shown to cause inhibition. Conrad et al (2000) observed inhibition during AD of rice roots when the TP concentration was higher than 1.9 g/L, while Xi et al. (2023) observed a decrease in CH_4 yield when the available P was increased from 0.1 to 0.28 g/L. In this study, the TP was below these inhibitory values for the crush and post-crush PWWS.

The optimal P requirements for AD are COD:N:P ratios of approximately 250-1000:5 to10:1-1.7 (Table 4-2). Based on these ratios, both crush PWWS (COD:N:P 213:16:1)

and post-crush PWWS (COD:N:P 210:9:1) theoretically contained sufficient balances of COD, N and P for optimal AD.

Table 4-2: Optimal parameters for anaerobic digestion

	Optimal	Reference
VS:TS	0.8	(Rasmeni et al., 2022)
C:N	20-30	(Berhe & Leta, 2019; Parra-Orobio et al., 2016)
COD:N:P	250-1000:5-10:1-1.7	(Amani et al., 2010; Hussain et al., 2015)
ISR	2-4	(Holliger et al., 2016)
VOA	50-500 mg/L	(Amani et al., 2010)
pH	6.8-7.4	(Cai et al., 2021)

TS=total solids; VS=volatile solids; COD = chemical oxygen demand; ISR = inoculum to substrate ratio; VOA= volatile organic acids

Amani et al. (2010) reported 50-500 mg/L VOA as optimal for AD, while Jiang et al., (2018) reported inhibition thresholds of 16.5-18.0 g/L for dry co-digestion of food waste and pig manure, above which reactor souring inhibits methanogenesis. In this case, the total VOA concentrations of the PWWS were well above the inhibitory range (Table 4-1), indicating a likelihood of bioreactor instability and failure during AD due to acidification. Acclimation of the inoculum to the intended substrate may have contributed to tolerance of the functional microbes to VOA. The acidification may also have been countered by the inherent buffering capacity offered by the speciation of NH_3/NH_4^+ and $CO_2/HCO_3^-/CO_3^{2-}$, in so doing maintaining pH within the optimal range.

4.2.2 Cationic inhibitors and micronutrients

Different studies report a broad spectrum of inhibitory concentrations of metals and other cations for AD. This variability stems from the degree of bacterial acclimation to the intended substrate/s, the interaction between different substances (either benefiting or hindering each other), and different operating conditions (Tshikantwa et al., 2018). The IC_{50} values have been reported as 30-350 mg/L for Cu, 1300 mg/L for Ni, >500-1500 mg/L for Zn, and > 5000 mg/L for Pb for acetogenic bacteria, and 5.6–53 g/L Na, 1000mg/L Al, 8.9-20.7 mg/L Cu, 35 mg/L Ni, and 7,7 mg/L Zn for methanogens (Mudhoo & Kumar, 2013; Czatzkowska et al., 2020; Chen et al., 2008).

The metal concentrations in the PWWS were all below these IC_{50} values for acetogenic bacteria. Similarly, concentrations were also below IC_{50} thresholds for methanogenic bacteria , with the exceptions of Cu and Zn. Given the complexity of AD reactor contents, metal inhibition may be mitigated by precipitation by HS^- or hydroxides, and/or sorption to the solid fraction, and/or the formation of complexes in solution (Estay et al., 2021).

Table 4-3: Elemental cation concentrations in primary winery wastewater sludge and inoculum for winery A

	Crush PWWS	Crush Inoculum 1	Crush Inoculum 2	Post-crush PWWS	Post-crush Inoculum 1	Post-crush Inoculum 2
Major elemental parameters (g/L)						
Fe	0.66 ± 0.02	0.92 ± 0.03	0.26 ± 0.06	0.89 ± 0.04	0.70 ± 0.12	0.59 ± 0.04
Al	1.92 ± 0.02	1.85 ± 0.06	0.64 ± 0.14	3.22 ± 0.07	1.89 ± 0.29	1.17 ± 0.14
Ca	1.28 ± 0.08	5.68 ± 0.05	0.65 ± 0.13	0.27 ± 0.04	1.49 ± 0.17	1.68 ± 0.35
K	8.96 ± 0.81	11.10 ± 0.36	1.84 ± 0.26	1.12 ± 0.04	8.92 ± 3.58	4.66 ± 1.84
Mg	0.28 ± 0.04	0.96 ± 0.05	0.14 ± 0.03	0.36 ± 0.12	0.32 ± 0.03	0.23 ± 0.02
Na	0.18 ± 0.03	7.94 ± 0.25	0.21 ± 0.01	0.05 ± 0.02	0.68 ± 0.23	0.44 ± 0.20
P	0.49 ± 0.02	1.35 ± 0.04	0.26 ± 0.06	1.63 ± 0.03	0.42 ± 0.01	0.44 ± 0.04
Si	0.16 ± 0.00	0.36 ± 0.03	0.04 ± 0.01	0.76 ± 0.16	0.23 ± 0.04	0.21 ± 0.04
Minor elemental parameters (mg/L)						
B	11.78 ± 8.36	14.03 ± 1.44	2.52 ± 0.47	BDL	6.57 ± 0.29	5.43 ± 0.29
Mn	6.87 ± 0.28	85.58 ± 0.98	5.79 ± 1.06	7.04 ± 0.66	10.80 ± 1.96	9.40 ± 1.32
Co	0.22 ± 0.09	4.34 ± 0.21	1.64 ± 0.36	BDL	2.96 ± 1.29	BDL
Ni	6.38 ± 0.85	2.98 ± 0.18	3.30 ± 0.75	3.88 ± 0.93	7.18 ± 0.78	4.21 ± 0.74
Cu	25.43 ± 2.20	43.18 ± 2.08	9.7 ± 2.15	36.12 ± 0.90	30.16 ± 4.39	23.05 ± 9.29
Zn	51.9 ± 2.60	370.9 ± 18.25	28.0 ± 6.23	106.6 ± 0.72	74.1 ± 11.98	45.1 ± 4.12
Sr	3.74 ± 0.29	23.23 ± 0.18	3.20 ± 0.60	20.20 ± 3.58	5.67 ± 0.44	7.85 ± 1.39
Mo	0.52 ± 0.22	1.29 ± 0.05	0.11 ± 0.03	ND	ND	ND
Pb	5.92 ± 1.11	4.91 ± 1.72	1.51 ± 0.45	10.44 ± 2.59	4.31 ± 0.91	4.11 ± 1.23
Ba	23.01 ± 1.49	11.00 ± 1.26	4.54 ± 1.20	21.01 ± 0.75	16.53 ± 4.29	13.34 ± 1.53

PWWS = primary winery wastewater sludge; ND = not determined

Micronutrients such as Co, Cu, Fe, Ni, Mo, Ba, Mg, Na are essential metabolic co-factors required in relatively small quantities by some microorganisms during AD. For example, trace concentrations of Na (0.23-0.35 g/L) are required for formation of ATP in methanogens, particularly AM, however high levels may inhibit activity and interfere with metabolism (Twigg et al., 2024). The concentration of Na in the crush PWWS (0.18 g/L) and post-crush PWWS (0.05 g/L) was well below the recommended value

and may have contributed to non-ideal CH_4 generation. Essential micronutrients, B and Co were below the analytical detection limit (0.05 mg/L B and 0.01 mg/L Co) in the post-crush PWWS and may also have been deficient in the post-crush PWWS, although the inocula used had higher levels of B (5.43-6.57 mg/L) and Co (2.96 mg/L) which likely accumulated during the acclimation stage. The high Fe concentrations in the PWWS could theoretically promote changes in speciation, co-precipitation, adsorption, and ion substitution of Co and Ni on FeS, reducing the bioavailability of these and other essential cation micronutrients (Myszograj et al., 2018).

4.2.3 Theoretical biomethane potential

Based on the Buswell equation described in Section 2.6, the theoretical CH_4 yield for crush PWWS and post-crush PWWS at STP was 363 $mLCH_4/gVS_{added}$ and 385 $mLCH_4/gVS_{added}$ respectively. At 37°C, the projected yield from the crush and post-crush PWWS was 412.1 $mLCH_4/gVS_{added}$ and 437.1 $mLCH_4/gVS_{added}$, respectively. Estimations based on the COD:VS predicted higher yields of 567 $mLCH_4/gVS_{added}$ and 490 $mLCH_4/gVS_{added}$ for crush PWWS (COD:VS 1.6) and post-crush PWWS (COD:VS 1.4) at 37°C.

4.3. Methane production and biodegradability of primary winery wastewater sludge from winery A

The BMP assay for crush and post-crush PWWS was conducted at ambient temperature (20.7°C ± 2.0°C) using a combination of two variables: ISR (2,3 and 4) and nutrient addition (nutrients/no nutrients) (Experiment 1) (Table 4-4). The combination of ISR and nutrients resulting in the highest specific biogas and CH_4 generation in Experiment 1 were repeated at ambient (19.4°C ± 1.77°C) and mesophilic (37°C) temperatures simultaneously to compare the effect of these temperatures on AD efficiency (Experiment 2). The ambient temperatures of experiment 1 and experiment 2 showed no significant statistical difference ($p>0.05$).

Table 4-4: Parameters used for Experiments 1 and 2

	Crush season PWWS			Post-crush season PWWS		
	Experiment 1	Experiment 2		Experiment 1	Experiment 2	
Temperature	Ambient	Ambient	37°C	Ambient	Ambient	37°C
ISR2	✓	×	×	✓	×	×
ISR2/N	✓	×	×	✓	×	×
ISR3	✓	×	×	✓	×	×
ISR3/N	✓	×	×	✓	×	×
ISR4	✓	×	×	✓	×	×
ISR 4/N	✓	✓	✓	✓	✓	✓

PWWS = primary winery wastewater sludge; ISR = inoculum to substrate ratio; N = nutrients (5 mg/L Cu^{2+}, 20 mg/L Ni^{2+}, 20 mg/L Co^{2+}); RT = ambient temperature

4.3.1 Effects of inoculum to substrate ratio and nutrient addition on methane generation at ambient temperature (Experiment 1)

This section evaluates the results of Experiment 1, which investigated the effects of operating temperature (ambient vs. 37°C), ISR, and nutrient addition on AD of crush and post-crush PWWS from winery A.

No lag phases were observed for either crush or post-crush PWWS (Figure 4-1). It was hypothesized that acclimation of the inoculum prior to experiments assisted in avoiding long lag phases. The highest cumulative CH_4 generation was obtained with an ISR of 4 and added nutrients for both the post crush (207.3 mL/gVS_{added}) and crush (206.4 mL/gVS_{added}) PWWS.

Inocula may contain residual biodegradable substrate that can contribute to CH_4 generation. Controls containing only inocula are therefore included as a requirement in standard BMP protocols (Holliger et al., 2016). Results are corrected by deducting the amount of CH_4 generated in the inoculum-only controls from the CH_4 generated in the test reactors. In this study, the results were corrected by deducting the amount of CH_4 generated in inoculum-only controls with and without nutrients from the relevant reactor results. After correction, the final cumulative CH_4 yields from AD of crush and post-crush PWWS optimal conditions (ISR4/added nutrients) were similar (Figure 4-2). However, CH_4 was generated at a faster rate, and the stationary phase was

reached earlier (21 days retention time) with the post-crush PWWS when compared with the crush PWWS (30 days retention time).

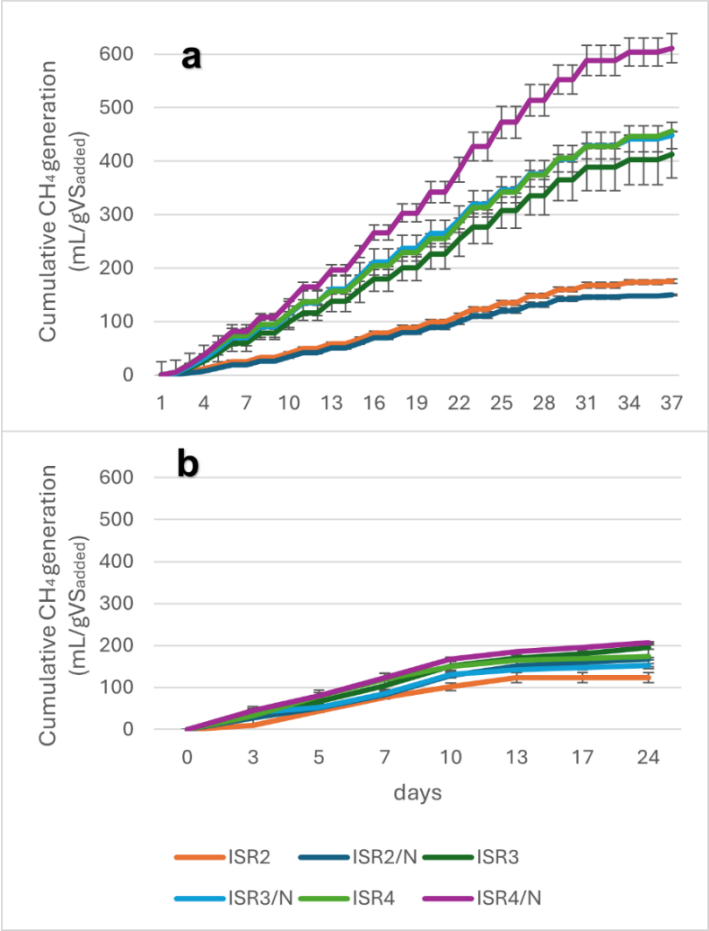

Figure 4-1: Cumulative methane generation with post-crush PWWS (a) and crush PWWS (b)

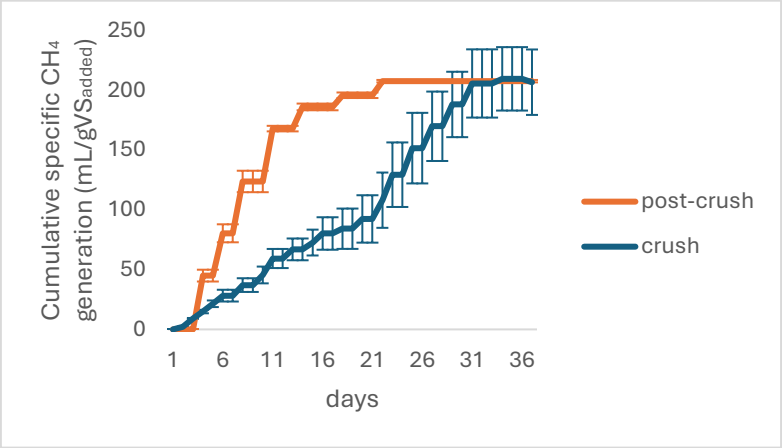

Figure 4-2: Corrected cumulative specific methane yields at ambient room temperature at ISR4 with added nutrients (experiment 1)

The corrected yields that were achieved were higher with the post-crush season PWWS (133 to 206 $mLCH_4/gVS_{added}$) than with the crush season PWWS (-182 to 205 $mLCH_4/gVS_{added}$), regardless of ISR or nutrient augmentation (Figure 4-3). This was attributed to differences in the inoculum, and/or the presence of lower concentrations of readily biodegradable organics such as VOAs in the crush PWWS (149 g/L COD, 29 g/L VOA) than in the post-crush PWWS (346 g/L COD, 104 g/L VOA).

The corrected CH_4 yields under optimal conditions (ISR4/nutrients) were 206 and 207 $mLCH_4/gVS_{added}$ for the crush and post-crush PWWS, respectively (Figure 4-2). Despite differences in the characteristics of crush and post-crush PWWS, the difference between the corrected yields was not statistically significant ($p>0.05$). For the post-crush season, reactors with ISR3/no nutrients had very competitive results producing 196 $mLCH_4/gVS_{added}$. If such results are scalable, this would mean that AD plants would be able to treat higher volumes of PWWS while saving on the costs of nutrient addition.

Correction resulted in negative CH_4 yields being obtained in reactors ISR2 and ISR2/N containing crush season PWWS. This is due to the use of an active inoculum in which the substrate had not completely depleted before use in the experiment. After correction, the lowest CH_4 yields were measured in reactors ISR2 (116 $mLCH_4/gVS_{added}$) and ISR2/N (-182 $mLCH_4/gVS_{added}$) for post crush season and crush season PWWS, respectively. This was likely due to the presence of residual substrate in the post-crush inoculum, which coupled with the low ISR, caused high levels of organic acids and reactor instability. At higher ISR, the addition of nutrients appeared to improve the CH_4 yield. Addition of nutrients enhanced biogas and CH_4 generation of the post-crush PWWS at ISR of 2 and 4 by 12.7% and 17.1% respectively, but had detrimental effect at ISR3, decreasing the CH_4 yield by 5.1%. In the post crush PWWS, nutrients enhanced CH_4 generation only at ISR of 4.

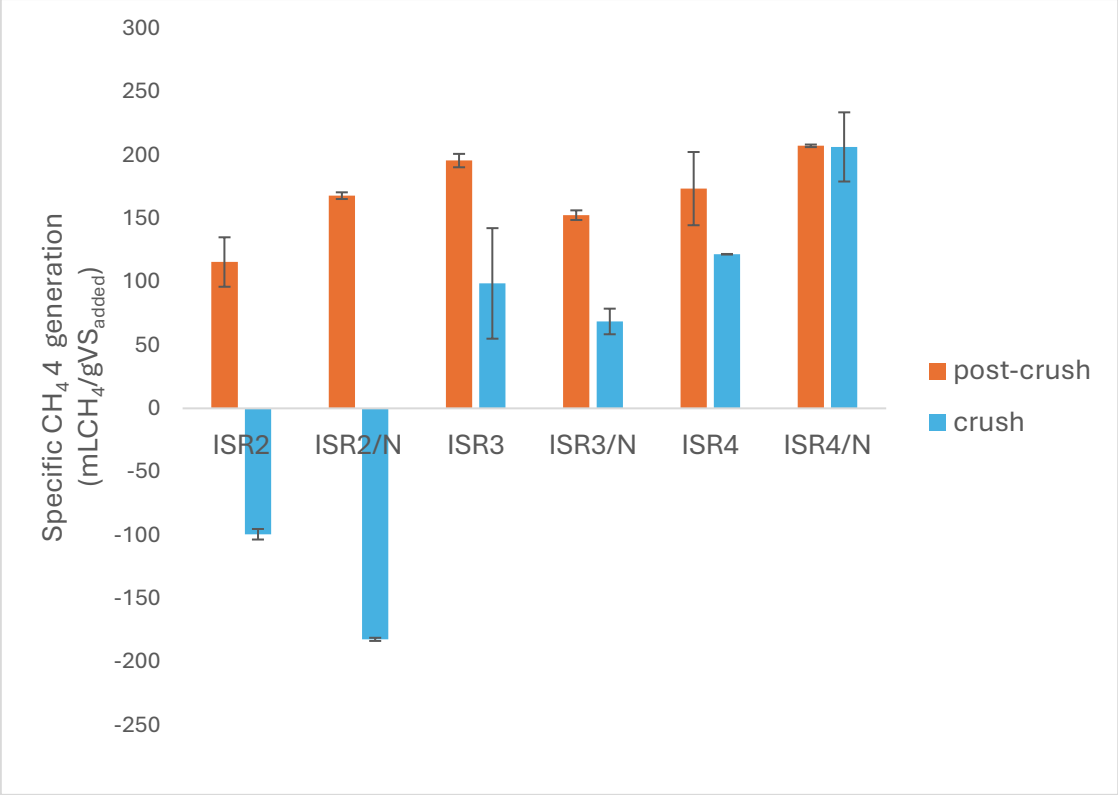

Figure 4-3: Corrected specific methane yields at varied inoculum to substrate ratios (2,3,4) with and without nutrients (Experiment 1)

Table 4-5: Summary of corrected CH_4 yields (average and standard error of the mean, n = 2)

	Nutrients	Crush	Post-crush
		$mLCH_4/gVS_{added}$	**$mLCH_4/gVS_{added}$**
Experiment 1			
ISR2	-	-99.4 ± 4.2	115.5 ± 19.5
ISR2/N	N	-182.4 ± 1.2	167.9 ± 2.6
ISR3	-	98.6 ± 43.6	195.6 ± 5.3
ISR3/N	N	68.6 ± 10.1	152.5 ± 3.8
ISR4	-	121.7 ± 0.22	173.5 ± 28.9
ISR4/N	N	206.4 ± 27.3	207.3 ± 1.0
Experiment 2			
ISR4/N/37°C	N	189.0 ± 28.1	166.3 ± 31.3
ISR4/N/ambient	N	137.3 ± 17.4	116.1 ± 11.3

N = 2.5 mg/L Cu, 10mg/L Ni, 10mg/L Co

4.3.2 Effect of temperature on methane generation at optimal inoculum to substrate ratio (Experiment 2)

At the previously determined optimal ISR of 4 (Section 4.3.1), the CH_4 yields were lower than those obtained in Experiment 1, even under the same conditions (Table 4-

5). The difference was assumed to be due to temporal changes in the microbial composition of the inoculum as the same batches of PWWS were used in both experiments. The inoculum for Experiment 2 was sourced from the reactors at the conclusion of Experiment 1. Between experiments, the inoculum was continuously fed with homogenised PWWS substrate, which likely led to the observed temporal shifts in the microbial community. This highlights how important the quality of the inoculum is for successful BMP experiments, and indeed full-scale AD (De Vrieze et al., 2015).

In this instance, short lag phases of 2-4 days were observed in the reactors incubated at ambient temperature, while no lag phases were measured in those incubated at 37°C (Figure 4-4).

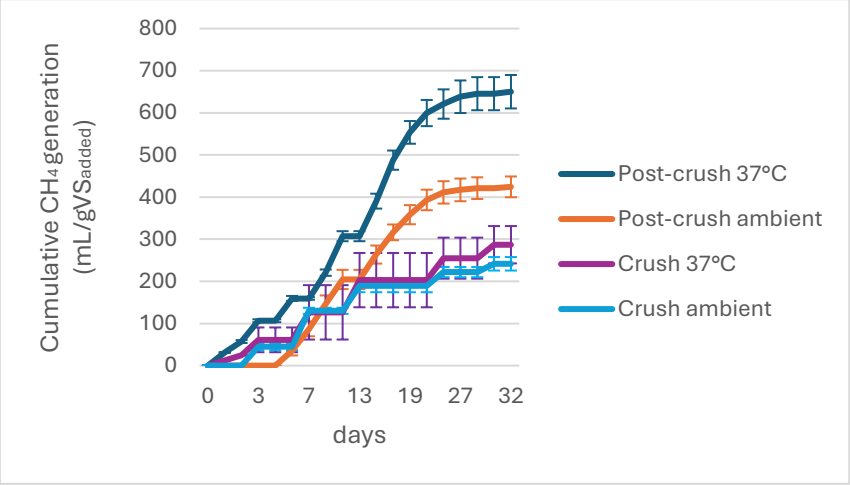

Figure 4-4: Cumulative methane yields (not-corrected) at ambient temperature and 37°C (Experiment 2)

Heating reactors to 37°C enhanced the CH_4 yields of both the crush and post-crush season PWWS by 37.7% and 43.2% respectively (Figure 4-6) Notably, the superior performance of post-crush PWWS was a consistent pattern observed under both thermal conditions, reinforcing that it is the more readily biodegradable substrate. However, while the CH_4 yield was notably higher at 37°C, the practical and economic implications require careful consideration. The decision to heat a full-scale digester would depend on a techno-economic analysis, balancing the capital and operational costs of heating against the revenue from the additional biogas.

To illustrate, a simplified energy balance for a 1 L glass reactor (thermal conductivity 1.0 W/mK and 4mm thickness) was conducted according to thermodynamic energy

balance methods described in Section 3.9. Assuming a working volume of 90%, and reactor content with the density and specific heat capacity of water (1 kg/L and 4.18 kJ/kg°C, respectively) the energy required to heat the reactor contents from 20°C to 37°C and maintain that temperature for 28 days would be ~19450 kJ, primarily due to the high surface-area-to-volume ratio, lack of insulation, and poor resistance offered by the glass reactor. In the case of crush PWWS, the 50.2 mL CH_4/gVS increase from ambient RT to 37°C would yield only ~20 kJ. At this lab scale, the increase in CH_4 from raising the temperature to 37°C is insufficient to compensate for the energy input required for heating, resulting in a net negative energy return.

However, this conclusion does not directly translate to full-scale systems, which benefit from much lower surface-area-to-volume ratios, heavy insulation, and often use waste heat to maintain temperature. Therefore, the decision for a winery to heat its digester would depend on a site-specific techno-economic analysis, with ambient temperature operation likely remaining the more practical approach for smaller operations or those in warmer climates.

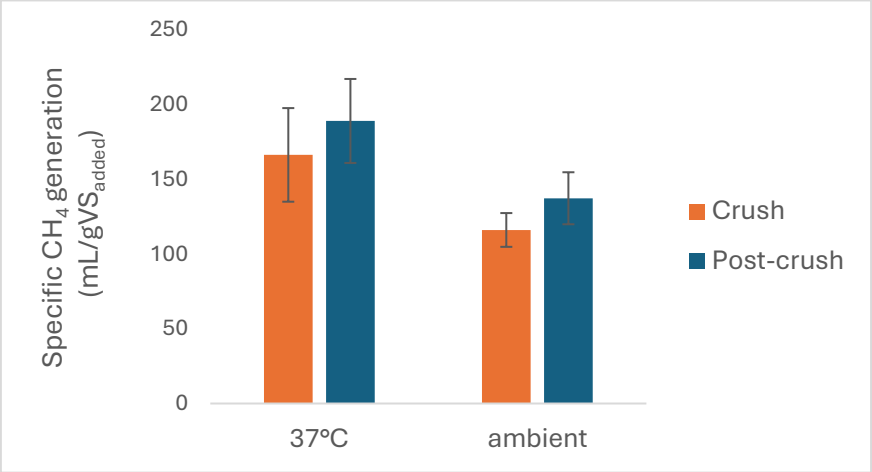

Figure 4-5: Corrected specific methane yields from primary winery wastewater sludge at inoculum to substrate ratio of 4 with added nutrients (experiment 2)

4.4. Characterisation and evaluation of primary winery wastewater sludge digestate

The management of ADg can pose a significant environmental challenge, since disposal methods like landfilling are unsustainable and costly. The use of ADg as a

soil additive is a popular disposal/beneficiation option and promotes a circular economy. However, few wineries (<9%) compost their by-products (Zhang et al., 2017b; Cáceres et al., 2012).

Detailed theoretical studies on fertilizers should ideally account for various soil types and plants, and preferably include experiments conducted in pots or in the field. However, this study had a narrower focus; it aimed to briefly assess whether PWWS digestate could potentially be beneficial when used as a fertilizer or soil additive. This was achieved by: i) comparing the macronutrient and micronutrient composition of the digestates with typical organic fertilizers, and ii) evaluating whether any elements were present in quantities that may be detrimental to plant and/or soil health. The PWWS digestate composition was compared with a range of four commercial agricultural organic fertilizers based on composted chicken manure (Table 4-6). Two of these were enriched, one with kelp and fishmeal, and the other with Ca and P.

Both inorganic and organic forms of C are important as much C is being lost from the soil due to agriculture, other anthropogenic land use practices and climate change across the globe (Dash et al., 2019; Lin et al., 2022). The total C concentrations in the were 2.0 to 2.6-fold higher in the PWWS ADg than in the commercial fertilizers, indicating that it would be a valuable source of C as a fertilizer. In terms of organic C, decayed organic material (humus) increases the water retention capacity and the cation exchange capacity of soils, assists with soil aeration and provides a reservoir for gradual release of plant nutrients (Zhang et al., 2019). All the PWWS ADg contained notable amounts of organic carbon (measured as TOC), and the amount was higher in the crush season PWWS ADg (average 261 g/kg) than in the post-crush season PWWS ADg (average 104 g/kg) (Table 4-6). Given the worldwide average soil organic carbon (SOC) concentration of 15.5 g/kg, even the post-crush PWWS ADg could be a valuable contributor of humic material, especially in the case of sandy soils (Deluz et al., 2020).

Table 4-6: Concentrations of essential plant nutrients, sodium and sodium adsorption ratio in the digestates from optimal anaerobic digestion at 37°C and ambient temperatures

	Crush PWWS		Post-crush PWWS		Commercial*
	37 °C	Ambient	37°C	Ambient	
MACRONUTRIENTS (g/kg dry weight)					
C	256.9±8.1	277.8±1.5	271.7±.9.1	229.2±4.9	106-115
H	40.9±3.7	43.8±2.6	30.7±1.1	51.0±1.9	-
N	25.5±1.4	25.6 ±0.8	27.7±0.1	21.5±0.5	26-34
S	3.0±0.2	3.0±0.02	5.1±0.5	3.6±1.1	9-10
Ca	10.5±0.24	9.8±1.57	10.36±0.83	9.69±1.66	35-60
K	38.3±2.11	37.9±3.47	38.42±1.91	27.67±6.63	30-33
Mg	2.20±0.07	2.00±0.01	2.93±0.09	2.38±0.55	6-7
P	3.4±0.01	3.3±0.51	5.15±0.43	4.47±0.69	17-27
MICRONUTRIENTS (mg/kg dry weight, **g/kg dry weight)					
Fe**	5.1±0.01	5.0±0.02	5.72 ±0.04	4.76±1.57	5
B	62±11	57±1.4	50±0.1	40±11	50
Mn	80±1.1	70±1.6	90±1.0	80±22	570-610
Cu	25±2.2	23±1.1	250±17	180±56	60
Zn	57±2.8	49±1.8	620±7.9	450±133	500-540
Mo	2.0±0.1	4.0±0.0	ND	ND	5
OTHER (g/kg dry weight)					
TOC	282±13.0	241±17.1	98.7±10.6	110±2.82	ND
Na	3.20±0.17	3.10±0.19	3.26±0.08	2.28±0.57	ND
RATIOS					
C:N	10.08	10.87	9.82	10.66	3.73
SAR	1.3	1.3	1.2	0.9	ND

*Range of organic fertilizers based on composed chicken manure (Bio ganic® Bio ganic crumble®), enriched with kelp and fishmeal (Bio ocean®), enriched with calcium and phosphate (Bio rock®) TOC = total organic carbon; SAR = sodium adsorption ratio; ND = not determined

Different plants require macro-nutrients in different quantities, and some soils themselves contain sufficient elements. For example, as the name suggests, calcareous soils have high Ca concentrations, but limited P and Zn availability (Akhtar et al., 2019). To cater for different requirements, fertilizers contain variable ratios of the macronutrients N, P and K, among other elements. The PWWS ADg contained similar amounts of N and K to the commercial fertilizers, but were deficient in P, Ca and S

(Table 4-6). Depending on the soil type and crop, the PWWS ADg may require supplementation with a source of these elements. Waste gypsum is an example of a sustainable source of Ca and S. In terms of micronutrients, both batches of PWWS ADg contained reasonable concentrations in comparison to the commercial organic fertilizers (Table 4-6), but both the commercial fertilizers and the PWWS ADg contained high concentrations of Fe. Although Fe deficiency can be detrimental to plant growth, it can be toxic to plants in highly acidic or hypoxic soils (usually formed via waterlogging) as Fe^{3+} is reduced to Fe^{2+} which is more bioavailable than the oxidized form (Delias et al., 2022). In well-aerated less acidic to alkaline soils, relatively high Fe concentrations are generally not problematic (Delias et al., 2022).

Unlike K, Na is not a plant nutrient and can negatively affect the soil structure by binding with negatively charged soil particles. This can be offset to some extent by the presence of divalent cations (Ca^{2+} and Mg^{2+}) that have more than one binding site and replace Na^{+} (Jesus et al., 2018). Although the Na concentrations in the digestates were high (Table 4-6), the concentrations in the PWSS before AD were relatively low in comparison to the inocula (Table 4-3), clearly demonstrating that most of the Na in the digestates emanated from the inocula. The original starter culture was taken from AD of highly saline tannery effluent, and thereafter the inoculum was fed constantly with WWW which also resulted in accumulation of Na over time. The low sodium adsorption ratio (SAR) (<1.3) in digestates indicated low risk of sodicity, likely offset by high concentrations of Ca and Mg. To put this in perspective, soils with SAR > 13 are considered sodic and those with SAR < 5 pose a low risk (Mohanavelu et al., 2021).

4.5. Summary

Biochemical methane potential assays were conducted to investigate the effect of ISR, nutrient addition and temperature on AD of PWWS from winery A. The results indicated optimal ISR was 4 with the addition of nutrients for both crush PWWS (206 $mLCH_4/gVS_{added}$) and post-crush (207 $mLCH_4/gVS_{added}$). Heating reactors to 37°C enhanced both the crush and post-crush season by 37.7% and 43.2%, respectively, under similar conditions. The theoretical yields were 560 $mLCH_4/gVS_{added}$ and 490 $mLCH_4/gVS_{added}$ at STP, and 907 $mLCH_4/gVS_{added}$ and 784 $mLCH_4/gVS_{added}$ at 37°C

for crush and post crush PWWS, respectively. The increase in yield at 37^{o}C did not justify the energy cost, therefore it is more practical for small wineries to operate digestors at ambient temperatures.

The variations seen with CH_4 yields of both crush and post-crush PWWS from Experiments 1 and 2 highlighted the importance of a well-acclimated microbial consortium for inoculating BMP experiments. Despite the high levels of VOA, Na, TN, Cu and Zn, which would otherwise inhibit methanogenesis, increased tolerance of the inocula assisted in successful AD. The effect of inocula is discussed more fully in Chapter 6.

Overall, the PWWS was a suitable substrate for AD. There was little to no lag phase, and complete digestion took place within 30-40 days, even under ambient conditions. The results suggest that PWWS could be converted without needing extra heating, especially since the peak waste generation coincides with warmer summer/autumn months.

The ADg was compared to commercial fertilizer and showed promise as an additive to improve C, N and supply essential nutrients to soils. Further studies are required using pot experiments to determine the effects of PWSS ADg on crop growth in different soils but were not within the scope of this study.

CHAPTER 5: INFLUENCE OF SOURCE VARIABILITY ON THE ANAEROBIC DIGESTION AND KINETIC MODELLING OF PRIMARY WINERY WASTEWATER SLUDGE

5.1. Introduction

The PWWS from winery A was comprehensively studied and promising results were obtained for AD of the PWWS and potential use of the ADg as an organic fertilizer. The results of this study were presented in Chapter 4. However, to assess the potential to operate a centralised plant for AD of PWWS, in-depth studies on AD of PWWS from different wineries are required. By understanding the variance in PWWS, the performance of an AD plant treating PWWS can be predicted more accurately. In this study, it was hypothesised that AD of PWWS from different sources would differ due to differences in wine making processes, the quality and quantity of cleaning materials that are used, and variations in other cellar practices. As such, PWWS from 4 sources (winery B, C, D and E) and 1 source of lees (winery F) were collected and characterised. Lees are sediments consisting of yeasts and other solids that are settled in fermentation tanks and removed once spent. Following AD of PWWS from wineries B-E and lees from winery F, the Logistic, Cone, Modified Gompertz (MG), First-Order, and Chen & Hashimoto (C&H) models were adopted to fit and evaluate the BMP process during AD of PWWS. The aim was to characterize and choose a suitable kinetic model for evaluating the AD of PWWS.

5.2. Characterization of primary winery wastewater sludge from wineries A-E

5.2.1 Organics, solids, macronutrients and theoretical methane yield

The high VS:TS ratios observed (0.5-0.87) (Table 5-1) indicates that there was a high proportion of organic matter in the PWWS and lees from the five wineries, all

theoretically favourable for AD (Wang et al., 2016). Similarly, the high COD:VS ratios of 0.68-3.88 and TOC values (6.8-20.2 g/L TOC) (Table 5-1), particularly from wineries B, E and F infers a high fraction of oxidisable organic matter which theoretically translates to good CH_4 yields (Shitophyta et al., 2023; Ruan et al., 2024). The theoretical CH_4 yields (Figure 5-1), which were calculated using the elemental composition from the CHNS analysis in the Buswell Equation (Eq. 4.1), reflect this potential. The yield from the lees (winery F) had a notably higher theoretical yield than the PWWS (winery B > winery E > winery D > winery C).

Despite the PWWS from winery C having a relatively high C:N ratio (19.8) and VS:TS ratio (0.87), the COD:VS ratio was low (0.68) in comparison to the PWWS from the other wineries (Table 5-1). This PWWS contained a high fraction of pomace, which could explain these results. Similarly, the PWWS from winery D had the second highest C:N ratio (10.8) and third highest VS:TS ratio (0.79), but an unexpectedly low theoretical yield (504 mL/gVS at 37°C) (Figure 5-1). The discrepancy may be due to foreign substances like leaves and twigs observed in the PWWS from Winery D. The relatively low C:N ratios in the PWWS (Table 5-1) indicate that the macronutrient balance in PWWS may be sub-optimal for AD, which is common for some agricultural wastes (Xiao et al., 2022). These wastes may benefit from co-digestion with carbon-rich substrates (Karki et al., 2021).

The COD:N:P ratios for Winery B, C, and D showed low levels of COD relative to N and P, indicating that there were sufficient amounts of macronutrients for AD of the organics present in these substrates. In contrast, the PWWS from Winery E and lees from winery F showed deficiencies in N relative to COD when compared to the optimal ranges often cited for AD (COD:N 300:1) (Hussain et al., 2015). Minor macronutrient deficiencies may not necessarily lead to process failure of AD as evidenced by a study by Britz et al. (1988) who achieved 95% COD removal from petrochemical effluent originating from a Fischer-Tropsch process with COD of 11660 mg/L, N of 45 mg/L, and P of 8 mg/L (COD:N:P 259:5.6:1).

The NH_4^+-N in the PWWS and lees was below inhibition ranges (<1.5 g/L), and within reasonable ranges for AD (Table 5-1) (Myszograj et al., 2018). Similarly, the Cl was well below inhibitory range reported in literature (17.1-20.0 g/L Cl) (Zupančič et al., 2023). The pH of the PWWS and lees (4.21-5.35) was acidic for all wineries, falling outside the range recommended for methanogenesis (Holliger et al., 2016; Myszograj

et al., 2018). The VOA of all but 1 PWWS (Winery D) was above the inhibition threshold for AD (16.5-18.0 g/L) (Jiang et al., 2018). Additionally, the VOA in all five substrates were above the optimum of 50 g/L (Amani et al., 2010). However, as discussed in Chapter 6, pre-acclimation of microbial communities in inocula may play a significant role in mitigating inhibition.

Table 5-1: Characteristics of primary winery wastewater sludge B-F, and inoculum (average and standard error of the mean, n =3)

Parameter	Winery B	Winery C	Winery D	Winery E	Winery F	Inoculum
Elemental CHNS analysis (g/kg dry weight)						
C	31.27 ± 0.44	47.81 ± 0.66	43.25 ± 0.58	42.06 ± 0.24	38.63 ± 1.56	27.14 ± 0.52
H	4.79 ± 0.17	6.73 ± 0.16	6.19 ± 0.03	6.28 ± 0.03	6.02 ± 0.08	4.11 ± 0.13
N	3.94 ± 0.09	2.41 ± 0.05	4.00 ± 0.18	5.07 ± 0.02	3.92 ± 0.17	2.78 ± 0.01
S	0.30 ± 0.00	0.22 ± 0.01	0.33 ± 0.02	0.24 ± 0.01	0.31 ± 0.01	0.47 ± 0.01
C:N	7.9	19.8	10.8	8.3	9.8	9.8
Characterisation (g/L wet weight homogenised sludge)						
COD	164.6 ± 8.4	66.9 ± 8.9	33.0 ± 1.2	181.7 ± 7.0	213.8 ± 1.0	46.4 ± 2.3
TOC	19.0 ± 1.0	13.3 ± 0.4	6.8 ± 0.7	20.2 ± 1.0	9.6 ± 0.3	8.5 ± 0.1
TP	0.20 ± 0.00	0.18 ± 0.00	0.10 ± 0.00	0.17 ± 0.00	0.32 ± 0.00	0.2 ± 0.0
TN	2.1 ± 0.1	1.1 ± 0.0	1.1 ± 0.1	0.3 ± 0.00	0.2 ± 0.0	3.0 ± 0.1
VOA	32.7 ± 1.0	16.0 ± 1.4	3.6 ± 0.5	50.0 ± 1.5	17.6 ± 0.2	8.6 ± 0.6
HS^-	0.03 ± 0.00	0.04 ± 0.00	0.01 ± 0.00	0.04 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
NH_4^+-N	0.37 ± 0.02	0.05 ± 0.01	0.06 ± 0.00	0.32 ± 0.01	0.06 ± 0.00	0.67 ± 0.05
Cl	2.52 ± 0.16	1.24 ± 0.02	0.32 ± 0.05	4.30 ± 0.08	2.70 ± 0.05	1.80 ± 0.11
TS	153.1 ± 5.1	107.0 ± 1.0	32.8 ± 0.5	143.3 ± 1.2	72.9 ± 2.5	85.3 ± 1.2
VS	93.0 ± 1.2	97.8 ± 8.7	26.0 ± 0.3	123.5 ± 1.5	55.1 ± 1.8	42.7 ± 0.7
COD:VS	1.77	0.68	1.27	1.47	3.88	1.09
VS:TS	0.57	0.87	0.79	0.86	0.76	0.5
COD:N:P	82.3:10:1	60.8:6.1:1	30:11:1	605:1.8:1	1069:0.6:1	15.5:15:1
pH	5.35	4.21	5.3	4.84	4.49	8.1

COD = chemical oxygen demand; TOC = total organic carbon; TP = total phosphate, TN = total nitrogen, VOA= volatile organic acids, TS=total solids; VS=volatile solids

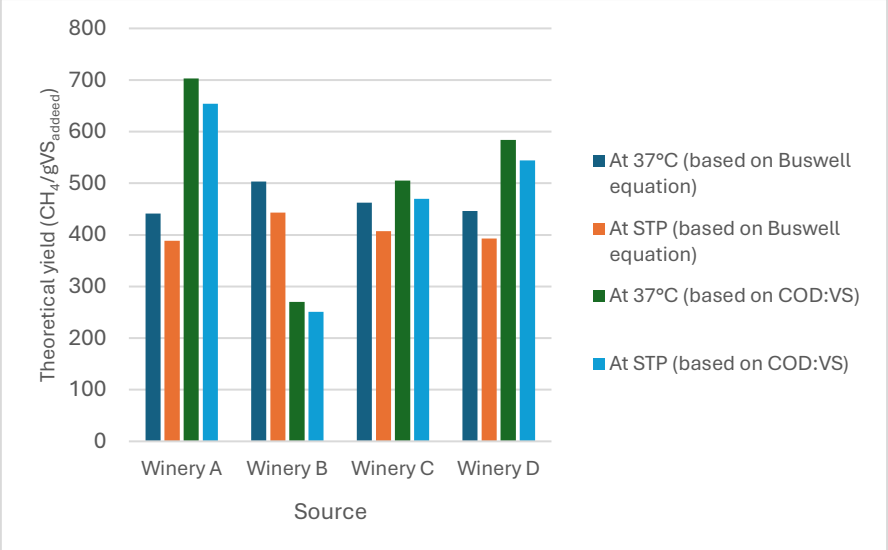

Figure 5-1: Theoretical specific methane yields at 37°C vs standard temperature and pressure

5.2.2 Cationic inhibitors and micronutrients

The IC_{50} values have been reported as 30-350 mg/L for Cu, 1300 mg/L for Ni, >500-1500 mg/L for Zn, and > 5000 mg/L for Pb for acetogenic bacteria, and the concentrations in the PWWS fell below these limits (Chen et al., 2008; Czatzkowska et al., 2020; Mudhoo & Kumar, 2013). The IC_{50} values for methanogens have been reported as 5.6–53 g/L Na, 1000mg/L Al, 8.9-20.7 mg/L Cu, 35 mg/L Ni, and 7,7 mg/L Zn (Chen et al., 2008; Czatzkowska et al., 2020; Mudhoo & Kumar, 2013). The IC_{50} of the PWWS and lees fell below these limits (Table 5-2). The cation concentrations reported in the PWWS across the wineries were therefore generally within safe limits for AD, and not likely to cause significant inhibition (Table 5.2).

Based on experiments conducted by Khan et al. (2021), who showed that during AD of molasses, the CH_4 yield was optimal at Ni concentrations of 2-4 mg/kg, Ni appeared to be deficient in all the PWWS and lees (0.01-0.17 mg/L Ni) but was present in sufficient quantities in the inoculum (Table 5-2). Concentrations of Co also appeared deficient, in all PWWS (<0.005 to 0.03 mg/L Co) from winery C and D (<0.005 mg/L Co), while the concentration of Cu was only deficient in the PWWS from winery C (0.51 mg/L) and winery D (0.12 mg/L). The low concentrations of Ni, Co as a micronutrient in all samples, including the inoculum, was concerning and it was hypothesized that AD of PWWS may benefit from addition of Co. For context, the optimal concentrations

for Ni, Co and Cu in AD are around 2-4 mg/L, 2 mg/L and 10 mg/L respectively (Chan et al., 2019; Khan et al., 2021; Li et al., 2023).

Table 5-2: Elemental cation concentrations of primary winery wastewater sludge and lees from winery B-F, and inoculum (average and standard error of the mean, n =3)

	Winery B	Winery C	Winery D	Winery E	Winery F	Inoculum
Major elemental parameters (g/L)						
Fe	82.67 ± 12.25	7.49 ± 1.07	1.97 ± 0.33	68.78 ± 1.43	18.95 ± 0.29	19.62 ± 5.12
Al	169.94 ± 24.50	11.77 ± 1.07	2.62 ± 0.33	161.93 ± 2.87	72.17 ± 1.46	48.62 ± 12.80
Ca	62.77 ± 1.53	24.61 ± 3.21	1.97 ± 0.00	42.99 ± 0.00	16.77 ± 0.00	27.30 ± 6.82
K	551.16 ± 15.31	78.11 ± 0.00	5.90 ± 0.33	278.00 ± 4.30	58.32 ± 0.73	197.90 ± 8.53
Mg	22.97 ± 3.06	5.35 ± 0.00	0.98 ± 0.00	22.93 ± 1.43	18.95 ± 0.00	7.68 ± 1.71
Na	18.37 ± 1.53	5.35 ± 0.00	1.64 ± 0.00	11.46 ± 0.00	5.83 ± 0.00	24.74 ± 1.71
P	50.52 ± 3.06	13.91 ± 0.00	1.97 ± 0.00	85.98 ± 2.87	19.68 ± 0.73	15.35 ± 2.56
Si	36.74 ± 3.06	14.98 ± 1.07	3.61 ± 0.33	37.26 ± 2.87	16.04 ± 0.73	13.65 ± 2.56
Minor elemental parameters (mg/L)						
B	0.52 ± 0.05	0.10 ± 0.01	0.01 ± 0.00	0.56 ± 0.01	0.33 ± 0.00	0.18 ± 0.05
Mn	0.53 ± 0.05	0.08 ± 0.01	0.02 ± 0.00	0.61 ± 0.01	0.11 ± 0.00	0.36 ± 0.10
Co	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.03 ± 0.00	0.00 ± 0.00	0.08 ± 0.02
Ni	0.17 ± 0.05	0.03 ± 0.01	0.01 ± 0.00	0.17 ± 0.03	0.03 ± 0.00	0.15 ± 0.04
Cu	2.48 ± 0.37	0.51 ± 0.05	0.12 ± 0.02	3.35 ± 0.17	2.33 ± 0.03	1.02 ± 0.26
Zn	4.52 ± 0.85	1.01 ± 0.07	0.24 ± 0.03	2.82 ± 0.09	0.14 ± 0.00	2.13 ± 0.62
Sr	0.35 ± 0.02	0.15 ± 0.01	0.01 ± 0.00	0.28 ± 0.01	0.06 ± 0.00	0.17 ± 0.04
Mo	0.02 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
Pb	0.48 ± 0.17	0.06 ± 0.01	0.01 ± 0.00	0.15 ± 0.02	0.01 ± 0.00	0.08 ± 0.02
Ba	1.41 ± 0.31	0.09 ± 0.01	0.03 ± 0.00	1.21 ± 0.08	1.03 ± 0.02	0.42 ± 0.11

5.3. Anaerobic digestion of primary winery wastewater sludge and lees from wineries B-F

5.3.1 Introduction

The AD of PWWS from wineries B-E and lees from winery F were conducted in 1L borosilicate bottles using the methods described in Chapter 3 and applying an ISR of 4 based on the optimum from the initial study with PWWS from winery A (Chapter 4). A complete setup of the BMP assay is detailed in Table A-4 of Appendix A. This study investigated the effects of: (i) variances related to the source of the substrates (different wineries), (ii) the type of substrate (PWWS versus lees), (ii) temperature (37°C versus ambient temperature of 15.4°C±2.3 (range: 9.7-21.1°C)), and (iv)

addition/non-addition of micronutrients. In contrast to the targeted addition of specific nutrient combinations explored in the first study (Chapter 4), this study investigated the impact of the addition of Micro 14, a commercial micronutrient blend manufactured by Bionetix®. This blend was selected to better represent nutrient supplementation practices more likely to be implemented in AD plants outside of a controlled laboratory setting. Micro 14 is a blend of 14 ingredients providing various sources of minerals, vitamins and microbial growth elements to enhances biogas production, improve microbial and enzyme activity, and reduce foul smells in AD. These include micronutrients such as Fe, S, Mn, Zn, Ni, Mo, Cu, I_2, Se and Co as well as vitamins and amino acids.

5.3.2 Cumulative biomethane yields and biomethane yields

The AD of PWWS from winery B and lees from winery F showed little to no lag phases operating at 37^{o}C with complete digestion achieved within 13 days (Figure 5-2a and Figure 5-2b). Increasing the temperature from ambient to 37^{o}C improved the maximum CH_4 production (observed between day 3 and day 13) and reduced the required retention time for all substrates. In contrast, under ambient conditions, lag phases of 3-5 days were noted (Figure 5-2c and Figure 5-2d). The overall rate of biogas production was also much slower, and even by day 30, significant amounts of CH_4 were still being generated. This is consistent with the principle that reduced microbial activity and AD efficiencies are found at lower temperatures (Nayeri et al., 2024). In comparison to the ambient temperatures in the previous experiments (20.7°C ± 2.0°C), the ambient temperatures were lower 15.4°C±2.3 (range: 9.7-21.1°C)) as the experiments were conducted at different times of the year and the environment was not temperature controlled. In general, the activity of methanogenic bacteria can be reduced by half for every 10°C reduction below the optimal 35°C (Nayeri et al., 2024).

In addition, at ambient temperature, the PWWS from wineries C, D and E, appeared to operate at an inhibited steady state (Figure 5-2c and Figure 5-2d), while the PWWS from winery B, C, D and E benefited from addition of nutrients at lower temperatures (Figure 5-2).

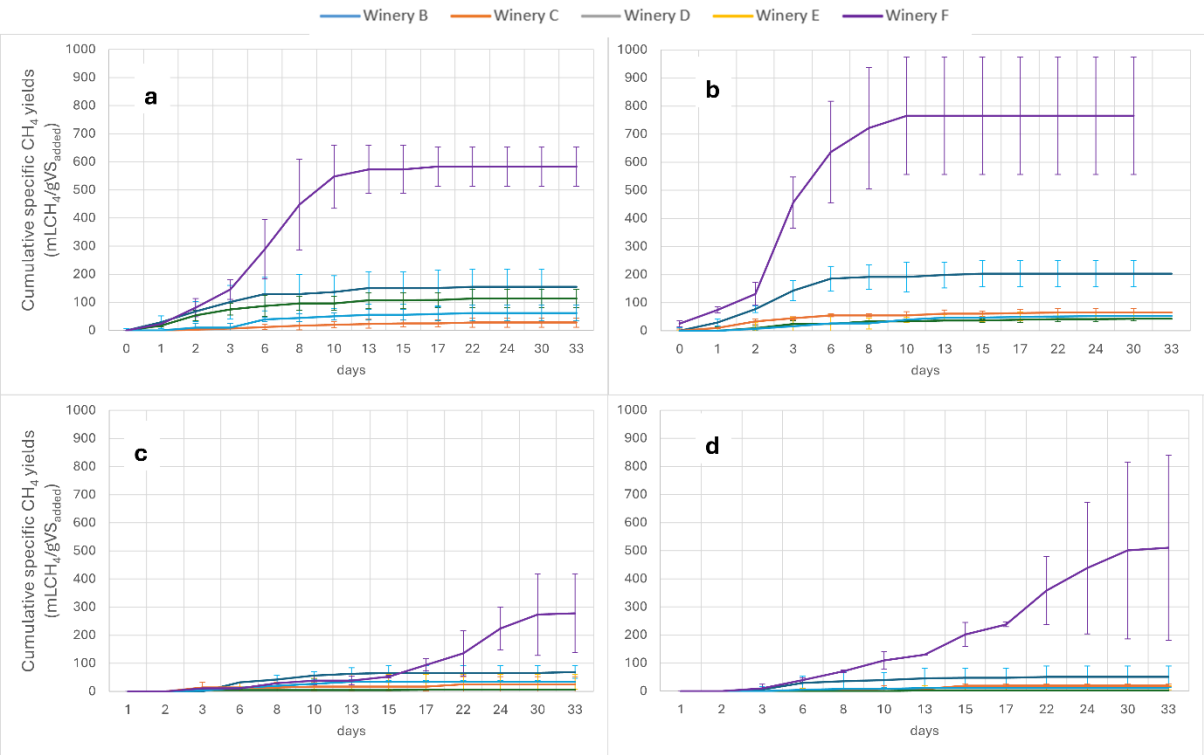

Figure 5-2: Corrected cumulative specific methane yields for wineries B-F at 37°C with nutrients (a) at 37°C without nutrients (b) at ambient temperature with nutrients (c), and at ambient temperature without nutrients (d) (error bars: standard deviation from the mean, n=2)

Theoretical CH_4 yields represent 100% conversion of all organic matter into CH_4 under ideal chemical conditions. However, not all VS are easily biodegradable and may contain recalcitrant compounds like lignin and polyphenols which microbes cannot easily break down. Additionally, a variable portion of the organic matter is not converted to CH_4 and other gases but is instead used by the microbes to proliferate (Jang et al., 2015). Process inhibition can slow down the activity of the methanogenic archaea. In this study, the actual specific CH_4 yields from AD of PWWS and lees followed a similar trend to the theoretical yields: winery F (lees) > winery B > winery D > winery E > winery C at 37°C (Table 5-3). At this temperature, the addition of nutrients unexpectedly reduced the CH_4 yields from AD of PWWS from winery B, C and lees from winery F by 24%, 57% and 24%, respectively, while nutrient addition improved the CH_4 yields from AD of PWWS from winery D and winery E by 163% and 16%, respectively. This is possibly due to inhibition by micronutrients, rapid acidification of the reactor due to overstimulation of acidogens by nutrient addition, or the formation of toxic NH_3 over the less harmful NH_4^+ at higher temperatures and pH.

Table 5-3: Theoretical and actual CH_4 yields of primary winery wastewater sludge and lees from winery B-F

	Winery B	Winery C	Winery D	Winery E	Winery F
Theoretical yield (CH_4/gVS)					
Ambient temperature	654	251	470	544	1434
37°C	703	270	505	584	1542
Actual yield (CH_4/gVS)					
With Nutrients					
Ambient temperature	68.7	25.4	5.9	34.4	277
37°C	154	27.8	113.5	61.7	584
Without Nutrients					
Ambient temperature	51.2	19.7	3.10	11.3	510
37°C	203	65.3	43.1	53.1	765

At ambient temperature (15.4°C ± 2.3, range: 9.7-21.1), the CH_4 yields of PWWS from wineries B-E and lees from winery F exhibited a different pattern (winery F > winery B > winery E > winery C > winery D (Table 5-3) to the results obtained at 37°C. The addition of nutrients increased the yield from AD of PWWS from wineries B, C, D and E by 34%, 29%, 28% and 204%, respectively. The lees from winery F was the only substrate that did not benefit from addition of nutrients at both ambient temperature and 37°C. Results provided clear evidence that the specific CH_4 yields from AD of PWWS at ambient temperature was improved by the addition of micronutrients, but not necessarily at 37°C, which only improved in 2 out of 4 cases.

It was hypothesised that addition of nutrients is more likely to cause inhibition due the increased bioavailability of nutrients (particularly N, P and micronutrients) at higher temperatures from accelerated mineralization and decomposition of organic matter (Dory et al., 2024; Nyang'au et al., 2024). Excess nutrients in such cases can lead to inhibition and be detrimental to AD systems.

The highest COD reduction for the BMP assay was 19.7% (at 37°C without nutrients) for PWWS from winery B, 10.2% (at 37°C without nutrients) for PWWS from winery C, 18.7% (at 37°C with nutrients) for PWWS from winery D, 7.4% (at 37°C without nutrients) for PWWS from winery E and 21.9% (at ambient temperature with nutrients)

for lees from winery F (Table A-5). The highest TOC reduction for the BMP assay was 19.5% (at 37°C without nutrients) for PWWS from winery B, 13.7% (at 37°C without nutrients) for PWWS from winery C, 9.4% (at 37°C without nutrients) for PWWS from winery D, 9.8% (at 37°C without nutrients) for PWWS from winery E and 40.4% (at 37°C with nutrients) for lees from winery F (Table A-5).

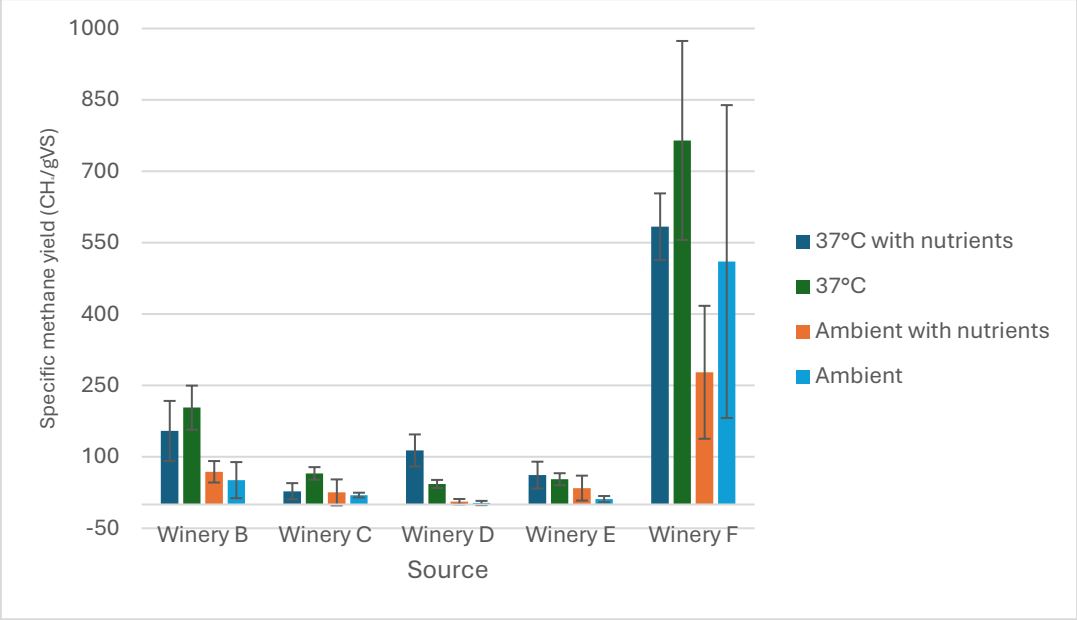

Figure 5-3: Specific methane yields from anaerobic digestion of primary winery wastewater sludge and lees at 37°C and ambient temperature with and without nutrients (error bars: standard deviation from the mean, n=2)

5.4. Economic justification for heating to mesophilic temperatures

To assess whether heating an anaerobic digester from ambient temperature (~15.4°C) to 37°C is economically justified, CH_4 yield data at both temperatures were evaluated. The analyses were based on methods detailed in Section 3.9 and the assumptions of a well-insulated 5000 L cylindrical reactor made from 50 mm low density polyetheylene (based on the dimensions of an easily accessible 'JoJo' tank) digestor with working volume of 4.5 L, VS content of 85 g/L, ambient temperature of 18°C, 28-day retention time, and 100% heater efficiency and assuming the reactor contents have heat capacity similar to water. The net energy gain was calculated by subtracting the energy required to heat the digester (129.7 kWh) from the energy content of the additional CH_4 produced due to increased temperatures.

The net energy balance from most samples was positive, indicating that heating to 37ºC could be economically viable (Figure 5-4). The benefit for heating was most pronounced for the PWWS from winery B as well as lees from winery F which both showed significant increases in CH_4 yield from ambient to 37ºC .

Samples of PWWS from wineries C and E (with and without nutrients) as well as PWWS from winery D treated without nutrients all showed low/negative benefits from heating. In these cases, the small CH_4 yield did not justify the energy required for heating.

Overall, these findings show that heating is only justified in cases where the expected increase in CH_4 is greater than ~30 $mLCH_4/gVS$. For smaller wineries, it may not be practical to supply additional heating. Such wineries can resort to other low-cost options such as insulation and painting digesters black to improve solar heat absorption and retention, especially during warmer seasons. Larger wineries, with higher volumes of organic waste could benefit from the additional energy produced. The additional energy recovered can help offset the cost of heating, improving overall energy self-sufficiency and sustainability.

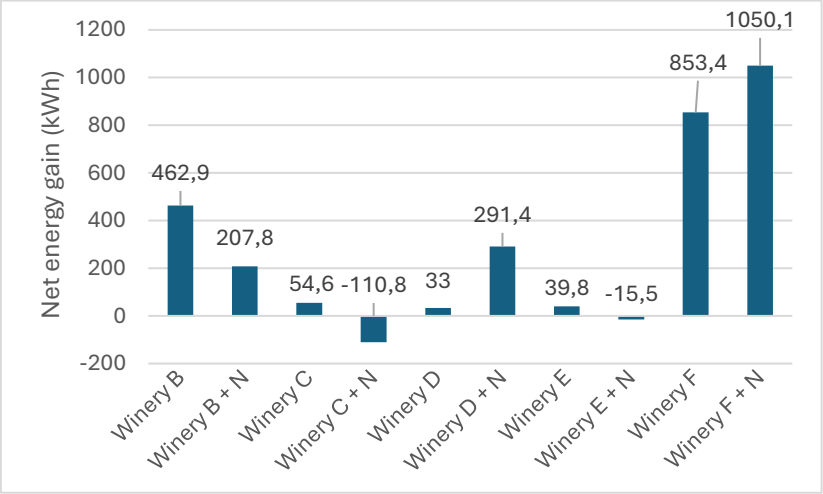

Figure 5-4: Net energy gain from heating from ambient temperature to 37°C

5.5. Kinetic models of anaerobic digestion of primary winery wastewater sludge and lees from wineries B-F

5.5.1 Introduction

In this study, the Logistic, Cone, MG, First-Order, and C&H models were adopted to fit and evaluate the biomethane production process during the AD of PWWS from wineries B-E and lees from winery F. The models were based on the assumption that CH_4 production is a function of bacterial growth during AD of PWWS and lees. The correlation coefficient (R^2), Akaike information criterion (AIC) and root mean square error (RMSE) were used to assess the fit and determine the most suitable model for the AD of the substrates under the study conditions. Multiple criteria were chosen to provide enough evidence of a valid model because a good R^2 fit alone does not. The determination of AIC and RMSE provide means of estimating errors and therefore the relative accuracy of statistical models for a data set. The kinetic parameters were determined by nonlinear regression, via the solver function in Excel to minimize the sum of squares error. The models from results obtained at ambient temperature are illustrated in Figure 5-5 and parameters are summarised in Table 5-4 and Table 5-5, while for mesophilic conditions (37°C), the models are illustrated in Figure 5.6 and parameters summarised in Table 5-6 and 5-7.

5.5.2 Kinetic model at ambient temperature

Without the addition of nutrients, the model fit statistics generally showed that the Cone and Logistic models provided the best fit (higher Adj R^2, lower AIC and RMSE) for most substrates, with some exceptions. For example, the Cone model consistently performed well for the PWWS from winery B (Adj R^2 = 0.995, AIC = 90.7, RMSE = 0.85) and Winery D (Adj R^2 = 1, AIC = -328, RMSE = 0). This suggests that CH_4 production under these conditions often follows a sigmoidal pattern or a pattern with a distinct lag, exponential, and stationary phase, which these models are good at capturing. The first-order and C&H models provided very high and unrealistic values for parameter A (57.8 LCH_4/gVS and 455.3 LCH_4/gVS) for lees from Winery F. This indicates that these models might not be suitable for lees under these conditions. Similarly, the AD of PWWS and lees with the addition of nutrients was well fit by the logistic and cone models (Adj $R^2 \geq 0.78$, AIC ≤ 252, RMSE ≤ 9.79). The first-order and

C&H again had unreasonably high values for parameter A (25.2 LCH_4/gVS and 2941.1 LCH_4/gVS) for lees from Winery F.

The MG and C&H models showed the highest error at ambient temperature. While most models also demonstrated a reasonable fit at ambient temperature, the MG model showed a significantly larger error particularly when nutrients were added to PWWS from winery B and winery C (Table 5-3). Additionally, the MG model could not account for the observed increase after the initial stationary phase observed in PWWS from winery C. Figure 5-5 illustrates that the C&H model did not predict the behaviour during the lag phase very well and appeared to perform better when there was no lag phase. Despite modelling the exponential phase reasonably well, the stationary region presented the largest error (up to 42.3%) when compared to other models,

When comparing the average errors in ultimate CH_4 yield at ambient temperature between the experimental and model results, the order of models by minimum error was Logistic > Cone > First Order > C&H > MG with average errors of 2.6%, 2.8%, 6.9%, 7.0% and 15.7%, respectively.

Table 5-4: Kinetic parameters and model fit statistics for anaerobic digestion of primary wastewater sludge and less at ambient temperature with added nutrients

	Model	Kinetic parameters					Adj R^2	AIC	RMSE
		A	μm	ʎ	K	n			
		($mLCH_4/gVS$)	($mLCH_4$ /gVSd)	(d)					
Winery B + N	Logistic	65.8	10.28	3.51			0.987	143	1.88
	Cone	67.3			0.15	3.7	0.995	113	1.19
	MG	20.6	15.22	2.71			0.546	260	11.04
	First order	74.9			0.10		0.903	210	5.19
	C&H	90.2	0.60		4.26		0.939	195	4.11
Winey C + N	Logistic	25.2	1.39	0.00			0.786	157	2.32
	Cone	39.0			0.06	0.93	0.86	143	1.88
	MG	6.6	4.74	1.52			0.291	197	4.22
	First order	27.1			0.09		0.856	145	1.94
	C&H	32.9	1.60		15.72		0.867	143	1.86
Winery D + N	Logistic	5.4	11.97	2.51			0.954	12.4	0.26
	Cone	5.4			0.37	24.6	0.954	12.4	0.26
	MG	2.0	6.34	2.72			0.954	12.4	0.26
	First order	5.8			0.22		0.769	66.9	0.59
	C&H	6.3	0.90		1.72		0.719	56.3	0.50
Winery E + N	Logistic	34.6	4.82	3.74			0.996	63	0.56
	Cone	35.3			0.14	3.85	0.994	73.8	0.66
	MG	12.9	1.73	6.10			0.996	62.2	0.55
	First order	39.2			0.10		0.906	166	2.64
	C&H	47.8	0.61		4.73		0.994	153	2.19
Winery F + N	Logistic	309.6	16.68	12.1			0.981	242	8.33
	Cone	395.2			0.04	3.33	0.974	252	9.79
	MG	145.8	5.20	20.8			0.974	252	9.75
	First order	25184.3			0.00		0.853	310	23.54
	C&H	2941080.5	0.15		41745		0.877	302	20.82

Adj R^2 = correlation coefficient; AIC = akaike information criterion; RMSE = root mean square error; MG = modified Gompertz; C&H = Chen and Hashimoto

Table 5-5: Kinetic parameters and model fit statistics for anaerobic digestion of primary winery wastewater sludge and lees at ambient temperature with no added nutrients

	Model	Kinetic parameters					Adj R^2	AIC	RMSE
		A	μm	ʎ	K	n			
		($mLCH_4$/gVS)	($mLCH_4$ /gVSd)	(d)					
Winery B	Logistic	49.2	6.97	2.56			0.977	140	1.78
	Cone	51.5			0.17	2.74	0.995	90.7	0.85
	MG	18.4	5.47	3.62			0.985	125	1.42
	First order	55.6			0.11		0.935	175	3.03
	C&H	65.6	0.67		4.14		0.968	152	2.15
Winey C	Logistic	20.3	2.13	6.94			0.952	110	1.14
	Cone	20.7			0.09	4.55	0.942	117	1.25
	MG	7.5	0.74	9.95			0.942	117	1.26
	First order	33.2			0.03		0.952	150	2.09
	C&H	41.1	0.42		10.78		0.872	144	1.89
Winery D	Logistic	3.1	5.33	11.7			1	-392	0
	Cone	3.1			0.09	48	1	-328	0
	MG	1.0	1.85	10			0.565	71	0.63
	First order	6.0			0.03		0.71	58.6	0.52
	C&H	7.2	0.34		9.70		0.749	53.8	0.48
Winery E	Logistic	11.3	1.64	3.60			0.975	40.4	0.4
	Cone	11.4			0.22	5.65	0.98	40.6	0.4
	MG	3.6	0.95	3.03			0.55	144	1.89
	First order	12.8			0.10		0.895	96.8	0.93
	C&H	15.6	0.59		4.45		0.929	83.8	1.89
Winery F	Logistic	536.6	28.09	8.31			0.994	244	8.59
	Cone	708.8			0.05	2.5	0.992	254	10.1
	MG	132.6	12.20	9.29			0.535	388	76.4
	First order	57775.9			0.00		0.946	318	26.5
	C&H	455373.7	0.25		5943		0.97	299	19.7

Adj R^2 = correlation coefficient; AIC = akaike information criterion; RMSE = root mean square error; MG = modified Gompertz; C&H = Chen and Hashimoto

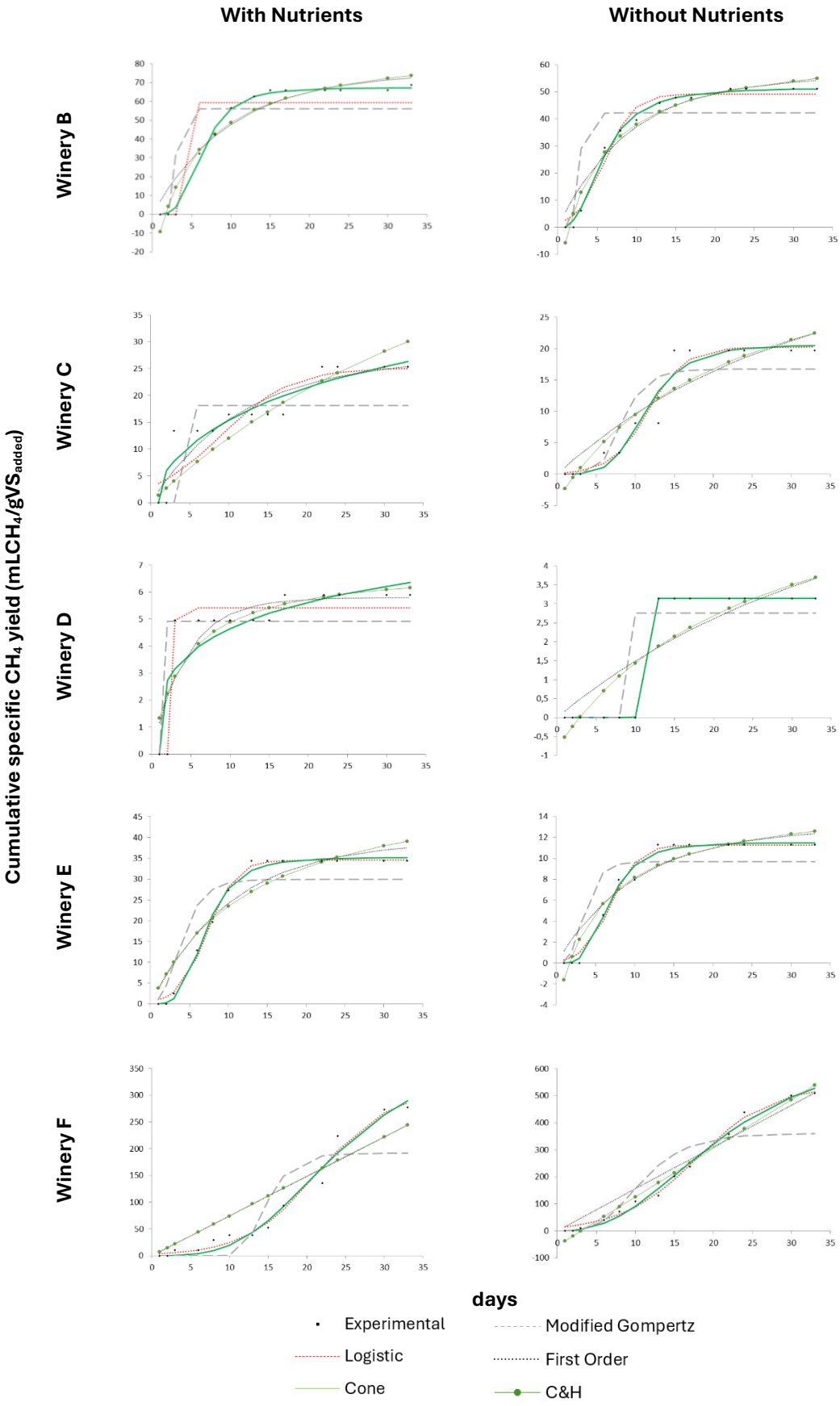

Figure 5-5: Comparison of cumulative specific CH_4 production and kinetic model predictions at ambient temperatures with and without nutrients

5.5.3 Kinetic model at 37°C

At 37°C, in the exponential zone, all the models appeared to match the experimental data, with the Logistic, cone, C&H, and linear models having consistently accurate fits, with a few exceptions (Figure 5-6). Without nutrients, MG had a poor fit for the PWWS from winery D (Adj R^2 = 0.24, AIC = 229, RMSE = 6.87), winery E (Adj R^2 = 0.39, AIC = 243, RMSE = 8,56) and lees from winery F (Adj R^2 = 0.48, AIC = 421, RMSE = 126) (Table 5-8). With the addition of nutrients, MG showed poor fit for PWWS from winery B (Adj R^2 = 0.13, AIC = 314, RMSE = 24.96) and winery C (Adj R^2 = 0.45, AIC = 200, RMSE = 4.41) (Table 5-7).

Table 5-6: Kinetic parameters and model fit statistics for anaerobic digestion of primary wastewater sludge and lees at 37°C with added nutrients

	Model	Kinetic parameters					Adj R^2	AIC	RMSE
		A	μ_m	λ	K	n			
		($mLCH_4/gVS$)	($mLCH_4$ /gVSd)	(d)					
Winery B + N	Logistic	147.6	32.00	0.00			0.949	210	5.18
	Cone	161.3			0.44	1.99	0.937	217	5.76
	MG	49.3	19.36	0.67			0.132	314	24.96
	First order	152.7			0.29		0.979	182	3.40
	C&H	165.7	1.53		2.61		0.991	154	2.21
Winey C + N	Logistic	27.2	2.45	1.49			0.984	82.3	0.75
	Cone	29.5			0.22	2.94	0.789	168	2.72
	MG	8.5	1.35	2.48			0.448	200	4.41
	First order	30.5			0.10		0.976	97.7	0.94
	C&H	36.7	0.82		6.37		0.987	76.3	0.68
Winery D + N	Logistic	105.8	27.18	0.24			0.914	201	4.93
	Cone	114.8			0.42	1.47	0.976	165	2.60
	MG	39.9	6.58	1.60			0.933	199	4.35
	First order	110.4			0.29		0.958	184	3.49
	C&H	119.1	1.41		2.30		0.977	138	1.73
Winery E + N	Logistic	59.3	7.77	1.63			0.976	148	2.02
	Cone	62.8			0.19	2.24	0.991	115	1.23
	MG	22.2	2.75	4.19			0.986	131	1.55
	First order	65.0			0.13		0.957	169	2.77
	C&H	75.6	0.83		4.21		0.991	151	2.10
Winery F + N	Logistic	585.3	75.10	1.84			0.995	246	8.94
	Cone	609.0			0.19	2.4	0.983	287	16.48
	MG	217.4	26.70	4.29			0.991	265	11.81
	First order	624.2			0.14		0.944	327	30.27
	C&H	731.0	0.88		4.37		0.929	323	28.72

Adj R^2 = correlation coefficient; AIC = akaike information criterion; RMSE = root mean square error; MG = modified Gompertz; C&H = Chen and Hashimoto

Table 5-7: Kinetic parameters and model fit statistics for anaerobic digestion of primary winery wastewater sludge at 37°C without added nutrients

	Model	Kinetic parameters					Adj R^2	AIC	RMSE
		A	μm	ʎ	K	n			
		($mLCH_4/gVS$)	($mLCH_4$ /gVSd)	(d)					
Winery B	Logistic	199.0	65.91	0.82			0.99	181	3.34
	Cone	203.0			0.44	2.46	0.994	166	2.64
	MG	74.7	25.16	1.86			0.993	168	2.74
	First order	205.0			0.31		0.958	228	6.8
	C&H	219.7	1.20		1.68		0.973	214	5.51
Winey C	Logistic	60.9	18.43	0.39			0.925	164	2.57
	Cone	64.4			0.48	1.64	0.969	135	1.66
	MG	22.5	6.65	1.55			0.934	159	2.4
	First order	63.0			0.32		0.925	155	2.26
	C&H	67.3	1.33		1.72		0.987	107	1.09
Winery D	Logistic	40.2	4.40	0.00			0.789	167	2.69
	Cone	44.8			0.23	1.42	0.945	142	1.85
	MG	12.9	1.86	0.10			0.238	229	6.87
	First order	42.5			0.16		0.935	149	2.03
	C&H	46.8	1.03		3.26		0.965	129	1.5
Winery E	Logistic	52.3	4.23	0.79			0.957	144	1.9
	Cone	58.7			0.22	2.46	0.984	124	1.39
	MG	16.2	2.20	2.12			0.391	243	8.56
	First order	57.2			0.10		0.969	147	1.98
	C&H	68.9	0.97		7.18		0.974	141	8.56
Winery F	Logistic	764.7	124.55	2.38			0.999	181	3.34
	Cone	781.1			0.20	3.12	0.995	264	11.6
	MG	247.1	125.73	2.33			0.481	421	126
	First order	819.6			0.14		0.924	357	48.1
	C&H	955.6	0.80		3.67		0.94	350	42.9

Adj R^2 = correlation coefficient; AIC = akaike information criterion; RMSE = root mean square error; MG = modified Gompertz; C&H = Chen and Hashimoto

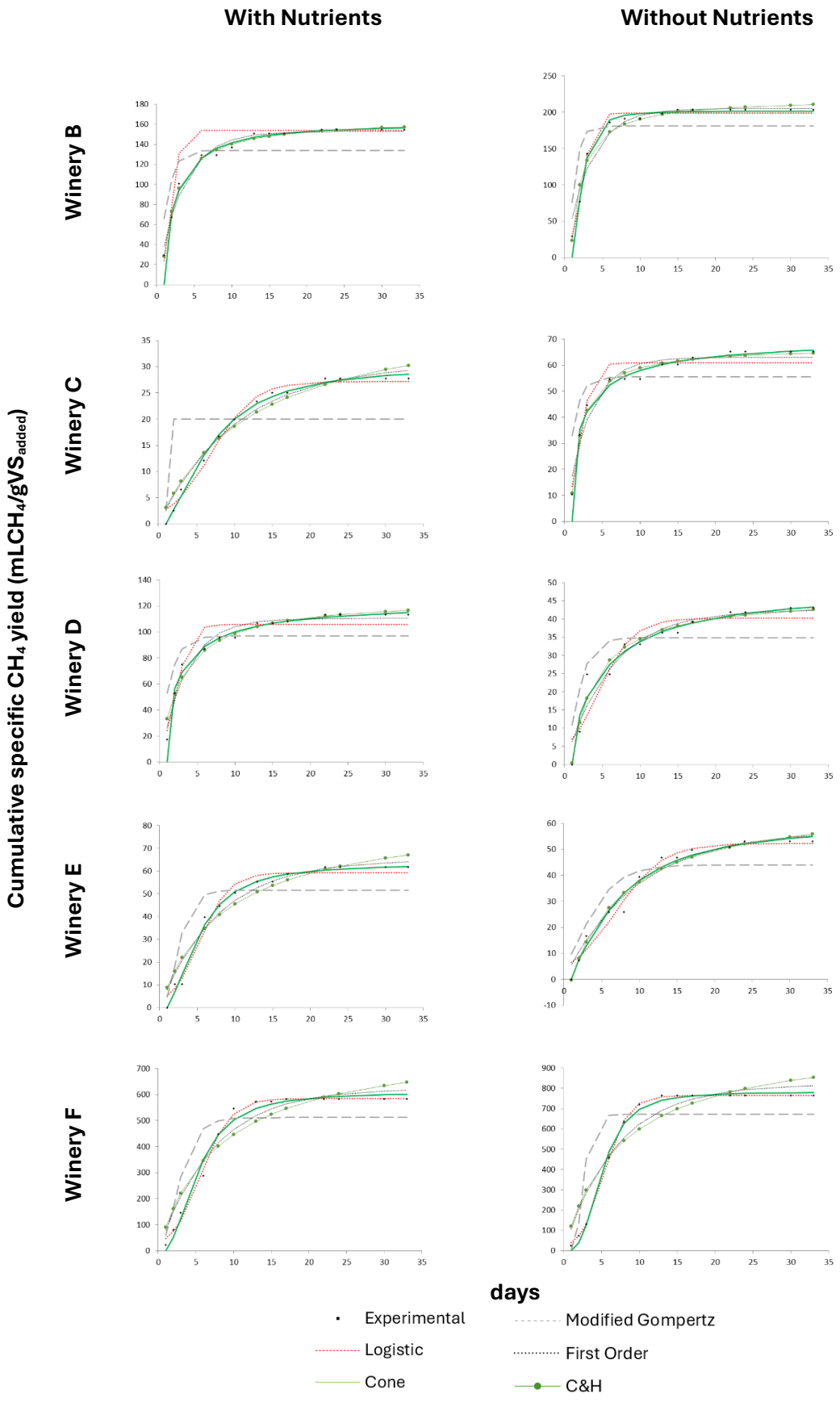

Figure 5-6: Comparison of cumulative specific CH_4 production and kinetic model predictions at 37°C with and without nutrients

5.5.4 Best fit summary for primary wastewater sludge from wineries B-E and lees from winery F

The best model for each scenario (source, temperature and nutrients) was selected by considering the highest value of Adjusted R^2 and lower value of AIC and RMSE (Table 5-9). Models with the best fit are summarized below:

- For PWWS from winery B, the cone model had the best fit. The C&H model performed better for PWWS from winery B with nutrients at 37^{o}C. This suggests that the Cone model robustly describes the CH_4 production kinetics, irrespective of nutrient addition or temperature within the tested range.
- For PWWS from winery C at 37^{o}C both with and without nutrients, the C&H model was the best model. At ambient temperature without nutrients, the logistic model was best, however the addition of nutrients at ambient temperature favoured the cone model. This indicates that both nutrient augmentation and temperature can influence the kinetic representation for PWWS from winery C.
- For PWWS from winery D at ambient temperature, both the logistic and cone models performed equally well, regardless of whether nutrients were added or not. The C&H fit the data more accurately at 37^{o}C, regardless of nutrient addition.
- For PWWS from winery E with the addition of nutrients, the MG and logistic models fit best at ambient temperature, and the cone model at 37^{o}C. The cone model also accurately predicted the curves without nutrients at both ambient temperature and 37^{o}C. The logistic model was comparable with the cone model at ambient temperature without nutrients.
- For lees from winery F at ambient temperature and 37°C, both with and without nutrients, the logistic model was the best fit. This consistent performance suggests that the Logistic model is well-suited for describing the sigmoidal growth kinetics for lees from winery F under the studied conditions.

The logistic model performed consistently well for lees, whereas the cone, C&H, and logistic models were effective for PWWS from wineries B-E indicating that they generally captured the AD dynamics of PWWS. The differences observed between ambient temperature and 37°C for some PWWS highlight the significance of

temperature-dependent kinetic behaviour. Similarly, the impact of nutrient supplementation, particularly evident in PWWS from winery B at 37°C, demonstrates its influence on the kinetics of AD. These results underscore the importance of selecting models based on the specific characteristics of the substrate and environmental parameters, rather than applying a one size fits all approach.

Table 5-8: Best models in terms of Adjusted R2, AIC and RMSE

Source	Adjusted R^2		AIC		RMSE	
	ambient	**37°C**	**ambient**	**37°C**	**ambient**	**37°C**
Winery B + N	Cone	C&H	Cone	C&H	Cone	C&H
Winery C + N	Cone	C&H	Cone	C&H	Cone	C&H
Winery D + N	Cone/ Logistic	C&H	Cone/ Logistic	C&H	Cone/ Logistic	C&H
Winery E + N	MG/ Logistic	C&H / Cone	MG/ Logistic	Cone	MG/ Logistic	Cone
Winery F + N	Logistic	Logistic	Logistic	Logistic	Logistic	Logistic
Winery B	Cone	Cone	Cone	Cone	Cone	Cone
Winery C	Logistic	C&H	Logistic	C&H	Logistic	C&H
Winery D	Cone/ Logistic	C&H	Cone/ Logistic	C&H	Cone/ Logistic	C&H
Winery E	Cone/ Logistic	Cone	Cone/ Logistic	Cone	Cone/ Logistic	Cone
Winery F	Logistic	Logistic	Logistic	Logistic	Logistic	Logistic

N = Micro14 nutrients ; AIC = Akaike Information Criterion ; RMSE = Root Mean Squared Error; AIC = akaike information criterion; RMSE = root mean square error; C&H = Chen and Hashimoto

5.6. Characterisation of primary winery wastewater sludge digestate and lees digestate from wineries B-F

5.6.1 Introduction

The use of ADg as a soil additive is a popular and cost-effective disposal/beneficiation option and promotes a circular economy. This study aimed to assess the potential use of ADg as a fertilizer or soil additive. Based on the seasonal intra-site variability discussed in Chapter 4.4, and the inter-site variability of PWWS from wineries B-E and lees from winery E discussed in Chapter 5.2, there is a need to assess the character

of digestate of each winery. This variability reflects differences in winery operations, and AD efficiencies.

The PWWS ADg composition of the best performing reactors for each substrate and digestion temperature was compared with a range of four commercial agricultural organic fertilizers based on composted chicken manure (Table 5-9). Two of these were enriched, one with kelp and fishmeal, and the other with Ca and P.

5.6.2 Potential use of anaerobic digestate as a fertilizer

Similar to the results obtained from winery A (Chapter 4.4), the total C concentrations in the PWWS ADg and lees ADg were 2.1 to 2.6 times higher than those found in commercial fertilizers (Table 5-9). This suggests that PWWS and lees ADg could serve as valuable sources of carbon when applied to soil. The TOC content of the PWWS ADg and lees ADg ranged from 333.9 to 611.6 g/kg, which was substantially higher than the values reported for winery A (98.7 to 282 g/kg) (Table 4-6). The elevated TOC levels are likely due to the presence of pomace and leaves in some of the PWWS, as well as lower CH_4 yields observed. Increased TOC, along with a greater proportion of humic substances, can be beneficial for soil structure by improving soil organic carbon, aeration and enhancing water retention (Zhang et al., 2019). Additionally, there is low risk of sodicity in ADg due to low SAR (<5) in all samples, likely offset by concentrations of Ca and Mg (Mohanavelu et al., 2021).

The ADg of PWWS and lees contained N concentrations comparable to those of commercial fertilizers. However, they were deficient in several essential elements including P, Mg, S and Mn (Table 5-9) . Depending on the specific crop and soil requirements, it may be necessary to supplement the application of ADg with additional sources of these nutrients to meet agronomic needs. All ADg samples, however, showed high levels of Cu (70.2-693.4 mg/kg) compared to commercial fertilizer (60 mg/kg), which may have implications for plant growth and increase the potential for accumulation in soils over time.

Micronutrient concentrations in the ADg of PWWS and lees varied widely and did not consistently align with the levels found in commercial fertilizers. In some cases,

concentrations were above commercial values, while in others they were within or below typical ranges. Iron (Fe) concentrations ranged from 1.4 to 14.9 g/kg in this study, compared to 5 g/kg in commercial fertilizers. Boron (B) levels ranged from 25 to 136.2 mg/kg, whereas commercial fertilizers contain 50 mg/kg. Zinc (Zn) concentrations, ranged from 101.4 to 1390.2 mg/kg, compared to a commercial fertilizers range of 500 to 540 mg/kg. Molybdenum (Mo) levels ranged from 0.6 to 7.1 mg/kg, while commercial fertilizers contained 5 mg/kg. This variability highlights the need for site-specific nutrient analysis when using ADg products in agricultural settings to accurately address deficiencies and avoid accumulation.

This study, when considered alongside the previous ADg characterization presented in Chapter 4.4, demonstrates that nutrient concentrations in ADg can vary widely depending on seasonal factors and differing winemaking practices across wineries. In theory, the nutrient composition of ADg could be optimized through the co-digestion of PWWS sourced from different wineries. By strategically combining these feedstocks, it may be possible to produce a more balanced ADg that more closely aligns with specific soil nutrient requirements, thereby enhancing its value as a biofertilizer.

Table 5-9: Concentrations of essential plant nutrients, sodium and sodium adsorption ratio in the digestates from optimal anaerobic digestion at 37°C and ambient temperatures

	Winery B		Winery C		Winery D		Winery E		Winery F		Commercial*
	37 °C	Ambient + N	37°^{0}C	Ambient + N	37°^{0}C + N	Ambient + N	37°^{0}C + N	Ambient + N	37°^{0}C	Ambient	
MACRONUTRIENTS (g/kg dry weight)											
C	266.8	282.4	234.1	284.7	283,0	273.2	299.7	271.9	278.3	282.2	106-115
H	42.2	45.2	42.3	43.7	43.8	42.1	45.3	41.5	42.4	43.1	-
N	29.2	28.8	24,0	26,0	26.5	27.3	33.2	28.4	28.2	28.6	26-34
S	4.8	5.34	4.3	4.9	5.4	5.1	5.1	5.2	5.7	4.8	9-10
Ca	18.9	6.4	8.0	15.4	3.3	8.4	2.5	18.3	7.4	14.9	35-60
K	146.0	126.7	98.3	119.7	120.2	99.1	124.6	126.8	94.6	128.8	30-33
Mg	5.5	2.1	2.4	4.7	1.1	2.6	0.8	5.3	3.0	4.6	6-7
P	10.7	7.3	6.6	9.1	6.2	7.0	7.3	11.6	7.0	9.3	17-27
MICRONUTRIENTS (mg/kg dry weight. **g/kg dry weight)											
Fe**	14.9	4.8	5.0	11.1	2.1	6.4	1.4	13.7	5.9	10.6	5
B	136.2	57.7	60.7	101.6	26.1	59.1	77.2	108.6	25.0	127.4	50
Mn	246.8	75.1	94.4	195.8	34.4	109.7	22.9	243.0	98.8	177.3	570-610
Cu	680.6	232.6	257.7	564.9	111.9	356.7	70.2	693.4	357.2	638.5	60
Zn	1390.2	401.7	449.5	1170.5	192.3	683.2	101.7	1327.4	482.9	980.9	500-540
Mo	7.1	2.2	2.4	5.9	1.0	3.2	0.6	7.1	3.3	6.0	5
OTHER (g/kg dry weight)											
TOC	451.5	611.6	430.2	450.6	504.7	518.5	464.8	498.9	333.9	347.9	ND
Na	17.2	13.8	12.6	15.5	15.5	13.0	14.9	15.8	11.8	16.3	ND
RATIOS											
C:N	9.1	9.8	9.8	11.0	10.7	10.0	9.0	9.6	9.9	9.9	3.73
SAR	0.7	1.6	1.2	0.8	3.6	1.2	4.6	0.7	1.1	0.8	ND

***** *Range of organic fertilizers based on composed chicken manure (Bio ganic® Bio ganic crumble®), enriched with kelp and fishmeal (Bio ocean®), enriched with calcium and phosphate (Bio rock®), N = added nutrients, TOC = total organic carbon; SAR = sodium adsorption ratio; ND = not determined*

5.7. Summary

Investigations to determine the variability in AD performance and to identify the most suitable kinetic models to predict cumulative specific CH_4 production of PWWS from four different sources (wineries B-E) and lees from one source (winery F were described in this chapter.

Most PWWS samples showed a high proportion of organic matter (high VS:TS and COD:VS ratios), indicating good potential for AD. The less from winery F consistently had the highest theoretical CH_4 yield, while the PWWS from wineries C and D showed lowest yields, possibly due to factors like mixed pomace in the PWWS from winery C or foreign substances in the PWWS from winery D. Macronutrient levels (TP, TN, NH_4^+-N) were generally within safe ranges, and while most VOA levels were initially above the toxicity threshold for methanogens. Prior work (Chapter 4) suggests that acclimation can mitigate inhibition. Metal concentrations were mostly below inhibitory levels, though Cu and Zn could be inhibitory in the PWWS from some wineries, some essential metabolic co-factors including and Co and Ni appeared deficient across many PWWS samples, suggesting potential benefits from micronutrient supplementation.

Increasing temperature from ambient to 37°C improved maximum CH_4 production and reduced retention time. The addition of a commercial micronutrient blend had varied effects on CH_4 yield which were highly dependent on temperature and PWWS source. At ambient temperatures, nutrient addition improved CH_4 yields for most sludges (by up to 204% for Winery E). At 37°C, the effect was mixed, benefiting some sludges while inhibiting others. The lees from winery F was the exception, showing no benefit from nutrient addition at either temperature.

Based on final characterization of the ADg from PWWS from winery B-E and lees from winery F, the residual TOC in the ADg was higher than values obtained from winery A (Chapter 4), being a good source of carbon if used as fertilizer.

Five kinetic models (Logistic, Cone, MG, First-Order, and C&H) were used to fit the experimental data using Adjusted R^2, AIC, and RMSE to determine the best fit for cumulative CH_4 production. The Logistic, Cone, and C&H models consistently

provided the best fit, accurately describing the lag phase, production rate, and maximum CH_4 generation potential. The first-order and MG models were generally poor predictors. The study found that there is no single "best" model for all cases, instead the most accurate model was highly dependent on the PWWS source, temperature, and nutrients.

In conclusion, the results presented in this chapter demonstrates that PWWS is a highly variable feedstock and understanding the diverse characteristics of PWWS from different sources is crucial for centralized AD plants. Furthermore, micronutrient augmentation and temperature can significantly impact CH_4 yields.

CHAPTER 6: EFFECT OF INOCULUM ACCLIMATION ON ANAEROBIC DIGESTION

6.1. Introduction

The results provided in Chapters 4 and 5 describe the effects of temperature, ISR, nutrients, and source of substrate during AD of PWWS. In addition, the variability in yields experienced during AD of PWWS from different sources was described, as well successful AD at previously established inhibitory concentrations of macro and micronutrients. It was been hypothesised that the inoculum played a pivotal role in the differences obtained during AD of PWWS, specifically: (i) differences in AD efficiencies obtained for experiment 1 and experiment 2 during AD of the same batch of PWWS from winery A under the same conditions, but conducted at different times, (ii) low and variable AD efficiencies in PWWS from different wineries with an inoculum that had been acclimated to PWWS from winery A only. Although it is recommended that MWAS is used as an inoculum for BMP experiments, acclimation to a specific substrate should theoretically enhance the inoculum's efficiency in degrading that same substrate, potentially leading to higher CH_4 yields (Hollinger et al., 2016). Conversely, a mismatch between the acclimation substrate and the test substrate might increase susceptibility to inhibition resulting in reduced AD performance. To validate this hypothesis, investigations on the influence of inoculum type and acclimation on CH_4 yield were conducted as described in this Chapter.

The experiments employed two distinct inocula, acclimated to either PWWS or MWAS for a period exceeding 60 days following the method described in section 3.3, as well as three substrates: PWWS, MWAS and secondary tannery wastewater sludge (STWWS). These were substrates were chosen due to their contrasting characterization profiles.

6.2. Characterization of inoculums and substrates

6.2.1 Organics, solids, macronutrients and theoretical methane yield

Notable similarities in many of the characteristics of PWWS-acclimated inoculum (PWWSi) and MWAS-acclimated inoculum (MWASi) were noted (Table 6-1). This suggests a comparable substrate baseline for microbial community structure and function. For example: C:N ratios (PWWSi = 7.8, MWASi = 7.2) and non-inhibitory concentrations of VOA (PWWSi = 0.6 ± 0.03 g/L, MWASi = 0.5 ± 0.02 g/L), NH_4^+-N (PWWSi = 0.36 ± 0.00 g/L, MWASi = 0.36 ± 0.01 g/L), and SO_4 (PWWSi = 0.13 ± 0.00 g/L, MWASi = 0.11 ± 0.00 g/L), among others. However, there were notable differences between PWWSi and MWASi for other parameters (Table 6-1). The COD (37.0 ± 0.8), TOC (6.2 ± 0.3), TS (36.0 ± 0.1), and VS (18.0 ± 0.1) values measured in the PWWSi were notably higher than those in the MWASi: COD (6.7 ± 0.7), TOC (2.3 ± 0.4), TS (18.2 ± 0.1), and VS (12.1 ± 0.1) (Table 6-1).

The characteristics of the substrates (PWWS, MWAS, and STWWS) varied widely, highlighting diverse potential for AD. The organic fraction of PWWS, measured by VS:TS ratio (0.86), COD (163.0 ± 26.7), TOC (22.7 ± 1.9), and TS (168.6 ± 1.3) and VS (144.8 ± 3.6) was notably higher than the other substrates. This indicates a very rich organic substrate with significant potential for CH_4 generation (Shitophyta et al., 2023; Ruan et al., 2024). Although organic parameters were lower in MWAS: COD (12.9 ± 0.6), TOC (3.9 ± 0.4), TS (24.1 ± 2.4), and VS (19.8 ± 2.0), it still contained considerable organic content compared to PWWS. The high VS:TS ratio for PWWS (0.86) and MWAS (0.82) further highlight their high organic fraction. In contrast, STWWS exhibited the lowest organic content: (TOC 2.1 g/L), COD (10.9 ± 0.9), TOC (2.1 ± 0.1), TS (8.0 ± 0.3), and VS (3.7 ± 0.2), and a low VS:TS ratio (0.46). The NH_4^+-N for all substrates was well below inhibition ranges (<1.5 g/L) (Myszograj et al., 2018). The VOA of PWWS (23.4 ± 1.3 g/L) was above the threshold for VOA inhibition (16.5–18.0 g/L) (Jiang et al., 2018). However, successful AD of PWWS was observed at VOA concentrations as high as 104 g/L when using PWWS-acclimated inoculum as described in Chapter 4.

Table 6-1: Characteristics of acclimated inoculums and substrates (average and standard error of the mean, n =3)

Parameter	PWWSi	MWASi	PWWS	MWAS	STWWS
Elemental CHNS analysis (g/kg dry weight)					
C	31.86 ± 0.34	30.78 ± 0.12	43.97 ± 0.45	39.07 ± 0.95	39.72 ± 0.14
H	4.84 ± 0.10	5.05 ± 0.09	7.16 ± 0.06	7.28 ± 0.07	7.31 ± 0.06
N	4.09 ± 0.05	4.30 ± 0.08	5.42 ± 0.05	6.44 ± 0.06	5.69 ± 0.03
S	0.39 ± 0.04	0.58 ± 0.02	0.23 ± 0.02	0.95 ± 0.09	1.82 ± 0.08
C:N	7.8	7.2	8.1	6.1	7.0
Characterisation (g/L wet weight homogenised sludge, g/L wastewater)					
COD	37.0 ± 0.8	6.7 ± 0.7	163.0 ± 26.7	12.9 ± 0.6	10.9 ± 0.9
TOC	6.2 ± 0.3	2.3 ± 0.4	22.7 ± 1.9	3.9 ± 0.4	2.1 ± 0.1
TP	0.13 ± 0.00	0.25 ± 0.00	0.32 ± 0.04	0.19 ± 0.03	0.03 ± 0.00
TN	3.4 ± 0.2	1.1 ± 0.0	4.6 ± 0.7	1.1 ± 0.02	0.6 ± 0.2
VOA	0.6 ± 0.03	0.5 ± 0.02	23.4 ± 1.3	1.1 ± 0.04	0.5 ± 0.08
HS^-	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
NH_4^+-N	0.36 ± 0.00	0.36 ± 0.01	0.35 ± 0.06	0.20 ± 0.02	0.06 ± 0.01
SO_4	0.13 ± 0.00	0.11 ± 0.00	0.10 ± 0.00	0.09 ± 0.00	0.76 ± 0.03
TS	36.0 ± 0.1	18.2 ± 0.1	168.6 ± 1.3	24.1 ± 2.4	8.0 ± 0.3
VS	18.0 ± 0.1	12.1 ± 0.1	144.8 ± 3.6	19.8 ± 2.0	3.7 ± 0.2
COD:VS	2.06	0.55	1.13	0.65	2.95
VS:TS	0.50	0.66	0.86	0.82	0.46
pH	7.93	7.65	4.60	6.88	7.65

PWWSi = primary winery wastewater sludge acclimated inoculum; MWASi = municipal waste activated sludge acclimated inoculum; PWWS = primary winery wastewater sludge; MWAS = municipal waste activated sludge; STWWS = secondary tannery wastewater sludge; COD = chemical oxygen demand; TOC = total organic carbon; TP = total phosphate, TN = total nitrogen, VOA= volatile organic acids, TS=total solids; VS=volatile solids

The C:N ratios of all the substrates were < 10 (Table 6-1). While these C:N ratios were below the optimal ratio for carbonaceous substrates (20-30), they fall within optimal range for proteinaceous substrates (6-9) (Berhe & Leta, 2019; Parra-Orobio et al., 2016). Despite this, successful AD was achieved with C:N ratios as low as 7.1 when digesting PWWS using PWWS-acclimated inoculum (Chapter 4).

The SO_4 concentration (0.76 ± 0.03 g/L) in the STWW was markedly higher than in the other substrates. This is expected, because sulfur-containing compounds are

commonly used in beamhouse processes, particularly during liming and unhairing. As a result, tannery wastewater often contains elevated levels of SO_4 (0.27-2.40 g/L) and S^{2-} (0.25-0.53 g/L) depending on the process and treatment stage (Mpofu et al., 2023). Elevated SO_4 levels can lead to the production of hydrogen sulfide (H_2S), which is inhibitory to methanogens and can also pose safety and corrosion issues. Studies by Kibangou et al. (2022) found that complete methanogenic inhibition was reached at SO_4 values > 1.96 g/L SO_4 when the inoculum was well acclimated and contained both sulfidogenic and methanogenic communities. Secondly, the TWWS also exhibited notably low macronutrient values, specifically TP (0.03 ± 0.00 g/L), and TN (0.6 ± 0.2 g/L). Based on this combination of low macronutrients, limited organic content, and higher SO_4, STWWS may not be a well-suited substrate for AD, potentially requiring pre-treatment (Mpofu et al., 2023).

Based on the Buswell Equation, the theoretical CH_4 yields at 37^oC were calculated as 964 mLCH_4/gVS, 827 mLCH_4/gVS, 856 mLCH_4/gVS for PWWS, MWAS and STWWS, respectively. Based on the COD:VS, the theoretical CH_4 yields at 37^oC were calculated as 449 mLCH_4/gVS, 258 mLCH_4/gVS, 1171 mLCH_4/gVS for PWWS, MWAS and STWWS, respectively.

6.2.2 Cationic inhibitors and micronutrients

Several key micronutrients were found to be deficient in the substrates, which could potentially impact AD. The concentration of Co was below the analytical detection limit of <2.50 mg/kg in both PWWS and MWAS substrates. This suggests a potential Co deficiency, which could negatively impact AD (Li et al., 2023). The inoculum, however, did contain some Co, particularly the MWASi (5.81 0.34 mg/g or 0.11 ± 0.01 mg/kg Co), suggesting it could mitigate against the deficiencies in the substrates.

The concentrations of Cu, Ni, Na, Zn and Pb in all substrates were well below the IC_{50} values for acetogenic bacteria (350 mg/L Cu, 1300 mg/L Ni, 1500 mg/L Zn, and >5000 mg/L Pb). The concentrations of Na and Ni are below the IC_{50} values for methanogens (5.6–53 g/L Na, 35 mg/L Ni, and 7,7 mg/L Zn) (Chen et al., 2008; Czatzkowska et al., 2020; Mudhoo & Kumar, 2013).

The PWWS and STWWS contained 0.53 ± 0.02 g/L Fe and 29.86 ± 0.70 g/L Fe, as well as 24.27 ± 0.32 g/L Zn and 24.46 ± 1.24 g/L Zn, respectively. High Fe and Zn levels can contribute positively to sulfide precipitation and reduce metal toxicity but may also reduce the bioavailability of other essential trace metals (e.g., Ni, Co) via co-precipitation or adsorption (Myszograj et al., 2018). Aluminium (Al) was also highest in PWWS (1.31 ± 0.04 g/L) and STWWS (0.24 ± 0.01 g/L). Excessive Al can also interfere with bioavailability other minor and major nutrients and microbial activity at very high levels.

Calcium (Ca) and Mg concentrations were variable, with Mg and Ca being higher in MWAS samples (0.24 ± 0.01 g/L Mg; 0.46 ± 0.02 g/L Ca) than in PWWS (0.22 ± 0.01 g/L Mg; 0.41 ± 0.05 g/L Ca) and STWWS (0.02 ± 0.00 g/L Mg; 0.03 ± 0.00 g/L Ca). Both cations are important for cell wall stability and granule formation (Mills et al., 2024). Na was above the required concentrations for ATP formation (0.23-0.35 g/L) for MWASi (1.50 ± 0.18 g/L) and STWWS (0.33 ± 0.02 g/kg). The optimal concentration of Ni is 2-4 mg/kg (Khan et al., 2021) and all substrates had insufficient amounts (1.73 ± 0.37 mg/L, 0.94 ± 0.06 mg/L, and 0.20 ± 0.00 mg/L for PWWS, MWAS and STWWS, respectively). The Cu and Al concentrations of PWWS (35.04 ± 2.21 mg/L Cu; 1.31 ± 0.04 mg/L Al) exceeded the methanogenic IC_{50} (8.9–20.7 mg/L Cu; 1 g/L Al) and could be detrimental to efficient AD (Chen et al., 2008; Czatzkowska et al., 2020; Mudhoo & Kumar, 2013).

The high concentrations of metals like Fe in the STWWS could theoretically promote speciation, co-precipitation, adsorption, and ion substitution, reducing the bioavailability of some essential micro and macronutrients (Myszograj et al., 2018). As discussed in Chapters 4 and 5, high concentration of metals does not necessarily inhibit AD of PWWS because inhibitory effects depend on bioavailability, microbial interactions, synergistic relationships, and other biochemical processes within AD systems.

Table 6-2: Elemental cation concentrations of acclimated inoculums and substrates (average and standard error of the mean, n =3)

	PWWSi	MWASi	PWWS	MWAS	STWWS
Major elemental parameters (g/L)					
Fe	0.22 ± 0.00	0.15 ± 0.00	0.53 ± 0.02	0.14 ± 0.01	0.24 ± 0.01
Al	0.50 ± 0.01	0.24 ± 0.01	1.31 ± 0.04	0.16 ± 0.01	0.24 ± 0.01
Ca	0.15 ± 0.00	0.34 ± 0.02	0.41 ± 0.05	0.46 ± 0.02	0.03 ± 0.00
K	0.92 ± 0.10	0.37 ± 0.04	3.29 ± 0.11	0.26 ± 0.03	0.01 ± 0.00
Mg	0.07 ± 0.00	0.11 ± 0.00	0.22 ± 0.01	0.24 ± 0.01	0.02 ± 0.00
Na	0.20 ± 0.01	1.50 ± 0.18	0.03 ± 0.01	0.21 ± 0.03	0.33 ± 0.02
P	0.14 ± 0.00	0.21 ± 0.01	1.01 ± 0.03	0.70 ± 0.02	0.04 ± 0.00
Si	0.10 ± 0.00	0.05 ± 0.00	0.40 ± 0.01	0.05 ± 0.00	0.02 ± 0.00
Minor elemental parameters (mg/L)					
B	1.34 ± 0.01	0.94 ± 0.02	5.03 ± 0.16	1.21 ± 0.04	0.06 ± 0.00
Mn	1.07 ± 0.39	4.63 ± 0.28	BDL	2.61 ± 0.09	7.77 ± 0.26
Co	BDL	0.11 ± 0.01	BDL	BDL	0.03 ± 0.00
Ni	0.87 ± 0.04	0.68 ± 0.00	1.73 ± 0.37	0.94 ± 0.06	0.20 ± 0.00
Cu	8.37 ± 0.12	7.06 ± 0.16	35.04 ± 2.21	3.69 ± 0.12	0.69 ± 0.10
Zn	15.61 ± 0.26	15.62 ± 0.48	24.27 ± 0.32	24.46 ± 1.24	5.98 ± 0.13
Sr	1.10 ± 0.00	2.16 ± 0.08	3.00 ± 0.17	3.98 ± 0.14	0.82 ± 0.01
Pb	1.88 ± 0.11	0.64 ± 0.01	2.16 ± 0.06	0.52 ± 0.03	0.26 ± 0.01
Ba	4.58 ± 0.29	1.90 ± 0.08	8.39 ± 0.36	2.74 ± 0.13	0.83 ± 0.02
Cr	2.86 ± 0.19	11.51 ± 0.27	2.56 ± 0.78	1.29 ± 0.11	1.72 ± 0.03

PWWSi = primary winery wastewater sludge acclimated inoculum; MWASi = municipal waste activated sludge acclimated inoculum; PWWS = primary winery wastewater sludge; MWAS = municipal waste activated sludge; STWWS = secondary tannery wastewater sludge; BDL = Below detection limit

6.3. Cross inoculation using acclimated inocula

6.3.1 Introduction

The AD of PWWS, MWAS and STWWS using PWWSi and MWASi was conducted in using the methods described in Chapter 3 and applying an ISR of 4 based on the optimum from the initial study with PWWS from winery A (Chapter 4). The substrates were sourced locally, the PWWS from winery A; the MWAS from a municipal wastewater treatment plant in the Western Cape; and the STWWS from a tannery wastewater treatment plant. Two inoculums were prepared using PWWS and MWAS. These inoculums were acclimated over a period of 60 days with periodic feeding as described in Chapter 3. Following the acclimation period, the acclimated inocula were

then cross inoculated with the three substrates (PWWS, MWAS, STWWS) at 37°C and the biogas and CH_4 production was measured. PWWS-acclimated inoculum (PWWSi) was fed with PWWS, MWAS, and TWAS in 500 mL borosilicate reactors as described in Chapter 3. A parallel setup was established for the MWASi, which was also subjected to the same cross-inoculation regimen. Three replicates were performed for each inoculum-substrate combination. Additionally, each inoculum assay included an acetate control, an inoculum control as well as a microcrystalline cellulose (MCC) control. A complete setup for the BMP assay is given in Table A-5 of Appendix A. This study investigated the effects of inoculum acclimation on specific CH_4 yield.

6.3.2 Biomethane yields

The reactors containing PWWSi showed methanogenic activity when fed with STWWS, PWWS, acetate, and MCC (Table 6-3). The CH_4 yields obtained with STWWS (5.7 $mLCH_4/gVS$) and PWWS (49.8 $mLCH_4/gVS$) amounted to only 5.2% and 0.7% of the theoretical yield calculated using Buswells' equation. Notably, no CH_4 was produced in the reactors containing MWAS which is generally considered a good substrate for AD.

Table 6-3: Corrected cumulative specific methane yields for MWAS, STWWS and PWWS using PWWSi and MWASi at 37°C

Substrate	Inoculum	
	PWWSi ($mLCH_4/gVS_{added}$)	**MWASi ($mLCH_4/gVS_{added}$)**
MWAS	0	30.4
STWWS	5.7	0
PWWS	49.8	0
Acetate	125.4	0
MCC	0	7.1

PWWSi = primary winery wastewater sludge acclimated inoculum; MWASi = municipal waste activated sludge acclimated inoculum; PWWS = primary winery wastewater sludge; MWAS = municipal waste activated sludge; STWWS = secondary tannery wastewater sludge; MCC = microcrystalline cellulose

During AD, hydrolysis is usually the rate-limiting step, with acetate, which is the main precursor for methanogenesis, being formed by acidogenic bacteria from the products of hydrolysis (Anukam et al., 2019). Microcrystalline cellulose is a positive control substrate used to validate inoculum activity and involves all stages of AD including hydrolysis (Holliger et al., 2016). The acetate control ensures that the methanogens are active, irrespective of hydrolytic and acidogenic efficiency (Ma et al., 2013). The CH_4 yield obtained with acetate (125.4 $mLCH_4/gVS$) was notably higher than the yields from STWWS and MWAS (Table 6-3). Since the CH_4 generation using the acetate control proves that the methanogens were active, and the MCC control did not produce any CH_4, the result implies that the efficiency of AD of STWWS and MWAS was restricted due to limited hydrolysis (Ma et al., 2013). The results obtained with the PWWSi suggest that AM and HM were the dominant methanogenic taxa responsible for CH_4 production in this inoculum. Acetoclastic conversion to CH_4 is achieved by AM such as *Methanothrix*, *Methanosarcinaceae* or *Methanosaetaceae* (Karakashev et al., 2006; Liang et al., 2024). The second mechanism for conversion of acetate to CH_4 involves acetate-oxidizing bacteria, such as *Clostridium* spp., whose products are converted to CH_4 by HM such as *Methanomicrobiales* or *Methanobacteriales* (Liang et al., 2024; Karakashev et al., 2006).

In contrast to PWWSi, the methanogenic activity was more focussed when MWASi was used as an inoculum, producing 30.4 $mLCH_4/gVS$ in reactors fed with MWAS and 7.1 $mLCH_4/gVS$ in reactors fed with MCC, but with no CH_4 being generated in reactors containing other substrates, including acetate. This suggests that in contrast to PWWSi, MWASi was dominated by MM and possibly HM, not AM.

Anaerobic digestion of STWWS was particularly challenging. It has been shown that successful AD of STWWS can be achieved with a well-acclimated inoculum, and efficiencies can be improved by co-digestion with other substrates (Mpofu et al., 2019). It has also been shown that AD of tannery effluent is possible, and that efficiency can be improved by pre-treatment to reduce HS^- (Horn et al., 2022; Horn, 2020). Horne et al. (2022) obtained 214 mL $CH_4/g\ COD_{consumed}$ from tannery effluent with an acclimated inoculum but were unable to obtain biogas using an inoculum from a highly active full-scale AD plant treating dairy effluent (Horn, 2020). This limited digestion of STWWS by PWWSi and lack of digestion by MWASi in this study is therefore not surprising. The fact that some CH_4 was produced with PWWSi could be attributed to the historical

exposure of PWWSi to STWW because the original inoculum included a variety of sources, including sludge from a digester treating STWW. It appears that both substrate types (PWWS and STWWS) are selective for different methanogenic taxa than MWAS. This is currently being investigated using amplicon sequencing.

Studies by Hupfauf et al. (2018) found that factors such as temperature and feedstock composition play roles in regulating the balance between the methanogenic pathways. The authors found that no significant difference in the amount of HM in reactors at psychrophilic temperatures when compared to mesophilic temperatures, but found higher HM contributions at thermophilic conditions, based on 16S rRNA amplicon sequencing analysis (Hupfauf et al., 2018; Xu et al., 2017). In addition, they found that the contribution of HM varied widely within reactors fed with the different substrate for either food waste, industrial wastewater, and municipal wastewater (Oliveira et al., 2025). This implies that contributions from methanogenic pathways may vary over time and are likely to change based on operating temperature and substrate.

The results of this study confirmed that acclimation of inocula is important for optimal AD. Without acclimation, inoculums performed poorly or did not produce any CH_4. Additionally, acclimation was able to assist in overcoming possible inhibition and deficiencies as discussed in Section 6.2 for PWWS and MWAS.

6.4. Summary

The effect of inoculum acclimation using two inocula (PWWSi and MWASi), and three substrates (PWWS, MWAS, and STWWS) are described in this chapter, which covers the characterization of these inocula and substrates and BMP assays of each inoculum and substrate combination. The results indicated that PWWS produced the highest specific CH_4 yields when using PWWSi and MWAS produced the highest specific CH_4 yields when using MWASi. This study also highlighted differences in the methanogenic pathways preferred by each inoculum, with PWWSi likely being dominated by AM and HM, while MWASi was likely dominated by MM. These findings underscore the critical importance of inoculum acclimation for achieving optimal AD and overcoming potential substrate inhibitions.

CHAPTER 7: CONCLUSION,RECOMMENDATIONS AND FUTURE WORK

7.1. Introduction

This research explored AD as a waste management option for PWWS. It includes characterization studies which highlight the intra- and inter-site variability of PWWS, as well as investigations into the effect of inoculum acclimation, PWWS source, operational conditions (ISR and operating temperature), and micronutrient supplementation on AD performance.

7.2. Conclusions

The PWWS quality from different wineries varied greatly in organic content and nutrient levels. The differences in character, and ultimately the CH_4 potential, was due to factors such as seasonal variations and winery specific practices. Some wineries, for instance, added pomace, leaves, stems, and other organic wastes into their WWW streams which contributed to the variations observed. The PWWS from winery A and B had the highest theoretical CH_4 yields (703 $mLCH_4/gVS_{added}$ at 37^oC). In contrast, PWWS from Winery C and D had lower yields, likely due to high concentrations of inhibitory substances and poor acclimation of the inoculum.

The study confirmed that acclimation of the inoculum has a significant effect on AD efficiency. Higher CH_4 yields were achieved when PWWSi or MWASi was paired with their acclimated substrate. Mismatched inocula-substrate pairs resulted in poor performance and increased risk to inhibition. Methane yields from PWWS increased from 0 to 49 $mLCH_4/gVS_{added}$ when the inoculum was switched from MWASi to PWWSi. Poor acclimation could have led to the poor AD performance of PWWS form wineries C and D.

While an ISR of 4 was found to be optimal for maximizing CH_4 yields, an ISR of 3 produced comparable results. Therefore, an ISR of 3 was identified as a more practical ratio for large-scale application, because it allows for the treatment of larger volumes of sludge within the same reactor capacity.

Temperature played an important role in improving the efficiency of AD. Increasing temperature from ambient to 37°C improved CH_4 yields and reduced retention times across all substrates. Nutrient addition had mixed effects on CH_4 yield depending on the operating temperature and PWWS source. Adding nutrients increased CH_4 production at ambient temperature. Under mesophilic conditions, addition of nutrients improved the CH_4 yield in only 50% of the PWWS studied. This inconsistency may stem from the use of a commercially produced nutrient blend across all substrates, regardless of the nutrient profile. The nutrient blend did not address specific nutrient deficiencies in the PWWS and may have contributed to inhibition, particularly at higher temperatures due to the increased bioavailability of certain micro and macro nutrients. Therefore, nutrient supplementation during AD of PWWS should be site-specific and aimed at addressing deficiencies.

This study also found that, although increasing operating temperature from ambient temperature to 37°C increased CH_4 yields, the energy expenditure does not justify the heating for increased yields below ~30 mL CH_4/gVS_{added}. Instead, smaller wineries could benefit from AD at ambient temperatures, especially when combined with simple, low-cost strategies such as insulating digesters and using black-coloured digestors to absorb solar heat.

Various kinetic models were tested for their ability to predict CH_4 production from PWWS and lees. The Cone and Logistic model had the best fits at ambient temperature. At 37°C, the C&H, cone and logistic models provided the best fit.

Anaerobic digestate was characterized and found to contain high levels of residual organic carbon when compared to commercial fertilizers. This implies that as a fertilizer, ADg could provide a source of C, thus improving the humic material and water retention capacity of soils. Additionally, the ADg could benefit soils by providing nutrients and improving the microbial activity.

Wineries in the Western Cape may benefit from a central AD plant. This will offer wineries an opportunity to cut costs (and other requirements like skills and personal) associated with landfilling, as well and building and maintaining an AD facility. A more ‘blended’ substrate could be advantageous to AD, and co-digestion with other agricultural wastes from the area is also a possibility. To achieve this, further studies involving co-digestion of PWWS from different wineries would have to be conducted.

Furthermore, a robust, well acclimated inoculum is required to handle the seasonal and site-specific variations in the PWWS.

7.3. Recommendations and limitations

Developing and maintaining a well-acclimated inoculum is critical to maximize CH_4 production during AD. Cross-inoculation strategies can be employed to mitigate inhibition and improve microbial resilience.

Periodic characterization of PWWS is required to identify variances in composition, to monitor known inhibitors, and to identify nutrient deficiencies before digestion. Adjustments to nutrient supplementation may be necessary to target deficiencies in PWWS identified through characterization.

7.3.1 Future research

To design a full-scale AD reactor for the treatment of PWWS and other winery waste streams, and to gain further insight into the biochemistry and physiochemistry of AD of PWWS, further investigations are recommended:

- Investigate the microbial community in greater depth to understand metabolic pathways dominating under different conditions.
- Explore pretreatment methods to reduce inhibition and improve nutrient availability.
- Investigate co-digestion of PWWS from different sources, and with other agricultural wastes to identify any benefits or shortfalls.
- Conduct an in-depth techno economic feasibility study to ascertain whether it is viable for local wineries to implement AD for production of biogas and ADg from PWWS.
- Quantify the amount of PWWS produced in the Western Cape to gain valuable insight into scaling PWWS treatment plants and to provide data for the techno-economic study.
- Investigate the use of ADg of PWWS as fertilizer using field experiments to understand the benefits and/or long-term effects on plants and soil health.

7.3.2 Limitations

Despite the findings presented in this study, several limitations should be acknowledged:

- The study was conducted at lab-scale reactors with maximum volume of 2L. Therefore, the findings do not account for the dynamics of full-scale, continuous-flow systems.
- The study sites were limited to the Western Cape. These results may vary in different regions and seasons due to temperature differences.
- The micronutrients used may not be suitable for different regions due the unique mineral profile of soil and water in that area.

This study highlights the need of a tailored approach to AD of PWWS. Considering factors like inoculum acclimation, substrate variability, operational conditions, and nutrient management. It is envisaged that the findings and recommendations of this study will assist in the adoption of AD in wineries. The adoption of AD will assist in diverting organic waste from landfills, reducing costs, and contributing to sustainable practices within the winery industry.

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APPENDIX A

Table A-1: Biomethane potential assay setup for post-crush season primary winery wastewater sludge (experiment 1)

Run	Inoculum (L)	Substrate (L)	Water (L)	N	ISR	Description
1	1,1	0,15	0,75	Yes	2	ISR2 + N
2	1,1	0,15	0,75	No	2	ISR2
3	1,1	0,15	0,75	Yes	2	ISR2 + N
4	1,1	0,15	0,75	No	2	ISR2
5	1,1	0,1	0,8	Yes	3	ISR3 + N
6	1,1	0,1	0,8	No	3	ISR3
7	1,1	0,1	0,8	Yes	3	ISR3 + N
8	1,1	0,1	0,8	No	3	ISR3
9	1,1	0,074	0,826	Yes	4	ISR4 + N
10	1,1	0,074	0,826	No	4	ISR4
11	1,1	0,074	0,826	Yes	4	ISR4 + N
12	1,1	0,074	0,826	No	4	ISR4
13	1,1	0	0,9	Yes	-	Inoculum control + N
14	1,1	0	0,9	No	-	Inoculum control
15	1,1	0	0,9	Yes	-	Inoculum control + N
16	1,1	0	0,9	No	-	Inoculum control
17	0	0,7	1,3	Yes	-	Substrate control + N
18	0	0,7	1,3	No	-	Substrate control
19	0	0,7	1,3	Yes	-	Substrate control + N
20	0	0,7	1,3	No	-	Substrate control
21	1,1	Ac	0,9	Yes	-	Acetate control + N
22	1,1	Ac	0,9	No	-	Acetate control

N = 5 mg/L Cu, 20 mg/L Ni, 5 mg/L Co; ISR = inoculum to substrate ratio; Ac = 3.6 g acetate

Table A-2: Biomethane potential assay setup for crush season primary winery wastewater sludge (experiment 1)

Run	Inoculum (L)	Substrate (L)	Water (L)	N	ISR	Description
1	1.25	0.7	0.05	Yes	2	ISR2 + N
2	1.25	0.7	0.05	No	2	ISR2
3	1.25	0.7	0.05	Yes	2	ISR2 + N
4	1.25	0.7	0.05	No	2	ISR2
5	1.25	0.45	0.3	Yes	3	ISR3 + N
6	1.25	0.45	0.3	No	3	ISR3
7	1.25	0.45	0.3	Yes	3	ISR3 + N
8	1.25	0.45	0.3	No	3	ISR3
9	1.25	0.34	0.41	Yes	4	ISR4 + N
10	1.25	0.34	0.41	No	4	ISR4
11	1.25	0.34	0.41	Yes	4	ISR4 + N
12	1.25	0.34	0.41	No	4	ISR4
13	1.25	0	0.75	Yes	-	Inoculum control + N
14	1.25	0	0.75	No	-	Inoculum control
15	1.25	0	0.75	Yes	-	Inoculum control + N
16	1.25	0	0.75	No	-	Inoculum control
17	0	0.7	1.3	Yes	-	Substrate control + N
18	0	0.7	1.3	No	-	Substrate control
19	0	0.7	1.3	Yes	-	Substrate control + N
20	0	0.7	1.3	No	-	Substrate control
21	1.25	Ac	0.75	Yes	-	Acetate control + N
22	1.25	Ac	0.75	No	-	Acetate control

N = 5 mg/L Cu, 20 mg/L Ni, 5 mg/L Co; ISR = inoculum to substrate ratio; Ac = 3.6 g acetate

Table A-3: Biomethane potential assay setup for crush and post crush primary winery waster sludge (experiment 2)

Run	Inoculum (L)	Substrate (L)	Water (L)	N	ISR	Temperature	Description
1	0.8	0.2	0	Yes	4	37°C	ISR4 + N
2	0.8	0.2	0	Yes	4	37°C	ISR4 + N
3	0.8	0	0.2	Yes	-	37°C	Inoculum control + N
4	0.8	0	0.2	Yes	-	37°C	Inoculum control + N
5	0.8	0*	0	Yes	-	37°C	Substrate control + N
6	0.8	0*	0.8	Yes	-	37°C	Substrate control + N
7	0.8	0.2	0.8	Yes	4	RT	ISR4 + N
8	0.8	0.2	0.8	Yes	4	RT	ISR4 + N
9	0.8	0	0.826	Yes	-	RT	Inoculum control + N
10	0.8	0	0.826	Yes	-	RT	Inoculum control + N
11	0.8	0*	0.826	Yes	-	RT	Substrate control + N
12	0.8	0*	0.826	Yes	-	RT	Substrate control + N

N = 5 mg/L Cu, 20 mg/L Ni, 5 mg/L Co; ISR = inoculum to substrate ratio; RT = ambient room temperature

A-4: Biomethane potential assay setup for primary winery waster sludge from winery B-E and lees from winery F (experiment 3)

Runs	I (L)	Substrate type	Substrate source	S (L)	Water (L)	Temperature	N	ISR
1, 2	0.7	PWWS	Winery B	0.09	0.21	37°C	Yes	4
3, 4	0.7	PWWS	Winery B	0.09	0.21	37°C	No	4
5, 6	0.7	PWWS	Winery C	0.08	0.22	37°C	Yes	4
7, 8	0.7	PWWS	Winery C	0.08	0.22	37°C	No	4
9, 10	0.7	PWWS	Winery D	0.29	0.01	37°C	Yes	4
11, 12	0.7	PWWS	Winery D	0.29	0.01	37°C	No	4
13, 14	0.7	PWWS	Winery E	0.06	0.24	37°C	Yes	4
15, 16	0.7	PWWS	Winery E	0.06	0.24	37°C	No	4
17, 18	0.7	Lees	Winery F	0.14	0.16	37°C	Yes	4
19, 20	0.7	Lees	Winery F	0.14	0.16	37°C	No	4
21, 22	0.7	-	-	0	0.3	37°C	Yes	-
23, 24	0.7	-	-	0	0.3	37°C	No	-
25, 26	0.7	PWWS	Winery B	0.09	0.21	RT	Yes	4
27, 28	0.7	PWWS	Winery B	0.09	0.21	RT	No	4
29, 30	0.7	PWWS	Winery C	0.08	0.22	RT	Yes	4
31, 32	0.7	PWWS	Winery C	0.08	0.22	RT	No	4
33, 34	0.7	PWWS	Winery D	0.29	0.01	RT	Yes	4
35, 36	0.7	PWWS	Winery D	0.29	0.01	RT	No	4
37, 38	0.7	PWWS	Winery E	0.06	0.24	RT	Yes	4
39, 40	0.7	PWWS	Winery E	0.06	0.24	RT	No	4
41, 42	0.7	Lees	Winery F	0.14	0.16	RT	Yes	4
43, 44	0.7	Lees	Winery F	0.14	0.16	RT	No	4
45, 46	0.7	-	-	0	0.3	RT	Yes	-
47, 48	0.7	-	-	0	0.3	RT	No	-

PWWS = primary winery wastewater sludge acclimated inoculum; I = inoculum volume; S = substrate volume; N = Bionetix® Micro 14 nutrient blend

Table A-5: Initial and final characterisation of biomethane potential assay setup for primary winery waster sludge from winery B-E and lees from winery F (experiment 3)

Runs	i-COD (g/L)	f-COD (g/L)	i-VOA (g/L)	f-VOA (g/L)	i-TOC (g/L)	f-TOC (g/L)	i-NH_4^+-N (g/L)	f- NH_4^+-N (g/L)	i-pH	f-pH
1, 2	47.3	45.9	9.0	4.6	7.7	7.2	0.5	0.6	7	8.4
3, 4	47.3	38.1	9.0	4.0	7.7	6.2	0.5	0.7	7	8.1
5, 6	37.8	36.2	7.3	8.6	7.0	6.6	0.5	0.7	7	8.0
7, 8	37.8	32.7	7.3	6.5	7.0	6.3	0.5	0.7	7	8.1
9, 10	42.1	34.2	7.1	6.6	7.9	7.3	0.5	0.7	7	8.0
11, 12	42.1	37.0	7.1	6.3	7.9	7.2	0.5	0.7	7	8.0
13, 14	43.4	41.0	9.0	7.8	7.2	6.8	0.5	0.6	7	8.0
15, 16	43.4	40.2	9.0	7.2	7.2	6.5	0.5	0.6	7	8.0
17, 18	62.4	49.2	8.5	3.1	7.3	4.4	0.5	0.4	7	8.2
19, 20	62.4	49.7	8.5	4.0	7.3	4.8	0.5	0.4	7	8.0
25, 26	47.3	41.5	9.0	4.4	7.7	8.4	0.5	0.6	7	8.2
27, 28	47.3	40.5	9.0	7.3	7.7	6.5	0.5	0.6	7	8.0
29, 30	37.8	38.3	7.3	9.0	7.0	6.5	0.5	0.7	7	8.0
31, 32	37.8	38.9	7.3	7.2	7.0	6.5	0.5	0.7	7	7.8
33, 34	42.1	38.1	7.1	9.1	7.9	7.5	0.5	0.7	7	8.0
35, 36	42.1	38.1	7.1	6.6	7.9	7.5	0.5	0.7	7	8.0
37, 38	43.4	43.3	9.0	8.4	7.2	7.3	0.5	0.7	7	8.0
39, 40	43.4	41.0	9.0	7.6	7.2	6.9	0.5	0.7	7	7.8
41, 42	62.4	48.8	8.5	4.3	7.3	4.9	0.5	0.5	7	7.9
43, 44	62.4	51.1	8.5	3.6	7.3	5.0	0.5	0.6	7	7.8

COD = chemical oxygen demand; VOA = volatile organic acids; TOC = total organic carbon; i =initial; f = final

Table A-6: Biomethane potential assay setup for cross inoculation using primary winery wastewater sludge acclimated inoculum and municipal waste activated sludge acclimated inoculum (experiment 4)

Run	Inoculum	Inoculum volume (L)	Substrate	Substrate (L)	Water (L)	ISR
1	PWWSi	0.35	MWAS	0.08	0.07	4
2	PWWSi	0.35	MWAS	0.08	0.07	4
3	PWWSi	0.35	MWAS	0.08	0.07	4
4	PWWSi	0.22	STWWS	0.27	0.01	4
5	PWWSi	0.22	STWWS	0.27	0.01	4
6	PWWSi	0.22	STWWS	0.27	0.01	4
7	PWWSi	0.4	PWWS	0.01	0.09	4
8	PWWSi	0.4	PWWS	0.01	0.09	4
9	PWWSi	0.4	PWWS	0.01	0.09	4
10	PWWSi	0.4	-	0	0.10	-
11	PWWSi	0.4	AC	AC*	0.10	-
12	PWWSi	0.4	MCC	MCC*	0.10	4
13	MWASi	0.43	MWAS	0.07	0.00	4
14	MWASi	0.43	MWAS	0.07	0.00	4
15	MWASi	0.43	MWAS	0.07	0.00	4
16	MWASi	0.27	STWWS	0.22	0.01	4
17	MWASi	0.27	STWWS	0.22	0.01	4
18	MWASi	0.27	STWWS	0.22	0.01	4
19	MWASi	0.48	PWWS	0.01	0.01	4
20	MWASi	0.48	PWWS	0.01	0.01	4
21	MWASi	0.48	PWWS	0.01	0.01	4
22	MWASi	0.48	-	0	0.02	-
23	MWASi	0.48	AC	AC*	0.02	-
24	MWASi	0.48	MCC	MCC**	0.02	4

PWWSi = primary winery wastewater sludge acclimated inoculum; MWASi = municipal waste activated sludge acclimated inoculum; PWWS = primary winery wastewater sludge; MWAS = municipal waste activated sludge; STWWS = secondary tannery wastewater sludge, Ac = acetate, Ac* = 0.9 g acetate, MCC = microcrystalline cellulose; MCC* = 1.8 g microcrystalline cellulose; MCC** = 1.45 g microcrystalline cellulose