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Investigating the potential of succulent plants to produce Volatile Fatty Acids

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Thesis submitted in fulfilment of the requirements for the degree of Master of Natural Resource Management at the Namibia University of Science and Technology

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I, Absalom Iyeleka Shitalangaho, hereby declare that the work contained in the thesis entitled:

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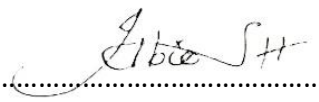
Signature:  Date: 30 January 2024

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List of Acronyms

AAD:	arrested anaerobic digestion
AD:	anaerobic digestion
CAM:	Crassulacean Acid Metabolism
DMC:	dimethyl carbonate
FAO:	Food and Agriculture Organization
FAMEs:	fatty acid methyl esters
GC-MS:	Gas Chromatography-Mass Spectrometry
HCl:	Hydrochloric Acid
$KHSO_4$:	potassium bisulfate
LN2:	Liquid Nitrogen
NCE:	Namibian Chamber of Environment
NIST:	National Institute of Standards and Technology
NUST:	Namibia University of Science and Technology
OLR:	organic loading rate
PET:	Terephthalate
PHAs:	polyhydroxyalkanoates
R-squared:	coefficient of determination
RT:	retention Time (hydraulic)
SRT:	substrate retention time
TPA:	terephthalic acid
VFAs:	volatile fatty acids
VS:	volatile solid

sTVFA: subtracted total volatile fatty acid

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Dedication

This thesis is dedicated to my namesake, Abie Amadhila for the name we share and cherish. To the parents of Abie, my closest friends, who have belief in me and named their son after me, this thesis will be a symbol of an unwavering connection that bridges the past and the future. With heartfelt gratitude, this work stands as a testament to the impact of your compassion and our commitment in the pursuit success.

Abstract

This research investigates the anaerobic digestion of succulent biomass grown in Namibia, focusing on *Portulacaria afra* and *Euphorbia mauritanica* as feedstock, to elucidate the volatile fatty acid (VFA) production dynamics under varying pH and temperature conditions. Employing Gas Chromatography-Mass Spectrometry (GC-MS) for VFA analysis, a comprehensive methodology involved substrate and inoculum collection, arrested anaerobic digestion (AAD), and analytical procedures. A relationship between concentration and GC-MS response was established by the calibration curve for standard VFAs, enabling accurate quantification in unknown AD samples. In *Portulacaria afra*, the optimal condition of 37 °C and pH 4 generated an estimated maximum VFA concentration of approximately 0.25 g of VFAs/g of dry *P. afra*. Conversely, *E. mauritanica*, under the same conditions, yielded an estimated VFA concentration of around 0.22 g of VFAs/g of dry *E. mauritanica*. The findings delineate distinct temperature and pH-dependent trends in VFA production for each species, providing crucial insights into optimal conditions and facilitating the estimation of biomass-to-VFA conversion rates. The optimal conditions for VFA production from *P. afra* and *E. mauritanica*, as determined in this study, align with similar trends reported in recent research, yielding above 5 g/L VFAs concentration. This research significantly contributes valuable data for refining bio-economical strategies focusing on succulent biomass, with potential applications extending to biofuel production or other VFA-based bioproducts.

Keywords:

Anaerobic digestion, Succulent biomass, Volatile fatty acids (VFAs), Gas Chromatography-Mass Spectrometry (GC-MS), *Portulacaria afra*, *Euphorbia mauritanica*

Chapter 1: Introduction

1.1 Background

In the context of a rapidly growing global population, pre-COVID-19 estimations pointed to a yearly increase of 1.1% (Nations 2019), which poses a significant challenge of overconsumption leading to the depletion of natural resources and environmental degradation (Ali *et al.* 2021, Wilmoth *et al.* 2022). Especially the depletion of fossil resources and the environmental consequences linked to the combustion of fossil fuel derivatives needs to be urgently addressed. To counteract these challenges, the quest for renewable energy sources garnered worldwide attention as the world moved toward sustainable practices and a greener economic model, all while addressing the pressing issue of climate change.

The endeavour of renewable energy research focuses on multiple approaches, encompassing various techniques like biogas generation through anaerobic digestion. Anaerobic digestion, a biological process traditionally known for its role in breaking down organic materials (such as carbohydrates, proteins, fats, and oils) within oxygen-deprived environments, has become a focal point in this pursuit. This process hinges on the activity of anaerobic microbes, which thrive in the absence of free oxygen radicals (O_2) to derive their nutrition from this breakdown process. A by-product of this activity is biogas, primarily composed of methane (CH_4), carbon dioxide (CO_2), and hydrogen sulfide (H_2S). This process happens through a sequence of events, commencing with fermentative bacteria that initiate the process, followed by acetogens and methanogens that progressively play pivotal roles (Senés-Guerrero *et al.* 2019).

By harnessing the capabilities of these microbes, the process can be emulated within a controlled environment referred to as a digester/reactor. This controlled setup enables the digestion of biomass to yield biogas, also recognized as biomethane. The residual output, termed slurry or digestate, comprises recycled nutrients that can be repurposed as a biofertilizer. Particularly, biomethane is a high-calorific energy compound boasting an approximate energy content of 39.8 MJ m^{-3}, equivalent to 11.06 kWh m^{-3} of electricity (Mitiku and Wubu 2018). Given the proper enhancements, biomethane finds applications ranging from vehicular fuel to cooking and heating.

Despite the promising potential of biogas production, economic hurdles are evident, and the viability of biogas as a competitor to conventional fossil fuels remains contingent on incentives (Lukitawesa *et al.* 2020). In addition to economic and distribution constraints, production constraints can be attributed to factors such as prolonged retention times, a complex interaction between pH, temperature, substrate composition, an accumulation of volatile fatty acids, difficulties to optimise organic loading rates, imbalances in carbon-to-nitrogen ratios, and the necessity for sizable reactor volumes to facilitate comprehensive metabolic reactions (Vo *et al.* 2018, Ramos-Suarez *et al.* 2021).

Anaerobic digestion can, however, result in a multitude of by-products generated through anaerobic digestion, which aligns with the principles of a green economy and thus makes this process even more attractive. One common application of residual sludge is as a valuable fertilizer. Other applications focus on the use of intermediate metabolites, such as volatile fatty acids (VFAs), that emerge during the anaerobic digestion process. These VFAs, formed from the biological conversion of lipids via microbial action in the acidogenic fermentation stage, have diverse industrial utilities. These include the creation of bioplastics, biodiesel, biohydrogen, consumables like food and cosmetics, textiles, bioenergy, pharmaceuticals, and even wastewater purification (Alok *et al.* 2016, Bedaso 2019).

Crucially, the feasibility of anaerobic digestion centres around the quantity of organic waste available—more waste equates to increased output. However, in nations like Namibia, where a sparse population coexists with a substantial poverty rate (42%) (Ashipala 2014), the supply of waste suitable for commercial anaerobic digestion is limited. To circumvent this challenge, the cultivation of succulent plants in regions with arid climates and scarce water resources offers a sustainable approach (Hawa *et al.* 2019). Several succulent plants exhibit remarkable resilience in dry conditions by employing Crassulacean Acid Metabolism (CAM) and thus present an opportunity to provide the essential green biomass for anaerobic digestion (Lueangwattanapong *et al.* 2020).

In a collaborative initiative involving the Namibia University of Science and Technology (NUST), Namibian Chamber of Environment (NCE), Otjikoto B2Gold, and Oxford University, the Succulent Bio-Economy Project has been established. The project's central goal revolves around exploring the cultivation of succulent plants within arid landscapes, in order to generate biomass suitable for diverse applications,

including serving as a feedstock for anaerobic digestion to facilitate the extraction of a spectrum of sustainable bioproducts and bioenergy derivatives (De Cauwer 2020).

The Succulent Bio-Economy aligns seamlessly with the notion of a global bio-economy, as delineated in the 2020 global bio-economy summit which is defined as the holistic production, application, preservation, and renewal of biological resources, accompanied by the associated knowledge, science, technology, and innovation (Issa *et al.* 2019, IACGB 2020), with the aim to stimulate sustainable solutions across diverse economic sectors, ultimately fostering the transition toward a sustainable economic model. A study into the utilization of succulent plant biomass for the generation of volatile fatty acids (VFAs) through the process of Anaerobic Digestion (AD) constitutes an integral component of the larger framework of the Succulent Bio-Economy Project.

1.2 Statement of the Problem

Namibia is a semi-arid country with erratic rainfall patterns making traditional agriculture in many cases unsustainable. Despite large areas of unproductive land, there are few initiatives that explore the cultivation of drought-tolerant plants, including succulents. Succulent plants could have the potential to feed into a diversified bio-economy that is more resilient to the impact of climate change. Anaerobic digestion, a universally proven practice that has the potential to derive organic products such as VFAs from renewable resources, is one of the processes within the proposed Succulent Bio-Economy Project. Anaerobic digestion is extremely dependent on microbes, but the microbial communities' complexity and metabolic pathways leading to the production of VFAs are not well understood and outcomes of several studies have not quite helped to maximize production in anaerobic digestion. The novelty of this study lies in the use of drought-resistant succulent plants grown in Namibia as a source of substrate for anaerobic digestion and the alteration of systems to optimize the yield of VFAs.

1.3 Research questions

- What are the optimal temperature and pH for the production of Volatile Fatty Acids from succulent plants?

- How much succulent material is required to produce a specific amount of Volatile Fatty Acids?

1.4 Aim and objectives of the study

1.4.1 Aim of the study

The study aims to explore the potential of biomass derived from two succulent plant species for the production of VFAs by adjusting operational conditions of the first, second and third (hydrolysis, acidogenesis, and acetogenesis) stages of anaerobic digestion.

1.4.2 Specific objectives:

- to determine optimal conditions for the production of VFAs from selected succulents;
- to determine VFA production potential from two succulent plant species that were grown in Namibia.

Chapter 2: Literature Review

2.1 Global Bioeconomy Summit's impact and Sustainable Bioeconomy Guidelines

Following the Global Bioeconomy Summit held in 2020, a significant development has taken place within the field of sustainable bioeconomy. The United Nations' Food and Agriculture Organization (FAO) has taken a proactive step by formulating and introducing guidelines for the establishment of a sustainable bioeconomy. One of the initiatives was the 'Towards Sustainable Bio-economy Guidelines' project. The core objective of this project was to extend valuable assistance to countries, as well as stakeholders involved in biomass and bioproducts, in their efforts to draft and implement strategies, policies, and programs that are firmly grounded in sustainability principles (Senapaty 2019).

2.1.1 Namibia’s step towards implementation bio-economy strategies

In consideration of the significance of agriculture and biomass utilization for various essentials like energy, food, feed, and fibre within Namibia, the existing approach involves a comprehensive array of programs and measures aimed at tackling complex challenges. This strategy leverages the advantageous impacts derived from other sectors, including agriculture, manufacturing, and health to address the identified pivotal concerns (FAO 2022).

Building upon the momentum generated by the Global Bioeconomy Summit and the subsequent FAO guidelines, Namibia has taken a decisive step forward. This step emerged in the form of 'The Bio-economy Strategy,' a comprehensive outline that sets the progression for Namibia’s activities in the bioeconomy domain (Mungeyi 2020). Scheduled for implementation over the period spanning from 2021 to 2026, this strategy is expected to function as the guiding framework (Mungeyi 2020). Its primary purpose is to provide the necessary conditions and prerequisites for the effective execution of various programs and initiatives, therefore these activities are strategically designed to position Namibia advantageously within the sphere of bioeconomy innovation and advancement (Heeren-Hauser *et al.* 2020).

2.1.2 Role of international organizations and bioeconomy focus in various countries

The bioeconomy, which is envisaged to be the key driver of economic growth and sustainability in the future, is increasingly becoming an important area of focus for many countries such as Germany, Malaysia, the United States of America, the United Kingdom, the Netherlands, Singapore, and India, among others (Mungeyi 2020). Although bioeconomy strategies are national issues, several international organizations are responsible for ensuring the implementation and success of the bioeconomy. These include the newly established International Advisory Council on Global Bioeconomy (IACGB), which is focused on the development and approval of policy recommendations to promote the development of a sustainable bioeconomy globally (Bößner *et al.* 2020). In Namibia, the FAO oversees the implementation of the Bio-economy strategies (FAO 2022).

While Namibia has shown strong political commitment towards the development of its bio-economy with vital policies and initiatives suitable for promoting sustainable management and utilization of its biodiversity, South Africa, on the other hand, developed a leading biopharmaceutical and biotechnology sector and has become an attractive place for investment (Ngige 2022). Ghana has also made advancements in its cocoa sector by diversifying its products and services. Most important to highlight is Uganda's commitment to a bio-economy strategy which resulted in Uganda becoming the regional leader in agricultural research and development. Select institutions in the country are leading in advancing food and agricultural biotechnology while also launching companies in this sector (Ngige 2022).

2.2 Succulents in anaerobic digestion and their potential in arid climate agriculture

Traditional energy crops require optimum rainfall and fertilized agricultural lands making them unsustainable in arid and semi-arid regions, especially under climate change scenarios. At the same time, many of the crops are used for animal and human consumption, therefore, their use as energy crops is in conflict with food security since scarce agricultural land and water could be used for energy production instead of food production, which will further hinder the accomplishment of the UN's SDG 2 (Mason *et al.* 2015). Consequently, there is a need to explore potential sustainable energy crops that can be grown on

poor soil or degraded lands and in areas with low rainfall, thereby reducing competition between energy and food (Hawa *et al.* 2019).

Several succulent plants employ the CAM mode of photosynthesis. They can use as little as 10% of the water that most conventional plants need and have inbuilt water storage (Gilman and Edwards 2020). Succulent plants are tolerant of very high temperatures and long periods of drought, making them suitable for areas changing towards more arid climates, such as south-western Africa (Mucina *et al.* 2006). Their nutrient uptake efficiency allows them to grow in nutrient poor or degraded soils. Hence, succulent plants thrive where conventional agriculture fails and could in future improve agriculture in dryland regions. They could be further harnessed in the production of biomass which can feed into several renewable resources techniques such as anaerobic digestion (Mason *et al.* 2015).

Opuntia ficus-indica and *Euphorbia tirucalli* are the two succulent plants that have been most investigated as sources of potential bioenergy and other products through anaerobic digestion. *Opuntia ficus-indica* has shown abilities to produce methane yielding up to 327 m^3 CH_4 Mg^{-1} volatile solids (VS) due to low fibre carbohydrates (Quiroz *et al.* 2021). It is anticipated that growing these plants on 100 – 380 million hectares of semi-arid land would make it possible to produce enough biogas to generate five trillion kilowatt-hours of electricity per year, equivalent to that generated from natural gas (George 2021). These findings indicate the potential of the two plants for VFA production, noting that microbes use VFAs to produce biogas. In addition, Alencar *et al.* (2022) have proven the potential of succulent plants in the production of bioethanol, through enzyme hydrolysis.

Lueangwattanapong *et al.* (2020) demonstrated the potential of succulents in the production of bioenergy and VFAs using five CAM crops namely, pineapple leaves (*Ananas comosus* (L.) Merr.), agave leaves (*Agave angustifolia* Haw.), opuntia cladodes (*Opuntia fragilis* (Nutt.) Haw.), kalanchoe leaves (*Kalanchoe daigremontiana* Raym.-Hamet & H. Perrier) and euphorbia stems (*Euphorbia virosa* Willd.). The results were compared to maize as a representative of a typical energy crop where *Agave angustifolia* showed 1.14 g/l sTVFA (subtracted total volatile fatty acid) concentration over 0.69 g/l sTVFA from maize in a period of 15 days. The lower lignin content of succulent biomass may have influenced this outcome. Additionally, there is no Namibian published study on the potential of CAM plants for the production of VFAs, making the current study a pioneering effort in Namibia.

2.3 Anaerobic Digestion in Biomass Conversion

Anaerobic digestion is a prominent renewable energy technology renowned for its capacity to simultaneously recover valuable nutrients from various biomass sources and generate green energy (Bautista Angeli *et al.* 2018). This biological process unfolds in the absence of dissolved oxygen, involving a complex interplay of diverse microbial communities. These communities consist of various groups of bacteria, including fermentative bacteria, hydrogen-producing acetogenic bacteria, hydrogen-consuming acetogenic bacteria, as well as archaea, with a particular focus on carbon dioxide-reducing methanogens and acetoclastic methanogens (Bautista Angeli *et al.* 2018).

2.4 The Four Stages of Anaerobic Digestion

The anaerobic digestion process can be broadly categorized into four stages: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. Each stage plays a pivotal role in the efficient conversion of organic materials into valuable end products, such as methane, a potent source of clean energy (Vanwonterghem *et al.* 2015). This process is illustrated in Figure 1 below.

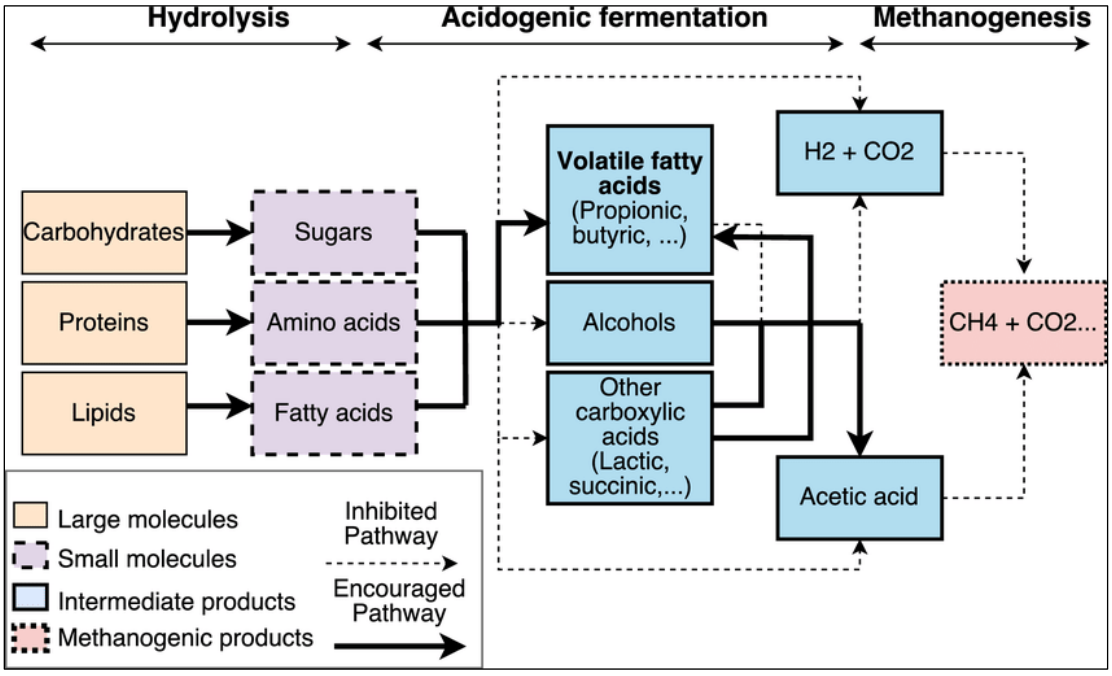

Figure 1. Anaerobic digestion steps and pathways (Source: Wainaina et al. 2019).

2.4.1 Hydrolysis

During the initial hydrolysis stage, the microbial community faces the challenge of assimilating complex biopolymers like proteins, carbohydrates, and lipids. To facilitate this breakdown, enzymes are deployed to cleave these polymers into simpler constituents, including amino acids, sugars, and fatty acids (Ramos-Suarez *et al.* 2021). Enzymes such as lipases, proteases, and cellulases are pivotal in this process. Hydrolytic members of the anaerobic microbiome, primarily bacteria, are responsible for the production and secretion of these enzymes. Notably, some hydrolysis by-products undergo concurrent transformation into compounds like acetic acid, ethanol, short-chain fatty acids, carbon dioxide, and hydrogen, facilitated by acidogenic bacteria operating under hydrolysis conditions (Bedaso 2019, Ramos-Suarez *et al.* 2021).

Several factors influence the rate of substrate breakdown during hydrolysis, including the size and chemical structure of the substrate, pH levels, enzyme production, the adsorption of enzymes onto substrate particles, and operating temperature. For instance, enhancing the hydrolysis efficiency of cellulose materials often involves pre-treatment, which may include subjecting the substrate to temperatures of approximately 60 °C and reducing particle sizes to augment the microbial surface area for enzymatic action (Jagadabhi 2011).

2.4.2 Acidogenesis

Following hydrolysis, the subsequent acidogenesis stage comes into play. In this phase, microorganisms efficiently ferment the products derived from the hydrolysis of complex biomolecules. Acidogenesis facilitates the diffusion of most final hydrolysis products into bacterial cells, where anaerobic digestion occurs (Qian *et al.* 2019). Key products of acidogenesis include VFAs such as acetic acid, propionic acid, butyric acid, isobutyric acid, and isovaleric acid, as well as hydrogen although the hydrogen output is relatively limited. To enhance the production of hydrogen, an alternative method involves using these VFAs through the process of photo-fermentation facilitated by photoheterotrophic bacteria, such as *Rhodobacter* species (Agnihotri *et al.* 2022). Additionally, smaller quantities of ethanol, lactate, and ammonia are generated.

The prevailing volatile fatty acids (VFAs) generated throughout the process of acidogenic fermentation predominantly comprise acetic acid at a range of 36–41%, followed by propionic acid at 18–22%, butyric acid at 13–21%, and valeric acid at 7–12% (Sukphun *et al.* 2021). However, the VFA composition is influenced by both the initial microbial population introduced into the bioreactors and the diverse physicochemical factors that impact microbial behaviour (Bedaso 2019, Wainaina *et al.* 2019).

Notably, approximately 20% of acetic acid and 4% of hydrogen are produced from primary molecules like sugars and amino acids during the acidogenesis stage (Sukphun *et al.* 2021). This transformative bioconversion process showcases the potential of anaerobic digestion in not only generating renewable energy but also yielding valuable biochemicals with diverse industrial applications (Agnihotri *et al.* 2022). (Agnihotri *et al.* 2022)

2.4.3 Acetogenesis

The acetogenesis stage of anaerobic digestion further contributes to the formation of volatile fatty acids (VFAs). Like in acidogenesis, acetic acid and hydrogen are the predominant products synthesized during acetogenesis, achieved through the dehydrogenation of long-chain fatty acids (LCFAs). This intricate biochemical process is facilitated by specific bacteria, including *Syntrophobacter wolinii* and *Sytrophomonos wolfei*. These obligate hydrogen-producing acetogenic bacteria specialize in breaking down propionate and butyrate, respectively, yielding acetic acid and hydrogen as primary products (Ghidotti *et al.* 2018, Moestedt *et al.* 2020).

Additionally, ethanol and lactate are transformed into acetic acid by acetogens and further into hydrogen by the action of *Clostridium formicoaceticum*, a process which is dependent on pressure conditions (Bellini *et al.* 2022). Optimal propionate degradation, for instance, occurs at remarkably low pressures (10^{-5} atm), a condition that can be achieved through co-cultivation with hydrogen-consuming bacteria (Gaur *et al.* 2020). This stage is often denoted as acetogenesis and dehydrogenation, highlighting its pivotal role in the transformation of diverse organic substrates into valuable VFAs (Bautista Angeli *et al.* 2018).

2.4.4 Methanogenesis

Methanogenesis constitutes a pivotal phase within this complex biochemical cascade of anaerobic digestion, where organic substrates are transformed into methane (CH_4), a potent renewable energy source. This process is facilitated by a group of archaea known as methanogens, which possess the unique metabolic capability to reduce carbon dioxide (CO_2) or acetate (CH_3COOH) to methane (CH_4) under anaerobic conditions (Gaur *et al.* 2020).

Carbon Dioxide Reduction to Methane

Methanogenesis can occur through two primary pathways: hydrogenotrophic and acetoclastic methanogenesis. In the hydrogenotrophic pathway, methanogens utilize hydrogen gas (H_2) as an electron donor to reduce carbon dioxide (CO_2) to methane (CH_4). This reaction is represented by the following equation:

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$$

Methanogens such as *Methanobacterium* and *Methanococcus* species are prominent hydrogenotrophic methanogens (Liu and Whitman 2008).

Acetate Conversion to Methane

Alternatively, in acetoclastic methanogenesis, methanogens convert acetate (CH_3COOH) to methane (CH_4) and carbon dioxide (CO_2). This pathway is characterized by the following reaction:

$$CH_3COOH \rightarrow CH_4 + CO_2$$

2.5 Anaerobic digestion renewable energy and climate change mitigation

Anaerobic digestion as a technology generates renewable energy and abates greenhouse gas (GHG) emissions by 3,290 to 4,360 Mt CO_2 eq., which is equivalent to 10-13% of the world's current greenhouse gas emissions (Jain *et al.* 2019). It also recovers organic nutrients and carbon for use in soil. Therefore, anaerobic digestion has the potential to help meet the climate change targets under the Paris Agreement(Jain *et al.* 2019). Methane production occurs as a result of the anaerobic decomposition of

organic matter within anaerobic environments such as landfills and manure lagoons. Anaerobic digestion facilities provide a means of harnessing methane or intermediate products which lead to methane such as VFAs. The capture of methane could negatively impact climate change when released uncombusted into the atmosphere (Styles *et al.* 2022).

2.6 Arrested anaerobic digestion (AAD) in prevention of methane production

Arrested anaerobic digestion (AAD) represents a modified form of anaerobic digestion (AD) where the crucial methanogenesis step is deliberately curtailed (Giduthuri and Ahring 2023). In the traditional AD process, organic materials undergo a sequence of microbial actions ending in the production of methane (CH_4). However, in AAD, the production of methane is intentionally inhibited through various control mechanisms, primarily by regulating the pH levels using substances like hydrochloric acid (HCl) and sodium hydroxide (NaOH) and operation temperature.

2.6.1 Controlling methanogenesis

The pivotal strategy in AAD is to arrest the activity of methanogenic archaea, which are the microorganisms responsible for methane production in AD. This inhibition can be effectively achieved by precisely managing the pH and temperature of the anaerobic reactor. By carefully regulating the pH using chemicals like HCl and NaOH, the conditions become unfavourable for the methanogens, impeding their metabolic processes. This control over pH and temperature is a key aspect of AAD, and it serves as a potent tool for suppressing methanogenesis (Giduthuri and Ahring 2023).

2.6.2 Role of methanogenic population

The population of methanogenic archaea is a critical factor in AAD. As the inhibition of methanogenesis progresses, the population of these methane-producing microorganisms naturally declines. With a reduced presence of methanogens, the production of methane experiences a substantial decline. Consequently, this reduction in methane formation leads to the accumulation of volatile fatty acids (VFAs) within the anaerobic fermentation broth (Wainaina *et al.* 2019).

2.6.3 Volatile fatty acids accumulation

The accumulation of VFAs within the fermentation broth is a distinctive hallmark of AAD. VFAs are intermediate organic compounds formed during the initial stages of anaerobic digestion, and in the absence of methanogenesis, they accumulate significantly. These accumulated VFAs, such as acetic acid, propionic acid, and butyric acid, play a crucial role in further suppressing methanogenesis. This is because the presence of VFAs in higher concentrations leads to a decrease in pH, creating an increasingly acidic environment within the reactor (Bedaso 2019).

2.6.4 Inhibiting methanogenesis

The acidic conditions resulting from VFA accumulation create an inhospitable milieu for methanogenic archaea. Methanogens are pH-sensitive microorganisms, and their activity is severely hampered in acidic environments. Hence, the lowered pH, brought about by the build-up of VFAs, further inhibits methanogenesis (Bedaso 2019). This intricate interplay between methanogen inhibition, VFA accumulation, and pH control is at the core of AAD.

In a study conducted by Vanwonterghem *et al.* (2015), it was revealed that the modulation of substrate retention time (SRT) and increasing the operational temperature are efficacious strategies in steering microbial communities towards the deliberate and precise synthesis of elevated quantities of volatile fatty acids (VFAs). These findings emphasize the potential for tuned control over anaerobic digestion processes, offering a means to optimize the production of specific VFAs for diverse industrial applications.

Arrested anaerobic digestion (AAD) represents a tailored approach to anaerobic digestion where the production of methane is intentionally restricted (Giduthuri and Ahring 2023). This is accomplished by inhibiting the methanogenic archaea responsible for methane generation through controlled temperature as demonstrated by Vanwonterghem *et al.* (2015) and careful pH control using substances like HCl and NaOH. As methanogenesis is suppressed, VFAs accumulate in the fermentation broth, creating an acidic environment that further hinders methane production. This innovative strategy offers a means to harness volatile fatty acids while reducing methane emissions, presenting potential applications in biogas production and wastewater treatment.

2.7 Factors influencing VFA production

The operational conditions of the anaerobic reactor influence the yield and composition of VFAs produced. The main and most studied operational parameters include pH, temperature, retention time, organic loading rate, substrate, and inoculum (Bedaso 2019, Qian *et al.* 2019, Ramos-Suarez *et al.* 2021). The factors affecting VFA production are discussed below.

2.7.1 pH

The degradation of organic matter by microbes takes place through enzyme activities. Each group of microbes is stable at a specific pH and if exposed to unfavourable pH, these enzymes (proteins) become inactive and can lose shape or denature, making them incompatible to break down substrates. Hence a slight shift in pH can affect the production of the end products, including the VFAs; it especially influences the hydrolysis and acidogenic processes (Zhou *et al.* 2021). Production of VFAs in acidogenesis releases protons and lowers the pH naturally to approximately 3.5 (Cetecioglu *et al.* 2022).

2.7.2 Temperature

Another important parameter in the production of VFAs is temperature because it influences microbial growth, enzymatic activities and the hydrolysis rate. Higher temperatures increase the rate of hydrolysis, but decrease the accumulation of VFAs (Zhou *et al.* 2021). Zhou *et al.* (2021) further found that the highest VFA accumulation was observed at 37 °C when the concentration of enzymes for VFA formation was also highest. Jiang *et al.* (2013) found that soluble chemical oxygen demand (SCOD) concentration increased when increasing temperature from 35 to 55 °C. VFA concentrations decreased from 41.34 g/l at 35 °C to 14.90 g/l at 55 °C.

2.7.3 Retention time (RT)

This is the average duration that the substrate remains in a reactor and should be long enough to achieve complete or maximum digestion and conversion of complex organic molecules to simple molecules. Furthermore, it is influenced by other factors such as pH, temperature, and substrate composition (Lukitawesa *et al.* 2020, Patinvoh *et al.* 2020, Sukphun *et al.* 2021). Complex substrates require a longer

retention time for hydrolysis. Lim *et al.* (2008) investigated the effect of various retention times (4, 8 and 12 days) for acidogenesis and observed that the production of VFAs increased with increasing retention time from 4 to 8 days, but there was no significant difference between retention times of 8 and 12 days. Quiroz *et al.* (2021) demonstrated that a retention time between 17 and 26 days showed a greater yield of VFAs from *Opuntia ficus-indica* cladodes. In an alternative AD investigation employing CAM plants as the substrate, an increase in retention time, favoured microorganisms to develop enhanced adaptability to low pH, leading to a subsequent improvement in the efficiency of acetate removal (Lueangwattanapong *et al.* 2020).

2.7.4 Organic loading rate (OLR)

The amount of substrate per unit reactor volume that can be fed on a daily basis into the reactor is defined as the organic loading rate (OLR) (Ramos-Suarez *et al.* 2021). OLR is one of the most important parameters in anaerobic digestion and should be high enough for the production of VFAs. An increase in OLR favours cell activity subsequently leading to increased methane production due to increased VFA production and improved degradation of the substrate in terms of biogas production (Patinvoh *et al.* 2020). A very high OLR can, however, cause process destabilization and a low yield in methane production (Jiang *et al.* 2020). This could be an advantage when the intention is to generate VFAs. Similar to RT, the OLR depends on the type of substrate fed into a reactor.

2.7.5 Other factors

Other factors to consider in anaerobic digestion include inoculum to substrate ratio (ISR) and product toxicity where undissociated VFAs become toxic to microbes (Bedaso 2019)**.** Lukitawesa *et al.* (2020) showed that a high amount of VFAs is attainable at an ISR of 1:3. The existence of high concentrations of volatile organic compounds such as undissociated VFAs will result in the reactor becoming toxic to the microbes. VFAs easily diffuse through the cell membranes of microbes and dissociate inside the cells, lowering the pH inside the cells. As a result, microbes start to divert most of their energy towards pH regulation instead of growth and reproduction (Bedaso 2019).

2.8 Uses of VFAs derived from anaerobic digestion

VFAs derived from anaerobic digestion can be used in various industrial applications such as biological nutrient removal from wastewater, biodegradable plastics (Polyhydroxyalkanoates), hydrogen, biodiesel and biogas production (Bedaso 2019). Similar research by Lueangwattanapong *et al.* (2020) has however, not indicated the presence of caproic (hexanoic) acid in AD reactors of succulent biomass and cow rumen inocula.

Acetic acid (CH_3COOH), a commercially attractive VFA, stands out as the shortest fatty acid and constitutes approximately 80% of all VFAs (Bedaso 2019, Agnihotri *et al.* 2022). Its versatility finds applications in adhesive production, plastics manufacturing, paper coating, latex paints, textile finishes, and enhancing aroma in cosmetics (Ghidotti *et al.* 2018, Agnihotri *et al.* 2022). In the food sector, it functions as both a solvent and food preparation agent, prominently found in vinegar and utilized as a preservative, acidity regulator, and flavour component. Its paramount role in the production of terephthalic acid (TPA) contributes to the manufacturing of various products, including polyethylene terephthalate (PET) packaging fibres, clothing, plastic bottles, and films. Additionally, it is used in the production of acetate esters serving as solvents for inks, paints, and coatings, reflecting its potential for further market expansion (Zacharof and Lovitt 2014, Xu and Ferdosian 2023).

Similarly, the applications of propionic acid and butyric acid encompass a broad spectrum of industries including the food, pharmaceutical, perfume, and polymer sectors. Propionic acid serves as a crucial building block chemical and preservative in the food and animal feed industries, while butyric acid and its derivatives find extensive use in the production of biofuels, flavouring and fragrance agents, and cellulose acetate butyrate in the polymer industry. The market for iso-butyric and valeric acid is also significant, driven by their applications in the flavour and fragrance industry, pharmaceuticals, and as chemical intermediates for various manufacturing processes. Finally, caproic acid is utilized in the production of esters for artificial flavours, plasticizers, antimicrobials, and as an additive in animal feed, with growing applications in the biofuel sector (Bedaso 2019, Wainaina *et al.* 2019, Agnihotri *et al.* 2022). Summarized potential applications of individual volatile fatty acids are shown in table 1 below.

Table 1 Molecular formula of individual volatile fatty acids and their potential uses (Wainaina et al. 2019).

VFA	**Molecular formula**	**Area of application**
Acetic acid	CH_3COOH	Food additives, plasticizers, dyes
Propionic acid	CH_3CH_2COOH	Resins, pharmaceuticals, paints
Isobutyric acid	$(CH_3)_2CHCOOH$	Pesticides, food additives, paints
Butyric acid	$CH_3CH_2CH_2COOH$	Perfumes, textiles, varnishes, plastics
Isovaleric acid	$(CH_3)_2CHCH_2COOH$	Pharmaceuticals, perfumes, fungicides
Valeric acid	$CH_3(CH_2)_3COOH$	Perfumes, plasticizers, lubricants
Caproic acid	$CH_3(CH_2)_4COOH$	Rubber, grease, tobacco flavour

Chapter 3: Methodology

The methodology employed in this study encompasses a comprehensive series of procedures, starting with the collection of succulent plant material and inoculum. Subsequently, thorough preparations are made for the setup of anaerobic digestion, followed by solvent extraction and the utilization of Gas Chromatography-Mass Spectrometry (GC-MS) for analytical purposes. These methodological steps form the foundation of investigation, enabling the exploration and analysis of succulent biomass as a substrate for anaerobic digestion processes. In the sections below, each of these methodological components is elucidated in detail, outlining the precise techniques and procedures employed to gather and analyse the essential data for this study.

3.1 Collection of substrate and inoculum

Two types of wet biomass substrates grown under arid conditions were harvested for investigation within the present study. The first was derived from *Euphorbia mauritanica*, an indigenous succulent species with rapid growth based on preliminary data, rendering it a promising biomass producer within the Namibian ecological context. The selection of this species aligns with its inherent potential to contribute significantly to biomass generation. Conversely, the second substrate originated from *Portulacaria afra*, a succulent species native to South Africa and acknowledged as a as potential biomass succulent (Mucina *et al.* 2006, Rezende *et al.* 2022). An additional attribute of particular significance is its non-invasive nature, which renders it particularly well-suited for cultivation in Namibian environments.

These substrates resulted from plants that were systematically cultivated as integral components of the Succulent Bio-Economy Project, an initiative undertaken at Erhardshof Farm, a part of the Otjikoto B2Gold mine (De Cauwer *et al.* 2022). The strategic choice of these specific succulent species and their cultivation is supported by ongoing research into their growth dynamics, ecological suitability, and potential contributions to the bio-economy. To provide precise location information, the field GPS coordinates for this cultivation initiative are designated as follows: 33° 55' 41.59" S 151° 12' 52.05" E.

For the inoculum, cow rumen fluid was collected from a local abattoir, and filtered using a 3mm pore sieve to separate solid components from the slurry. The inoculum underwent a maturation period of 16 days

at room temperature (20 - 26 °C), a process aimed at removing easily degradable volatile solids, as previously described by Hawa *et al.* (2019) and Lukitawesa *et al.* (2020).

3.2 Arrested Anaerobic Digestion (AAD)

3.2.1 Sample preparation

Succulent material, both indigenous and non-indigenous, was carefully chopped into pieces averaging 1 cm^3 each. These pieces for individual species, were blended at maximum speed using a kitchen blender and stored in plastic containers at around 4 °C for later use. To ensure fluidity in the blender, the succulents were blended with distilled water in the weight proportions of 562 g : 368 g, respectively. The inoculum and substrate were then mixed in a ratio of 1:3 v/v respectively, to set up a 200 mL working volume in 250 mL Schott bottles.

Table 2. Summary of experimental parameters for the anaerobic digestion of P. afra and E. mauritanica, including initial biomass, blend concentration, and dry matter content

Parameter	Value	Units
Initial species mass (wet)	562	g
Water volume (used in blending)	370	mL
Concentrations in blends	1.52	g/mL
Blends volume added to reactor	150	mL
Species mass in digesters	228	g
Dry matter content of *P. afra*	11.3	%
Dry matter content of *E. mauritanica*	10.8	%
Initial *P. afra* biomass (dry)	25.74	g
Initial *E. mauritanica* biomass (dry)	24.56	g
Inoculum volume	50	mL
Final reactor volume	200	mL

3.2.2 Experimental design

For each succulent plant species, the production of VFAs was measured in 9 trials at different pH ranges (3 – 3.9, 4 – 4.9 and 5 – 6) and temperatures (35 °C, 50 °C, 55 °C and 60 °C) as per table 2.

Table 3 pH ranges and temperatures of the VFA productionexperiment for each succulent species.

Trials	**No. replicate/trial**	**Temperature**	**pH**		
1 – 3	3	35 °C	3 – 3.9	4 – 4.9	5 – 6
4 – 6	3	37 °C	3 – 3.9	4 – 4.9	5 – 6
7 – 9	3	39 °C	3 – 3.9	4 – 4.9	5 – 6

3.2.3 Incubation and shaking

Reactors (250 mL Schott bottles) were placed in shaker-incubators equipped with an orbital shaker (mrc) – LOM 80, under controlled pH and temperature conditions, following the procedure established by Hawa *et al.* (2019).

Control triplicates containing only matured inocula were prepared for comparison. Throughout the AAD process, the pH was monitored using a pH meter at 48-hour intervals, and adjustments were made using Sodium Hydroxide (NaOH) and Hydrochloric Acid (HCl) to maintain constant pH levels within each set of triplicate flasks. Additionally, oxygen was removed from the flask surfaces using Liquid Nitrogen (LN_2) by adding 1 mL with a Polyethylene Transfer Pipette, and the flasks were sealed once anaerobic conditions were achieved.

3.3 Analytical methods

3.3.1 Solvent extraction using dimethyl carbonate (DMC)

Extraction of samples

Digestate samples were weighed (200 mg) and placed in conical centrifuge tubes (volume 2 mL). A saturated solution of $KHSO_4$ (500 mg in 100 mL of deionized water), an internal standard solution (2-ethyl butyric acid at 0.1 mg mL^{-1} in deionized water), and 600 µL DMC were added sequentially. DMC was the solvent chosen for its distinctive property in effectively extracting VFAs from aqueous solutions (Ghidotti *et al.* 2018). The mixture was vortexed and then centrifuged at 15000 rpm to separate solid and aqueous phases from the DMC layer. The suspended layer containing fatty acid methyl esters (FAMEs) was extracted and transferred to GC vials for analysis.

Extraction of standards

The calibration of multianalyte solutions involved dissolving pure VFAs (99% acetic acid, 99% propionic acid, 99% butyric acid, 99% iso-valeric acid, 99% valeric acid, and 99% caproic acid) in deionized water and performing serial dilution with deionized water. These solutions were combined with the saturated solution of $KHSO_4$ and the internal standard, and then extracted with DMC similarly to sample extraction to establish a calibration curve spanning a concentration range of 1 mg mL^{-1} to 6 mg mL^{-1} (6 points).

3.3.2 Gas Chromatography-Mass Spectrometry (GC-MS) analysis

Analysis was conducted every 48 hours using a PerkinElmer GC-MS system (Clarus 690 SQ 8 T, Optima 8000). DMC extracts were injected into the split injector of a Clarus 690 SQ 8 T gas chromatograph. The analytes were separated using the PerkinElmer universal column (30 m length, 0.25 mm i.d, 0.25 mm film thickness), with helium gas flow of 1mL min^{-1} and detection was carried out using the Clarus SQ 8 T mass spectrometer, operating under electron ionization at 70 eV with full scan mode acquisition at 1 scan s^{-1} in the 40 – 450 m/z range. The syringe of the autosampler was programmed to take 1 ml of the solution to be injected at a fixed height from the bottom of the vial (10 mm), corresponding to the layer of DMC extract.

The starting GC oven temperature was held at 40 °C for 1 minute and increased to 150 °C at a rate of 15 °C min^{-1} before it was ramped up to 300 °C at a rate of 50 °C min^{-1} where it was held for 10 minutes. Peak identification was based on the MS retention time, the amount of time in seconds it takes for a specific compound to travel through a chromatography column and reach the detector in a GC-MS, and mass spectra of the standards and by National Institute of Standards and Technology (NIST) library mass spectra matching. Quantification was made by the component peak area, a metric that is directly proportional to the concentration of the target compounds, and obtained by integration of characteristic ions from the total ion current.

3.3.3 Calibration curve

The standards were extracted and analysed in triplicate using the same GC-MS conditions outlined in 3.3.2. Values obtained were used to plot a calibration curve of a concentration range of 1 mg mL^{-1} to 6 mg mL^{-1}. The calibration curve was then used to determine the unknown concentrations of total VFAs in extracted AD samples. The linear regression model for the calibration curve established a quantitative relationship between the concentration of VFAs and the corresponding GC-MS response. The model represents the change in GC-MS response for a one-unit increase in VFA concentration. The y-intercept was set to 0 to avoid impractical interpretations.

3.3.4 Wet and dry mass

Triplicate samples of each plant species were collected from the substrate, weighed for wet mass, subjected to controlled oven drying at a temperature below 45 °C, and weighed every 24 hours until the weight was constant to determine dry mass. This method facilitated the determination of the dry/wet biomass ratio, allowing for relating the biomass of each species to the amount of VFAs produced.

Finally, the generated data from GC-MS analyses was continuously analysed and compared to predefined study parameters to draw meaningful conclusions from the experimental results.

3.4 Statistical methods

The dataset underwent comprehensive analysis using R software, wherein a diverse range of statistical methodologies were applied. These included the computation and examination of descriptive statistics, linear regression model for calibration curve which served as a tool for converting measured GC-MS responses into meaningful concentrations of volatile fatty acids in AD samples, the visualization of data patterns, linear and exponential regression models that provided an understanding of the effects of pH and temperature, and enabled estimation succulent biomass in VFAs production.

A summary of methods used in this study is illustrated in Figure 2.

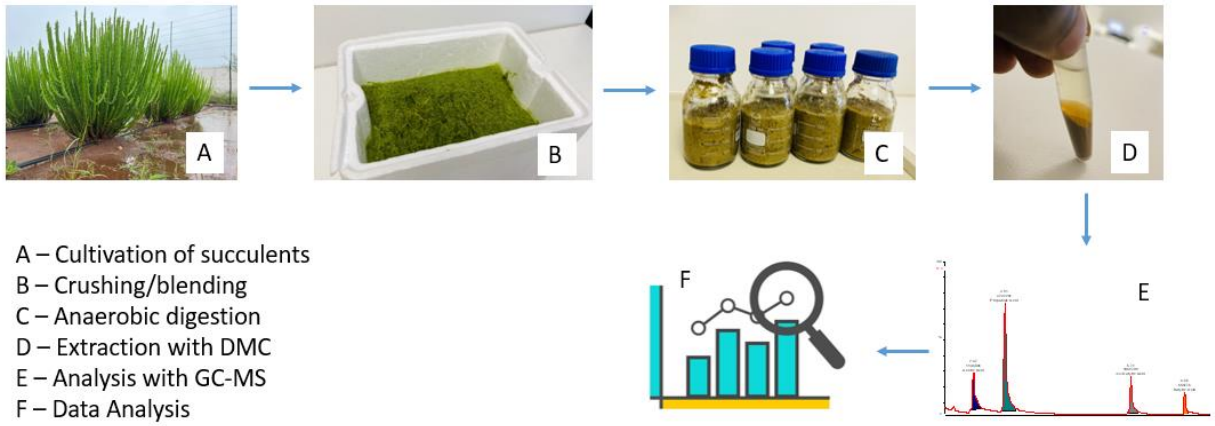

Figure 2 Summary of methods used in this study.

Chapter 4: Data Collection and Analysis

4.1 Calibration Curve for VFAs Analysis by GC-MS

The calibration curve was constructed to establish a relationship between VFA (volatile fatty acids) concentration and the corresponding GC-MS response, measured as peak area. The calibration standards were prepared with known VFA concentrations (standards), and the resulting data is presented in Table 3.

Table 4 Values of a calibration curve for total volatile fatty acids analysis by GC-MS.

VFAs Concentration (mg/mL)	GC-MS Response (Peak Area)
0	0
1	2964602.2
2	4571204.4
3	6821806.6
4	9042408.8
5	11403011.0
6	13163613.2

4.1.1 Calibration Curve Parameters

In this study, a linear regression model was constructed with the y-intercept set to 0. Hence,

$$\text{GC} - \text{MS Response} = 2249877 \times \text{VFA Concentration (mg/mL)} \qquad \textit{Equation 1}$$

This substantial coefficient signifies the estimated change in the GC-MS response for a one-unit increase in VFA concentration, assuming a starting point of 0 for GC-MS response. The statistical significance of this coefficient was established through a t-test with a t-value of 64.75 and *p*-value less than 0.001.

The goodness of fit of the model was assessed through the r-squared value. The linear regression model, without an intercept, is therefore a meaningful and valid representation of the calibration curve. In summary, the calibration curve equation is given by equation 1 and the curve is shown in Figure 3.

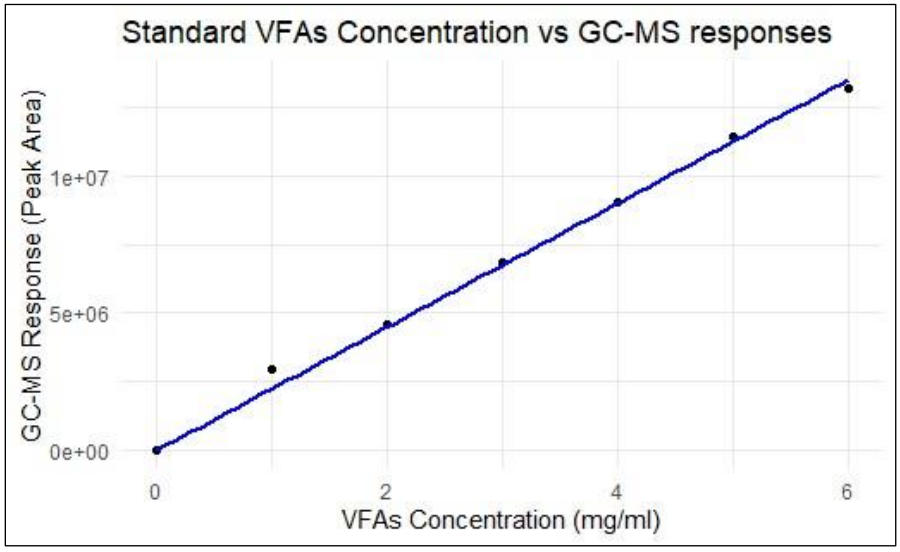

Figure 3 A standard calibration curve of VFAs (volatile fatty acids) concentrations against their corresponding GC-MS responses.

In the calibration curve for the concentration of VFAs in anaerobic samples, the decision to set the y-intercept to 0 was driven by the recognition that the best-fit curve would otherwise yield a higher intercept. This elevated intercept would imply negative concentrations for unknown samples, especially during the incubation period when microbial activity is initiating. From a practical standpoint, attributing negative concentrations to these samples lacks meaningful interpretation. Therefore, setting the y-intercept to 0 enhances the practical relevance and interpretability of the calibration curve in the context of real-world applications.

4.1.2 Inoculum Analysis

The GC-MS analysis of control triplicates revealed an absence of VFAs, attributed to the inoculum maturation period of 16 days at room temperature prior to utilization. The GC-MS chromatogram of a control sample, illustrated in Figure 4, exclusively displays a solvent (DMC) peak. These findings, devoid of any VFAs presence, were deemed inconsequential to VFAs production and thus considered negligible for the scope of this study.

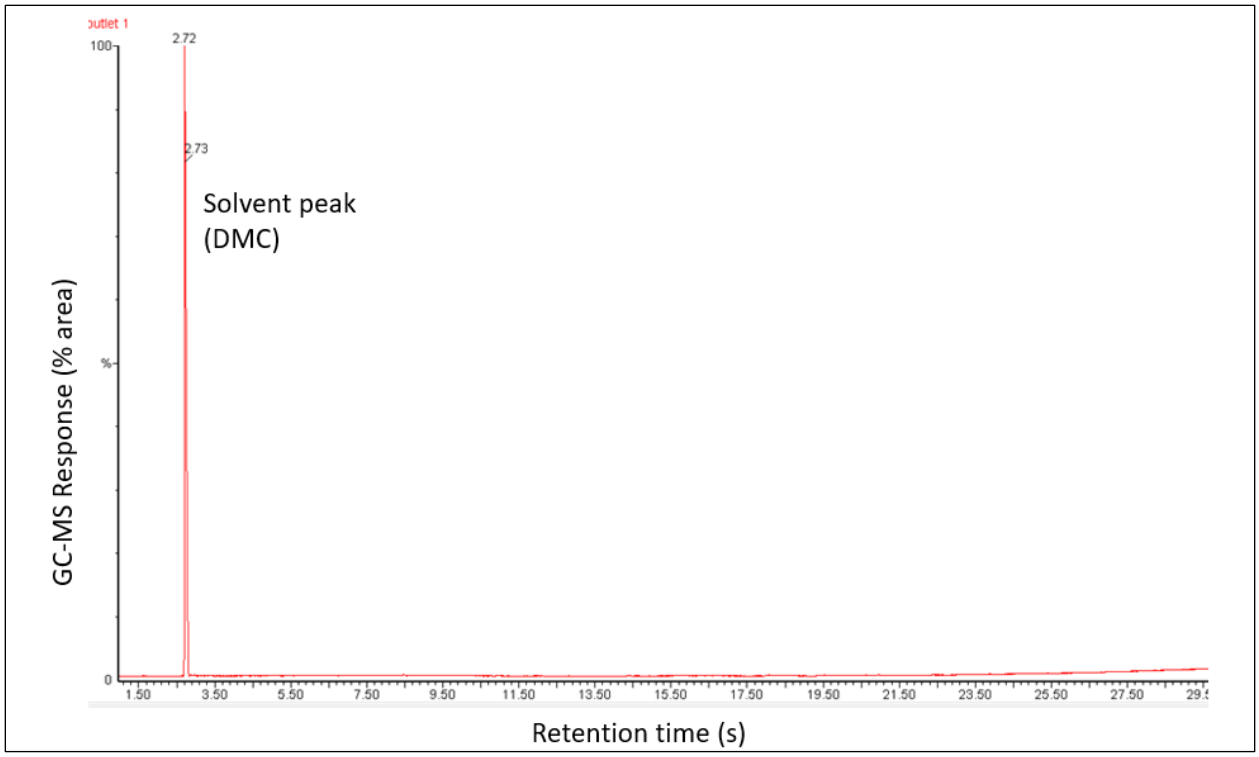

Figure 4. GC-MS chromatogram of control triplicates showing only a solvent (DMC) peak, indicating the absence of VFAs presence.

4.2 AD samples analysis

The volatile fatty acids identified within the anaerobic digestion DMC-extracted analytes comprised acetic acid at a retention time of 3.08s, propionic acid at 3.65s, butyric acid at 3.98s, isovaleric acid at 4.47s, valeric acid at 4.61s, and caproic acid at 5.47s, respectively, in sequential elution (Figure 5).

The observed sequence of detection closely mirrored that of pure VFAs standards, aligning with the ascending order of their respective boiling points: 118 °C, 141.3 °C, 154 °C, 164.1 °C, 179 °C, 186.4 °C, and 205.7 °C, respectively. This concurrence highlights the analytical precision of the methodology employed, as the elution sequence corresponded both to the standards and the boiling point hierarchy.

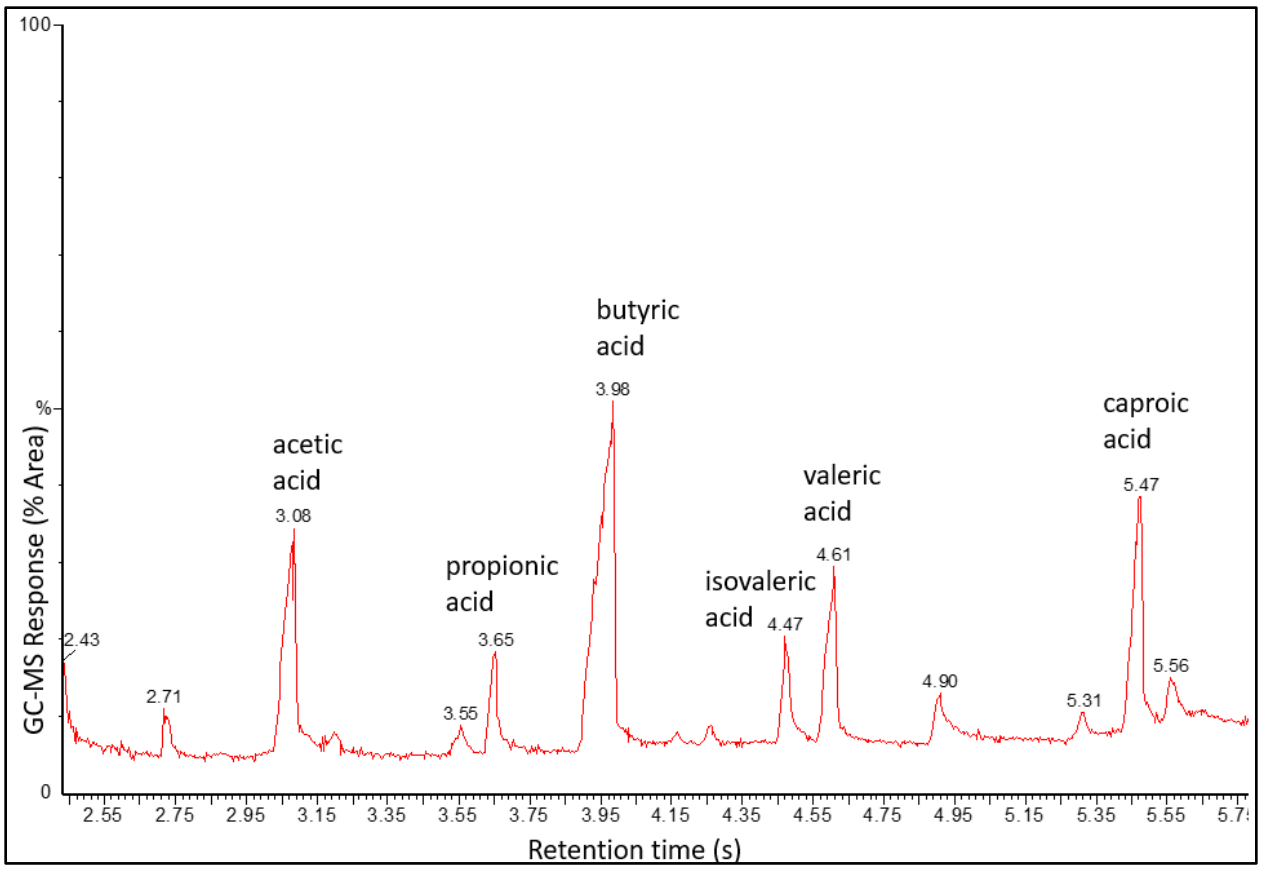

Figure 5 Gas chromatography-mass spectrometry (GC-MS) peak chromatogram of VFAs in anaerobic digestion of Portulacaria afra.

Figure 6 presents the GC-MS chromatogram of volatile fatty acids (VFAs) detected in the anaerobic digestion of *Euphorbia mauritanica*. As observed in the *Portulacaria afra* anaerobic digestion analysis, the chromatogram exhibits distinct peaks corresponding to various VFAs. The retention time (x-axis) serves as a unique identifier for each compound within the GC column, enabling their unequivocal identification.

Understanding the calibration curve parameters was crucial for accurately quantifying VFA concentration in unknown AD samples based on their GC-MS responses (total peak area) using equation 1, where y-intercept is 0 and slope is 2249877.

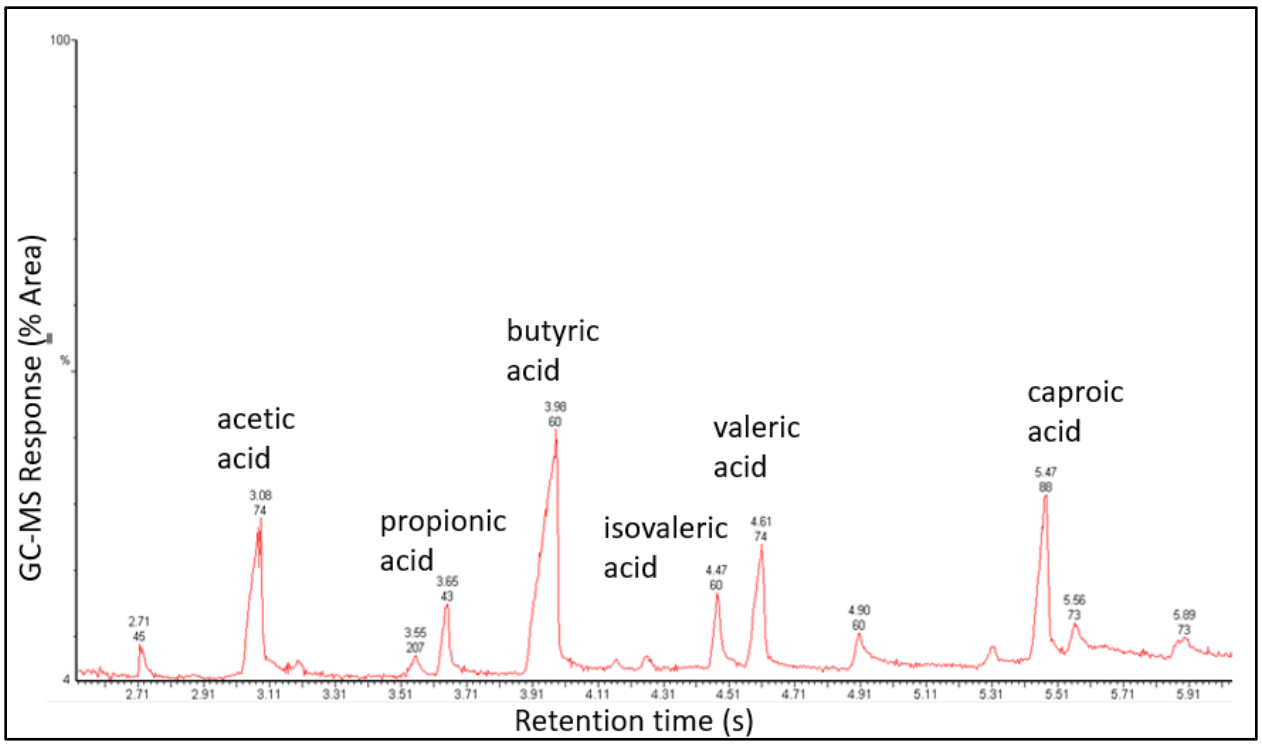

Figure 6. Gas chromatography-mass spectrometry (GC-MS) peak chromatogram of VFAs in anaerobic digestion of Euphorbia mauritanica.

4.3 Data analysis

4.3.1 Data visualization and interpretation

Means and standard deviations

Time-series plots in Figure 7 unveil the dynamic interplay of key parameters in *Portulacaria afra* and *Euphorbia mauritanica* under varying conditions, revealing species-specific biotechnological potential through temporal trends and variability.

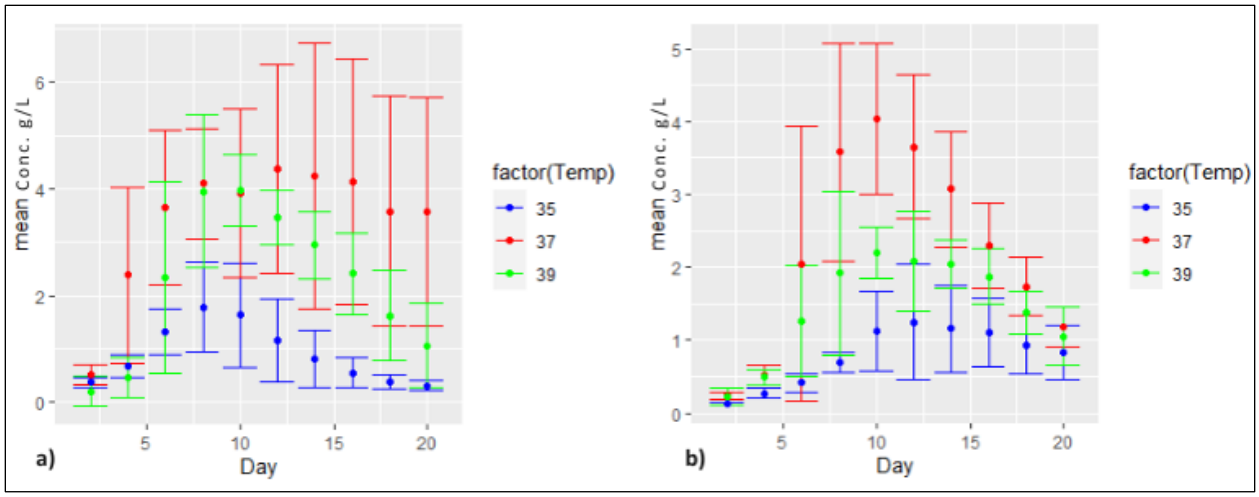

Figure 7. Mean concentration of VFAs (g/L) changes over days at various temperatures (35 °C, 37 °C, and 39 °C), and pH (3, 4, and 5) for anaerobic digestion of (a)Portulacaria afra and (b)Euphorbia mauritanica.

The temporal analysis in Figure 7(a) revealed distinct trends in mean concentration of VFAs for each temperature category for anaerobic digestion of *Portulacaria afra*. At 35 °C, a gradual decline of 16% was observed from 0.38 g/L on day 2 to 0.32 g/L on day 20, likely reflecting the slowdown of acidogenesis due to decreased thermal fitness of the microbial population. Conversely, at 37 °C, the mean concentration exhibited a rapid increase of 365% by day 4 (2.38 g/L compared to 0.53 g/L), followed by sustained growth until day 10, suggesting optimal conditions for acidogenesis fermentation and subsequent VFA production. This trend then decreased towards day 20, potentially indicating substrate depletion or accumulating inhibitory products. The larger error bars at 39 °C (compared to 35 °C and 37 °C) indicate higher variability in concentration, likely due to the destabilizing effect of higher temperatures on the microbial population and its metabolic activity. These divergent patterns suggest a complex interplay between temperature and underlying mechanisms driving concentration changes.

Meanwhile, the plot visualization of mean concentration in Figure 7(b) *Euphorbia mauritanica'*s anaerobic digestion for VFA production showed similar trends. At 35 °C, a slow start with a mean VFA concentration of 0.24 g/L, was followed by a peak mean concentration of about 1.20 g/L on day 12. A standard deviation of 0.54 g/L suggests a potential transition to a more active phase of substrate assimilation or heightened process heterogeneity within the digester. The mean concentration eventually decreased to around 0.80 g/L on day 20. At a temperature of 37 °C, a slow start just under 0.5 g/L was observed on day 2 leading to a steep ascend to a peak mean concentration just above 4 g/L on day 10 before a gradual fall to about

1.20 g/L on day 20. Finally, at 39 °C, a moderate mean VFA concentration was recorded in comparison to 35 °C and 37 °C, peaking on day 10 at about 2.20 g/L and eventually descended to about 1.0 g/L on day 20. This complex temporal pattern hints at a confluence of factors potentially influencing VFA production, including substrate depletion, accumulation of inhibitory metabolites exceeding a threshold (Chen *et al.* 2008, Venkiteshwaran *et al.* 2015) and adaptation of the microbial community within the digester.

Another contributing factor may be the rapid deviation of pH from the optimal range during the anaerobic digestion phases, particularly in the acidogenesis stage characterized by an exponential increase in mean Volatile Fatty Acid (VFA) production in Figure 7. The observed pH drop occurred more swiftly than in other phases, leading to an accelerated accumulation of VFAs within the 48-hour period when pH adjustments are conventionally made. The accelerated accumulation during acidogenesis might have caused the fluctuations observed in the mean concentration change plots.

While identifying the optimal conditions for VFA production requires further investigation incorporating temperature and pH data alongside statistical analysis, the plots unravel the complex dynamics of plant biomass conversion through anaerobic digestion, paving the way for future research and optimization strategies to maximize bioenergy yields from this versatile plant species.

Mean VFA concentrations at various temperature and pH

The multifactorial line plots with error bars in figures 8 and 9 depict the temporal evolution of mean VFA concentrations during anaerobic digestions of *Portulacaria afra* and *Euphorbia mauritanica* at three different temperatures (35 °C, 37 °C, and 39 °C) and pH (3, 4, and 5). Analysing the trends across pH levels reveals variations in VFA production.

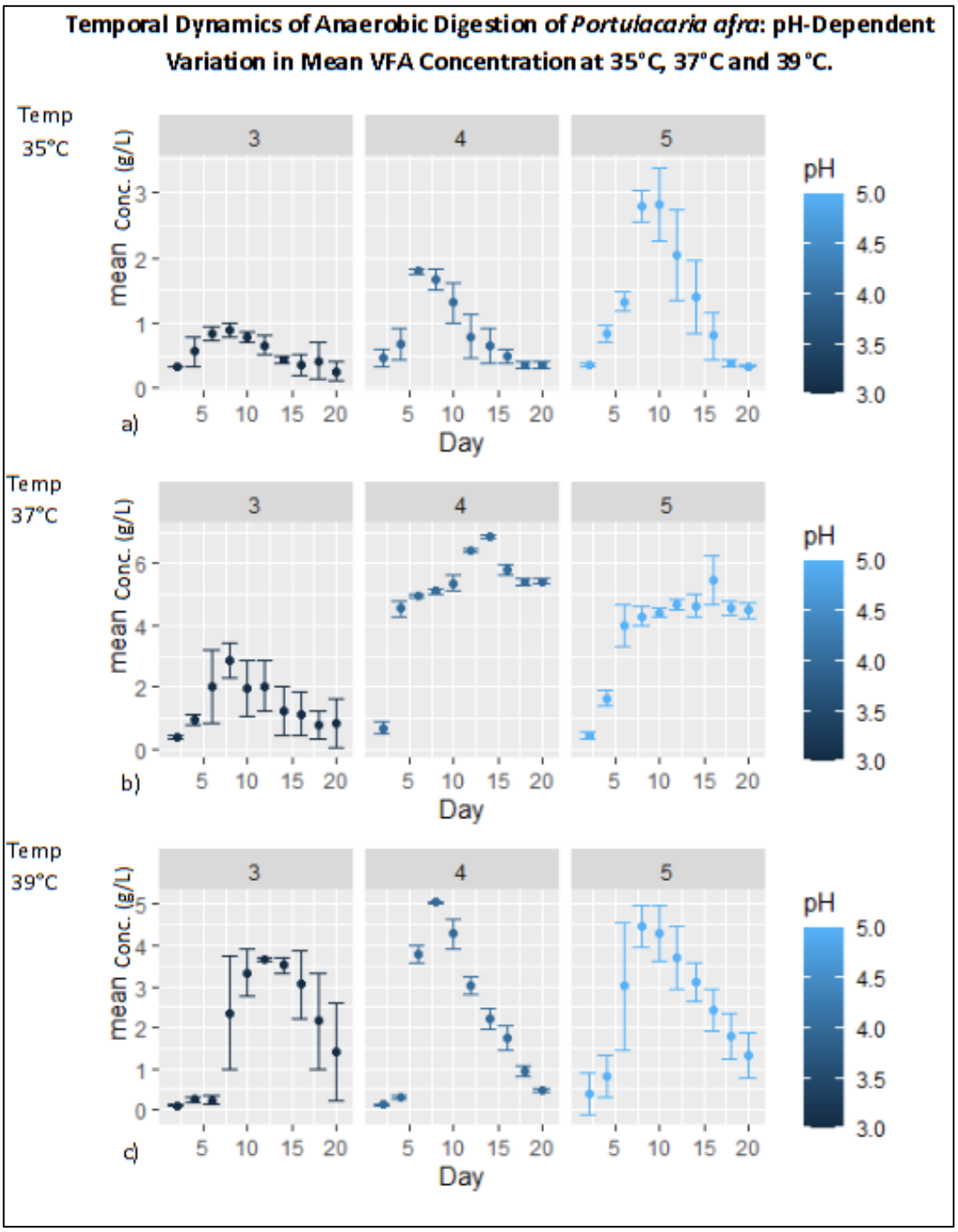

Figure 8 Temporal dynamics of Portulacaria afra anaerobic digestion: pH-dependent variation in volatile fatty acids mean concentration at 35 °C, 37 °C and 39 °C.

Figure 8(a) visualizes the temporal evolution of mean concentration during anaerobic digestion of *Portulacaria afra* at 35 °C where the initial phase (days 2-6) displayed a uniform rise in concentration for

all pH levels, with pH 4 exhibiting the highest mean concentration of 1.79 g/L on day 6. However, fluctuations around day 8 lead to a slight decrease from day 10 onwards, with pH 3 recording the lowest concentration of 0.43 g/L on day 14.

Under a temperature of 37 °C in Figure 8(b), the pH-dependent trends demonstrated vital differences. In the initial days from 2 to 6, pH 4 surpasses other levels, reaching a mean concentration of 6.42 g/L highlighting a superior efficiency for this condition. Subsequently, a stabilization or slight decrease from day 10 onwards was observed, with pH 3 recording the lowest concentration of 0.78 g/L on day 10.

At 39 °C depicted in Figure 8(c), the pH-dependent trends again exhibit variations where pH 4 showed the highest mean concentration of 5.00 g/L on day 6 during the initial phase (days 2-6). The transition around day 8 appeared to fluctuate before a stabilization or slight decrease from day 10 onwards, with pH 3 recording the lowest concentration of 3.91 g/L on day 8.

Generally, the optimal conditions for VFA production from *Portulacaria afra* vary across temperatures and pH levels. At 37 °C and pH 4, the highest production of 6.42 g/L on day 6 was observed, making it an ideal anaerobic digestion condition for maximum VFA production, followed by a temperature of 39 °C at pH 4 which showed the highest mean concentration of 5.00 g/L on day 6.

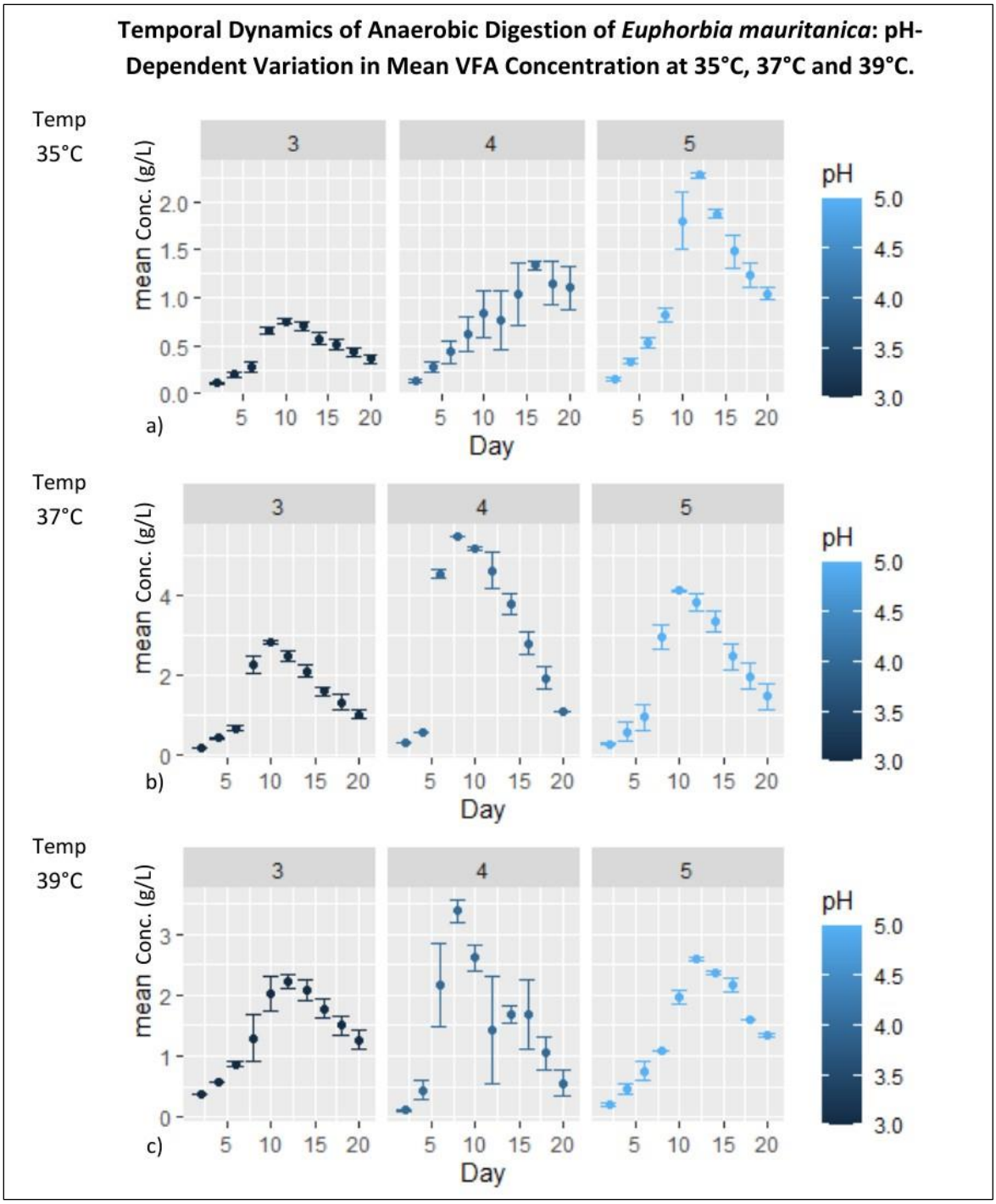

Figure 9 A temporal dynamics visualisation of Euphorbia mauritanica anaerobic digestion: pH-dependent variation in volatile fatty acids mean concentration at 35 °C, 37 °C and 39 °C.

The trends observed in the anaerobic digestion of *Euphorbia mauritanica* (figures 8 a, b, &c) align with those seen in *Portulacaria afra*, although with some variations. Both species show pH-dependent patterns in VFA production, with pH 4 generally resulting in higher concentrations at all three temperature points. At 35 °C, pH 4 exhibits a steady increase in VFA concentration, peaking around day 10, followed by a slight decline towards day 20. Meanwhile, at 37 °C, pH 4 also shows a notable increase in concentration, with fluctuations around day 8 and stabilization thereafter.

However, the influence of temperature on VFA production dynamics is evident, with higher temperatures generally leading to higher concentrations. For instance, at 37 °C, the overall VFA concentrations are higher compared to those observed at 35 °C, regardless of pH levels. This indicates that temperature plays a significant role in influencing the rate and extent of VFA production, with higher temperatures generally favoring increased VFA concentrations.

Overall, while pH-dependent patterns remain consistent across temperatures, the specific concentrations and timing of fluctuations vary, highlighting the complex interplay between pH and temperature in VFA production during anaerobic digestion.

4.4 Relation of biomass to VFAs production

The blend of *P. afra* and water (used during blending) weighed 263.3 g in a volume of 150 mL. As indicated in methods, section 3.2.1, the ratio of *P. afra* to water during blending was determined as 562 g: 370 mL. Accounting for the water (in the blend), the weight attributed solely to wet *P. afra* amounted to 228 g. Subsequently, through the process of oven drying (see methods, section 3.3.4), it was found that 142.5 g of wet *P. afra* yields 16.1 g of dry *P. afra*. Extrapolating from this, the calculated weight of 228 g for *P. afra*, factoring in the drying process, equates to approximately 25.74 g of dry *P. afra* in the reactor. The same concept was applied to *E. mauritanica* which accounted for approximately 24.56 g in its reactors. Below are the corresponding VFAs production to dry biomass of each species.

4.4.1 *Portulacaria afra*

In optimal conditions, specifically at a temperature of 37 °C and pH 4, with an inoculum-substrate ratio of 1:3, the results of this investigation indicate that wet biomass (228 g), which is approximately 25.74 g of dry *P. afra*, has the capability to generate an estimated VFA concentration of approximately 6.42 g/L.

Hence, assuming a linear relationship between the dry weight of *P. afra* biomass and VFA production, it is possible with *equation 2* to approximate the VFA production in known amounts of *P. afra* biomass, where 0.249 is the VFA production rate (g VFA/g dry *P. afra*).

$$VFA\ production\ (g) = 0.249 \times Dry\ P.afra\ biomass\ (g) \qquad \textit{Equation 2}$$

4.4.2 *Euphorbia mauritanica*

During oven drying *E. mauritanica,* an average of 156.2 g of wet biomass is equivalent to 15.3 g of dry biomass, consequently resulting in 24.56 g of dry *E. mauritanica* present in the reactors. While *P. afra* showed a slightly higher outcome, the findings of this study suggest that approximately 24.56 g of *E. mauritanica* dry weight has the potential to yield an estimated VFA concentration of approximately 5.52 g/L in optimal conditions, specifically at a temperature of 37 °C and pH 4, with an inoculum-substrate ratio of 1:3. Therefore *equation 3* allows the estimation of VFA production in known amounts of *E. mauritanica* biomass, where 0.225 is the VFA production rate (g VFA/g dry *E. mauritanica*).

$$VFA\ production\ (g) = 0.225 \times Dry\ E.mauritanica\ biomass\ (g) \qquad \textit{Equation 3}$$

4.4.3 Comparison to other AD substrates

The observed optimal conditions for VFA production from *Portulacaria afra* and *Euphorbia mauritanica* at different temperatures and pH levels align with recent findings in a study by Sun *et al.* (2021) which reported similar trends, with pH 4 favouring VFA production in anaerobic digestion of lignocellulosic biomass at both 35 °C and 37 °C. Similarly, an investigation by Wang *et al.* (2018) observed enhanced VFA yields at pH 4 for the digestion of microalgal residue at 39 °C. These findings suggest that a pH of 4 might be a critical factor for maximizing VFA production from various feedstock across a range of mesophilic temperatures.

Furthermore, the two semi-arid plant species as feedstocks for AD under optimized AD conditions, exhibited promising VFA production capabilities, reaching a VFA concentration of 6.42 g/L and 5.52 g/L, respectively. These values compare favourably to the 6.3 g/L reported by Vanwonterghem *et al.* (2015) for a mixture of microalgal residue and lignocellulosic biomass. These findings suggest that *Portulacaria afra* and *Euphorbia mauritanica*, represent viable alternative feedstocks for AD.

Additionally, in a study conducted by Kullavanijaya and Chavalparit in 2019, the production of volatile fatty acids (VFAs) from Napier grass was investigated using an anaerobic leach bed process. Kullavanijaya and Chavalparit's findings indicated a VFA yield of 0.26 g VFA/g VS from Napier grass. In contrast, the outcomes of this study revealed virtually identical VFA yields for *P. afra*, with a yield of 0.25 g VFAs/g VS. However, *E. mauritanica* exhibited a slightly lower VFA yield of 0.22 g VFAs/g VS compared to Napier grass. These results suggest that the anaerobic digestion process applied to *P. afra* may offer a comparable or slightly more efficient means of VFA production compared to Napier grass under similar conditions, while *E. mauritanica* may offer a slightly less efficient alternative.

A distinction between this study and that of Lueangwattanapong et al. (2020) lies in the observed VFA concentrations. This study demonstrated significantly higher VFA concentrations, with *P. afra* achieving an estimated 6.42 g/L, while Lueangwattanapong et al. (2020) reported a maximum sTVFA concentration of approximately 1.14 g/L for *Agave angustifolia* using rumen fluid inoculum. This discrepancy can be attributed to several factors, including potential differences in substrate biodegradability between *P. afra* and *E. mauritanica* compared to the CAM plants employed in Lueangwattanapong et al. (2020). Furthermore, the use of a matured inoculum in the present study, in contrast to the fresh rumen fluid and sludge inocula utilized by Lueangwattanapong et al. (2020), likely influenced VFA production rates due to pre-existing VFAs, and variations in microbial community composition and activity. Finally, differences in experimental conditions, such as temperature, pH, and inoculum-substrate ratios, could also contribute to the observed variations in VFA production.

In a separate study using food waste, the maximum production of VFAs was observed at pH 10, resulting in a concentration of 6.3 g/L (Sukphun *et al.* 2021). These results complement the findings of our study, wherein concentrations of 6.42 g/L and 5.52 g/L were respectively obtained for *P. afra* and *E. mauritanica*, corroborating the efficacy of anaerobic digestion conditions in fostering VFA production.

However, the influence of temperature on VFA production dynamics seems to be feedstock-specific. The current study highlights variations in the timing and specific concentrations of VFA peaks obtained from *Portulacaria afra* and *Euphorbia mauritanica* at different temperatures. This observation is supported by Wang *et al.* 2018, where the optimal temperature for VFA production varied depending on the type of lignocellulosic substrate used. Therefore, while pH 4 appears to be a promising factor for optimizing VFA

production, further research is needed to elucidate the relationship between temperature, pH, and feedstock characteristics for specific biomass conversion processes which could potentially unlock their full potential for biofuel production or other VFA-based applications.

Chapter 5: Conclusions and Recommendations

5.1 Conclusion

This study aimed to explore the potential of succulent biomass, specifically *Portulacaria afra* and *Euphorbia mauritanica*, as substrates for anaerobic digestion (AD) to produce valuable volatile fatty acids (VFAs). Specific goals included identifying optimal conditions for VFA production from these succulents and evaluating the VFA production potential of two succulent plant species cultivated in Namibia.

The findings revealed that both *Portulacaria afra* and *Euphorbia mauritanica* exhibit substantial bio-economical potential through the generation of VFAs, including acetic acid, propionic acid, butyric acid, and others through anaerobic digestion. In *Portulacaria afra*, the optimal conditions of 37 °C and pH 4 led to an estimated maximum VFA concentration of approximately 6.42 g/L, utilizing 25.74 g of dry weight. Conversely, under the same conditions, *Euphorbia mauritanica* yielded an estimated VFA concentration of around 5.52 g/L, utilizing 24.56 g of Euphorbia mauritanica dry weight. Temperature exerted the most significant influence on VFA production, whereas pH consistently influenced the process to a lesser extent.

5.2 Recommendations

Building on the insights gained from this study, several recommendations are proposed for future research and practical applications. This study primarily focused on the physicochemical aspects influencing VFA production and found opportunities for more in-depth exploration.

While this study offered valuable insights into the physicochemical factors influencing VFA production, potential future research could involve a more detailed examination of the microbial communities involved in anaerobic digestion to understand microbial dynamics and how these communities respond to varying conditions which could provide additional insights, potentially optimizing VFA yields.

The findings of this study highlighted the significance of specific combinations of pH and temperature in maximizing VFA production for each succulent species. To further enhance bioenergy yields and rationalise the anaerobic digestion process, additional optimisation studies could be undertaken to fine-

tuning these parameters based on degradation capability succulent biomass. This could lead to more efficient and sustainable VFA production.

Future studies could also consider specific phase-related pH adjustment time intervals in anaerobic digestion processes which could address the fluctuation in mean VFA concentrations observed in exponential phases of AD.

To broaden the applicability of the research findings, future studies could involve comparative analyses with other biomass feedstocks. Exploring how VFA production varies across different substrates would contribute to a more comprehensive understanding of anaerobic digestion processes. Identifying common trends and specificities in VFA production could inform more universal approaches to bioenergy production.

Finally, assessing the feasibility of scaling up succulent biomass anaerobic digestion for practical applications is crucial. A techno-economic analysis is recommended to evaluate the economic viability and potential challenges associated with large-scale VFA production from succulent biomass. This analysis would provide valuable insights for stakeholders and decision-makers considering the implementation of anaerobic digestion on a broader scale.

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Appendices

Appendix 1 Lab data entry sheet

	Plant.Species	Replicate	RT	Concentration	Day	Temp	pH
469	EM	2	Day 18	1.5909254	18	39	3
470	EM	2	Day 20	1.3750144	20	39	3
471	EM	3	Day 2	0.3687536	2	39	3
472	EM	3	Day 4	0.5681874	4	39	3
473	EM	3	Day 6	0.9147823	6	39	3
474	EM	3	Day 8	1.7329729	8	39	3
475	EM	3	Day 10	2.2849569	10	39	3
476	EM	3	Day 12	2.1022898	12	39	3
477	EM	3	Day 14	1.8977470	14	39	3
478	EM	3	Day 16	1.5909254	16	39	3
479	EM	3	Day 18	1.3238776	18	39	3
480	EM	3	Day 20	1.0795564	20	39	3

	Plant.Species	Replicate	RT	Concentration	Day	Temp	pH
481	EM	1	Day 2	0.1258652	2	39	4
482	EM	1	Day 4	0.2704458	4	39	4
483	EM	1	Day 6	1.4772881	6	39	4
484	EM	1	Day 8	3.3555908	8	39	4
485	EM	1	Day 10	2.8303490	10	39	4
486	EM	1	Day 12	0.3984428	12	39	4
487	EM	1	Day 14	1.7937180	14	39	4
488	EM	1	Day 16	2.2934615	16	39	4
489	EM	1	Day 18	1.0795564	18	39	4
490	EM	1	Day 20	0.5889029	20	39	4
491	EM	2	Day 2	0.1000968	2	39	4
492	EM	2	Day 4	0.4716915	4	39	4
493	EM	2	Day 6	2.1992294	6	39	4

	Plant.Species	Replicate	RT	Concentration	Day	Temp	pH
494	EM	2	Day 8	3.5817061	8	39	4
495	EM	2	Day 10	2.4169031	10	39	4
496	EM	2	Day 12	1.9128173	12	39	4
497	EM	2	Day 14	1.7164418	14	39	4
498	EM	2	Day 16	1.5933251	16	39	4
499	EM	2	Day 18	1.3115010	18	39	4
500	EM	2	Day 20	0.7542041	20	39	4
501	EM	3	Day 2	0.1013905	2	39	4
502	EM	3	Day 4	0.5740121	4	39	4
503	EM	3	Day 6	2.8282310	6	39	4
504	EM	3	Day 8	3.1986631	8	39	4
505	EM	3	Day 10	2.5824174	10	39	4
506	EM	3	Day 12	1.9465279	12	39	4

	Plant.Species	Replicate	RT	Concentration	Day	Temp	pH
507	EM	3	Day 14	1.5122211	14	39	4
508	EM	3	Day 16	1.1685437	16	39	4
509	EM	3	Day 18	0.7752739	18	39	4
510	EM	3	Day 20	0.3257118	20	39	4
511	EM	1	Day 2	0.2102290	2	39	5
512	EM	1	Day 4	0.5681874	4	39	5
513	EM	1	Day 6	0.8636454	6	39	5
514	EM	1	Day 8	1.0795564	8	39	5
515	EM	1	Day 10	2.1022898	10	39	5
516	EM	1	Day 12	2.5625220	12	39	5
517	EM	1	Day 14	2.3579792	14	39	5
518	EM	1	Day 16	2.2954787	16	39	5
519	EM	1	Day 18	1.5909254	18	39	5

	Plant.Species	Replicate	RT	Concentration	Day	Temp	pH
520	EM	1	Day 20	1.3750144	20	39	5
521	EM	2	Day 2	0.1897744	2	39	5
522	EM	2	Day 4	0.4131292	4	39	5
523	EM	2	Day 6	0.5681874	6	39	5
524	EM	2	Day 8	1.0795564	8	39	5
525	EM	2	Day 10	1.8977470	10	39	5
526	EM	2	Day 12	2.5664174	12	39	5
527	EM	2	Day 14	2.3466156	14	39	5
528	EM	2	Day 16	2.1022898	16	39	5
529	EM	2	Day 18	1.5909254	18	39	5
530	EM	2	Day 20	1.3238776	20	39	5
531	EM	3	Day 2	0.2357976	2	39	5
532	EM	3	Day 4	0.4291973	4	39	5

	Plant.Species	Replicate	RT	Concentration	Day	Temp	pH
533	EM	3	Day 6	0.8125086	6	39	5
534	EM	3	Day 8	1.0795564	8	39	5
535	EM	3	Day 10	1.8977470	10	39	5
536	EM	3	Day 12	2.6136128	12	39	5
537	EM	3	Day 14	2.4091160	14	39	5
538	EM	3	Day 16	2.1022898	16	39	5
539	EM	3	Day 18	1.5909254	18	39	5
540	EM	3	Day 20	1.3238776	20	39	5