BIOMETHANE AND ACIDOGENIC POTENTIAL OF AGRI-FOOD WASTE STREAMS: TOWARDS THE PRODUCTION OF VOLATILE FATTY ACIDS (VFAs).

Lappeenranta- Lahti University of Technology (LUT)

Master's Thesis

Erasmus Mundus Joint Master's Degree Program in Sustainable Biomass and Bioproducts Engineering (SBBE).

2025

Marlon Elian Bello Sepúlveda

Examiner(s): Francisco Javier Pinar Pérez, PhD. (R&D CLAMBER Biorefinery).
Francisco Jesús Fernández Morales, PhD. (UCLM University).
Tuomas Koiranen PhD. (LUT University).
Samuel Pérez Vega, PhD. (LUT University).

ABSTRACT

Lappeenranta–Lahti University of Technology LUT

LUT School of Engineering Sciences

Erasmus Mundus Master Program Sustainable Biomass and Bioproducts Engineering

In co-operation with partner universities: Wrocław University of Science and Technology (Poland), Universidad de Castilla-La Mancha (Spain)

Marlon Elian Bello Sepúlveda

Biomethane and acidogenic potential of agri-food waste streams: towards the production of volatile fatty acids (VFAs).

Master's Thesis

2025

55 pages, 17 figures, 11 tables.

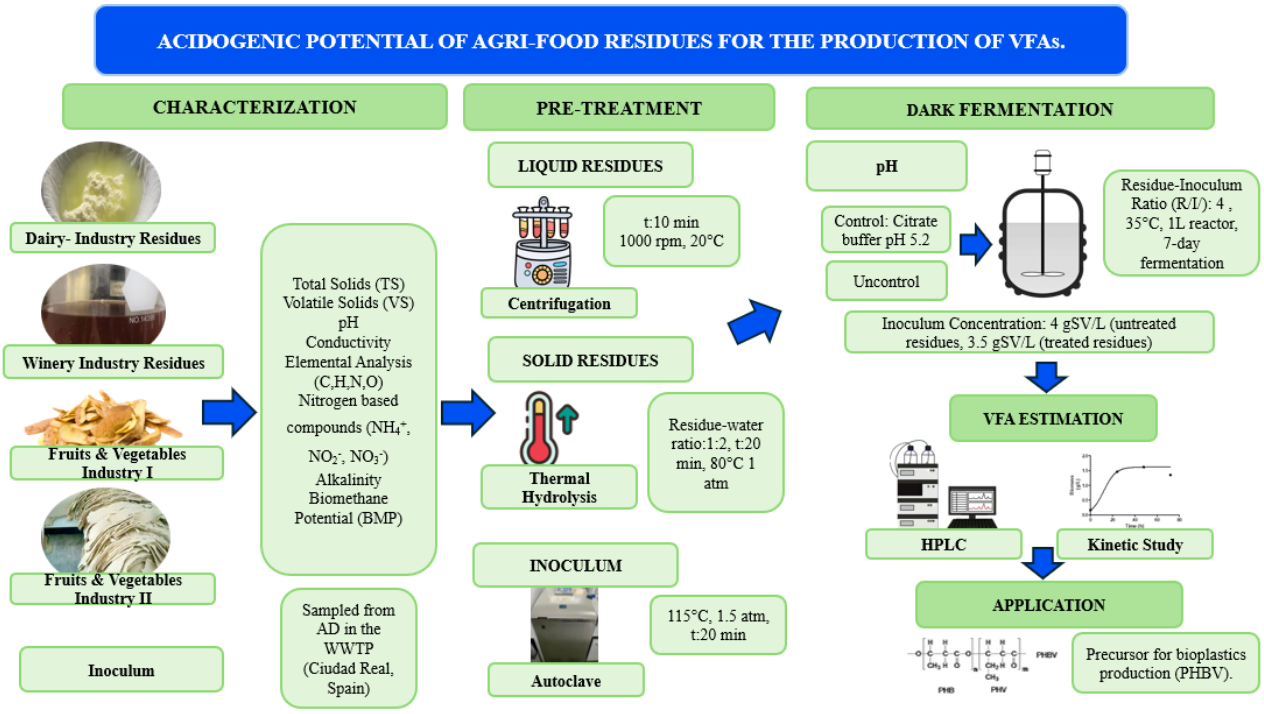
Examiner(s): Francisco Javier Pinar Pérez, PhD. (R&D CLAMBER Biorefinery), Francisco Jesús Fernández Morales, PhD. (UCLM), Tuomas Koiranen, PhD. (LUT), Samuel Pérez Vega, PhD (LUT).

Keywords: Dark fermentation, Volatile Fatty Acids, Yield, Pretreatment, Agri-Food Residues

Dark fermentation results in an appropriate alternative in the valorization of residues derived from agri-food industry, through the transformation of simple sugars into value-added products. CLAMBER R&D Biorefinery is a public Spanish research center that is currently working in the assessment of agri-food residues to produce volatile fatty acids (VFAs), which then might be used in the production of bioplastics such as poly3-hydroxybutyrate-co-valerate (PHBV). This study investigates the acidogenic potential of agri-food residues as precursors in the production of VFAs. Four agri-food wastes (AFW) (Dairy Industry Residues (AFW1), Winery Industry Residues (AFW2), and two fruits and vegetable residues (AFW3 & AFW4)) were initially characterized to determine physico-chemical and biological properties. Centrifugation and thermal hydrolysis were applied to liquid and solid residues respectively as pretreatment prior to the dark fermentation experiments, which were carried out under mesophilic conditions (35°C). Experiments were run for residues with and without pretreatment, as well as for operating conditions with and without pH control. Quantification of VFAs was performed by using HPLC Chromatography, and kinetic parameters of the study were estimated using a second-order model. Results showed an important improvement on the final VFA yield for the four residues when pH control was implemented, with a percentage increase of 423% for AFW1, 367% for AFW2, 103% for AFW3 and 33% for AFW4 in comparison with experiments without pH control. Centrifugation resulted in a proper option for pretreatment of liquid residues, with improvement of 24% for AFW1 and 53% for AFW2 in VFA yield. Thermal hydrolysis showed poor potential as a pretreatment of solid residues, with a decrease of 4% for AFW3 and 25% for AFW4 in VFA yield. The maximum VFAs yield was determined for each residue, resulting in

values of 0.927 gVFAs/gVS for AFW1, 0.668 gVFAs/gVS, for AFW2, 0.936 gVFAs/gVS for AFW3 and 0.841 gVFAs/gVS for AFW4. The results of this work provide useful information about the potential of the four tested residues, becoming products of interest for implementation in further analysis to produce PHBV.

GRAPHICAL ABSTRACT



ACKNOWLEDGEMENTS

First of all, I would like to thank God for allowing me to live this wonderful experience and for making this time a good challenge for my professional and personal development, with the ups and downs I faced along the journey.

Special thanks to PhD. Francisco Javier Pinar for his invaluable support in mentoring, monitoring, and correcting this Msc thesis, providing me with resources and useful information for the suitable development of this work, and for his warm reception during my stay at CLAMBER's facilities. Likewise, I want to express my deep appreciation for the PhD. Francisco Jesús Fernández for his contribution from his area of expertise to the successful development of this thesis.

In addition, I want to express my infinite gratitude to the CLAMBER staff who worked closely with me to make the development of this thesis possible, especially M.Sc. Marta Arriaga, PhD. Irene Izarra, PhD. Jesús del Amo, and PhD. Javier Mena, for their advice and recommendations throughout every step of the process.

To my SBBE colleagues, who were an important pillar in this adventure, and with whom I shared unforgettable moments, stressful nights, and who were a backbone in times of weakness. To all the teachers and people who contributed to the academic improvement of my professional career during my stay in Poland, Spain, and Finland.

Last but not least, I am deeply grateful to my family for being a source of encouragement despite the miles that separated us these 2 years, motivating me to keep going and persevering.

ABBREVIATIONS

FW (Food Waste)
AFW (Agri-Food Waste)
Bio-Based Industries Consortium (BIC)
Circular Bio-based Europe Joint Undertaking (CBE JU)
Dark Fermentation (DF)
Greenhouse Gases (GHG)
Anaerobic Digestion (AD)
Polyethylene terephthalate (PET)
Polyhydroxyalkanoates (PHA)
Microbial Fuel Cells (MFC)
COD (Chemical Oxygen Demand)
Volatile Solids (VS)
Hydraulic Retention Time (HRT)
CLAMBER (Castilla-La Mancha Bioeconomy Region)
Brewer Spent Grain (BSG)
Wastewater Treatment Plant (WWTP)
Refractive Index (RI)
Residue/Inoculum (R/I)

TABLE OF CONTENTS

INTRODUCTION 9

1. BACKGROUND AND THEORETICAL DESCRIPTION 11

1.1 Organic Waste Production: Agrifood Sector Case 11

1.2 Circular Economy in Agrifood Industry: Towards a Climate Neutrality 12

1.2.1 General Overview and Agri-food perspective 12

1.2.2 Drivers and Barriers 13

1.3 Value-added products and processes from bioconversion of AFW 14

1.4 Volatile Fatty Acids (VFAs). 15

1.4.1 VFAs Overview 15

1.4.2 Anaerobic metabolic pathways for VFA production 17

1.4.3 Operational parameters that affect VFAs yield. 17

1.4.3.1 pH 17

1.4.3.2 Temperature 18

1.4.3.3 Initial Organic Load 18

1.4.3.4 Retention Time 19

1.4.4 AFWs as raw material for VFAs production. 19

1.4.5 Pre-treatment technologies for VFAs production. 21

1.5 CLAMBER and context of the Master 22

2. AIM AND SCOPE 23

3. MATERIALS AND METHODS 24

3.1 Materials and Equipment 24

3.2 Experimental Methods. 25

3.2.1 Characterization of Residues & Inoculum 25

3.2.2 Pretreatment of Inoculum 29

3.2.3 Pretreatment of Residues 29

3.2.4 VFAs production via Dark Fermentation without pH control 30

3.2.5 VFAs production via Dark Fermentation with pH control 30

3.2.6 Characterization of VFAs. 31

3.2.7 Kinetics of Fermentation 32

4. RESULTS AND DISCUSSION 33

4.1 Initial Characterization of Residues 33

4.2 Dark Fermentation without pH control 36

4.3 Dark Fermentation with pH control 39

4.4 Kinetic Parameters and Optimal Conditions 40
CONCLUSIONS 44
RECOMMENDATIONS FOR FUTURE STUDIES 45
References 46
LIST OF TABLES 54
LIST OF FIGURES 55

INTRODUCTION

The global population reached nearly 8.2 billion by mid-2024 and is expected to grow by another two billion over the next 60 years [1]. This growth is accompanied by rapid economic and technological development, which leads to a massive increase in waste generation over 2 billion tons of municipal solid waste annually, estimating a rise of 70% by 2050. Most of the waste derives from developing countries, caused by the lack of trustworthy waste collection services, the restricted source separation of waste types, and the dependence on uncover landfills for discarding, resulting in garbage piles that contaminate the water, pollute the air and accelerate climate change [2]. Concerns about the expansion in the volume of waste have led to the search for treatments that allow the appropriate use of waste. Nevertheless, an appropriate treatment will depend on the waste type. In general, organic waste, including food waste (FW) and forest residues, represents the major source of waste production and is considered the main target for management. The improper management of FW leads to the release of 3.3 billion tons of CO_2 per year, representing 8% of the greenhouse gas emissions caused by human activity [3]. Several alternatives have been studied for the valorization of organic waste by different entities to develop products of high utility for different applications. Through advanced processing techniques, FW are transformed into value-added products, the most common of which include the production of bioactive compounds [3], biosurfactants [4], bioplastics [5] and energy [6], becoming a revolutionary solution for waste reduction and the generation of new economic opportunities.

The European Union, in cooperation with the Bio-Based Industries Consortium (BIC), developed the Circular Bio-based Europe Joint Undertaking (CBE JU) initiative, with around €2 billion to finance projects in circular bio-based industries in Europe. The development of renewable bio-based products and materials contributes considerably to the goal of climate neutrality proposed for 2050, while creating green jobs and promoting sustainable economic development in Europe [7]. Additionally, CBE JU pretends to unlock the future of Europe's circular bioeconomy by focusing on innovative solutions to solve the technological and regulatory challenges of the bio-based sector. Nowadays, one common challenge in the field is the reduction of waste caused by the over-production of single-use plastic through the progressive transition towards biodegradable options such as bioplastics [8].

Bioplastics are bio-based materials that represent one important alternative in the replacement of fossil-based polymers, becoming a good opportunity for the protection of the planet, due to challenges related to microplastic contamination and islands of plastic formed in large bodies of water, such as lakes, oceans, and seas [9]. Currently, dark fermentation (DF) has proved to be a suitable option to produce Volatile fatty acids (VFAs), which are the main precursors of polyhydroxyalkanoates (PHAs), a family of biodegradable bioplastics that includes polyhydroxybutyrate (PHB) and poly3-hydroxybutyrate-co-valerate (PHBV) [10].

PROMOFER is a project funded by CBE JU, having as a central point the production of bioplastics from two types of feedstocks (food-waste and lignocellulosic material) [11]. The Regional Institute of Castilla-La Mancha for Agri-Food and Forest Research and Development (IRIAF) consists of eight different research centers dedicated to the study of activities and procedures related to the forest and agri-food sectors. Among these centers is CLAMBER R&D

biorefinery [12], a research center whose main aim is the valorization of lignocellulosic and fermentable biomass to develop high-added-value bioproducts and to scale bioprocesses up to the demonstrative phase. This center is one of the partners within the PROMOFER project working group, and its main role in the project is the lab-scale optimization of VFA production and PHBV at a demonstrative scale. As part of the research carried out in CLAMBER, this master's thesis was developed to identify potential agri-food wastes that can be used as carbon sources to produce VFAs via dark fermentation and the effect of the pretreatment of the substrate in the final VFA yield. Initially, this report analyzes the potential of four different wastes to produce VFAs when temperature is the sole control parameter, comparing the results when the substrate is pretreated with the non-pretreated one. Later, the effect of pH is studied to determine improvements in the VFA yield. Finally, it is expected to assess the mixture of different substrates as an alternative to enhance VFA production.

1. BACKGROUND AND THEORETICAL DESCRIPTION

This part of the document gathers the essential information to grasp the context and final objective of the master's thesis.

1.1 Organic Waste Production: Agrifood Sector Case

The constant increase in the worldwide population leads to a higher demand in food products, resulting in the generation of biowaste. The current biowaste production from agricultural practices, primarily agrifood waste (AFW) and forestry residues, is projected to be 140 Gt/yr [13]. While some residues are used for agronomic applications, most of them end up in landfills or dumpsites for uncontrolled burning [14]. The proper management of these large waste streams poses a significant challenge for governments, which must invest in alternatives to mitigate the impact of waste on the earth. Additionally, approaches for suitable AFWs management are neglected due to harmful environmental impacts associated with high nutrient content, producing dioxins during combustion [15]. For that reason, the real discussion focuses on how to optimize the necessary amount of food without exhausting resources.

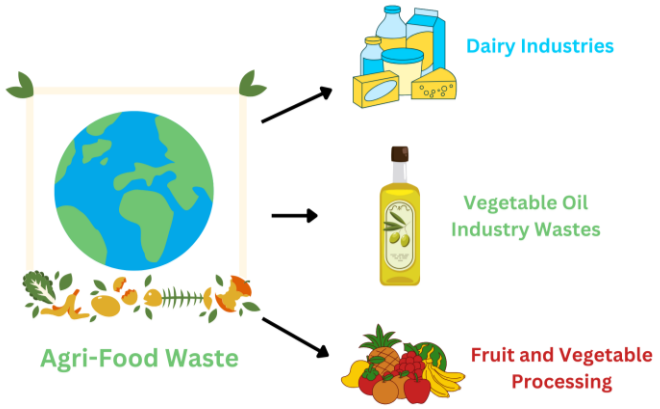


Figure 1. Main types of AFWs found in industry**.**

AFWs comprise a diversity of materials, generally coming from dairy industries, vegetable oil industry, and fruits and vegetable processing (Figure 1), including bagasse, husks, molasses, shells, spent grains, straw, peels, plant debris, whey, and animal and poultry manure. The agri-food sector has a substantial impact on environmental damage and greenhouse gas (GHGs) emissions at every stage, from production, processing, and consumption, to its end as waste, representing approximately 19-29% of the global GHGs production [16]. Processing and packaging contribute to the loss of food due to contamination or inappropriate packaging, being also the least affected stage of the food chain. Meanwhile, during the distribution, food products lose their quality due to the spoilage caused by the inefficiency in cooling storage or during transportation, resulting in food unfit for consumption. On the other hand, human consumption contributes to the largest quantity of waste within the food chain, mainly caused by food overbuying, improper storage and food expiration. Moreover, proper management of AFW requires economic investment, making it more affordable to deposit food waste in landfills instead of developing a zero-residue strategy [17]. Regardless, AFWs have a high potential in the extraction of plant-based chemicals, especially from fruits and vegetables, including active biomolecules, which include carotenoids, flavonoids, and phenolic acids, providing a wide

range of right options for applications in different sectors, with a focus on pharmaceutical, cosmetic, health, and food industries.

1.2 Circular Economy in Agrifood Industry: Towards a Climate Neutrality

1.2.1 General Overview and Agri-food outlook

The circular economy is becoming a topic of interest not only in industry, but also in academia and policymaking, as an alternative system that fights against the augmentation of emissions and waste. The central idea of the circular economy is based on a production and consumption model, involving activities such as reusing, renovating, and recycling the available materials and products at maximum potential to reduce waste, as a replacement of the traditional linear economy, based on the single use of products that eventually end up as unusable waste [18]. With the purpose of adopting more sustainable development strategies, the agri-food industry needs to follow the circular economy principles to confront potential obstacles related to recycling and the recovery of resources [19].

The agri-food industry supply chain differs from other industries due to exclusive characteristics that make it different to manage. For example, almost every stage of the supply chain is subject to time limitations: crop growth is dependent on the season. Additionally, it takes time the collection of both crops and livestock resources. Also, the rapid expiration of crops, livestock resources, and their products leads to specific treatment in storage and inventory. Furthermore, the product's quantity and quality is affected by biological variations, as well as possible hazards caused by the weather and pests [20]. Apart from that, agricultural processes frequently produce undesirable co-products that are then disposed of in the trash, for example, fertilizers, stems, and leaves from crops. Considering the presence of such waste, the agri-food sector might take advantage of the adoption of the circular economy, in both environmental and economic terms. On the environmental side, circular economy can reduce the GHG level and improve the fertility of the soil [21]. On the economic side, residues might be valorized to transform biomass into biofuels, as well as a precursor of manure-based organic fertilizers [22]. From the above, it is possible to identify the capacity for self-sufficiency of the agri-food industry, thanks to the diversity of the processes, making it a good candidate for the application of the circular economy model [20].

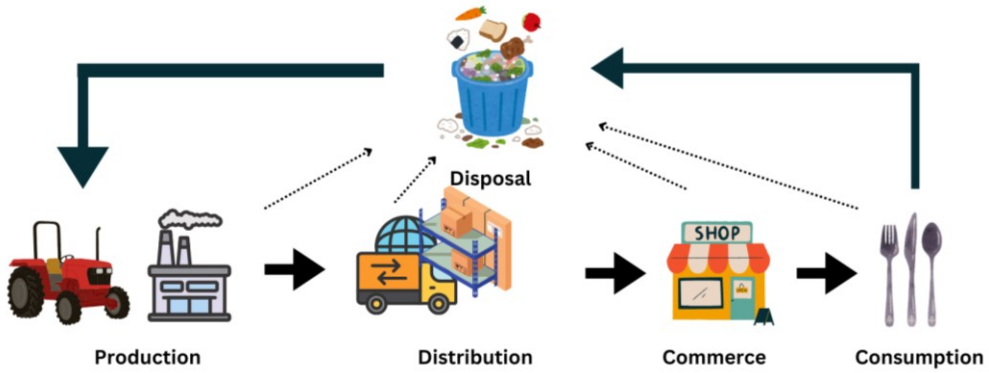


Figure 2. Representation of Agri-food supply chain.

The main elements in the agri-food supply chain are known production, distribution, commerce, consumption, and final disposal (Figure 2). The exploration of these elements permits the identification of the potential of the supply chain as a business strategy and an optimal approach for residue minimization, ensuring efficiency not only in agri-food processing, but also in food security [23].

1.2.2 Drivers and Barriers

Food waste production represents the main issue in the current agri-food supply chain. Likewise, Food waste is also an area of high interest in the circular economy worldwide, being directly correlated with environmental, economic, and social impacts [18]. Progress towards shift to a circular economy involves the identification of the drivers and barriers present during the application of the model. Generally, limited progress throughout the transition is determined by barriers related to "harder" and "softer" factors. More complex factors are classified as critical aspects that influence the transition and are easier to define, including technical and economic issues, while softer factors include those elements that also affect the correct implementation of the model, however they are difficult to describe, since they depend more on history and tradition, such as cultural and regulatory issues (Table 1.) [24].

Table 1. Drivers and Barriers in the implementation of a circular economy model. Source: [24].

FACTORS		**DRIVERS**	**BARRIERS**
COMPLEX	Technical	Accessibility of Technologies.	Unsuitable technology. Inadequate training and technical support.
	Economic	Demand-side trends. Supply-side trends.	Large capital requirements. Unpredictable profit and return.
SOFTER	Cultural	Rising in environmental legislation and standards.	Lack of incentives. Deficient institutional structure.
	Regulatory	Social Awareness. Environmental knowledge. Modifying consuming preferences.	Non-flexibility in consumer behaviour and business routines.

The categories mentioned in the previous table are not exclusive to themselves. Commonly, the contributions from different factors allow transition towards the circular economy in function of local conditions. One example of that is the European Union, where it is evident not only the economic development, but also innovation, research, and even the level of institutional quality [24]. In general, Agri-food supply chain complexity is higher than that of other supply chains and promotes a circular supply chain in the agri-food sector. Future research should focus on the integration of the principles of the Circular Economy throughout various stages of the supply chain by using robust empirical evidence. Hence, upcoming research needs to have a focus on the final stages of the supply chain, since it is the section with the highest production of waste, but at the same time, the least explored section in terms of circular economy [25].

1.3 Value-added products and processes from bioconversion of AFW

Historically, AFWs have been managed through traditional methods, which include the conversion into biofuels, animal feed, and organic manure. However, new strategies and techniques are now being implemented to convert AFWs and their co-products into value-added products. During the process of valorization of residues, food security is assured, as well as sustainable agriculture and revenue generation [26]. Through advanced techniques, bioactive compounds found in AFW might be either extracted or transformed into value-added products (Figure 3), helping in the minimization of agri-food loss, and even in the reaching of the zero-waste goal, becoming a benefit for companies, consumers, and society in the long term [27].

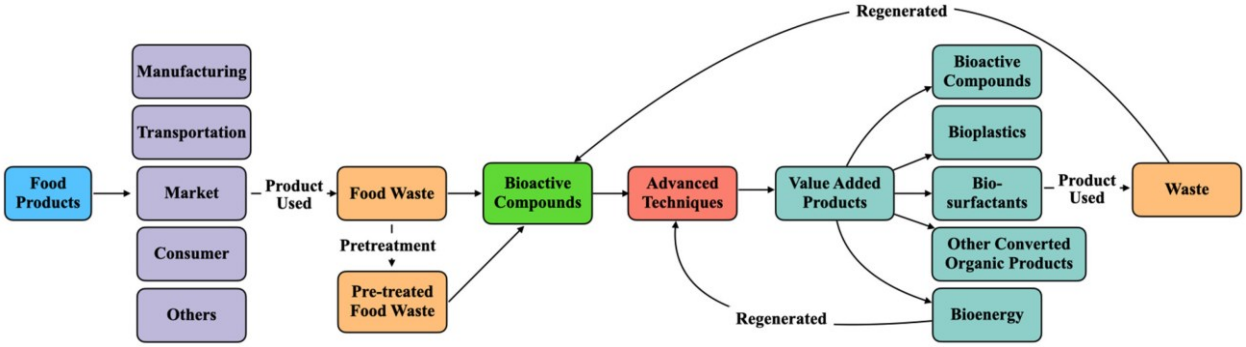


Figure 3. Opportunities of conversion of AFWs into value-added products. [3]

Depending on the final product to be generated, different biological, thermochemical, and physico-chemical technologies have been studied for years with the purpose of mitigating the effect of the agri-food sector on natural ecosystems and the environment [28]. Table 2 highlights most of the technologies currently used in industry to obtain value-added products.

Table 2. Available technologies used for valorization of AFWs. [15]

BIOLOGICAL	THERMOCHEMICAL	PHYSICOCHEMICAL
• Anaerobic Digestion: Biogas, Biofertilizers. • Fermentation: Organic acids, alcohols, and VFAs. • Composting & Vermicomposting: Organic Fertilizers	• Pyrolysis: Biochar, bio-oil, syngas • Gasification: Syngas • Hydrothermal Carbonization: Hydro char • Torrefaction: Biofuels	• Supercritical fluid extraction: Polyphenols • Solvent Extraction: Essential oils.

The use of one technology or another will depend on the desired end-product, and the variety of technologies gives the option to choose the ideal selection for the valorization of a specific residue. Several studies agree on the use of fermentation and anaerobic digestion (AD) as the most proper technology in AFW management. From these processes, it is possible to obtain alcohol, organic acids, and even biofuels with high calorific value, such as biomethane and biohydrogen [29]. Moreover, researchers show a big interest in the potential of fermentation technology due to the presence of additional value-added products, for example, VFAs (which

are precursors in the production of bioplastics), enzymes, bio-surfactants, and biochar [30]. Nevertheless, the remaining technologies have the same level of importance and applicability in industrial processes, considering each one is designed to obtain detailed products. For example, thermochemical processes are mainly addressed to the production of energy, becoming a good valorization alternative due to the use of the whole residue to generate value-added products, offering high energy efficiency in shorter times [31]. On the other hand, physicochemical technologies lead to the recovery of high-value compounds, including antioxidants, polyphenols, essential oils, vitamins, and other types of molecules, which are then used to produce fragrances, pharmaceuticals, adhesives, etc. [15].

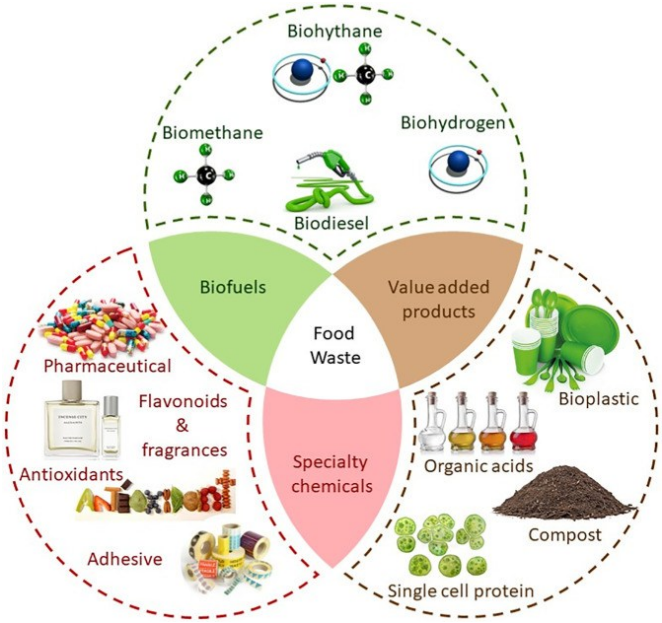


***Figure 4*. Bioconversion of AFWs and their variety of products.** [29]

Figure 4 summarizes the diversity of products obtained from the conversion of AFW. The implementation of technologies mentioned above reinforces the idea of the potential of AFWs as a precursor of valuable products. In fact, biorefineries consider the establishment of integrated systems to take full advantage of the potential of waste [13]. Most of the research is focused on the integration of biological and thermochemical processes, with the goal of valorizing the sequence by-products, nevertheless, this approach is still in a development phase. The main challenge is based on the engineering scaling necessary for competitiveness with well-defined industries and the adoption of these technologies to produce valuable products [32].

1.4 Volatile Fatty Acids (VFAs).

1.4.1 VFAs Overview

VFAs, or volatile fatty acids, consist of short-chain fatty acids commonly found in nature, with the advantage of being distilled at atmospheric pressure and with high solubility in water. VFAs are also denoted by other terms, for example, low-weight carboxylic acids and volatile organic acids [33]. Table 3 presents a list of the most common VFAs found in nature, as well as some of the characteristics of these compounds.

***Table 3*.** Properties of Volatile Fatty Acids (VFAs) [34]**.**

Common Name	Formula	Structure	Molecular Weight (g/mol)
Formic	CH_2O_2	HCOOH	46.03
Acetic	$C_2H_4O_2$	CH_3COOH	60.05
Propionic	$C_3H_6O_2$	$CH_3–CH_2$-COOH	74.08
Isobutyric	$C_4H_8O_2$	$CH_3–CH(CH_3)$-COOH	88.11
N-butyric	$C_4H_8O_2$	$CH_3–(CH_2)_2$-COOH	88.11
Isovaleric	$C_5H_{10}O_2$	$CH_3–CH(CH_3)-CH_2$-COOH	102.13
n-valeric	$C_5H_{10}O_2$	$CH_3–(CH_2)_3$-COOH	102.13
Isocaproic	$C_6H_{12}O_2$	$CH_3–CH(CH_3)$ $–(CH_2)_2$-COOH	116.16
Hexanoic	$C_6H_{12}O_2$	$CH_3–(CH_2)_4$-COOH	116.16
Heptanoic	$C_7H_{14}O_2$	$CH_3–(CH_2)_5$-COOH	130.18

The most industrially produced VFAs include acetic, propionic, and butyric acid. During the year 2020, the global demand for these acids was projected at 18.5 Mt [35]. Acetic acid, for example, is used in several sectors, such as the food industry in the form of vinegar, and as a preservative. In addition, it is the precursor in the synthesis of terephthalic acid, which is the feedstock to produce polyethylene terephthalate (PET) [36]. On the other hand, propionic acid is well-used in the pharmaceutical industry, mainly to obtain herbicides used in the manufacturing of perfumes. Butyric acid is used in the production of thermoplastics and in the pharmaceutical industry to synthesize drugs with powerful therapeutic effects [35]. In general, VFAs are potentially used as feedstocks for bioplastics synthesis, such as polyhydroxyalkanoates (PHAs) [2], electricity production in microbial fuel cells (MFCs) [37], and hydrogen and biofuels production [38]. Even more complex molecules might be derived from VFAs, for instance, hydrocarbons, N-acylethanolamines, or lycopene. [35]

VFA's demand has been historically supplied via petroleum-based chemical synthesis. Some of the strategies include reactions like methanol carbonylation to produce acetic acid, propionaldehyde oxidation to produce propionic acid, or butyraldehyde oxidation to produce butyric acid [33], [39]. However, the main problem lies in the availability and costs of the oil, considering that it is a non-renewable resource, as well as the recent concerns about the environmental impacts of petroleum-based resources on climate change and pollution, moving the strategy towards the use of more eco-friendly alternatives in the generation of VFAs [40]. The use of biological technologies to replace chemical alternatives is an object of study. Some of the problems lie in low process yields and high expenses compared with chemical production. The current role of researchers is focused on the assessment of strategies that improve the VFAs production, including [33]:

- Operational parameters that improve VFA efficiency.
- Pre-treatment of substrates and inoculum to increase microbial activity.
- Co-fermentation to improve the digestion potential of substrates.
- Best reactor configuration that presents the higher VFA production.

1.4.2 Anaerobic metabolic pathways for VFA production

Biologically, VFAs are produced in the AD of organic matter. AD technology involves the breakdown of biodegradable material by microorganisms' action in the absence of oxygen. This process takes place in the following stages: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. However, when the process is interrupted in the acidogenesis step, the name is modified to *dark fermentation* [35]. Figure 5 illustrates both dark fermentation and the anaerobic digestion process. In the hydrolysis step, complex polymers present in waste are reduced to low-molecular-weight substances, for instance, simple sugars, fatty acids, and amino acids. Then, in the acidogenic stage, hydrolysis products are transformed into VFAs, alcohols, and other products such as carboxylic acids, by action of multiple fermentative reactions. The focus in this work is based just on the dark fermentation route, since the study is addressed towards the production of VFAs.

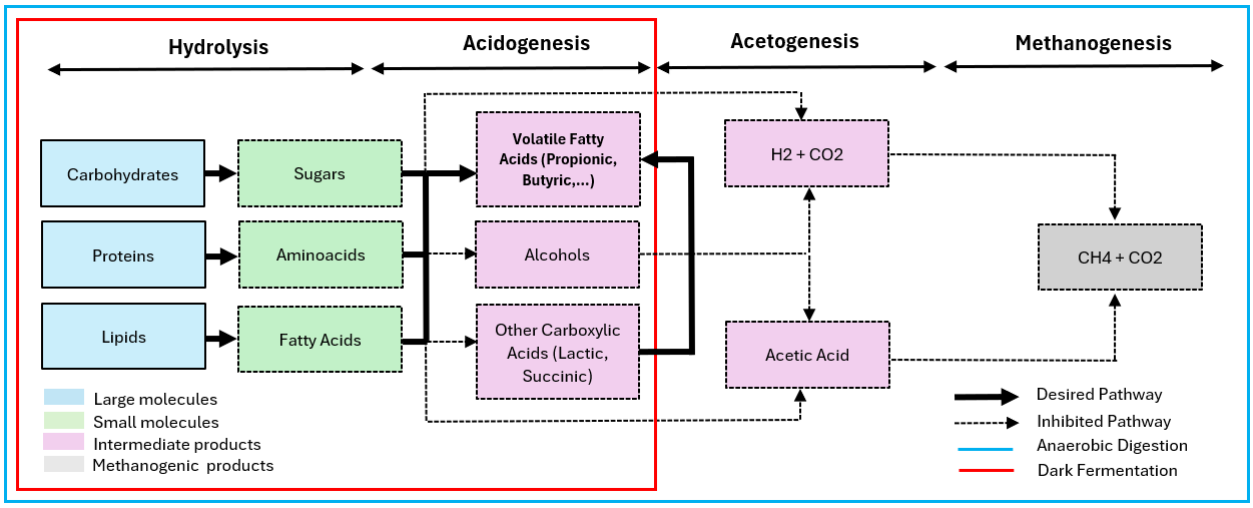


Figure 5. Anaerobic Digestion routes and steps: The desired pathway is underlined in bold, and the dark fermentation process is highlighted in red. Modified from [41].

1.4.3 Operational parameters that affect VFAs yield.

To maximize the VFA yield in a batch fermentative process, it is necessary to control operational parameters. However, this step will depend on the scope of the research. Most studies focus on the control of these four parameters: temperature, pH, initial organic load and the retention time. The above-mentioned parameters affect every step of anaerobic digestion, especially if it is desired to be selective on the pathway production of VFA, to support the inhibition of undesirable products, such as CO_2 and H_2. Every parameter will be discussed separately.

1.4.3.1 pH

The pH in the dark fermentation is one of the most important parameters to produce VFAs, due to acidogenic bacteria cannot survive under extreme pH conditions (lower than 4 and higher than 12). The lack of pH control leads to inadequacy in microbial growth, reducing the efficiency in the decomposition of substrates, causing augmentation of non-dissociated species of VFAs,

which is not desirable since destabilizing the process and decreasing the yield [42]. An optimal pH range for VFA production fluctuates between 5.3-11.0, nevertheless a more specific value will depend on the type of waste used as a substrate. For example, in the case of sludge, alkaline conditions improve hydrolysis due to the ionization of the charged groups present in the extracellular matrix of the sludge, releasing more carbohydrates and proteins to the environment, resulting in more availability of soluble substrates to produce VFA [33]. In contrast, acidic conditions in food waste and wastewater lead to high solubility of carbohydrates, proteins and lipids, with optimum values between 5.3-6.0, therefore a high production of VFAs. Additionally, the pH can improve the production of specific VFAs. For example, in ranges of pH between 4.0-4.5, propionic acid production is favored, whereas at a pH range between 6.0-6.5 it is more possible to obtain acetic and butyric acid [43].

1.4.3.2 Temperature

Temperature needs to be monitored in VFA production, since it influences the metabolic rates and the microorganism's growth. The temperature value will depend on the microbial consortium and the waste composition, working under psychrophilic (0-20°C), mesophilic (20-45°C), thermophilic (45-65°C), or extra thermophilic conditions (65-80°C) [43]. Experimental setups using mesophilic conditions are the most studied for researchers, showing improved values of VFA yield in comparison with the rest of the conditions due to hydrolytic and acidogenic enzymes show higher activity, resulting in more availability of carbohydrates, proteins and lipids to produce VFAs. In fact, mesophilic conditions are preferable to thermophilic and psychrophilic conditions, considering the lower power consumption, making the process economically more sustainable. In comparison to pH, the temperature effect over VFA production is slight. This argument tests the current idea about the effect of temperature, which states that microbial activity is highly affected by temperature and changing the focus to the use of similar microbial species at different temperatures without affecting the production of VFA [33] .

1.4.3.3 Initial Organic Load

The Initial Organic Load (OL) represents the amount of residue fed to the reactor, normally in terms of Volatile Solids (VS) or COD (Chemical Oxygen Demand). The influence of the initial OL on VFAs production has not previously been studied in detail, but the available information shows that there is no direct correlation between the variables except for the presence of optimal values [43]. For instance, in the acidogenic fermentation of agri-food residues, the VFAs production increased in an initial OL range of 5g/L – 13g/L. Nevertheless, the operation at higher initial OL values became unstable due to the increase in the viscosity of the broth, considerably affecting the efficiency of the fermentation. As conclusion, the linearity of the initial OL is preserved until the VFA production reaches an optimal, for then dropping out [44]. In continuous mode, the feeding frequency might also improve the VFA production. A study found that feeding substrates to the fermentation broth 3 times daily lead to higher VFA content compared to feeding 24 times daily [43].

1.4.3.4 Retention Time

This parameter is determined separately for the substrate (Hydraulic Retention Time (HRT)) and bacteria (Solid Retention Time). Both HRT and solid retention time are important parameters that affect VFA production, since proper control of them can reduce the loss of slow-growing microbes, beneficial in hydrolysis and acidogenesis steps for the breaking down of sugars [45]. Overall, an increase in the retention time enhances the production of VFA, however, excessive prolongation in the retention time stops the formation of VFAs. For example, in the fermentation of food waste to obtain VFAs, production increased when the retention time increased from 92 h to 196 h, but then at 288 h, there was no important formation [44].

1.4.4 AFWs as raw material for VFAs production.

Agri-food wastes (AFWs) comprise a wide variety of residues from production to consumption, such as fruits, vegetables, dairy residues, fish, etc. The potential of these residues as precursors of VFAs has been studied along with other sources of waste, for example, sludge and the biodegradable part of municipal waste. Practically any agri-food waste can be used as substrate in dark fermentation to produce VFAs [43]. However, it is necessary to take into account some points: for example, the accessibility to the raw material and the waste potential, concerning the availability of carbohydrates, proteins and lipids that are then transformed into VFAs. In this study, four different wastes are objects of interest to produce VFAs: Dairy Industry Residues (AFW1), Winery Industry Residues (AFW2), and two fruits and vegetable residues (AFW3 & AFW4) (See Table 4).

Dairy Industry Residues: Generally, residues from dairy industry are liquids with a yellow-green aspect, which comes from the preparation of cheese obtained after the precipitation of milk casein. The added value of this waste lies in the high nutritional content, principally attributed to the existence of residual proteins [46]. The biomolecules content in dairy residues mainly depends on the source of the milk used for the preparation of the cheese. In the case of whey derived from sheep's milk, the protein content is higher than in cow's whey, including β-lactoglobulin, α-lactalbumin and serum albumin. The goat whey composition changes from cow whey, with higher levels of α-lactalbumin and minimal levels of β-lactoglobulin [47]. In general, whey contains considerable carbohydrate content (such as lactose, 47 g/L), lipids (0.2 %), proteins (7 g/L), and total suspended matter (maximum 22 g/L) [48]. Molinuevo-Salces *et al*. evaluated the production of VFAs using dairy residues as substrate, and how the pH affects the fermentation process, employing a methodology that uses gas membranes. Results conclude that up to 54% of conversion is obtained under acidic conditions; however, under basic conditions, conversion of organic matter tends to decrease [49]. In general terms, Whey is a good waste source to produce VFAs thanks to the high content of fermentable organic matter.

Winery Industry Residues: The residues generated during wine production come mainly from the grape marcs, which are distilled to obtain alcohol. After winemaking process, a large portion of the marc remains unused, becoming a potential waste to be valorized [50]. They present a high content of carbohydrates, specifically glucose (410 mg/L), sucrose (1280 mg/L) and glycerol (3000 mg/L) [51]. In comparison with the other wastes of study, they might contain VFAs, for instance acetic, propionic, butyric and iso-butyric acid. For that reason, it is important

to perform an initial quantification of VFA content to measure the real production obtained during the dark fermentation.

***Table 4*.** Operating conditions used to produce VFAs via dark fermentation. Modified from [46].

Waste	pH	T(°C)	Retention time (days)	Additional Considerations	VFA Concentration
Dairy Residues	6	35	10	-	-
Fruits Residues	7	37	5	-	-Acetic acid: 0.29 g COD/ $gVS_{fed.}$ -Propionic acid: 0.18 g COD/$gVS_{fed.}$ -Butyric acid: 0.16 g COD/ gVS_{fed} Hexanoic acid: 0.89 g COD/ $gVS_{fed.}$
Vegetable Residues	-	35	5	-	-Acetic acid: 0.23 g/gVS -Butyric acid: 0.09 g/gVS Propionic acid:0.06 g/gVS
Winery Residues	6	30	7	Pretreatment of hydrolysis to Brewer Spent Grain (BSG)	-
Dairy and Winery Residues	6.5	35	48	Recirculate the obtained CO_2 and H_2 to improve propionic acid formation.	-Acetic acid: 10.6 g COD/L -Propionic acid: 3.9 g COD/L -Butyric acid: 20.4 g COD/L -Valeric acid: 3.8 g COD/L -Caproic acid: 13.0 g COD/L

Fruits and Vegetable Industry Residues: This industry represents one of the largest areas in food industry, reaching up to 350 million tons in 2020 [46]. Therefore, waste production is a factor to consider, since it might cause environmental pollution. For that reason, it is important to find strategies to minimize the waste volume. Several studies confirm the high potential of fruits and vegetable residues as feedstock for producing value-added products. For example, Liang et al performed a characterization of potato peels, with results concluding that peels contain a high value of carbohydrates (63%), proteins (17%) and lipids (1.2%) [52]. This confirms that organic waste that comes from the processing of fruits and vegetables is an optimal carbon feedstock in a fermentation process to obtain VFAs. By performing a dark fermentation, the hydrolysis and acidogenesis steps take place, promoting the formation of VFAs, and being even more valuable than the final products of methanogenesis, for example, CO_2 and CH_4.

Starch: It is the second most common biopolymer in nature, serving as a key ingredient in food, pharmaceutical and paper manufacturing industries. Starch comes from different sources, including cassava, corn, barley and potato, and may be utilized for VFA production. Starch is the main molecule in tubers, representing 80 % of dry solids and 20% of total mass [53] Several studies confirm the potential of starch as carbon source for VFA production. For example, Bhargav *et al* tested samples starch thermally treated to assess the impact on the production of VFAs, resulting in higher yield (0.482 mg VFA/mg COD) for a retention time of 2 days, having acetic acid and butyric acid as dominant products [54]. Mohadmania *et al* valorized starch using anaerobic acidification to produce VFAs. Results displayed an acetic acid yield of 0.23g/gVS for a retention time of 5 days [55].

1.4.5 Pre-treatment technologies for VFAs production.

Pretreatment methods are commonly used to eliminate inhibitors and inert matter and increase the solubilization of organic matter to enhance the hydrolysis step, which affects directly the entire fermentation process, then increasing the final concentration of VFAs [43]. Typical pre-treatment methods include physical, chemical, thermal and biological techniques.

- *Physical Pretreatment:* This type of treatment is needed in fermentation because it enhances the physical properties of the substrate, making the organic matter from complex biomass easier to digest in shorter retention times. Physical pretreatment not only decreases the substrate size but also raises the contact area with the available material, improving the fermentation efficiency, and reducing the mass and heat transfer rate related to large particles [33]. Some techniques include chipping, grinding, cavitation, ultrasonication and centrifugation. The main disadvantage of physical treatment is energy consumption and the inability of lignin remotion, making necessary high investment and the use of additional pre-treatment methods, such as thermal, chemical or biological [56].
- *Thermal Pretreatment:* This method breaks the hydrogen bonds of cellulose, allowing access to a larger surface area for microorganisms to consume organic matter, therefore increasing the efficiency of the fermentation [15]. Conventional heating, steam explosion, and thermal hydrolysis are some examples of thermal pretreatment. Nevertheless, experiments with high temperatures might promote the generation of harmful phenolic molecules, for example 5-hydroxymethyl-2-furfural, inhibiting hydrolysis, and reducing the VFA production [33].
- *Chemical Pretreatment:* The use of acids and alkalis help to break the cross-linkages of the biomass and reduce the crystallinity of cellulose [57]. This improves the digestibility of the substrate and improves the VFA production. However, the formation of inhibitory compounds like furfural, hydroxymethyl furfural, ferulic acid, p-coumaric acids and organic halogens represent an obstacle in the fermentation process, because it requires an additional step to remove pollutants [33].
- *Biological Pretreatment:* This technique is the most selected for pretreatment of biomass, with several advantages that include lower financial investment, working operation under mild conditions, low formation of inhibitory compounds and no post-treatment for chemicals removal [58]. The principle of this pretreatment is the use of microorganisms or enzymes to break the lignocellulosic matrix of biomass, resulting in lower retention time during dark fermentation. The main problem lies in the large area necessary for pretreatment;

however, this issue is compensated for by the benefits offered in terms of economy, environment, and eco-friendliness [59].

1.5 CLAMBER and context of the Master

R&D CLAMBER (Castilla-La Mancha Bioeconomy Region) Biorefinery is a public demonstration facility founded in 2015 and focused on scientific research, execution of scale-up experiments, and development of new bioprocesses and bioproducts based on the use of wet fermentable or lignocellulosic biomass. Likewise, the biorefinery was designed to carry out research on the physical, chemical and biological processes performed in a near industrial-scale biorefinery, in order to determine whether the processes of transformation of biomass into bioproducts of interest is technically and economically feasible, as well as to establish the optimal operating parameters of such bioprocesses for the subsequent design and construction of the industrial-scale plant.

Currently, CLAMBER is working on the development of several projects, mainly focused on the valorization of lignocellulosic waste and wet fermentable biomass to produce value-added products by using different technologies, such as extraction, steam explosion, and fermentative processes like aerobic and anaerobic digestion. PROMOFER is an ongoing project in CLAMBER, and it aims to use specific biobased wastes from different industries, generated in significant amounts in Europe, to be valorized into PHBV and 2,3-butanediol. The role of CLAMBER in this project is the lab-scale optimization and validation of VFA production from agri-food streams by dark fermentation, for then being scaled up to demonstrative scale and used as a source to PHVB production. To accomplish this purpose, it is necessary to identify, assess, and pretreat the agri-food streams that will be used as a substrate in the fermentation process, and identify the suitable operational conditions (pH, T, etc.). This step is crucial for understanding the process and determining the possibility of its industrialization in the near future.

2. AIM AND SCOPE

This study aims to assess the potential of four industrial agri-food residues (Dairy Industry Residues (AFW1), Winery Industry Residues (AFW2), and two fruits and vegetable residues (AFW3 & AFW4), for their valorization as raw materials in a fermentative process to obtain volatile fatty acids (VFAs), and how the substrate pretreatment might enhance VFA yield, which then will be employed as feedstock in the production of BHPV.

Having established the aim of the project, the following outlines the scope of the study:

- The pretreatment of the residues used as substrate in the production of VFAs and the comparison of the final VFA yield obtained with a non-treated residue.
- The effect of pH control on the VFA production and the mass distribution of acids in the final mixture.
- The lab-scale study of the fermentation of agrifood residues at acidogenesis conditions to produce VFA in 1L discontinuous reactors.

3. MATERIALS AND METHODS

The materials and methods section highlights the analytical techniques and the procedures necessary to meet the objective of the present research work. All the experimental section is conducted in the CLAMBER R&D Biorefinery, located in Puertollano (Castilla-La Mancha, Spain).

3.1 Materials and Equipment

Tables 5 to 6 summarize the reagents, materials, and equipment used to carry out the project.

Table 5. Reagents and materials employed in experimental methodology.

Material/Reagent	Formula	Purity	Supplier	Origin
Type I water	H_2O	Conductivity 0.056 μs cm^{-1}	See Table 6	Spain
Nitrogen	N_2	100%	Carburos Metálicos S.A	Spain
Sulfuric Acid	H_2SO_4	95%-97%	Scharlab	Spain
Volatile Fatty Acids Standard	Different Acids, see Table 3	10 mM	Sigma- Aldrich	USA
Hydrochloric Acid	HCl	37%	Scharlab	Spain
Citric Acid Anhydrous	$C_6H_8O_7$	>99.5%	Scharlab	Spain
Tri-sodium citrate dihydrate	$C_6H_5Na_3O_7 \cdot 2H_2O$	99.0%	Scharlab	Spain
Sodium Hydroxide	NaOH	99.5%	Scharlab	Spain

Table 6. Equipment used in the experimental section.

Equipment	Manufacturer	Model Type	Additional Information
Milli-Q Integral Water Purification System	Millipore	5 A 10	Water Type I for HPLC Analysis
HPLC Chromatograph with autosampler	Hitachi- Shimadzu	5260	Integrated Wash Pump + holds 100 1.5 mL vials & refraction index detector.
Incubator with forced convection	Binder	BF53	Range Temperature from 0°C to 100°C.
Centrifuge	Orto Alresa	Digtor 22 R	4 x 750 mL with a maximum speed of 14300 rpm.
Stirrer Agimatic	Selecta	ED-C	Temperature Control
Multi-parameter modular system	HANNA	HI-6000	Measurement modules for pH/ORP, pH/ORP/ISE
Autoclave for steam sterilization	Selecta	4001758	Range Temperature from 115°C to 134°C.
Drying Oven	Selecta	2000251	Range Temperature from 40°C to 250°C.
Muffle Furnace	Selecta	R-8-L	Range Temperature from 25°C to 1100 °C
GC Chromatograph	Shimadzu	2030	FID Detector
Precision Scale	VWR	LP4202-C	Maximum Capacity: 450 g.

3.2 Experimental Methods.

3.2.1 Characterization of Residues & Inoculum

The residues used in the dark fermentation process were initially characterized, determining volatile solids (VS), total solids (TS), pH, and conductivity (for liquid samples). This step was important because it helped to determine the quantities to be fed into the reactor according to the operational conditions defined and explained in section 3.2.5. Additional parameters such as nitrogen-compounds tests, elemental analysis, biochemical methane potential (BMP), and chemical oxygen demand (COD) were also tested. Experiments to determine VS and TS were performed using the internal procedures defined by CLAMBER Biorefinery [60], based on international standards APHA 2540, USA.

Figure 6. Drying Oven (left) and ***Figure 7.*** Muffle furnace (right) used for VS and TS determination.

Total Solids (TS): Total solids are the residues of material remaining in a vessel after evaporation of a sample and subsequent drying at a defined temperature. Initially, a porcelain beaker was tared at 105 °C for 2 hours in the drying oven (Figure 6). Then, the beaker was transferred to a desiccator for 20 min until it cooled down. The empty beaker was weighed, and this value was recorded as the initial weight ($W_{initial}$). Enough sample (between 2 and 3 g) was taken and deposited in the tared empty beaker, recording the weight of the sample plus the beaker as W_{sample}. Then, the beaker was introduced to the oven at 105°C for 6 hours, and after that, it was removed and cooled down for 20 minutes in the desiccator. Finally, the beaker was weighed with the final sample, recording this weight as $W_{finalTS}$. TS is then calculated using Equation 1:

$$TS(\%) = \frac{W_{finalTS} - W_{initial}}{W_{sample} - W_{initial}} * 100 \qquad \text{Eq 1.}$$

Volatile Solids (VS): Volatile solids are defined as the portion of total solids that is evaporated at high temperatures (550°C), and it is used to measure the overall organic matter content in a sample. The first part of the procedure to obtain VS in a sample is the same followed in the determination of TS. After this, the beaker was placed in a muffle furnace (Figure 7) at 550 °C for 30 minutes, then removed and cooled down for 20 minutes in a desiccator. The beaker was weighed, and this value was recorded as $W_{finalVS.}$ VS is the calculated using Equation 2:

$$VS(\%) = \frac{W_{finalVS} - W_{initial}}{W_{sample} - W_{initial}} * 100 \qquad \text{Eq 2.}$$

pH and conductivity: For liquid samples, the pH and conductivity were measured by using the multi-parameter modular system HI-6000 (Figure 8). Measurements were performed three times.

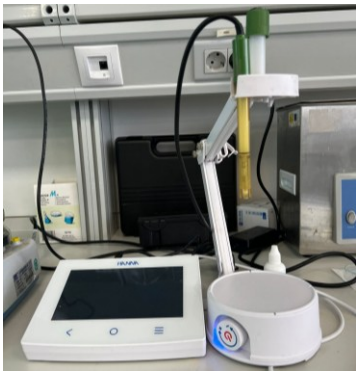


Figure 8. Multiparameter system HI-6000 for pH and conductivity measurements.

Biochemical Methane Potential (BMP): This parameter permits identifying the potential of biodegradability of biomass in terms of production of methane (CH_4), therefore, it provides a preliminary outline about the acidogenic potential of the substrate. Initial conditions are displayed in Table 7. The residue-inoculum ratio was defined according to experimental conditions proposed by Tian *et a*l [61], under batch operation mode.

Table 7. **Operating conditions used to produce VFAs via dark fermentation.**

Parameter	**Units**	**AFW3**		**AFW4**		**AFW1**		**AFW2**	
		N.P	P	N.P	P	N.	P	N.P	P
Residue-Inoculum Ratio (R/I)	gSV/gSV	4	4	4	4	4	4	4	4
Inoculum Concentration	gSV/L	4	3.5	4	3.5	4	3.5	4	3.5
Temperature	°C	35							
Reactor Volume	L	1							
Broth Volume	L	0.45							

N.P: Non-pretreatment. P: Pretreatment.

Experiments were performed under mesophilic conditions in 1L glass reactor vessels with three necks as shown in Figure 9. The center-positioned neck was used to add broth fermentation at the beginning of the process, and the lid is coupled to an OXITOP® reactor, in order to monitor the internal pressure of the reactor. The left-positioned neck was set with a lid with needle insertion, and the right-positioned neck was set with a three-way valve to take liquid samples. Calculations were estimated considering the estimation of VS and TS performed earlier, for a broth volume of 0.45 L.

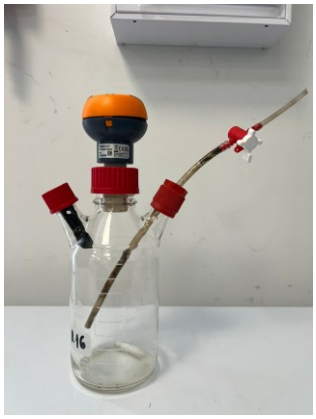


Figure 9. 1L glass r*eactor* OXITOP® *model.*

First, substrate and inoculum were weighed and mixed separately in glass beakers to homogenize the solution, and the broth volume was made up with distilled water. After that, a nitrogen stream was used to inert the reactors and ensure anaerobic conditions. The reactors were then placed in the heating chamber at 35°C with agitation at 150 rpm, and the experiments were set for a fermentation time of 6-7 days. Every experiment was repeated 3 times to ensure reproducibility of the results.

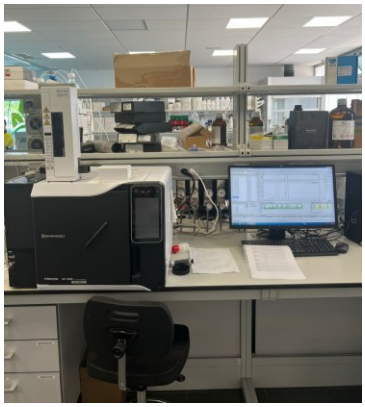


Figure 10. Gas Chromatograph GC-2030 for methane quantification.

The BMP quantification was performed in terms of milliliters of methane produced per gram of volatile solids for a specific residue, using a GC chromatograph as in Figure 10. coupled to a Flame Ionization Detector (FID).

Chemical Oxygen Demand (COD), NH_4^+, NO_2^- *&* NO_3^- *Test:* Photometric commercial methods were employed for COD quantification, nitrates, nitrites, and ammonium (Merck Darmstadt, Germany, ISO 15705, ISO 23695, ISO 6777 and ISO 23696-1, respectively).

Elemental Analysis: Samples were sent for analysis to the Laboratory of Soils and Residues located in the Institute of Chemical and Environmental Technology (ITQUIMA), in UCLM, Ciudad Real, Spain, following internal procedures.

Alkalinity: A titrimetric commercial method was applied for alkalinity quantification (Merck Darmstadt, Germany, ISO 9963).

3.2.2 Pretreatment of Inoculum

The inoculum was sampled from the anaerobic digester section in the wastewater treatment plant (WWTP) situated in Ciudad Real, Spain. The sample was stored at 4°C before pretreatment, which consisted of thermal hydrolysis using an autoclave (Figure 11). The use of thermal pretreatment inhibits the methanogenic bacteria found in the inoculum, therefore enhancing VFA production [62]. The inoculum was placed in glass bottles, leaving the lid half open to avoid over-pressurization of the bottle.

Figure 11. *Autoclave* 4001758 *for inoculum pretreatment.*

Autoclave conditions were set according to the procedure followed by Al-Haddad *et al* [63], at 115 °C for 20 min, reaching an internal pressure of 1.5 bar. Once the cycle was finished, the inoculum was allowed to temper and stored again under refrigeration for the respective experiments. A 10 mL sample was taken to measure VS and TS.

3.2.3 Pretreatment of Residues

The pretreatment of four residues was performed as an initial step in the dark fermentation to produce VFAs. The pretreatment method differed for solid and liquid residues. Solid residues (AFW3 & AFW4) were submitted to thermal hydrolysis, whereas liquid residues (AFW1 & AFW2) were pre-treated using centrifugation. Thermal hydrolysis, when applied to solid samples, aids in cellular breakdown and release of proteins and polysaccharides useful for fermentation [64]. On the other hand, centrifugation drives the removal of suspended material that is not fermentable and reduces the concentration of fats and oils from animal waste [65]. The scheme of pretreatment of residues is represented in Figure 12.

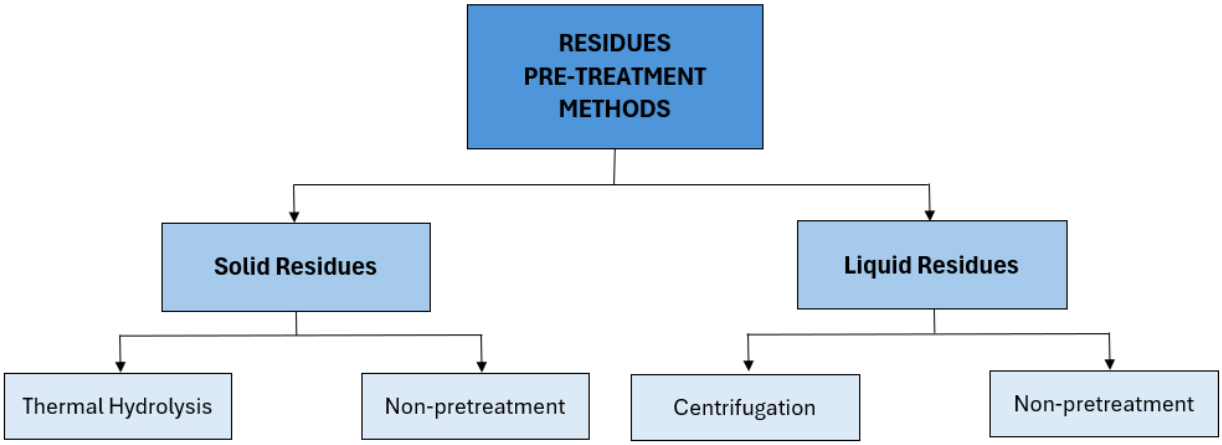


Figure 12. Pretreatment Methods Scheme for residues used in dark fermentation.

Thermal Hydrolysis: The residue to be treated was mixed with water until reaching a ratio of 1:2. The sample is placed on a heating plate with magnetic agitation at 300 rpm to maintain homogeneity of the mixture. The temperature was controlled until it reached 80°C, and this value was kept for 20 minutes. After that, water was filtered, and the pretreated sample was analyzed for VS, TS and pH measured following the procedure explained in section 3.2.1.
Centrifugation: Liquid residues were placed in 50 mL falcon tubes and positioned in centrifuge spaces. The centrifugation cycle was set at 1000 rpm for 10 min. The supernatant was discarded, and the liquid was analyzed for VS, TS and pH following the procedure explained in section 3.2.1.

3.2.4 VFAs production via Dark Fermentation without pH control

The procedure to produce VFAs via dark fermentation was followed as for the estimation of BMP in section 3.2.1. This section of the document differs from BMP methodology in the analysis of the samples, since it was intended to determine the daily and final production of VFAs as a first approach to the acidogenic potential of the residues.

3.2.5 VFAs production via Dark Fermentation with pH control

This section was intended to determine the impact of pH control on the final VFAs yield result. As mentioned in section 1.4.2, pH between 5.0 and 6.0 enhances the acidogenesis; therefore, it was important to find a proper buffer that keeps the pH in the suitable region for VFA production. A citrate buffer solution with pH 5.2 ± 0.1 was prepared according to instructions provided by the provider of reactants used in the preparation of the buffer (Scharlab, Spain). Prior the performance of the experiments, a preliminary test was conducted to assess the performance of the buffer over the whole length of the fermentation. Figure 13 shows the experimental setup to monitor the pH behaviour.

Figure 13. Experimental Set-up for dark fermentation with pH control**.**

The preparation of the samples was followed as in section 3.2.1 under the same conditions, with the difference in the addition of the buffer solution, equivalent to 70 mL per reactor. As the buffer did not act immediately, pH was first adjusted to pH 5.2 using either HCl or NaOH, depending on the case.

3.2.6 Characterization of VFAs.

During the 7 days of the acidogenesis fermentation, reactors were monitored, taking daily samples of 5-10 mL to track the VFA production and VFA profile. pH was measured using the multi-parameter modular system. The VFAs obtained during fermentation were quantified using a HPLC Chromatograph with Refractive Index Detection (RI) (Figure 14) and a column Agilent Metacarb 67H 300x6.6 mm.

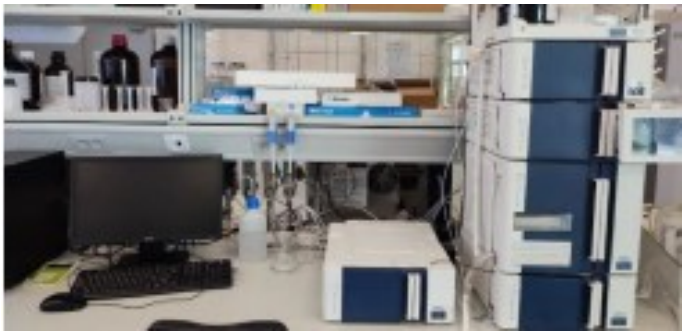


Figure 14. HPLC equipment for VFAs characterization.

The operational conditions of the equipment were established as follows: temperature of 65 °C, a 0.65 mL/min flow, an injection volume of 10 uL, and a mobile phase consisting of a H_2SO_4 solution 0.01 M [66]. The solution used as reference was a volatile free acid mix solution (Sigma Aldrich, USA). The reference solution includes all the acids mentioned in Table 3, with a concentration of 10 mM for each single acid. An initial standard sample was injected to identify every single acid peak, and then several dilutions of 1 M, 2 M, 4 M, 6 M, 8 M, and 10 M were prepared to draw the calibration curve for VFAs calculations. The preparation of fermentation samples was done in a dilution 1:2, and after that the samples were filtered with disposable syringe filters of 0.22 micron and placed in HPLC vials.

3.2.7 Kinetics of Fermentation

The experimental data were fitted following a second order model, as in Equation 3. Y represents the VFA production efficiency in terms of yield (gVFAs/gVS), k'' represents the second-order production rate constant (h^{-1}), and Y_{max} is the maximum possible yield. The Matlab® R2025a (MathWorks, Massachusetts, U.S) software was implemented to adjust the experimental data to the second order model, using the tool *curve fitter.*

$$Y(t) = \frac{k'' * Y^2 * t}{1 + k'' * Y_{max} * t} \quad \text{Eq 3.}$$

The second-order model is widely used to fit data in experiments for VFA production, easy to implement and provide trustable results with high accuracy, optimal for exponential behaviors in experimental data [67].

4. RESULTS AND DISCUSSION

The results and discussion section displays the outcomes obtained after assessing the acidogenic potential of the following agri-food residues: AFW1, AFW2, AFW3 & AFW4. The information is organized in four sections, starting from the initial characterization of the treated and non-treated residues before acidogenic test execution. Then the results of the dark fermentation without pH control are exhibited, including the pH behaviour, the VFA yield and the mass distribution of VFAs. The third section includes the results for the dark fermentation experiments with pH control, also displaying the results for VFA yield and distribution of VFAs as in the previous section. The final section summarizes the metabolic pathway and kinetic parameters extracted from the dark fermentation experiments. These results provide information that represents the potential of the residues to produce VFAs, which will serve as a starting point for scaling up the process in the future.

4.1 Initial Characterization of Residues

A complete characterization of the raw residues was performed to identify the initial properties of the residues. Table 8 summarizes the results of the initial characterization for the four raw residues. For the treated residues, it was necessary just the estimation of VS, TS and pH, which are displayed in Table 9.

Table 8. Initial Characterization for untreated residues.

Parameter	AFW1	AFW2	AFW3	AFW4
Total Solids (TS) %	6.6	2.6	14.0	44.0
Volatile Solids (VS) %	6.2	2.2	13.1	43.7
pH	4.8	3.7	6.9	6.9
Conductivity (µS/cm)	4.9	4.0	0.2	0.4
COD (mg O_2/L)	89650	39900	42800	89750
C %	22.5	35.61	55.17	59.79
N %	0.95	1.69	1.50	2.03
C/N	23.7	21.1	36.8	29.5
NH_4^+ (mg/L)	12.5	66.8	11.7	83.8
NO_2^- (mg/L)	22.7	33.1	3.6	0.9
NO_3^- (mg/L)	42.0	163	64	N.D
Alkalinity (mg/L as $CaCO_3$)	1.3	1.3	0.7	1.4
BMP (mL CH_4/g VS)	402.2	232.0	485.3	428.9

N.D: Non-Detectable

Table 9. Initial Characterization for treated residues.

Parameter	AFW1	AFW2	AFW3	AFW4
Total Solids (TS) %	6.2	2.3	16.5	40.3
Volatile Solids (VS) %	5.7	1.9	13.6	40.1
pH	4.7	3.5	6.8	6.7
Conductivity (µS/cm)	0.5	0.5	0.2	0.2

A preliminary characterization provides initial information on the characteristics of the residues, as well as considerations for sample preparation, such as in the case of experiments with pH control, to determine if the pH had to be adjusted with NaOH or HCl. Likewise, VS content affects the quantities of mass residue to be used per reactor, considering if the R/I (ratio Residue/Inoculum) was to be preserved for all the experiments, it was necessary to add more residue in cases where VS content was lower (for example in AFW1 and AFW2) compared to samples with a higher VS content (AFW3 and AFW4). In industrial processes, this results in higher volumes of residue needed to produce value-added products, then more mass to treat, affecting the process in economical and design terms. Initial COD results considerably differ between residues, with higher values for AFW1 and AFW4, and lower values in the remaining residues. This information helps to identify the potential organic matter in the residues, thus possible carbon sources which will then be transformed into VFAs. Values reported in the literature for COD were compared with those obtained during the characterization of residues. For example, reported COD values for AFW1 and AFW2 present similar values with the experimental results reported in Table 8 (90000 mg/L [68] and 45000 mg/L [69] respectively). However, in the case of AFW3 and AFW4, results differ considerably with the values found in literature (approximately 10000 mg/L [70]).Several explanations might give a response to the difference in COD values, including the origin of the residue, modifying the composition in the sample, as well as the species of vegetables analyzed, changing the initial organic load [54].

The ratio C/N plays an important role in all fermentation processes, since it guarantees the proper balance of nutrients for a correct transformation of sugars into valuable products. In DF, it is reported that an ideal C/N ratio range between 15-30 enhances the acidogenesis step, then the formation of VFAs [71]. From the tested residues in this study, the only one that did not meet the requirement was the AFW3. The high value obtained for the sample mentioned above might affect the fermentation process, because the nitrogen deficiency directly affects cell growth, and the sugars are partially transformed because microorganisms cannot convert the organic matter efficiently.[54]. Nevertheless, the range mentioned above is not strictly mandatory for a suitable performance of the anaerobic fermentation, especially when it means to methane production. A study performed by Choi *et al* [72] evaluated the influence of the type of carbon and C/N ratio on the biochemical methane potential, and they concluded for experiments with C/N ratio of 40, maximum production of methane was exclusively affected by the carbon type (cellulose, starch, xylan), but production rate is affected by both C/N ratio and carbon type. On the other hand, initial pH gives information about the initial composition of the residue. For example, in the case of AFW2, the low pH might be explained by the presence of organic acids as result of alcohol distillation, including acetic, butyric and lactic acid [73]. A similar case occurs with AFW1, where microorganisms present during cheese production ferment the sugars in AFW1 to form lactic acid, gently reducing the pH in the solution. The variation on pH explains the adjustment made at the beginning on pH to keep a value in proper conditions for acidogenesis (pH between 5.0 and 6.0 [74]).

Additional parameters such as conductivity, alkalinity, and nitrogen-containing compounds content might also help to understand how the nature of the residues affects the fermentation process. For example, conductivity indicates the presence of essential nutrients and minerals for microbial growth (including nitrogen-containing compounds, Mg, and Ca), but also the presence of sub-products such as non-dissociated organic acids that must be considered when calculations are performed, leading to overestimation of VFAs [75]. Furthermore, the characterization

presented in Table 9 showed minor differences compared with the raw residues. In the liquid residues, TS and VS content reduced slightly (explained by the removal of solid particles during the pretreatment) along with pH, and the conductivity experienced a more drastic drop, without considerably affecting the properties of the residue. As in the case of the TS and VS, conductivity is affected by the ion's concentration (Na^+, Cl^-, NH_4^+) present in the suspended particles [76]. When removed during centrifugation, the decrease in conductivity will be reflected in the sample. In contrast, solid residues remained stable values for pH and conductivity, with an increase in VS and TS content for AFW3 and the opposite for AFW4. The rise in VS and TS in AFW3 is explained by the decrease in the content of inorganic fraction (mainly salts and K), which are dissolved in the water used during the thermal treatment, increasing the ratio of organic fraction in the solid sample [77]. However, in the case of AFW4, the decrease in these parameters is due to a possible gelatinization process that occurs at temperatures up to 80 °C, encapsulating the organic material, increasing the viscosity, and hindering the homogenization of the sample. A possible solution to mitigate the effect is to carry out thermal hydrolysis with pressure, allowing the breaking of the cell walls without achieving gelatinization [54].

BMP was performed to determine the potential of biodegradability of the residues, giving insights about the amount of organic matter transformed by fermentative reactions at anaerobic conditions into value-added products AFW3 have the highest value for BMP, followed by AFW4 and AFW1. Nevertheless, BMP result for AFW2 is quite low when it is compared with the other residues. A study conducted to determine the acidification risk of dairy residues reported BMP values between 389-436 mL CH_4/g VS [78], resulting in a range where the BMP obtained in this study for AFW1 fits. Another study whose main goal was the estimation of biomethane production by using winery residues as a substrate reported a range for BMP between 215-324 mL CH_4/g VS [79]. Then, BMP for an experimental setup using AFW3 as substrate reported a value of 217 mL CH_4/g VS [80], as well as the BMP value obtained during the fermentation of AFW4 was about 480 mL CH_4/g VS [81]. The main finding was the difference between the reported values and the ones obtained in this work for AFW3. These differences might be explained by the availability of biodegradable material to be transformed during fermentation, due to factors such as substrate pretreatment, as well as operating conditions, and even the nature of the substrate [71]. For example, the pH evolution during experiments in AFW3 reported in literature were affected by the pretreatment of the substrate (acid hydrolysis). The report shows local minimum between days 5-7 of fermentation, with pH values lower than 6.5, which might be caused by the presence of remained acid from the pretreatment, disfavoring the methanogenesis step, taking place the acidogenesis step, and the available sugars are transformed to VFAs rather than CH_4 [80]. In any case, AFW3 still proves its potential as an organic matter source for the production of VFAs.

Residues like AFW1 and AFW4 reflect the correlation between the results obtained for initial characterization and their potential for methane production. In the case of AFW2, the same situation occurs; however, the result for BMP is around 50% lower than any other residue. The explanation falls mainly into the chemical toxic species for methanogens. The most common includes oxygen, nitrates, nitrites, sulfides and antibiotics, these last two largely found in sugarcane fermentation (where this residue comes from), attacking the bacteria, and inhibiting the methanogenic activity [82]. Winery industry residue production yields are typically low [83], and that explains the necessity of addition of higher quantities of substrate to meet the VS

requirements described in Table 7. For AFW3, although C/N ratio is out of the recommended range for anaerobic fermentation, it does not affect considerably the methanogenic potential of the residue as explained in earlier paragraphs, since the efficiency of anaerobic fermentation for C/N>30 was acceptable, and other factors play a more important role, such as the carbon type. Therefore, C/N ratio becomes a more flexible parameter as long as the remaining operating parameters are on specifications.

4.2 Dark Fermentation without pH control

The performance of the VFA production via dark fermentation was first evaluated without pH control in the 1L discontinuous reactors. The pH profile for experiments with the residues are presented in Figure 15. Graphs tendency helps to identify the final pH reached in the reactor in the experiments performed for the tested residues. The pH stabilization time differs for every residue, and it is directly correlated with the production of VFAs. In most cases, pH rapidly dropped in the first 24 hours, until remaining constant in the value range between 3.5-4.5, caused by the presence of the VFAs produced, and indicated that DF was completed. However, these pH values are not suitable to enhance the acidogenesis step, considering they are out of the optimal range for VFA production mentioned in section 4.1.

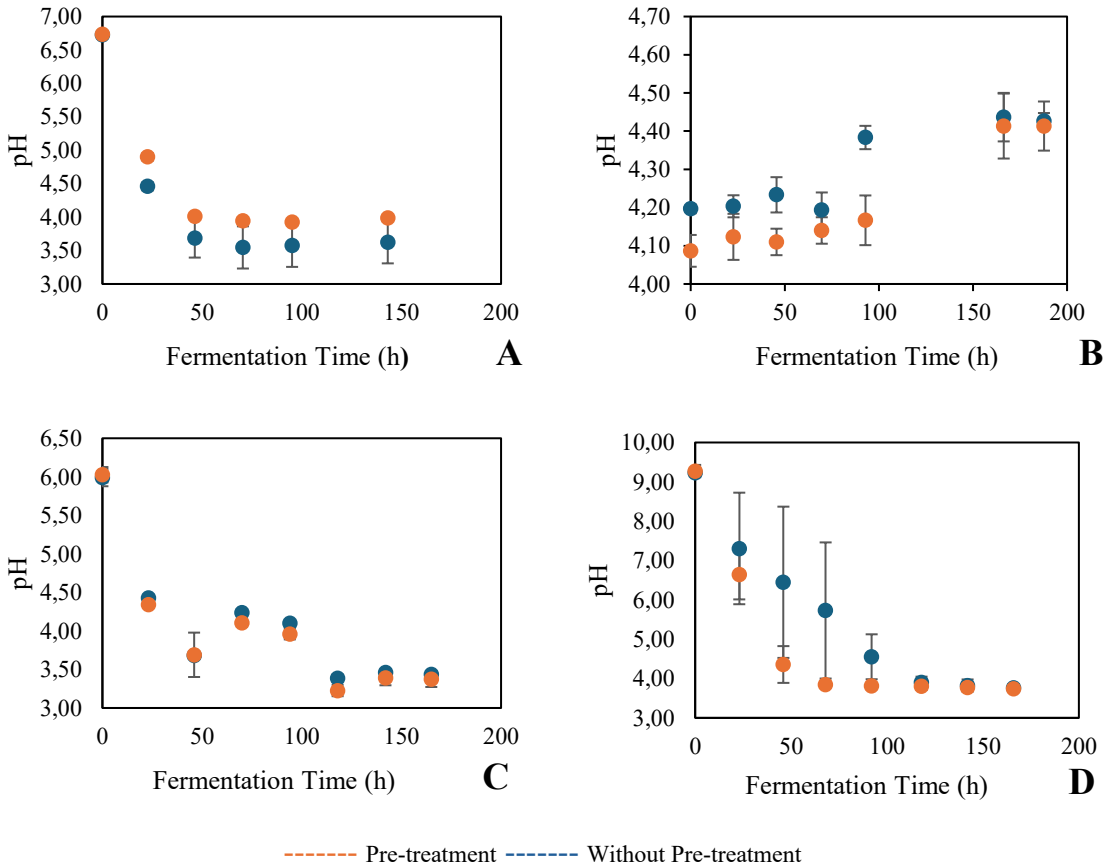


Figure 15. pH evolution during the uncontrolled pH DF. A) AFW1 B) AFW2 C) AFW3 & D) AFW4.

The influence of pre-treatment on the pH is more evident halfway through the experiment, however, in general, the effect was not strong enough to make a big difference at the end of the DF. In all cases (except AFW1), pretreatment tends to cause a minor decrease on pH compared with the samples without pre-treatment. The initial pH is also an important parameter to discuss, since in most experiments, values range from conditions close to neutrality (pH 7), meaning a low or even a lack of presence of acid compounds, except for the AFW2's experiments. An initial pH of 4.1-4.2 is proof of the existence of acids in the fermentation broth, and the additional production of VFAs did not significantly affect the pH profile, with similar values throughout the fermentation process.

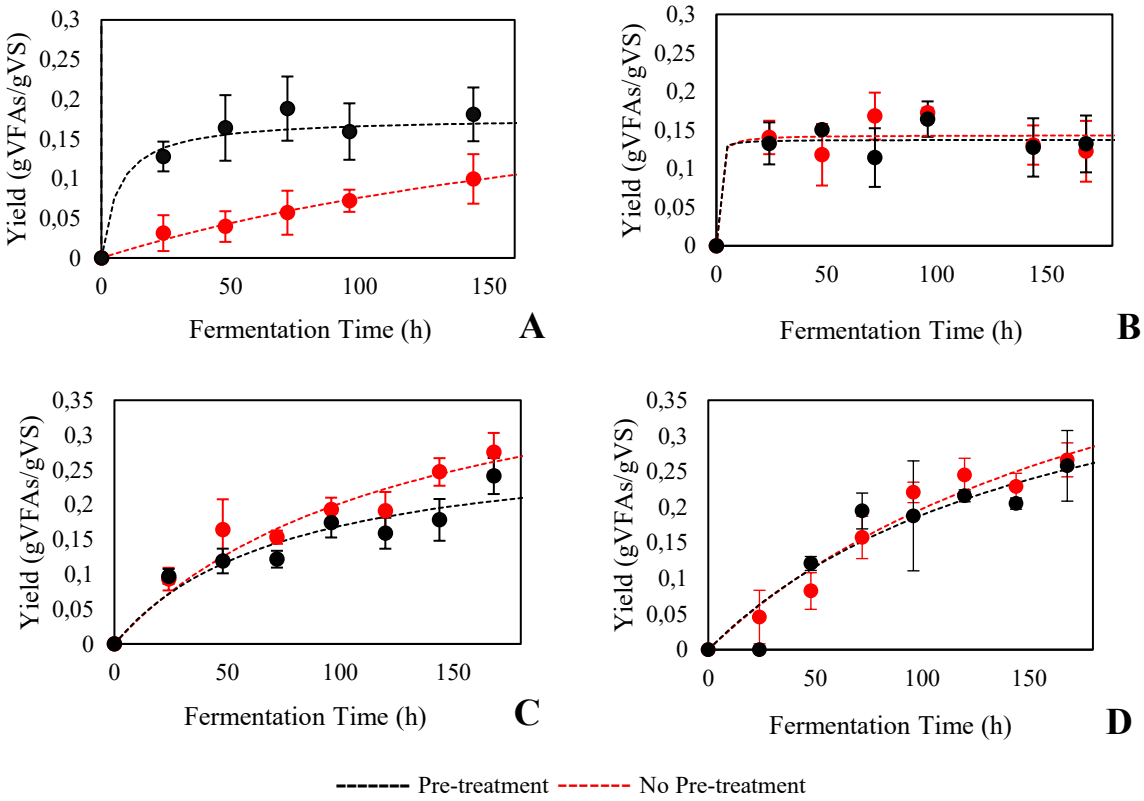


Figure 16. Yield evolution during the uncontrolled pH DF. A) AFW1 B) AFW2 C) AFW3 & D) AFW4.

The application of pH-uncontrolled fermentation provides operational and economic advantages to the production process of VFAs. First, the use of acids and bases is avoided. Another important advantage is the simplicity of the process design, which is easier to control as it does not require pH monitoring equipment. Possible growth inhibition using contaminated reagents is also reduced. Finally, operating costs are reduced, since no additional equipment is needed to monitor the pH of the process. The production of VFAs was determined as a yield in terms of grams of VFA produced per gram of VS available for fermentation. Figure 16 shows the VFA yield behaviour over time for every residue with and without pretreatment. Experimental results for time zero were subtracted for each time, as they correspond to VFAs that were not formed during the DF tests; however, they were quantified by the HPLC equipment. The general overview shows low values in VFA yields for all experiments, with results that barely approach

0.3 gVFAs/gVS. Acidogenic potential is higher for solid residues, with values reaching 0.25 gVFAs/gVS, compared to liquid residues, where values did not reach 0.2 gVFAs/gVS. Liquid samples showed a faster stabilization in the final VFAs yield in comparison with the solid samples, where there is no evidence of clear maintenance of a settled result. This deceleration on the transformation of organic matter to VFAs is explained by the availability of organic matter. In liquid residues, the organic matter is well-distributed due to miscibility of the organic matter in the fermentation broth [84], making it more accessible to acidogenic microorganisms to transform it into VFAs. However, in solid samples, the release of organic material takes place progressively as factors such as agitation, temperature, and even micro-organisms are responsible for capturing the material to be transformed. This influences the fermentation rate, making the process slower, and although pretreatment helps to break down the cell walls of the substrate [56], a longer fermentation time is still necessary to reach a more stabilized value. Ramos-Suarez *et al* [41] collected information about the acidogenesis potential of some food industry wastes, including AFW1 and AFW2. This research reports yield values of 0.48 g/gVS and 0.39 g/gVS, respectively, and both analyses were performed under pH control, maintaining the pH at 6 by the addition of NaOH 1M. Bhargav *et al* [54] performed the dark fermentation of heat-treated AFW4 to produce VFAs with control of pH using a sodium bicarbonate buffer that regulates the pH at 6.0, with a result of 0.48 g/gVS. Lu *et al* [85] studied the volatile fatty acid production by vegetable waste as substrate, plus automatic adjustment of pH at 6.0 using NaOH 4M, and with yield results of 0.63 g/gVS. The information reported in literature agrees on the use of control of pH to enhance the VFA yields; however, the analysis will be done in section 4.3.

For liquid substrates, pre-treatment improves the production of VFAs, especially for the AFW1 profile, with an increasement up to 15% in the final VFA yield from 0.15 gVFAs/gVS to 0.17 gVFAs/gVS, while for AFW2, there are minimal improvement at the end of the fermentation process, with an increment in 2% respect to the untreated residue, from 0.13 gVFAs/gVS to 0.14 gVFAs/gVS. According to Ruffino *et al* [86], centrifugation is a good option to improve VFA production, since it enhances the organic matter solubilization due to bacteria cell disintegration, and disaggregation of suspended materials. There are not enough results available about mechanical pretreatment for VFA production; however, it is demonstrated that the efficiency of most mechanical technologies is upgraded when it is coupled with other methods, such as thermal pretreatment. On the contrary, the tendency in solid residues shows a slight decrease in VFA yield for pre-treated samples, without compromising considerably the final value compared to the non-treated samples. For example, in AFW3, reduction on VFA yield was estimated to be 7%, from 0.27 gVFAs/gVS to 0.25 gVFAs/gVS, whereas for AFW4, the decrease was considered negligible (less than 1%). According to Bhargav *et al* [54], thermal treatment at temperatures between 80°C – 140°C might lead to the formation of less biodegradable compounds from carbohydrates, then reducing the amount of available organic matter. Instead, it is more recommendable to use low temperature thermal pretreatment to reduce the probabilities of formation of non-desired compounds. The quantity of water added for the treatment may also affect the results. Absence of enough water promotes the overcooking of the substrate (and in some cases, gelatinization), decreasing the accessibility of sugars for microorganisms, and the formation of complex compounds. The factors mentioned above affect directly fermentation efficiency, decreasing the VFA yield. Fermentation process evolves over time differently for each residue, with faster stabilization times for liquid residues, while for

solid residues it is more evident a non-completed fermentation for the fermentation time established at the beginning of experimentation.

4.3 Dark Fermentation with pH control

The strategy of controlling pH is centered on the goal of preserving the fermentation broth under optimal acidogenic conditions. As general advantages, carrying out fermentation with pH control improves microbial growth, providing a suitable environment for the microorganisms to work under optimal conditions (in this case acidogenic), thus increasing the productivity of the biomass to be transformed into products of added value. Likewise, pH control avoids inhibition due to extreme conditions of acidity or alkalinity, reducing the possibility of favoring metabolic pathways contrary to the target pathway. The buffer dosage was added, and the pH was adjusted with NaOH to 5.2. The buffer worked properly over the fermentation of all samples with minimal variations within the acidogenesis tolerance range. Figure 17 displays the yield results for VFAs production experiments with pH control.

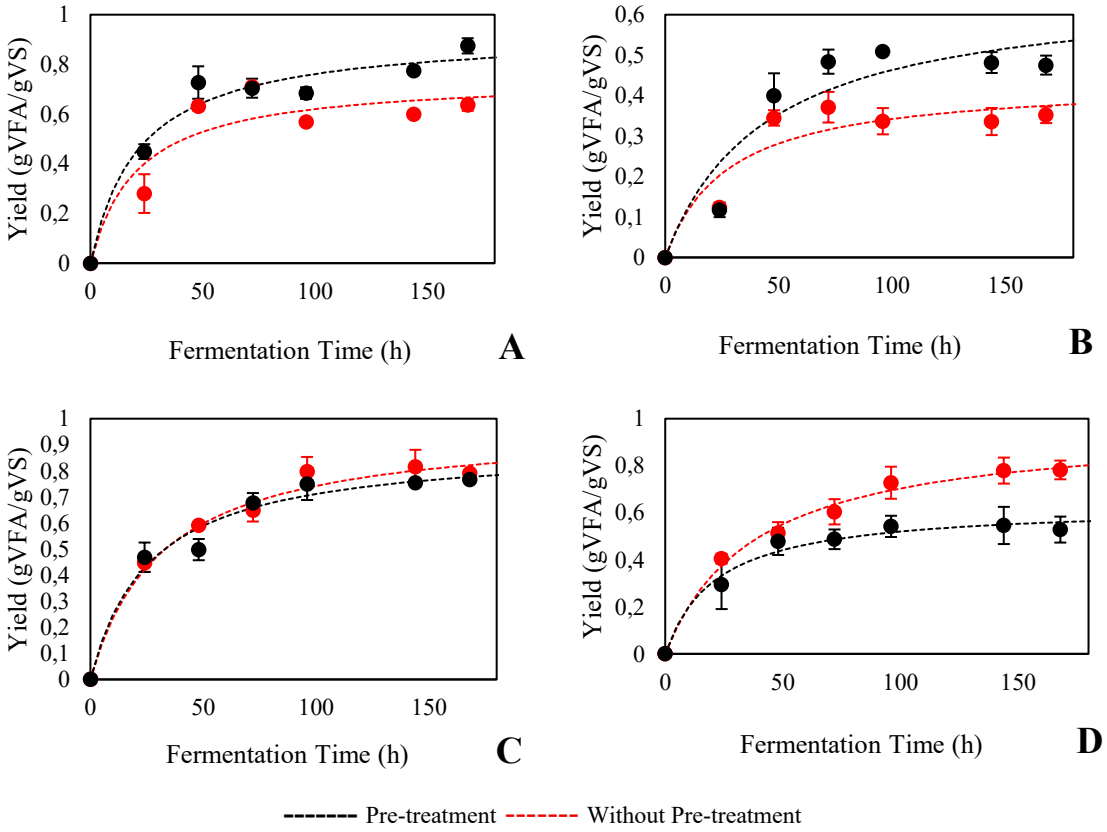


Figure 17. Yield evolution during the controlled pH DF. . A) AFW1 B) AFW2 C) AFW3 & D) AFW4.

In general, the DF results with pH control show better performance in comparison with the ones obtained in the samples without pH control. For example, AFW2 reached the lower yield from the four residues tested, with values around 0.35-0.50 gVFAs/gVS, being even higher than all the tested samples for previous experiments with uncontrolled pH. For the remaining residues, yield VFA values move around 0.70-0.90 gVFAs/gVS, which is clear evidence of the positive

effect of control of pH over the fermentation. Pretreatment in liquid samples shows a wide improvement in final yield, with more impact over the AFW2's residue, that shows upgrading in final yield when the pretreatment was carried out, contrary to what occurred with the sample with uncontrolled pH, where there were no significant differences between the treated and the non-treated sample. For example, in the case of AFW1, the control of pH increased the final VFA yield in the untreated sample from 0.15 gVFAs/gVS to 0.62 gVFAs/gVS, which means that yield increased four times in comparison with the sample without pH control. For AFW1 samples with pretreatment, improvement was also considerably important, moving from 0.17 gVFAs/gVS to 0.90 gVFAs/gVS. Likewise, the improvement in AFW1 samples with pretreatment over samples without, is reflected in the increase of 45% on the final VFA yield. A similar behaviour occurs with AFW2, where untreated samples with pH control showed a more positive result compared to the samples without pH control, with a jump from 0.13 gVFAs/gVS to 0.35 gVFAs/gVS, representing almost three times the value obtained for uncontrolled pH. In the pretreated samples, the final VFA yield moved from 0.14 gVFAs/gVS to 0.52 gVFAs/gVS. The comparison between samples with and without pretreatment, and with pH control showed an improvement of 48% by performing a pretreatment in the AFW2 sample. All information mentioned above supports the statement discussed in section 4.2, since setting pH under optimum conditions for DF highlights the effect of centrifugation over the VFA yield, and proves the necessity of additional methods to get better results for fermentation of liquid substrates.

In solid residues, however, as occurred in the experiments with uncontrolled pH, the pretreatment did not show again any evidence as a good alternative for enhancing the production of VFAs, with higher yields for samples without pre-treatment. For instance, in AFW3 samples, pretreatment decreased the final VFA yield by 4%, whereas in AFW4 the decrease was higher, resulting in 40% respect to the untreated sample. In AFW3, the pH control improved the VFA yield from 0.27 gVFAs/gVS to 0.78 gVFAs/gVS for samples without pretreatment, and from 0.25 gVFAs/gVS to 0.75 gVFAs/gVS for samples with pretreatment. Finally, for AFW4, control of pH increased the VFA yield in untreated samples from 0.26 gVFAs/gVS to 0.78 gVFAs/gVS, and in treated samples from 0.26 gVFAs/gVS to 0.50 gVFAs/gVS. pH control also exposed advantages in terms of stabilization of the process, with a clearer reaching of the steady-state condition than the experiments without pH control. Some other factors might affect the correct development of the fermentation, such as growth inhibitors. A high content of salts, furans, and even some carboxylic acids affect glycolytic enzymes necessary for the metabolism of microorganisms, reducing cell viability and damaging membranes, resulting in a prolonged lag phase, and decreasing fermentation rate [87].

4.4 Kinetic Parameters and Optimal Conditions

The kinetic parameters as well as the correlation coefficients (r^2) are shown in Table 10. The different operating conditions exposed r^2 above 0.90, demonstrating that the model adjusts properly with the experimental data. The experimental data for AFW1 shows a good fit with the second order model, and the Y_{max} results higher for experiments with substrate pretreatment. Additionally, the values for the rate constant (K) show that, in general, fermentation is slow in terms of the established fermentation time proposed in the experimental section, with the lowest values for experiments with control of pH. This is important to consider because it determines the length of the experiment, which in some cases will need a higher fermentation time to reach a stable VFA yield value. In the case of the AFW2 experiments, rate constants in samples without

pH control are higher than any other experiment with different substrates, which means that available organic matter in the residue was rapidly consumed by the microorganisms and then transformed into VFAs. Nevertheless, in AFW2's samples with pH control occurs the same behaviour as in AFW1's samples, with low values of K and an incomplete DF for the defined experimentation time. Likewise, AFW2 samples result in the lowest values for r^2, which is reflected in the shape of the models in Figure 16B and 17B.

Table 10. Parameters of second-order models for DF experiments.

Residue	pH control	No pretreatment			Pretreatment		
		K (h^{-1})	Y_{max} (gVFAs/gVS)	r^2	K (h^{-1})	Y_{max} (gVFAs/gVS)	r^2
AFW1	**No**	0.013	0.153	0.985	0.846	0.177	0.945
	Yes	0.064	0.750	0.904	0.050	0.927	0.964
AFW2	**No**	10.817	0.137	0.906	25.582	0.143	0.911
	Yes	0.083	0.436	0.908	0.034	0.668	0.901
AFW3	**No**	0.017	0.462	0.916	0.044	0.298	0.904
	Yes	0.032	0.936	0.984	0.043	0.897	0.976
AFW4	**No**	0.007	0.632	0.963	0.012	0.504	0.901
	Yes	0.027	0.841	0.991	0.073	0.632	0.978

On the other hand, in AFW3, data fits well with the model, and the pretreatment (contrarily to liquid residues) accelerates fermentation time compared with the non-treated experiments, where additional time is required to complete the transformation of organic matter, and with no improvements in the value of Y_{max} for pretreated samples. Finally, for AFW4, the rate constant was enhanced when pretreatment was carried out, due to poor values of K for samples without pretreatment. In addition, the Y_{max}, did not show any increase by the implementation of pretreatment on AFW4 samples, where apparently, better results were obtained for experiments without pretreatment. To improve the adjustment to the model, more experimental data must be taken in shorter periods of time, considering that this kind of fermentation is relatively fast, and the production of VFAs is already important by the second day of fermentation [80].

The assessment of the most suitable conditions to produce VFAs by using agri-food residues was based on several factors, including the final VFA yield, the required fermentation time and even economical and technical. To begin with, it was more than evident the effect of pH control over the results for VFA production. For the four residues, considerable improvements were noticed, with an increase on the final yield of 423% for AFW1, 367% for AFW2, 103% for AFW3 and 33% for AFW4. The worthy increase on final VFA yield for all residues reflects the importance of maintaining acidogenic conditions on the reactor to enhance the production of VFAs. In experiments without pH control, the decrease on pH affected negatively the microbial activity of acidogenic bacteria, that works with pH higher than 5.0 and might promote additional routes that are activated at lower pH, such as the lactate route (pH between. 3.5 and 4.5) [88].

Additionally, although in liquid samples the value of K is lower in experiments with pH control, the tendency shows a proximate reaching in stabilization of the VFA yield, according to Figure 17A and 17B. For solid samples, K promises a faster stabilization of the final VFA yield with pH control. In a study executed by Swiatkiewicz *et al* [89] to assess the production of VFAs from DF of kitchen waste, they found that a steady state was reached on day 12 of fermentation. Therefore, literature supports the idea of modifying the fermentation time for a longer term. On the other hand, the effect of pretreatment varied differently for all the residues, making the decision of selecting a pretreatment to be implemented, not necessary in all cases. For instance, in liquid residues, Y_{max} was improved when the pretreatment was applied on the samples, with a percentage increase of 24% and 53% for AFW1 and AFW2 respectively, whereas for solid residues, the pretreatment reduced Y_{max} to 4% for AFW3 and 25% for AFW4. The effect of the fermentation time is negligible for untreated and treated samples, since K values are quite similar between samples, different when it is compared the effect of pH with and without control. However, it is also recommended the implementation of longer fermentation times to have a more stable steady condition, as mentioned in the previous paragraph.

The economic aspect plays an important role in process design, as it becomes to be a limiting factor in the development and implementation of technologies to improve process efficiency. Some fermentation processes do not implement the use of pH control to reduce operating costs caused by using neutralizing agents, which might incur technical issues due to the increase in salinity downstream. Furthermore, installation of automatic dosing pumps, pH sensors, and the calibration and periodic calibration and revision of this equipment makes investment higher compared with the scale-up of a, anaerobic fermentation system without pH control [90]. Nevertheless, it was more than clear, the benefit of pH control over the final VFA production, justified by the increase on the final yield in all experiments. Moreover, pretreatment is not recommendable for all cases as a previous step in DF of agri-food residues. In liquid samples (AFW1 and AFW2), the improvement on the final yield makes convenient the investment on centrifugation prior the DF. As for any other pre-treatment technique, additional expenses are necessary for installation and setting up. Centrifugation, in fact, includes expenses related to operating costs, management waste after pretreatment, and the equipment itself. Even so, the improvement on the VFA yield (just like in pH control experiments) when centrifugation was performed, justify the implementation of this technology for AFW1 and AFW2. On the other hand, thermal hydrolysis did not show any upgrade on the pretreatment of AFW3 and AFW4. In fact, there was a reduction on the final VFA yield, and no improvements on the kinetic of the process. Having described all the above, the optimal conditions to implement in the DF of agri-food residues studied in this thesis are described in Table 11, as follows:

Table 11. Final and optimal conditions for implementation in the DF of agri-food residues.

Residue	Recommended conditions	Y_{max} (gVFAs/gVS)	K (h^{-1})	Comments
AFW1	Centrifugation + pH control	0.927	0.050	High yield, important improvements with centrifugation
AFW2	Centrifugation + pH control	0.668	0.034	Acceptable yield, high residues quantities needed.
AFW3	No pretreatment + pH control	0.936	0.032	Highest yield, possible thermal inhibition on pretreatment.
AFW4	No pretreatment + pH control	0.841	0.027	High yield, possible gelatinization in pretreatment affects yield.

CONCLUSIONS

In this study, the assessment of acidogenic potential of dairy, winery, and fruits and vegetables industry residues, through the implementation of DF under mesophilic conditions in 1L discontinuous reactors were performed. Likewise, the effect of two pretreatments methods (thermal hydrolysis and centrifugation) was evaluated to identify improvements on the final VFAs yield, as well as the execution of pH control as an additional enhancement on the process. VFAs produced as of these residues can be used then in future processes to produce PHBV, as expected in the main objective of this master thesis. With the information mentioned above, the main findings are listed as follows:

- The control of pH has a positive effect on the final VFA yields for all experiments, with a percentage increase of 423% for AFW1, 367% for AFW2, 103% for AFW3 and 33% for AFW4, in comparison with the experiments without pH control.
- In liquid samples, application of centrifugation as a step prior fermentation improves VFA yield, showing an increasement of 24% for AFW1 and 53% for AFW2.
- Contrarily, the application of thermal hydrolysis did not show any advantage over the final VFA yield, with a decrease of 4% for AFW3 and 25% AFW4.
- Kinetic parameters for DF experiments were evaluated with a second-order model, driving the identification of flaws on the experimentation processes, such as the need to increase the fermentation time to 12 days to guarantee steady-state conditions.
- Although the implementation of technologies such as pH control and the use of agrifood residues pretreatment increases the operating costs of the process, they are necessary to make the most of the available organic material and ensure that conversion to VFAs is as high as possible.

Considering all the points discussed above, the key goal of this study was met, demonstrating the acidogenic potential of four agri-food residues, with maximum VFAs yield of 0.927 gVFAs/gVS for AFW1, 0.668 gVFAs/gVS, for AFW2, 0.936 gVFAs/gVS for AFW3 and 0.841 gVFAs/gVS for AFW4, at the optimal conditions tested during the experimental section for each residue. This thesis results helped CLAMBER R&D Biorefinery to meet the objectives set for the PROMOFER project. Additionally, the results give an understanding of the potential of these residues to produce bioplastics, specifically PHBV, promising a more sustainable future with the implementation and use of biodegradable products that allow the reduction of single-use plastics.

RECOMMENDATIONS FOR FUTURE STUDIES

To upgrade the scaling-up of the dark fermentation process to estimate the acidogenic potential of agri-food residues, the following recommendations for the future were proposed:

- Performance of a semi-continuous dark fermentation and the optimization of fermentation time that maximizes the VFA yield production while minimizing the operative costs.
- A techno-economical assessment is highly recommended to determine the real cost-benefit of implement technologies such as pH control and pretreatments on the production of VFAs via DF.
- Carrying out the co-fermentation of multiple mixtures of agri-food residues, to improve properties such as the C/N ratio and enhance the VFA yield.
- Validation of the process at pilot scale to evaluate pros and cons of the dark fermentation process when is carried under real conditions.

References

[1] United Nations, “World population to reach 8 billion on 15 November 2022,” Department of Economic and Social Affairs. Accessed: Mar. 02, 2025. [Online]. Available: https://www.un.org/en/desa/world-population-reach-8-billion-15-november-2022

[2] C. Vidal Antich, “Food waste and waste activated sludge conversion into volatile fatty acids to produce bioplastics,” Universitat de Barcelona, 2022. [Online]. Available: www.tdx.cat

[3] Z. Liu, T. S. P. de Souza, B. Holland, F. Dunshea, C. Barrow, and H. A. R. Suleria, “Valorization of Food Waste to Produce Value-Added Products Based on Its Bioactive Compounds,” Mar. 01, 2023, *Multidisciplinary Digital Publishing Institute (MDPI)*. doi: 10.3390/pr11030840.

[4] V. K. Gaur *et al.*, “Production of biosurfactants from agro-industrial waste and waste cooking oil in a circular bioeconomy: An overview,” Jan. 01, 2022, *Elsevier Ltd*. doi: 10.1016/j.biortech.2021.126059.

[5] Z. Ali *et al.*, “Organic waste-to-bioplastics: Conversion with eco-friendly technologies and approaches for sustainable environment,” *Environ Res*, vol. 244, Mar. 2024, doi: 10.1016/j.envres.2023.117949.

[6] N. Nwokolo, P. Mukumba, K. Obileke, and M. Enebe, “Waste to energy: A focus on the impact of substrate type in biogas production,” Oct. 01, 2020, *MDPI AG*. doi: 10.3390/pr8101224.

[7] European Union, “Circular Bio-based Europe Joint Undertaking: The Organisation,” CBE. Accessed: Mar. 03, 2025. [Online]. Available: https://www.cbe.europa.eu/organisation

[8] M. Ghasemlou, C. J. Barrow, and B. Adhikari, “The future of bioplastics in food packaging: An industrial perspective,” Jun. 01, 2024, *Elsevier Ltd*. doi: 10.1016/j.fpsl.2024.101279.

[9] A. Visco *et al.*, “Agri-Food Wastes for Bioplastics: European Prospective on Possible Applications in Their Second Life for a Circular Economy,” Jul. 01, 2022, *MDPI*. doi: 10.3390/polym14132752.

[10] L. Urbina *et al.*, “Acidogenic fermentation of dairy by-products for the utilization of volatile fatty acids IN PHBV production by Haloferax mediterranei †,” 2025, doi: 10.1039/d4□00279b.

[11] PROMOFER, “Boosting upstream and downstream processes to maximize yield of PHB production and 2,3-butanediol.” Accessed: Feb. 20, 2025. [Online]. Available: https://promofer-project.eu/

[12] CLAMBER, “Castilla- La Mancha Bio-Economy Región.” Accessed: Feb. 20, 2025. [Online]. Available: https://clamber.castillalamancha.es/

[13] F. O. Kassim, C. L. P. Thomas, and O. O. D. Afolabi, “Integrated conversion technologies for sustainable agri-food waste valorization: A critical review,” *Biomass Bioenergy*, vol. 156, Jan. 2022, doi: 10.1016/j.biombioe.2021.106314.

[14] S. K. Bhatia, H. S. Joo, and Y. H. Yang, “Biowaste-to-bioenergy using biological methods – A mini-review,” *Energy Convers Manag*, vol. 177, pp. 640–660, Dec. 2018, doi: 10.1016/j.enconman.2018.09.090.

[15] S. A. Ansari, T. Kumar, R. Sawarkar, M. Gobade, D. Khan, and L. Singh, “Valorization of food waste: A comprehensive review of individual technologies for producing bio-based products,” *J Environ Manage*, vol. 364, Jul. 2024, doi: 10.1016/j.jenvman.2024.121439.

[16] J. Paini, V. Benedetti, S. S. Ail, M. J. Castaldi, M. Baratieri, and F. Patuzzi, “Valorization of Wastes from the Food Production Industry: A Review Towards an Integrated Agri-Food Processing Biorefinery,” *Waste Biomass Valorization*, vol. 13, no. 1, pp. 31–50, Jan. 2022, doi: 10.1007/s12649-021-01467-1.

[17] C. V. Berenguer, C. Andrade, J. A. M. Pereira, R. Perestrelo, and J. S. Câmara, “Current Challenges in the Sustainable Valorisation of Agri-Food Wastes: A Review,” Jan. 01, 2023, *Multidisciplinary Digital Publishing Institute (MDPI)*. doi: 10.3390/pr11010020.

[18] F. Khatami, E. Cagno, and R. Khatami, “Circular Economy in the Agri-Food System at the Country Level—Evidence from European Countries,” *Sustainability (Switzerland)*, vol. 16, no. 21, Nov. 2024, doi: 10.3390/su16219497.

[19] S. Poponi, G. Arcese, F. Pacchera, and O. Martucci, “Evaluating the transition to the circular economy in the agri-food sector: Selection of indicators,” *Resour Conserv Recycl*, vol. 176, Jan. 2022, doi: 10.1016/j.resconrec.2021.105916.

[20] R. Nattassha, Y. Handayati, T. M. Simatupang, and M. Siallagan, “Understanding circular economy implementation in the agri-food supply chain: the case of an Indonesian organic fertiliser producer,” *Agric Food Secur*, vol. 9, no. 1, Dec. 2020, doi: 10.1186/s40066-020-00264-8.

[21] Z. Xu-Liang, Z. Zhao-Hui, X. Zong-Jun, Y. Hai-Yan, and Z. Ke-Fang, “Developmental Mode for Circular Economy in the Yellow River Delta,” *International Journal of Environmental Science and Development*, pp. 662–667, 2013, doi: 10.7763/ijesd.2013.v4.434.

[22] D. Huybrechts, A. Derden, L. Van den Abeele, S. Vander Aa, and T. Smets, “Best available techniques and the value chain perspective,” *J Clean Prod*, vol. 174, pp. 847–856, Jan. 2018, doi: 10.1016/j.jclepro.2017.10.346.

[23] Z. Irani and A. M. Sharif, “Food security across the enterprise: a puzzle, problem or mess for a circular economy?,” *Journal of Enterprise Information Management*, vol. 31, no. 1, pp. 2–9, 2018, doi: 10.1108/JEIM-03-2017-0045.

[24] R. Paltrinieri, S. Spillare, and F. Savoia, *Circular Economy in the Agrifood Sector Circular Economy in the Agrifood Sector The SinCE-AFC ebook*. 2022. [Online]. Available: http://bit.ly/francoangeli-oa

[25] A. Mehmood, S. Ahmed, E. Viza, A. Bogush, and R. M. Ayyub, “Drivers and barriers towards circular economy in agri-food supply chain: A review,” *Business Strategy and Development*, vol. 4, no. 4, pp. 465–481, Dec. 2021, doi: 10.1002/bsd2.171.

[26] C. V. Berenguer, R. Perestrelo, J. A. M. Pereira, and J. S. Câmara, “Management of Agri-Food Waste Based on Thermochemical Processes towards a Circular Bioeconomy Concept: The Case Study of the Portuguese Industry,” Oct. 01, 2023, *Multidisciplinary Digital Publishing Institute (MDPI)*. doi: 10.3390/pr11102870.

[27] J. Paini, V. Benedetti, S. S. Ail, M. J. Castaldi, M. Baratieri, and F. Patuzzi, “Valorization of Wastes from the Food Production Industry: A Review Towards an Integrated Agri-Food Processing Biorefinery,” *Waste Biomass Valorization*, vol. 13, no. 1, pp. 31–50, Jan. 2022, doi: 10.1007/s12649-021-01467-1.

[28] F. Demichelis *et al.*, “Agro-food waste conversion into valuable products in the Italian scenario: current practices and innovative approaches,” Apr. 01, 2025, *Elsevier Ltd*. doi: 10.1016/j.jece.2025.115458.

[29] R. Yukesh Kannah *et al.*, “Food waste valorization: Biofuels and value added product recovery,” Sep. 01, 2020, *Elsevier Ltd*. doi: 10.1016/j.biteb.2020.100524.

[30] T. P. C. Ezeorba, E. S. Okeke, M. H. Mayel, C. O. Nwuche, and T. C. Ezike, “Recent advances in biotechnological valorization of agro-food wastes (AFW): Optimizing integrated approaches for sustainable biorefinery and circular bioeconomy,” Jun. 01, 2024, *Elsevier Ltd*. doi: 10.1016/j.biteb.2024.101823.

[31] C. V. Berenguer, R. Perestrelo, J. A. M. Pereira, and J. S. Câmara, “Management of Agri-Food Waste Based on Thermochemical Processes towards a Circular Bioeconomy Concept: The Case Study of the Portuguese Industry,” Oct. 01, 2023, *Multidisciplinary Digital Publishing Institute (MDPI)*. doi: 10.3390/pr11102870.

[32] I. Prado-Acebo, J. Cubero-Cardoso, T. A. Lu-Chau, and G. Eibes, “Integral multi-valorization of agro-industrial wastes: A review,” *Waste Management*, vol. 183, pp. 42–52, Jun. 2024, doi: 10.1016/j.wasman.2024.05.001.

[33] V. K. Varghese, B. J. Poddar, M. P. Shah, H. J. Purohit, and A. A. Khardenavis, “A comprehensive review on current status and future perspectives of microbial volatile fatty acids production as platform chemicals,” Apr. 01, 2022, *Elsevier B.V.* doi: 10.1016/j.scitotenv.2021.152500.

[34] F. Raposo *et al.*, “First international comparative study of volatile fatty acids in aqueous samples by chromatographic techniques: Evaluating sources of error,” 2013, *Elsevier B.V.* doi: 10.1016/j.trac.2013.07.007.

[35] A. Vázquez-Fernández, M. E. Suárez-Ojeda, and J. Carrera, “Review about bioproduction of Volatile Fatty Acids from wastes and wastewaters: Influence of operating conditions and organic composition of the substrate,” *J Environ Chem Eng*, vol. 10, no. 3, Jun. 2022, doi: 10.1016/j.jece.2022.107917.

[36] I. Baumann and P. Westermann, “Microbial Production of Short Chain Fatty Acids from Lignocellulosic Biomass: Current Processes and Market,” 2016, *Hindawi Limited*. doi: 10.1155/2016/8469357.

[37] T. N. Pham, W. J. Nam, Y. J. Jeon, and H. H. Yoon, “Volatile fatty acids production from marine macroalgae by anaerobic fermentation,” *Bioresour Technol*, vol. 124, pp. 500–503, Nov. 2012, doi: 10.1016/j.biortech.2012.08.081.

[38] X. Song, G. Chen, F. Wang, J. Zhang, Y. Liu, and J. Zhao, “Potential of volatile fatty acids production from banana waste juice: Impacts of storage, substrate phase, operation conditions, and microbial community analysis,” *Fuel*, vol. 366, Jun. 2024, doi: 10.1016/j.fuel.2024.131294.

[39] S. Agnihotri *et al.*, “A Glimpse of the World of Volatile Fatty Acids Production and Application: A review,” 2022, *Taylor and Francis Ltd.* doi: 10.1080/21655979.2021.1996044.

[40] S. Aghapour Aktij, A. Zirehpour, A. Mollahosseini, M. J. Taherzadeh, A. Tiraferri, and A. Rahimpour, “Feasibility of membrane processes for the recovery and purification of bio-based volatile fatty acids: A comprehensive review,” Jan. 25, 2020, *Korean Society of Industrial Engineering Chemistry*. doi: 10.1016/j.jiec.2019.09.009.

[41] M. Ramos-Suarez, Y. Zhang, and V. Outram, “Current perspectives on acidogenic fermentation to produce volatile fatty acids from waste,” Jun. 01, 2021, *Springer Science and Business Media B.V.* doi: 10.1007/s11157-021-09566-0.

[42] W. Jie, Y. Peng, N. Ren, and B. Li, “Volatile fatty acids (VFAs) accumulation and microbial community structure of excess sludge (ES) at different pHs,” *Bioresour Technol*, vol. 152, pp. 124–129, 2014, doi: 10.1016/j.biortech.2013.11.011.

[43] W. S. Lee, A. S. M. Chua, H. K. Yeoh, and G. C. Ngoh, “A review of the production and applications of waste-derived volatile fatty acids,” Jan. 01, 2014. doi: 10.1016/j.cej.2013.09.002.

[44] S. J. Lim, B. J. Kim, C. M. Jeong, J. dal rae Choi, Y. H. Ahn, and H. N. Chang, “Anaerobic organic acid production of food waste in once-a-day feeding and drawing-off bioreactor,” *Bioresour Technol*, vol. 99, no. 16, pp. 7866–7874, Nov. 2008, doi: 10.1016/j.biortech.2007.06.028.

[45] H. Xiong, J. Chen, H. Wang, and H. Shi, “Influences of volatile solid concentration, temperature and solid retention time for the hydrolysis of waste activated sludge to recover volatile fatty acids,” *Bioresour Technol*, vol. 119, pp. 285–292, Sep. 2012, doi: 10.1016/j.biortech.2012.05.126.

[46] M. Arriaga *et al.*, “Valorization of Agri-Food Waste into PHA and Bioplastics: From Waste Selection to Transformation,” Feb. 01, 2025, *Multidisciplinary Digital Publishing Institute (MDPI)*. doi: 10.3390/app15031008.

[47] A. Hejtmánková, V. Pivec, E. Trnková, and H. Dragounová, “Differences in the composition of total and whey proteins in goat and ewe milk and their changes throughout the lactation period,” 2012.

[48] F. Carvalho, A. R. Prazeres, and J. Rivas, “Cheese whey wastewater: Characterization and treatment,” Feb. 05, 2013. doi: 10.1016/j.scitotenv.2012.12.038.

[49] B. Molinuevo-Salces, V. da Silva-Lacerda, M. C. García-González, and B. Riaño, “Production of Volatile Fatty Acids from Cheese Whey and Their Recovery Using Gas-Permeable Membranes,” *Recycling*, vol. 9, no. 4, Aug. 2024, doi: 10.3390/recycling9040065.

[50] K. ichi Hatano, S. Kikuchi, T. Miyakawa, M. Tanokura, and K. Kubota, “Separation and characterization of the colored material from sugarcane molasses,” *Chemosphere*, vol. 71, no. 9, pp. 1730–1737, Apr. 2008, doi: 10.1016/j.chemosphere.2007.12.019.

[51] A. F. R. Silva, Y. L. Brasil, K. Koch, and M. C. S. Amaral, “Resource recovery from sugarcane vinasse by anaerobic digestion – A review,” Oct. 01, 2021, *Academic Press*. doi: 10.1016/j.jenvman.2021.113137.

[52] S. Liang, A. G. McDonald, and E. R. Coats, “Lactic acid production with undefined mixed culture fermentation of potato peel waste,” *Waste Management*, vol. 34, no. 11, pp. 2022–2027, Nov. 2014, doi: 10.1016/j.wasman.2014.07.009.

[53] S. Palakawong Na Ayudthaya, A. H. P. Van De Weijer, A. H. Van Gelder, A. J. M. Stams, W. M. De Vos, and C. M. Plugge, “Organic acid production from potato starch waste fermentation by rumen microbial communities from Dutch and Thai dairy cows,” *Biotechnol Biofuels*, vol. 11, no. 1, Jan. 2018, doi: 10.1186/s13068-018-1012-4.

[54] M. Bhargav and K. Singh, “Volatile Fatty Acid Production Using Heat-Treated Potato Starch: Impact of Hydraulic Retention Time,” *Journal of Environmental Engineering*, vol. 146, no. 10, Oct. 2020, doi: 10.1061/(asce)ee.1943-7870.0001779.

[55] S. Mohamadnia, A. Thygesen, P. Ghofrani-Isfahani, A. P. Monachese, B. Valverde-Pérez, and I. Angelidaki, “Valorization of potato starch wastewater using anaerobic acidification coupled with Chlorella sorokiniana cultivation,” *J Appl Phycol*, vol. 35, no. 6, pp. 2645–2658, Dec. 2023, doi: 10.1007/s10811-023-03046-4.

[56] D. P. Maurya, A. Singla, and S. Negi, “An overview of key pretreatment processes for biological conversion of lignocellulosic biomass to bioethanol,” Oct. 22, 2015, *Springer Verlag*. doi: 10.1007/s13205-015-0279-4.

[57] L. Lin and X. yan Li, “Effects of pH adjustment on the hydrolysis of Al-enhanced primary sedimentation sludge for volatile fatty acid production,” *Chemical Engineering Journal*, vol. 346, pp. 50–56, Aug. 2018, doi: 10.1016/j.cej.2018.04.005.

[58] H. M. Zabed *et al.*, “Recent advances in biological pretreatment of microalgae and lignocellulosic biomass for biofuel production,” May 01, 2019, *Elsevier Ltd*. doi: 10.1016/j.rser.2019.01.048.

[59] E. Rouches, S. Zhou, J. P. Steyer, and H. Carrere, “White-Rot Fungi pretreatment of lignocellulosic biomass for anaerobic digestion: Impact of glucose supplementation,” *Process Biochemistry*, vol. 51, no. 11, pp. 1784–1792, Nov. 2016, doi: 10.1016/j.procbio.2016.02.003.

[60] CLAMBER, “Determinación de Sólidos Votátiles y Totales. ,” 2017.

[61] L. Tian, L. Pan, and L. Wang, “Effect of Inoculum Pretreatment and Substrate/Inoculum Ratio on Acidogenic Fermentation of Chemically Enhanced Primary Treatment Sludge,” *Sustainability (Switzerland)* , vol. 16, no. 8, Apr. 2024, doi: 10.3390/su16083347.

[62] B. Saha, M. Khwairakpam, and A. S. Kalamdhad, “Thermal pre-treatment – A prerequisite for the reduction of hydrolysis stage during anaerobic digestion of Ageratum conyzoides,” *Mater Sci Energy Technol*, vol. 4, pp. 34–45, Jan. 2021, doi: 10.1016/j.mset.2020.12.002.

[63] S. Al-Haddad, C. K. Okoro-Shekwaga, L. Fletcher, A. Ross, and M. A. Camargo-Valero, “Assessing Different Inoculum Treatments for Improved Production of Hydrogen through Dark Fermentation,” *Energies (Basel)*, vol. 16, no. 3, Feb. 2023, doi: 10.3390/en16031233.

[64] X. Huang and Z. Xiang, “Enhanced volatile fatty acids production in thermal hydrolysis pretreatment with constant pH regulation: Effects evaluation and mechanism elucidation,” *J Environ Chem Eng*, vol. 12, no. 6, Dec. 2024, doi: 10.1016/j.jece.2024.114830.

[65] M. Pérez-Rangel, E. Villanueva-Galindo, and I. Moreno-Andrade, “Hydrogen production from lactic acid-rich effluent of food waste fermentation: Influence of pH, type and inoculum concentration, and physical pretreatment of the substrate,” *J Environ Chem Eng*, vol. 13, no. 2, Apr. 2025, doi: 10.1016/j.jece.2025.116033.

[66] J. Castilla-Archilla, S. Papirio, and P. N. L. Lens, “Two step process for volatile fatty acid production from brewery spent grain: Hydrolysis and direct acidogenic fermentation using anaerobic granular sludge,” *Process Biochemistry*, vol. 100, pp. 272–283, Jan. 2021, doi: 10.1016/j.procbio.2020.10.011.

[67] Y. A. da Fonseca *et al.*, “Enhancing Biobased Volatile Fatty Acids Production from Olive Mill Solid Waste by Optimization of pH and Substrate to Inoculum Ratio,” *Processes*, vol. 11, no. 2, Feb. 2023, doi: 10.3390/pr11020338.

[68] S. Ana Estikomah, . S., A. Susilowati, and M. Masykuri, “Cheese Whey Wastewater: Characterization and Value,” *KnE Social Sciences*, Aug. 2023, doi: 10.18502/kss.v8i14.13858.

[69] M. F. Soto, C. A. Diaz, A. M. Zapata, and J. C. Higuita, “BOD and COD removal in vinasses from sugarcane alcoholic distillation by Chlorella vulgaris: Environmental evaluation,” *Biochem Eng J*, vol. 176, Dec. 2021, doi: 10.1016/j.bej.2021.108191.

[70] G. Shang *et al.*, “A cathodic electro-fermentation system for enhanced methane production from high-concentration potato starch industrial wastewater,” *Journal of Water Process Engineering*, vol. 59, Mar. 2024, doi: 10.1016/j.jwpe.2024.105006.

[71] R. Jariyaboon *et al.*, “Thermophilic Dark Fermentation for Simultaneous Mixed Volatile Fatty Acids and Biohydrogen Production from Food Waste,” *Fermentation*, vol. 9, no. 7, Jul. 2023, doi: 10.3390/fermentation9070636.

[72] Y. Choi, J. Ryu, and S. R. Lee, “Influence of carbon type and carbon to nitrogen ratio on the biochemical methane potential, pH, and ammonia nitrogen in anaerobic digestion,” *J Anim Sci Technol*, vol. 62, no. 1, pp. 74–83, 2020, doi: 10.5187/JAST.2020.62.1.74.

[73] L. A. Ordaz-Díaz and A. M. Bailón-Salas, “Molecular identification of microbial communities in the methane production from vinasse: A review,” *Bioresources*, vol. 15, no. 2, pp. 4528–4552, Feb. 2020, doi: 10.15376/biores.15.2.diaz.

[74] M. E. Ibáñez-López, E. Díaz-Domínguez, F. J. Fernández-Morales, and J. L. García-Morales, “Enhancing dark fermentative biohydrogen and VFA production via ozone pre-treatment,” *Bioresour Technol*, vol. 419, Mar. 2025, doi: 10.1016/j.biortech.2025.132107.

[75] S. Chan, K. Nishi, M. Koyama, T. Toda, T. Matsuyama, and J. Ida, “Combined effects of various conductive materials and substrates on enhancing methane production performance,” *Biomass Bioenergy*, vol. 178, Nov. 2023, doi: 10.1016/j.biombioe.2023.106977.

[76] N. Parsa, G. Khajouei, M. Masigol, H. Hasheminejad, and A. Moheb, “Application of Electrodialysis Process for Reduction of Electrical Conductivity and COD of Water Contaminated By Composting Leachate,” *Civil Engineering Journal*, vol. 4, no. 5, pp. 1034–1045, Jun. 2018, doi: 10.28991/cej-0309154.

[77] D. Vescovo *et al.*, “The Valorization of Potato Peels as a Functional Ingredient in the Food Industry: A Comprehensive Review,” Apr. 01, 2025, *Multidisciplinary Digital Publishing Institute (MDPI)*. doi: 10.3390/foods14081333.

[78] M. Casallas-Ojeda *et al.*, “Understanding the acidification risk of cheese whey anaerobic digestion under psychrophilic and mesophilic conditions,” *Heliyon*, vol. 10, no. 5, Mar. 2024, doi: 10.1016/j.heliyon.2024.e26476.

[79] J. C. de Carvalho *et al.*, “Biomethane Production from Sugarcane Vinasse in a Circular Economy: Developments and Innovations,” Apr. 01, 2023, *MDPI*. doi: 10.3390/fermentation9040349.

[80] S. Achinas, Y. Li, V. Achinas, and G. J. W. Euverink, “Biogas potential from the anaerobic digestion of potato peels: Process performance and kinetics evaluation,” *Energies (Basel)*, vol. 12, no. 12, Jun. 2019, doi: 10.3390/en12122311.

[81] C. Fang, K. Boe, and I. Angelidaki, “Biogas production from potato-juice, a by-product from potato-starch processing, in upflow anaerobic sludge blanket (UASB) and expanded granular sludge bed (EGSB) reactors,” *Bioresour Technol*, vol. 102, no. 10, pp. 5734–5741, May 2011, doi: 10.1016/j.biortech.2011.03.013.

[82] J. C. de Carvalho *et al.*, “Biomethane Production from Sugarcane Vinasse in a Circular Economy: Developments and Innovations,” Apr. 01, 2023, *MDPI*. doi: 10.3390/fermentation9040349.

[83] M. F. Ramos-Reyes *et al.*, “Exploring the potential of distillery vinasses through upcycling: Pathways to a circular economy,” May 01, 2025, *Elsevier B.V.* doi: 10.1016/j.eti.2025.104072.

[84] X. Huang and Z. Xiang, “Enhanced volatile fatty acids production in thermal hydrolysis pretreatment with constant pH regulation: Effects evaluation and mechanism elucidation,” *J Environ Chem Eng*, vol. 12, no. 6, Dec. 2024, doi: 10.1016/j.jece.2024.114830.

[85] Y. Lu, Q. Zhang, X. Wang, X. Zhou, and J. Zhu, “Effect of pH on volatile fatty acid production from anaerobic digestion of potato peel waste,” *Bioresour Technol*, vol. 316, Nov. 2020, doi: 10.1016/j.biortech.2020.123851.

[86] B. Ruffino *et al.*, “Improvement of anaerobic digestion of sewage sludge in a wastewater treatment plant by means of mechanical and thermal pre-treatments: Performance, energy and economical assessment,” *Bioresour Technol*, vol. 175, pp. 298–308, Jan. 2015, doi: 10.1016/j.biortech.2014.10.071.

[87] M. M. Ishola, “Novel Application of Membrane Bioreactors In Lignocellulosic Ethanol Production: Simultaneous Saccharification Filtration and Fermentation,” 2014. [Online]. Available: https://www.researchgate.net/publication/271326803

[88] Y. Ren, X. Wang, Y. Li, Y. Y. Li, and Q. Wang, “Lactic Acid Production by Fermentation of Biomass: Recent Achievements and Perspectives,” Nov. 01, 2022, *MDPI*. doi: 10.3390/su142114434.

[89] J. Swiatkiewicz, R. Slezak, L. Krzystek, and S. Ledakowicz, “Production of volatile fatty acids in a semi-continuous dark fermentation of kitchen waste: Impact of organic loading rate and

hydraulic retention time," *Energies (Basel)*, vol. 14, no. 11, Jun. 2021, doi: 10.3390/en14112993.

[90] D. Zagklis, M. Papadionysiou, K. Tsigkou, P. Tsafrakidou, C. Zafiri, and M. Kornaros, "Effect of pH on the economic potential of dark fermentation products from used disposable nappies and expired food products," *Applied Sciences (Switzerland)*, vol. 11, no. 9, May 2021, doi: 10.3390/app11094099.

LIST OF TABLES

Table 1. Drivers and Barriers in the implementation of a circular economy model. Source: [24]. 13
Table 2. Available technologies used for valorization of AFWs. [15] 14
Table 3. Properties of Volatile Fatty Acids (VFAs) [34]. 16
Table 4. Operating conditions used to produce VFAs via dark fermentation. Modified from [46]. 20
Table 5. Reagents and materials employed in experimental methodology. 24
Table 6. Equipment used in the experimental section. 25
Table 7. Operating conditions used to produce VFAs via dark fermentation. 27
Table 8. Initial Characterization for untreated residues. 33
Table 9. Initial Characterization for treated residues. 33
Table 10. Parameters of second-order models for DF experiments. 41
Table 11. Final and optimal conditions for implementation in the DF of agri-food residues.....43

LIST OF FIGURES

Figure 1. Main types of AFWs found in industry.....11
Figure 2. Representation of Agri-food supply chain. 12
Figure 3. Opportunities of conversion of AFWs into value-added products. [3] 14
Figure 4. Bioconversion of AFWs and their variety of products. [29].....15
Figure 5. Anaerobic Digestion routes and steps: The desired pathway is underlined in bold, and the dark fermentation process is highlighted in red. Modified from [41].....17
Figure 6. Drying Oven (left) and Figure 7. Muffle furnace (right) used for VS and TS determination. 26
Figure 8. Multiparameter system HI-6000 for pH and conductivity measurements. 27
Figure 9. 1L glass r*eactor* OXITOP® *model.*.....28
Figure 10. Gas Chromatograph GC-2030 for methane quantification. 28
Figure 11. *Autoclave* 4001758 *for inoculum pretreatment.*.....29
Figure 12. Pretreatment Methods Scheme for residues used in dark fermentation.....30
Figure 13. Experimental Set-up for dark fermentation with pH control.....31
Figure 14. HPLC equipment for VFAs characterization.....31
Figure 15. pH evolution during the uncontrolled pH DF. A) AFW1 B) AFW2 C) AFW3 & D) AFW4. 36
Figure 16. Yield evolution during the uncontrolled pH DF. A) AFW1 B) AFW2 C) AFW3 & D) AFW4. 37
Figure 17. Yield evolution during the controlled pH DF. . A) AFW1 B) AFW2 C) AFW3 & D) AFW4. 39