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Recovery of biofuels, biobased products and nutrients in agricultural biogas plants

S.S.D. ICHI-02/A – Impianti Chimici

Coordinator: Prof.ssa Flavia Guzzo

Signature _____

Tutor: Prof. David Bolzonella

Signature _____

Doctoral Student: Dott. Fabio Rizzioli

Signature _____

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Contents

Abstract

This PhD thesis focuses on the conceptualization and laboratory-scale implementation of a biorefinery model for the simultaneous production, purification, and recovery of high-value compounds and biofuels from *Opuntia ficus-indica* (OFI) and agricultural digestate. A biorefinery integrates biomass conversion processes to produce bio-based products, such as biofuels, biochemicals, biomaterials, and bioenergy, utilizing renewable biomass instead of fossil resources.

The proposed biorefinery model is centered around anaerobic digestion (AD), a well-established biotechnology in the European Union, which converts organic carbon into biomethane, volatile fatty acids (VFAs), and fertilizers. The selected feedstocks were: (i) OFI cladodes, considered a third-generation energy crop, and (ii) agricultural digestate, an AD by-product. OFI cladodes were tested under various operational conditions to optimize the production of biogas, VFAs, and middle-chain fatty acids (MCFAs). The best VFAs and MCFAs outputs were purified using membrane filtration technologies to remove unwanted organic matter. Concurrently, agricultural digestate, rich in macronutrients, was processed through sequential filtration steps to recover nitrogen and phosphorus compounds. These purified VFAs and MCFAs were then combined with the recovered nutrients to create optimal conditions for polyhydroxyalkanoates (PHAs) synthesis using *Thauera* spp.

This biorefinery model has multiple potential applications. First, it can contribute to energy security by producing domestic biomethane, reducing reliance on Russian natural gas, as outlined in the REPowerEU Plan. Second, it enables the valorization of agricultural digestate by recovering essential nutrients for bio-fertilizer production. Additionally, this thesis extends the biorefinery concept beyond biogas and nutrient recovery, demonstrating a flexible system for VFAs, MCFAs, and PHAs production, adaptable to various economic sectors.

Thesis goal and objectives

This thesis is centered on the conceptualization and implementation at laboratory scale of a **biorefinery** line for the simultaneous production, purification and recovery of high added value compounds and biofuels from *Opuntia ficus-indica* (OFI) and agricultural digestate. With the term "biorefinery" we refer to a facility or system that integrates biomass conversion processes to produce a range of bio-based products, such as biofuels, biochemicals, biomaterials, and bioenergy. Unlike the traditional refinery that processes fossil resources, such as crude oil or natural gas, into multiple valuable products, a biorefinery utilizes renewable biomass (e.g., agricultural residues, forestry waste, energy crops, algae, or organic waste) as a feedstock to maximize value and minimize wastes (Conteratto et al., 2021).

The proposed biorefinery model in this PhD thesis, has been built around the Anaerobic Digestion (AD) technology, the most consolidate and diffused biotechnology in the European Union (EU), used for the conversion of the organic carbon into valuable biomethane, Volatile Fatty Acids (VFAs) and fertilizers. The two organic feedstocks used for this process were: i) OFI cladodes, a cactus that can be regarded as a third-generation energy crop, and ii) agricultural digestate, the AD by-product. Specifically, OFI cladodes were tested at different operational parameters to optimize biogas, VFAs and Middle Chain Fatty Acids productions. The best outstream in VFAs and MCFAs were purified by pressure driven membrane technologies in order to remove the other organic matter. In the meantime, the agricultural digestate, rich in macronutrients, was processed according to different sequential filtration steps to recover nitrogen and phosphorous compounds. Then, the purified VFAs and MCFAs from OFI fermentation were combined with the recovered macronutrients from agricultural digestate to create the optimal conditions for the synthesis of polyhydroxyalkanoates (PHAs) in pure culture of *Thauera* spp.

By this way, the potential impacts and applications of this biorefinery model are multiple. These two substrates can be exploited to address: i) the energetic crisis started from the Russian invasion of Ukraine with the production of "domestic biomethane" in order to replace the natural gas imported to Russia as expected by the REPowerEU Plan (European Commission, 2022b), and ii) the valorization of the huge amounts of agricultural digestate, the main byproduct from AD, for the recovery of high added value nitrogen, phosphorus and potassium macronutrients which can represent the precursors for the synthesis of bio-fertilizers or other bio-compounds. Moreover, this thesis will also try to extend the biorefinery concept over biogas and nutrients recovery, offering an example of flexible biorefinery process to produce VFAs, MCFAs and PHAs. This demonstrates the adaptability of the biorefinery in different economic sectors.

With specific reference to the temporal evolution of the following thesis project during the first of this three-year PhD project, I focused my attention on OFI cladodes characterization and optimizing the AD. During the second and third years, after the AD optimization, I worked on the recovery process of the digestate in the AD output, which can be rich on nutrients and VFAs, depending on the operational parameters of the AD reaction. In order to avoid expensive digestate treatment processes, typical of classic nutrient recovery, this work process was based on sequential pressure-driven membrane filtration. As final products, biogas or VFAs, and nutrients were obtained. Finally, to close the circular economic approach, VFAs obtained were tested for PHAs production, which are polymers able to synthesize a completely biobased and biodegradable plastic material.

In order to better explain these research years, this thesis is divided into two chapter classes: i) **theoretical chapters**, needed to introduce and explain the basics and features of technologies used in this thesis; ii) **experimental chapters**, where the technologies, explained in the theoretical chapters, were applied on the substrates of interest of this research project. First theoretical chapters focuses on AD and its substrates, in particular bioenergy crops; next chapters will address the state-of-art for digestate management, and its most recent technological updates; last chapters are dedicated to VFAs purification and utilization for PHAs production. Experimental chapters have a scientific article structure, as most of the methods and data written in this thesis were submitted or published on international academic journals.

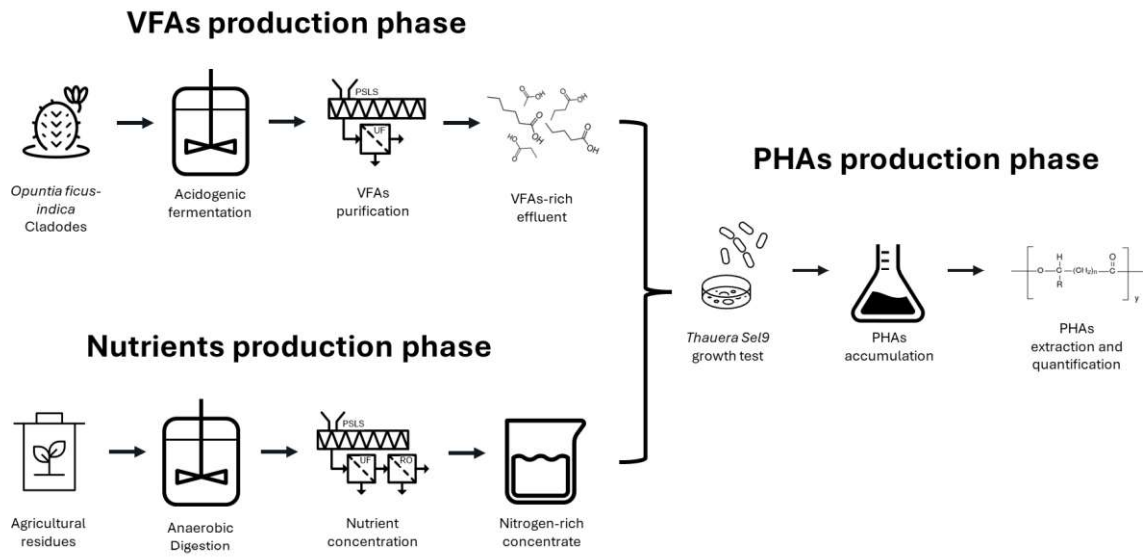

Figure 1. Schematic representation of this PhD thesis processes.

Introduction

The purpose of the following introduction chapters is to give insights into the environmental and energetic crisis, which is currently ongoing across the EU. In particular, the first three introduction chapter focuses on the crisis characterization, the future consequences, and the actions planned by the EU to respond to the crisis environmental threats. The successive introduction chapters will treat one of the key processes for this thesis, and for the EU emergency energetic plan, which is the AD for biogas and biomaterials production (Chapter 4 and 5). Once the AD process has been explained, the next chapters will focus on digestate (AD by-product) management (Chapter 6), to minimize pollution risks and recover most of its nutrients using a pressure-driven membrane filtration process, which will be the second key process of this thesis. Next chapters will introduce and elaborate the nutrient recovery process, firstly treating the best substrates for the production of both biogas and VFAs (Chapter 7), individuating OFI cladodes as a valid solution to face ethical and environmental limitations for the cultivation of bioenergy crops (Chapter 8). Finally, the last introduction chapters will give an example of circular economy approach, turning the nutrients and bioproducts recovered into PHAs polymer, which are a biobased, biodegradable plastic that can re-enter the economic market as new material.

1. Environmental and energetic crisis

Human environmental impact caused an overshot of at least six of nine "planetary boundaries" which are needed to preserve the ecosystem well-being. As described by Richardson et al. (2023), "planet boundaries" defines nine processes critical for Earth system stability and resilience. These boundaries are:

1. **Climate Change:** expressed by atmospheric CO_2 concentration (ppm CO_2) and total anthropogenic radiative forcing at top-of-atmosphere (W m^{-2});
2. **Change in biosphere integrity:** expressed by genetic diversity (extinctions per million species-years (E/MSY)) and by genetic functional integrity (% of human appropriation of the biosphere net primary production);
3. **Stratospheric ozone depletion:** global average of stratospheric O_3 concentration;
4. **Ocean acidification:** carbonate ion concentration, expressed as average global surface ocean saturation state compared to aragonite;
5. **Biogeochemical flows:** represented by phosphorus and nitrogen cycles. In particular, phosphate flow from freshwater to the ocean (global), from fertilizers to erodible soils (regional), and industrial and intentional fixation of nitrogen (global). Expressed by Tg of P (or N) per year;
6. **Land system change:** % area of forested land remaining compared to preindustrial Holocene base value;

7. **Freshwater change:** human-induced disturbance of global and plants-available water flows;
8. **Atmospheric aerosol loading:** interhemispheric difference in aerosol optical depth;
9. **Novel entities:** defined as percentage of synthetic chemicals released to the environment without adequate safety testing.

Except for stratospheric ozone depletion, atmospheric aerosol loading, and ocean acidification, all the remining plant boundaries are exceeding the safe operating space. Four of them, as described in Figure 2, are in a high-risk zone (Richardson et al., 2023).

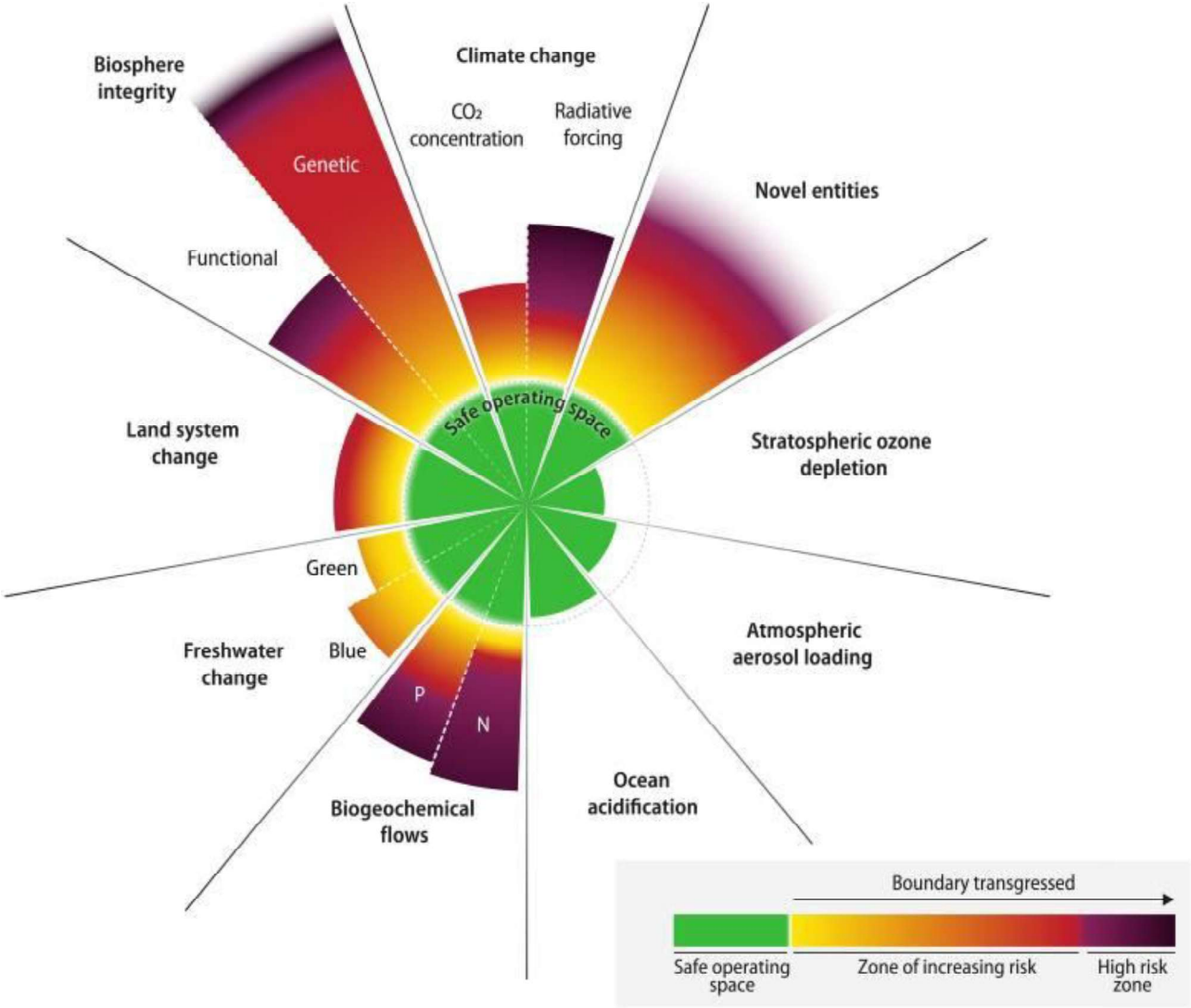

Figure 2. Current status of control variables for all nine planet boundaries (Richardson et al., 2023).

The direct consequence is climate change, loss of biosphere integrity, and the whole Earth biocycle has been irreversibly altered by human activities and high raw material usage (Sariatli, 2017; Steffen et al., 2015).

Actual estimation indicates that global raw material consumption will increase from 89 Gt of 2017 to 167 Gt before 2060. This rise will include biomasses, fossil fuels, metals and mineral materials, whose consumption will increase by 73%, 63%, 126%, and 97% respectively. This will cause more environmental consequences due to the increase of raw material extraction, processing and then waste management. According to the Organization for Economic Co-operation and Development (OECD) report (OECD, 2019), greenhouse gases emissions and their consequent impact on soil, air and water pollution will duplicate, and some of these consequences are starting to act from the start of 2020' years.

Climate change has posed significant threats to the EU environment and, consequently, economy. In particular, 2022 was a significative year regarding the occurrence of droughts, with low precipitation in over half of Europe. According to the Single Market Emergency Instrument (SMEI) Study of EU (European Commission, 2023), by August 2022, a drought warning was issued for 64% of EU land and 17% was put on drought alert. Maximum temperatures from June to August exceeded 40°C and were at least 10 °C higher than the normal maximum for that time of the year. The consequent impact was very dire, with over 19,200 fatalities and over 644,000 hectares of burnt areas due to wildfires. The estimated economic loss was 20.6 billion €.

On the European single market, droughts affected the supply of strategic important goods, such as food and energy. Food production was hindered due to low precipitation and high heat stress. Amongst food and oil crops, the most affected by drought were maize, soybean, and sunflowers, with a reduction in yield of 16%, 15%, and 12% respectively. Low precipitation and drought affected also different forms of energy production. Low levels of water in rivers and reservoirs, especially in Bulgaria, France, Italy, Portugal and Romania, reduced hydropower energy generation up to 20% as the case of France. Water scarcity also affected nuclear power plants cooling system and river shipping.

According to the European State of Climate, 2023 was the warmest year on record, along with 2020, with 1.0 °C above average, and 2.6°C above the pre-industrial level. Although precipitations overall increased by 7%, temperature and precipitations were contrasting from one month to the next: from June saw above-average temperatures across much of northern Europe and below-average temperatures, in July this pattern was reversed, with many regions experiencing heatwaves while others saw floodings. 2023 "extended summer" from June to September saw heatwaves and droughts on 41% of southern Europe. Soil moisture across Europe was drier-than-average, affecting vegetation growth and agricultural yields.

2. Circular economy

It is then clear that the classic linear economy model, namely “take-make-dispose”, where new raw materials are continuously extracted to form new products that will become just a waste after been used, is not sustainable anymore.

Figure 3. Comparison between linear and circular economy (European Commission, 2016).

It is then necessary to transitate to a circular economy model, where resources are used in a sustainable mode and an end-of-life product can re-enter the economic cycle, minimizing waste production. The direct consequence is a reduction of the environmental impact and an increase of planet resources availability, which is also crucial considering that the human population is increasing and currently, in 2024, is over 8 billion people.

Waste disposal and management are regulated by EU Directive 2008/98/EC (European Council, 2008), which indicates principles to reduce waste generation and optimize waste management, in order to decrease the impact on human health, environment, and raw material consumption. Notably, Article 4 of this directive introduces the “Waste Hierarchy” concept, which defines 5 actions, ordered by its importance, for good waste management. Top priority should be regarded on waste **prevention**, followed by waste reduction by reuse, recycle, energy recovery, and, lastly, landfill disposal or incineration without energy recovery, as explained in Figure 4.

Figure 4. Waste management hierarchy.

The following EU Directive 2018/851 amends the Waste Directive, focusing on a better definition of waste typology, reinforcing the EU transition towards circular economy, culminating in 2019 with the EU Green Deal (European Commission, 2019). The Green Deal defined a circular economy strategy for EU Member states, planning to reduce greenhouse gases emissions by 55% before 2030, and becoming the first "carbon-neutral" continent by 2050. To realize this project, the EU must implement a regenerative growth strategy, which can decrease resource consumption and therefore restore the "Planetary Boundaries" limit with greener sustainable production methods alternatives to the linear economy ones. The Green Deal strategy will also contribute to achieving United Nations Sustainable Development Goals and fulfill the global warming limit of +1.5 °C implemented by the Paris Agreement by UN COP26 and reaffirmed during Baku COP29.

3. Energy and waste

Facing global climate change, bioenergy production was selected as the main candidate alternative to fossil fuels and for decarbonizing global economy (Field et al., 2020). Compared to the other renewable energy sources, for example solar, wind and geothermal, which depends on the environmental and seasonal characteristics, biomass-derived energy can be produced steadily, representing at date 10% of global energy supply. Estimation on sugar and oil crops, algae, lignocellulosic biomasses, agricultural and municipal organic wastes, can fulfill up to 60% of global energy demand (Duarah et al., 2022). According to the State of the Energy Union Report 2023, bioenergy from agricultural, forestry and organic waste accounts for 59% of the total EU renewable energy consumption in 2021. Of this share, solid biofuels account for 70.3%, followed by liquid biofuels (12.9%) and biomethane (10.1%). Especially, the 2022 Russian invasion of Ukraine started and the consequent economic sanctions towards Russia Federation led to a complete closure of natural gas supply from Russia. Therefore, all EU member states faced an energetic crisis and found

themselves in a condition of looking for new natural gas suppliers or of producing energy on their own. Before the start of the war, EU imported 90% of the total gas consumption, 40% of which was provided from Russia. Oil and coal imports also depend for 27% and 46% respectively, from Russia. After the Ukraine invasion, EU issued a new energetic plan, called REPowerEU to reach an independence from Russian gas as soon as possible (European Commission, 2022b). To do so, European energetic supply should have diversified supplier states and, most importantly, EU member states should boost their own production of energy, with more emphasis on renewable energy generation. In this topic, the planned objective is the production of at least 35 billion cubic meters (bcm) of biomethane per year by 2030.

On this topic, AD is a consolidated technology for municipal and agricultural waste management to produce biogas, which can be upgraded to biomethane. Today, the EU biogas power plants are 20,000. Italy is the second-best EU country for overall plants number with 1655, concentrated especially in the north of Italy, on the Po River basin. However, following the "waste hierarchy" pyramid, biogas production is regarded as energy recovery and, therefore, not ideal for a circular economy model except in particular situations, for example the REPowerEU, which triggered an emergency response to an energy supply sudden interruption. When the geopolitical situation stabilizes, using AD for biogas production will not be as convenient as today, and AD should be modified accordingly to meet different market demands.

AD process can obtain also biobased products with higher value than biogas: VFAs and Medium-chain Fatty Acids (MCFAs), in particular caproic acid, which are synthesized during the middle phases of the process. Shifting the process equilibrium towards acidogenic microorganisms, despite methanogenic ones, is possible to accumulate VFAs in the effluent stream. Recovering these VFAs increases the value of the AD process, shifting from "energy recovery" to "recycling" on the waste hierarchy pyramid. On this topic, the AD by-product, namely digestate, can be recycled recovering most of the nutrients, in particular nitrogen, phosphorus and potassium, to produce a stable and storable fertilizer.

4. Anaerobic Digestion

AD is a form of organic matter stabilization in absence of oxygen (Bolzonella et al., 2006). Products of this process are (i) biogas, which is a mixture of various gases, mainly methane and carbon dioxide; and (ii) digestate, which is the remaining stabilized semi-solid organic matter. Naturally, this process occurs in ruminants stomach, swamps and human-made landfills or municipal sewers. Generally, in AD organic compounds are converted into inorganic matter: most of the organic carbon is volatized to CH_4 and CO_2; nitrogen, phosphorus and sulfur, mostly from organic matter proteins, are mineralized to inorganic compounds such as ammonium (NH_4^+), phosphate (PO_4^{3-}) and sulfide (HS^-) (Possente et al., 2022). Compared to aerobic treatments, another well-established

forms of organic matter stabilization, AD processes are technically simpler to build and, most importantly, can produce high-grade energy (biogas) and compounds. Moreover, aerobic treatments require energy for aeration and produces more than 50% of new sludge from the COD converted (van Lier et al., 2023). AD, which obviously does not require aeration, can overcome this issue by saving up to 1.0 kWh/kgCOD removed and producing up to 13.8 MJ CH_4energy/kgCOD removed or 1.5 kWh electricity, assuming 40% of electric conversion efficiency (van Lier et al., 2023). The main drawback of AD regards pollutant removal, which can be low compared to aerobic processes.

AD is a multi-step process, formed by four stages: 1) hydrolysis, 2) acidogenesis, 3) acetogenesis, and 4) methanogenesis. The number of stages and reactions, which are represented i, are indicatives of a complex ecosystem of multiple microorganism species.

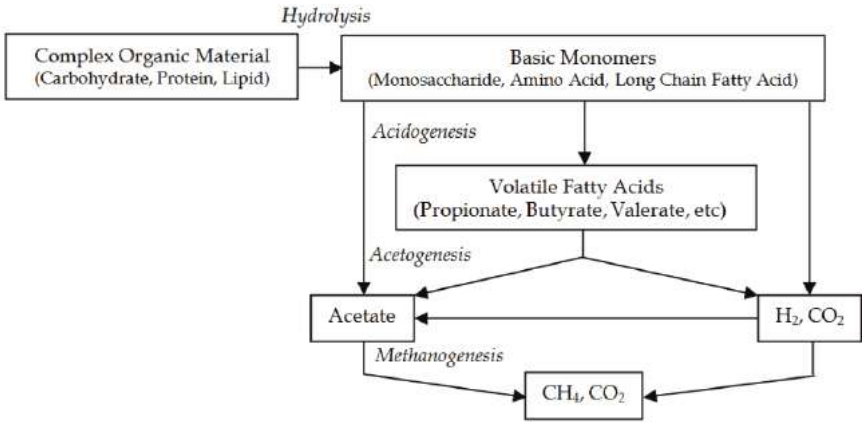

Figure 5. AD reaction scheme (Evren et al., 2011).

4.1 Substrate pretreatment

Pretreatments are usually considered as the upstream processes, which serve to prepare the organic substrates for the main unit operations. As reported above, pretreatments help the hydrolysis and the hydrolysis of the most complex materials, making them more accessible to microorganisms. By this way, positive effects can be achieved on the process, such as the increasing of conversion yield an rates, shorter HRT, reduction of the reactor volume and less overall costs (EBA, 2022). In particular, pretreatments are highly recommended in biomasses rich in lignocellulosic residues, due to the difficult accessibility of cell wall to microorganisms. Cell walls of lignocellulosic cells are formed by nanofibrils of crystalline cellulose in an amorphous matrix of lignin and hemicellulose, which blocks the actions of enzymes (Himmel et al., 2007). Pretreatments destabilize lignin-hemicellulose barrier and reduce cellulose crystallinity (da Costa Sousa et al., 2009).

Specifically, lignocellulosic materials, such as agricultural residues, forestry waste, and dedicated energy crops, represent a vast and renewable source of biomass for bioenergy production. However, their complex structure poses a significant challenge for AD. Lignocellulose is composed mainly

of three key components: cellulose, hemicellulose, and lignin. While cellulose and hemicellulose are polysaccharides that can be hydrolyzed into fermentable sugars, lignin is a highly recalcitrant polymer that acts as a physical and chemical barrier, limiting microbial access to these carbohydrates. As a result, direct AD of untreated lignocellulosic biomass often results in low biogas yields due to inefficient hydrolysis, which is the rate-limiting step of the process. To overcome these limitations, various pretreatment methods have been developed to enhance the solubilization of lignocellulosic materials, making them more accessible to microbial degradation. The goal of pretreatment is to disrupt the intricate structure of the biomass, reduce cellulose crystallinity, break down hemicellulose, and partially remove or modify lignin. By achieving these modifications, pretreatment improves hydrolysis efficiency, accelerates microbial digestion, and ultimately enhances methane production.

The effectiveness of various pretreatment methods on the degradation of cellulose, hemicellulose, and lignin in lignocellulosic biomass is pivotal for enhancing the efficiency of subsequent AD processes. Each pretreatment technique uniquely impacts these components, facilitating their breakdown to varying extents.

Physical Pretreatment methods, such as milling and grinding, primarily reduce the particle size of biomass, thereby increasing the surface area accessible to microbial and enzymatic actions. While these methods enhance the physical accessibility of cellulose and hemicellulose, they do not significantly alter the chemical structure of lignin. Consequently, the lignin component remains relatively intact, potentially limiting the overall digestibility of the biomass.

Chemical Pretreatment approaches involve the application of acids, alkalis, or oxidative agents to disrupt the lignocellulosic matrix. Acid hydrolysis, for instance, effectively solubilizes hemicellulose and can partially degrade lignin, thereby increasing the accessibility of cellulose for enzymatic hydrolysis. Alkaline pretreatments, utilizing agents like sodium hydroxide, are particularly effective in delignification, significantly reducing lignin content and enhancing the digestibility of both cellulose and hemicellulose. Oxidative pretreatments employ oxidizing agents to break down lignin structures, further facilitating the hydrolysis of cellulose and hemicellulose.

Physicochemical Pretreatment techniques, such as steam explosion and ammonia fiber expansion (AFEX), combine physical and chemical processes to alter the biomass structure. Steam explosion involves subjecting biomass to high-pressure steam followed by rapid decompression, which disrupts the lignocellulosic matrix, leading to hemicellulose solubilization and partial lignin degradation. AFEX treatment uses ammonia under high pressure to swell and partially solubilize lignin, thereby increasing the accessibility of cellulose and hemicellulose to enzymatic actions.

Biological Pretreatment utilizes microorganisms, such as fungi, to degrade lignin and hemicellulose, leaving cellulose relatively intact. White-rot fungi, for example, are known for their

lignin-degrading capabilities, which enhance the subsequent hydrolysis of cellulose. However, biological pretreatments generally require longer processing times compared to other methods and may be less predictable due to the variability in microbial activity.

Integrating multiple pretreatment methods can significantly improve biomass degradation efficiency, reduce energy consumption, and minimize the use of chemical reagents. Such integrated approaches have shown promise in enhancing the overall effectiveness of lignocellulosic biomass pretreatment.

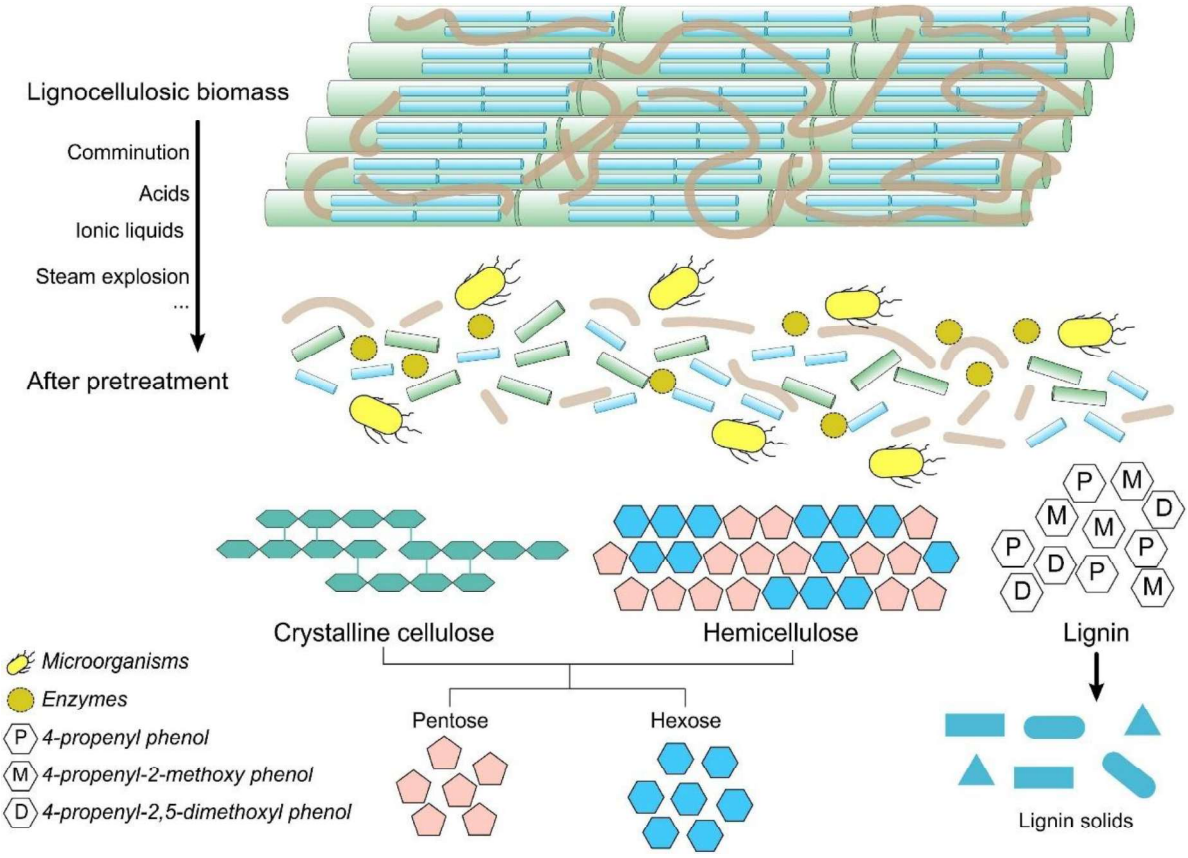

Figure 6. Pretreatments effects on lignocellulose structure (B. Zheng et al., 2022).

A more detailed description of the above-cited categories of pretreatments is provided in the next sections.

4.1.1 Physical pretreatments

Physical pretreatments include mechanical and thermal pretreatments. With mechanical pretreatments such as milling, the main goal is to reduce the diameter of the biomass particles and therefore increase the surface to volume ratio, consequently, more biomass areas can be attacked by biodegrading enzymes. Thermal pretreatments, with temperatures ranging from 50 to 250 °C, can disintegrate the cellular membrane as well as solubilize hemicellulose and lignin on lignocellulosic substrates. Pressure differences can be used as well, as is the case of steam explosion

pretreatments, where the biomass is firstly heated at high temperature (160 – 260 °C), pressurized with water vapor (0.69 – 4.83 MPa) and then rapidly decompressed to break the cell wall and hydrolyze the hemicellulose in monomers, thus enhancing biomass degradability (Y. Zheng et al., 2009).

4.1.2 Chemical pretreatments

Chemical pretreatments lead to a dissolution of organic compounds using strong acids or bases. This pretreatment is usually indicated with lignin-rich compounds, causing the hydrolysis of hemicellulose to the corresponding monosaccharides, while lignin precipitates (Fernandes et al., 2009; Hendriks & Zeeman, 2009).

Acid pretreatments can be done with either concentrated or diluted acids, with a broad range of temperatures (40 – 230 °C). It is possible to use both inorganic and organic acids, mainly sulphuric (H_2SO_4), hydrochloric (HCl), nitric (HNO_3), phosphoric (H_3PO_4), acetic, and malic acid. Using a strong concentrated acid is highly efficient for cellulose and hemicellulose hydrolysis, however, it can lead to the destruction of lignin and monomers carbon chain, and to the synthesis of toxic inhibitors for AD microorganisms (Battista et al., 2021). These inhibitors are divided into three main groups: i) phenols (vanillin, syringaldehyde and coniferyl aldehyde), ii) toxic acids (formic acid, levulinic acid), and iii) furfurals (furfural, 5-hydroxymethylfurfural) (Juarez et al., 2018). Moreover, the reactor structure and construction materials should be compatible with strong concentrated acids that can damage the reactor hull. Instead, using diluted acid can also hydrolyze hemicellulose into its monomers (xylose, arabinose, and galactose), but is inefficient to completely degrade the lignin (C. Zhang et al., 2014).

Alkaline pretreatment is performed using strong bases, mainly sodium hydroxide (NaOH), calcium hydroxide ($Ca(OH)_2$), and potassium hydroxide (KOH) to degrade lignin, solubilize cellulose and hemicellulose. In particular, during the pretreatment, lignin-carbohydrates bonds were saponified and cleaved, causing the collapse of lignin structure (C. Zhang et al., 2014). This pretreatment can be done at room temperature (J. S. Kim et al., 2016).

4.1.3 Biological pretreatments

Biological pretreatments are aerobic processes (e.g. composting or micro-aeration), which can hydrolyze complex substrates due to the biological production of enzymes (Lim & Wang, 2013). In particular, White Rot Fungi have been studied as a potential biological agent in the pretreatment of lignocellulosic waste due to the enzymes they release into the medium, although pretreatment times ranging from several weeks to several months may be required for significant lignin degradation (Wan & Li, 2012). Carbohydrases, proteases, and lipases produced by industrial fermentation processes can also be used (H. J. Kim et al., 2006). In the case of lignocellulosic substrates, the

enzymes used are cellulases and hemicellulases; however, being expensive, their use is often limited (C. Zhang et al., 2014).

Compared to physical and chemical pretreatments, biological pretreatments usually require less energy and chemicals, and are carried out under much milder conditions, resulting in fewer inhibitors that could negatively affect AD. However, the long retention times of biological pretreatment make it less advantageous compared to physical and chemical pretreatments, and marginal increases in biogas may not justify the high cost of enzymes (C. Zhang et al., 2014). Additionally, there is competition for carbohydrates between pretreatment and downstream biogas production because microorganisms during biological pretreatment require certain levels of carbohydrates. On the other hand, cellulose accessibility is increased after pretreatment, which can improve biogas production. Therefore, one of the main objectives of biological pretreatment is to minimize carbohydrate loss and maximize lignin removal to provide highly digestible raw materials.

4.2 Hydrolysis

Microorganisms and bacteria cannot use complex organic biopolymers and particulate directly as a substrate. Therefore, lipids, polymeric carbohydrates and proteins must be hydrolyzed by fermentative bacteria into small and soluble molecules, respectively long-chain fatty acids (LCFAs), monosaccharides and amino acids (C. Zhang et al., 2014), which can be transported inside the cellular membrane. Hydrolytic enzymes can be directly secreted (exo-enzymes) or be membrane-bound. Hydrolysis rate is mostly influenced by temperature, pH, type of particulate to be hydrolyzed and the concentration of the hydrolytic biomass, as well as their enzymes (Veeken & Hamelers, 1999). The kinetic model which predicts the most reaction rate is the enzyme-absorption model (ABK model). In this model, the substrate hydrolysis rate is directly proportional to the specific area of the substrate available for the enzyme adsorption. Due to the difficulty of hydrolyzing complex organic compounds, this step is often the rate-limiting step, hence, pretreatments may be needed.

4.3 Acidogenesis

The products of hydrolysis (monosaccharides, amino acids and LCFAs) can diffuse through the cell membrane into the cytosol of bacterial cells. Inside the cell membrane, these compounds are fermented and anaerobically oxidized, producing VFAs such as acetate, propionate and butyrate, molecular hydrogen (H_2) and carbon dioxide. This reaction, also known as dark fermentation, is the most rapid step for AD process. Therefore, when an anaerobic reactor is overloaded or intoxicated, it is subjected to a rapid pH decrease that further enhance methanogenesis inhibition. In normal

conditions, the pH decrease is buffered by the release of ammonia from the degradation of amino acids.

4.4 Acetogenesis

In this step, all the short-chain fatty acids, mostly propionate and butyrate, are converted to acetic acid, hydrogen and carbon dioxide. Fatty acids were converted to acetic acid by β-oxidation reactions. The β-oxidation is a crucial for the AD process to continue, because requires an association between acidogenic and methanogenic microorganisms. In particular, the β-oxidation of fatty acids produces molecular hydrogen, which is an inhibitor of the reaction itself. Therefore, hydrogen must be removed to keep the free energy level negative. This can occur by either manually removing the hydrogen or can be used by methanogenic microorganisms for CH_4 production, keeping the H_2 level below 10-4 atm. On the other hand, if the H_2 concentration is too high, the free energy of the β-oxidation reaction becomes positive, obtaining its reverse reaction called **chain elongation** (CE), which can also be exploited for the accumulation of medium-chain fatty acids, a high-value compounds, despite a drastic reduction of CH_4 from methanogenesis.

4.5 Methanogenesis

In this stage, the products from the previous AD stages are converted into an insoluble compound, biogas, which is a mixture composed by two main gases: methane and carbon dioxide, alongside more residual gases whose compositions are listed in Table 1. Acetate and CO_2 were reduced to CH_4 by two different methanogenic pathways. The acetoclastic methanogenesis decarboxylate the acetate to form carbonate and CH_4, while the hydrogenotrophic methanogenesis reduces CO_2 to CH_4 using hydrogen as electron donor (Li et al., 2018). Although these two pathways co-exist, approximately 70% of methane is produced by acetoclastic methanogenesis, while only a minority of methane is produced by CO_2 and H_2. This step is carried out by mainly two genera of microorganisms: *Methanosarcina* spp. and *Methanosaeta* spp., both of them converts mostly acetate, although *Methanosarcina* spp. can also follow the hydrogenotrophic pathway. These microorganisms are characterized by a slow growth rate, with a doubling time up to several days, that require a very long start-up of the anaerobic reactor (Pramanik et al., 2019).

Type of gas	**Percentage present in biogas (%)**
Methane (CH_4)	55 – 70
Carbon Dioxide (CO_2)	35 – 40
Nitrogen (N_2)	0 – 3
Hydrogen (H_2)	<1
Oxygen (O_2)	<1
Hydrogen Sulfide (H_2S)	<1

Table 1. Biogas composition (Jameel et al., 2024).

4.6 AD operational parameters

4.6.1 HRT and SRT

The principal parameter for AD bioreactor continuously operating, with constant inlet and outlet flow, is the **Hydraulic Retention Time** (HRT). This parameter indicates the average time of residence of a soluble compound inside the reactor, and the minimum time for the replacement of all reactor volume. HRT (day) is defined as the ratio between reactor volume V (m^3) and the inlet flow rate Q ($m^3 day^{-1}$):

$$HRT = \frac{V}{Q}$$

The HRT reciprocal defines the **Dilution Factor or Dilution Time**, indicating the percentage of bioreactor volume that is replaced each day:

$$D = \frac{1}{HRT}\% = \frac{Q}{V}\%$$

Whenever the HRT value is inferior to the duplication time of a microorganism present in the bioreactor, it will wash out and, after the bioreactor stabilization, that microorganism will not be present. The wash out should be carefully considered for certain species of microorganisms, in particular methanogenic ones, which are characterized by slow metabolic kinetics and duplication time. Therefore, changing the HRT on a bioreactor can be used to select the optimum microbiome for acidogenic or methanogenic phases.

If the bioreactor allows solids recirculation, HRT separates from the **Solid Retention Time** (SRT), which represents the average residence of the solid part of the influent inside the reactor and is defined by the following formula:

$$SRT = \frac{V \cdot X}{Q_R \cdot X_R + Q_S \cdot X_S}$$

where X is the solid concentration in the reactor (kgVS m^{-3}), Q_R and X_R are the recirculation flow rate (m^3 day^{-1}) and solid concentration (kgVS m^{-3}), Q_S and X_S are the sedimentation unit effluent flow rate and solid concentration.

4.6.2 Temperature

AD process optimal temperature is based on the microorganisms consortia present in the bioreactor. Is possible to distinguish between 3 different operating temperature: mesophilic (25 – 40°C, most common), thermophilic (45 – 60 °C), and psychrophilic (<20°C). Temperature influences microorganisms growth and overall metabolic activity. Generally, metabolic activity is higher at the maximum temperature allowed for microorganisms. However, the microbial population is sensible

to temperature variation over the optimal range. Variation of 2 – 3 °C over the optimum range can greatly influence the overall performance of AD process (Angelidaki & Sanders, 2004).

4.6.3 pH

AD requires an overall neutral pH (6.0 – 8.3). The pH value is mainly determined by carbonate (CO_3^{2-}), ammonia (NH_3), and VFAs presence. pH control is crucial to the well-being of the AD process, and little pH variation can influence microorganisms metabolic activity or inhibit some phases of the process. Acidic pH values (<6.0) can inhibit methanogens and favor acidogenic bacteria, which will increase the production of VFAs until pH drops below 5, where they are also inhibited, leading to a bioreactor stall due to substrate excess (Angelidaki & Sanders, 2004).

4.6.4 OLR

The quantity of substrate which enter in the bioreactor each day is called **Organic Load Rate** (OLR):

$$OLR = \frac{Q \cdot S}{V}$$

where Q is the inlet flow rate (m^3 day^{-1}), S is the substrate inlet concentration (kg m^{-3}), and V is the reactor volume (m^3).

Increasing the OLR in an AD bioreactor leads to an increased total VFAs production, due to the fast metabolic kinetics of acidogenic microorganisms. Methanogenic microorganisms cannot consume VFAs fast enough and, therefore, VFAs accumulates as pH decreases, further inhibiting the methanogenic phase.

4.6.5 Biogas composition

Monitoring the composition of the produced biogas is important to evaluate bioreactor stability, or to troubleshoot inhibition or process issues. In stable operating conditions, the biogas should be composed mainly of CO_2 and CH_4, with a concentration and production stable over time. Increasing CO_2 and H_2 concentration, with a coincident decrease in CH_4 concentration, can indicate a substrate inhibition or a methanogens wash-out.

4.6.6 VFA production

VFAs concentration can also be an indicator of AD process well-being and stability. Typically, in a bioreactor for biogas production, the average VFAs concentrations are in the range between 200 to 2000 mg/L (Strazzera, Battista, Tonanzi, et al., 2021). Higher concentrations represent the prevalence of acidogenic microorganisms over methanogens, caused by substrate inhibition or wash-out. On the contrary, low VFAs concentration can reduce biogas output due to substrate scarcity.

Alternatively, to biogas production, this anaerobic process can be finalized to VFAs production. Mixed microbial cultures (MMC) can be redirected to acidogenic fermentation by modifying operating conditions, in particular HRT and OLR. It is also possible to split acidogenic and methanogenic fermentations in two different reactors (Two Stage Anaerobic Digestion, TSAD), allowing to optimize both VFAs and biogas production.

5. Chain Elongation

The AD process can go beyond biogas production, if properly managed, and be suitable for VFAs production, usually requiring fossil sources for their synthesis (Battista et al., 2024). Biological VFAs production are a topic of growing interest in the biorefinery field (Battista et al., 2022) and are "building blocks" for a high number of biobased products, especially MCFAs. MCFAs then can be applied in food, chemical, pharma, biofuel and bioplastic industries, allowing the increasing of potential economic revenue of the biorefinery platforms (Menon & Lyng, 2021; Scarborough et al., 2018).

Caproic acid, known also as hexanoic acid, is a 6-carbon saturated carboxylic acid, with its molar mass of 116.158 g/mol, is the smallest of the MCFAs. Compared to the shorter VFAs, the hydrophobic carbon chain prevails on the carboxylic group of the acid, hindering its water solubility to 10.3 g/L at 25°C. Caproic acid is usually synthetized from fossil resources or extracted from vegetal oils like palm, coconut or ricin. Major drawbacks of these processes are poor sustainability and low yields (8-15% of total fatty acid extracted) (Venkateswar Reddy et al., 2020a). The low yields translate to a limited production of 25 Kton per year (Debergh & Van Dael, 2022). For these reasons, the scientific community focused its attention on other biological ways to produce caproic acid and other MCFAs, in particular producing VFAs through acidogenic fermentation (the second AD step) and use them to obtain caproic acid through CE process.

The CE process, as previously discussed, is the reverse reaction of fatty acids β-oxidation. In an anaerobic reactor, certain environmental conditions can favor this reaction: i) presence of reduced compounds with high energy, in particular ethanol or lactate, as electron donors to provide reducing equivalents (NADH) and acetyl-CoA; ii) high H_2 partial pressure to prevent carboxylates oxidation. Both conditions are met during the first phases of AD, in particular during acidogenesis and acetogenesis. For this reason, CE can represent a solution to revamp AD, recovering fatty acids, with higher value than classic energy recovery (biogas) (Battista & Bolzonella, 2024).

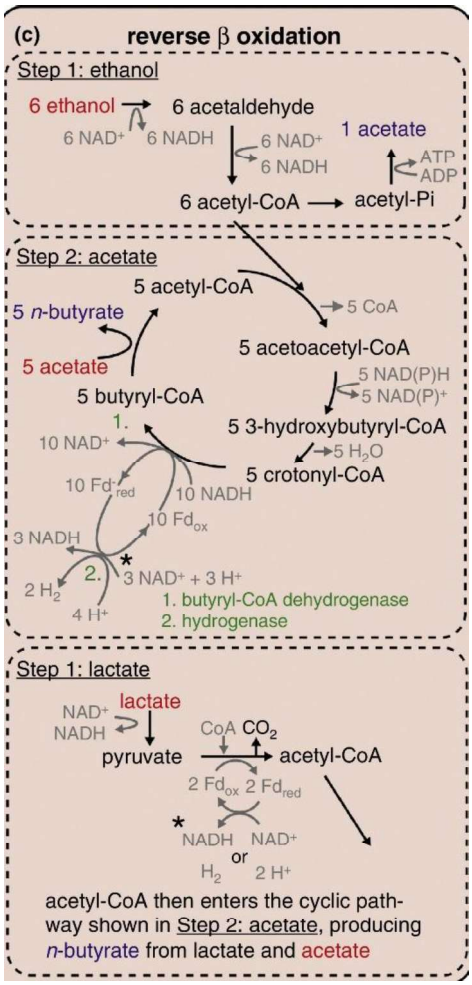

Figure 7.Chain elongation pathway through reverse β-oxidation (Spirito et al., 2014).

CE through reverse β-oxidation is a 2-step pathway as illustrated in Figure 7. In the first step, lactate or ethanol are oxidized to pyruvate and acetaldehyde respectively, producing NADH as reducing equivalents. Pyruvate and acetaldehyde are then oxidized again to form acetyl-CoA and NADH. In the second step, acetyl-CoA, formed in step 1, enters in the true reverse cycle of fatty acid β-oxidation, adding two carbon atoms to the carbon chain of a carboxylate. Thermodynamically this pathway is feasible, with a negative Gibbs free energy variation of -185 kJ/mol and -60 kJ/mol for ethanol and lactate as electron donors respectively, at pH 7 with temperatures ranging from 25 to 55 °C. For ethanol and lactate, the overall reactions are described in the following equations, respectively:

$$6\ ethanol + 4\ acetate^{-} \rightarrow 5\ butyrate^{-} + H^{+} + 2\ H_2 + 4\ H_2O$$

$$Lactate^{-} + acetate^{-} + H^{+} \rightarrow butyrate^{-} + CO_2 + H_2O$$

5.1 Parameters influencing Chain Elongation

CE process is highly influenced by chemical and physical parameters of operation of the bioreactor. Optimal conditions in the bioreactor favor the growth of CE-capable microorganisms, in particular *Clostridium kluyveri* and *Megasphaera elsdenii*, which can use ethanol and lactate as substrate respectively.

5.1.1 Temperature

Temperature can influence microorganism growth and activity. For CE, the temperature optimum is in the range 34 – 37 °C for *C. kluyveri* and *M. elsdenii*. Although the thermodynamics of CE reaction can allow a higher temperature, Spirito et al. (2014) noted that no products were formed with temperatures over 55 °C.

5.1.2 pH

The pH is one of the most important parameters for the process. As reported by P. San-Valero et al. (2019), neutral pH range (6.8 – 7.5) is the optimum for CE process and associated with higher caproate productions. In neutral pH, only 1% of the total caproic acid is in undissociated form. This form is highly toxic for microorganisms, therefore, working with pH close or below to the pKa of the caproic acid (4.9) can reduce process yields.

5.1.3 Organic Load Rate

Accumulation of VFAs concentration in the bioreactor can lead to the inhibition of methanogens microorganisms (Li et al., 2018). Overloading of organic matter generally happens when the microbiome is slower in substrate degradation than the substrate load. An overloading can be obtained by increasing the Organic Load Rate (OLR) or the Inoculum to Substrate ratio (I/S, $gCOD_{fed}/gCOD_{inoculum}$), as it defines the substrate feed, expressed as COD or volatile solids, compared to the biomass of the inoculum.

5.1.4 Hydraulic Retention Time

Short HRT favor the selection of CE microorganisms in spite of methanogenic microorganisms, whose growth kinetics are slower. Methanogenic microorganisms have a duplication time of about 20 days, therefore, a shorter HRT washout them. HRT should not be too short to give time for the elongation of the carbon chain past butyric acid to yield caproic acid. Therefore, the optimum HRT is around 10 days (Contreras-Dávila et al., 2020).

5.1.5 Readily fermentable organic fraction

Depending on how easily the organic fraction is accessible to the microorganisms, the substrate can be indicated for VFAs production for CE or for biogas. In particular, substrates derived from livestock effluents, organic fraction of municipal solid waste (OFMSW), or paper industry waste

allows VFAs production and CE. On the other hand, recalcitrant substrates, for example vegetal residues, secondary sludge and depleted industrial waste, are more indicated for biogas production due to the long hydrolysis time of these substrates, which is compatible with minimum HRT of 20 days. To enhance substrate availability, and solubilize lignocellulosic residues, it is possible to pretreat the substrate.

6. Digestate management

Nitrogen (N), Phosphorus (P) and Potassium (K) are the main components of fossil-based, mineral fertilizers (Drosg et al., 2015). Because of their fossil-based and non-renewable origin, the production of conventional fertilizers is unsustainable, with concerns over long-term availability (Mehta et al., 2015). The conventional, and most used, ammonia-based fertilizer production process is represented by the Haber-Bosch process, which was developed more than one century ago, in 1913. Atmospheric molecular nitrogen (N_2) is converted to ammonia by reacting with hydrogen (H_2), at pressures and temperatures up to 100 bar and 500 °C, respectively, at the presence of a heterogeneous catalyst, usually iron. N_2 is extracted from air, while H_2 is produced by steam-methane reforming, from fossil natural gas, with temperatures up to 1,000 °C. The Haber-Bosch process produces 1.5 tonnes CO_2 per tonne of produced NH_3, with an energy consumption of around 6.4×10^{12} MJ/year, 2% of worldwide total energy consumption (Noriega-Hevia et al., 2020; Razon, 2014), and accounting for 0.93% of worldwide greenhouse gas emission (Bicer et al., 2016). With specific reference to conventional phosphorus-based fertilizers, these are produced by extraction of mineral phosphate rocks, generally as impure calcium phosphate. The extracted rock is then treated with sulphuric acid to produce raw phosphoric acid (H_3PO_4) and gypsum as a waste. The raw phosphoric acid, which can contain pollutants and heavy metals, is refined in order to remove such pollutants, and to obtain more soluble and thus bioavailable phosphate fertilizers (Kohl & Nielsen, 2007). Phosphate rocks are non-renewable and highly requested sources. According to Mehta et al. (Mehta et al., 2015), by 2033 the demand of phosphoric fertilizers will be higher than the availability, causing a rapid depletion of reserves. Moreover, 90% of phosphate rock reserves are located in few countries, especially Morocco, Iraq, China, Algeria, and Syria. Regarding the greenhouse gas emissions, superphosphate ($Ca(H_2PO_4)_2$) and ammonium phosphate ($(NH_4)_3PO_4$) fertilizers produce 0.4-1.6 and 1.3-8.9 $kgCO_2/kgP_2O_5$ respectively (Walling & Vaneeckhaute, 2020). The energetic consumption corresponds to 7700 kJ/kg phosphate (Gellings & Parmenter, 2016). In addition, phosphorus is considered strategically and critical raw material for the EU (Maniscalco et al., 2020).

The EU accounts for 133.9 million hectares of fertilized agricultural land (Fertilizers Europe, 2021), mainly cultivated intensively. Europe consumed 11.2 million tonnes of mineral fertilizers in 2020, with a consumption increase of 6.9 and 21.9 % for N and P respectively compared to 2010 (Eurostat,

2022c). Moreover, 30%, 68% and 85% of N, P and K respectively of the total nutrient consumption was imported, mainly from Russia and Belarus (Fertilizers Europe, 2022), with a total import of 26 Mt of mineral fertilizer between 2019 to 2021. As discussed previously, the recent Ukrainian-Russian war remarked how EU is not independent in the supplying of resources. The Russian Federation, economically sanctioned by the EU, is the largest natural gas and fertilizers exporter to the EU, with over 155 billion cubic meters (bcm) of natural gas, which is a key element for nitrogen-based fertilizers, and 1.1 billion € of fertilizers exports (Fertilizers Europe, 2022; IEA, 2022).

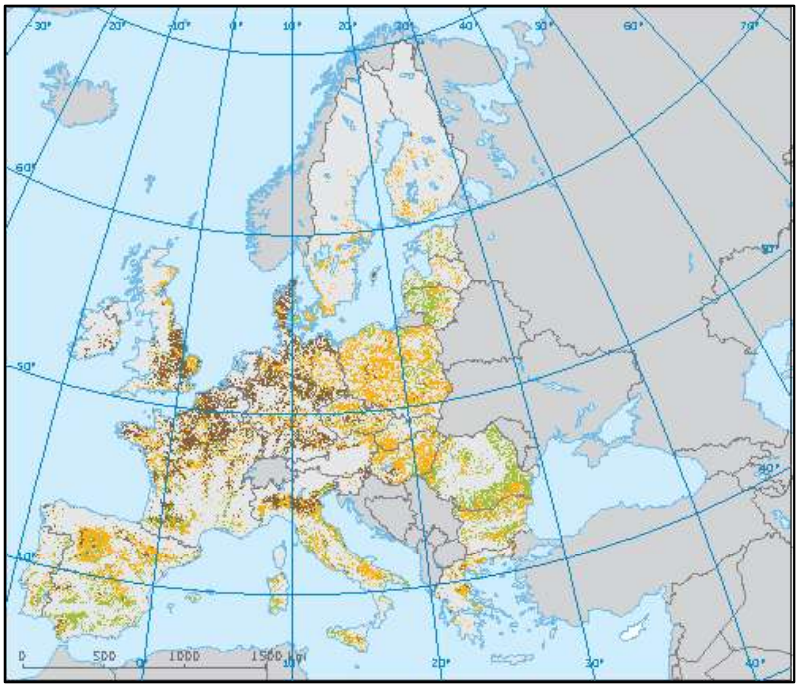

Figure 8. Agricultural land use intensity (EEA, 2016). Green: extensive used arable land; Yellow: moderately intensive used arable land; Brown: intensive used arable land; Light gray: non agricultural land; White: no data; Dark gray: outside coverage.

The unsustainability of the classic fertilizers production, the depletion of the conventional P-based fertilizer reserves, combined with the increasing instability of the geopolitical scenario, highlight that the research of new biobased alternatives for fertilizers production is now urgent and necessary. The intensive agriculture and livestock concentration across Europe could offer a valid source of nutrients and an alternative to classic synthetic fertilizers. In 2020, the EU crops production reached over 285 and 61 million tonnes of cereals and fresh vegetables respectively, with a consequent generation of over 4 million tonnes of vegetal wastes (Eurostat, 2018, 2020). Regarding the EU livestock production, in 2021, there were over 1.5 billion poultries, 141 million pigs, 75 million bovines and 75 million sheep and goats (Eurostat, 2022b; Flotats et al., 2013), with a global production exceeding 1.4 billion tonnes per year of manure between 2016 and 2019 (Köninger et al., 2021). The organic wastes derived from these agricultural and farming activities are often

treated by AD for biogas production, whose main byproduct is represented by digestate rich in nutrient compounds.

6.1 Agricultural residues and manure characterization

The typical physical-chemical characteristics of crop residues and livestock manure are reported in Table 2. The vegetal residues, like straw of different origin, are rich in Total Solids (TS), thus carbon, but show limited levels of nutrients, while livestock effluents generally show high content of nitrogen and phosphorus (Battista et al., 2019). The chemical composition of livestock manure heavily depends on the animal feed used and on the quality of feed application, thus enhancing the characterization variability of livestock effluents (Dadrasnia et al., 2021).

Source	Total Solids	Volatile Solids	Total Nitrogen	Ammonia	Total Phosphorus	Reference
Crop Residues						
Barley	26.31 %w/w	25.44 %w/w	12.31 mg/g	0.53 mg/g	n.d.	(Morales-Polo et al., 2021)
	25.8 – 66.3 %w/w	25.1 – 59.1 %w/w	7.0 – 19.9 g/kg	n.d.	0.8 – 3.9 g/kg	(Garcia et al., 2019)
Barley Straw	86.83 ± 0.13 %w/w	81.37 %w/w	0.76 %TS	n.d.	n.d.	(Meyer et al., 2022)
Corn	20.1 – 40.4 %w/w	18.3 – 36.6 %w/w	4.0 – 5.8 g/kg	n.d.	0.3 – 0.6 g/kg	(Garcia et al., 2019)
Corn Straw	90.0 ± 0.2 %w/w	81.3 ± 0.1 %w/w	1.3 ± 0 %TS	n.d.	n.d.	(S. Wang et al., 2022)
	66.91 ± 0,95 %w/w	76.22 ± 0.54 %TS	0.78 ± 0.03 %TS	n.d.	n.d.	(Guo et al., 2022)
Fresh Vinegar	32.56 ± 1.32 %w/w	31.01 ± 0.97 %w/w	1.92 ± 0.05 %TS	n.d.	n.d.	(L. Chen et al., 2022)
Rice Husks	90.7 %w/w	74 %w/w	25.5 g/kg	n.d.	3.5 g/kg	(Garcia et al., 2019)
Triticale	30 – 30.8 %w/w	27.9 %w/w	13.5 g/kg	n.d.	0.7 g/kg	(Garcia et al., 2019)
Livestock Effluents						
Cows	15.9 ± 0.2 %w/w	78.4 ± 0.2 %TS	25.9 mg/gVS	n.d.	n.d.	(Coarita Fernandez et al., 2020)
	9.50 %w/w	80 %TS	0.351 %w/w	n.d.	0.082 %w/w	(Aravani et al., 2022)
	208.4 ± 1.9 g/kg	166.4 ± 1.8 g/kg	6.1 ± 0.3 g/kg	2.0 ± 0.0 g/kg	1.9 ± 0.3 g/kg	(D. Kim et al., 2022)
	15.6 – 47.7 %w/w	13.5 – 32.1 %w/w	3.2 – 7.1 g/kg	n.d.	0.2 – 1.5 g/kg	(Battista et al., 2019)
	19.97 ± 0.12 %w/w	11.95 ± 0.25 %w/w	4.02 ± 0.02 %TS	n.d.	n.d.	(Z. Yang et al., 2022)
Horses	34 %w/w	n.d.	0.82 %TS	n.d.	1.76 g/kg	(Parvage et al., 2015)
Pigs	39.2 g/L	27.4 g/L	12.5 g/L	5.0 gN/L	0.2 g/L	(Jo et al., 2022)
	27.5 ± 0.7 %w/w	22.0 ± 0.3 %w/w	3.9 ± 0.3 %TS	n.d.	n.d.	(S. Wang et al., 2022)
	27.13 ± 0.27 %w/w	19.89 ± 0.20 %w/w	5.32 ± 0.01 %TS	n.d.	n.d.	(Z. Yang et al., 2022)
	25.95 ± 1.17 %w/w	20.18 ± 0.84 %w/w	2.27 ± 0.03 %TS	n.d.	n.d.	(L. Chen et al., 2022)
	36.1 %w/w	35.9 %w/w	n.d.	n.d.	n.d.	(Garcia et al., 2019)
Pig slurry	1.9 ± 0.3 %w/w	61.1 ± 2.8 %w/w	0.28 %w/w	0.22 %w/w	0.061 %w/w	(Häner et al., 2022)
Poultry	20 %w/w	75 %TS	1.032 %w/w	n.d.	0.413 %w/w	(Aravani et al., 2022)
	25 ± 0.4 %w/w	66 ± 3.3 %TS	1.4 ± 0.6 %w/w	8.0 ± 1.3 g/kg	8.7 ± 1.0 g/kg	(Rizzo et al., 2022)
	37.76 ± 0.62 %w/w	26.45 ± 0.24 %w/w	2.36 ± 0.12 %w/w	0.55 ± 0.15 %w/w	n.d.	(Buivydas et al., 2022)
	31.5 – 78.3 %w/w	21.3 – 51.7 %w/w	2.3 – 38.9 g/kg	n.d.	5.2 – 15.3 g/kg	(Battista et al., 2019)
	23.00 ± 0.53 %w/w	67.52 ± 0.89 %TS	4.84 ± 0.37 %TS	1.11 ± 0.05 g/kg	n.d.	(Guo et al., 2022)
	48.3 %w/w	69.5 %TS	4.7 %TS	0.8 %TS	n.d.	(Wedwitschka et al., 2020)
Rabbits	21.8 %w/w	18.1 %w/w	1.7 g/kg	n.d.	n.d.	(Garcia et al., 2019)

Table 2. Agricultural residues and manure physical-chemical characterization. n.d.: not determined

Typically, vegetal residues and manure are directly applied to fields as a soil improver (Myers, 2020). However, the direct application of manure for soil amending and fertilization is regulated and can be applied on specific seasonal windows, due to odor and environmental pollution issues (Sommer & Knudsen, 2021). As an alternative, vegetal residues and livestock effluents can be used as substrates for biogas production, through AD processes (Valenti et al., 2018). This process could assume a valid and strategic role for biogas and fertilizers production (European Commission, 2022b).

In Europe in 2019 a total of 18,943 biogas plants were present, according to the European Biogas Association (EBA) (EBA, 2020), producing 167 TWh of biogas and 180 million tons of digestate annually (Corden et al., 2019). The AD plants are mainly located in Germany (11,269), followed by Italy (1,710), United Kingdom (1,233) and France (890) (EBA, 2020). Regarding the feedstock composition, over 70% (13,477) of biogas plants in Europe used agricultural substrates, mainly from energy crops, manure and food crops residues (i.e., maize triticale, wheat straw and rice husk) (EBA, 2020). The main byproduct from AD is represented by digestate, which includes the stabilized organic matter, nitrogen, and phosphorous compounds, potentially exploitable for bio-based fertilizer production. According to the 2023 EBA Statistical Report, the total digestate production in 2022 was 27.1 Mt dry matter, containing 1.5 Mt of nitrogen, 0.3 Mt phosphorus and 0.2 Mt potassium.

6.2 Characterization of typical agricultural digestates

As discussed above, during the AD process all the easily accessible organic carbon is converted into methane (CH_4) and carbon dioxide (CO_2). Thus, the digestate is the remaining part of the substrate and is primally composed by recalcitrant lignocellulosic residues, and nutrients, especially N, P and K. Digestate characteristics are variable, depending on the origin and characteristics of manure fed to the digester and as well as the operational parameters applied to the AD reactor (Sheets et al., 2015). Table 3 reports the characterization of digestates from the most common agricultural substrates. The pH is usually slightly basic, ranging from 7 to 8.5. Overall, pH increases when ammonia is present, VFAs are degraded and when basic cations are released; pH typically decreases when carbonate and phosphate precipitation reaction occur (Drosg et al., 2015; Hjorth et al., 2010) or VFA accumulate in the system. The TS content varies between 1 to 25%, due to the variability of input substrates biodegradability. Generally, higher TS content is due to feeding of high levels of lignocellulosic substrates and low digestibility, typical of agricultural residues (Drosg et al., 2015). When the AD substrate is rich in easily biodegradable substances, the digestate will exhibit a lower TS content and VS/TS ratio (Barampouti et al., 2020). The degradation of organic nitrogen, mostly derived from the protein and aminoacidic content of the substrate, by the AD process leads to an accumulation of ammonium, which represents a major part of the total nitrogen

content. Digestates from manure and protein rich substrates are expected to have a higher ammonium content compared to digestates from vegetal residues. The phosphorus content is not altered by the AD (Drosg et al., 2015). The environment inside an AD bioreactor favors the formation of phosphates, that can precipitate as magnesium or calcium salts (Fricke et al., 2007). During the AD, the organic P is converted into orthophosphate. However, 90 % of phosphate interacts and precipitates with Ca^{2+} and Mg^{2+} cations, thus increasing P concentration in the solid fraction of the digestate (Christensen et al., 2009).

Substrate	pH	Total Solids (TS)	Volatile Solids (VS)	Total N	Ammonia (NH_3)	Total P	Total K	Ref.
Cattle manure + pig slurry	7.9	6.20%	81.50 %TS	3.7 kg/m^3	1.6 kg/m^3	0.7 kg/m^3	2.7 kg/m^3	(Kuusik et al., 2017)
Cattle manure	8.3	7.10%	81.30 %TS	3.8 kg/m^3	1.8 kg/m^3	0.6 kg/m^3	3.3 kg/m^3	(Kuusik et al., 2017)
Manure (unspecified)	7.3 – 8.6	2.2 – 9.2 %	67.8 – 75.0 %TS	0.05 – 0.62 %TS	0.255 – 1.01 %TS	0.034 – 0.221 %TS	0.03 – 0.43 %TS	(Barampouti et al., 2020)
Diary cows digestate	n.d.	70 ± 3 g/kg	49 ± 2 g/kg	3.35 ± 0.3 gN/kg	1.73 ± 0.1 gN/kg	1.64 gP/kg	n.d.	(Bolzonella et al., 2018)
Cow dung slurry	8.89	n.d.	n.d.	38.4 mg/g	23.58 mg/g	6.16 mg/g	12.73 mg/g	(Walsh et al., 2018)
Pig digestate	n.d.	32 ± 3 g/kg	21 ± 2 g/kg	2.25 ± 0.4 gN/kg	1.16 ± 0.3 gN/kg	0.36 ± 0.01 gP/kg	n.d.	(Bolzonella et al., 2018)
Pig Slurry	8.4	4.80%	63.90 %TS	5.2 kg/m^3	1.6 kg/m^3	1.5 kg/m^3	2.1 kg/m^3	(Kuusik et al., 2017)
Energy Crops + Manure	7.7 – 8.1	6.1 – 8.3 %	4.4 – 6.3 %	7.6 – 9.6 kgN/t	4.9 – 6.1 kgN/t	n.d.	n.d.	(Drosg et al., 2015)
Energy Crops	7.6 – 8.0	6.6 – 9.3 %	4.8 – 6.9 %	3.6 – 4.9 kgN/t	1.3 – 2.4 kgN/t	n.d.	n.d.	(Drosg et al., 2015)
Agricultural Feedstock	7.5 – 8.4	7.4 – 24.0 %	69 - 74 %	22 - 88 gN/kg	6 - 45 gN/kg	2 - 66 gP/kg	9 - 100 gK/kg	(Selvaraj et al., 2020)
Agricultural Feedstock	7.5 – 8.4	6.41 - 24 %	69 - 77 %	0.14 – 2.1 % TS	0.04 – 1.71 %TS	0.058 – 2.400 %TS	0.324 – 0.392 %TS	(Barampouti et al., 2020)
Digestate (unspecified)	7.5 - 9	1.5 – 45.7 %	38 - 77 %TS	0.005 – 5.04 %TS	0.052 – 2.75 %TS	0.002 – 2.400 %TS	0.001 – 2.52 %TS	(Barampouti et al., 2020)
Digestate (unspecified)	8.1 – 8.6	4.98 – 12.0 %	2.8 – 7.6 %	0.17 – 0.75 %	0.52 – 3.41 g/L	0.14 – 0.65 %	0.20 – 0.50 %	(Vaneeckhaute et al., 2017)

Table 3. Digestate characterizations; n.d. not determined.

6.3 European legal framework for digestate management

The conventional use of digestate is the direct application on farmland soils as conditioner/fertilizer. This use, if not properly managed, can lead to several pollution issues, mainly nutrient surplus leaching, with contamination and eutrophication of surface and ground-waters (Levine et al., 2013) and greenhouse gas emissions, especially ammonia (NH_3) and nitrous oxide (N_2O) (Hou et al.,

2017). Ammonia gas in atmosphere can then lead to particulate formation, mainly as ammonium sulfate, one of the major issues for atmospheric pollution (micro-particulate) in both urbanized and rural areas (Y. Yang et al., 2018) .

These pollution issues are particularly concentrated in the EU's agricultural areas, which accounts for around 47% of total EU land use (EU27 + UK) (Eurostat, 2022a). According to the EU report 2021/1000 (European Commission, 2021), the nutrient balance of an agricultural area is the difference between nutrient input, usually with the use of fertilizers or manure, and nutrient output, represented by crops and fodder. A shift from neutrality of the nutrient balance could lead to two scenarios: (i) nutrients deficit, when the balance is negative; (ii) nutrients surplus, with a positive balance. These two conditions lead to soil infertility, nutrient leaching, and dispersion in the environment, respectively. Regarding the EU, the nutrients balance on agricultural land is dramatically positive: 47.1 kg/ha of N surplus and 1.1 kg/ha for P surplus in 2014 (Eurostat, 2022a). Moreover, EU has passed the limits for N and P cycles, which are 2.1 and 0.07 Tg/y (teragrams per year) respectively, by a factor 3.3 for N (6.8 Tg/y) and 2 for P (0.14 Tg/y) (EEA, 2020).

The EU started to regulate digestate application from 1991, with the European Council (EC) directive concerning the protection of waters against pollution caused by nitrates from agricultural sources, otherwise named "Nitrate's Directive" (European Council, 1991). This directive imposed to all Member States to monitor the welfare of surface and ground waters and the concentration of nitrates, in order to designate "Nitrate Vulnerable Zones" (NVZs), where there is high risk of nitrate pollution. This monitoring must be periodic, with a maximum of 4 years between each control. Moreover, this directive requires Member States to implement measures to prevent and reduce nitrates pollution, with an annual limit of 170 kg/ha of manure-derived nitrogen. Figure 8 illustrates the actual NVZs for EU.

The NVZs are coincident with European plains, flat areas, and river drainage basins. Consequently, a majority of livestock production sites and farms are concentrated in NVZ in Europe, leading to an excess of digestate that cannot be directly applied on farmland. Moreover, even if the manure and digestate application follows the Nitrate Directive regulations, another issue is represented by P accumulation. The stoichiometric N/P ratios documented for soil microorganisms and plants (around 6 – 8 (Cleveland & Liptzin, 2007)) are higher than the N/P ratios of most types of manure (typically < 5 gN/gP, Table 2 and 3). This indicates that even manure applied to land in line with the Nitrates Directive contributes significantly to the observed P accumulations in agricultural ecosystems that receive high manure loads.

Therefore, it turns out evident that digestate needs to be treated both to favor a further stabilization of organic matter and to recover the nitrogen and phosphorous compounds, leading to a proper fertilization product to be applied on non-vulnerable or nutrient deficient areas. However, until 2019

the EU failed to issue a legislation act that covers nutrient recovery, biobased fertilizers production and their market. The EU Regulation 2003/2003 (European Council, 2003) only covered inorganic fertilizers, mined or chemically produced. Thus, this regulation ignored fertilizers derived from organic waste materials or organic-inorganic mixed products, as well as soil improver or conditioner. For this reason, organic fertilizers were regulated and commercialized, inside and outside the origin country, in accordance with the National Legislation, thus discouraging the whole process of bio-fertilizers production at EU level. To overcome all the gaps in the old regulation, the European Parliament and the EU Council approved in 2019 the new Fertilizing Product Regulation 1009/2019 (European Council, 2019) that has entered into effect on 16 July 2022. This new regulation opened the access to the EU Single Market for biobased fertilizers, with a harmonized legislation that removes all the issues and costs regarding the mutual certification of the national rules. The biobased fertilizers can receive the "CE mark", making it easier to be commercialized and, consequently, promoting the production. This promotion is needed to foster the circular economic development and allowing a more efficient resources usage, while reducing the EU dependence from other countries.

Digestates are classified in two different Component Material Categories (CMC): CMC 4 for digestates from crops growth exclusively for biogas production (i.e., energy crops); CMC 5 for digestate derived from (i) bio-waste according to the directive 2008/98/EC (European Council, 2008) and (ii) derived products referring to the EU Regulation 1069/2009 (European Council, 2009), which includes manure and digestion residues from transformation into biogas. To be considered as organic fertilizer, the final product shall have a minimum quantity of N, P (as phosphorus pentoxide, P_2O_5) and K (as potassium oxide, K_2O). For solid, single-component fertilizers, this threshold is 2.5, 2 and 1 % w/w of N, P and K respectively. For liquid, single-component fertilizers, the threshold is 2, 1 and 2 % w/w for N, P and K respectively. In case of multi-component fertilizers, both solid and liquid fertilizers shall have at least 1 % w/w for each element. The process line for digestate nutrient recovery must be isolated and, in addition, is forbidden to physically mix the input and output streams of the processing line. Regarding the chemical-physical properties of the digestate, the regulation sets an Oxygen Uptake Rate (OUR) limit of 25 mmol O_2/kg VS per hour; with a maximum biogas production potential of 0.25 L/g VS (European Council, 2019).

7. Energy crops and biogas production

The world population will reach 9.7 and 11.2 billion by 2050 and 2100 respectively, 34 and 53 percent more than the current level (FAO, 2018; ONU, 2019). At the same time the most recent demographic forecasts emphasized that population boost will mainly involve the developing Countries, and in particular their urban areas: about 70 percent of the world population will live in

cities in 2050. This migration from rural to urban areas, and the subsequent difference of working and living conditions, will significantly affect the dietary requirements and food demand of the population (FAO, 2019).

In order to feed this larger, more urban and richer population, crops production yield is expected to increase by around 30% in 2050 (FAO, 2019). The cereal production increase is projected to 336 Mt in the next decade (OECD/FAO, 2021). The request for animal proteins will increase too (Bertasini et al., 2022): according to the OECD-FAO Agricultural Outlook 2021 – 2030, meat consumption will increase by 14% by 2030, reaching 35.4 kg/y in retail weight equivalent. Therefore, the production of crops for livestock feeding will increase as well, reaching almost 2 billion tonnes by 2030. As a consequence, the current global demand for fertilizers is 200 million tonnes, with a progressive reduction of supply and demand balances over the years (FAO, 2018).

To overcome this situation several activities should be undertaken: to enlarge the availability of arable land, to improve agricultural efficiency, and, importantly, to increase the availability of fertilizers. In addition, all these actions have to take into consideration the environmental impact, implementing a sustainable intensification of agriculture, reducing nutrient losses, crop protection chemicals and greenhouse gases emissions (Buckwell et al., 2015; FAO, 2018).

In this topic, agricultural biomass plays the most important role, as it expands from vegetal food portions to the whole structure of the plant and livestock wastes. Agricultural biomass can obtain energy by (i) crops production, yielding carbohydrates (starch, sugar, cellulose) and oil; (ii) AD for biogas production, which can be upgraded to biomethane or burnt through cogeneration for heat or electricity; (iii) fermented to extract high-value compounds, for example VFAs or lipids which can be turned in bioplastics or biofuels.

Bioenergy crops are plants producing high amounts of biomass, in particular lipids and carbohydrates, that can be easily converted into biogas, biomethane and biofuels. Wever et al. (Wever et al., 2020) distinguish 3 generations of Bioenergy Crops.

7.1 First-generation energy crops

First-generation crops are annual plants such as maize, sorghum and sugarcane. These plants are also used as human and animal food. Due to their annual lifespan, these crops require a high energy effort, especially in soil tilling and seeding, that hinders their potential on the industrial side for biogas and biofuel production. Moreover, first-generation of bioenergy crops raises ethical concerns due to the reduction of fertile land for food production, which is already reduced due to climate change.

7.1.1 Maize

Maize, or corn (*Zea mays*) is a cereal plant, originated from the Andean region of South America, used prevalently for food both for human and animal consumption, but also is used for bioethanol production. This plant can survive above 15°C, with an optimum of 20°C of mean daily temperature in the growing season. As long enough water is provided, maize can withstand temperature up to 45°C. Corn also requires a high amount of fertilization, reaching up to 200 kg N/ha, 80 kg P/ha and 100 kg K/ha. Regarding water requirements, a medium maturity grain crop requires 500 – 800 mm of water. The overall yield is 6 – 9 ton/ha under irrigation, with a water utilization efficiency of 0.8 – 1.6 kg/m^3. Worldwide corn production amounts to 1,102 million tonnes, with a potential bioethanol production of 46.6 million tonnes corresponding to a reduction of 106.44 million tonnes of CO_2 equivalents (Aghaei et al., 2022). Focusing on the energy balance, based on CH_4 and H_2 energy outputs, maize can produce 121 GJ/ha per year of net energy output (Christiansen et al., 2018).

Figure 9. *Zea mays*.

7.1.2 Sorghum

Sorghum (*Sorghum bicolor*) is a C4 plant originated from Sahel area of Africa. This plant is cultivated for its grains and stalks which are used as human and animal food. The capacity of this crop to withstand extreme climates and atmospheric events, for example drought and floods, render its cultivation versatile and widespread, becoming one of the top five worldwide most cultivated crops (Ameen et al., 2024). Fertilizer requirements are up to 180 kg N/ha, 45 kg P/ha and 80 kg K/ha. Compared to maize, sorghum is relatively more drought-resistant, with a requirement of 450 – 650 mm water depending on the climate. Under irrigation, this plant can yield from 3.5 to 5 ton/ha, with a water utilization efficiency between 0.6 and 1.0 kg/m^3. Besides food, sorghum can serve as energy crop as a cogeneration partner with bagasse and for the production of ethanol from starch, sugars and cellulosic material. This plant yields up to 3500 L/ha of ethanol (Ameen et al., 2024). Overall, the net energy output reaches 223 GJ/ha per year.

Figure 10. *Sorghum bicolor*.

7.1.3 Sugarcane

Sugarcane (*Sacccarum* spp.) is a perennial grass used for sugar production, originated from southeast Asia and India. This plant prefers warm temperate and tropical regions. Therefore, its cultivation diffused worldwide with a total production of over 1.9 billion tonnes in 2020, with Brazil leading with 40% of total sugarcane production (K. Ahmad & Ming, 2024). Optimum growth requires mean daily temperature between 22 to 30°C. Regarding the soil nutrients, sugarcane needs a high quantity of nitrogen and potassium with up to 200 kg N/ha and 160 kg K/ha, less phosphorus is needed (90 kg P/ha). As a tropical plant, sugarcane needs up to 2500 mm of water, therefore, its growth without frequent irrigation greatly reduces the overall production yield, which corresponds to a maximum of 150 ton/ha per year.

Figure 11. *Saccharum officinarum*.

7.1.4 Sunflower

Sunflower (*Helianthus annuus*) is cultivated as an annual oil crop alongside soybean. This plant can grow from arid to temperate climates under irrigation conditions, with an optimum of 18 –

25°C. Sunflower growth requires fertilization, with about 50 – 100 kg N/ha, 20 – 45 kg P/ha and 60 – 125 kg K/ha. Water requirements for this plant vary between 600 to 1000 mm based on climate.

Figure 12. *Helianthus annuus*.

7.2 Second-generation energy crops

Second-generation bioenergy crops like miscanthus, poplar, willow and switchgrass can represent a solution to the drawbacks of first-generation crops, in particular the decreasing to fields dedicated for food production. Second-generation bioenergy crops are perennial plants and can grow in soils usually unfeasible for food production. Perennial plants do not require annual soil tilling, and the plant roots can grow deeper in the soil, improving nutrients and water availability. Nutrients level and biomass yield in a soil cultivated with perennial plants did not decrease over long periods of time (Glover et al., 2010). For this reason, the energy input for plant growth can be reduced up to 11.75 fold, thus maintaining a similar biomass production. Addressing the ethical concerns, second-generation bioenergy crops can grow, due to the advanced root systems, in soils poorer of nutrients than soils ideal for first-generation bioenergy crops. Moreover, these crops are historically less used as human food, thus socially accepting their use as biomass crops instead of food crops. However, first- and second-generation of bioenergy crops cannot resolve entirely the ethical dispute between food or fuel, especially in a world dominated by food insecurity and with many finite resources already degraded.

7.2.1 Miscanthus

Miscanthus (*Miscanthus* spp.), originating from southeast Asia, is a perennial C4 plant which can grow also in cooler environments. Due to being perennial, after the first 2 to 3 years, Miscanthus can be harvested annually with an average lifespan of 20 years, producing up to 15 tonnes/ha of biomass per year, even in a generally cool environment (Hodgson et al., 2024). Miscanthus is used principally as combustion fuel for energy generation but can also be used to produce biofuels through AD, gasification and fermentation. As analyzed by (Olba-Zięty et al., 2021), Miscanthus

has a high economic value, with a mean revenue of 404.11 €/ha per year, which are almost double the mean revenue of other important perennial energy crops like willow and poplar.

Figure 13. *Miscanthus sinensis*.

7.2.2 Poplar

Poplars (*Poplus* spp.) are deciduous trees that are growing in popularity for woody biomass production which can be converted into biofuels, electric energy and heat through combustion or gasification. Compared to other trees, poplars are fast-growing and can reach maturity in 20 years. Its growth rate implies also a high rate of carbon sequestration and soil bioremediation, thus becoming an important ecologic actor for the world (Jaafari, 2023). Poplar trees require nutrient and well-drained soil, with a constant water source in a temperate environment. This tree has a dry matter biomass yield of 9.68 tonnes/ha per year, with a mean revenue of 180.91 €/ha per year (Olba-Zięty et al., 2021).

Figure 14. *Poplus nigra*.

7.2.3 Weeping Willow

Weeping Willow (*Salix* spp.) is a tree which grows worldwide with a native variety of species in each continent. Willows are important biomass producers due to their high growth rate and cellulose content (36-65%), coupled with relatively low lignin ranging from 17 to 22 %. Moreover, willow can be used in a biorefinery cycle due to the numerous active compounds, most importantly salicylic glucosides, which can be extracted from willow bark to produce medical and veterinary compounds; the remaining part of the harvested biomass can be turned into raw material for biofuels and heat generation (Baker et al., 2022). Willow trees can yield an average of 10.71 tonnes/ha of dry matter per year, accounting for a mean revenue of 236 €/ha per year (Olba-Zięty et al., 2021).

Figure 15. Weeping Willow (*Salix babylonica*).

7.2.4 Switchgrass

Native to North America, switchgrass (*Panicum virgatum*) is a species of grass which, since the mid-1980s, has been grown as a renewable energy source, in particular for biofuels production through AD or direct combustion. Environmentally, switchgrass can sequestrate atmospheric carbon, increasing the soil carbon content, increasing it of 6.49 tonnes/ha in the top 30 cm of soil in 15 years (Bai et al., 2022).

Figure 16. *Panicum virgatum*.

7.3 Third-generation energy crops

Third-generation energy crops are plants that can provide both energy and food. In particular, the new crop generation should be flexible between food and biomass production, in order to adapt the cultivation to the changes in bioeconomy that these transitioning years, between fossil and renewable energy use, produces. Third-generation energy crops, as descripted by (Wever et al., 2020), should have these three characteristics:

1. **Nutrients independence**. To avoid competition with food crops and other ecosystems, these plants should not be irrigated, except for the initial establishment of the cultivation. For the same principle of irrigation, also fertilizer use should be avoided and therefore, the plant must provide all the nutrients by itself (i.e. nitrogen fixation). For these reasons, the plant must be perennial, with deep roots that can increase nutrient availability.
2. **Ecosystem services**. Third-generation energy plants must provide multiple services to the ecosystem, namely provision, regulating and supporting services. For "provisioning services", the plant must be flexible to the production of both food and industrial raw materials in the same or alternate years of harvest. For example, these types of crops can be harvested for food during summer and for raw materials during autumn. This possibility allows farmers to respond accordingly to the market request, while boosting food security. For "regulating services", the plant must directly contribute to the well-being of the ecosystem. For example, reducing soil erosion or increasing carbon sequestration. For "supporting services", the plant must indirectly contribute to the ecosystem vitality by supporting other flora and fauna, by providing floral resource for pollinators or nesting animals. For this reason, a third-generation energy crop should be native, or well-established, in the territory.
3. **Increased landscape diversity**. Third-generation energy crops should differ from the common crops cultivated in the territory. This point focuses the attention on plants that are

less cultivated than common crops. For example, the cultivation of a cactus plant as alternative to common first-generation energy crops (i.e. maize) in an arid territory. In order to maintain the soil productivity besides atmospheric events and climate change disruption.

7.3.1 Kernza

Intermediate wheatgrass (*Thinopyrum intermedium*), also commercially named Kernza, is a perennial, rhizome-producing, grass that can produce wheat-like grain. Kernza is mainly cultivated for gran but, due to be smaller in size than regular wheat, can be grown also for forage production. As reported by (Pugliese et al., 2019), harvesting Kernza for both grain and forage production is stimulating for the growth of the plant and for the strengthening of its roots. Moreover, carbon and nitrogen storages in the soil are not affected by the multiple harvesting, concluding that Kernza can provide a productive and profitable way for perennial grain adoption as a third-generation energy crop.

Figure 17. *Thinopyrum intermedium*.

7.3.2 Cup Plant and Sida

Silphium perfoliatum, also known as Cup plant is a perennial plant which is a promising third-generation energy crop, particularly for biogas production through AD. Due to its nutrient use efficiency, source of nectar and pollen for insects, and positive influence on soil structure as well as reduction of soil erosion, this plant can fulfill almost all the services required to a third-generation energy crop. The lack of food production can be resolved by hybridization with another Cup plant species: *S. integrifolium* for future production of edible oil and fodder. Regarding the biogas production, this plant is still inferior to maize, with a specific methane yield of 259 – 273 L CH_4/kgVS (Wever et al., 2019).

Figure 18. *Silphium perfoliatum* (U. Schmidt).

Sida hermafrodita is a perennial plant cultivated as fodder and fiber source, which recently was studied also as biogas source. With a potential of 280 - 293 L CH_4/kgVS, this plant is similar to Cup Plant and Miscanthus (Jablonowski et al., 2017).

Figure 19. *Sida hermaphrodita* (Fritzflohrreynolds).

The main drawback of both Sida and Cup plant is the difficulty to offer a food alternative, thus compromising its definition of "Third generation" as energy crops. Therefore, for my PhD project I focused my attention to another plant that can fit all the three services needed for the definition of Third-generation energy crop. This is the OFI case, that due to its properties explained in detail in the next section, can be used for both food and energy production and also can grow in arid environment.

8. *Opuntia ficus-indica*

Opuntia ficus-indica (OFI), commonly known as prickly pear or nopal cactus, thrives in high-temperature and low-precipitation environments due to its crassulacean acid metabolism (CAM). This photosynthetic metabolism originated from plants growing in arid regions in order to prevent

water loss during the day (M. Chen & Blankenship, 2021). In the nighttime, when the air temperature is lower, thus lowering the overall water loss as well, CAM plants open their stomata for CO_2 uptake, instead of opening them during the day. CO_2 is then used for malic acid synthesis, storing it in the vacuole. During the day, malic acid is decarboxylated back to CO_2, which can enter the Calvin-Benson cycle and thus carbohydrates are reformed by photosynthesis. The CAM process is similar to the classic C4 photosynthesis pathway with a time-separation between dark and light phases.

Figure 20. *Opuntia ficus-indica.*

OFI is diffused in all the arid and semi-arid regions of the world (USA, Central and south America, China, India, north Africa and Mediterranean zones), although is originally from Mexico. OFI is cultivated mostly for its fruit for human consumption, and for the extraction of numerous compounds, for example pectins, flavonoids, polyunsaturated fatty acids (PUFAs), that can be used in cosmetic or pharmaceutical industries (Cushman et al., 2015).

In Italy, OFI is grown over 8600 ha of available agricultural area, in particular in the Sicily Island (90 %) with the rest in Puglia and Calabria regions. However, OFI is commonly present in the wild in all the southern regions of Italy (FAO, 2019; ISTAT, 2024). Italy is the second-best country for the production of OFI, after Mexico (Andreu-Coll et al., 2020). Overall productivity depends on the climate. In ideal conditions, the dry mass productivity reached 50 t/ha per year (Liguori et al., 2014). However, normal growth condition in the mediterranean areas, with less water availability and drought periods, overall productivity is much lower, reaching 6.2 t/ha of dry matter per year. This low productivity can still surpass the other C3 and C4 crops if grown in the same arid conditions (Mondragón-Jacobo & Pérez-González, 2001).

8.1 OFI chemical composition

Following an analysis made by Kuloyo et al. (2014) and Calabrò et al. (2018), OFI biomass is characterized by high water and carbohydrates content, which can reach up to 95 %FW and 42 %TS

respectively. This carbohydrate content is lower than classic crops, for example sugar cane, maize and barley. However, the carbohydrate fermentable fraction of 34.3 %TS is similar to the other first-generation energy crops. The lignin content of 7.9 %TS is significantly lower than other crops, which are ranging from 18.6 to 25.2, and this can allow less pretreatment of the substrate, more energy production and less toxic inhibitor due to the release of phenolic compounds and furfurans. Due to the OFI cladodes being a structural part of the plant, the lignin and carbohydrates content varies with the age of the cladode: older cladodes have less easily accessible carbohydrates than new ones. This composition suggests that OFI can be a good candidate for AD. The VS content defines a high content of biodegradable biomass, and the C/N ratio is in the range for AD (Panizio et al., 2020). Moreover, the water content can improve substrate availability to microorganisms (Sadaka & Engler, 2003).

8.2 Water use efficiency

Water-use efficiency (WUE) is a benefit:cost index, in particular the ratio of carbon dioxide fixed through photosynthesis to water lost by transpiration. Regarding OFI, WUE corresponds to 0.022 mol CO_2 fixed per mol H_2O lost. This corresponds to a net CO_2 uptake of 1.14 mol/m^2 day, with a water loss of 51.3 mol/m^2 day. Compared to other crops, OFI WUE is 3 times higher than maize and sugar cane, which are C4 highly productive plants, and 5 times higher than C3 plants such as alfalfa, cotton and wheat (Nobel, 1991). The high WUE is due to the CAM process explained before, which can lose only 20 – 35% as much water as do C3 or C4 plants in the same environmental conditions.

OFI, besides CAM process, can save water also by physiological adaptation for arid environments where it usually lives. OFI stems are covered in a waxy cuticle 5 – 30 µm thick, preventing water loss. Moreover, OFI has a low stomatal frequency of 20 – 30 stomata/mm^2, reducing the surface area where water vapor can move from the plant to the environment. During drought periods, when the water soil potential is lower than the plant water potential, OFI can use water stored in the parenchyma tissue to maintain vital chlorenchyma water levels. Roots systems in OFI are optimized for a quick response to light rainfall, with a mean depth of 15 cm and representing only 12% of the total plant biomass. In drought conditions, OFI can uptake CO_2 for photosynthesis even after 2 months, when other C3 and C4 crops stop the uptake, with a net loss of CO_2 after one week of drought. Indicating that OFI is extremely suited to arid and semi-arid environments, where water can be absent for over 2 months.

8.3 Temperature relations

Extreme temperatures can lead to cell plant injury and death, with high temperature also influencing metabolic processes and CO_2 uptake. Being acclimatized to temperature day/night of 50/40°C, OFI

is extremely resistant to high temperature, withstand temperature up to 65°C for 1 hour. However, OFI is not resistant to low temperatures, and is damaged by temperatures below -5°C. Due to CO_2 uptake during nighttime, night temperatures are more important for CO_2 uptake, with an optimum of 15°C to uptake 80% of maximal net carbon dioxide. When the nighttime temperature rises over 30°C, OFI reduce water transpiration by closing 70% of stomata, thus reducing also CO_2 uptake.

8.4 Nutrient relations

Nutrients, soil salinity and texture influence OFI net CO_2 uptake, growth and productivity. Nitrogen content on soil affects OFI growth, with the maximum growth achieved with 0.3 %TS of nitrogen content. Therefore, OFI could require nitrogen fertilization, to increase maximum production, in soils with less than 0.07 %TS of nitrogen, which is typical of arid and semi-arid lands. Phosphorus levels are also important for OFI growth; however, phosphorus fertilization is usually not needed, due to the minimum requirement for 50% OFI maximum growth rate is achieved with 5 ppm of phosphorus by TS. Salinity is dangerous for OFI growth. Cactus Biomass accumulation is reduced by 50% with a soil sodium content of 150 ppm, with a linear inhibition.

8.5 Bioenergy crop potential

A life cycle assessment (LCA) approach has been employed to quantify the environmental impacts of biogas production from *Opuntia* biomass. Findings from an LCA study comparing organic versus conventional farming systems revealed that the use of organic fertilization could reduce the global warming potential by over 22%. However, trade-offs were observed in terms of acidification and eutrophication potentials, which increased due to specific agricultural practices (Ramírez-Arpide et al., 2018).

In Italy, especially in Sicily, the potential use of uncultivated marginal lands for *Opuntia* cultivation has been explored. With an estimated dry matter yield of 8.5 t/ha per year and a methane potential of approximately 300 Nm^3 per ton of dry matter, the regional production could reach over 600 million Nm^3 of biogas annually. This can be valorized as biomethane or transformed into electricity and heat, supporting local energy independence and rural development (Dubeux et al., 2021).

In conclusion, *Opuntia ficus-indica* represents a sustainable feedstock for biogas and VFA production. Its integration into circular bioeconomy strategies, particularly in water-scarce environments, offers significant environmental and socio-economic benefits. Nevertheless, careful consideration of cultivation and processing practices is essential to optimize its overall life cycle sustainability.

9. Pressure-driven membrane filtration

Pressure-driven membrane filtration is a physical-based process, where pressure is exerted on a solution to separate the flow in a solid fraction, also named concentrate or retentate, and in a liquid fraction that passes through the membrane, called permeate. Depending on the pressure applied and the type of membrane, pressure-driven membrane filtration is distinguished in 4 categories:

- **Microfiltration (MF):** referring to a separation using a membrane with porosity of 0.1 – 10 µm in diameter, able to retain bigger viruses, microorganisms, cellular debris and other suspended solids (Davis, 2019).
- **Ultrafiltration (UF):** with a pore range of 0.001 – 0.05 µm, the membrane used for UF have a dense skin layer which, along the smaller porosity, increase the hydrodynamic resistance of the liquid suspension. The operating pressure usually is 2 – 5 bar. UF application is similar to MF but the smaller porosity enables the removal of macromolecules such as proteins, enzymes and all the viruses. Therefore, MF/UF are used for potable water treatment (Singh & Hankins, 2016).
- **Nanofiltration (NF):** membrane porosity has a range between 200 to 1000 Da. NF is able to separate divalent ions with a high rejection, however NF is not suited for the rejection of monovalent ions. Industrial application includes recovery of alkaline solution in the food industry, recovery of heavy metal ions in water treatment processes, and recovery of acid solution in mining applications (Bargeman, 2021).
- **Reverse Osmosis (RO):** with a porosity of 0.5 – 1.5 nm and an applied pressure of 20 – 70 bar, this process can reject all the molecular ions, including monovalent ones, and letting thought only small neutral molecular compounds such as water and ammonia. Therefore, this process can remove 95 – 100% of inorganic salts and charged compounds. Its main application is water purification and desalination (Sivaranjanee et al., 2022).

For MF and UF, the primary separation principle is sieving, or size-exclusion. For NF the charged surface is crucial to set up electrostatic repulsion towards multivalent ions and small charged compounds. Lastly, solution-diffusion is the main separation mechanism for RO (N. N. R. Ahmad et al., 2021).

For high impurities solutions, wastewater or digestates, membrane technologies involve several filtration steps. The first steps are rough solid-liquid separations, performed commonly in screw-presser, centrifuges or sieves. The pre-treated liquid fraction can enter MF or UF to remove all suspended solids and microorganisms. The liquid permeate can then enter on the final filtration, which is often a NF or RO step to obtain a nutrient rich retentate and pure, particle and pathogen free, water as permeate. As final products, a solid fertilizer, N and P rich (8.2-12.0 kg t^{-1} TN; 5.6-

10.4 kg t^{-1} P_2O_5), can be obtained from the solid fraction, while a liquid fertilizer, rich in ammonium and K (2.9-5.6 kg t^{-1} NH_4^+; 6.2-9.2 kg t^{-1} K^+), can be obtained from UF and RO concentrates . Membrane fouling is the main disadvantage of these technologies. Periodic maintenance or substitutions in order to maintain adequate separation performance are required (Monfet et al., 2018). To reduce maintenance costs, ceramic membranes can be used instead of less expensive organo-polymeric membranes, as they can be easily cleaned and are more resistant to pressure and chemicals (Fangueiro et al., 2017). According to Gienau et al. (Gienau et al., 2018), the UF step is the most energetically expensive, with a consumption of 10 – 15 out of 20 – 30 kWh/m^3 of digestate. In order to reduce the energy cost, they suggested the adoption of an enzymatic pretreatments (1 g/L of amylase, pectinase, cellulase and protease) able to decrease the viscosity of the digestate. With the same purposes, ozone treatment can be applied (Gienau et al., 2020). The application of membrane technologies to a digestate from the AD of animal manure, recovery yields of 75 – 96% of Total Ammoniacal Nitrogen (TAN) and of 100% TAN were achieved at pH 8 and at pH 4, respectively (Masse et al., 2007). Regarding the phosphorus removal yields, 87 – 98 % w/w of P removal were achieved (Pinelli et al., 2022; Zielińska & Mikucka, 2021). The membrane technologies have a TRL level of 9, with the total cost for a full-scale plant between 4 to 12 €/m^3 (Fangueiro et al., 2017).

10. Purification and extraction of VFAs

The main challenge in fermentative production of VFAs lies in their separation from the fermentation medium and subsequent concentration. Several techniques have been proposed to address this issue: i) VFAs precipitation; ii) liquid-liquid extraction, where VFAs are separated using organic solvents; iii) Membrane filtration, through molecular weight cut-off; iv) Adsorption, where VFAs interact with active sites on a solid matrix (Aghapour Aktij et al., 2020).

Precipitation and liquid-liquid extraction are easy to implement and scale up, offering high extraction yields. However, the production of solid waste and the use of organic solvents pose significant environmental and safety concerns (Atasoy et al., 2018). On the other hand, membrane-based methods such as electrodialysis, ultrafiltration, and reverse osmosis provide high yields but can face challenges such as severe membrane fouling when applied to real bioreactor effluents. In particular, pressure-driven membrane filtration is able to purify a VFAs mixture from other, high molecular weight organic compounds and microorganisms, thus sterilizing the VFAs-rich permeate (Tao et al., 2016). Ion-exchange adsorption, based on interactions between the negatively charged carboxylic groups of VFAs and positively charged groups on a solid matrix (e.g., amines), offers relatively high selectivity and is simple to perform (Reyhanitash et al., 2017). This makes it a promising method for industrial-scale VFAs extraction, particularly from wastewater or fermentation effluents.

In recent years, researchers have investigated the performance of various solid matrices for VFAs adsorption. For instance, Da Silva & Miranda (2013) studied the adsorption of single- and multi-component VFAs mixtures (acetic, propionic, and butyric acids) using Purolite A133S (a resin functionalized with tertiary amines) and granular activated carbon (GAC). They found that the resin provided 35% higher adsorption yields than GAC. Similarly, Rebecchi et al. (2016) examined VFAs adsorption from grape pomace digestate using different resins and identified Amberlyst A21 (tertiary amine) as an effective candidate, achieving adsorption yields of ~61% for acetic acid and ~11% for real grape pomace digestate. They also observed a desorption efficiency of ~99% using NaOH in a mixture of ethanol and water. Reyhanitash et al. (2017) tested different resin functionalizations and found that non-functionalized resins were more selective than functionalized ones (primary, secondary, or tertiary amines).

Despite these advancements, desorption remains a critical step for achieving industrial-grade VFAs concentrations (70–100 g/L). Desorbents must be cost-effective, efficient, non-toxic, and environmentally friendly. Da Silva and Miranda proposed ethanol and n-propanol for desorbing VFAs from Purolite A133S, reporting ~99% desorption efficiency, with n-propanol requiring a smaller volume to achieve this yield. After desorption, further processes are often necessary to concentrate VFAs. Rebecchi et al. suggested distillation to recover the basified ethanol used during desorption, minimizing VFAs losses. Reyhanitash et al. employed nitrogen gas stripping to regenerate the adsorbent matrix, recovering the VFAs using a condenser.

11. EU Policy Framework on Biobased, Biodegradable, and Compostable Plastics

In November 2022, the EU released a new policy framework, addressing the challenges and opportunities associated with biobased, biodegradable, and compostable plastics (European Commission, 2022a). As part of the EU transition to a circular, resource-efficient, and climate-neutral economy, the policy framework emphasizes the need to improve plastic sustainability while addressing issues of greenhouse gas emissions, waste management, and biodiversity protection, enhancing the previously discussed Waste Directive and EU Strategy for Plastic in the Circular Economy (European Commission, 2018).

In particular, this directive defines the types of bio-plastic, differentiating them in: i) biobased, which are derived from biomass, offering a partial reduction in fossil resource use but requiring sustainable sourcing; ii) biodegradable, which are plastics designed to decompose under specific environmental conditions; iii) compostable, which are degradable plastics that require industrial composting under controlled condition of heat and humidity, and therefore not suitable to be degraded in classic environmental conditions.

This policy framework also emphasizes the sustainability of the feedstocks used for bioplastic production. In particular, producers should prioritize organic waste and by-products over primary biomass, and when the latter must be used, it is important to ensure its environmental sustainability and eventual ecosystem harming effects.

12. PHAs

PHAs are biodegradable polyesters synthesized by a variety of microorganisms, as energy reserve in case of nutrient scarcity on the environment (C. S. K. Reddy et al., 2003). PHAs formation and accumulation inside the cell happens in stress conditions and nutrient imbalance, for example during scarcity of nitrogen or phosphorus when carbon is instead abundant (Anderson & Dawes, 1990). PHAs accumulation can reach up to 90% of cell TS, giving to PHA-producing microorganisms a high resistance to environmental stresses (C. S. K. Reddy et al., 2003). PHAs are formed by over 150 monomers, which consequently gives a high variety of polymers, in terms of structure and properties. Polymer typology heavily depends on accumulation condition, substrate composition and microorganism species (Mannina et al., 2020). Is it possible to accumulate PHAs using different natural resources like carbohydrates (starch, cellulose), triacyclglycerols and VFAs (acetic, propionic and butyric acid).

As PHAs have mechanical properties similar to fossil-based plastics, they can serve as a biodegradable alternative to classic polymers, to various applications, ranging from packaging to medical uses. However, the production costs are still bigger than classic plastics by 20 – 80% (Tan et al., 2014). For this reason, the scientific community is heavily involved in the research of methods and production processes to increase substrate purity, consequent conversion yields, and polymer extraction (De Donno Novelli et al., 2021; Fernández-Dacosta et al., 2015). As reported by (Getino et al., 2024), the production cost of polyhydroxybutyrate (PHB), the most important PHA, range from 5380 to 18300 $/ton of pure product, which 70% of cost is influenced by the raw feedstock price, with the other 30% by the extraction process.

12.1 Structure and properties

PHAs are hydroxyacid polyesters with the structure illustrated in Figure 21. The alkyl group -R is variable from a single methyl group up to a pentadecyl (C_{15}) group as described in Table 4. The chain can be lengthened to 35,000 units in R configuration due to the stereospecificity of the PHA synthase. Monomers can be divided into 3 classes based on the -R chain:

- Short-chain PHAs (scl-PHA), with side chain 3-5 C long.
- Medium-chain PHAs (mcl-PHA), with side chain 6-14 C long.
- Long-chain PHAs (lcl-PHA), with side chain over 15 C long.

Functional group type and side chain length heavily influence polymer physical properties and, therefore, its final application. For example, scl-PHAs confers rigidity to the final product, while mcl-PHA gives elastic and adhesive properties (Mannina et al., 2020).

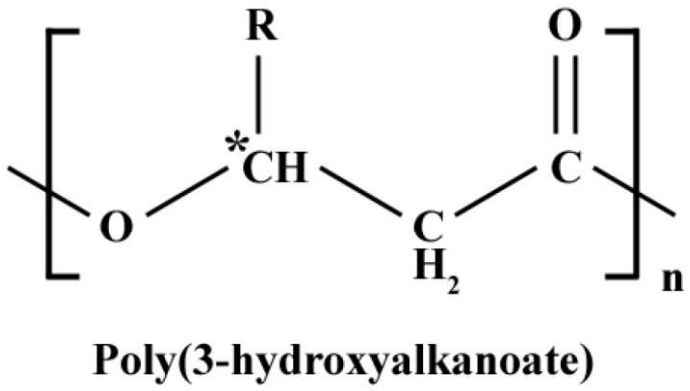

Figure 21. PHAs chemical structure, adapted from (Tan et al., 2014). R group is listed in Table 4.

Chain	R group	Carbon no.	PHA polymer
Short	Hydrogen	C_3	Poly(3-hydroxypropionate)
	Methyl	C_4	Poly(3-hydroxybutyrate)
	Ethyl	C_5	Poly(3-hydroxyvalerate)
Medium	Propyl	C_6	Poly(3-hydroxyhexanoate)
	Butyl	C_7	Poly(3-hydroxyheptanoate)
	Pentyl	C_8	Poly(3-hydroxyoctanoate)
	Hexyl	C_9	Poly(3-hydroxynonanoate)
	Heptyl	C_{10}	Poly(3-hydroxydecanoate)
	Octyl	C_{11}	Poly(3-hydroxyundecanoate)
	Nonyl	C_{12}	Poly(3-hydroxydodecanoate)
	Decyl	C_{13}	Poly(3-hydroxytridecanoate)
	Undecyl	C_{14}	Poly(3-hydroxytetradecanoate)
Long	Dodecyl	C_{15}	Poly(3-hydroxypentadecanoate)
	Tridecyl	C_{16}	Poly(3-hydroxyhexadecanoate)
	Pentadecyl	C_{18}	Poly(3-hydroxyoctadecanoate

Table 4. Nomenclature and carbon number of various PHA polymers, adapted from Tan et al. (2014).

As discussed, the most used PHAs is PHB. Dure to be a scl-PHA, product formed with PHB are typically rigid and frail, with low thermal stability and high crystallinity. To enhance its physical and mechanical properties, 3-hyproxyvalerate (3HV) is often incorporated in the polymer chain, giving a polymer similar to polypropylene (PP) (Andreolli et al., 2022). PHB is insoluble in water, can resist UV radiation and is impermeable to oxygen.

12.2 PHA Biosynthesis

Biosynthetic pathways for PHAs production are strictly connected to central metabolism of microorganisms, sharing common metabolic intermediates. During nutrients abundance, high content of free coenzyme A (CoA) inhibits β-ketothiolase (PhaA), allowing the acetyl-CoA to be used for energy production and cellular growth, thus blocking PHAs accumulation. When carbon is present, but nitrogen quantity is low, CoA is not produced at inhibitory level, therefore PhaA is not inhibited and redirects acetyl-CoA to the PHAs accumulation pathways. The common pathways for PHAs production are 3:

1. *Cupravidus necatur* pathway, which involves the condensation of 2 acetyl-CoA molecules from tricarboxylic acid cycle (TCA) using PhaA, with consequent formation of acetoacyl-CoA. Acetoacyl-CoA is then reduced by acetoacyl-CoA reduttase (PhaB) to 3-hydroxybutyl-CoA. Then, PHA synthase (PhaC) polymerizes 3 molecules of 3-hydroxybutyl-CoA to poly-3-hydroxybutyrate (P(3HB)).

2. *Pseudomonas* spp. pathway. This is based on the generation of substrates by β-oxidation of fatty acids, which can be polymerized by the PHA synthase. In *Aeromonas caviae*, *trans*-2-enoyl-CoA is converted to (R)-hydroxyacyl-CoA by a specific hydratase.
3. Third pathway can use simple monomers, such as glucose, sucrose and fructose, to generate monomers for PHAs synthesis. In particular, the (R)-3-hydroxyacyl intermediates from FA biosynthesis are converted from their acyl carrier protein (ACP) form to the CoA form by acyl-ACP-CoA transacylase.

12.3 PHAs Biocompatibility

PHAs are superior to other bioplastics due to their complete biodegradability, particularly in marine environments (Zhou et al., 2023). Their biotic degradation is 8–20 times faster than abiotic processes (G. Wang et al., 2021). Numerous bacteria and fungi capable of degrading PHAs have been identified in environments such as soil, compost, and marine ecosystems (D. Y. Kim & Rhee, 2003).

Under aerobic conditions (e.g., soil and marine), PHAs degrade into carbon dioxide and water, while under anaerobic conditions (e.g., sediments and landfills), they degrade into carbon dioxide and methane. Microbial exo-enzymes play a critical role in breaking down insoluble PHAs into smaller, water-soluble molecules (oligomers), which are then used as carbon and energy sources by microorganisms for complete degradation (Meereboer et al., 2020).

Microorganisms degrade PHAs by a dedicated extracellular PHAs depolymerase, producing monomers to be used as carbon and energy source (Mas-Castellà et al., 1995). Polymer crystallinity, granules superficial area, microbial activity, pH, temperature, humidity and other nutrient presence influence PHAs degradability. Moreover, biocompatibility is enhanced by the lack of toxic intermediates produced during PHAs degradation, a factor which allows the application of this polymer to medial uses (D. Y. Kim & Rhee, 2003).

12.4 PHAs applications

PHAs offer a promising solution to the increasing plastic waste problem by providing biodegradable alternatives that reduce dependency on fossil fuels and support sustainable resource use in a circular economy (Zhou et al., 2023).

PHAs can be applied on a broad range of topics, from medical uses to agricultural uses due to their high variability in terms of properties and polymer combinations. In the medical field, numerous studies have explored the use of PHAs in drug delivery, tissue engineering, and medical devices. Due to their biocompatibility and biodegradability, PHAs are non-toxic to the human body, making them excellent candidates for medical applications (X. Zhang et al., 2022). MCL-PHAs can be used

as scaffolds for skin regeneration or bone tissue engineering. Additionally, the hydrophobic nature of PHAs makes them suitable for controlled drug delivery systems (V. U. N. Reddy et al., 2022). Notably, PHB has shown potential in cancer detection, as certain cancer cells adhere to it while normal cells do not. Potential medical devices include surgical needles, suture materials, bone tissue replacements, and biodegradable carriers that deliver drugs over time (O'Connor et al., 2013).

Regarding disposable daily applications, PHAs can replace conventional single-use plastics, being utilized in products like plastic bags, food packaging, cosmetic containers, drinking straws, cups, lids, forks, and utensils. Their complete degradability and performance against oxygen and water make them superior to traditional plastics (Nanda et al., 2022). With the EU implementation of disposable conventional plastic ban, PHAs have significant market potential, despite current price limitations, leading towards a sustainable and circular economy (Gupta et al., 2022).

In agriculture, PHAs can be used as biodegradable mulch films that improve soil structure, retain water, prevent contamination, and enhance crop yield (Sintim et al., 2021). Unlike traditional plastics, which can pollute the soil, PHAs degrade without negative environmental impact. PHAs-based growth bags reduce toxicity, prevent root deformity, and promote faster plant growth with better immunity against pathogens (El-malek et al., 2020). Their low oxygen permeability also allows PHAs to be used in agricultural films and coatings, benefiting seed germination and plant protection (Zhou et al., 2023).

The unique properties of PHAs (biodegradability, versatility, and environmental friendliness) position them as crucial materials in advancing sustainable practices across various industries. Their potential to replace conventional plastics, contribute to medical innovations, and support sustainable agriculture makes them invaluable for future developments. It is fascinating to consider how the adoption of PHAs could revolutionize not only waste management but also achieve significant progress in medicine and agriculture. The shift towards biodegradable plastics like PHAs could reshape consumer behaviors and industrial practices, leading to a more sustainable and eco-friendly future (Zhou et al., 2023).

Opuntia ficus-indica: modulating operational parameters for biogas and VFAs production

OFI cladodes, as previously discussed, represent a valid alternative to classic, first- and second-generation bioenergy crops cultivation. Compared to first generation bioenergy crops (wheat and maize) OFI, which can produce edible fruits and fermentable biomass (cladodes), do not require large amounts of water and nutrients to grow, and can survive in arid fields. Moreover, being a perennial plant, less effort on soil management is needed, which is a peculiar quality of second-

generation bioenergy crops. The first part of this thesis experimental work had the ambition to express the full potential of the AD of OFI, as never done before, for the simultaneous production of different bioproducts and biofuels by the simple modulation of the operating conditions of the anaerobic process. Firstly, some batch tests were carried out to identify the OFI-water mixture able to reduce the high apparent viscosity of the minced OFI, which can negatively affect the rheology of the reactor (Battista et al., 2018). Then, the best OFI-water mixture was used for the semi-continuous tests, evaluating the influence of different HRT on VFAs, caproic acid and biomethane productions. Finally, the attention was focused on the caproic acid recovery, due to its high economic value. Therefore, the reaction medium from the tests with the highest caproic acid and VFAs productions was sent to sequential pressure driven membranes to increase the purity of the medium, removing all the non-VFAs compounds. Finally, the caproic acid was separated from the other VFAs by adsorption on Powdered Activated Carbon (PAC) and concentrated by the following desorption step using a lower amount of eluent.

1. Materials and methods

1.1 Substrate and inoculum characterization

The OFI cladodes substrate was sourced from the company "Assoro Biometano s.r.l." in Assoro, Enna (Sicily, Italy). The inoculum used for the tests was obtained from a full-scale AD plant in Isola della Scala (Italy), having a capacity of 1,000 kW and treating a mixing of cow manure and lignocellulosic residues from the energy crops at mesophilic condition (35°C). The inoculum was firstly treated *in loco* with a screw press to remove bigger solid particles.

	Substrate (OFI)	**Inoculum (Digestate liquid fraction)**
Total Solids (TS)	7.37 ± 0.26 % w/w	6.86 ± 0.37 % w/w
Volatile Solids (VS)	5.01 ± 0.20 % w/w	4.53 ± 0.19 % w/w
VS/TS	68.05 ± 0.38 %	66.02 ± 0.28 %
Chemical Oxygen Demand (COD)	573.64 ± 0.53 mgO_2/gTS	644.56 ± 12.03 mgO_2/gTS
Total Kjeldahl Nitrogen (TKN)	14.50 ± 0.39 mgN/gTS	6.97 ± 0.42 gN/L

Table 5. Substrate and inoculum characterization.

1.2 Batch tests for the optimization of the rheological features of the reaction medium

OFI cladodes were minced through a professional mixer to reduce the particle dimensions and increase the specific superficial area. The apparent viscosity of the minced OFI was measured by a Viscotech Hispania S.L.U “VR3000” viscometer. It was observed that minced OFI cladodes, used

as substrate, were characterized by a high apparent viscosity, due to the interaction between the protein fraction and the polysaccharide content, which forms intermolecular bonds (Stintzing & Carle, 2005). A high apparent viscosity can lead to mixing problems inside the bioreactor, increasing the energetic output needed for the system (Holtzapple et al., 2022a; Venkateswar Reddy et al., 2020a), reducing the process yield. Consequently, Bio-Methane Potential (BMP) batch tests were carried out at different water dilutions of the minced cladodes. The tested water concentrations in the minced OFI-medium were 5, 13, 24, 51 and 64 %w/w, corresponding to TS contents of 6.79, 6.32, 5.39, 3.49, 2.54 % w/w, respectively. The BMP tests were performed accordingly to Angelidaki et al. (2009) and Holliger et al. (2016) protocols. The tests were performed in a 1 L sealed glass bottle with an Inoculum:Substrate VS ratio of 2, at 37°C. The apparent viscosity was measured at the beginning and at the end of the BMP trials.

1.3 VFAs, caproic acid and biomethane optimization on semi-continuous systems

Semi-continuous tests at different HRT were tested to evaluate its influence on VFAs, caproic and biomethane productions, adopting the OFI-water mixture with the best biomethane production along batch tests.

The semi-continuous systems were inoculated using the same agricultural digestate used for the batch trials according to an Inoculum:Substrate VS ratio of 2:1. The semi-continuous systems were tested at different dilution rates (D), HRTs and Organic Load Rates (OLR), as listed in Table 6. Daily, a volumetric amount equal to the dilution factor of the reactor was removed and replaced with a mixture of minced OFI cladodes at 5 %w/w water dilution. The reactor pH was checked daily and corrected to 7 with 1M NaOH. The tests were performed at laboratory-scale in 1 L glass bottles, with a working volume of 720 mL. The temperature was kept constant at 37°C. The duration of each semi continuous test was about 3 HRT.

D (d^{-1})	0.95	0.40	0.20	0.10	0.05	0.03	0.025
HRT (d)	1.05	2.5	5	10	20	30	40
OLR ($gVS*L^{-1}*d^{-1}$)	38.5	16.2	8.1	5.7	2.8	1.8	1.1

Table 6. Operational parameters tested during the semi-continuous trials.

The performance of both biomethane and VFAs productions were evaluated by the corresponding substrates conversion yields, referred to the daily inlet COD amount (g_{CODin}) (Equations 1 and 2):

$$Y_{CH_4} = \frac{mL_{CH_4}/d}{gCOD_{in}/d} \quad (1)$$

$$Y_{VFAs} = \frac{(gCOD_{VFAs}/d) * V_{out}}{gCOD_{in}/d} \quad (2)$$

The productivity was also determined by multiplying the specific production of biomethane and VFAs with the dilution rate (D) of the corresponding test.

Besides the specific yield and the productivity, the experimental data were used to interpolate the kinetic parameters of the process. Kinetic models are used in anaerobic processes to check the initial hypothesis and to predict the system performance. Consequently, they also represent a tool for the scale-up of the process. The modified Stover–Kincannon model is one of the most common models to evaluate the organic substance removal rate as a function of organic loading rate at steady state (de la Lama et al., 2017). The model can be described as reported in Equation 3.

$$\frac{HRT}{S_0 - S_e} = \frac{k_b}{R_{max} - S_0} HRT + \frac{1}{R_{max}} \quad (3)$$

Where:

S_0 and S_e are the VS concentration (g_{VS}/L) for the inlet and the outlet, respectively. R_{max} is the maximum substrate removal rate (g_{VS}/L d) and K_b is the saturation value constant (g_{VS}/L d).

According to this model a plot of the quotient $\frac{HRT}{(S_0 - S_e)}$ versus HRT should result in a straight line with slope equal to $\frac{k_b}{R_{max} - S_0}$ and intercept equal to $\frac{1}{R_{max}}$.

1.4 VFAs purification by sequential steps of pressure-driven membranes

At the end of the fermentation (both acidogenic and methanogenic) and of the CE processes, the reaction medium was rich in VFAs and caproic acid. But it contained also the most recalcitrant organic matter, which was not or partially converted, and the bacteria, which promoted the biological processes (Rizzioli et al., 2021). The reaction medium from the HRT with the highest VFAs and caproic acid yields was sent to a purification step. Membrane processes were adopted due to their physical nature, which do not require the addition of chemicals to facilitate the separation of the reaction medium into a solid fraction, also named retentate (RET), and in a liquid fraction that passes through the membrane, called permeate (PER).

The purification steps involved a first phase of centrifugation at 4,000 rpm for 10 minutes to remove the bigger solid particles. Then, the supernatant was sent to different sequential pressure driven membranes for micro and ultrafiltration (0.45 µm, 300kDa and 1 kDa). The RET obtained from each membrane was removed from the purification line, while the PER, rich in VFAs and caproic acid, was kept in the purification line. The 0.45 µm, 300kDa and 1 kDa membranes were purchased by SANI membranes A/S (Denmark), which operated at 0.6 bar, 2 bar and 2.5 bar, respectively. For

each filtration step the distribution of the inlet into the RET and PER streams was calculated. Moreover, the purity degree (PUR) for each stream was determined accordingly to the following equation:

$$\text{PUR (\%, w/w)} = \frac{amount\ of\ VFAs\ and\ caproic\ acid\ (g)}{amount\ of\ the\ total\ COD\ which\ enters\ in\ the\ membrane\ (g)} \quad (4)$$

1.5 Recovery and concentration of caproic acid

The caproic acid was recovered through solid-matrix adsorption, using Powdered Activated Carbon (PAC) 7440-44-0, supplied by Merck. PAC chemical and physical properties were listed in Table 7. PAC was chosen for its higher affinity for hydrophobic compounds than hydrophilic ones (Rizzioli et al., 2021). Consequently, PAC are the best candidates to adsorb longer-chain and hydrophobic fatty acids, such as valeric and caproic acid, allowing their separation from smaller-chain FAs such as acetic of butyric acid. The PAC adsorption capacity is around 200 to 500 mg of VFAs per gram of PAC (Metcalf & Eddy, 1981).

	PAC 7440-44-0
Chemical composition	Carbon
Particle size (mm)	0.001 – 0.150
Approx. pore volume (cm^3/g)	0.65
Approx. surface area (cm^2/g)	0.12

Table 7. PAC chemical and physical composition.

The tests were made in 15 mL falcon vials, with a working volume of 10 mL of purified fatty acids from the 300 kDa PER, using different amounts of PAC: 0.10, 0.25, 0.50, and 0.75 g. The adsorption tests were performed at room temperature for 3 h; the vials were shaken in an orbital mixer for the entire adsorption process, to favor VFA diffusion in the vials and their adsorption on the solid matrices.

At the end of the adsorption step, the PAC was separated from the starting mixture through vacuum filtration. Then, the VFAs and caproic acid were desorbed from the PAC testing two different eluents: i) distilled water (1M NaOH); ii) ethanol (1M NaOH). The eluent volume for the desorption tests was 2 mL.

1.6 Analytical methods

For all the tests, the biogas production was analyzed through the water displacement method. The biomethane concentration was obtained with a GeoTech® Biogas 5000 gas analyzer. Methane production and concentration are average values referred to the steady state period of the tests, usually the last two HRT. VFAs concentrations were obtained with a ThermoFischer Dionex ICS-

1100 ion chromatography system, reported as gCOD/L. Total and Volatile Solids, Chemical Oxygen Demand, and Total Kjeldahl Nitrogen determination were performed accordingly with standard methods (APHA, 1998). Ethanol and lactate concentrations were determined by Megazyme kits.

2. Results and discussion

2.1 Batch tests

The aim of the first part of the experimental campaign was to evaluate the minimal minced OFI-water dilution able to reduce the system apparent viscosity in batch tests. The performances of the tests were evaluated in terms of biogas production.

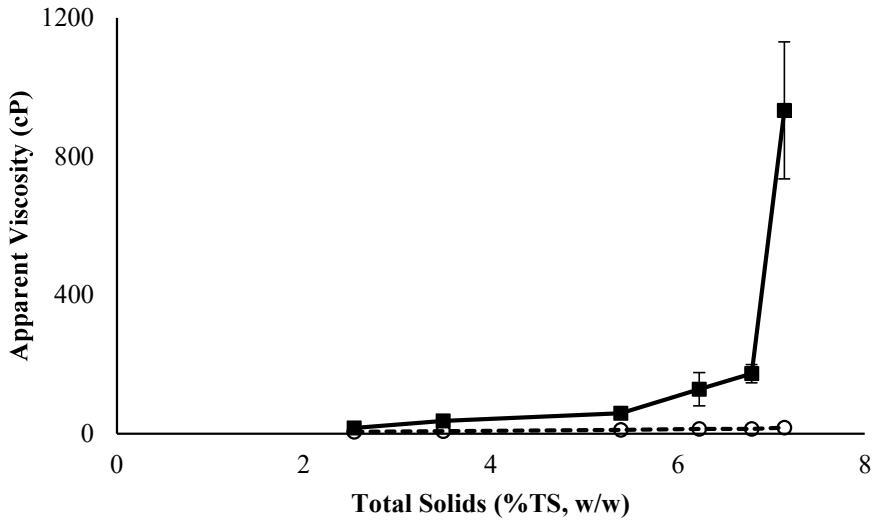

Figure 22. Apparent viscosity of the substrate at various TS. Black squares, continuous line: viscosity at the start of the BMP test; empty circles, dashed line: viscosity at the end of BMP test.

Minced OFI dilution was necessary due to its high apparent viscosity. As reported in Figure 22, the apparent viscosity of the undiluted minced OFI, which corresponded to a TS concentration of 7.37 %w/w, was 932.94 ± 197.45 cP at 25°C. These values are usually not suitable for stirred bioreactors. Even if the rheological performances of the system differ from case to case, depending on the substrates, the geometry of the reactor and of the mixing system, and the operational conditions of the reactor, previous studies showed a system inhibition caused by high apparent viscosity. As reported by Battista et al. (2016), focusing on AD biogas production from olive pomace, an apparent viscosity of 200-300 cP caused an inadequate mixing of a bioreactor using conventional impellers, for example Rushton or marine types. Further research on enzymatic hydrolysis for fermentable sugars production from wheat straw, demonstrated that the reaction medium, having an apparent viscosity of about 450 cP, had bad rheology performances even with complex impellers, such as a double helicoidal impeller (Battista et al., 2018). Moreover, it was observed that high apparent

viscosity can cause solids sedimentation and layering of fibers in the superior part of the reaction medium. This aspect prevents the biogas passage from the liquid phase towards the gaseous phase of the reactor headspace, inhibiting the fermentation processes (Ao et al., 2020; Q. He et al., 2017).

As expected, it was challenging to mix undiluted minced OFI with the impellers adopted conventionally in AD plants, like Rushton and marine impellers.

It was observed that a dilution as small as 5%, corresponding to a TS concentration at 6.79 %w/w, allowed to reduce the apparent viscosity down to 173.58 ± 23.33 cP (Figure 22), under the critical range of 200-300 cP, able to guarantee an adequate mixing performance for OFI. By this way, it was possible to solve the mixing issue with very low water addition, which means with low environmental and economic impacts on the process. The apparent viscosity was also measured at the end of the AD process. It was observed that all the tests had a significant apparent viscosity reduction of the reaction medium, with values close to the water viscosity of 1 cP (Figure 22). This can be attributed to the effect of the hydrolysis process. In this first AD process stage, microorganisms break down complex compounds like polysaccharides and cellulose, resulting in lower molecular weight compounds (Ribeiro et al., 2010), (Romio et al., 2022).

Regarding the biomethane production, the different dilutions seemed to not have a significant influence on the specific methane productivity: the biomethane specific production ranged from 180 to 220 mL/gVS for all the tests, with the maximum value observed in correspondence of the lowest dilution of 5% w/w. The dilution seemed to act on the kinetics of the tests. The batch test with the highest initial apparent viscosity required about 50 days to reach the final methane production value, while the other tests achieved the final methane amount in around 25-30 days. This demonstrates that the hydrolysis of the compounds is inhibited with high apparent viscosity. Probably it is due to a lower mass transfer in the reactor.

2.2 Semi-continuous tests

Considering the trends of the batch tests, the 5 % w/w water dilution of the minced OFI had apparent viscosity lower than 200 cP, which was able to assure an adequate reactor mixing with the conventional impellers used in the anaerobic digesters, as explained above. Moreover, the 5% w/w water dilution led to the highest biomethane production among the batch tests (Figure 23). For these reasons, this dilution was used for the semi-continuous tests at different HRT.

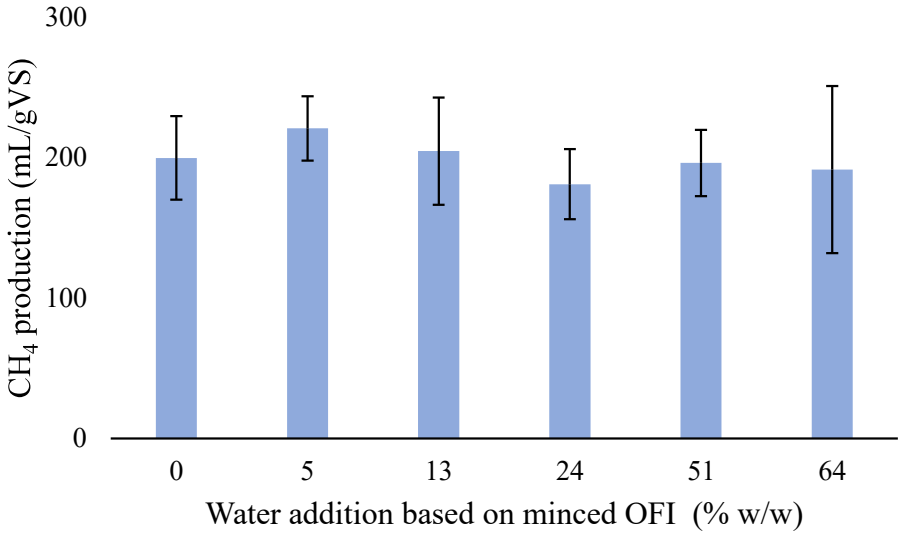

Figure 23. Biomethane production at various water content.

2.2.1 Discussion on the methane production trend

Table 8 shows the average production of methane, and VFAs and caproic acid concentrations of the semi-continuous tests, when steady state conditions were reached.

HRT (d)	CH_4 (L/kgVS)	VFAs (gCOD/L)	Caproic Acid (gCOD/L)
1.05	0.00 ± 0.00	3.82 ± 1.41	0.20 ± 0.06
2.50	0.00 ± 0.00	16.32 ± 2.35	0.50 ± 0.11
5.00	0.00 ± 0.00	26.09 ± 2.46	7.85 ± 1.02
10.00	1.86 ± 0.52	23.16 ± 1.07	1.55 ± 0.21
20.00	210.20 ± 20.73	12.68 ± 1.73	0.37 ± 0.04
30.00	201.59 ± 12.83	8.30 ± 0.66	0.00 ± 0.00
40.00	176.66 ± 17.52	4.47 ± 0.99	0.00 ± 0.00

Table 8. Biomethane, VFAs and caproic acid production from the semi-continuous tests.

The biomethane production is null or negligible at lower HRTs, below 5 days, while it was obtained at higher HRTs (20-40 d). The specific methane productions for the tests at HRT 20, 30 and 40 days, reported in Table 8, were referred to the reaching of the steady state condition, which occurred about 30-40 days after the beginning of the tests. It is fundamental to emphasize that in the first 30-40 days the biomethane production was 15-25% lower than the values reported in Table 8. This trend can probably be explained considering the heterogeneous chemical composition of the OFI, rich in carbohydrates easily degradable by methanogenic bacteria. At the same time, hemicellulose, and cellulose, the other two major components in OFI (Ribeiro et al., 2010), are known to be

recalcitrant, and their conversion required from 3 to 5 weeks (Battista et al., 2020). Consequently, their contribution to biomethane production started to be observed after about 30 days. With specific reference to biogas production, the best biomethane yield of 210 L/kgVS was achieved at HRT of 20 d. Higher HRT led to a slight decrease in the biomethane production, probably due to the lack of micro- and macro-nutrients which began after 45-50 days of the process, when the C:N ratio increased at around 76. This value was far too high compared to the optimal value of 15 – 30 (Panizio et al., 2020). The unbalanced C:N ratio was due to the feeding stream, constituted by a single substrate, the OFI cladodes which are rich in carbon content and low in nitrogen compounds (proteins). Nitrogen was essentially supplied just at the beginning of the test with the inoculum of the reactors (Danzì et al., 2020). These results emphasized that the mono-digestion of OFI is not sustainable. Therefore, in practical terms, co-digestion is an obligatory choice. A co-digestion of OFI with rich nitrogen substrates, like livestock effluents, will be evaluated in the future steps of this research. A recent work confirmed that co-digestion can improve biomethane production. Espinosa-Solares et al. demonstrated that OFI and cow manure co-digestion can lead to a biomethane production 80% higher than cow manure mono-digestion, obtaining the best result of 152.7 L/Kg VS biomethane with 50:50 cow manure to OFI ratio. Cow manure mono-digestion only produced 79.5 L/Kg VS of biomethane. As an alternative to co-digestion, pretreatments can help the degradation of the most recalcitrant OFI compounds in methane. Iris Saldovan Rojas et al. (Rojas et al., 2023) tested different chemical, physical and thermal pretreatments on OFI. Demonstrating that a combination of mechanical, chemical, and thermal operations increased the methane production by 160%, obtaining around 200 L/Kg VS. This is an interesting result similar this study, which were achieved by a simple water dilution, without expensive and high environmental impacting pretreatments. Finally, very interesting is the work by Tarrisse et al. (2022), which focused the attention on a new OFI feature. Their work showed methane production in the range of 200-350 L/kgVS. This large range can be justified considering the tested OFI came from different countries, which can have differences in the soil calcium content and, consequently, in the cladodes. The authors emphasized calcium action as being able to facilitate the precipitation of CO_2 bubbles as calcium carbonate, increasing the methane content in the biogas.

The biomethane productivity was also evaluated. Clearly it had a consistent trend with the specific biomethane production: its value was maximized at the HRT of 20 days, with about 10 $mLCH_4/gVS*d$ (Figure 24A), decreasing to 4.5-7 $mLCH_4/gVS*d$ at HRT of 40 and 30 d, respectively. Having achieved the best biomethane yield, the mass balance was performed for this test. Regarding the calculation, it was considered the COD daily flow entering the system and the biomethane theoretical conversion of 350 $mLCH_4/gCOD$. This yield is referred to a biogas composed for 55-60% v/v in methane and 40-45% v/v in carbon dioxide. So, the same theoretical yield referred to biogas corresponds to about 580-600 mL/gCOD (Labatut et al., 2011).

Consequently, a biomethane specific yield of 232.59 ± 41.53 mLCH_4/gCODin was found. Figure 24 reported the daily COD inlet in the system, the COD converted into biomethane and the COD which left the reactor in the effluent streams. The COD amount sum leaving the reactor fitted perfectly with the entering COD amount, validating the consistence of the experimental data and of the mass balance.

Besides the COD, it is interesting to observe that the VS/TS ratio evolution before and after the AD at HRT 20 d. The minced OFI had a VS/TS ratio of about 70% before entering the reactor. This value is low compared to other organic substrates, due to the high presence of inert and minerals in the OFI, especially calcium and magnesium (Contreras-Padilla et al., 2011). At the end of the AD, the VS/TS ratio was about 45-50%, demonstrating the degradation of only the most edible organic compounds. The most recalcitrant compounds were not or partially degraded. Besides the cellulose and the hemicellulose, OFI has also a good number of mucilaginous polysaccharides, high molecular weight heteropolysaccharides composed of up to 30,000 different sugars, which are not easily degradable (Mannai et al., 2023).

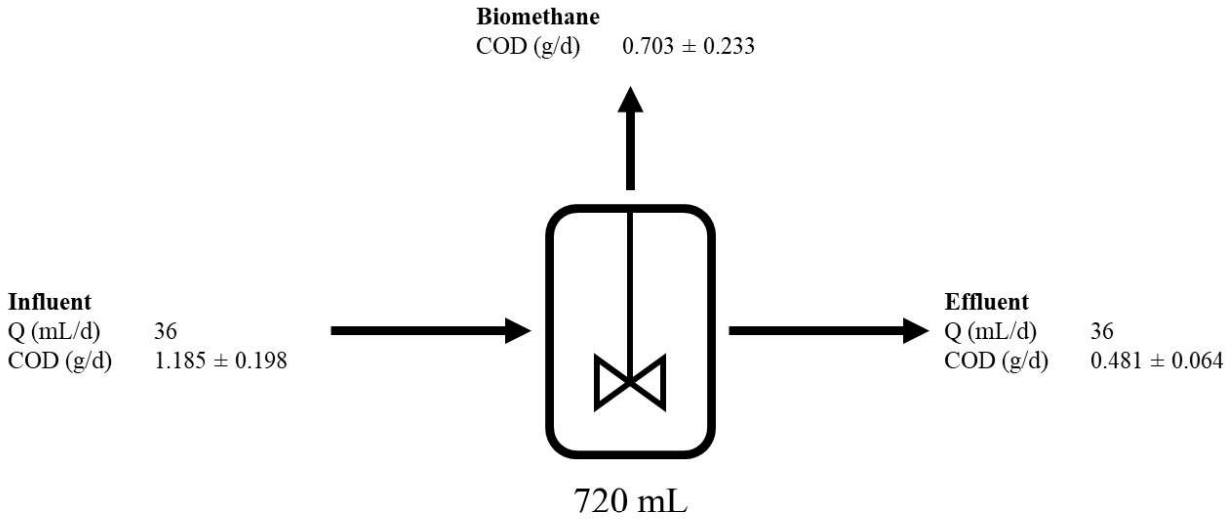

Figure 24. Mass balance for HRT 20.

2.2.2 Discussion on VFAs and caproic acid production trends

VFAs and caproic acid production showed a reverse trend than the biomethane yield, achieving the best concentrations at low HRTs, typically below 10 days. VFAs and caproic acid concentrations peaked at HRT 5 with 26.09 and 7.85 gCOD/L respectively. Regarding the VFAs productivity (Figure 25B), the best value was at HRT 2.5 with around 6 gCOD / (Ld), closely followed by HRT 5 test, which had the best caproic acid productivity. In particular, the best VFAs yield of 79% on influent COD was achieved by the HRT 5, followed by HRT 10 with 70 %. Focusing on the caproic acid production, the best yield was 30 %, obtained at HRT 5 too.

The overall results of both biomethane and VFAs yields are consistent with the acidogenic and methanogenic steps of the AD process. In fact, HRTs of 1 – 10 days leads to the wash out of methanogenic bacteria, which have higher reproduction time than the considered HRTs (Possente et al., 2022). Instead, acidogenic bacteria have smaller reproduction time, ranging from few hours to 5 days and, for this reason, they were not washed out by low HRT. In particular, low HRT in combination with a high OLR represent the optimum condition for VFAs production (Strazzera, Battista, Andreolli, et al., 2021).

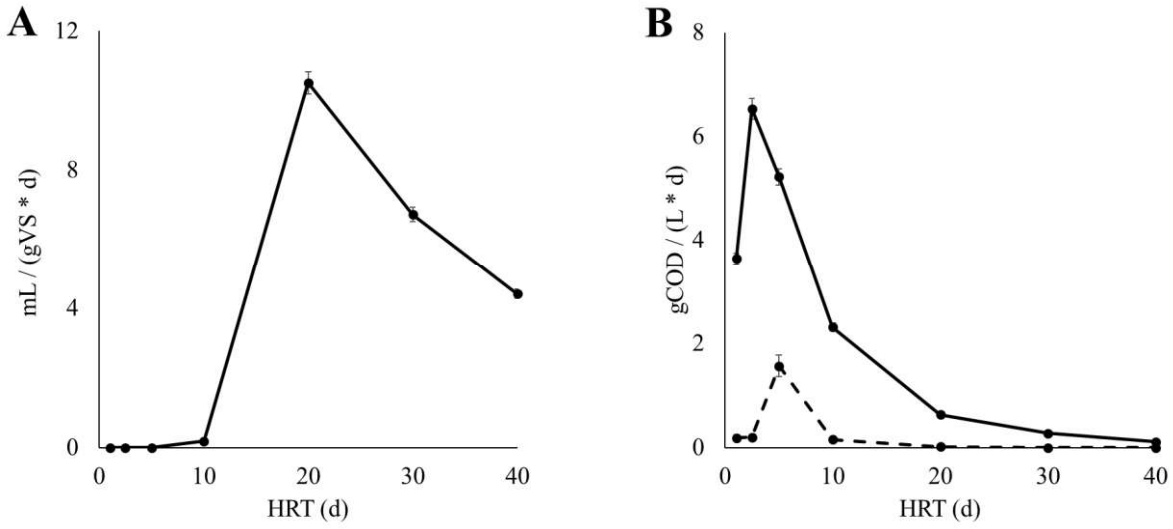

Figure 25. A: Biomethane productivity. B: VFAs (continuous line) and Caproic Acid (dashed line) productivity.

The washout of the methanogenic bacteria at low HRT also allowed for the instauration of the reverse β-oxidation processes for the CE of short VFAs. It was observed that the acetic acid appeared immediately, at the very beginning of the fermentation, while the butyric acid accumulation in the bioreactor started after 4 – 5 days. In the first 5 days since the beginning of the test, the total concentration of the VFAs was 13.7 gCOD/L; acetic acid represented 84% w/w of them, followed by propionic and butyric acids which accounted for the 11% and 5% w/w, respectively. Then, the appearance of valeric and caproic acid production after 7 – 10 days since the beginning of the tests was observed, demonstrating the occurring of the implementation of the reverse β-oxidation reactions. The process consists in the elongation of the VFAs chain as effect of the acetyl-CoA molecule, supplied by an electron donor compound, typically ethanol or lactic acid. These electron-donor compounds allow for the increase of the VFAs by two carbons (C_2) at a time. Consequently, acetic acid and propionic acids are converted into butyric and valeric acids in a first cycle elongation, respectively. Then butyric acid is further elongated into caproic acid along the second cycle (Holtzapple et al., 2022b; Venkateswar Reddy et al., 2020b). Consequently, when the steady state of the HRT 5 test was reached, the total concentration of the VFAs was 26.09 gCOD/L,

as previously reported. Regarding the VFAs composition, acetic acid represented 41.5% w/w, caproic acid 30.2% w/w, butyric acid 15.6 % w/w, valeric acid 6.9% w/w and propionic acid 5.8% w/w of the total VFAs.

If the HRT 5 showed the best condition for caproic acid accumulation, the HRT 2.5 led to an accumulation of acetic and butyric acids, meaning that longer HRT are necessary for the complete instauration of the CE process with OFI (Andersen et al., 2015).

Besides the VFAs presence, the reverse β-oxidation reactions require an electron donor compound. Olokede et al. (2022) reported a decrease in ethanol and lactic acid concentrations, which can be justified by the instauration of the CE process. Similarly, the HRT 5 test of the present research work showed the presence of one electron-donor compound, the lactic acid which accounted at about 67.5 g/L. Lactic acid presence can be easily explainable by the hydrolysis of the carbohydrates and cellulose contained in OFI into glucose, which was further converted into lactate through both different metabolic routes: the glycolysis, the bifidus and the 5 P-gluconate pathways (Kandler, 1983). Considering the acetic acid concentration of about 12 g/L, the molar ration of the acetate and lactate was 1: 3.8. The molar concentration between acetic acid and the electron donor compound (ethanol or lactate) is one of the fundamental parameters for the instauration of the reverse β-oxidation (Venkateswar Reddy et al., 2020b). Different authors found the optimal molar ratio between the VFAs, and the electron donor compound is in the range 1:3 – 1:6 (Cavalcante et al., 2017; Tang et al., 2022), which fits well with the one found along the HRT 5 test.

Even if there are few works on VFAs and caproic acid productions, the comparison with scientific literature highlights the very promising results obtained by the present work. Medjekal et al. (2023) performed batch acidogenic fermentation of OFI obtaining a VFAs yield of about 0.6 g/gTS. But, analyzing the VFAs profile, it emerged that they were essentially due to acetic (71%) and propionic (21%) acids. Thus, the process was not optimized for CE which led to longer and higher value fatty acids. Tenci et al. (2023) adopted *Euphorbia tirucalli* as substrate for VFAs production in batch test. *E. tirucalli* is a CAM plant and has similar chemical characteristics to OFI. The VFAs productivity was 0.3-0.4 gVFAs/gCOD when E. tirucalli was fermented alone, while reached 0.65 gVFAs/gCOD in co-digestion with pig blood, characterized by high proteins content. After 16 days of fermentation, the VFAs profile was characterized by around 50% w/w of acetic acid, followed by 15-20% w/w of butyric acid. Valeric and caproic acids were detected in lower concentrations (5-10% w/w). It demonstrated that, even if co-digestion can increase VFAs yield, this work demonstrated that the simple mono-fermentation of OFI performed in semi-continuous mode at optimized HRT can lead to higher production yields of high added value compounds.

2.2.3 Determination of the kinetical parameters of the process

The experimental data were interpolated to find the kinetical parameters of the fermentation process according to the modified Stover-Kicannon model (Figure 26), whose equation was reported before.

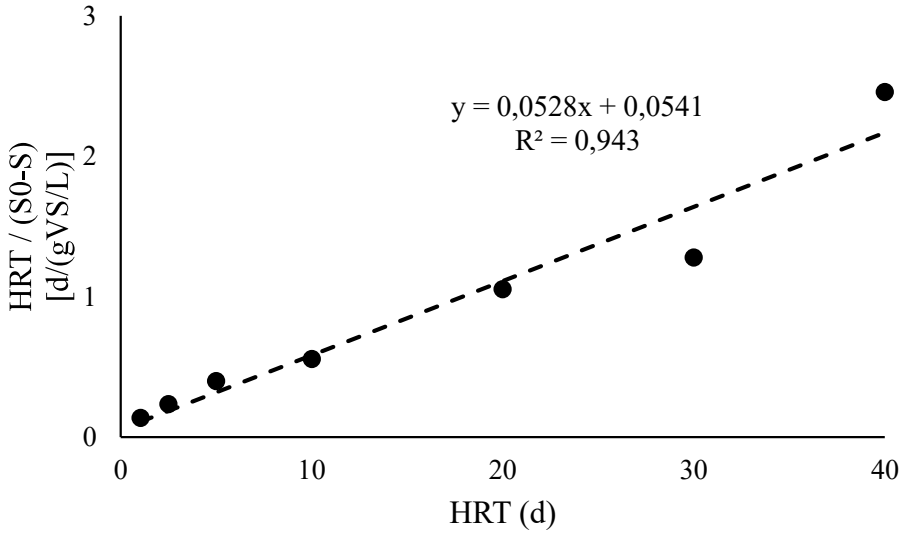

Figure 26. Modified Stover–Kicannon model plot.

As shown in Figure 26, the trend line, obtained from the experimental data, had a slope value of 0.0528 and an intercept of 0.054. From these values, the kinetic parameters were calculated as K_b = 67.64 g_{VS}/L d and R_{max} = 32.15 g_{VS}/L d, respectively.

The modified Stover-Kicannon model plot can be used to predict the theoretical effluent concentrations of the reaction medium ($S_{e\ theor}$) for the different HRT considering the following Equation 4:

$$S_{e\ theor} = S_0 - \frac{Rmax\ S0}{kb+(\frac{S0}{HRT})} \quad (4)$$

The deviation between the theoretical values and the experimental ones were in the range 5-15% for all the HRT. These differences are due to biological variations affecting microbial activity because of environmental factors like temperature and pH. The presence of biofilm alters mass transfer, impacting solid concentrations. Flow dynamics, including turbulence and stratification, influence substrate and biomass distribution. Biomass washout occurs with changes in retention time, modifying microbial growth (Karapinar Kapdan & Aslan, 2008). Moreover, the regression value of 0,943 strongly demonstrated the suitability of the modified Stover–Kicannon model to predict the effluent concentration and ultimately the process performance (B. He & Wang, 2019).

2.3 VFAs purification by sequential steps of pressure driven membranes

The reaction medium, daily discharged, was centrifuged according to the procedure reported in paragraph 2.3. The supernatant had a PUR degree of 86.11 % (Figure 27), indicating $gCOD_{VFA}/gCOD_{total}$, as described in paragraph 1.4.

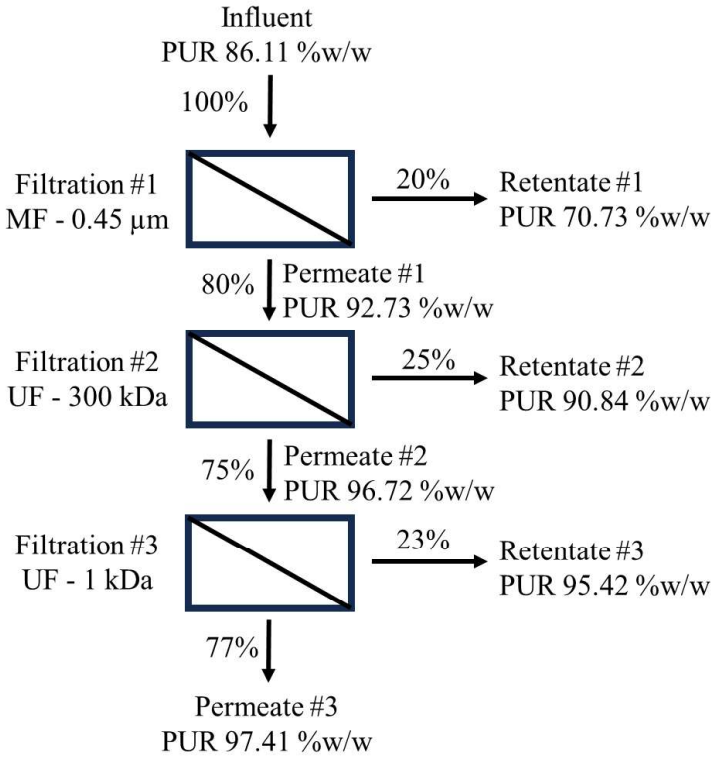

Figure 27. The distribution and purity degree of the purification line streams.

The first filtration step at 0.45 µm led to two streams, the RET, which accounted for the 20% of the inlet supernatant, and the PER, whose represented the remaining 80% w/w of the inlet supernatant. It is interesting to observe that the 0.45 µm filtration step was very effective in the increasing the purity degree of the supernatant: the PUR was of about 92.73 and 70.73% w/w for the PER and the RET, respectively (Figure 27). It means that no-VFAs organic compounds were largely retained by the 0.45 µm filter. A further filtration step (300 kDa) led to an improvement of the PUR which almost reached 96.72 % w/w for the PER. While the PUR was 90.84% for the RET, slightly lower than the value from the supernatant PER coming from the previous filtration stage. It demonstrated that 300 kDa filter was able to retain the smaller non-VFAs organic matter. On the contrary, no significant differences in PUR parameters were observed in the PER and RET streams from the last filtration step (1 kDa), meaning that the highest possible purity degree were already achieved along the previous 300 kDa stage and the few remaining no-VFAs compounds have dimension similar to the VFAs.

Regarding the distribution of the VFAs between the PER and RET along the purification line, no significant difference was detected. The relative concentrations of the different acids remained almost constant, showing very close values to those reported in the previous paragraph. It demonstrated that micro and ultrafiltration were not able to perform a first separation between VFAs caproic acid. For this reason, the final step of adsorption on PAC, followed by desorption were performed.

2.4 Adsorption/desorption of caproic acid on/from PAC

An adsorption test was performed on the 300 kDa permeate. As mentioned above, the adsorption resin used was PAC, which has a higher affinity with hydrophobic compounds, such as caproic acid whose chemical features differ from shorter fatty acids (acetic, propionic, and butyric ones) (Bhadra et al., 2015) (Rizzioli et al., 2021). The adsorption tests resulted in a complete adsorption of the caproic acid (Table 9), while lower chain and less hydrophobic VFAs had lower adsorption yields, as expected. Going in detail, caproic acid adsorption was higher with 0.5 g PAC (corresponding to 50g PAC/L) ranging from 97.7 to 99.1%, and lower for 0.25 and 0.75 gPAC/L, demonstrating that 0.5 was the ideal PAC amount.

Regarding the desorption, the overall VFAs desorption yield was 69.5 and 57.7 % for ethanol and water respectively. The very interesting fact was that desorption yield with ethanol was around 70-72%, higher than desorption yield which consider all the VFAs, while it was very low when water was adopted as eluent. This attitude can be explained considering the caproic acid solubility, which is low in water (10.8 g/L), complete in ethanol (PubChem, 2022). Consequently, adsorption and desorption test in ethanol allowed both to reach a very high caproic acid purity degree and to concentrate it until 3.5 times the concentration in the 300 kDa PER, passing from about 7.5 g_{COD}/L to about 26 g_{COD}/L.

PAC (g)	Adsorption yield (% w/w)		Desorption Eluent	Desorption yield (% w/w)	
	Caproic acid	Total VFAs		Caproic Acid	Total VFAs
0.25	90.8	20.6	H_2O (1M NaOH)	26.5	66.9
0.50	97.7	25.9		3.3	60.3
0.75	96.3	33.8		0.76	60.1
0.25	94.7	33.2	Ethanol (1M NaOH)	72.9	75.8
0.50	99.1	46.0		70.2	58.9
0.75	96.6	42.4		70.9	76.9

Table 9. Adsorption and desorption yield at different eluent and PAC quantity, regarding caproic acid and all the VFAs.

3. OFI AD optimization conclusions

This part of the project tried to express the full potential of the AD applied to OFI to produce different bioproducts through the simple modulation of the HRT. Moreover, sequential downstream processes were optimized to purify and concentrate the obtained compounds. Considering the high apparent viscosity of the minced OFI, preliminary batch tests were carried out to find the best water-OFI mixture. A 5 % w/w (based on minced OFI amount) of water addition was enough to decrease the apparent viscosity from about 1,000 cP to about 200 cP, which was able to assure an adequate mixing of the bioreactor. This water dilution led to the highest biomethane production of 220 $mLCH_4/gVS$. Consequently the 5% w/w of water addition was used for the implementation of the biorefinery model to produce VFAs, caproic acid and biomethane. The influence of different HRT was evaluated on the typologies of the produced compounds and the process yields.

It emerged that HRT modulation can effectively lead to different bioproducts and biofuels. The best result for biomethane was obtained at HRT 20, with a specific production of 210 $mLCH_4/gVS$ and 232.59 $mLCH_4/gCOD_{in}$. While the VFAs and caproic acid accumulation reached the best yields at HRT 5, with concentrations of 26.09 and 7.85 g_{COD}/L of VFAs and caproic acid, corresponding to a COD conversion yield of 79 and 30 % w/w, respectively.

This part of the work also investigated the downstream processes to purify and concentrate the VFAs and caproic acid from the semi continuous test at HRT 5. Firstly, pressure-driven membrane filtration was implemented to remove all non-VFAs compound. The process allowed to pass from a purity degree of 86.11 %w/w to 97.41 %w/w of the 300 kDa permeate. Finally, the purified 300 kDa permeate was sent to adsorption process on PAC to separate caproic acid from the other VFAs. It emerged that caproic acid had a very high affinity with PAC, demonstrating that this solid matrix can be used to separate it from the other VFAs. The following desorption test showed a good yield with ethanol (1 NaOH), while water had very poor performance because of the low solubility of caproic acid in it.

AD Digestate nutrient recovery from pressure-driven membrane filtration

AD Digestate, as discussed previously, is rich in nutrients, mainly N, P, and K. Normally, the digestate is directly applied in the agricultural soils for fertilization purposes. However, due to the high water and nutrient content, the direct application poses serious pollution risks for soils, atmosphere and, most importantly, surface waters. With the new REPowerEU energetic plan, which

boosted biogas production, consequently increasing the digestate output, it is crucial to find alternative digestate management, while at the same time avoiding nutrient losses.

To answer this issue, the same process scheme used for VFAs recovery from OFI anaerobic fermentation and described in the previous chapter of this thesis, can also be applied for the recovery of nutrients, most importantly ammonium, potassium and phosphorus. In this second part of the work, the agricultural digestate used as inoculum in the previous chapter undergoes a first step of Primary Solid/Liquid Separation (PSLS), using combined mechanical filtration and centrifugation. For the second step, the liquid part obtained from the PSLS was separated into 3 different lines, using different combinations of pressure-driven membrane filtration, including MF, UF and NF. The second step liquid phase was then tested in a RO module to recover a nutrient-rich concentrate and water as permeate.

The aim of this work was the selection of the best combination of different pressure-driven membrane filtrations able to maximize the nutrients and water recovery from all the process and from the concentrate of the RO at laboratory scale. The middle steps served as solid and microorganisms removal steps to purify and stabilize the liquid phase to be feasible for RO filtration. The RO concentrate rich in ammonium, was used as nitrogen source for the growing medium of PHAs-producer microorganisms which will be described in the next chapter of this work. Moreover, the apparent viscosity of the various process effluents has been monitored to further characterize digestate rheology and filtration efficiency.

1. Materials and methods

In order to achieve the best condition of the liquid phase for RO filtration, the agricultural digestate (AGRD) was characterized by its physical and chemical properties. Most of the methodology and instruments used in this part of the work were already described in the previous chapters. New methods used for this part of the work will be written below.

1.1 Digestate characterization

The digestate used in this work was from the same facility described in the previous chapter materials and methods. However, the characterization reported here in Table 10 corresponds to the value of the raw digestate.

	Raw digestate
Total Solids (TS) (% w/w)	7.76 ± 0.16
Volatile Solids (VS) (% w/w)	5.45 ± 0.24
VS/TS (%)	70.23 ± 0.05
pH	7.93 ± 0.02

Conductibility (mS/cm)	37.01 ± 0.14
Total Kjeldahl Nitrogen (gN/L)	4.08 ± 0.30
NH_4^+-N (gN/L)	3.52 ± 0.19
PO_4^{3-}-P (gP/L)	0.21 ± 0.02
Total Phosphorus (gP/L)	1.05 ± 0.09
Total Potassium (gK/L)	4.01 ± 0.23

Table 10. Digestate nutrient characterization.

1.2 Filtration process set-up

As the raw digestate has high solid contents, it was filtered and centrifuged, at laboratory scale, in order to remove bigger solid particles and fibers. The PSLS is composed by a mechanical filtration using 3 different filters, having a mesh size of 2, 0.5 and 0.1 mm respectively. The liquid part obtained was then centrifuged (CF) for 20 minutes at 4000 rcf using the centrifuge Eppendorf® 5810R (Germany). By this way, the biggest solid particles, the fibers and the colloidal particles were removed in order to reduce fouling and wearing of the following step membranes.

For the second step, the supernatant (CF-SUR) entered three different combinations of pressure-driven filtration in order to evaluate the best one in term of TS, nutrients, and water recovery from an initial mass of AGRD of 2.00 kg. Specifically, the three filtration lines tested were the following:

a) Line 1: PSLS + CF + MF + UF + RO;

b) Line 2: PSLS + CF + UF + RO;

c) Line 3: PSLS + CF + NF + RO.

For the microfiltration (MF) step, DuraPES 450 (3M Membranes), polyethersulfone (PES) membrane with 0.45 mm pore size was used; the filtration area was 35 cm^2 with an operative Trans-Membrane Pressure (TMP) of 2 bar. The ultrafiltration (UF) step used a 300 kDa PES membrane, LX MAX (Synder Filtration), with 35 cm^2 of filtration area and operative TMP of 2 bar. The TMP was maintained using two peristaltic pumps and the membrane area was 35 cm^2. MF and UF were performed using "Vibro-Lab 35P filtration system" by SANI membranes A/S (Denmark) in crossflow mode. The NF step used a FilmTec™ NF270 (Dupont), polypiperazine-amide thin film composite (PA-TFC) membrane, with 17 cm^2 of filtration area and operative TMP of 4.5 bar.

The liquid permeate obtained from the second step entered the third and final step, composed by a RO module. The membrane used was Polyamide TRISEP® ACM2 High Rejection membrane, supplied by MANN+HUMMEL, with a TMP of 40 bar. Both NF and RO were performed in dead-end mode using Steriltech (United States) HP4750 High Pressure Stirred Cell, a technical-grade nitrogen cylinder was used to pressurize the filtration cell.

When the permeability decreased under 10% of the steady state value, the membranes were cleaned following the factory guidelines of the filtration system, which involved a caustic wash at pH 11 for 30 minutes, followed by an acid wash at pH 2 for 15 minutes, both steps at 55°C temperature.

1.3 Parameters for the evaluation of the filtration lines

The following parameters were used for the selection of the best filtration line:

a) Global nutrient recovery (% w/w) = $\frac{effluents\ nutrient\ mass}{inlet\ (AGRD)\ nutrient\ mass}$ % (1)

b) RO nutrient recovery (% w/w) = $\frac{RO{-}RET\ nutrient\ mass}{inlet\ (AGRD) nutrient\ mass}$ % (2)

c) RO water recovery (% w/w) = $\frac{RO{-}PER\ water\ mass}{inlet\ (AGRD)\ water\ mass}$ % (3)

d) Volume Reduction Factor, VRF (% v/v) = $\frac{PER,i}{PER,i{-}1}$ % (4)

Where $PER_{,i}$ represents the permeate volume of each specific pressure driven filtration step and $PER_{,i-1}$ is the volume of the permeate flux coming from the previous filtration step (Zhang Y et al., 2010).

e) Crossflow Velocity, CFV (m/s) = $\frac{Volumetric\ inlet\ flow\ (\frac{m^3}{s})}{membrane\ area\ (m^2)}$ (5)

CFV represents the linear velocity of the flow tangential to the membrane surface.

Finally, the best filtration line emerged from the evaluation of the previous parameters (Equations 1, 2 and 3) were also tested in terms of permeability to verify flux stability over time.

d) Permeability = $\frac{permeate\ mass\ (kg)}{membrane\ area\ (m^2) * TMP\ (bar) * time\ (h)}$ (6)

2. Result and discussion

2.1 TS and water recovery

The removal of solids, fibrous and colloidal materials is crucial to avoid RO membrane fouling and to decrease the osmotic pressure, allowing a longer membrane operational life and performance. Along the primary solid/liquid separation and the following UF, TS concentration changes were used to evaluate the efficiency of the process.

The TS removal was almost complete in all the filtration lines: the residual TS amount in the RO permeate was 0.26, 0.34 and 0.06% w/w for the filtration line #1, #2 and #3, respectively. Figure 28 shows the TS distribution on the different output streams of the three filtration lines.

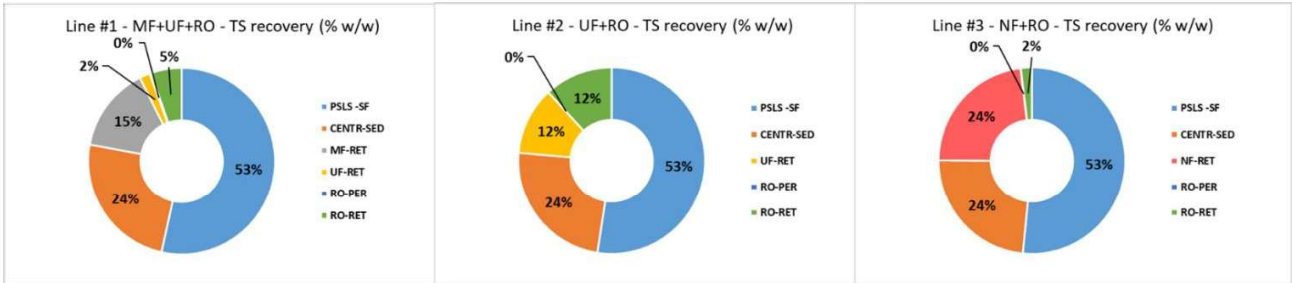

Figure 28. The distribution of the Total Solids along the three filtration lines

It is interesting to note that the PSLS and the CENTR stages were able to remove the 53 and 24% w/w, respectively, of the TS amount initially present in the AGRD. Specifically, PSLS step allowed the retention of the largest solid particles, such as lignocellulosic fibers from AGRD, composed by a mixture of energy crops residues and bovine manure, which also includes fibers derived from the animal feeding. The PSLS separation can remove the phosphorus-based compounds too, as commented in the next paragraph of the manuscript. The PSLS-LF was then treated by the CENTR step, which was able to retain in the CENTR-SED the colloidal and fine particles (Van Puffelen et al., 2022). Colloidal materials are compressible, have a density close to the water and are viscous. These features could make them a problem for the MF and UF membranes as they can cause fouling (Bowen & Jenner, 1995). Consequently, their preventive removal can help the next pressure driven filtration units, mitigating membrane fouling and assuring a constant permeability over time. It is important to emphasize that a CENTR step cannot assure the full removal of the colloids as their dimensions range is very large: from 0.1-10 µm (Bowen & Jenner, 1995). It means that their retention requires a further MF or UF step to avoid the fouling of the RO membrane.

The distribution of the remaining TS depended on the configuration of the next steps of the filtration lines. Line 1 assured the recovery of about 15% w/w of the TS in the MF retentate, while the next steps of UF and RO completed the TS removal with a recovery yield of 2 and 5% w/w, respectively. Specifically, the MF step led the removal of the suspended solids and microorganisms (Popova et al., 2024), and of the organic nitrogen, mainly proteins and urea, allowing the passage in the permeate flux of the inert compounds (EBA, 2024), such as the ammonium and potassium salts, which can be recovered and concentrated through the final RO stage (Bolzonella et al., 2018). Considering the performance of Filtration Line 1, the UF step can be neglected as it recovered only the 2% w/w of the TS.

Instead, Line 2 excluded the MF step and involved only the UF. The TS recovered in the UF retentate were 12% w/w, similar to the 15% w/w achieved by the MF in Line 1. The following RO step led to a TS retention of 12% w/w, more than double what was obtained by RO in Line 1. It demonstrated that Line 2 allowed a better fractioning of the TS based on their nature: suspended solids and microorganisms were blocked by the UF membranes, while the ammonium and

potassium salts were mainly recovered in the RO step. Instead, the presence of two pressure driven membranes (MF and UF) in Line 1 reduced the quantity of salts arriving to the RO step, reducing the overall process yield as RO concentrate can be considered the high added value products from AGRD (Phosphorus Platform, 2024). Finally, Line 3 adopted NF after the PSLS and CENTR steps. However, CENTR-SUR, which had a 3% w/w TS content, caused a rapid fouling of the NF membrane, mainly due to accumulation of colloidal solids and small particulate material on the membrane surface (Mohammad et al., 2015). This led to a permeating stream which was only 12% w/w of the inlet stream. On the contrary, in the previous filtration lines #1 and #2, the permeate represented the 70-75% w/w of the inlet stream both for the MF and UF processes. Consequently, all the residual TS content from the CENTR-SUR was in the NF retentate, meaning that the RO led to the recovery of only the 2% w/w of TS in the AGRD.

Another important issue in the digestate treatment is represented by water, which is another element to be recovered, especially in arid and semi-arid regions. The RO step, while concentrating the nutrients in RO retentate and reducing the transport costs of these compounds, allowed the recovery of water in the permeate stream. The three filtration lines were different in terms of water recovery yields from the AGRD: the best one was Line 2 with about the 33% w/w, then Line 1 achieved a 25% w/w, while the worst yield belonged to Line 3 with a recovery water amount lower than 5% w/w. These results confirmed that: i) higher are the membrane units in a filtration line, lower is the water (and the nutrients amount, see next paragraph) which arrived at the RO step. It was a consequence of the increasing number of streams generated from each membrane. Moreover, ii) NF membrane is rapidly clogging without a previous step able to act a prior removal of colloids, suspended solids and lower particles, such as MF or UF.

2.2 Nitrogen and potassium recovery from the filtration lines

The distribution of the TKN, ammonium and potassium compounds in the output streams of the three filtration lines was reported in Figure 29a, 29b and 29c, respectively.

Figure 29. Distribution of Total Kjeldahl Nitrogen (A), ammonium (B) and Potassium as K2O (C).

The distributions of the TKN, ammonium and potassium compounds were very similar along all the filtration lines. It can be explained considering that ammonium represented more than 85% w/w of TKN in the AGRD (Table 10), while potassium compounds followed the same distribution of ammonium being both little molecules.

As commented above, Line 2 was the best one for the recovery of these compounds in the RO concentrate (Table 11).

	LINE 1 - MF+UF+RO		LINE 2 - UF+RO		LINE 3 - NF+RO	
	Total Recovery (%)	**Recovery from RO-RET (%)**	**Total Recovery (%)**	**Recovery from RO-RET (%)**	**Total Recovery (%)**	**Recovery from RO-RET (%)**
Water (from RO permeate)	25.09 ± 1.25		32.75 ± 1.86		4.75 ± 0.14	
TKN	92.65 ± 3.69	26.29 ± 1.27	91.18 ± 3.29	41.01 ± 1.87	94.15 ± 2.25	5.63 ± 0.21
N-NH_4^+	96.55 ± 3.21	26.36 ± 0.25	97.42 ± 1.30	46.50 ± 1.14	99.97 ± 0.01	6.41 ± 0.75
TP	92.78 ± 4.85	7.77 ± 0.04	94.54 ± 3.58	14.98 ± 0.70	94.49 ± 3.01	1 .00 ± 0.04
K as K_2O	93.99 ± 3.55	22.95 ± 1.31	99.98 ± 0.00	44.32 ± 0.42	99.21 ± 0.02	4.59 ± 0.33

Table 11. Recovery yields of nutrients and water from the filtration lines

The recovery yields for TKN, ammonium and potassium from the RO concentrate were 41.0, 46.5 and 44% w/w, respectively. The UF-PER had an ammonium-TKN ratio in the range of 97-99% (Table 11), which demonstrated that organic nitrogen, characterized by a lower solubility in the aqueous phase, was separated already in the PSLS and CENTR steps and in a little fraction in the UF-RET. Finally, the ammonium-TKN ratio remained constant in the RO-RET, meaning that the final step allowed almost exclusively the water molecules passage (Carter et al., 2015). Potassium recovery had no significative difference compared to ammonium one as they are both small molecular weight compounds: consequently, it was recovered mainly in the RO-RET. The ammonium and potassium concentrations in the RO-RET were similar too: 11.20 and 12.50 g/L.

The filtration Line 1 led to lower performances with TKN, ammonium and potassium recovery yields in the range of 23.0- 26.5% w/w for the three parameters. Even if the filtration mechanism for these compounds was similar to the one described for Line 2, the addition of a filtration membrane, the MF was negative as it created a further output stream, the MF-RET, causing the loss of about 15-20% w/w of the recovery performances in the RO concentrate. Being composed mainly by water, MF concentrate also retained a quote of ammonium and potassium compounds, characterized by high solubility in aqueous medium (Fechter et al., 2023).

Finally, the worst recovery performances belonged to Line 3. As already mentioned, it can be explained by the low permeation amount generated from the NF step due to the rapid membrane fouling, demonstrating that the direct application of this filtration was not convenient. Anyway, some authors (Ma et al., 2024; Mohammad et al., 2015) emphasized that NF can be very useful for the fractioning of divalent and monovalent ions. Specifically, The NF and RO membranes consist both of micropores. NF membranes can remove less than 50% of the monovalent ions and more than 90% of divalent ions. Nevertheless, RO membranes can remove more than 98% of monovalent

ions (Ibrahim et al., 2020; Shenvi et al., 2015). Clearly, the failure of Line 3 did not allow us to appreciate this difference between mono and divalent ions recovery in the present research work.

2.3 Phosphorous compounds recovery from the three filtration lines

With nitrogen and potassium, phosphorus is the other essential macronutrient for the synthesis of fertilizers. But, more than nitrogen and potassium, phosphorous is also considered a strategic element as it derives from phosphate rocks, which are located in few countries, i.e. Marocco and China (Azam et al., 2019; Daneshgar et al., 2018). Then, its recovery is important for EU energy and food independence and the avoiding of geo-political tensions. The distribution of this element along the outputs from the three filtration lines is shown in Figure 30.

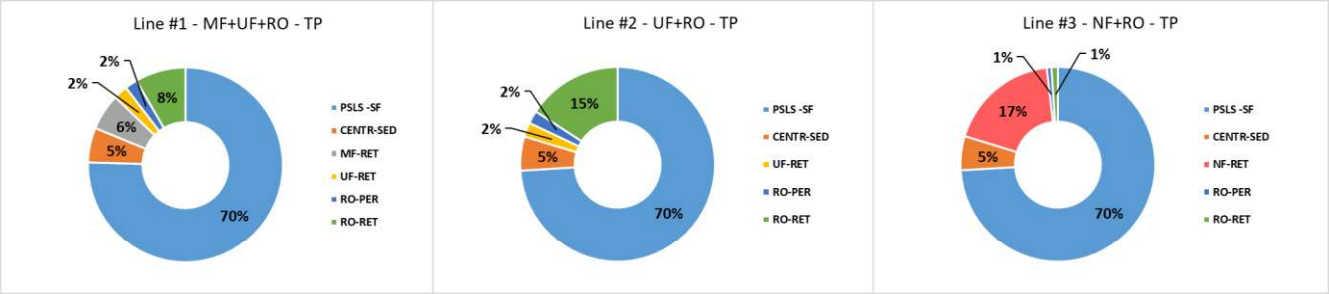

Figure 30 The distribution of the Phosphorous compounds along the three filtration lines.

In contrast to the nitrogen and potassium compounds, phosphorous was already recovered at the first mechanical separation step (PSLS). Specifically, over the 70% w/w of P compounds was retained in the SF generated from the PSLS, consistently with previous research (Mazzini et al., 2020; Van Puffelen et al., 2022). It is important to emphasize that phosphorus separation in the SF can be further improved by the adoption of coagulants and flocculants, which help to bring non-settleable particles together into larger, heavier clumps of solid material that can be easily removed (Nav et al., 2024; D. Wang et al., 2013). Similarly to nitrogen, total phosphorous (TP) compounds involve organic phosphorous and inorganic one, such as phosphate compounds (PO_4^{3-}). Organic phosphorous has a similar separation trend of organic matter and TS, going in the SF of digestate, while phosphate can be found more in the LF and can be separated through the next filtration steps. With reference to the present work, the pressure driven membranes (MF, UF and NF) assured the recovery of the phosphate. Phosphate content in the AGRD was about 0.20 g/kg, that means less than 20% w/w of the TP compounds. MF (Line 1) and UF (Lines 1 and 2) were not efficient in their recovery, being the dimension of the membranes too large to retain phosphate, which was concentrated in the RO-RET. While NF (Line 3) and RO (Line 2) led to a 15-17% w/w of recovery, assuring almost the complete removal of the TP (Table 11) and, mainly, its separation from the ammonium and potassium compounds. As commented for the ammonium recovery, NF and RO operations were the best for the recovery of ions, with NF demonstrating good removal yields for

plus-valent ions, i.e. PO_4^{3-}, and RO both for plus-valent and monovalent ones (Bowen & Jenner, 1995).

Tables 12, 13, and 14 reported the detailed characteristics of the different streams for Line #1,2 and 3, respectively.

Line 1	TS (% w/w)	COD (g/kg)	TKN (g/kg)	Ammonium (g/kg)	TP (g/kg)	K_2O (g/kg)
AGRD	7.76%	718.39	4.08	3.52	1.05	4.10
PSLS -LF	5.01%	621.34	4.19	3.78	0.38	4.00
PSLS -SF	11.80%	919.28	3.44	2.92	2.45	4.20
CENTR-SUR	2.92%	512.33	4.05	3.97	0.35	4.10
CENTR-SED	14.43%	1,127.12	3.82	2.67	0.50	3.50
MF-PER	1.30%	422.90	4.29	3.99	0.33	4.40
MF-RET	4.97%	612.56	3.81	3.78	0.30	3.40
UF-PER	1.16%	337.11	3.87	3.79	0.32	3.60
UF-RET	1.53%	638.45	3.60	4.80	0.29	5.50
RO-PER	0.07%	6.27	0.87	0.57	0.08	0.65
RO-RET	5.60%	1,507.07	17.10	16.80	1.30	15.00

Table 12. Characterization of the different streams in Line #1.

Line 2	TS (% w/w)	COD (g/kg)	TKN (g/kg)	Ammonium (g/kg)	TP (g/kg)	K_2O (g/kg)
AGRD	7.76%	718.39	4.08	3.52	1.05	4.10
PSLS -LF	5.01%	621.34	4.19	3.78	0.38	4.00
PSLS -SF	11.80%	919.28	3.44	2.92	2.45	4.20
CENTR-SUR	2.92%	512.33	4.05	3.97	0.35	4.10
CENTR-SED	14.43%	1,127.12	3.82	2.67	0.50	3.50
UF-PER	1.40%	337.11	3.87	3.79	0.38	3.80
UF-RET	6.73%	1,175.32	3.60	4.80	0.20	5.50
RO-PER	0.07%	5.27	0.50	0.27	0.07	0.01
RO-RET	5.60%	1,053.07	11.70	11.20	1.10	12.50

Table 13. Characterization of the different streams in Line #2.

Line 3	TS (% w/w)	COD (g/kg)	TKN (g/kg)	Ammonium (g/kg)	TP (g/kg)	K_2O (g/kg)
AGRD	7.76%	718.39	4.08	3.52	1.05	4.10
PSLS -LF	5.01%	621.34	4.19	3.78	0.38	4.00
PSLS -SF	11.80%	919.28	3.44	2.92	2.45	4.20
CENTR-SUR	2.92%	512.33	4.05	3.97	0.35	4.10
CENTR-SED	14.43%	1,127.12	3.82	2.67	0.50	3.50
NF-PER	2.10%	201.00	3.90	3.85	0.20	3.40
NF-RET	3.01%	554.21	4.05	4.00	0.35	4.20
RO-PER	0.09%	25.00	0.70	0.55	0.16	0.65
RO-RET	7.45%	665.00	13.12	12.90	0.60	10.75

Table 14. Characterization of the different streams in Line #3.

2.4 VRF and permeability evaluation for the filtration lines

VRF is a very useful parameter to demonstrate the eventual fouling of the membrane. According to our opinion, an acceptable VRF is in the range of 0.90-0.65, which means that the membrane can assure the recovery in the retentate of the molecules bigger than the mesh size of the membrane, assuring the passage of the smaller compounds in the permeate. Higher values can mean the membrane is not recovering compounds as it allows the passage of the inlet flux, while lower VRF values demonstrate the inadequacy of the membrane as consequence of its partial or total fouling.

Line #1 had two sequential pressure driven membranes, the MF and the UF followed by RO. They showed a VRF of 66.7, 80.0 and 80.1% v/v, demonstrating the good working of all the separation steps. Line #2, which used only an UF step followed by RO, achieved a comparable VRF of approximately 80% (v/v). This outcome is noteworthy, as it not only confirms the UF step effectiveness, but also eliminates the need for the preceding MF step used in Line #1. The UF step provided sufficient filtration of the inlet flux, yielding a dual benefit. First, it reduces energy consumption by optimizing the operation of pressure-driven membranes. Second, it prevents the creation of an additional retentate flux (MF-RET), which would otherwise reduce the volume of recoverable water in the RO-PER and increase management costs in a full-scale plant. Finally, Line #3 was characterized by NF and RO. The VFR was very low, about 11%, demonstrating the complete fouling of the membrane and its total inadequacy to assure nutrients recovery without previous separation step.

The permeability parameter over time was determined for Line #2, which emerged as the best one for water and nutrients recovery. This parameter can be defined as the flux of permeate, passing

through a membrane under the action of a force gradient, such as the application of a pressure gradient (Childs & Mika, 2003). The permeability of the UF membrane was tested both using deionized water and CENTR-LP. Figure 31 shows the trend of the UF membrane permeability over time.

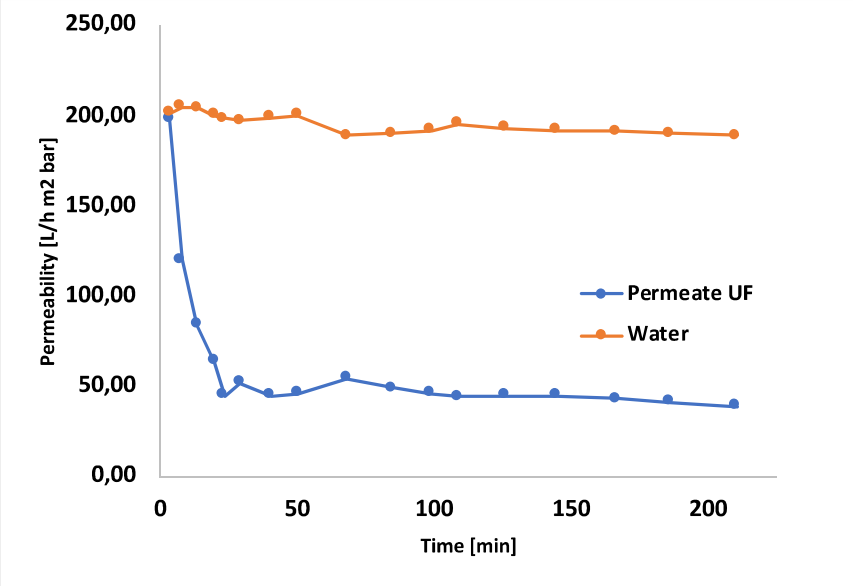

Figure 31. The trend of permeability for water and UF permeate over time

The membrane permeability with distilled water was around 200 L m^{-2} h^{-1} bar^{-1}, very close to the value reported in the technical sheet of the UF membrane. It demonstrated the good status of the UF membrane at the beginning of the test. The water permeability remained stable for the entire test, which lasted over 3 hours, which demonstrated the good working of the UF process. Instead, the pumping of the CENTR-SUR to the UF membrane (Line #2) led to quick dropping of the membrane permeability which reached a steady state value of about 50 L m^{-2} h^{-1} bar^{-1} after 30 minutes since the beginning of the test. The permeability reduction was the consequence of the cake formation on the membrane which caused a partial UF membrane fouling. Considering the structure of the lab-scale UF apparatus, it was not possible to manage the membrane in the attempt to improve the UF permeability, by an increase of the filtration area, which was of 35 cm^2, as reported in the Materials and Methods chapter. The permeability decreased below 10% of the steady state value after 3 hours since the start of the UF operation. The washing of the UF allowed to come back at around 50 L m^{-2} h^{-1} bar^{-1}.

With reference to the permeability of Line #1, MF had a similar trend to the one observed in Line 2 (Figure 31): it started at 210 L/m^2h bar decreasing until a steady state value of 65-70 L/m^2h bar, slightly higher than UF permeability. However, the configuration in Line #1 was worst in terms of nutrient recovery in the RO retentate, as previously commented, as it was coupled to UF which led to the emission of two RET fluxes. It is important to emphasize that the only MF step would not be

able to avoid the fouling of following RO. So, Line #1 was not considered convenient for the scale-up of the process. Finally, regarding Line #3, having the only NF it was observed that after 5 minutes from the beginning of the filtration, membrane permeability dropped to very low values 1.41 L/m^{2}h bar, slowly decreasing until 60 minutes, when the filtration was stopped with a permeability of 1.18 L/m^{2}h bar. It was further confirmation of the fouling of NF membrane.

CFV represents another key parameter in filtration processes as it provides information about the fluid dynamics on the surface area and the choice of the pump in the scale-up of the process. The best filtration line in terms of nutrients recovery (Line #2), characterized by the UF membrane, had a CFV of about 0.2 m/s in steady state condition, which is close to the conventional CFV values for pressure driven membranes according to Sterlitech website (Sterlitech, 2024).

3. Digestate nutrient recovery conclusions

Three different combinations of mechanical separation and pressure driven membranes technologies were tested in order to recover water and nutrients (nitrogen, phosphorous and potassium compounds) from AGRD. The MF did not lead to a complete separation of the nutrients and of the removal of colloids and suspended solids. Consequently, this filtration step can be removed to avoid losing nutrients to be recovered in the RO concentrate. The direct application of NF was neither able to assure good performances as it was characterized by low mesh dimension which led to the rapid fouling of the membrane. Instead UF was able to assure the finest and colloidal particles removal, assuring the passage of the ammonium and potassium compounds in the permeate flux and then in the RO steps, where they were recovered and concentrated. In this study, we focused primarily on evaluating the initial performance and efficiency of the nutrients recovery membranes, given the time constraints and the scope of the research.

PHA production from *Opuntia ficus-indica* fermentation effluents and anaerobic digestate

The previous experimental chapters are focused on the valorization of two substrates, respectively OFI cladodes and anaerobic digestate, using a pressure-driven filtration process. The end products of these processes are: i) a carbon-rich effluent, represented by VFAs, from OFI cladodes; ii) a nutrients-rich effluent, composed mainly by N, P, and K, from the filtration of AD digestate. Although both products can be used singularly for various applications, which were previously discussed, in this last chapter I focus my work to use both of them in a single application for the

production of PHAs using a pure culture of *Thauera* spp. microorganisms. In particular, VFAs from OFI fermentation can be used as carbon source, while concentrated AD digestate can be used as nutrient source, creating a perfect growth environment for microorganisms. PHAs are polymers with great potential applications as biobased, biodegradable plastics, and therefore sought in the new EU policy framework as an alternative to classic fossil-based plastics. Moreover, as the starting substrates for this application originates as by-products, this PHAs production represents an interesting circular economy approach as foster by EU Waste Management directive and Green Deal.

1. Materials and methods

The substrate used as carbon source for PHA accumulation is the purified fermentation effluent of OFI cladodes, obtained from the HRT 5 bioreactor, which was the best in terms of VFAs production (Table 15).

	Concentration (gCOD/L)
Acetic acid	11.97 ± 0.79
Propionic acid	1.87 ± 0.42
Butyric acid	6.36 ± 0.57
Valeric acid	0.82 ± 0.06
Caproic acid	7.85 ± 1.02
Total VFAs	26.09 ± 2.46

Table 15. VFAs concentration from HRT 5 of OFI fermentation semi-continuous tests.

The nutrient source for the growth test was obtained from the RO of AD digestate (Line #2), discussed in the previous chapter. The concentration of N, P, and K is reported in Table 16.

	Concentration
Total Kjeldahl Nitrogen (gN/L)	11.70 ± 0.17
Ammonium (gNH4+-N/L)	11.20 ± 0.04
Phosphorus (gP/L)	0.72 ± 0.06
Potassium (gK/L)	12.50 ± 0.33

Table 16. Nutrient concentration from the RO stream of AD digestate filtration process.

For this study, a bacterial strain of the genus *Thauera* in pure culture was used for PHAs production. The strain was selected from a mixed microbial culture (MMC) producing PHAs. Microbiological analysis showed 99% similarity with *Thauera butanivorans* (NBRC 103042T), hence the strain used is called *Thauera* sp. Sel9 (Botturi et al., 2020a; Frison et al., 2021a).

In general, the genus *Thauera* belongs to the class of β-proteobacteria. It was first identified first identified by (Macy et al., 1993) and comprises 14 species with validly published names. *Thauera* is one of the three bacterial genera most commonly found in mixed microbial cultures selected as accumulators of PHA (Dubbels et al., 2009), with others strains where *Azoarcus* and *Paracoccus*. *Thauera butanivorans*, formerly known as *Pseudomonas butanovora*, is a bacterium Gram-negative

non-spore-forming bacterium with a rod shape (0.6-0.8 μm wide, 1.1-2.4 μm long) capable of able to move due to the presence of a polar flagellum. It has a chemo-organotrophic metabolism facultative anaerobic: it can use molecules such as O_2^-, NO_3^-, NO_2^-, N_2O as electron acceptors and during aerobic growth is able to utilize C2-C9 alkanes as a source of carbon and energy. It is not able to break down aromatic hydrocarbons or open-chain terpenoids open chain under anaerobic conditions(Dubbels et al., 2009).

1.1 *Thauera* Sel9 growth test

In order to evaluate the performance of the OFI substrate on the growth of *Thauera Sel9* microorganisms, a growth test must be done. The colony of *Thauera Sel9* from a petri dish was pre-inoculated in a vial of rich medium overnight. The following day, the cells grown in the vial were collected and washed with physiologic water. The washed cells were inoculated in the growth medium, which is a variation of Brunner medium where the carbon and nitrogen sources are replaced with: I) a VFAs rich effluent, obtained from the filtration of OFI fermentation; II) digestate RO concentrate as a nitrogen source. Table 17 shows the exact composition of the growth medium. For this phase, the carbon to nitrogen ratio used was 10. The *Tahuera Sel9* cells were tested with 1 gCOD/L and 2 gCOD/L in two duplicated 250 mL flasks, along with negative control without carbon and nitrogen sources. During the test, every day, the microorganisms colon-forming units (CFUs) were counted through sequential dilution and petri dish inoculum. The test lasted 4 days. The microorganisms grew at 27 °C in an orbital shaker.

	Concentration (g/L)
Na_2HPO_4	2.44
KH_2PO_4	1.52
$MgSO_4 \cdot 7H_2O$	0.20
$CaCl_2 \cdot 2H_2O$	0.05
SL - 4	10 mL

Table 17. Brunner medium composition.

1.2 *Thauera* Sel9 accumulation test

For PHAs accumulation test, it is crucial to start with an inoculum at the top of the microorganisms growth curve, to start with the maximum quantity of cells available. To do so, a *Thauera Sel9* pre-inoculum was made in a 5 L flask with rich medium and kept growing in an orbital shaker at 27 °C until the time of maxium growth, identified during the growth test. At that time, the cells were collected through centrifugation and washed from the rich medium with physiological water. The collected cells were then inoculated in two 5L flasks with OFI VFAs medium as carbon source, without nitrogen source to force PHAs accumulation. Every day, 100 mL of accumulation broth were stored and analyzed for: i) average cell diameter; ii) VFAs content in the medium; iii) PHAs extraction. The cellular diameter was analyzed trough a particle counter supplied by Beckman

Coulter Inc. (USA), equipped with a 100 µm capillar analyzer. VFAs content were analyzed through ionic chromatography with ThermoFischer (USA) Aquion Chromatographer, equipped with an ICE AS 1 column and conductibily detector. PHA extraction were performed according to the Braunegg's method, which will be explained in the next section. The accumulation test lasted 4 days.

1.3 PHAs extraction and quantification

PHAs are accumulated as granules inside the microorganism. Therefore, these granules must be extracted and quantified based on total cell mass. The collected cells were firstly centrifuged at 3900 rpm for 15 minutes. Then, the pellet obtained was lyophilized overnight. The pellet was then prepared for quantification using the method described in Braunegg et al. (1978): the freeze-dried pellet was dissolved in 1 mL of chloroform, 2 mL of acidified methanol (3% v/v H_2SO_4), adding finally 1 mL of 0.5 %w/w benzoic acid in chloroform. The samples were digested at 100 °C for 4 h. After digestion, 1 mL of distilled water was added to better separate aqueous and organic phases. The PHAs esters, obtained by digestion, were concentrated on the organic phase, which was transferred to the GC vials. Using the same method, a calibration curve was prepared using pure poly(3-hydroxybutyric acid-*co*-3-hydroxyvalertic acid). PHAs values were calculated as percentage over the sample VS (Bastianelli et al., 2023).

2. Results and discussion

2.1 *Thauera* Sel9 growth test

To evaluate PHAs production and accumulation, it is crucial to understand how the microorganisms grow with the same carbon source and condition that will be used for the accumulation test. As illustrated in Figure 32, the starting CFUs, after the pre-inoculum with rich medium, were around log 6.8 CFUs/mL for all the tests. For the test with 1 gCOD/L of OFI Substrate (blue line), the microorganisms grow to log 8.30 CFUs/mL after 1 day of incubation, reaching a maximum level of log 8.70 CFUs/mL at the second day, before the CFUs started to decrease due to the progressive depletion of the substrate. For the test at 2 gCOD/L of OFI Substrate (gray line), the growth started after 1 day of lag phase, due to excessive substrate inhibition. On the second day of incubation, CFUs/mL reached log 8.70 CFUs/mL before peaking to log 8.81 CFUs/mL the third day. The negative controls (orange and yellow line), without carbon sources, were unable to grow new cells, thus removing an eventual error due to substrate contamination by other carbon sources. Moreover, the growth curves were in line with previous work from Andreolli et al. (2022), which tested *Thauera* Sel9 PHAs production capability in the same conditions, using a synthetic VFAs substrate. This confirmed the good health status and performance of the inoculum, and therefore its use as PHAs producer for the next accumulation test.

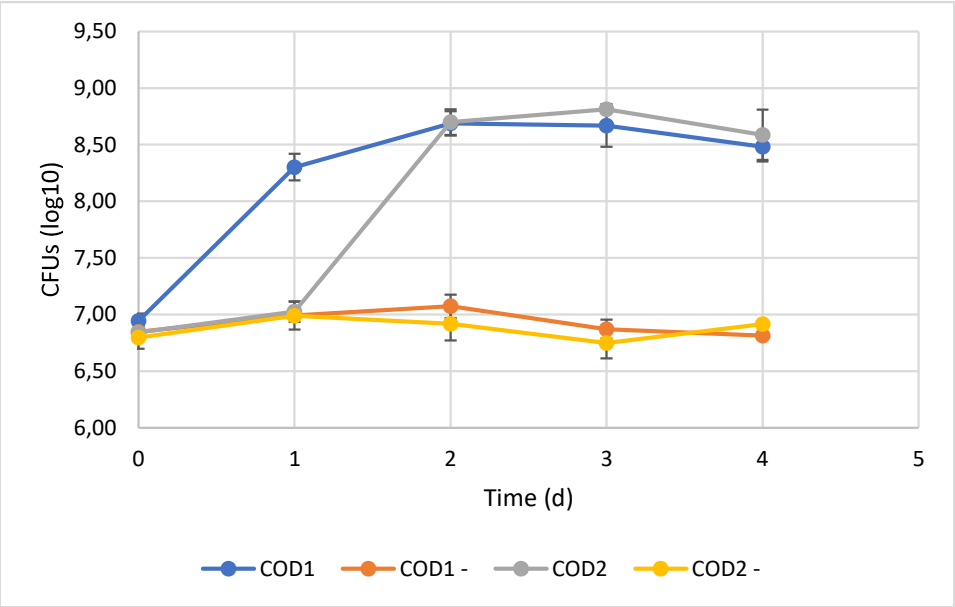

Figure 32. Thauera Sel9 growth test.

2.2 PHAs accumulation test

When there is an abundant carbon source but scarcity of other nutrients, in particular nitrogen, cell growth cannot happen. PHAs producer microorganisms sense this nutrient scarcity and therefore interrupts cell duplication and starting to accumulate carbon as PHAs to survive these hostile environmental condition. Therefore, until carbon is present, *Thauera Sel9* will keep accumulate PHAs inside the cell membrane and thus increasing the overall cell diameter. When all the substrate is depleted, the microorganism will consume stored PHAs to survive, thus decreasing its cell diameter. As illustrated in Figure 33 blue line, primary axis, *Thauera Sel9* cell diameter increased from 0.83 µm to 0.89 µm after the first day of accumulation. After the first day, the cell diameter started decreasing until reaching almost starting condition after 3 days, when the test was interrupted. The VFAs content in the substrate were totally consumed after the first day of accumulation. Therefore, the microorganisms consumed all the substrates in one day, accumulating PHAs, and then in the following second and third day consumed all the PHAs accumulated. This trend was confirmed by the PHAs quantification analysis, described in Figure 33 orange line, secondary axis, where the PHAs concentration peaked at day 1 with 23.81 % of cell TS.

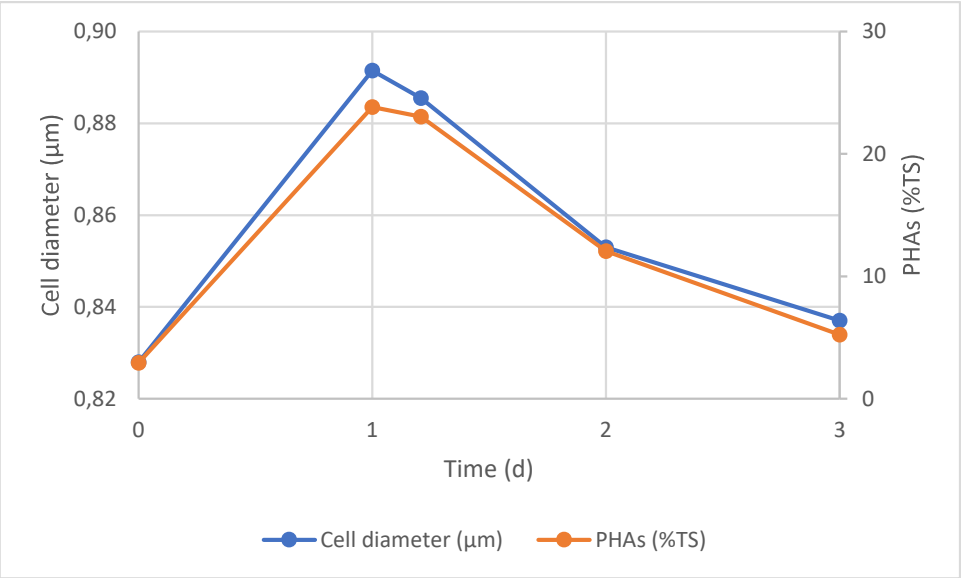

Figure 33. Cell diameter (blue line) and PHAs concentration (orange line, secondary axis).

3. Conclusions on PHAs production

This chapter focused on PHAs production through a pure culture of *Thauera* spp. Sel9 microorganism. VFAs from the fermentation trials of OFI, after pressure-driven membranes purification, were used as carbon source for the microorganisms. To assure the optimum grow conditions besides carbon, nitrogen was provided from an anaerobic digestate, previously treated with pressure-driven filtrations up to RO. The best growing condition were obtained with 1 gCOD/L of substrate, with a C:N ratio of 10. This conditions were applied for the PHAs accumulation test, without nitrogen source to produce a nutrient scarcity stress, obtaining a maximum PHAs percentage of 23.81 % based on cell TS. However, the percentage of PHAs produced is lower than other studies, which reported values around 50% (Andreolli et al., 2022). This can be attributed to multiple factors: i) variations in culture conditions, such as carbon source availability and the C/N ratio, can significantly influence PHA synthesis, favoring biomass production instead of polymer accumulation; ii) additionally, specific metabolic responses of *Thauera* to environmental stressors, such as nutrient limitations or aeration dynamics, could play a crucial role in modulating PHAs storage (Botturi et al., 2020b; Frison et al., 2021b). The observed discrepancy underscores the necessity for further investigation into optimization strategies, including refining growth conditions and assessing strain-specific genetic regulation to enhance PHA yield in future applications .

This trial confirmed the feasibility of OFI VFAs-rich fermentate and AD digestate as valid substrates for PHAs production, thus closing the circular economy loop, obtaining a new raw material, biobased biodegradable plastic, which can re-enter the economic cycle.

Conclusions

The environmental and energy crisis is one of the most urgent challenges facing our planet, with the overshoot of six of the nine 'planetary boundaries' threatening the stability of the global ecosystem. The increasing consumption of raw materials and the impact of climate change, already evident in extreme events such as droughts and fires, require a paradigm shift in the economic model. In particular, the transition to a circular economy is a necessary solution to reduce the exploitation of natural resources and mitigate environmental effects. The adoption of more sustainable waste management strategies, as indicated by EU legislation, and the promotion of alternative energy sources, such as bioenergy, are key steps towards achieving climate neutrality by 2050. In this context, the use of technologies such as AD not only favors the production of biogas but can also be optimized to obtain high value-added products such as VFAs, thus contributing to a more efficient model of resource recycling and valorization.

This PhD thesis is centered on the implementation of a laboratory-scale biorefinery for the simultaneous production, purification and recovery of high-value compounds and biofuels from OFI cladodes and agricultural digestate. The proposed biorefinery is based on AD technology, used to convert organic carbon into biomethane, VFAs, MCFAs, and fertilizers. FAs-rich effluents and AD digestate were refined using a pressure-driven membrane filtration process. In the last part of the thesis, the refined effluents were used as carbon and nitrogen source for the production of PHAs by pure culture of *Thauera* spp. Sel9 microorganisms.The first part of the experimental trials explored the potential of AD applied to OFI cladodes for biogas and VFAs production by modulating the HRT and optimizing the purification processes. Preliminary tests established that the addition of 5% w/w water reduced substrate viscosity, improved mixing and increased biomethane production by up to 220 $mLCH_4/gVS$. The results showed that the variation of HRT significantly affected the final products: the highest biomethane yield (210 $mLCH_4/gVS$) was obtained with a HRT of 20 days, while the accumulation of VFAs and caproic acid reached the highest values with a HRT of 5 days (26.09 gCOD/L for VFAs and 7.85 gCOD/L for caproic acid). For purification, membrane filtration increased the purity of VFAs from 86.11% to 97.41% w/w. Caproic acid was then separated by adsorption onto PAC, demonstrating a high affinity for this material. The desorption test revealed that ethanol with NaOH was the most effective solvent, while water showed poor performance due to the low solubility of caproic acid.

In the second experimental part of this thesis, three different combinations of mechanical separation and pressure membrane technologies were tested for the recovery of water and nutrients (N, P, and K) from the AD digestate. The best result was obtained in Line #2, which used UF and RO. This line proved effective in the removal of fine and colloidal particles, allowing N and K to be recovered and concentrated in the RO retentate, with over 40 % and 44% of N and K, respectively recovered.

The last experimental phase regarded the PHAs production from pure culture of *Thauera* spp. Sel9, using the previously recovered VFAs and nutrients, obtaining a maximum of 23.81 % of PHAs based on dry cell TS.

The results demonstrate the potential of the biorefinery to address energy and environmental challenges, contributing to the production of biomethane to reduce dependence on gas imports and facilitating the recovery of nutrients for biofertilizer applications. Furthermore, the use of VFAs for the synthesis of PHAs highlights new opportunities in the production of biodegradable bioplastics, expanding the scope of biorefinery applications in different industrial sectors.

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3. Battista F., Zeni A., Andreolli M., Salvetti E., **Rizzioli F**., Lampis S., Bolzonella D.; Treatment of food processing wastes for the production of medium chain fatty acids via chain elongation ;(2024) Environmental Technology and Innovation, 33, art. no. 103453

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- **Thessaloniki 2021 – 8th International Conference on Sustainable Solid Waste Management**

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- **Corfù 2022 - 9th International Conference on Sustainable Solid Waste Management**

  Presenter: "Nutrient Recovery from agricultural digestate at high TRL".

- **Chania 2023 - 10th International Conference on Sustainable Solid Waste Management**

  Presenter: "A biorefinery case study based on the valorization of Opuntia Ficus Indica for biofuels and bio-compounds productions".

- **Istanbul 2024 - 18th IWA World Conference on anaerobic Digestion**

  Poster presentation: "Nutrients recovery from agricultural digestate by sequential pressure driven membranes".

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Figure summary

Table summary

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