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Production of biofuels and biochemicals from waste biomass

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Turin, October 31, 2025

Summary

In recent years, climate change has opened our eyes to the importance of energy transition and waste management. These two issues have become intertwined with the development of new sustainable technologies and the production of biofuels to address ecological challenges that we can no longer ignore. On the one hand, the growing production of plastic materials, including those labelled as biodegradable, raises questions about their effective compatibility with existing biological treatment systems: some of them do not degrade within the timeframes and under the conditions typical of anaerobic digestion plants (according to ISPRA (2023) data, 67% of Italian organic waste ends up in anaerobic and mixed treatment plants), creating inefficiencies and accumulating residues. According to Legislative Decree 28/2011 (Italian Republic, 2011), waste biomass is "biodegradable components of products, waste and residues of biological origin from agriculture, forestry, fishing and related industries," including municipal solid organic waste that arrives at composting and anaerobic digestion plants.

Such biogenic waste is an excellent source of renewable energy because it is not and will not be subject to depletion as long as humans exist on Earth. Indeed, as the global population grows, waste management is becoming an increasingly pressing issue that requires space, facilities, and investment. What if we changed the paradigm so that these investments were not only made to manage waste but also to benefit from it? In my PhD research, I sought to answer this very question by studying and testing the potential of different types of biomass, from agricultural to urban waste (both organic and non-organic) to obtain biomolecules for the formulation of biofuels and, at the same time, to valorise the non-recyclable plastic component, which is now an integral part of organic fraction of municipal solid wastes (OFMSW) but still separated and mostly sent to landfill due to a lack of suitable treatment facilities. Traditional non-biodegradable plastics, and even worse microplastics, are found everywhere: from the soil to the marine environment, even

finishing up in the food we consume and therefore in our bodies. And if finding an alternative is no longer an option but an imperative, we must also understand how to make the most of all the plastic waste that already exists. This is where the waste problem can itself become the solution to our dependence on non-renewable fossil fuels. If properly pre-treated, plastics can be integrated into biological processes such as anaerobic digestion, whose gaseous product, biogas, can be converted into biomethane and used as a biofuel. Despite plausible doubts about biofuels being the ultimate solution to decarbonization, in some sectors, they still appear to be the only option in the short to medium timeframe. Although electrification is now widespread in the road transport sector, the aviation and shipping sectors, which jointly account for almost 10% of global greenhouse gas emissions (Ertelt, Kask & Breslin, 2024), remain difficult to electrify. These sectors require high-energy-density liquid fuels that cannot be replaced by electricity or solid fuels. This study focused on this issue, combining biological and thermochemical processes using agricultural and organic wastes and by products.

This PhD route aims to contribute to solving today's environmental and energy challenges by taking a holistic approach to waste management and fuel production. In a nutshell it involves optimising and combining innovative approaches to the valorisation of second and third generation streams, with the ultimate aim of converting them into value.

The first study concerns the anaerobic digestion of biodegradable polymers, previously treated biologically and chemically, to accelerate the degradation of PBAT within the conventional timeframes required for the management of organic municipal solid waste (OFMSW), obtaining the three constituent elements: adipic acid, terephthalic acid, and 1,4-butanediol. On a pilot scale, 24% polymer degradation was achieved after 24 hours of enzymatic pre-treatment with esterase and amylase, and 97% in terms of weight loss after 55 days of anaerobic digestion.

The second chapter examines the synergistic effect of anaerobic digestion in combination with the hydrothermal carbonization (HTC) process for the non-biological fraction of OFMSW. Hydrothermal treatment produces a liquid and a solid fraction, which, when added up to a maximum of 40% v/v and 10% w/w, respectively, increase biogas production and decrease H_2S production.

Finally, the third chapter compares different techniques for extracting triglycerides (TAGs) obtained from a two-step fermentation process using syngas produced from the gasification of biogenic residues. The final yield of the process is 26.5% of extracted TAGs.

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This thesis is the result of three years of work as a PhD student apprentice at Environment Park S.p.A. During my PhD studies, I took part in various activities and projects of the Green Chemistry group, in particular:

- SATURNO Project: funded by the Piedmont region through the Bioeconomy Technology Platform. The experimental results of this project constitute the experimental part of the first chapter of this thesis.
- METHAREN Project: this project received funding from the European Union's Horizon Europe research and innovation program under Grant Agreement No. 101084288. The experimental results of this project constitute the experimental part of the second chapter of this thesis.
- BioSFerA Project: this project received funding from the European Union's Horizon 2020 research and innovation program under grant agreement No. 884208. The experimental results of this project constitute the experimental part of the third chapter of this thesis.

My role in the projects included bibliographic research, extraction and pre-treatment methods, drafting experimental protocols, data analysis, and report writing. I thank the project partners for their fruitful collaboration.

Throughout this process, I was supported by the members of the Green Chemistry group, both in the experimental phases and in reviewing the documents to be shared with the project partners. For this reason, I would like to express my heartfelt gratitude to Dr. Paola Zitella, manager of the group and company tutor for my PhD program. Her availability, professionalism, and the trust she placed in me and my abilities were constant incentive for me to improve and enabled me to fully experience the company environment, thus enriching my thesis work. Thank you for making this path of growth possible.

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Chapter 1

Anaerobic digestion and bioplastics degradation

1.1 Regulations on organic waste management

The main regulations governing organic waste management in Italy are the Waste Framework Directive 2008/98/EC (European Parliament and Council, 2008), later amended by Directive (EU) 2018/851 (European Parliament and Council, 2018), and the Italian Consolidated Environmental Act Legislative Decree 152/2006 (Italian Republic, 2006), in particular Articles 183-184-185, which classify types of waste, processes, and what is excluded from the definition of waste.

In Italy, organic waste is treated in anaerobic digestion plants, composting plants, or integrated aerobic/anaerobic digestion plants, where the two types of processes are "integrated" in the same industrial site. Figure 1.1 shows the distribution of the organic waste load treated in the different types of plants (ISPRA, 2023). As can be seen, although anaerobic treatment plants alone account for only 9% of total organic waste treatment, this value rises to 67% when integrated management plants are also considered.

Anaerobic digestion is a biochemical process performed by microorganisms in environments without oxygen. These microorganisms break down and convert biomass, producing a sludge known as "digestate". Over time, technological implementation has adapted this biological mechanism for the degradation of organic material, aiming to recover energy from waste by producing biogas.

Before entering the biodigester, the waste arriving from the urban collection system must undergo preliminary processes aimed at eliminating all components that could hinder fermentation processes but have ended up (by mistake or due to improper individual household management) in the organic waste collection.

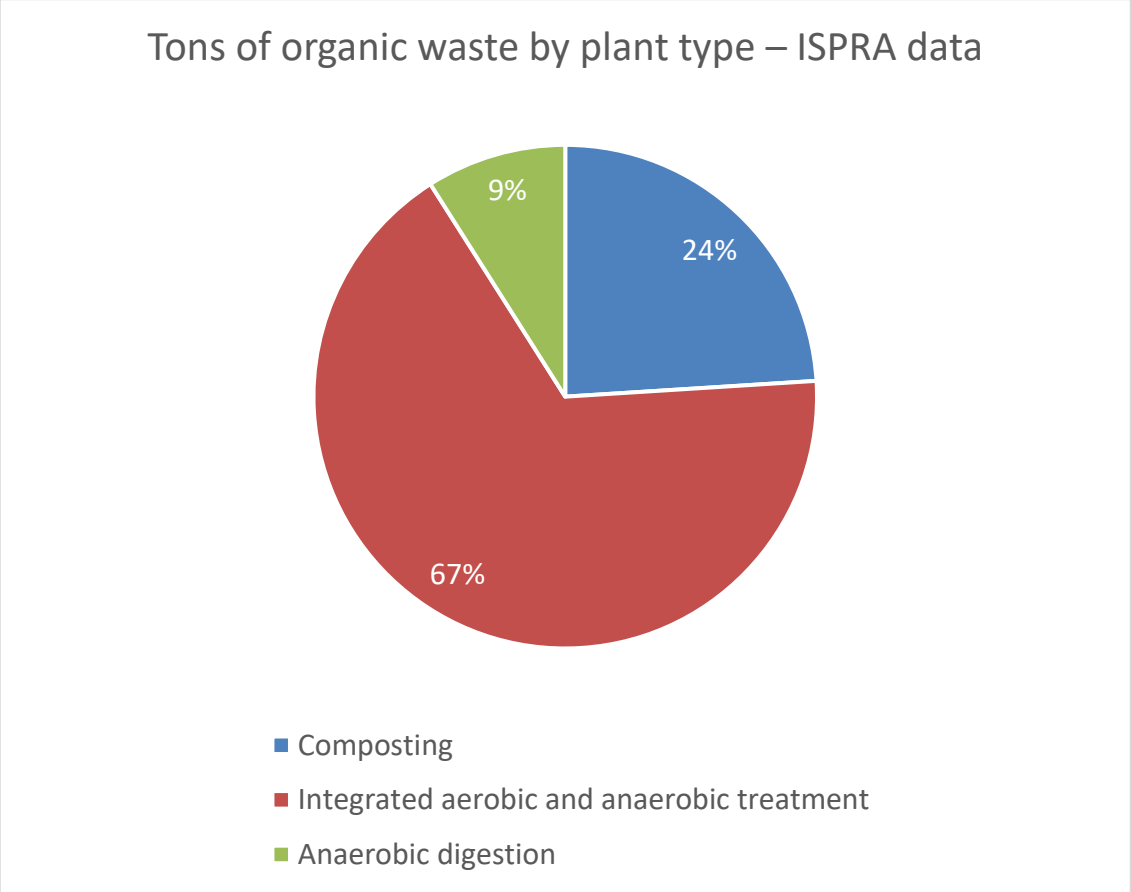


Figure 1.1: Percentage of waste managed by different types of plants

An example of a typical applied treatment path is as follows (ACEA Pinerolese, 2025):

- Bag breaking with a bag-breaking machine
- Initial screening to remove light components (e.g., plastics, both bio and non-bio)
- Deferrization to remove any ferrous waste
- Possible second screening
- Addition of water to the organic mixture is for the purpose of bringing the biomass to the right consistency and volatile solid content.

At this stage, anaerobic fermentation process takes place under continuous agitation. The process conditions depend on the type of biomass, the selected technology and the solids content (Piccinini, 2006; CRPA di Reggio Emilia, n.d.):

- Mesophilic (approximately 35°C): 15-40 days of hydraulic retention time (HRT)
- Thermophilic (approx. 55°C): 20-25 days of HRT
- Psychrophilic (10-25°C, simplified process conditions): 30-90 days of HRT

At the end of the anaerobic digestion process, two main products are obtained: biogas and digestate. The digestate is then treated in the sludge management plant, where it is mainly separated into two fractions. The liquid component can be sent for water treatment. The solid component, on the other hand, is mixed with grass clippings, foliage, and pruning residues and sent to the composting process, where it is left to macerate under aerobic conditions for 45–60 days (Piccinini, 2006).

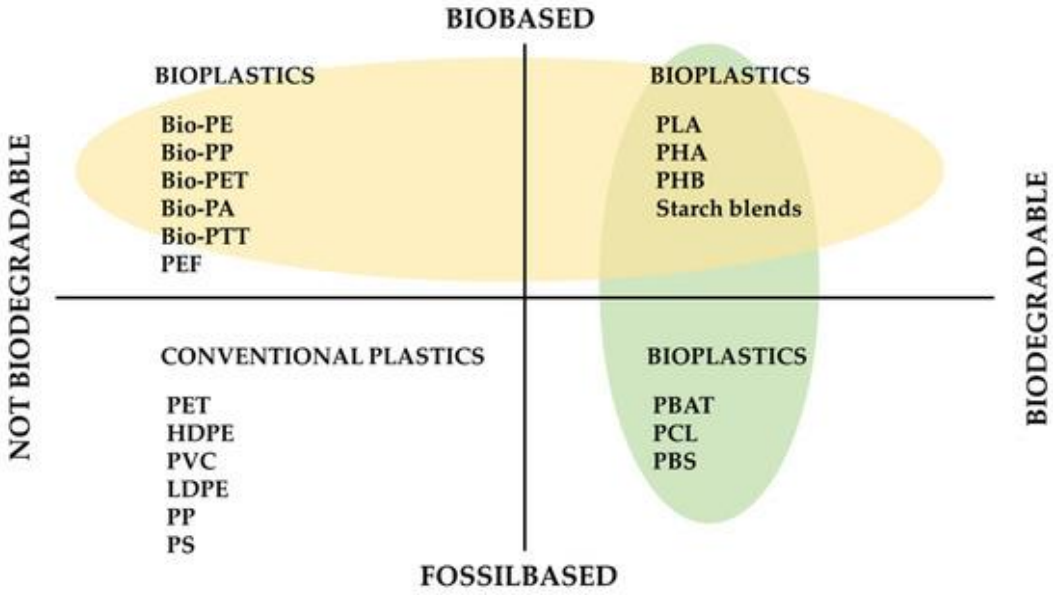
1.2 The growing prevalence of bioplastics in organic waste

The term "bioplastics" refers to all products that are biobased, biodegradable, or have both characteristics (European Bioplastics, n.d). Indeed, the origin of the product, whether from renewable (biobased) or fossil sources, is not strictly linked to the concept of "biodegradability". According to European Standard EN 13432 (CEN, 2000), biodegradability describes "the ability of compostable material to be converted into CO_2 and water under the action of microorganisms in the presence of oxygen". To demonstrate complete biodegradability, a level of biodegradation of at least 90% must be achieved in less than 6 months, measured using the European Standard EN 14046 (CEN, 2003) standard laboratory test. For a product to be biodegradable, therefore, it must decompose into its building blocks when subjected to the appropriate environmental conditions. Figure 1.2 shows a diagram that systematizes the origin of bioplastics and their biodegradability.
With the introduction of European Directive 2019/904, known as the "SUP Directive" (European Parliament and Council, 2019), the use of single-use plastics has been banned: no more plastic bags, cutlery, and cups. The reason is simple: unbridled production, excessive consumption, and careless disposal of plastic products have led to a global problem: animals killed by plastic bags mistaken for food, uncontrolled extraction of fossil materials, and microplastics in the environment that end up in the products we put on our tables. If a reversal of this trend is what the scientific community around the world is calling for, then the search for alternative solutions is something we can no longer ignore. In this regard, Italy has transposed the SUP Directive with Legislative Decree No. 196 of November 8, 2021 (Italian Republic, 2021), which came into force on January 14, 2022, and which deviates from the European directive in terms of the definition of plastic items, the definition of single-use, and the introduction of an exemption that allows the placing on the market of biodegradable and compostable plastic products composed of at least 60% renewable raw material in cases where (Centro Studi per l'Economia Circolare – CONAI, 2022):

- Single-use plastic packaging cannot be replaced by reusable products.
- The use of such packaging is intended for controlled circuits that supply waste to the public service through separate collection, such as canteens, healthcare, or social welfare facilities.

- Possible alternatives do not provide sufficient guarantees in terms of hygiene and safety in relation to the specific circumstances in which they are used.
- Considering the specific variety of food or beverages.
- When there are many people.
- If, according to a life cycle analysis by the manufacturer, the environmental benefit of reusable packaging is less than that of biodegradable and compostable single-use alternatives

Clarification is needed on the potential solutions that bioplastic materials could offer. As mentioned above, the prefix “bio” is very misleading because it is incorrectly linked to the concept of biodegradability and, even more erroneously, to simple methods of disposal. However, products made from bio-PE, bio-PP, and bio-PET are not biodegradable, as shown in Figure 1.2, but still fall into the category of bioplastics.



Vinci et al. Sustainable Management of Organic Waste and Recycling for Bioplastics: A LCA Approach for the Italian Case Study, Sustainability (2021)

Figure 1.2: Characteristics of bioplastics compared to traditional plastics

This first example alone makes it clear that it is impossible to think of a single disposal solution, precisely because we are dealing with different products with different characteristics.

As is often the case, however, legislation clashes with reality: not all anaerobic digestion and/or composting plants are suitable for treating compostable plastic products, or at least not in any quantity. This means that, for reasons related to excessive amounts, inadequate dimensions or process parameters, biodegradable

plastic often does not degrade or cannot be managed in organic treatment plants, and instead finally goes with unsorted waste. In Italy, there is a risk of having compost that does not meet specifications, i.e., that does not comply with the provisions of Legislative Decree No. 75 of April 29, 2010 (Italian Republic, 2010), which sets a limit of 0.5% w/w for the presence of pollutants such as metals, glass shards, and plastic, without currently distinguishing between traditional plastics and bioplastics (Cucina et al., 2021; Sorino et al., 2024).

The statistics speak for themselves: in 2020, 111000 tons of bioplastic products were produced (Assobioplastiche, 2020), accounting for 3.7% of biomass in organic waste collection (CIC & COREPLA, 2020). Therefore, there is a clear need to find an alternative solution for the disposal of these new valuable and innovative products, for which demand is constantly growing.

1.3 Experimental part

The following paragraphs will illustrate the chemical and enzymatic pretreatment techniques applied to bioplastic (BP) bags to facilitate the subsequent anaerobic digestion phase. The data presented in this chapter have also been previously published in the article "Management and possible valorization of bioplastics separated from the organic fraction of municipal solid waste" (Pellegrino et al., 2023). The tests described in this chapter were performed as part of the SATURNO project funded by the Piedmont region through the Bioeconomy Technology Platform.

Various pretreatments can be employed to enhance the degradation of BPs and their subsequent digestion by microorganisms (Mohee et al., 2008). Calabrò et al. (2020) tested Mater-Bi after various pretreatment procedures, including mechanical grinding, chemical pretreatment with NaOH for 24 hours, and aerobic digestion with an inoculum from an activated sludge treatment plant. Mechanical grinding had little effect, but chemical preparation with NaOH was the most effective because it made the polymer more accessible to enzymes.

The literature mentions several hydrolase enzymes generated by bacteria that attach themselves to the surface of the substrate and destroy it (Muroi et al., 2017; Suzuki et al., 2014; Zumstein et al., 2017). RoL lipase derived from Rhizopus oryzae (Sigma-Aldrich, Catalog No. 80612) and a FsC-like cutinase isolated from Fusarium solani (Novozymes, Catalog No. 51032) are two enzymes widely available on the market (Urbanek et al., 2020). These two enzymes completely degrade very thick Mater-Bi films in 20 hours at a pH of 6 and a temperature of 20 °C.

1.3.1 Materials and Methods

Pretreatment tests were performed on European Standard EN 13432 (CEN, 2000) certified compostable bioblastic bags, composed of 70% polybutylene adipate co-terephthalate (PBAT), 20% starch, and 10% additives (Venturelli et al., 2021), which were used for municipal solid organic waste collection. PBAT is a copolyester of three monomers: adipic acid, terephthalic acid, and 1,4-butanediol.

For the chemical pretreatment tests, HCl and NaOH supplied by Sigma Aldrich were used, as well as amylase enzymes from *Bacillus licheniformis*, amylase from *Bacillus amyloliquefaciens*, and esterase from *Bacillus subtilis*, used for enzymatic pretreatment, which were supplied by Sigma Aldrich.

The bacterial consortium used to initiate anaerobic digestion, consists of the digestate from the Acea Pinerolese company. Acea Pinerolese Industriale is a multi-utility company based in northern Italy, responsible for the treatment of mucipal organic waste to produce biogas and compost and processes 60000 t/y of municipal bio-waste to produce more than 6000000 Nm^3/y of biogas in anaerobic digestors.

Laboratory tests were performed in 500 ml Duran bottles. Pilot-scale tests were carried out in a 7 l glass reactor (LAMBDA Minifor Fermenter). The reactor is equipped with a control base, on which it stands, allowing the monitoring of operating conditions, such as temperature, agitation, pH, and the presence of O_2, thanks to the two probes with which it is equipped. Agitation is achieved by a shaft with vibrating rubber discs, which moves axially in a vertical direction. Heating is provided by a coil located on the control base of the reactor itself. Finally, management of pH variation is through automatic dosage of acid and base by two peristaltic pumps. Activation of these is based on the process set point and the pH detected by the probe. The reactor is also equipped with a metal tube that reaches the bottom of the vessel for sampling liquids and for gas injection or sampling. Figure 1.3 shows the reactor used for pilot tests.

The samples were tested by LabAnalysis. Here is the procedure they used, which is also in the article by Pellegrino et al. (2023)

To assess weight reduction, the samples were mixed with water and left to infuse overnight. After removing the upper liquid part, the solid residue was washed repeatedly with water and filtered again. The solid was then placed in an oven at 80 °C for drying and subsequently weighed.

To quantify terephthalic acid, the water from each sample was analyzed directly by HPLC-UV, using a Phenomenex Sinergi Hydro 250 x 4.6 mm, 4 µm column and eluting with a combination of water +0.1% phosphoric acid/acetonitrile. The wavelength set for detection was 254 nanometers.

For the quantification of adipic acid, this was converted to methanolic acid and then analyzed by GC-MS, using a VF-17ms column.

Finally, for 1,4-butanediol, methanol was injected directly into a GC-MS system equipped with a VF17ms column to dilute the sample.

In addition, LabAnalysis conducted infrared microscopy tests to verify the compatibility of BP samples with PBAT samples, thereby ruling out the possibility of other microplastics being present.

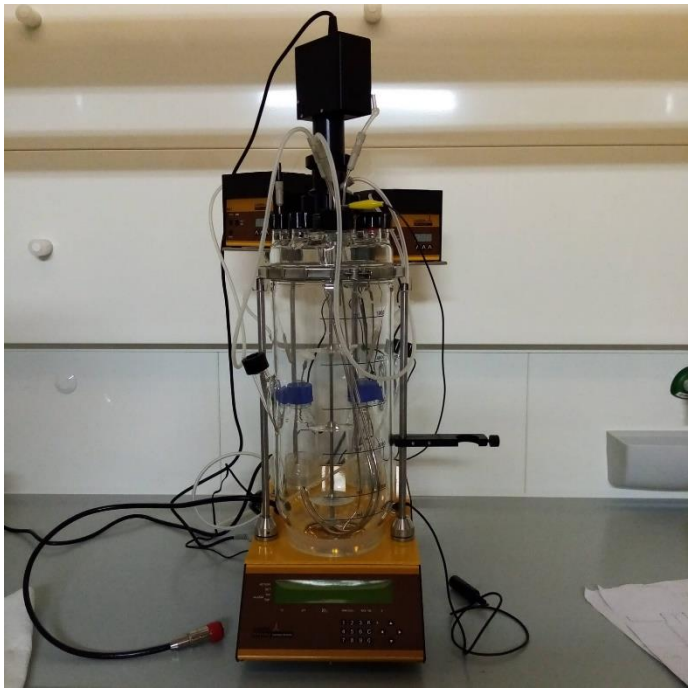


Figure 1.3: Reactor used for pilot-scale testing

In this regard, once the sample had been taken from the reactor during the anaerobic digestion reaction, a portion of the supernatant was filtered through a silver filter. This filter was then placed on PerkinElmer Spotlight 400 equipment for inspection and recording of the IR spectra. The same procedure was followed with the PBAT sample used as the starting material.

1.3.2 Chemical pretreatment and anaerobic digestion

Several chemical pretreatment tests were conducted, both with NaOH and HCl at different pH, temperature, and residence time conditions. Table 1.1 shows a summary of the most significant test conditions (Pellegrino et al., 2023).

The BP samples, cut with scissors to simulate breakage with bag breakers, were all approximately the same size and had a total weight between 6.89g and 8.63g.

It was not possible to determine whether weight loss was influenced by chemical pretreatment due to the deterioration of the samples themselves. The potential breakdown of the bond, with consequent separation of the polymer into its monomers, was therefore studied by searching for the presence of 1,4-butanediol, adipic acid, and terephthalic acid in the hydrolysate. Only 1,4-butanediol could be measured in the treated hydrolysates. Its concentration varied between 0.265% and 0.626%, depending on the pretreatment. The maximum values are equivalent to a conversion rate of 30% of PBAT to 1,4-butanediol. The results obtained by Hobbs et al. (2019) using PLA-based BP were similar.

Table 1.1: Operating conditions of chemical pre-treatments

Test	***Water (ml)***	***pH***	***Temperature (°C)***	***HRT (h)***
NaOH 25%	330	13.5	50	384
NaOH 25%	330	11	50	144
NaOH 25%	330	11.5	50	144
NaOH 25%	330	12	50	144
NaOH 25%	330	12	50	192
NaOH 25%	330	10	50	168
NaOH 25%	330	12.3	50	96
HCl 37%	330	3	25	144
HCl 37%	330	5	25	144

However, in the cited study, the polymer underwent almost complete solubilization (approximately 97%) within 15 days of alkaline pretreatment at 21 °C.

The tests carried out at a temperature of 50 °C may have accelerated the treatment time, breaking down the internal bonds of the polymer more quickly and rendering the internal BPs invisible in just 4 days. Treatments with NaOH proved to be more effective than those with HCl.

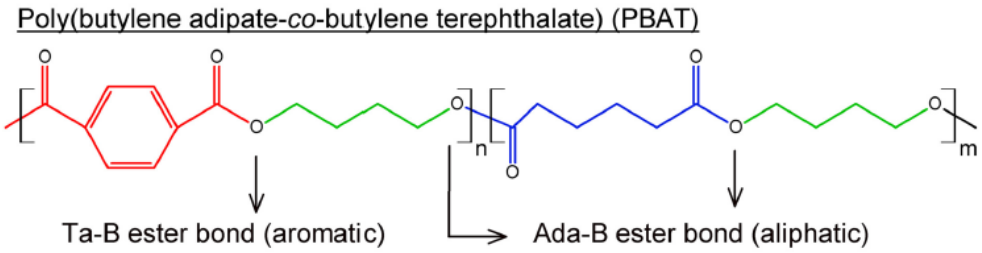
After establishing the optimal pretreatment requirements based on the results obtained by monitoring the reduction in polymer mass, the test phase continued by duplicating the experiment before proceeding with anaerobic digestion. To perform the pretreatment test and anaerobic digestion, 10 ml of 25% NaOH was mixed with 7 grams of BP in water for about one minute at a pH of 12.2, then heated to 50 °C. This test lasted 96 hours (Pellegrino et al., 2023). Subsequently, the addition of 10% v/v of digestate initiated the anaerobic digestion process. This final anaerobic digestion process lasted 16 days. Finally, the BPs were no longer detectable.

1.3.3 Enzymatic pretreatment on a laboratory scale

1.3.3.1 About esterase and amylase

The PBAT molecule (Figure 1.4) is composed of three monomers: adipic acid, terephthalic acid, and 1,4-butanediol. The three monomers are linked together by an ester bond. The most suitable type of enzyme to break that bond is esterase.

Esterase is an enzyme belonging to the hydrolase class, whose function is to catalyze the hydrolysis reaction of specific bonds in the presence of water.



Müller et al. Discovery of polyesterases from mossassociated microorganisms, Appl Environ Microbiol, 2017

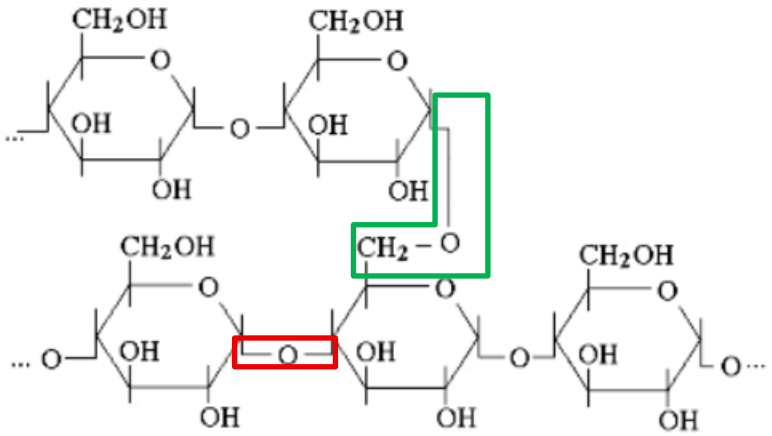
Figure 1.4: PBAT structure

In this case, the breakdown of the ester bond leads to the formation of a carboxylic acid and an alcohol (Prashant et al., 2022):

$$R - CO - O - R' + H_2O \rightleftarrows R - COOH + HO - R'$$

Plants, animals, and microorganisms produce esterases. They are functional in cellular activities. Indeed, they are responsible for the hydrolysis of lipids and complex molecules in general, in which an ester bond is present. The main bacteria that produce esterases, which are also used industrially, include, but are not limited to, *Bacillus, Pseudomonas*, and *Rhodococcus* (Akram et al., 2024). In this work, an esterase from *Bacillus subtilis* was used.

As described above, however, the BP films tested in this work are also composed of 20% starch, which must be degraded and broken down into its building blocks to allow for complete digestion of the product. Starch (Figure 1.5) is composed of long chains of n glucose molecules, linked together linearly without branching via α-1,4-glycosidic bonds (amylose) or branched chains via α-1,6-glycosidic bonds (amylopectin). The enzyme capable of catalyzing the hydrolysis of α-glucosidic bonds is α-amylase, which acts on the polysaccharide bonds of complex sugars, such as starch, to convert them into simpler sugars, including maltose and glucose. α-Amylase can be found in the human body, especially in saliva and blood, but it is also produced by microorganisms such as bacteria. In industry, α-amylase is produced by bacteria of the genus *Bacillus*, such as *Bacillus stearothermophilus*, or *Bacillus licheniformis* and *Bacillus amyloliquefaciens*, used in this study. (Azevedo et al., 2003; Monteiro de Souza & de Oliveira Magalhães, 2010).



Aloisi, Carboidrati: struttura, classificazione e caratteristiche, https://www.biopills.net/carboidrati/, 29/07/2025

Figure 1.5: Structure of starch. In red, α-1,4-glycosidic bond; in green, α-1,6-glycosidic bond

1.3.3.2 Description of tests and results

As mentioned above, Sigma Aldrich supplied amylases from *Bacillus licheniformis* and *Bacillus amyloliquefaciens*, as well as esterases from *Bacillus subtilis*. For the tests, 500 ml Duran bottles were used. In the enzymatic pretreatment, three flasks were prepared for amylase (two to test different concentrations and one as a control), two flasks of esterase with two different amounts of BP, and two more in another setup with amylase from *Bacillus amyloliquefacien*s plus esterase added at two different times so that their synergy during treatment could be studied. A summary of the operational details, as pH and temperature, test duration, buffer used, and test setup, is shown in Table 1.2.

Table 1.2: Summary of the process parameters of the enzymatic tests performed

Enzyme	***Buffer***	***pH***	***Temperature (°C)***
Bacillus licheniformis	citrate	5	50
Bacillus amyloliquefaciens	phosphate	7	50
Bacillus subtilis	phosphate	7.5	30

Each plastic bag was cut into equal parts and weighed. After 30 minutes in an oven at 55 °C, the pieces were left in a dryer for another 30 minutes before being weighed again. Each bottle was filled with a specific amount of BP (Table 1.3). Then, the bottles were placed in a temperature-controlled shakers at 50 °C or 30 °C (Table 1.2).

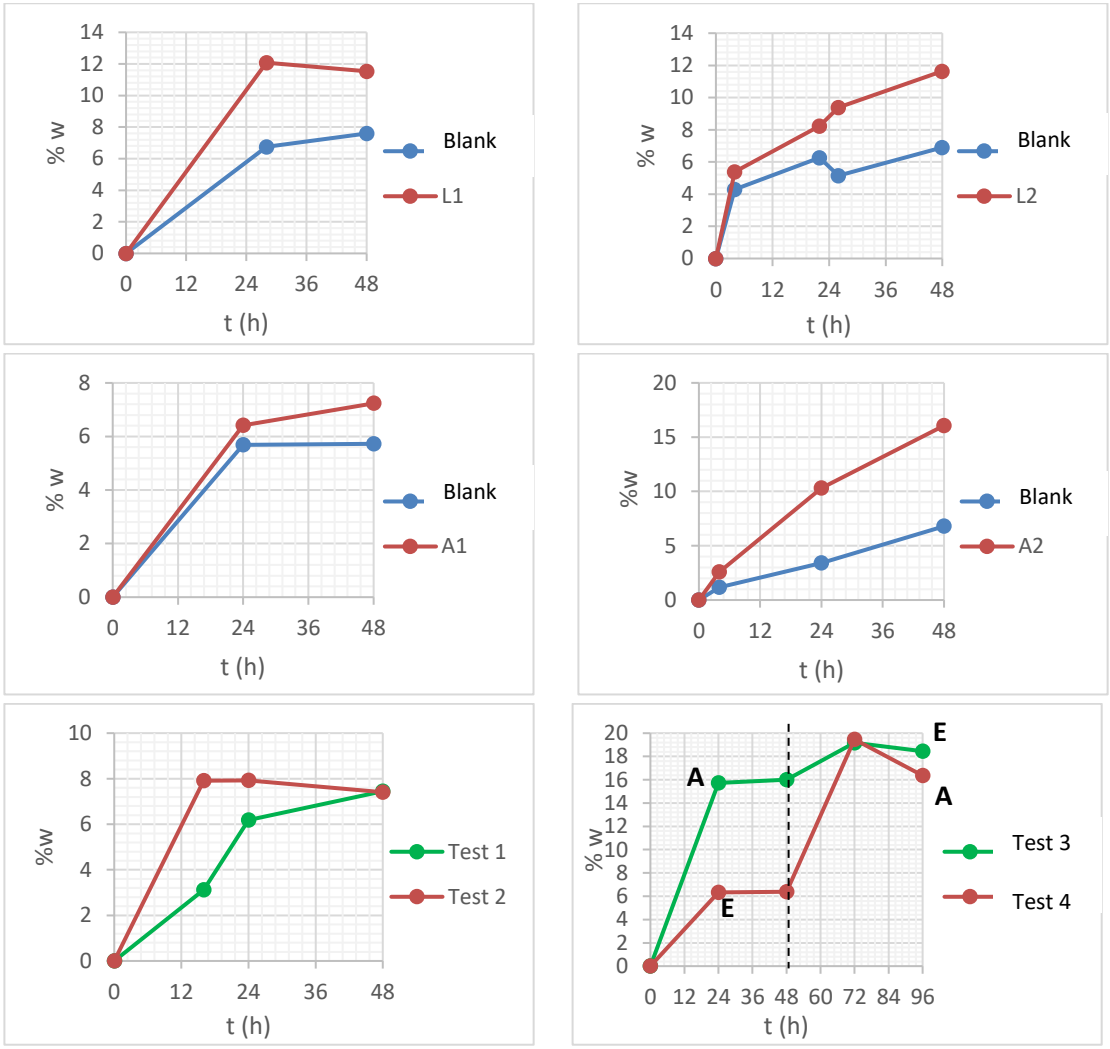
Table 1.3: Tests carried out with the different enzymes studied

Test	***Type of enzyme***	***Enzyme quantity***	***Biopolymer quantity (g)***	***HRT (h)***
L1	Amylase (*B. licheniformis*)	2 mg	7	48
L2		4 mg	7	48
A1	Amylase (*B. amyloliquefaciens)*	20 µl	7	48
A2		40 µl	7	48
Test 1	Esterase (*B. subtilis*)	2.5 mg	3.5	24
Test 2		2.5 mg	1.7	24
Test 3	Amylase + Esterase	20 µl + 2.5 mg	3.5	48 + 48
Test 4	Esterase + Amylase	2.5 mg + 20 µl	3.5	48 + 48

After 24 hours, a piece of BP was extracted from each test bottle and one from the control bottle. After being rinsed with distilled water, the samples were left in an oven at 55 °C overnight and in a dryer for 30 minutes. This procedure was followed every 24 hours and at the end of the experiments. Using the Megazyme KMASUG kit, the presence of sugars was tested and measured in 8 ml of liquid from the test and control bottles during each BP sampling. In tests with both enzymes, esterase and amylase (Test 3 and Test 4), the bottles were shaken at 50 °C for two days and then sampled daily. Finally, the samples were weighed, and the amount of weight lost after drying was determined.

Figure 1.6 shows the weight loss trend of BPs in both bottles containing enzymes and blank bottles without enzymes. Examining *Bacillus licheniformis* (L1 and L2), an increase in the amount of enzymes does not result in a direct reduction in the weight of the BP. Indeed, there is no significant difference between the controls and the tests with enzymes. The weight loss in experiments A1 and A2 (*Bacillus amyloliquefaciens*) is directly proportional to the amount of enzyme. Specifically, 40 µl of amylase can degrade 16% of the BP in just 48 hours. Test 2, with the enzyme *Bacillus subtilis*, conducted to study the breakdown of ester bonds, does not demonstrate clearly efficacy in plastic hydrolysis experiments. Similar to the control tests, this trend is observed. In tests 3 and 4, esterases were studied as enzymes capable of enhancing the effectiveness of amylases, in synergy, by breaking ester bonds. Of the two esterases available, *B. amyloliquefaciens* was chosen as it was more effective than *B. licheniformis*. It was found that alternating the activity of these enzymes, allowing one to act first and then the other and vice versa, did not lead to substantial differences in weight loss. However, the combined results are still unexpected, with a weight loss of just under 20%. The amount of maltose produced was analyzed in the pretreatment experiments. The weight loss results are consistent with the amounts of maltose measured in the hydrolysates (Figure 1.7).

As explained above, amylase acts on starch, breaking it down and releasing maltose and glucose into solution. Starch accounts for 20% of the total weight of the sample (in this case, 1.4g).

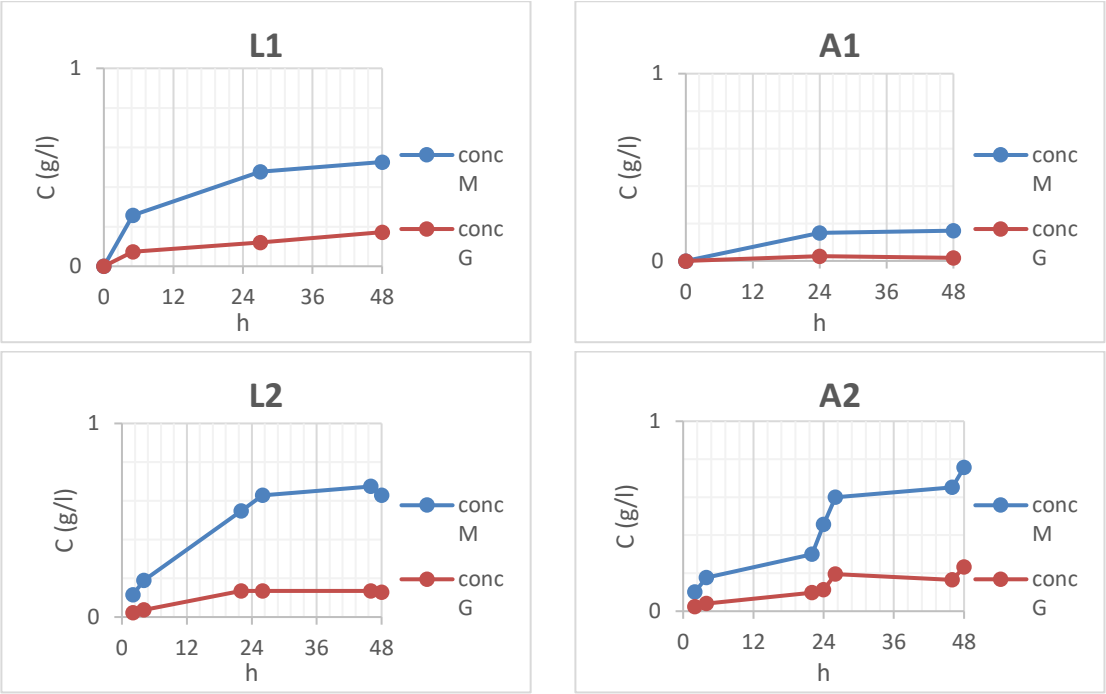


Pellegrino et al., Management and possible valorization of bioplastics separated from organic fraction of municipal solid waste, Environmental Engineering and Management Journa (2023)

Figure 1.6: Weight decreases over time

Taking the best test (A2) as an example, we can see that with a 16% weight loss after 48 hours, a maltose concentration of 0.2 g/g BP is achieved, which is in line with other similar experiments reported in the literature (Mostafa, 2010).

Table 1.4 shows the percentage of starch degraded and the concentration of maltose and glucose obtained after 48 hours of testing with the two amylases.



Pellegrino et al., Management and possible valorization of bioplastics separated from organic fraction of municipal solid waste, Environmental Engineering and Management Journa (2023)

Figure 1.7: Trend of maltose and glucose concentration in the hydrolysate

Table 1.4: Key results of amylase tests

		Maltose	***Glucose***
Sample	***% degraded starch***	***g/g BP***	***g/g BP***
L1	59	0.151	0.049
L2	58	0.186	0.037
A1	36	0.046	0.005
A2	81	0.216	0.066

1.3.4 Anaerobic digestion test on a laboratory scale

After completing tests on the effectiveness of enzymatic pretreatments in the decomposition of BPs, several anaerobic digestion tests were conducted. The aim was to understand the effect of these pretreatments on the kinetics of BPs biodigestion. The tests were carried out at 50 °C with groups of methanogenic bacteria and at 37 °C with groups of Clostridia bacteria, both with and without the addition of organic fraction of wastes (OFMSW), using two specific media, one for clostridia and one for methanogens (Perz et al., 2016; Yagi et al., 2014). For comparison, BPs that had not undergone any pretreatment were used. As with the

pretreatments, weight reduction was estimated as the key indicator of degradation effectiveness. Table 1.5 summarizes the tests performed.

Table 1.5: Summary of anaerobic digestion conditions tested

Test	***Colture***	***pH***	***T (°C)***	***Medium (ml)***	***OFMSW (ml)***	***Water (ml)***	***Digestate (ml)***	***Total volume (ml)***	***Pretreatment***	***HRT (d)***
D1	Clostridia	5	37	337.5	-		37.5	375	-	111
D2	Clostridia	5	37	-	-	337.5	37.5	375	-	111
D3	Methanigenic	7	50	337.5	-		37.5	375	-	76
D4	Methanigenic	7	50	-	-	337.5	37.5	375	-	76
FD1	Clostridia	5	37	37.5	300	-	37.5	375	-	111
FD1*	Clostridia	5	37	37.5	300	-	37.5	375	A1 o L1	111
FD2*	Methanigenic	7	50	37.5	300	-	37.5	375	A1 o L1	56
FD4*	Methanigenic	7	50	37.5	250	-	87.5	375	A2 o L2	86
FD5*	Clostridia	5	37	37.5	250	-	87.5	375	A2 o L2	86
FD6*	Clostridia	5	37	37.5	300	-	37.5	375	Prova 1	49
FD7*	Clostridia	5	37	37.5	300	-	37.5	375	Prova 3	49
FD8*	Methanigenic	7	50	37.5	300	-	37.5	375	Prova 2	49
FD9*	Methanigenic	7	50	37.5	300	-	37.5	375	Prova 4	49

The untreated BP fractions were placed in an oven at 55 °C for 30 minutes and then in a dryer for another 30 minutes. After drying, the BPs were weighed. In the bottle test without pretreatment, 0.6 g of BP was added per bottle.

In the pretreatment test, the amount of BP remaining after the enzymes' action was used. Fresh organic waste originating from household waste and consisting exclusively of fruit and vegetable peelings, was shredded. Culture media for Clostridia and methanogenic bacteria were prepared according to the quantities shown in Table 1.6.

Table 1.6: Medium for Clostridia and methanogenic bacteria

Reagent	***Medium Clostridia (g)***	***Medium methanigenic (g)***
Distillate water	675	675
$NaHCO_3$	0.324	3.375
NH_4Cl	0.206	0.675
KH_2PO_4	0.102	0.202
$FeCl_3$ 6H2O	0.007	-
Yeast extract	0.021	-
NaCl	-	0.405
Glucose	-	13.5

The anaerobic digestion tests were performed in 500 ml Duran bottles, each filled to a total volume of 375 ml (Pellegrino et al., 2023). Under an extractor hood, each bottle was saturated with nitrogen for a quarter of an hour, creating an oxygen-

free environment. Finally, the bottles were sealed with an airtight cap. The bottles were then placed in a thermostatic bath at 37°C or on an orbital shaker set at 50°C, depending on the requirements of the two different bacterial cultures.

At each sampling, the pH was noted to monitor changes and understand the current stage of anaerobic digestion, and the previous anaerobic conditions were immediately restored. Once washed, the BP samples were placed in an oven at 55 °C for two to three hours, or overnight if they had undergone enzymatic treatment, to allow them to dry completely; they were then transferred to a dryer for half an hour. Once dry, the samples were weighed to quantify weight loss over time.

Tests with methanogenic bacteria show that enzyme pretreatment helps decompose BPs during anaerobic digestion. Enzymes such as amylases or esterases break down polymers into smaller, more easily utilized components by microorganisms in the digestate (Abraham et al., 2021). After fermentation, plastics that were pretreated and digested with methanogens were no longer detectable. For plastics without enzymatic pretreatment, the maximum weight reduction detected was 70%. This reduction is still notable compared to other studies (Abraham et al., 2021). However, plastics digested anaerobically with clostridia bacteria lost negligible weight compared to the start of the test.

The degradation rate may vary depending on factors related to different digestion plants, such as the microorganisms present or the characteristics of the digestate. It will be necessary to investigate these factors to make the method more reproducible on an industrial scale. In addition, tests with esterase pretreatment (whose initial effect appeared to be minimal) showed an acceleration of degradation in subsequent tests with methanogens. After 49 days, a time similar to many industrial processes (Ruggero et al., 2020), the plastic had completely dissolved, a result achieved with other enzymes after 56 and 86 days.

1.3.5 Enzymatic pretreatment on a pilot scale and anaerobic digestion

The enzymatic treatment of BPs on a pilot scale was carried out in a 7 l pilot reactor (LAMBDA Minifor Fermenter), as also described in the work of Pellegrino et al. (2023).

The BP bags were cut into 10 x 10 cm squares, weighed (with an initial weight of 0.21 g each), and subjected to enzymatic pretreatment to facilitate disintegration during anaerobic digestion experiments by methanogenic bacteria. The 7 l reactor was filled with 35 g of cut bags (approximately 166 squares), 3.3 l of phosphate buffer (100 mM, pH 7), 200 µl of α-amylase from *Bacillus amyloliquefaciens*, and 25 mg of esterase from *Bacillus subtilis*, following the protocol developed after the laboratory-scale enzymatic pretreatment tests described in section 1.3.4. The layout of the reactor for enzymatic pretreatment is shown in Figure 1.8.

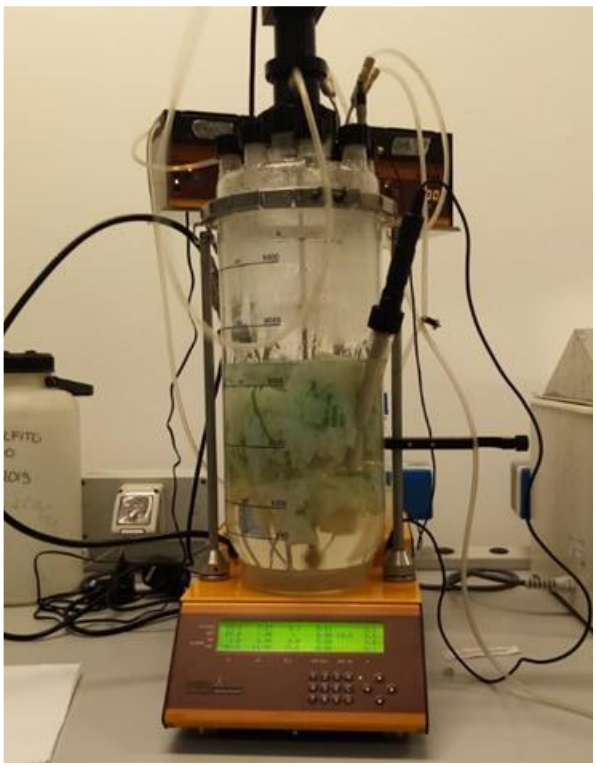


Figure 1.8: Reactor layout for enzymatic pretreatment

The reactor was kept agitated at a constant temperature of 50 °C for 48 hours. BP samples were taken from the reactor at 18, 24, 36, and 48 hours to assess weight variation (Pellegrino et al., 2023).

After 48 hours of enzymatic treatment, the reactor was emptied. The BP was reintroduced into the reactor, and the buffer was discarded. The OFMSW, consisting mainly of fruit and vegetable waste, was shredded and slightly diluted with water. To the reactor already containing the pretreated BPs, for a total volume of approximately 4 l, were added:

- 3 l of OFMSW
- 375 ml of digestate containing methanogenic bacteria
- 375 ml of the medium (recipe indicated in Table 1.6).

Finally, a temperature of 50 °C and continuous agitation were set. To recreate anaerobic conditions and stimulate initial agitation of the mixture, nitrogen was introduced into the reactor from the bottom for approximately one hour. The layout of the reactor for anaerobic digestion is shown in Figure 1.9.

Anaerobic digestion test samples were collected 30 and 55 days after the start of the test and sent to a qualified laboratory to measure the weight change and evaluate the decomposition of PBAT into its three monomers: adipic acid, terephthalic acid, and 1,4-butanediol.

Figure 1.9: Reactor layout for anaerobic digestion

The data reported in Table 1.7 reveal that enzymatic pretreatments reduced the weight of BPs by 27%, in line with the results of laboratory-scale experiments, as also highlighted by Pellegrino et al. Table 1.7 also shows that most of the degradation occurs within the first 24 hours, resulting in a 24% reduction in weight.

Table 1.7: Weight variation during BP enzymatic treatments

Pretreatment duration	***Dry weight (g)***	***Weight loss (%)***
18 h	0.2049	2.4
24 h	0.1595	24.0
36 h	0.1553	26.0
48 h	0.1530	27.1

With a view to optimizing the process, therefore, an operating protocol lasting 24 hours could be developed, rather than 48 hours.

In the following anaerobic digestion, after 10 days of fermentation, the digestate showed a significantly hydrolization, while the BPs appeared shrivelled, indicating that the hydrolysis phase had begun. Another study reported similar results after 30 days of batch anaerobic digestion (Kosheleva et al., 2023).

Infrared spectrometric examination revealed that 100% of the microparticles present were PBAT microplastics (Figure 1.10).

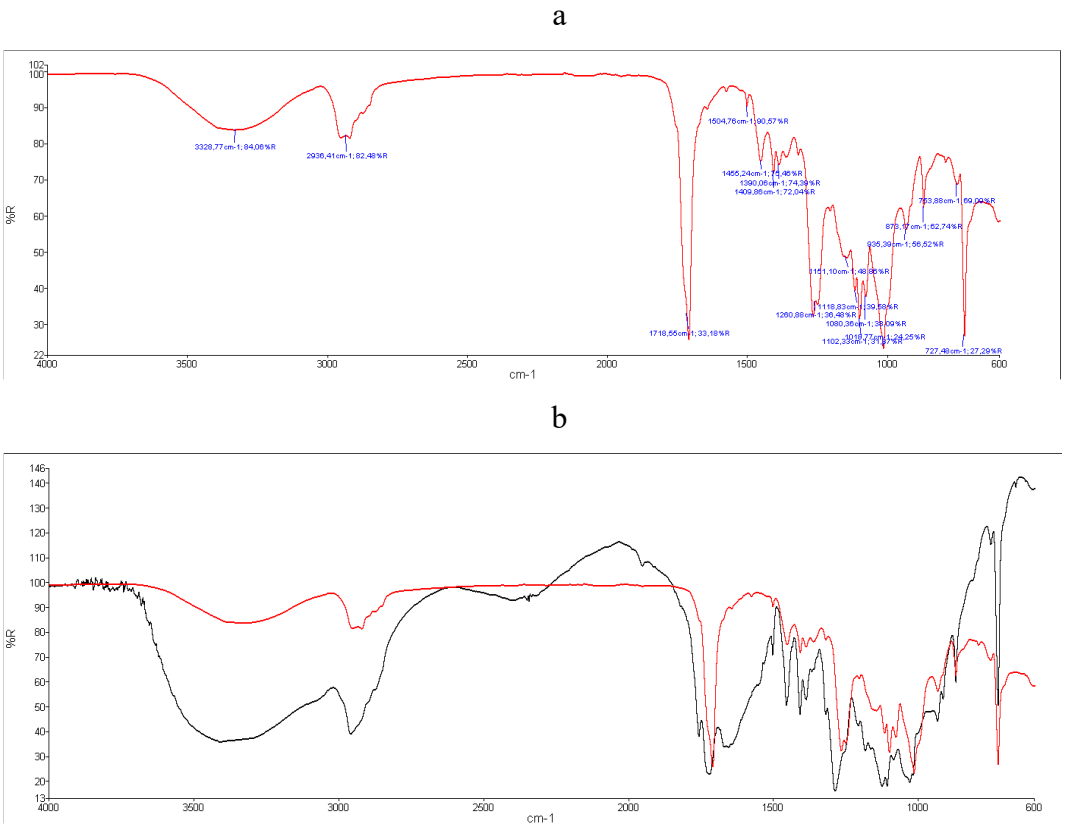


Figure 1.10: IR spectra of a) PBAT film sample, b) BP sample in the reactor

In all samples, the IR spectra show the following absorption bands:

- At approximately 3360 cm^{-1}
- At approximately 2945 cm^{-1}
- At approximately 1760 and 1730 cm^{-1}
- At approximately 1505 cm^{-1}
- At approximately 1450, 1410, and 1390 cm^{-1}
- At approximately 1290, 870, and 730 cm^{-1}

Therefore, for all samples analyzed, the microparticles are composed of the same material. When compared with the IR spectrum of the original plastic film (PBAT), a close similarity can be observed in the principal absorption bands. In the microparticle spectrum, the most pronounced band at 3400 cm^{-1}, together with the

band around 1660 cm^{-1}, could be derived from residual water content after filtration. The differences between 1000 and 1300 cm^{-1}, on the other hand, are less pronounced and could result from the partial breakage of some bonds in the PBAT film, which also causes the carbonyl band to broaden to around 1700 cm^{-1}. In essence, the microparticles found are PBAT microplastics.

The test results are presented in Table 1.8.

Table 1.8: BPs degradation rate and concentration of adipic acid, terephthalic acid, and 1,4-butanediol

	After 30 days	***After 55 days***
Degradation (%)	67	97
Adipic acid (mg/l)	130	167
Terephthalic acid (mg/l)	13.6	21.3
1,4-butanediol (mg/l)	33.8	76.6

The concentrations of adipic acid, terephthalic acid, and 1,4-butanediol were provided by LabAnalysis and obtained according to the methodology described in section 1.3.1 of this thesis. The percentage of degradation was calculated based on sample weight loss, also provided by LabAnalysis. Considering an initial concentration of 8750 mg/l of BP in the reactor, after enzymatic pretreatment, a weight loss of 27.1% resulted in a concentration of 6379 mg/l of BP.

After 30 days of anaerobic digestion, the weight of the BP sample decreased by 67%, from 6379 mg/l to 2106 mg/l, as it degraded into its three monomers, most of which were adipic acid. Based on the results obtained and comparing the results of chemical and enzymatic pretreatment, it was found that enzymes are more effective in breaking the internal bonds of the polymer, allowing the release of all three compounds, albeit in different ways, thus promoting the digestion of PBAT by methanogenic bacteria.

Conversely, chemical pretreatment resulted in the complete dissolution and depolymerisation of the sample, with the release of 1,4-butanediol into the solution. This could be used directly as a raw material for preparing new polymers.

1.4 Ecotoxicity of PBAT and its building blocks

Industrially, once the anaerobic digestion process is complete, the fermentation broth is sent for sludge treatment, where any non-degradable pollutants remaining in the mixture are separated from the digestate, which is then treated to become compost. There is no consensus among scientists on the potential impact of PBAT, or its possible residues, on the environment, due to a lack of scientific evidence.

In his study, Serrano-Ruiz et al. (2023) compared the effect of six different blends of PBAT and other polymers or starches on the growth rate of stems and

leaves, and the chlorophyll and proline content of tomatoes and lettuce. The experiment was conducted in the controlled environment of a greenhouse and using a negative control, i.e., a jar without bioplastics. The results showed that all PBAT films delayed the growth of both plant species to some extent, also having adverse effects on the "aesthetic" appearance of the plants themselves and the concentration of chlorophyll and proline. A similar study, (Liu et al., 2022) investigated the impact on the growth of the *Arabidopsis* plant's stems and leaves, as well as on its root microbiome, caused by both the PBAT polymer and its building blocks: adipic acid, terephthalic acid, and 1,4-butanediol. Again, as the concentration of PBAT increased from 0.25% to 1%, the growth of *Arabidopsis* decreased considerably compared to the control sample, and the situation worsened when the building blocks were examined. The worst appears to be terephthalic acid at a concentration of 1%, which did not allow even a single sprout to grow after 5 weeks of cultivation. Yu et al. (2023), on the other hand, analyze the impact of PBAT on the roots of soybean and maize plants. Once again, the effect is strongly adverse: the total length of the roots, their surface area, and their biomass decrease by up to 58%, 54%, and 40% for soybeans and 71%, 71%, and 64% for maize, respectively, compared to the control sample based on PBAT concentrations (from a minimum of 0.1% to a maximum of 1%). For Martínez et al. (2024) the reason for this toxicity lies in its ability to transfer electrons. Some products of PBAT biodegradation, such as 1,4-butanediol and adipic acid, are good electron donors, while terephthalic acid and the polymers involving it, and therefore PBAT itself, are good electron acceptors. Electron acceptors steal them from other molecules, oxidizing them and increasing the oxidative stress of the plant itself.

However, if we consider a slightly broader approach, such as that used in Sforzini et al. (2016), the results differ. Ecotoxicological tests were conducted on bacteria and protozoa, as well as on the green algae *Pseudokirchneriella subcapitata*, the plants *Sorghum saccharatum* and *Lepidium sativum*, and on certain invertebrates that had grown in soil or soil eluates containing 1% bioplastic bags that had been left to degrade for six months in the soil itself under controlled conditions. Specific standard reference protocols were followed for each test, and different parameters were evaluated according to the type of organism, such as inhibition of bioluminescence in the case of bacteria, germination rate in the case of plants, and mortality and reproduction rate in the case of invertebrates. In all tests, no toxic effects due to the bioplastic presence were found compared to the control samples. Liu et al., (2025) also tested, including germination rate and morphology of the *Arabidopsis thaliana* plant, subjected to the effects of PBAT metabolites at different concentrations, aimed at simulating the possible accumulation of these substances in the soil over 1 to 10 years. The concentration of intermediates corresponding to the accumulation over 10 years showed a strong inhibitory effect on plant root growth. An accumulation equal to 5 years can potentially inhibit root length but has no significant effect on leaf growth and plant morphology. The accumulation that did not show any adverse effects occurred after 2 years, equivalent to 76 mg/l of degraded PBAT film. Regarding germination, after 48 hours, no significant decrease was observed with either the reaction

intermediates or PBAT itself in any of the accumulation cases tested, compared to the control. In all cases, the germination rate remained in the range of 80% to 100%. By increasing the concentration by just one order of magnitude, as done in Macan et al. (2024) study, the maximum germination rate of *Arabidopsis thaliana* remains quite high, averaging above 80% compared to the control carried out with water. In the same article, the effects of PBAT building blocks, released after 7 days, were evaluated, initially with a very low concentration, in the order of µg/l for adipic acid and terephthalic acid, and in the order of mg/l for 1,4-butanediol. In this case, the maximum germination rate reached 100%. However, if this concentration is multiplied by 1000, the maximum germination rate drops to around 25%.

Souza et al. (2020), on the other hand, used the aqueous extract of soils containing both pure and 'aged' PBAT (post-biodegradation test) with a very low concentration of 0.2% by mass on *Lactuca sativa* and evaluated the germination rate, root length, and hypocotyl length, comparing the results with those obtained from PBAT-free soil. In the study, two additional controls were used in addition to PBAT-free soil alone: a negative toxicity control consisting only of ultrapure water and a positive toxicity control consisting of a zinc sulfate solution. The results showed that the samples containing PBAT showed increases in germination and root and hypocotyl growth very similar to those of only soil and very different from the positive controls. This suggests that PBAT has no positive effects on the study targets, but at least not negative effects either. We can speak of a null effect.

From this brief bibliographic research on the effects of PBAT and its building blocks, it is clear why there is no single answer to the dilemma of the ecotoxicity of this polymer and its monomers. There are many variables at play: the species tested, the amount of PBAT used, and the different parameters studied. Martin-Closas et al. (2014) emphasize the importance of considering each case on its own merits. Indeed, in his study, the results obtained with tests on lettuce and tomatoes were very similar but not identical, indicating that each species has a different sensitivity. His tests aimed to evaluate the effects on germination and dry weight of roots and shoots grown in vitro with concentrations of 5, 50, and 500 mg/l of adipic acid or butanediol. The results showed that germination was successful with all treatments except for 500 mg/l adipic acid, where only 73% of the seeds germinated. As for tomatoes, root and shoot growth was strongly negatively affected by concentrations of 50 and 500 mg/l of adipic acid. It was positively impacted by the presence of 5 mg/l of adipic acid, probably, according to the authors, because this provided acetyl-CoA to the plant metabolism. This increase in roots and shoots, however, was not noticed in lettuce. As regards the use of butanediol, while no concentration tested was found to be toxic for tomatoes, and indeed 500 mg/l of butanediol appeared to increase tomato root growth, in the case of lettuce, only 500 mg/l was a positive concentration for the roots, with the other two inhibiting growth or having almost no effect. These latest results confirm that the sensitivity of the tested species is a parameter to be considered for final assessments.

1.5 Conclusion

Anaerobic digestion appears to be a very effective method for degradation of BP-based waste if it is appropriately sized, managed and complemented. In particular, chemical or enzymatic pretreatment enhances the process efficiency.

The results show a degradation efficiency of up to 30% after 96 hours of alkaline treatment and approximately 20% after 72 hours of biological processing. The pilot test results validated the degradation capabilities of the enzymes and supported the concept of possible industrial use: the effects were evident after only 24 hours of treatment when all enzymes were added simultaneously.

An anaerobic digestion test was then performed, with a degradation efficiency of 67% after 30 days of digestion. Studies on the same polymer gave fewer positive results, with degradation rates reaching 15% after 55 days at 50 °C (Abraham et al., 2021). These results are very encouraging and prove that the retention period in industrial anaerobic digestion plants can be compatible with a high degradation rate of bioplastics digested together with OFMSW, provided that enzymatic pretreatment is performed. Enzymatic pretreatment before anaerobic digestion, followed by aerobic digestion, should ensure the complete degradation of bioplastics.

These results pave the way for further testing to confirm and improve the conclusions reached and described in detail in this research, as well as for the development of complete and efficient industrial supply chains dedicated to the valorization of bioplastics. Comparing the two pretreatments, chemical and enzymatic, both processes could be integrated into existing anaerobic digestion plants. Chemical treatments can be incorporated through a pH control system. Enzymatic treatments require dosing the input quantity based on the bioplastic content, which can be adjusted according to the plant's specific needs. Moving from laboratory to pilot scale, enzymes appear more effective at degrading bioplastics. If adopted industrially, the high cost of enzymes must be considered. This would require finding a method to recycle, recirculate, or immobilize them. Each option presents challenges, such as loss of enzyme activity, accumulation of solid residues, or additional costs for these activities.

As regards the ecotoxicity of PBAT and its building blocks, the situation is not entirely clear, and further studies are needed to understand whether it is necessary to add a separation step for the presumed pollutants or whether the issue can be resolved by keeping the concentrations of the polymer and its monomers under controlled levels.

Chapter 2

Use of hydrothermal carbonization products from plastic waste in organic matter in the anaerobic digestion process

2.1 Microplastics: they are among us (and we often do not know it)

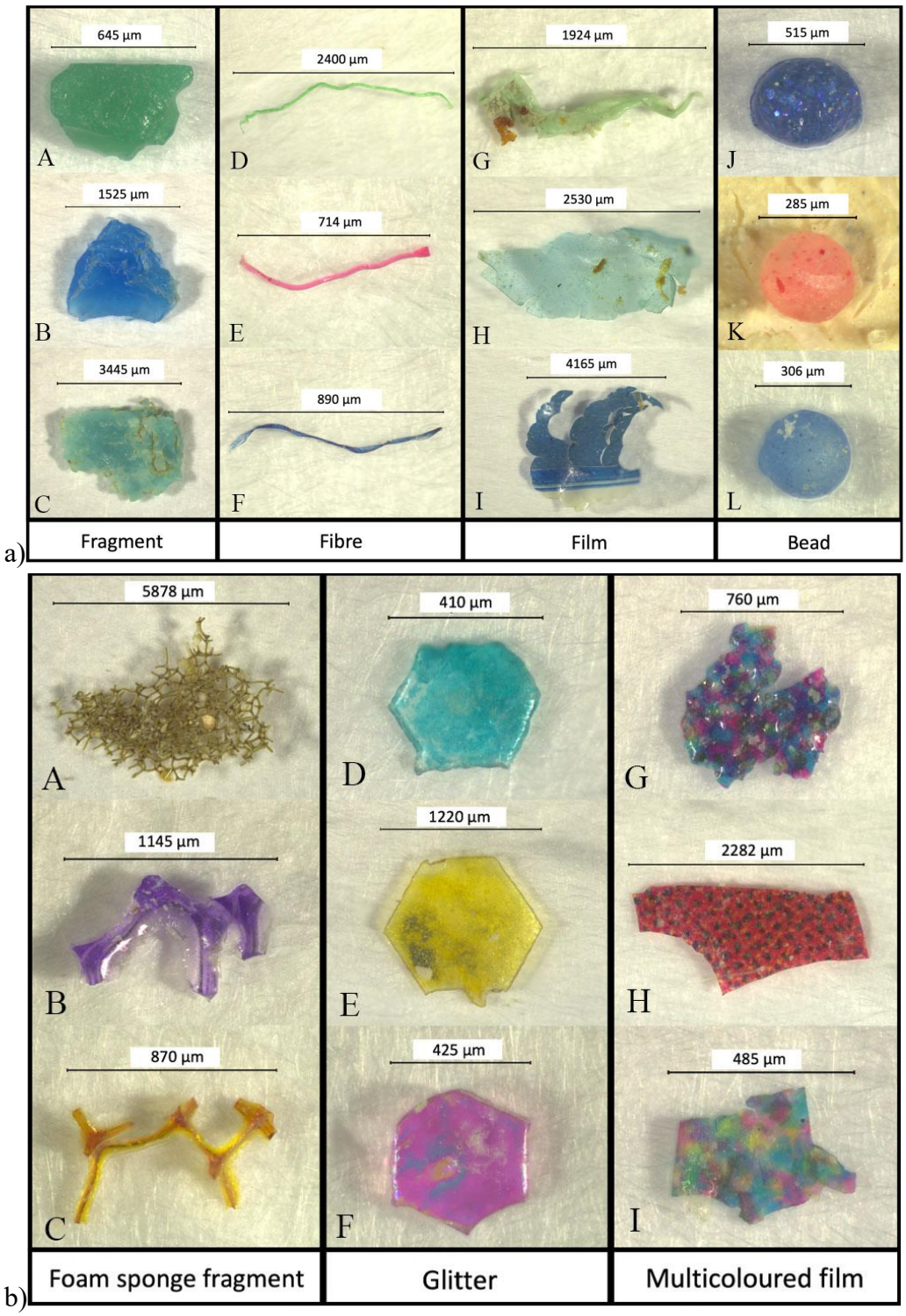
The spread of plastic products since the 1950s has made us dependent on this super-convenient and versatile material: lightweight yet strong, solid yet easily moldable, suitable for contact with food and chemicals, and even for creating textile blends. There is a plastic polymer for most industrial and everyday applications, and it's hard to imagine a world without plastic today, given how ubiquitous it is.

Several studies have detected the presence of microplastics in the most unimaginable places: from Arctic glaciers (Peeken et al., 2018) to Everest (Napper & Elmore, 2020) to the Mariana Trench (Jamieson et al., 2019); in all animals, both wild and domestic (Bahrani et al., 2024); in table salt (Karami et al., 2017); and inevitably in the human body, particularly in the blood, placenta, and lungs (Leslie et al., 2022; Ragusa et al., 2021).

Although this study will analyze how to valorize the macroscopic component of non-biodegradable plastics within the organic fraction of municipal solid waste, many of the articles referred to deal with microplastics. This is because there are no readily available data on the percentage of plastic contamination, broken down by type of plastic, present in OFMSW. The Italian Composting Consortium (CIC e Biorepack, 2024) reports that 7.1% of organic municipal waste is non-compostable, which means that 21.9% of the waste arriving at treatment plants is discarded because it is contaminated with material that is not suitable for anaerobic digestion

and/or composting. Indeed, it is not possible to effectively eliminate all pollutants without also eliminating a percentage of organic material during operations, which complies with specifications when taken individually but remains adhered to the pollutants themselves. However, this data is cumulative and represents the percentage of impurities that includes plastics, glass, and metals (CIC e Biorepack, 2024). The idea of finding plastic in organic waste may seem improbable. However, there are many ways in which this can happen, not only due to errors or misinformation about separate waste collection. For example, often all unsold food from supermarkets arrives at the treatment plant still wrapped in plastic packaging (Öling-Wärnå et al., 2023).

Then again, more data can be found by analyzing the products obtained after anaerobic digestion and composting. A study on compost produced by a plant in Germany showed that one ton of compost contains between 7000 and 440000 microplastic particles (K-Online, 2021). Another study on field compost carried out in Geneva shows that the presence of microplastics ranges from 194 to 1315 particles/kg of compost depending on the size analyzed (between 1.25 mm and 5 mm and above) (Berset & Stoll, 2024). The authors of the study report similar work in other articles, as does Portefriield et al. (2022). The values are all different because there are many factors that contribute to the presence of these pollutants: social factors, screening methods upstream of digestion and composting operations, and the primary substrate of these operations (agricultural waste, urban waste, selected waste, etc.). The type and composition of these residues also vary from one study to another. Ruffel et al. (2025) analyzes the types of microplastics found in four types of compost: biosolids (solid organic material derived from wastewater treatment), vermicompost (natural organic soil conditioner obtained through the transformation of organic matter by earthworms), bulk compost, and bagged compost. Microplastics were found in all sample types, with a concentration ranging from 1.1 to 2.71 microplastics/g of compost. Classification showed that plastic fragments were the most abundant type (62.7%), followed by 24.7% film, 12.2% fibers, and 0.4% beads (Figure 2.1a). The fragments in question were mainly pieces of sponge, glitter, and multicolored pieces (Figure 2.1b). In terms of composition, they consisted primarily of polypropylene (29.3%), polyethylene (26.3%), and polymethyl methacrylate (16.2%). The remainder was composed of polyethylene terephthalate and polyurethane. Again, a review of similar articles by the same author revealed a complex picture: no single polymer stands out in terms of frequency of occurrence precisely because, as mentioned above, there are so many variables. It is worth reflecting, however, that if microplastics are present in such high quantities downstream of anaerobic digestion and composting processes, this means that plastic is also present upstream. It is no coincidence that, as described in the first chapter, after the bags are broken, an initial screening step is performed to remove light pollutants (such as plastic films), followed by a deferrization phase and finally a second screening to remove other heavy components (such as metals or other plastics).



Ruffell et al. Quantification of microplastics in biowastes including biosolids, compost, and vermicompost destined for land application Water Emerg. Contam. Nanoplastics 2025

Figure 2.1: a) Examples of microplastic morphotype classifications of microplastics in biowaste samples. Fragments (A-C), fibers (D-F), b) Examples of microplastic foam sponge fragments (A-C), glitter (D-F), and multicolored films (G-I) in biowaste samples

The principal screening methods are (dePackaging Equipment, 2025; Recycling Today, 2025)

- Manual: Operators manually remove all macroscopic contaminants. This procedure must be performed in combination with other methods that require machinery to be as efficient as possible. The disadvantage is the hygiene conditions of the operators, who work in filthy conditions and risk inhaling plastic substances or substances that are generally harmful to health.
- Mechanical screening: Screens with holes of different sizes (from 3 to 100 mm) and with various shapes and functions are used to separate pollutants with jets of air, based on density, or based on size with rotating drums or star screens.
- Physical separators: Hydrocleaners, which use buoyancy to separate light plastics from the rest, which sink to the bottom, or sand removal systems, designed to remove heavy particles.
- Optical separators: The waste mix is transported by a belt inside a dark chamber where, through an optical system of filters and lenses, monochromatic light at a specific wavelength is focused on the waste group. Based on the sample's response in terms of reflectance intensity, the instrument identifies the type of waste (plastic, paper, metals, etc.) and generates an output (compressed air jet, mechanical arm that activates, etc.) to physically convey the specific waste to the waste collection group with the same characteristics. In the study on plastic and organic waste identification, an optical separator based on a multispectral imaging system was used to separate impurities present in OFMSW. The study showed that the optimal wavelength, equal to 710 nm and corresponding to infrared radiation, allows for differences in reflectance not only between macro types of waste, enabling them to be separated, but also between the main types of plastics analyzed: HDPE, PET (with and without labels), transparent and colored PP.
- Unpackers: Integrated systems in which a single solution breaks the bags, either by cutting or hammering in the case of more rigid packaging and separates organic waste from lighter waste using a vortex separator.

Furthermore, recovered plastic is not always compatible with recycling methods: for example, some plastic polymers are not recyclable (see PMMA), and contamination of plastic with other waste is an obstacle to its recyclability (Dakota Academy, 2024). Therefore, the problem of plastic in organic waste needs to be managed alongside the complexity of the degradation of biodegradable plastics in anaerobic digestion plants designed before biopolymers were introduced to the market.

The fate of non-biodegradable plastic is to end up in waste-to-energy plants, as Solid Recovered Fuel (SRF) or in landfills. Although the first two solutions seem far removed from the concept of a circular economy, they are complementary

alternatives to mechanical or chemical recycling, and not in competition with them, as they valorize a plastic component that cannot be recycled due to reasons related to cleanliness, composition, available technologies, and market requirements. Furthermore, the thermochemical conversion of plastics reduces the demand for virgin fossil fuels, contributing to the achievement of the target set for the transport sector (Ciotti et al., 2021). To effectively close the loop on waste recovery, the techniques employed must be highly efficient, safe, and environmentally sustainable. This is not always possible, as the combustion of plastic leads to the formation of dioxins and therefore temperatures above 850 °C are required to prevent their formation. In the next section, an alternative method for the thermal recovery of plastics will be evaluated, using the hydrothermal carbonization process.

2.2 Hydrothermal carbonization process

Hydrothermal carbonization (HTC) is a thermochemical conversion process in which wet biomass or biomass in aqueous suspension is treated at a temperature between 180-250 °C, a pressure of 2-6 MPa, and a residence time between 30 minutes and 8 hours (Gómez et al., 2020; Sivaprasad et al., 2021). Waste biomass, which can be of any type, from lignocellulosic biomass to agro-industrial residues and plastics, is converted into three fractions:

- a solid carbonaceous product, called hydrochar, which can be used as a secondary solid fuel, soil conditioner, or pollutant adsorbent such as activated carbon;
- a liquid fraction, resulting from the massive use of water in the process, containing both organic and inorganic compounds;
- a small amount of gas of low commercial interest, composed mainly of CO_2.

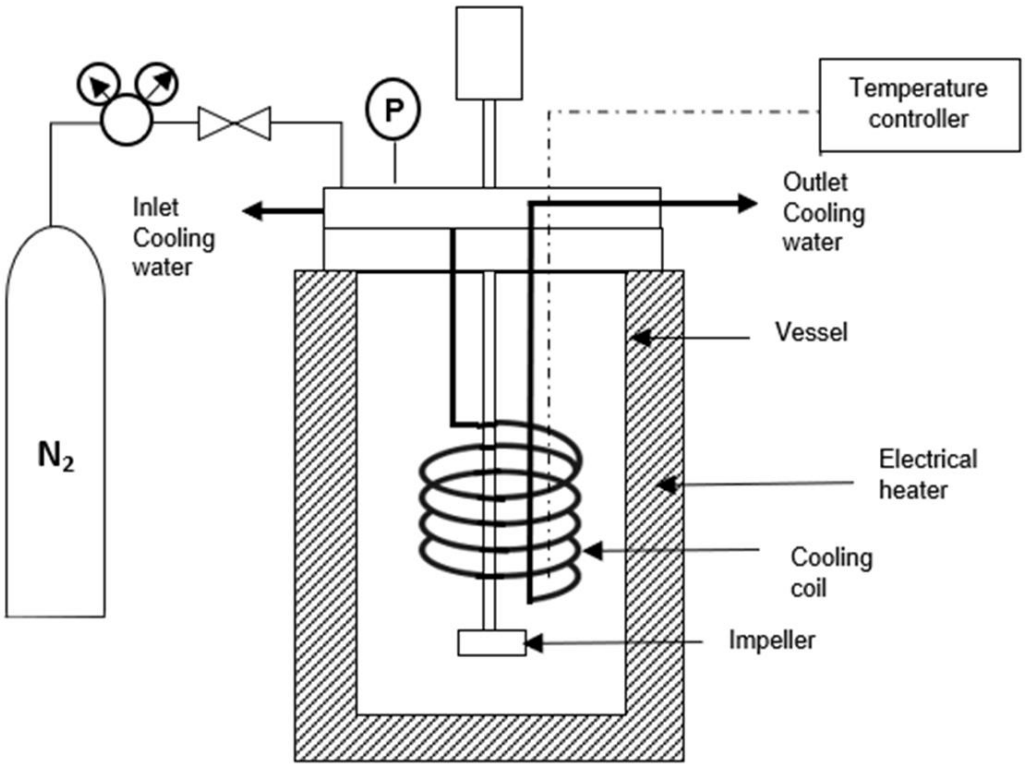
High-temperature, high-pressure water is an excellent reaction medium, as it allows the internal bonds of biomass to be broken efficiently and in an environmentally sustainable manner, without the need for additional solvents. Furthermore, since water is already present in the process conditions, HTC can be used with any wet biomass without the need for prior drying, unlike pyrolysis and gasification processes.

The process occurs as follows:

- The reactor is filled with biomass in aqueous suspension
- A stirring system (with rotating blades, impeller, or axial shaft) keeps the suspension in continuous motion
- The system is gently pressurized by injecting nitrogen

- A heating system (jacketed or electric) raises the temperature (and consequently the pressure) of the process to the desired level
- These process conditions are maintained for the necessary time
- A cooling system brings the temperature back to ambient conditions
- The treated biomass is sent to a filtration system, and the recovered biochar is dried.

An example of a reactor for HTC is shown in Figure 2.2.



Roslan et al., Hydrothermal Carbonization of Sewage Sludge into Solid Biofuel: Influences of Process Conditions on the Energetic Properties of Hydrochar, Energies, 2023

Figure 2.2: HTC reactor

As mentioned earlier, this process can also be used with plastic waste. An industrial example is reported in the article of Shekoohiyan et al. (2025), where, however, a slightly different process is described. In this specific case, the biomass was not in an aqueous solution. Water was introduced into the reactor in the form of superheated saturated steam, which quickly brought temperature and pressure to the desired conditions.

The hydrochar produced from plastic waste seems to be more advantageous from a thermal viewpoint compared to that produced from another biomass. Indeed, due to the high energy density of some plastic polymers, this type of hydrochar

could have a higher heating value. Furthermore, plastics generally have less moisture, which is beneficial for combustion efficiency (Shekoohiyan et al., 2025). Moving away from the energy aspect, the use of char could also be interesting in the context of pollutant absorption, thanks to its spherical and porous structure and its high specific surface area. Furthermore, thanks to its ability to retain inhibitors, hydrocarbon has been shown in the literature to have positive effects on methane production during fermentation. Solid residues derived from HTC can be considered an economical adsorbent capable of increasing methane production in anaerobic digestion. Thanks to its porous structure, coal can absorb harmful and resistant substances, reducing their inhibitory effect. A desorption effect caused by the difference in concentration between the adsorbent and the liquid medium is also visible. As a result, the adsorbents can act as buffers, gradually releasing the effluent chemicals.

A disadvantage of using the HTC is the massive use of water in the process, which results in a liquid effluent containing organic, inorganic, and pollutant substances, often underutilized (Shekoohiyan et al., 2025; Sivaprasad et al., 2021**).**

This chapter will investigate the valorization of hydrochar and the liquid fraction of the HTC process carried out on reject fractions resulting from treatment of organic fraction of urban waste, mainly composed of non-recyclable plastics, solid fraction of digestate and scraps from compost production.

2.3 Substances that inhibit anaerobic digestion

The following paragraphs describe the anaerobic digestion tests aimed at valorizing the solid and liquid fractions of the HTC process of non-recyclable plastics from municipal solid waste.

Anaerobic digestion, as mentioned in the previous chapter, is a complex biological process. To clarify the experimental part of this chapter, it is necessary to examine anaerobic digestion in greater detail, including its biological aspects. At the plant level, the process takes place inside a digester (or bioreactor or anaerobic reactor). Pressure, temperature, pH, and nutrient content must meet the specific requirements of individual microbial groups to allow all microorganisms involved to thrive simultaneously. Biogas produced by anaerobic fermentation is the result of a series of processes in which, in the first stage, macromolecules are broken down into their constituent monomers. These are transformed into short-chain acids through a process called acidogenesis. This step is followed by acetogenesis, which produces acetic acid and a small amount of H_2. The final stage, methanogenesis, consumes these compounds to generate CO_2 and CH_4. The methanogenesis stage, therefore, consumes the hydrogen and fatty acids produced in the previous stages.

The bacteria involved in the different stages may be sensitive to certain unusual substances called inhibitors. A material can be considered an inhibitor if it interferes with bacterial development or alters the microbial population. For example,

furfuraldehydes are considered standard inhibitors as they are slightly more dangerous to microbial metabolism. From a microbial viewpoint, however, they are considered metabolic inhibitors, and 5-HMF is considered the hexose equivalent of furfural. According to Dias et al. (2021), a concentration higher than 1 g/l of furfurals or HMF inhibits methanogenesis, prolonging the lag phase. As part of the detoxification process, furans are converted into their respective alcohols, to which bacteria are usually less sensitive. Acetaldehyde also has an inhibitory effect on bacteria involved in anaerobic digestion, acting on the breakdown of single and double DNA strands at concentrations of 1.56 mM and 100 mM, respectively (Jilani & Olson, 2023).

Chen, Cheng and Creamer (2008) in their paper, tested toxic levels of ammonia for bacterial consortia typically found in anaerobic digesters. Methanogenic consortia were inhibited with an increase in ammonia between 4051 and 5734 mg(NH3-N)/l (56.5% loss of activity), and acidogenic populations remained virtually unchanged when concentrations were increased to this level. Among the methanogenic strains typically present in digesters, *Methanospirillum hungate*i showed the highest sensitivity to inhibition at a concentration of 4.2 g/l; the other strains were resistant to ammonia at concentrations above 10 g/l. In general, nitrogen is essential for the survival of anaerobic microorganisms; therefore, ammonia at concentrations below 200 mg/l is generally considered beneficial for an anaerobic process (Sheng et al., 2013). Higher concentrations, however, between 1.7 and 14 g/l, reduce methane production by 50%.

Reagards possible sulfur effects, the literature suggests an ideal concentration between 1 and 25 mg(S)/l (Chen, Cheng and Creamer, 2008). The IC50 values (or inhibitory concentration) for microbial population inhibition in suspended sludge are 50-125 mg(H_2S)/l, at pH 7-8 (Chen, Cheng and Creamer, 2008)

For decades, the toxicity of salt on microorganisms has been studied. The reason why bacteria dehydrate when exposed to high concentrations of salt is due to osmotic pressure and cell plasmolysis, which induces death in the bacterial cell. However, it has been discovered that sensitivity to cations is the main inhibiting factor. Although the addition of common table salt can stimulate biogas production, there is still an optimal concentration of Na^+, beyond which the effects of the cation are inhibitory. The study of Li et al. (2019) mentions approximately 350 mg/l of Na^+ and 230 mg/l of Na^+ as the optimal concentrations for methane production with mesophilic hydrogenotrophic methanogens and mesophilic acetoclastic methanogens, respectively. Inhibition begins to occur when the concentration of Na^+ is in the range of 3.5-5.5 g/l, while reaching 8 g/l of Na^+ means strongly blocking methane production. On the other hand, ionic chlorine is also harmful to bacteria. It is no coincidence that many commercially available disinfectants are chlorine-based. In the study of Shao et al. (2021), the effect of various chlorine-based disinfectants on the anaerobic digestion of pig slurry was tested. The results showed that these compounds prolong the lag phase of methanogenesis, and this effect is proportional to their concentration.

A 59-day exposure to 1000 mg/l of $Al(OH)_3$ reduced the specific activity of methanogenic bacteria by 50% and that of acetogenic bacteria by 73% (Chen,

Cheng and Creamer, 2008); according to another study, anaerobes could survive at concentrations of up to 2500 mg/l of Al^{3+} (Singh, 2023)

As regards Ca^{2+}, concentrations of up to 7000 mg/l do not inhibit anaerobic digestion. Sensitivity to Mg^{2+} is even greater, with inhibition blocking bacterial growth at just 400 mg/l (Singh, 2023).

It has been found that acetate-utilizing methanogens are 50% inhibited by 0.15 M K^{+}. At mesophilic temperatures, sodium salt concentrations between 3500 and 5500 mg/l showed a moderately inhibitory effect on methanogens, while 8000 mg/l showed a strongly inhibitory effect (Chen, 2003)

Some heavy metals can also inhibit methanogens and prevent the subsequent use of digestate in the soil. The inhibitory concentration is already in the ppm range and varies slightly depending on the type of metal, with Cr and Pb being more tolerable than Cd and Ni. According to Singh (2023)., the presence of Cu^{2+}, Zn^{2+}, $Cr^{6+,}$ and Pb^{2+} decreases microbial activity and methane yield by 27-36%.

2.4 Biogas production through anaerobic digestion

Through an anaerobic trophic chain involving various metabolic groups of bacteria that differ in both their substrates and the products of their metabolism, complex organic substrates are converted into methane. The biodegradation process consists of four stages (Zhu et al., 2016; Mishra et al., 2019):

1. Hydrolytic phase: In this phase, larger molecules are broken down into smaller molecules so that other bacteria can use them. Especially for matrices with a high organic content, the hydrolytic phase can be the limiting factor in the degradation process.
2. Acidification phase: So called because bacteria produce carbon dioxide, hydrogen, ammonia, mainly short-chain organic acids such as pyruvate, lactic acid, volatile fatty acids, and other products such as ethanol and ketones from sugars, fatty acids, and amino acids.
3. Acetogenesis: In this phase, acetogenic bacteria convert organic acids into additional carbon dioxide, hydrogen, and ammonia
4. Methanogenesis: Methanogenic archaea can produce methane through two pathways: hydrogenotrophic (reduction of carbon dioxide with hydrogen) and acetoclastic (anaerobic dismutation of acetic acid), which are responsible for the only non-reactive product of the anaerobic digestion process. The resulting substance is the end product of this process and marks the conclusion of aerobic digestion.

The gas production phases begin after the initial growth period of the inoculum, called the “lag time,” when the organic load is elevated compared to the number of bacteria. The first phase produces only hydrogen and carbon dioxide. The second phase produces methane in place of hydrogen, along with carbon dioxide and some

other contaminants, depending on the substrates. The final phase is characterized by a decrease in gas productivity due to an increase in the number of bacteria in relation to the carbon present. At this point, the process continued by feeding new biomass, thus passing to a continuous process, or the fermentation will end with the death of the consortium.

The anaerobic digestion test could be ideally divided into four main phases:

1. start-up phase;
2. rich hydrogen biogas production phase
3. rich methane biogas production phase
4. decreasing of biogas production due to low carbon content

2.5 Experimental part

The tests described in this chapter were carried out as part of the METHAREN project. This project has received funding from the European Union's Horizon Europe research and innovation program under grant agreement No. 101084288.

2.5.1 Materials and Methods

The liquid phase and solid phase (hydrochar) used for anaerobic digestion testing, supplied by the French Atomic Energy and Alternative Energies Commission (CEA), are the product of the HTC process of three streams from the waste selection and separation phases carried out at Acea Pinerolese (ACEA): the stream known as “F-waste,” obtained by separating waste with a 60 mm screen and consisting mainly of plastic bags used for separate collection; the stream known as “D-waste,” obtained by separating waste with a 6 mm screen and consisting mainly of the coarsest fraction of waste not digested by the digester, such as plastic residues, shells, or pits; 40 mm compost. The effluent stream from the HTC process contains several potential inhibitory organic molecules, some of which, due to their high concentrations, could be the primary cause of anaerobic digestion failures. For this reason, the liquid phase was characterized to assess the presence of inhibitors and nutrients to be balanced with OFMSW for anaerobic digestion purposes. The results are shown in the Table 2.1. As can be seen, acetaldehyde, total polyphenols, and chlorides are present in quantities sufficient to prevent fermentation processes. To allow methanization testing, the effluent should be mixed with fractions of organic waste.

ACEA also supplied the mixed bacterial consortium rich in methanogens used as inoculum for the anaerobic digestion tests.

Anaerobic digestion tests were performed in Duran bottles of 250, 500, or 750 ml. The bottles were placed inside a thermostatic orbital stirrer, and the agitation

speed and temperature were set. Each bottle was equipped with a perforated cap and connected via a plastic tube to the Ritter MilliGascounter MGC-1 PMMA flow meter (Figure 2.3), equipped with RIGAMO software.

Table 2.1: Analysis of HTC aqueous phase

Substance	**Quantity**
2,3 PENTADIONES (mg/kg)	4.6
2,5 HEXANEDIONE (mg/kg)	5
2-PYRROLIDONE (mg/kg)	56
5-Hydrosymethylfurfural (mg/kg)	117.4
5-METHYL-FURFURAL (mg/kg)	11
ACETALDEHYDE (mg/kg)	136
ACETOIN (mg/kg)	93
BUTYRIC ACID (mg/kg)	16
PROPIONIC ACID (mg/kg)	58
PENTANOIC ACID (valeric acid) (mg/kg)	4.9
AMMONIA (NH4) (mg/kg)	232.7
TOTAL NITROGEN (%)	0.12
ETHANOL (mg/kg)	20
FURFUROL (mg/kg)	98.1
INDOLE (2,3-benzopyrrole) (µg/kg)	23
METHANOL (mg/kg)	224
PHENOL (mg/kg)	8.8
Pyridine (mg/kg)	11
TOTAL POLYPHENOLS (come acido gallico) (mg/kg)	2180
SIRINGALDEHYDE (mg/kg)	9.77
SIRINGOL (mg/kg)	35.5
VANILLIN (mg/kg)	30
ACETIC ACID (g/kg)	1.71
FUMARIC ACID (g/kg)	0.04
CITRIC ACID (g/kg)	0.04
MALIC ACID (g/kg)	0.3
LACTIC ACID (g/kg)	5.4
TARTARIC ACID (g/kg)	0.01
CHLORIDE (expressed as Cl^-) (mg/kg)	1357
PHOSPHATES (mg/kg)	76
SULPHATES (mg/kg)	134

The same gas was then collected in TEDLAR bags and subsequently analyzed in the ETG 6500 biogas analyzer (Figure 2.4), which quantifies the presence of oxygen, nitrogen, carbon dioxide, methane, and hydrogen as a percentage, and the presence of sulfuric acid in terms of ppm.

Figure 2.3: Ritter MilliGascounter MGC-1 PMMA flow meter

Figure 2.4: ETG 6500 biogas analyzer

In addition, liquid samples were taken and analyzed throughout the test to monitor pH and FOS/TAC ratio.

Indeed, a parameter used to monitor the progress of anaerobic digestion is FOS/TAC, obtained by titration with 0.1 N sulfuric acid.

This parameter indicates the ratio between volatile organic acids (FOS = Flüchtige Organische Säuren) and alkaline buffer capacity (TAC = Totales Anorganisches Carbonat) expressed as mg/l of calcium carbonate equivalent. The procedure involves testing 20 ml of a representative sample of the digestion broth, from which any solid impurities have been removed. The sample was collected in a beaker containing a magnetic stir bar and placed on a shaking plate, next to a burette for titration containing 0.1 N sulfuric acid. Monitoring with a pH meter, the pH was adjusted to 5, and the volume of acid consumed was recorded. The pH was then brought to 4.4, and the volume of acid consumed to reach pH 4.4 was noted again. With these data available, the FOS and TAC values were calculated as follows:

$$TAC = 250 V_{pH5}(H_2SO_4)$$

$$FOS = 500(1.66V_{pH4.4}(H_2SO_4) - 0.15)$$

Where $V_{pH5}(H_2SO_4)$ is equal to the volume of sulfuric acid (in ml) needed to bring the sample to pH 5, and $V_{pH4.4}(H_2SO_4)$ is equal to the volume of sulfuric acid (in ml) required to bring the sample from pH 5 to pH 4.4. The FOS/TAC ratio is a key parameter for evaluating the fermentation process. Measuring it allows you to quickly identify factors that interfere with the process and can lead to an imbalance in the digester biology, enabling you to take immediate countermeasures. Indeed, the accumulation of FOS (formic, acetic, propionic, lactic, and butyric acids), which are formed during acidogenesis and converted into methane and carbon dioxide during acetogenesis and methanogenesis, inhibits the anaerobic process. A FOS/TAC ratio of 0.3-0.4 indicates that anaerobic digestion is proceeding under ideal organic load conditions. A higher or lower ratio indicates unfavorable process conditions, with excessive or insufficient organic load, respectively, and new biomass must be added, otherwise the consortia gradually stop producing gas. (Lossie & Pütz, n.d.)

2.5.2 Tests performed

Tests were conducted in 3 different periods, using 3 to 5 reaction bottles. The tests were repeated to validate the results. The general procedure followed in all tests is as follows:

1. Fresh OFMSW collected in the previous days was shredded and diluted with water until it reached a semi-liquid consistency.
2. The bottles filled with OFMSW (and, where applicable, with the effluents to be treated) were autoclaved at 121 °C for 20 minutes. Although industrial processes are not carried out under sterile conditions, as these are laboratory-scale tests to identify the best reaction conditions for a certain bacterial consortium, it is necessary to ensure that there are no other microbial interferences that could compromise the test results.
3. The inoculum was then added to the autoclaved bottles and placed on a shaking plate.
4. The initial pH was measured and adjusted when necessary, considering an optimal reaction pH of around 6.
5. Nitrogen was blown into the bottles, allowing it to bubble through the liquid, until all the oxygen in the bottle was eliminated. With this step, we replaced the oxygen with nitrogen and recreated anaerobic conditions. This step of the procedure was repeated after each liquid sample was taken.

6. The bottles were placed in the orbital shaker under thermophilic conditions (50 °C), with the perforated cap connected to the flow meter and/or TEDLAR bags for collecting the gas produced.

The specific description of the tests conducted is provided below.

2.5.2.1 First test period

The first tests were conducted on 250 ml bottles. The composition of the bottles (also described in Table 2.2) included the use of 10% v/v inoculum (ACEA digestate) in all bottles.

Table 2.2: Bottles composition for the first period

Bottle	Volume (ml)	T (°C)	pH	Liquid phase (ml)	OFMSW (ml)	Digestate (ml)	TS (g/kg)	TVS (g/kg)
Bottle 1	250	50	7.11	0	225	25	6.9	61.6
Bottle 2	250	50	6.96	25	200	25	54.8	54.2
Bottle 3	250	50	7.13	50	175	25	61.6	61.0

In one bottle, 10% v/v of HTC aqueous phase was also added, and in another, 20% v/v. A third bottle was left without the aqueous phase to serve as a control. Everything was brought to volume with easily digestible OFMSW, consisting of fruit and vegetable peels, without seeds, pits, or woody components. This step also balanced the C/N ratio, which must greater than 30 for optimal anaerobic digestion. Once the pH was adjusted, the bottles were placed in an incubator. The test lasted about 60 days. Figure 2.5 shows the arrangement of the three bottles during the test.

As shown in Table 2.2, the total and volatile solids present in the fermentation broth were also calculated. These values give an idea of the degradable fraction present in the effluent.

Figure 2.5: Set up tests for the first period

2.5.2.2 Second test period

In the second test period, the test was repeated with 10% v/v aqueous phase and a mixture of 10% v/v aqueous phase and 10% w/w solid phase (char) was also tested (Table 2.3).

Table 2.3: Bottles composition for the second period

Bottle	Volume (ml)	T (°C)	pH	Liquid phase (ml)	OFMSW (ml)	Digestate (ml)	Char (g)
Bottle 1	700	50	4.88	0	630	70	-
Bottle 2	700	50	5.51	70	560	70	-
Bottle 3	700	50	5.38	70	560	70	20.5

The tests were performed with 1 l bottles. In this case, the bottles were not placed in the orbital shaker but on three magnetic heating plates, which were also connected to the gas collection bags. Figure 2.6 shows the arrangement of the three bottles in the second test period.

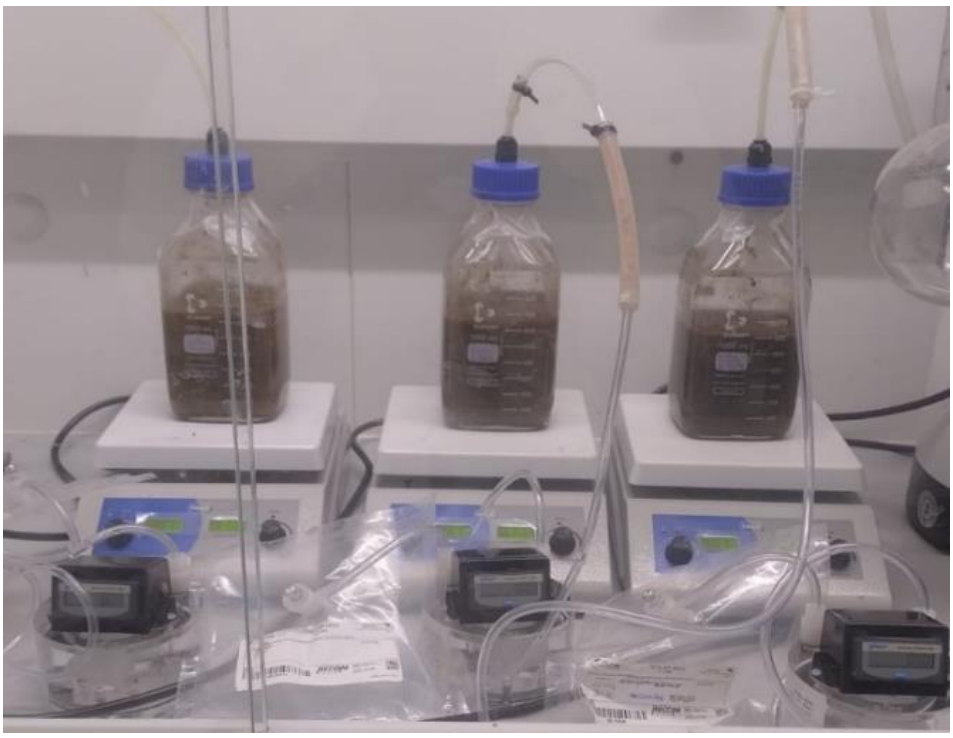


Figure 2.6: Set up tests for the second period

2.5.2.3 Third test period

In the third test period, tests were performed in 500 ml bottles with the composition shown in Table 2.4 then a control bottle (bottle 1), a bottle with 20% v/v aqueous phase (bottle 2), a bottle with 40% v/v aqueous phase and no OFMSW (bottle 3), a bottle with 20% v/v aqueous phase and 10% w/w char (bottle 4), and a bottle with 40% v/v aqueous phase, 0% OFMSW, and 10% char w/w.

Table 2.4: Bottles composition for the third period

Bottle	Volume (ml)	T (°C)	Medium (ml)	Liquid phase (ml)	OFMSW (ml)	Digestate (ml)	Char (g)
Bottle 1	500	50	50	0	200	250	-
Bottle 2	500	50	50	100	100	250	-
Bottle 3	500	50	50	200	0	250	-
Bottle 4	500	50	50	100	100	250	14.6
Bottle 5	500	50	50	200	0	250	14.6

During these tests, some experiments were repeated to confirm the results, and new ones were conducted, such as a 40% v/v aqueous phase use with and without char. In addition, a higher inoculum quantity was used, equal to 50% v/v, and 10% v/v of medium for methanogenic bacteria was added to improve the reaction conditions and place the bacteria in the most favorable conditions for gas

production, given the high quantity of wastewater to be tested. Table 2.5 shows the recipe for the medium for methanogens.

Table 2.5: Recipe for 1 liter of medium for methanogens

Substance	Quantity
NaCl	0.6 g
NH_4Cl	1 g
$NaHCO_3$	5 g
KH_2PO_4	0.3 g
K_2HPO_4	0.3 g
Yeast Extract	5 mg
Sucrose	20 g

2.5.3 Results

2.5.3.1 First test period

Figure 2.7 shows the FOSTAC trend.

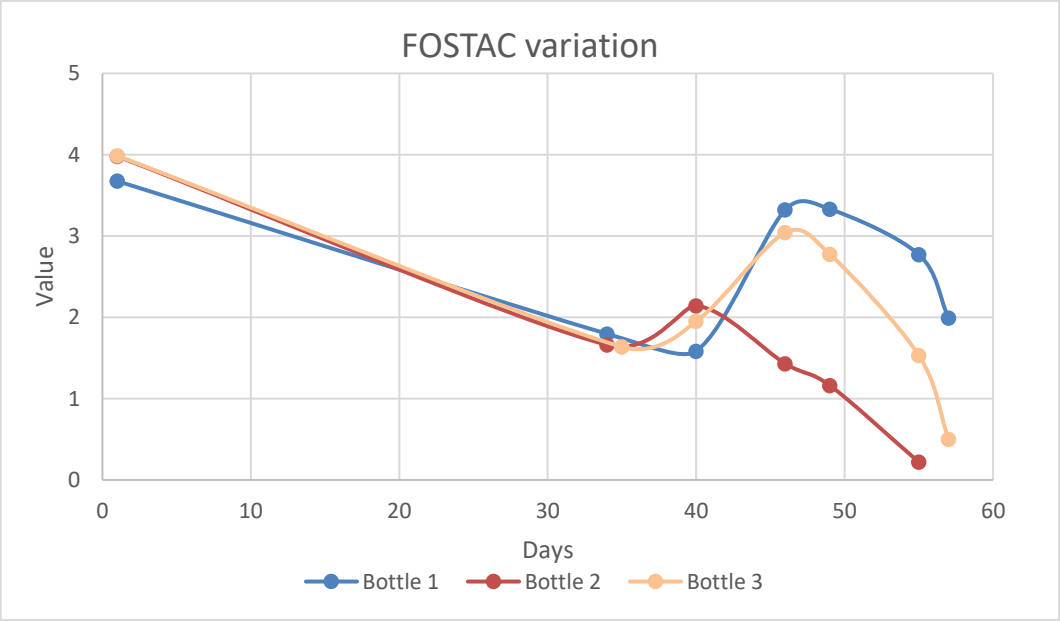


Figure 2.7: FOSTAC variation of first test period

The trend is consistent across all three bottles, indicating that the anaerobic digestion process is working correctly. Indeed, given the very high organic load (FOSTAC values around 4), it took more than 30 days for the bacterial consortium to acclimatize properly and start producing gas (see Figure 2.8).

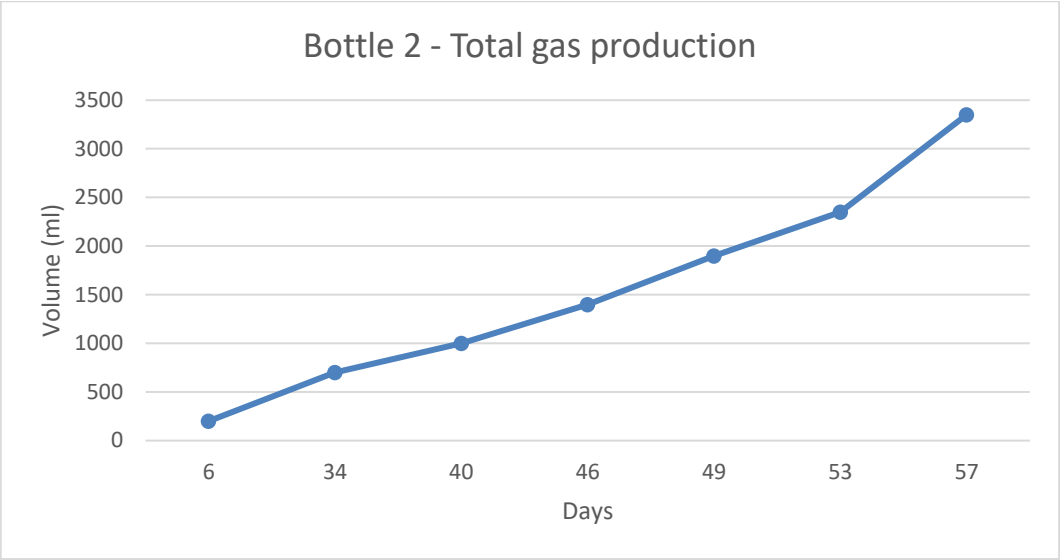


Figure 2.8: Total gas production of bottle 2 of first test period

High FOSTAC levels indicate a very high concentration of volatile organic acids, expressed as mg/l of acetic acid. However, although acetic acid is the precursor of methane and carbon dioxide production (the end product of anaerobic digestion), it is also true that too high concentrations of this nutrient lead to bacterial inhibition due to excess "food." Rosato (2015) defines this phenomenon with a typically human term, namely "indigestion." In his article, he also explains that there may be other types of organic acids besides acetic acid, such as propionic acid, which is commonly used as a food preservative and has strong antibacterial properties. It is usually converted into acetic acid, but if something in the process does not go as intended (e.g., a lack of bacteria suitable for conversion), the accumulation of this or other types of organic acids could lead to a slowdown in biogas production, which explains why it took more than 30 days to evaluate initial gas production.

Monitoring pH is also an excellent method for assessing the "well-being" of anaerobic digestion. The ideal pH for an anaerobic digester is between 6.8 and 7.2. The process can also function at pH values between 6.5 and 8. Anaerobic digestion stabilizes when the pH is maintained at around 7. During anaerobic digestion, the CO_2 produced partially dissolves in the liquid phase to form carbonic acid, which then dissociates depending on the pH to form bicarbonate and carbonate ions. This step, together with the presence of ammonia and volatile fatty acids, determines the pH of the reaction. The carbonic acid equilibrium is always the predominant buffer system, and its presence guarantees a pH within the appropriate range. As can be seen from Figure 2.9, the pH trend is also consistent with what is expected from a properly conducted anaerobic digestion process.

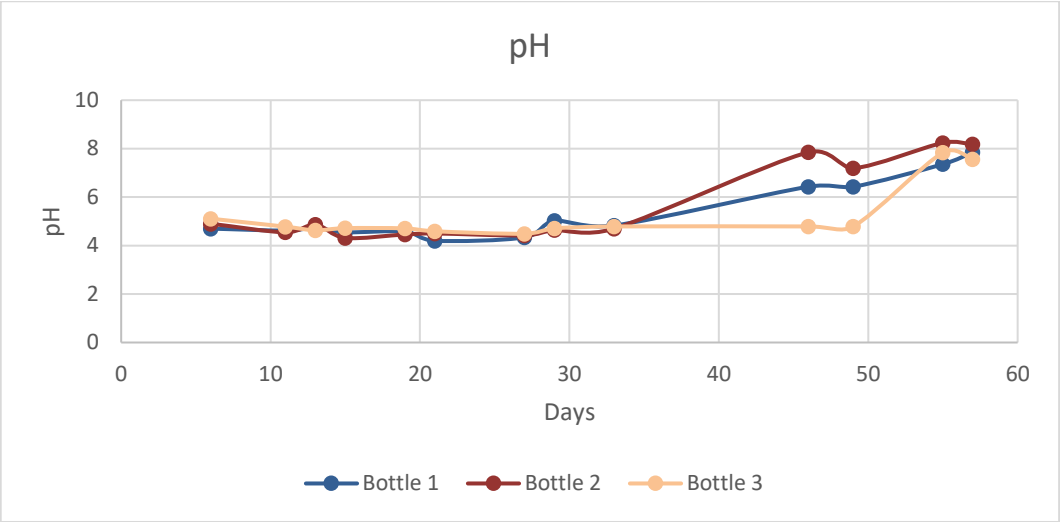


Figure 2.9: pH trend during first test period

Despite all the favorable conditions, however, only bottle 2, containing a 10% aqueous phase, produced biogas understood as a mixture of CO_2/CH_4. Bottle 1, containing only OFMSW, which was the reference bottle, produced only 900 ml of gas after 34 days, composed mainly of CO_2 (48%), H_2 (28%), and 837 ppm of H_2S. The remainder consists of 3% O_2 and 21% N_2. The composition and quantities of total gas produced and H_2S are very similar to those of bottle 2 (Figure 2.8, Figure 2.10 and Figure 2.11) obtained over the same period.

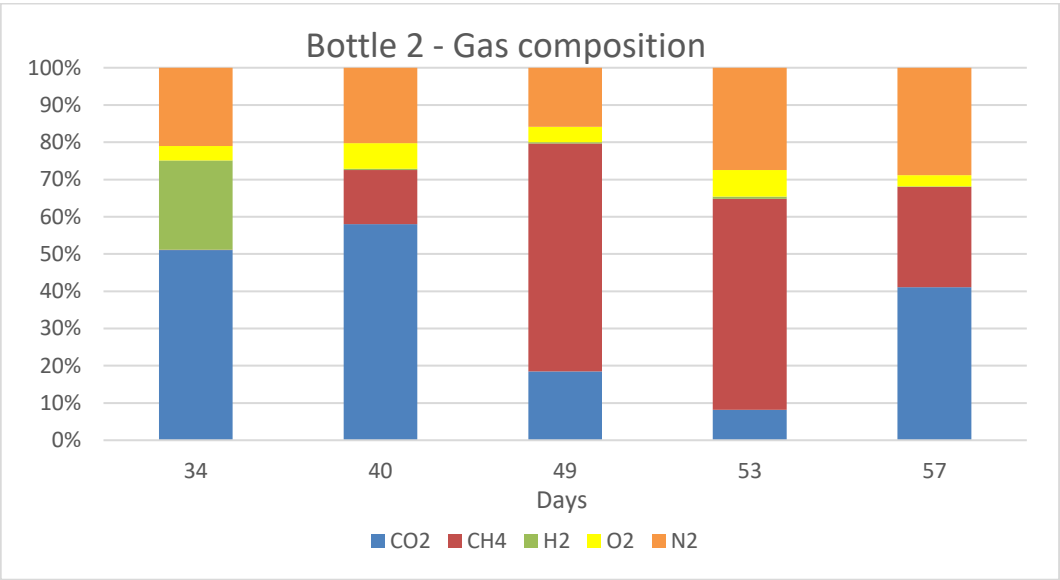


Figure 2.10: Gas composition of bottle 2 of first test period

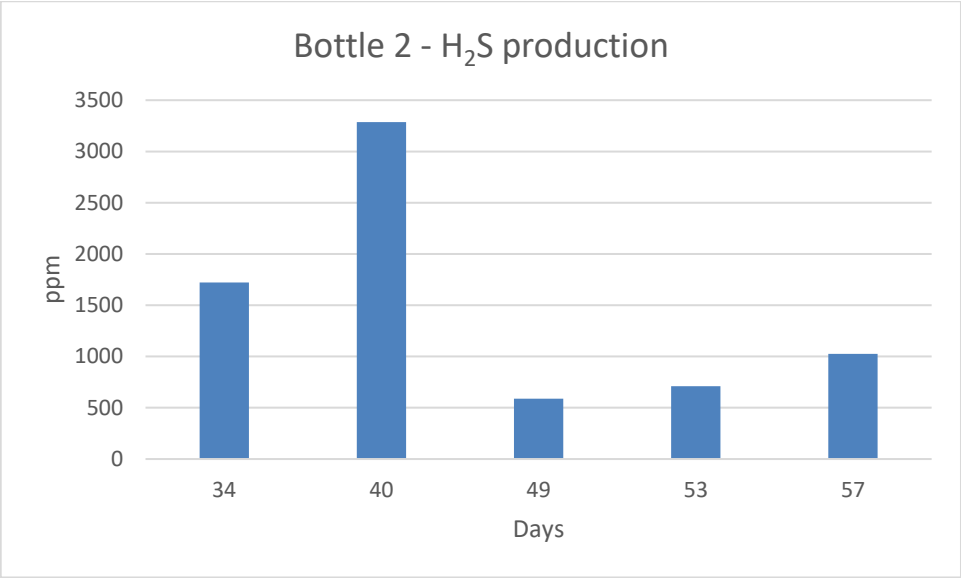


Figure 2.11: H_2S production in bottle 2 of first test period

In both cases, the gas concentrations obtained are consistent with the first steps of the anaerobic digestion process. After a long hydrolytic phase, which, as mentioned above, represents the typical phase of the process and lasted about 30 days, the acidification phase began, whose main gaseous products are CO_2 and H_2. Subsequently, production in bottle 1 stopped. A plausible reason could be inhibition of the bacterial consortium due to sampling followed by a problem occurred in maintaining anaerobic conditions.

As for bottle 2, 3.35 l of gas was produced after 57 days, with a composition of 41% CO_2, 27% CH_4, 3% O_2, 29% N_2 and 1027 ppm H_2S. As can be seen from Figure 2.10 and Figure 2.12 the composition of the gas produced varied during the process. A strong correlation between the concentration of CH_4 and that of H_2S can be observed. Indeed, as the concentration of H_2S increases, that of CH_4 decreases and vice versa. The reason for this is that during the anaerobic digestion process, the bacterial consortia have the same biomass available for their metabolites and therefore compete with each other. It means that when there is a higher production of H_2S, the methanogenic bacteria are in the minority compared to the sulphur-producing bacteria, and the concentration of CH_4 decreases. Looking at the amount of methane produced, after 49 days, the maximum rate obtained during this test was equal to 61%. Adding up all the percentages of gas produced, we obtain 875 ml of CH_4 in 57 days, with 250 ml of fermentation broth, thus a ratio of 3.5 l_{CH4}/l fermentation broth.

The data shown in Figure 2.12, on the other hand, are the result of a reprocessing in which the concentration of CO_2, CH_4, and H_2 was re-parameterized by eliminating N_2 and O_2, which were identified by the analyzer but are useless for the purpose of evaluating the biogas produced.

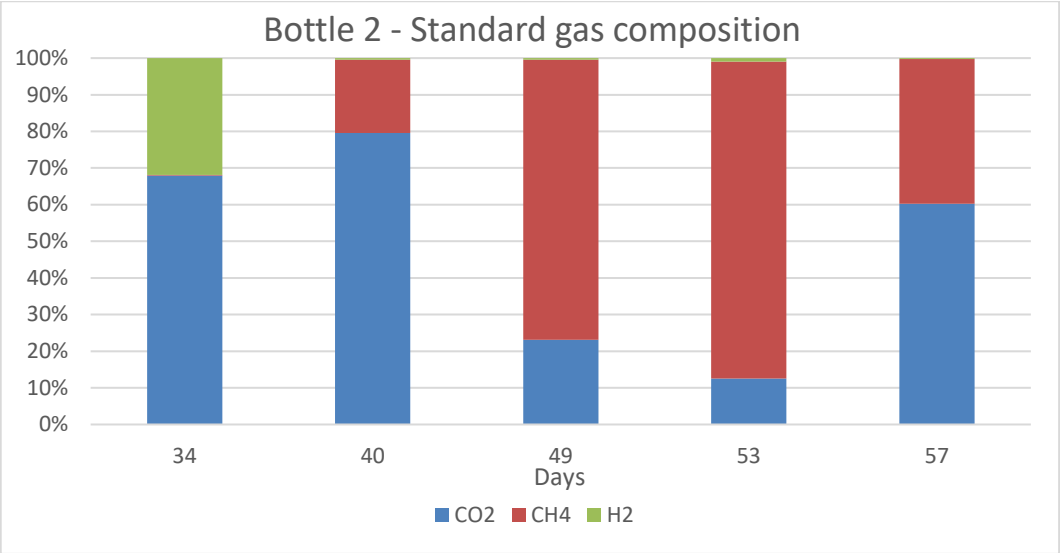


Figure 2.12: Standard gas composition of bottle 2 of first test period

The presence of O_2 and N_2 can be attributed to the possibility of small air leaks during sampling or gas analysis. The fact that O_2 and N_2 are not in a stoichiometric ratio in the air, however, is justified by the fact that anaerobic digestion is performed by blowing in N_2, so its presence over oxygen is very plausible. Fortunately, oxygen is always present, but in very low concentrations. Considering these percentages, it is possible to evaluate not only the gas produced in terms of CH_4, but more generally in terms of biogas, even if the ratio obtained is not the standard $CH_4/CO_2 = 1.5$. Therefore, 1.6 l of biogas were produced in 57 days with 250 ml of fermentation broth, resulting in a ratio of 6.4 l of biogas per 1 l of fermentation broth.

At the same time, bottle 3, containing a 20% aqueous phase, did not produce any gas. This data will be repeated later to confirm that this event was due to inhibition caused by a too high concentration of inhibitors in the aqueous phase and not to problems encountered during the trial.

2.5.3.2 Second test period

Figure 2.13 shows the FOSTAC trend. In this case too, the trend is generally consistent in all three bottles: after an initial decrease in the FOSTAC value, there was a rise due to the increase in volatile fatty acids, followed by a further decrease and the start of gas production. At this point, liquid sampling was interrupted to avoid opening the bottle and interrupting the process. The beginning and end of the FOSTAC test did not coincide in the three cases because, as can be seen from Figure 2.14, the initial pH of bottles 2 and 3 was too low to perform the test. It was necessary to adjust the pH back to values more suitable for anaerobic digestion several times by adding 0.1 M NaOH.

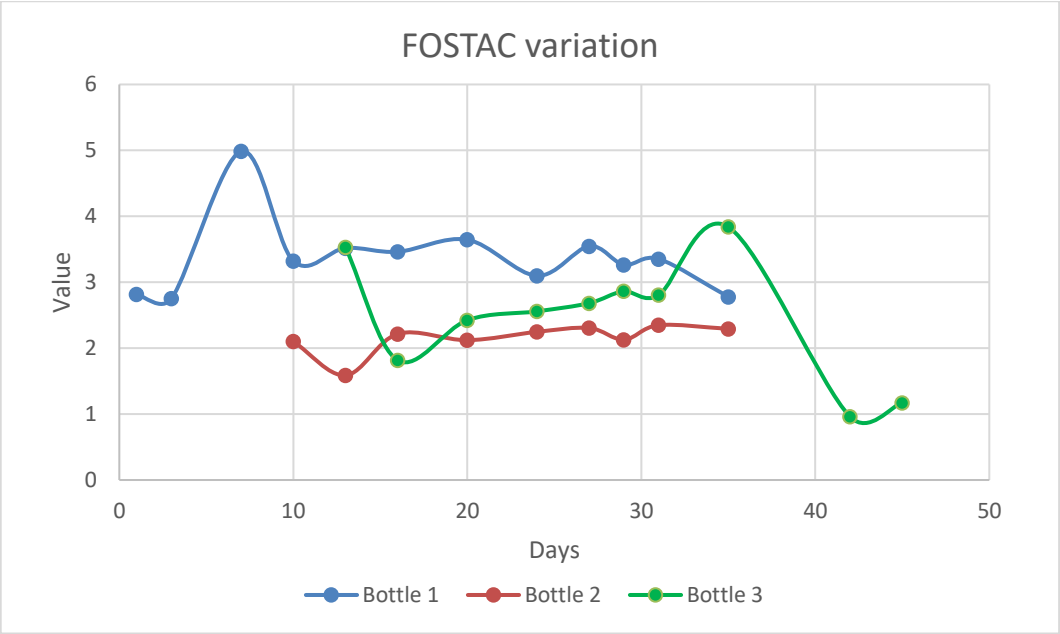


Figure 2.13: FOSTAC variation of second test period

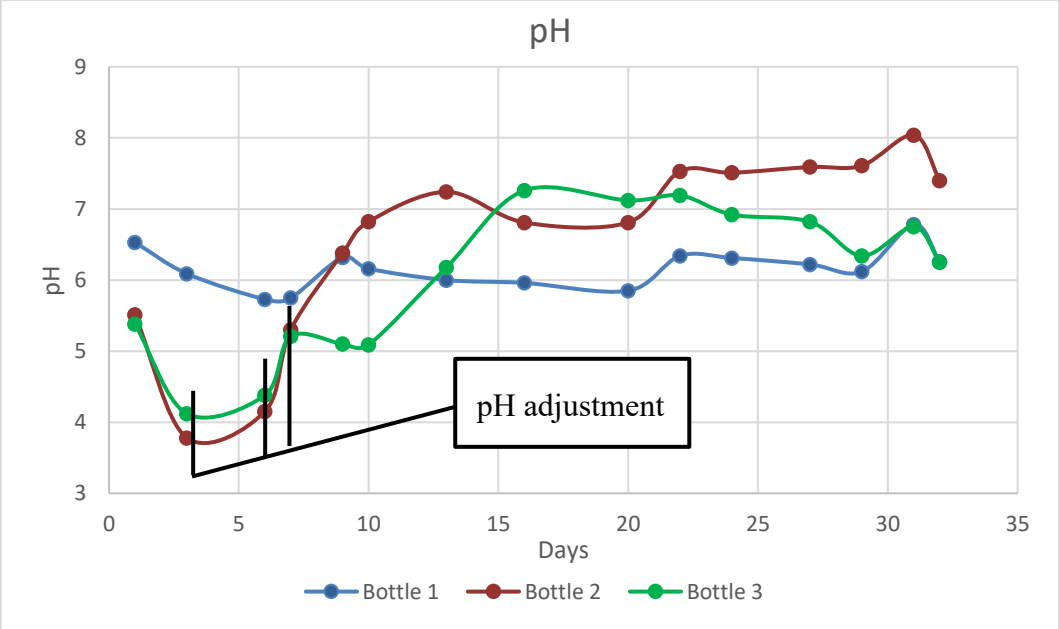


Figure 2.14: pH trend and adjustment during second test period

This phenomenon is explained by the fact that the pH drops during the acidogenesis and acetogenesis phases of batch fermentation because acid fermentation occurs before methanogenic fermentation rather than simultaneously especially during the startup phase of the anaerobic digestion before steady state operation. As a result, acetic acid accumulates. The pH can therefore drop to levels between 4.5 and 5.

Occasionally, it may be necessary to adjust the alkalinity externally when a low pH is reached during the stagnation phase; in these cases, NaOH is used to facilitate

the resumption of the reaction. The pH returns to near-neutral levels when the methanogenesis phase begins, and the process continues to proceed stably.

Figure 2.15 shows the gas production.

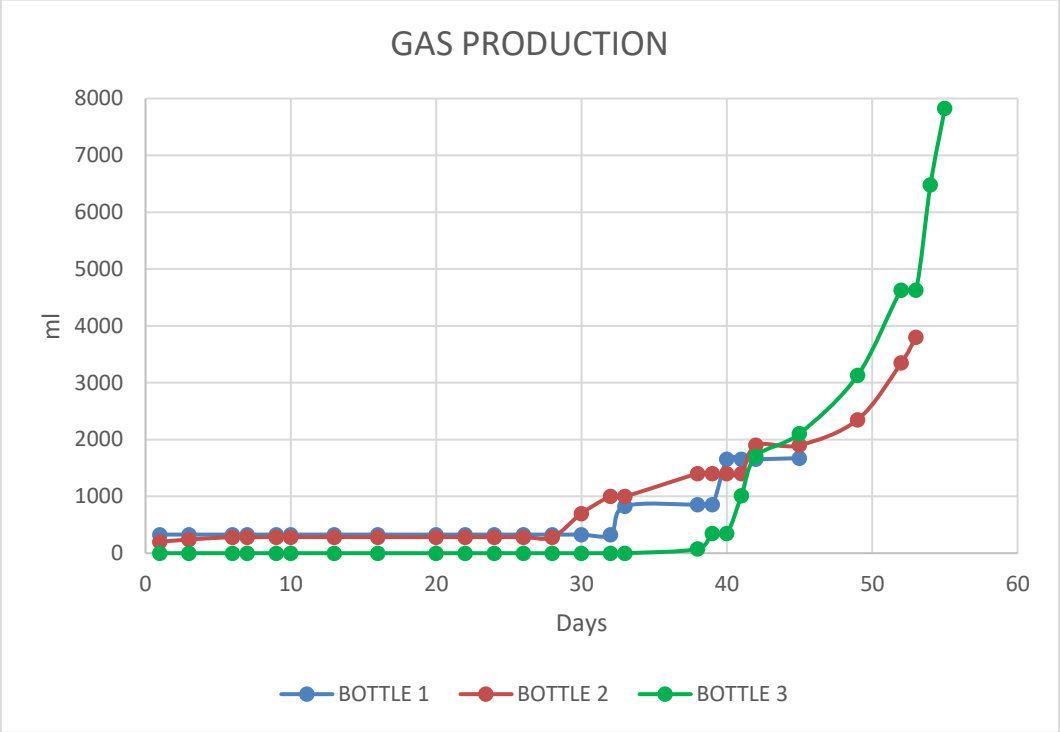


Figure 2.15: Total gas production of second test period

Again, the trend is very similar in the three bottles, with the only difference being that the bottle with char (bottle 3) struggles a little more to start fermentation, which is in line with the FOSTAC analysis results.

Bottle 1 showed a similar trend to the previous test: a small initial production, followed by a more substantial production after around 30 days, reaching 1.67 l of gas produced after 45 days. Although this test yielded better results than the previous one in terms of quantity and quality of gas produced (approximately 38% CH_4), the reaction always stops earlier than in the two bottles with the effluents to be tested. However, the fact that the reference reaction performed worse than those to be tested should not be discouraging: it is an indication of the complexity of biological reactions, in which several variables contribute to the success of the test and in which the laboratory scale shows its limitations.

Bottle 2 confirmed the result of the previous test: 3.8 l of gas were achieved in 53 days.

Figure 2.16, Figure 2.17 and Figure 2.18 show the composition (both total and normalized) of bottle 3. Here too, gas production began with CO_2 and H_2 after 33 days and ended on the 55th day with 7.8 l of gas produced and a composition of 14% CO_2, 79% CH_4, 5% N_2, 2% O_2, and only 72 ppm of H_2S, the best composition of the test in terms of CH_4 and H_2S concentration.

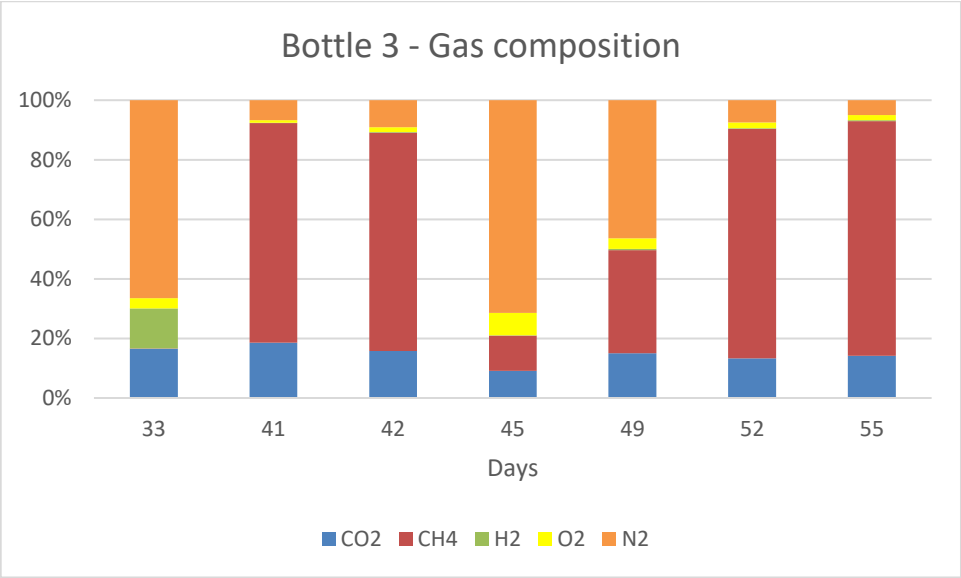


Figure 2.16: Gas composition of bottle 3 of second test period

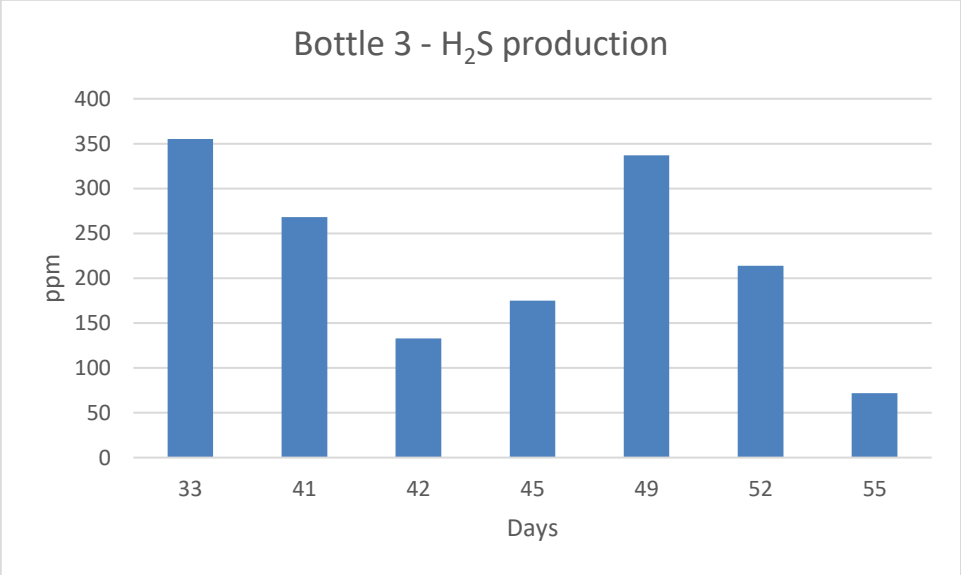


Figure 2.17: H_2S production of bottole 3 of second test period

Once again, the gas composition trend is highly variable over time, but two related things stand out compared to the previous period's tests:

- The high CH_4/CO_2 ratio
- The very low concentration of H_2S

This behavior could be due to the presence of char, which adsorbs H_2S and promotes the production of CH_4 and biogas. This data will need to be confirmed, so the test will be repeated to rule out the possibility that this was just a one-off and that there are other causes.

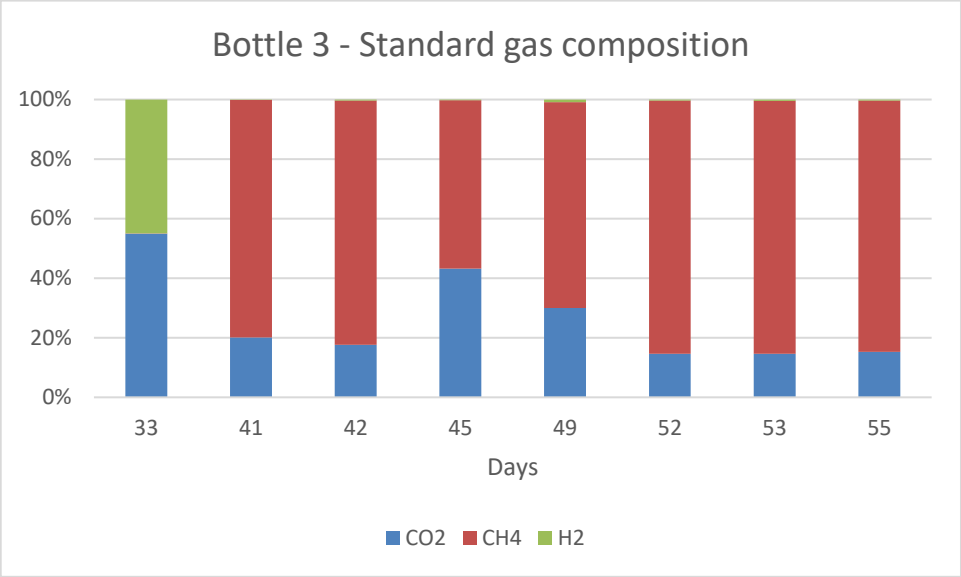


Figure 2.18: Standard gas composition of bottle 3 of second test period

Considering all the CH_4 produced, 3.6 l of CH_4 were obtained with 700 ml of fermentation broth, with a ratio of 5.14 l_{CH4}/l fermentation broth, higher than that obtained with bottle 2 of the previous test, containing only the aqueous phase and no char. Speaking of biogas in general, however, 4.6 l of biogas were produced with 700 ml of fermentation broth, equal to a ratio of 6.57 l_{biogas}/l fermentation broth, in this case not too dissimilar to the test mentioned above.

2.5.3.3 Third test period

Table 2.6 shows the FOSTAC values of the bottles containing the effluents to be tested.

Table 2.6: FOSTAC variation of the third test period

Days	Bottle 2	Bottle 3	Bottle 4	Bottle 5
7	1.38	1.00	1.57	1.04
17	0.277	0.273	0.332	0.305

The values appear very low right from the start. This phenomenon is likely due to the choice of higher inoculum content compared to previous tests, which allowed for faster consumption of the organic load after only 7 days of digestion. Finally, after 17 days, the FOSTAC values were already within the optimal range expected by the method. Only two samples were taken because almost all the bottles started

producing gas almost immediately (Figure 2.19). However, it was significant to check the FOSTAC trend even with a higher inoculum quantity.

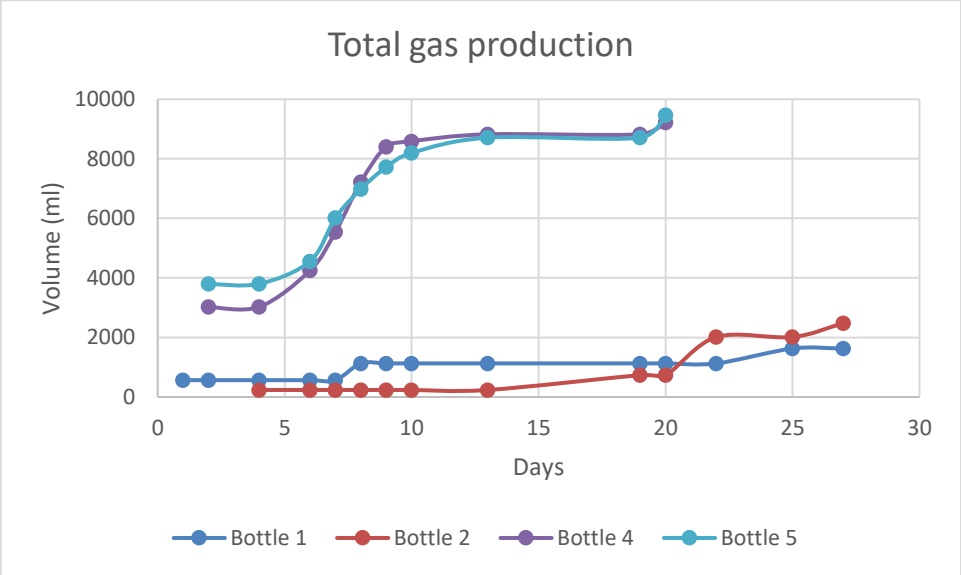


Figure 2.19: Total gas production of third test period

As can be seen from the figure, bottle 1 confirmed its maximum gas production already obtained in the previous test, amounting to 1.63 l, but after only 27 days. Then, the production stopped. The addition of the medium and the increase of inoculum percentage appear to have had a positive effect on the kinetic of steady state gas production, but not on the quantity.

Bottle 2 has a very similar trend to the control bottle. This data is positive because it refutes the negative results of the first test period, in which the bottle with 20% v/v of aqueous phase did not produce gas.

As for bottle 3, despite the test being repeated twice in the third test period, no gas production was observed.

Bottles 4 and 5, containing 10% w/w char, showed extremely high gas production. Indeed, there is a visible difference between the bottles with char (bottle 4 and bottle 5) and those without char (bottle 1 and bottle 2). Apparently, char accelerates and increases gas production compared to the control bottle (bottle 1) and the bottle with liquid phase but without char (bottle 2). It is also interesting to note that although bottle 3, containing 40% v/v aqueous phase, 0% OFMSW and no char, did not produce gas, when 10% w/w char was added to that composition (bottle 5), gas production was comparable to the bottle with 20% v/v aqueous phase, also containing OFMSW (bottle 4). The latter two bottles reached a gas volume of 9.2 l and 9.5 l, respectively, the highest production recorded in the three tests.

Figure 2.20, Figure 2.21, Figure 2.22 and Figure 2.23 show the compositions of bottle 1, bottle 2, bottle 4, and bottle 5, respectively.

Looking at the graphs, you can see that in all four of them, there is a transition from the acidification phase, where H_2 is produced, to the methanation phase, where biogas is produced.

Bottle 2 produced 2.48 l of gas, of which 1.77 l was biogas in 500 ml of fermentation broth, with a ratio of 3.54 l biogas/l fermentation broth. Specifically, 1.06 l of CH_4 was obtained in 500 ml of fermentation broth, with a ratio of 2.12 l_{CH4}/l fermentation broth.

Looking at the graphs showing the composition of bottles 4 and 5, the remarkable gas production results is somewhat reduced: for bottle 4, from day 10 onwards, more than 50% of the gas composition is made up of N_2 and O_2; the same can be seen for bottle 5, where this phenomenon is observed between days 13 and 20. However, the data are still excellent. If we imagine stopping production between day 9 and day 10, we would still have obtained between 8.2 l and 8.4 l of gas with a CH_4 composition of between 50% and 69% in just 9-10 days. In the previous test, such compositions were obtained after 40 days. Bottle 4 therefore produced 6.8 l of biogas, of which 3.5 l was CH_4, giving a ratio of 13.4 l_{biogas}/l fermentation broth and 7 l_{CH4}/l fermentation broth. With bottle 5, on the other hand, 7.08 l of biogas were obtained, of which 3.26 l were CH_4, with a ratio of 14.2 l_{biogas}/l fermentation broth and 6.52 l_{CH4}/l fermentation broth.

Figure 2.24, Figure 2.25 and Figure 2.26 show the concentrations of H_2S in bottles 1, 2, and 5, respectively. There is no graph for bottle 4 because no H_2S production was detected in the 20 days of reaction, from the start to the end of the test. This result is truly astonishing. A marked decrease in H_2S production had already been noted in the previous test with the bottle containing char. By repeating the test with the same liquid/char proportions and the usual percentages by volume, but increasing the amount of inoculum, it would appear that the ideal recipe for eliminating H_2S production has been found. However, the instrument used analyzes H_2S down to ppm levels. The char adsorbent capacity towards H_2S is also confirmed by the results on H_2S production in bottle 5. Although there was a small amount of H_2S production in the first 9 days, particularly when compared to the other bottles or other tests, production subsequently ceased completely. This phenomenon is illustrative that it is not the presence of char alone that reduces H_2S production, but a combination of several factors, such as the liquid phase/char ratio, the presence of additional nutrients, and the amount of inoculum. The presence of char is therefore a necessary but not sufficient condition for H_2S production abatement.

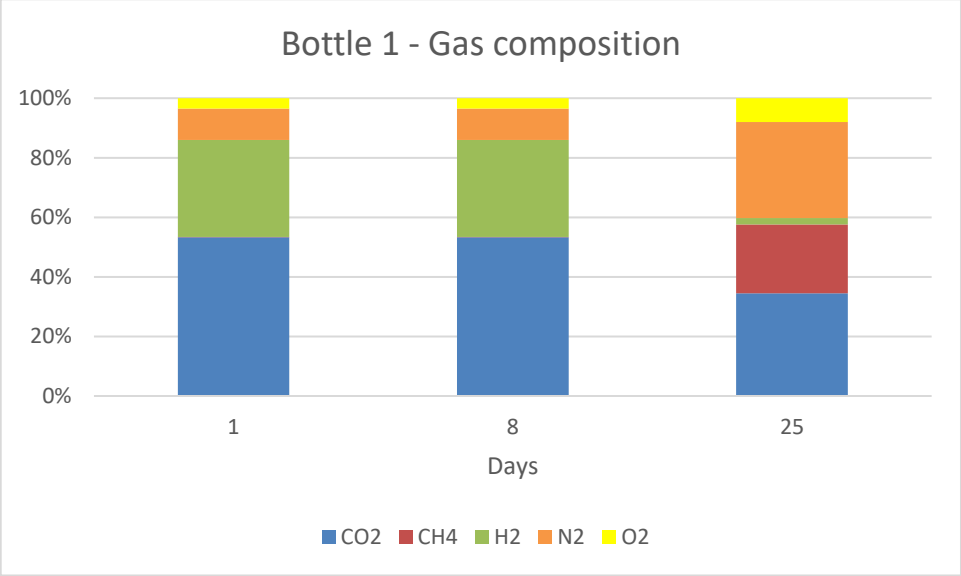


Figure 2.20: Gas composition of bottle 1 of third test period

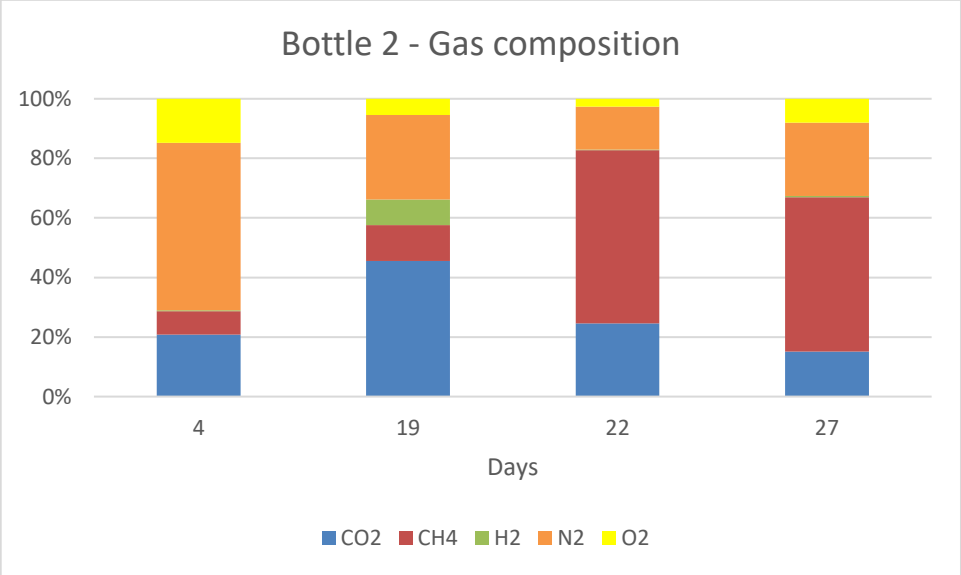


Figure 2.21: Gas composition of bottle 2 of third test period

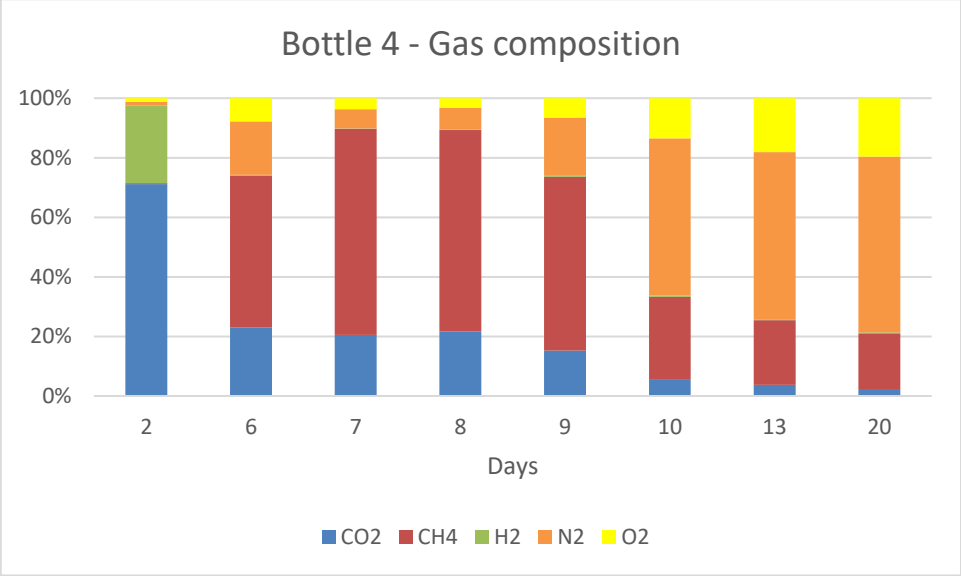


Figure 2.22: Gas composition of bottle 4 of third test period

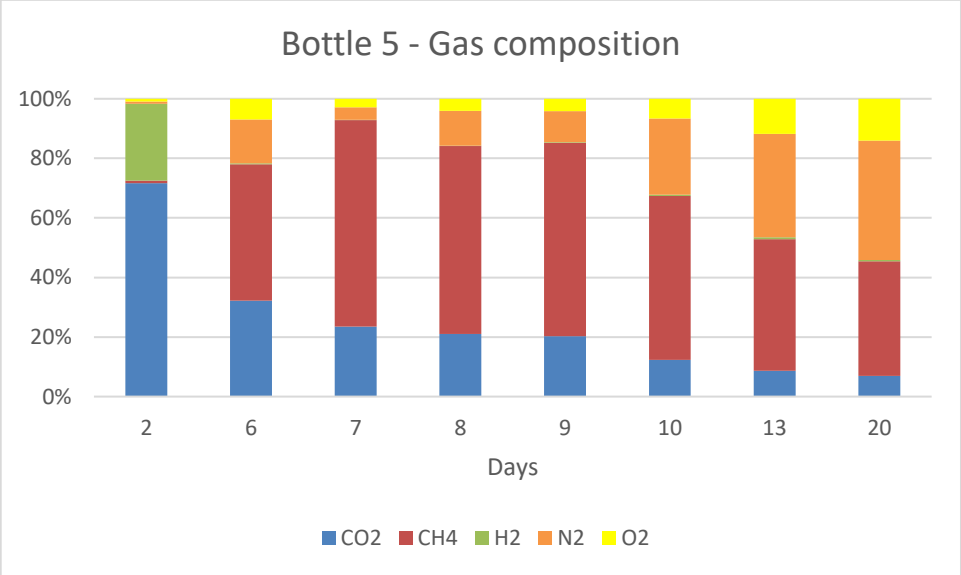


Figure 2.23: Gas composition of bottle 5 of third test period

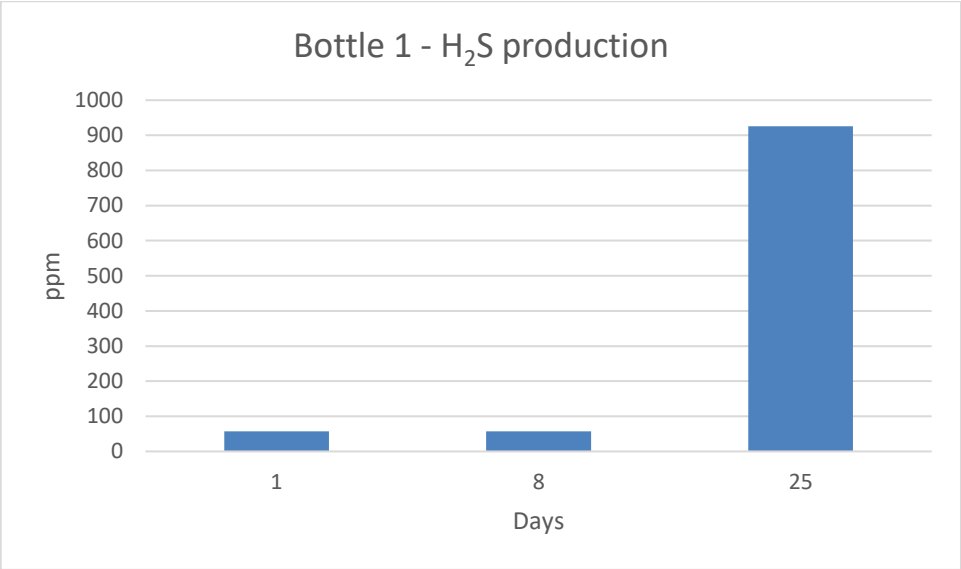


Figure 2.24: H_2S production of bottle 1 of third period test

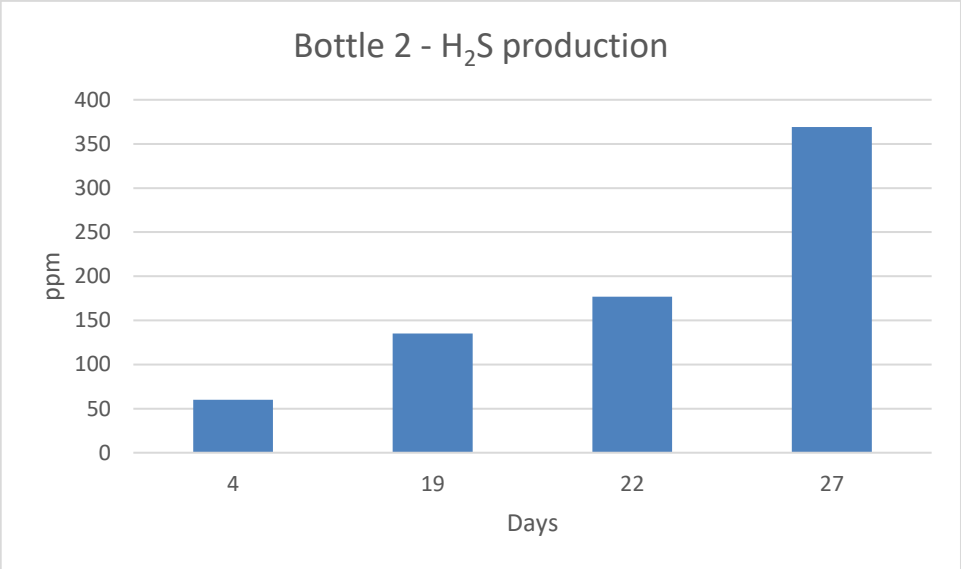


Figure 2.25: H_2S production of bottle 2 of third period test

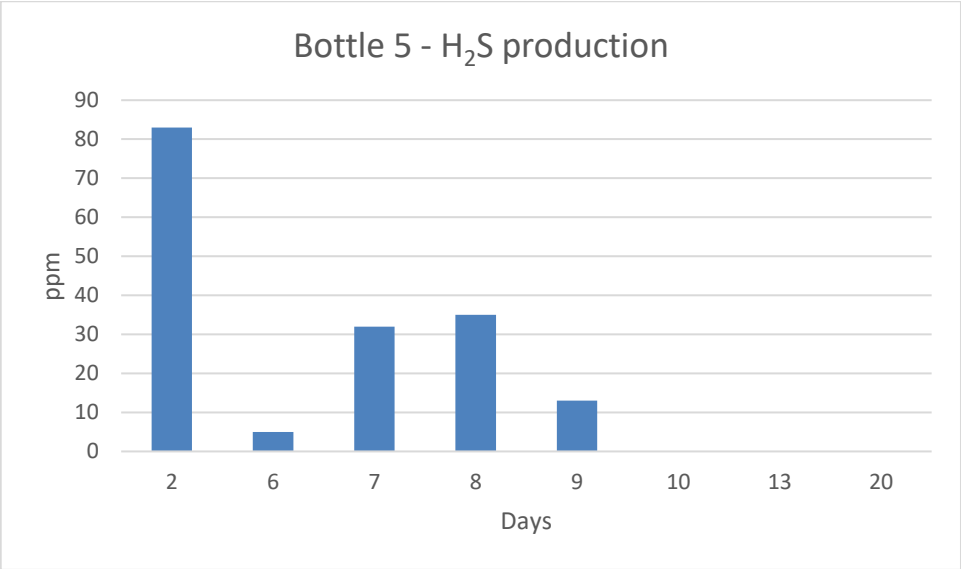


Figure 2.26: H_2S production of bottle 5 of third period test

2.6 Conclusions

Plastic has become integrated into everyday life and, due in part to current management, it has also found its way into our organic waste. The problem arises when this organic waste containing plastic (micro and macro) is sent for anaerobic treatment and/or composting, causing problems for the plant and producing compost that does not always meet the quality criteria set by European legislation. Thanks to screening systems, plastic that ends up in the wrong bin might be identified, separated, and recovered, either through recycling or thermal recovery. A viable solution to be implemented could be hydrothermal carbonization. HTC is a process that transforms wet biomass into three principal products: a carbonaceous solid called hydrochar, a liquid fraction, and a small amount of gas. The HTC process can be applied to plastic waste. The hydrochar obtained from plastic waste has advantages because it has a porous structure that makes it useful for absorbing pollutants and enhancing methane production in fermentation. In this chapter, tests were conducted to recover hydrochar and the liquid derived from the HTC of plastics recovered from organic waste to finally produce biogas. The results showed that, when dosed in appropriate quantities with OFMSW, the aqueous phase of the HTC process can be successfully integrated into the anaerobic digestion process up to 20% of the total volume, not hindering but rather promoting biogas production. The amount of aqueous phase can even increase up to 40% v/v when integrated with 10% w/w char. When properly dosed to a large and active bacterial consortium, char promotes biogas production and reduces the production of H_2S, a toxic by-product of anaerobic digestion, making even higher concentrations (up to a maximum of 40% v/v) of the aqueous phase tolerable.

Chapter 3

From biogenic waste to biofuels: triglyceride extraction from the yeast *Yarrowia lipolytica* for the production of sustainable fuels for aircraft and ships

3.1 Biogenic residues

Biogenic wastes are all organic waste resulting from human activities, such as agriculture, the maintenance of public green spaces, woods, and gardens, forestry, etc. Population growth and the waste produced amount are two interlinked factors. Indeed, as the population increases, so do all the related human activities, aimed not only at sustaining the population itself, but also at achieving well-being and a longer life expectancy. According to EUROSTAT data, in 2020, agriculture, forestry, and fishing generated 21 million tons of waste (Soares, 2025). In particular, agriculture produces 70% of the total biomass in Europe, but inedible residues are also considered agricultural waste. Indeed, 46% of this biomass is transformed into waste, mainly leaves, stems, straw, peels, seeds, and pomace. (European Commission, 2025; Soares, 2025). Currently, the main route for this type of waste is landfill or field incineration, both of which lead to increased CO_2 emissions or negative effects on the environment, as not all this waste biodegrades quickly (European Commission Joint Research Centre, 2020; Soares, 2025). Woody biomass, on the other hand, accounts for 60% of total European renewable energy production. Of this biomass, 49% is of secondary origin, i.e., by-products of forestry and wood processing. The remaining 51% is primary wood, which poses a threat to biodiversity: if the demand for woody biomass for energy purposes were

to increase, the cultivation of a certain type of fast-growing tree would be preferred to have primary biomass available at short notice, to the detriment of other slow-growing species (European Commission, 2025). However, there is a European directive, the recast Renewable Energy Directive (European Commission, 2025), which regulates the sustainable management of agriculture and forestry for energy purposes. However, this document also has its limitations, as it refers to areas that are off-limits for agriculture, but for forestry, it only prohibits protected areas or those with ancient trees. It is therefore necessary that the same criteria adopted for agriculture be transferred to forestry to avoid "aggressive reforestation" in favour of energy use but at the expense of biodiversity, just as it is important to establish criteria for the use of forest residues in the field (European Commission, 2025). Some of these residues are used to preserve the soil and keep it fertile, so even uncontrolled clearing of this waste can be harmful (European Commission, 2025). Wood waste also includes demolition waste, which is very difficult to recycle due to the presence of paint or ferrous materials, so the simplest solution is often energy recovery. However, this is always 'simple energy recovery', mainly combustion, from which heat and electricity are obtained. Anything beyond simple combustion requires not only industrial infrastructure, but also everything behind it, from the logistics of waste collection and sorting to the creation of specific standard protocols for each material flow (Kircher, 2023).

Indeed, a whole world of high-added-value substances can still be extracted from this biogenic waste, and the European Union is pushing hard towards this research through its funding programs, which take these technologies beyond the TRLs of the laboratory. The most sensible approach is a cascade approach: extract as many high value-added products as possible, then move on to thermal valorisation. From agroforestry waste, for example, it is possible to extract sugars, lignin, and solvents such as Cyrene. One example is the SWEETWOODS project, whose objective was to design an industrial-scale plant for the extraction of lignin and sugars from woody biomass, to be used instead of fossil raw materials for the foams for pipe insulation production, rigid polyurethane panels, and polymers for molding. Thanks to enzymatic pretreatment, 90% of the potentially extractable sugars and high-quality lignin were obtained (Sweetwoods, n.d.)

Lignin is a natural glue that holds fibers together, which is why it can replace fossil-based glues. The extraction of sugars, on the other hand, can be useful in the production of biofuels, such as bioethanol from yeast fermentation.

In the ReSolute project, however, the goal was the industrial-scale production of a non-toxic solvent, Cyrene, whose production had already been optimized on a pilot scale. Thanks to its safety and high efficiency, Cyrene can replace other solvents considered toxic, such as NMP (N-methyl-2-pyrrolidone), DMF (dimethylformamide), and DMAc (dimethylacetamide) (European Commission, n.d.).

From agro-industrial and domestic organic waste, such as fruit and vegetable peels, countless multifunctional substances can be extracted, such as polyphenols, flavonoids, anthocyanins, carotenoids, antioxidant molecules, sugars, essential oils, and dietary fibers, which can be reused in the cosmetics, nutraceuticals, and food

industries themselves in the form of supplements. The problem in this case lies in the high heterogeneity and seasonality of the starting biomass: there is currently no procedure for dividing organic waste according to the starting matrix. Indeed, organic waste collection is already complicated, as seen in the first chapter, with all the steps involved in separating pollutants to obtain high-quality digestate and compost. This greatly slows down the spread of wide-ranging 'cascade' solutions. However, there are still excellent opportunities for research and investment to be applied to agricultural businesses that supply a limited variety of products and that could find interesting business opportunities in extracting these products from their waste.

For these reasons, energy or thermal recovery remains the easiest route to take, especially since, once dried, it also accepts mixed streams. By exploiting this concept, however, other thermal techniques can be adopted that valorize such waste without stopping at energy recovery alone. This is the case with pyrolysis or gasification, in which biogenic waste is treated at high temperatures (between 400 °C and 800 °C) in a sub-stoichiometric oxygen atmosphere or in the total absence of oxygen, to obtain three main products: syngas, fuel oil, and biochar. In particular, syngas can be used both as a gaseous fuel and as a feed gas in anaerobic fermentation to produce intermediates, such as acetate.

This is the starting point for the BioSferA project (BioSferA, n.d.), a project funded by the European Union, of which this work is an important part. As part of the BioSferA project, a process has been developed for producing biofuels from biogenic waste and residues, including olive and vine prunings, cereal straw, and wood residues. The biomass is converted into syngas using a double-fluidized bed reactor. Syngas is used as feed gas for a two-stage fermentation process: the selected *Moorella thermoacetica* bacterial strain is used to produce acetate under anaerobic conditions; the acetate produced is fermented aerobically to produce triglycerides (TAG) lipids using *Yarrowia lipolytica*, an oil-producing yeast engineered to increase TAG production. Once the TAG lipids have been produced, a lipid extraction and purification process is performed. The deoxygenated linear chain paraffin is hydrocracked and selectively isomerized, yielding highly branched alkanes used as drop-in biofuel for aviation and marine applications. This work specifically explores the steps involved in the extraction and purification of TAGs. Subsequently, deoxygenated linear chain paraffin is hydrocracked and selectively isomerized to form highly branched alkanes. The ultimate goal is the production of sustainable fuels for the aviation and shipping sectors. Figure 3.1 shows a summary diagram of the process developed in BioSferA.

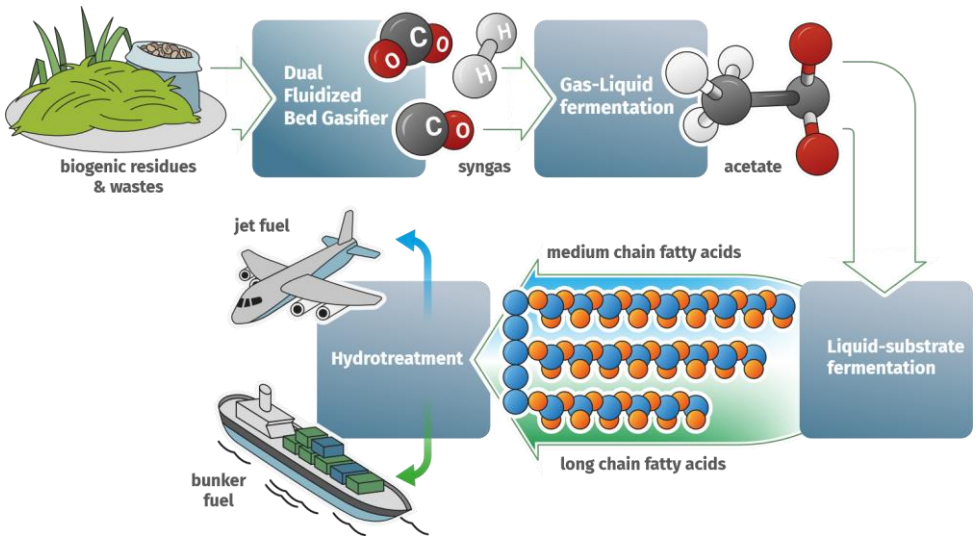


Figure 3.1: BioSFerA process diagram

3.2 Sustainable aviation and marine fuels

The transportation sector is still too closely tied to petroleum-derived fuel use, leading the way in greenhouse gas (GHG) emissions worldwide. While urban transportation is expected to decarbonize through the electrification of cars, decarbonizing maritime and aviation sectors in the short medium time requires a different kind of innovation due to limits imposed by long-distance travel and amount of energy required. The production of biofuels is crucial for the decarbonization of this vehicle type. Moreover, given the uncertain global geopolitical situation, energy autonomy would be a strong point in the European landscape. Often, however, the advantages of this type of renewable energy clash with ethical limits: the production of biofuels is linked to crops such as wheat and barley, typical food grains. The risk would be to take land away from food use and convert it to biofuel feedstock production. One solution to this problem could be the production of biofuels using biogenic residues and wastes as the source of biomass, which as a waste material, has a low carbon footprint compared to traditional sugar, starch, and oil plants used in the production of liquid biofuels.

According to a study by the International Maritime Organization (IMO, 2023) on greenhouse gas emissions, the shipping sector emitted 1056 million tons of CO_2 in 2018, accounting for about 3% of global anthropogenic emissions. If this trend were followed, without using any corrective action to reduce CO_2 emissions, the development of the shipping sector would lead to 90-130% of 2008 emissions in 2050. IMO has implemented a series of actions over the past ten years to reduce GHG emissions in the maritime sector, to reduce total annual GHG emissions by up to 50% and reduce CO_2 emissions by up to 70% compared to 2008, in compliance with the temperature targets of the Paris Agreement. In this regard, the second Symposium on alternative low- and zero-carbon fuels for shipping was

organized in 2021, highlighting emerging new fuel technologies and discussing how to make them globally accessible.

Members of the International Air Transport Association (IATA, 2022) at its 77th Annual General Meeting also highlighted the importance of aligning aviation with the goals of the Paris Agreement to limit global warming. The main goal is to achieve zero net emissions by 2050. The travel demand of 10 billion people is expected to be met in 2050, with at least 1.8 Giga tons of CO_2 to be abated. In addition, the aviation sector has had a strong rebound after the shutdown due to the COVID-19 pandemic. In addition to the need for travel, the airline sector has proven to be crucial for transporting vaccines, masks, and all medical devices useful in dealing with the pandemic emergency. To handle this sector development, it is essential to break free from the constraint of fossil fuels and produce affordable biofuels, the so-called sustainable aviation fuels (SAFs), on an industrial scale. This technology is already in use in Europe, North America and Asia and can reduce GHG emissions by 80% compared to fossil fuels, considering the entire production chain (Neste, 2023). SAFs are mainly produced from waste oils or other waste, such as animal and plant biomass (European Alternative Fuels Observatory, 2025).

This approach overcomes the ethical problem of using crops for human consumption or converting land to produce biomass specifically for biofuels. Although the combustion of SAFs also emits CO_2, converting waste biomass into energy reduces overall CO_2 emissions. This is especially true for plant biomass, whose combustion returns to the atmosphere only the CO_2 absorbed during photosynthesis.

Currently, the main techniques for producing SAFs are (European Union Aviation Safety Agency [EASA], n.d; Richter et al., 2018; Sagentia Consulting, n.d.; SkyNRG, n.d.; Song et al., 2023;):

- Hydroprocessed esters and fatty acids: This process utilizes hydrogen to break the double bonds in unsaturated triglycerides, thereby producing paraffins. The paraffins are then hydrocracked and isomerized to produce alkane chains suitable for use as jet fuel. Currently, all SAFs on the market are produced using this process.
- Alcohol-to-Jet: A process that converts alcohols (mainly bioethanol and bioisobutanol) into alkane chains through deoxygenation and oligomerization.
- Synthesized Iso-Paraffins: A biological process in which C6 chain sugars (such as glucose, a product of photosynthesis in plants) are converted via fermentation into farnesane, which is then hydrogenated into jet fuel.
- Gasification and Fischer-Tropsch synthesis: Biomass is subjected to gasification at temperatures of 800 °C - 1800 °C. This produces syngas, composed mainly of H_2 and CO. At this point, the Fischer-Tropsch process occurs, involving the reduction of CO by H_2 in the presence of suitable catalysts, which leads to the formation of alkane chains. These chains are then isomerized and converted into jet fuel.

- Power-to-Liquids: In this process, the energy (power) is used by the electrolyzer to obtain H_2 from water, combined with CO_2 (obtained from exhaust gases, but also from the air itself) to obtain syngas to be converted into fuel using the Fischer-Tropsch process.

There is also a counterpart to SAFs for marine fuels: sustainable marine fuels (SMFs). In this case, too, we can talk about biofuels obtained from the same oils and waste as SAFs, albeit produced using different methods and with different characteristics (Manikandan, Vickram, & Devarajan, 2025).

Solutions for a path towards sustainability exist, and Europe demands that we invest in them and implement them: by 2030, all flights departing from European airports must use a fuel blend containing 5% SAF. In 2020, SAF production could only cover 0.05% of jet fuel demand. It is therefore important to invest time, energy, and resources in researching these alternative fuels to meet the climate targets set for 2030 (European Alternative Fuels Observatory, 2025)

3.3 Experimental part

This chapter presents the results obtained in the BioSFerA project on the extraction and purification of TAGs from *Yarrowia lipolytica* yeast broth. This project has received funding from the European Union's Horizon 2020 research and innovation program under grant agreement No. 884208.

The main extraction techniques studied were:

- Pretreatment of biomass with microfiltration or centrifugation to reduce the amount of excess water in the broth;
- Steam explosion fractionation to break the yeast cell wall and recover the TAGs;
- Enzymatic hydrolysis of TAGs, the results of which were compared with those obtained with steam explosion;
- Combination of Steam explosion and enzymatic hydrolysis;
- Purification of TAGs with membrane filtration technology;
- Extraction with different green solvents;
- Separation of the oily substance obtained by distillation and a filter press

Within the project, this work represented an alternative to that developed by the other project partner, Bio Base Europe Pilot Plant (BBEPP), which involved cell disruption by homogenization.

3.3.1 Materials and Methods

The engineered yeast *Yarrowia lipolytica* was provided by BioSferA project partner BBEPP in the form of culture broth with a TAG concentration of 29.6 g/l.

TAGs were quantified both by a certified laboratory and using a spectrophotometric kit (Analytical KIT Chronolab Triglycerides-LQ GPO-POD Liquid).

The method used by the analysis laboratory is as follows:

- Sample preparation
    - Liquids: 2 ml of sample extracted with 5 ml of chloroform, shaken for 5 minutes on a vortex, centrifuged, lower phase (chloroform) removed and diluted twice with methanol. Filtered with PVDF filters for analysis
    - Solids: 2 g weighed and diluted with H_2O to a volume of 5 ml, then extracted as liquid samples.

- Instrumental method
    - Column: Agilent Poroshell 120 2.7 um 3.0x100mm
    - eluents: 2-propanol (A), acetonitrile (B), water 25 mM ammonium formate (C) in gradient
    - detection with q-orbitrap mass spectrometer, HESI source, in positive mode
    - quantification with standard for triolein (OOO), trilinolein (LLL), trilinonenin (LnLnLn), tristirin (SSS), trimystirin (MMM), tripalmitin (PPP)
    - screening for mixed triglycerides

Analytical results, obtained by spectrophotometric kit, show the total amount of TAG present in the test sample. The kit uses a lipase enzyme, lipoprotein lipase (LPL), which breaks down triglycerides by hydrolyzing the bond between glycerol and fatty acids. The glycerol is then converted several times through conversion reactions catalyzed by enzymes such as kinases and oxidases. The kit uses a colorimetric method: in the last conversion, a reaction with chlorophenol occurs in the presence of peroxidase, which gives the sample a more or less red color. A more intense color indicates a higher TAG concentration and vice versa.

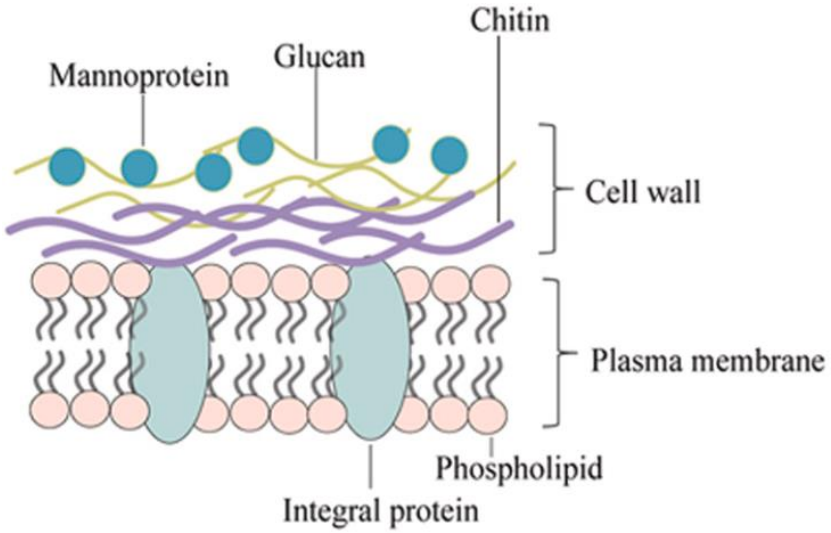
The enzymes papain from *Carica papaya* and Alcalase were supplied by Sigma Aldrich, while the enzyme zymolyase was supplied by VWR International srl.

3.3.1.1 *Yarrowia lipolytica*

Yarrowia lipolytica is an oil-producing yeast with a high lipid content: it can accumulate up to 20% of its dry weight in lipids, reaching up to 80% in the case of

specially engineered yeasts (Konzock, Zaghen, & Norbeck, 2021) and 90% when grown with glucose (Ko et al., 2018). Precisely because of this ability, *Y. lipolytica* is one of the most studied yeasts in the field of biofuels produced from oils and fats (Ko et al., 2018; Ledesma-Amaro & Nicaud, 2016).

Y. lipolytica cells have an outer wall composed mainly of glucans (β-1,3-D-glucan and β-1,6-D-glucan), mannoproteins, and chitin (Figure 3.2).



Extract from Zhang et al. Cell wall engineering of Yarrowia lipolytica to improve extraction efficiency of functional lipids, Food Bioscience (2025)

Figure 3.2: Y. lipolytica cell wall

Mannoproteins are mainly located towards the outer part of the matrix, allowing the cell to be permeable to both nutrients from the outside and waste substances from the inside. The layer closest to the cell membrane, on the other hand, is composed of chitin and β-1,3-D-glucans, which form a complex interwoven structure that provides rigidity to the entire wall. Chitin is responsible for this rigidity. In the wall of *Y. lipolytica*, it accounts for 7%, which is 1.5 times more than in *S. cerevisiae*, making the yeast very resistant to mechanical stress and complicating the extraction of TAGs (Zhang et al., 2025).

The situation worsens in the case of engineered yeasts, especially if modified to increase the content of lipids produced. In their article, Pomraning et al. (2015) found a thickening of the cell wall in *Y. lipolytica* cells during lipid accumulation.

Given the scarcity of information in the literature, during this thesis work, reference was initially made to articles dealing with the cell rupture of microalgae (Lorente et al., 2015; Onumaegbua et al., 2018; Steriti et al., 2014) which also have a very thick and resistant cell wall. Subsequently, the focus was adjusted thanks to the results obtained along the way.

Table 3.1 shows the characteristics of the TAGs present in untreated *Y. lipolytica*. As can be seen, the TAGs in *Y. lipolytica* are mainly triolein and

tristirine, together with mixed triglycerides composed of fatty acids such as linoleic acid (L), linolenic acid (Ln), oleic acid (O), stearic acid (S), and palmitic acid (P). These data are also present in deliverable 3.6 of the BioSFerA project (BioSFerA Consortium, 2023).

Table 3.1: Characterization of TAGs

TAG	**Concentration (mg/kg)**
LLL	2.12
LLnL/OLnLn	1.56
LPL/OPLn	0.75
OLL/OLnO/SLLn	1.61
OLLn/SLnLn	1.99
OLO/LSL/OSLn	1.29
OLP/PSLn	0.64
OOO	16.5
OSO/LSS	0.53
PLLn	0.98
PLnLn	0.97
SLnS/OLS	0.86
SSS	14

3.3.2 Steam explosion

The steam explosion plant utilizes saturated or slightly superheated steam to treat biomass for a specified period. When the steam passes from high temperature and pressure conditions to atmospheric conditions, expanding suddenly, it causes the internal bonds of the treated biomass to break.

The plant consists of a 30 l jacketed batch reactor (Figure 3.3) that can operate with saturated steam at a maximum pressure of 26 bar and a maximum temperature 230 °C. The process parameters are maintained by a remote-control system, which also controls the opening and closing of reactor valves, the expansion vessel, and the steam supply. When the valve separating the reactor from the 300-liter expansion vessel (Figure 3.4) below is opened, the system, which is also connected to a chimney in the atmosphere, returns the pressure to atmospheric conditions, and the material expands into the vessel below. The expansion takes place under almost isentropic conditions, usually within a few seconds.

When the expansion tank is completely depressurized and the internal temperature is low enough to allow an operator to intervene, again using the remote-control system, the lower butterfly valve is opened to collect the waste materials.

Table *3.2* summarizes the four stages of the process.

Figure 3.3: Steam explosion reactor

Figure 3.4: Steam explosion expansion vessel

Table 3.2: Description of the steam explosion process

Stage	Description
Stage 1 Loading the biomass	To reduce material losses, the biomass was loaded manually into the reactor after the system had been preheated
Stage 2 Pressure operation	In stage 2, by opening the control valve for steam injection, the reactor was brought from the original pressure (1 bar) to the ideal process parameters in a short time (in the order of minutes)
Stage 3 Maintaining process conditions	Once the operating conditions inside the reactor were reached, the temperature and pressure were maintained at the required levels for the necessary pretreatment time using the remote-control software
Stage 4 Collection of hydrolyzed material	The expansion valve is opened via the remote-control system, and the biomass is expanded. It is then allowed to cool and finally collected

The severity factor (R), which depends exponentially on the temperature inside the pressurization vessel and linearly on time, is the main parameter that must be adjusted for the steam explosion process, as shown in (1):

$$\boldsymbol{R = t \cdot e^{\frac{T-100}{14.75}}} \qquad (1)$$

Where R is the severity factor, t is time, T is the temperature and 14.75 is a parameter determined empirically based on first-order kinetics assumptions, which quantifies the activation energy for the breaking of bonds within the biomass.

A total of seven steam explosion tests were performed, varying conditions such as:

- Temperature and pressure
- Volume of biomass treated
- Any pre-treatment aimed at reducing excess water in the treated fermentation broth (membrane separation and centrifugation)

Table 3.3 shows a summary of the test conditions used. The tests were conducted in chronological order. The first three tests were conducted during an initial period. The operating conditions for the last four tests were determined after reflecting on the initial period.

Table 3.3: Summary of steam explosion test conditions

	Test 1	Test 2	Test 3	Test 4	Test 5	Test 6	Test 7
Pretreatment	-	-	-	-	MF	CF	CF
Temperature (°C)	174	174	210	150	150	150	150
Pressure (bar)	10	10	20	5	5	5	5
Duration (min)	10	10	10	10	10	10	10
Severity (min)	1529	17899	2228	301	301	301	301
Volume in (l)	4	20	20	20	4	5	15
Volume out (l)	28	23	23	23	28	27	27

Between the first and second tests, only the amount of biomass input changed. This factor correlated with the degree of reactor filling and the amount of steam input. A reactor that is emptier than full leads to a higher degree of dilution.

In the third test, on the other hand, a higher reactor filling level was maintained, and the influence of pressure on the ability to break the outer cell wall of the yeast and thus release more TAG was tested.

In the first three tests, the more severe process conditions were justified by the attempt to break the yeast cell wall to release all the intracellular products. Since *Yarrowia lipolytica* is an engineered yeast, it is assumed that it has a cell wall that is very difficult to break, like that of algae, so it was necessary to use high temperatures and pressures.

However, initial results, compared with the initial characterization of untreated *Yarrowia*, showed that following steam explosion, the total amount of TAG increased by 24% following test 2, but decreased by 24% following test 3 (Table 3.4).

Table 3.4: Total amount of TAG before and after steam explosion treatment in tests 2 and 3

Sample	**TAG quantity (mg)**
Untreated *Y. lipolytica*	882
Test 2	1093
Test 3	673

This indicates that, while severe conditions are necessary to break the cell wall and release more TAG than the analysis can detect in the untreated sample (this is due to the analysis extraction protocol that sometimes is less efficient respect to the steam explosion extraction), overly severe conditions lead to hydrolysis of the bond between glycerol and fatty acids, breaking down the triglyceride, which is then no longer detected during analysis.

This hypothesis has also been confirmed in the literature. According to Vecchio et al. (2008), the carboxylic chains of triglycerides degrade at temperatures between 160 and 380°C. For this reason, tests 4 to 7 were conducted at 150°C for 10 minutes to prevent the breakdown of triglycerides.

As shown in Test 5, pretreatment was performed using microfiltration (MF) membranes. The concentrate was then subjected to steam explosion. In tests 6 and 7, pretreatment was performed using a three-phase centrifuge (CF). In these cases, the concentrate and permeate from centrifugation were treated with steam explosion, respectively. Figure 3.5 shows the appearance of *Y. lipolytica* after steam explosion treatment.

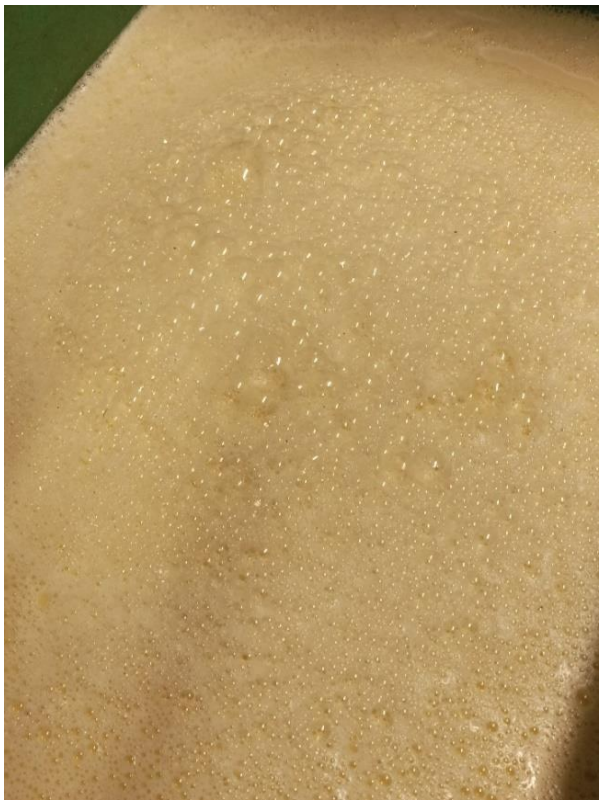


Figure 3.5: Foamy appearance of Y. lipolytica after steam explosion treatment

The best pretreatment was found to be that performed in test 5, consisting of an initial separation of excess water using a microfiltration membrane, the permeate of which was then subjected to steam explosion and finally further microfiltered.

Table 3.5 summarizes the concentrations involved.

The addition of a filtration phase prior to steam explosion therefore allows for more efficient cell disruption, even at low temperatures. Therefore, the best procedure involves an initial microfiltration phase, followed by a steam explosion at 5 bar for 10 minutes. To avoid problems of excessive dilution, an additional filtration phase is considered necessary, which significantly reduces the volumes involved. Following this path, an 18% increase in TAGs was obtained compared to untreated *Yarrowia* and a 98% increase compared to the concentrate from which the steam explosion begins.

Table 3.5: Concentration and amount of TAGs obtained as the type of treatment performed varies

Sample	**Volume (l)**	**Concentration (g/l)**	**TAG (g)**
Untreated *Y. lipolytica*	20	29.6	592
Concentrated *Y. lipolytica*	4	88	352
Y. lipolytica after test 5	28	24.9	697
MF Concentrate after test 5	7	80.5	564

Finally, by microfiltering the hydrolysate, it is possible to achieve a separation efficiency of 81%, yielding 564 g of concentrated TAG in a volume four times smaller than that of the hydrolysate from the steam explosion, which is also convenient given the industrial scalability. From this point on, when referring to the steam explosion product or hydrolysate, we are referring to the product of this specific test, test 5.

3.3.3 Filtration

A PVFD (polyvinylidene fluoride) microfiltration membrane with a cut-off of 0.2 μ and a polyamide composite nanofiltration membrane with a cut-off of 300 Da were used with an overpressure of 1-1.5 bar to perform the biomass treatment process in a pilot membrane filtration plant supplied by Idea3 (Figure 3.6).

The membranes were initially used downstream of the steam explosion to reduce the water content and concentrate the TAGs. Subsequently, the microfiltration membrane was used upstream of the steam explosion in test 5 as a pretreatment to concentrate the fermentation broth and assess whether this would allow the steam to act more effectively on the cell wall of *Yarrowia* to break it down. Indeed, two products can be obtained from the filtration system: the permeate fraction, composed of all molecules smaller than the membrane pores and therefore very "watery," and the concentrate fraction, which, on the contrary, has a thicker and "milky" appearance (Figure 3.7). In the case of TAGs, it has been noted that the molecular cut-off of nanofiltration is not selective enough to allow TAGs to be concentrated in just one of the two output products. Furthermore, given the presence of material such as cell residues, which also makes the starting broth viscous, the risk of using membranes with too tight meshes is that of the fouling effect, in which the membrane clogs very easily and the entire product is lost during washing because it remains adhered to the membrane itself during treatment or need to increase the process pressure too much in order to proceed with filtration. Therefore, after an initial screening using the product of steam explosion test 1, separation and concentration tests were conducted only with microfiltration membranes.

Figure 3.6: Pilot membrane filtration plant

Figure 3.7: Samples of permeate and concentrate after microfiltration

3.3.4 Centrifugation

The centrifugation process was performed in continuous in a three-phase centrifuge (CLARA 20 – Alfalaval) (Figure 3.8) with a flow rate of 0.24 m^3/h, a pressure of 1.8 bar and a bowl rotation speed of 9200 rpm.

In this case too, the products are called supernatant and sedimented. Figure 3.9 shows examples of the products obtained.

Figure 3.8: Centrifuge

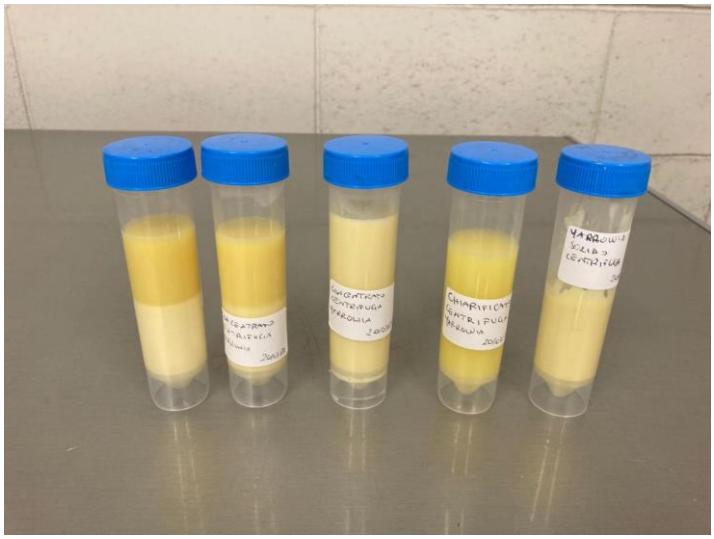


Figure 3.9: Centrifuge products

3.3.5 Enzymatic extraction

To break down the outer wall of yeast and release accumulated TAGs, a biological approach was attempted. Special enzymes, such as proteases and glucanases, were used. These enzymes attack the macrostructures of the wall, including proteins and glucans. *Y. lipolytica*'s outer part (see Figure 3.2) mainly consists of mannoproteins (proteins linked to mannose) and β-glucans. Proteases hydrolyse the peptide bonds of mannoproteins, releasing mannans. This step exposes β-glucans to glucanases, which then solubilise them (Gautério et al., 2023). In this study, the effects of three commercial enzymes (papain, alkalase, and zymolyase) were compared. The tests were conducted on both concentrated *Y. lipolytica* and the product of test 5 of the steam explosion.

3.3.5.1 Papain

Papain is an enzyme belonging to the protease class, hydrolases that catalyze the hydrolysis of peptide bonds in proteins and amino acids and is extracted from the fruit of the papaya (*Carica papaya*) (Amri & Mamboya, 2012).

The tests were carried out following the protocol described in Vukasinovic et al. (2007), with slight modifications. The tests were performed in 500 ml Duran glass bottles. In one bottle, 450 g of concentrated *Y. lipolytica* was added, while in the other, the same amount of post-SE hydrolysate was added. To both bottles, 2.5% w/w papain enzyme and 10% v/v phosphate buffer at pH 6 were added. The two bottles were stirred at 40 °C for 72 hours at a speed of 150 rpm. At the beginning and end of the reaction, a sample was taken, boiled at 100 °C for 10 minutes to deactivate the enzyme, and the TAG concentration was measured using the Chronolab spectrophotometric kit.

To avoid false measurements due to enzyme interference with the kit reagents, the first sample taken at the beginning of the test was subtracted from the subsequent samples, acting as a "blank."

Both tests led to a very similar increase in TAG content, equal to 4.84 g/l and 4.31 g/l, respectively.

3.3.5.2 Zymolyase

Zymolyase is a complex enzyme preparation and is potentially capable of breaking down the cell wall by acting on multiple compounds in the structure. Indeed, it mainly contains β-1,3-glucanase and β-1,3-glucanlaminaripentaidrolase, which convert glucans into glucose and pentoses. In smaller quantities, proteases and mannanases are also present, which affect the protein part of the cell wall (United States Biological, n.d.)

To carry out the tests, the enzyme sheet protocol (United States Biological, n.d.) was followed, with slight modifications. The tests were performed in 500 ml or 100 ml Duran glass bottles. In one bottle, 450 g of concentrated *Y. lipolytica* was added, while in the other, 90 g of post-SE hydrolysate was added. 10 ml and 2 ml of zymolyase enzyme preparation and 10% v/v phosphate buffer at pH 7.5 were added to both bottles, respectively. The two bottles were stirred at 30°C for 60 minutes at a speed of 150 rpm. Samples taken during the reaction were heated to 60°C for 5 minutes to deactivate the enzyme, and the TAG concentration was measured using the Chronolab spectrophotometric kit.

To avoid false measurements due to enzyme interference with the kit reagents, the first sample taken at the beginning of the test was subtracted from the subsequent samples, acting as a "blank."

Both tests led to a very similar increase in TAG content, equal to 6.59 g/l and 6.47 g/l, respectively. Despite the complexity of the enzyme preparation, the extraction yield proved rather disappointing. In this case, as with the previous enzyme, this may be because the enzyme preparation weakens the cell wall, but a further step is then necessary to break it down completely. In some articles, indeed, the next step consisted of further mechanical treatment with glass beads (Ma et al., 2022**;** Qiao et al., 2017). However, as we only wanted to compare the action of different enzymes on a pretreated and untreated cell wall, this additional step was not carried out in this study.

3.3.5.3 Alcalase

Alcalase is an enzyme preparation composed of 50% glycerol, 41% water, and 9% proteases extracted from *Bacillus licheniformis* (Tacias-Pascacio et al., 2020).

The tests were performed following the Takalloo et al. (2020) protocol, with slight modifications. The tests were performed in 500 ml Duran glass bottles. 450 g of concentrated *Y. lipolytica* was added to one bottle, while the same amount of post-SE hydrolysate was added to the other. 0.2% w/w of proteases from Alcalase enzyme preparation and 10% v/v of phosphate buffer at pH 7 were added to both bottles. The two bottles were stirred at 55 °C for 48 hours at a speed of 150 rpm. Samples taken during the reaction were boiled at 100 °C for 5 minutes to deactivate the enzyme, and the TAG concentration was measured using the Chronolab spectrophotometric kit.

In this case too, especially given the presence of glycerol in the Alcalase enzyme preparation, which could have interfered with the kit and given a false positive value, the sample taken at the start of the test, immediately after the addition of Alcalase, was considered a "blank" and its value was subtracted from the subsequent measurements. During the test, a total of 5 samples were taken, including the blank, at intervals of 9 or 24 hours from each other, and named T0-T4. Figure 3.10 shows the progress of the test over time.

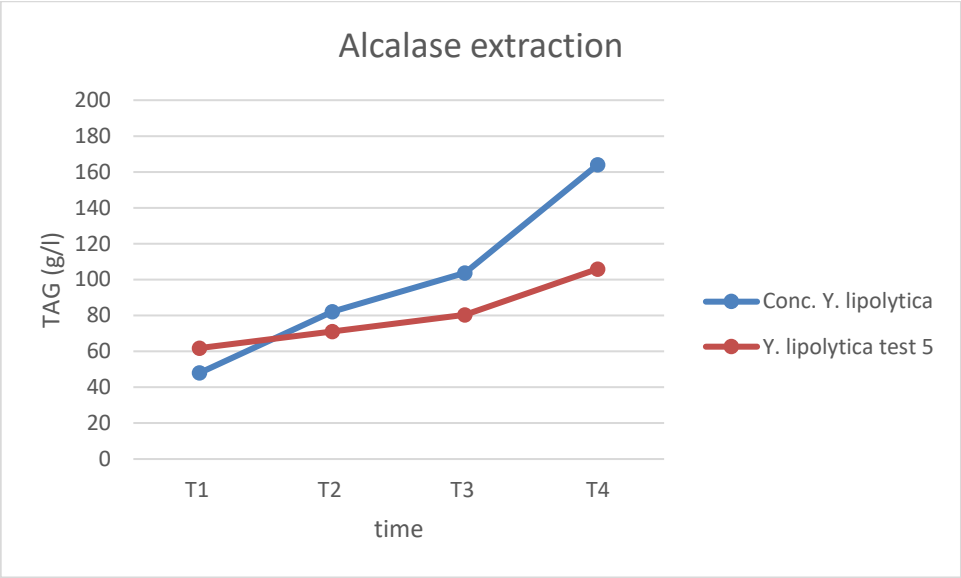


Figure 3.10: Extraction with Alcalase

In this case, the increase in extracted TAGs is significantly more appreciable, reaching 164 g/l and 106 g/l of extracted TAGs in concentrated *Y. lipolytica* and post-steam explosion *Y. lipolytica*, respectively. However, the use of Alcalase on biomass pretreated with steam explosion does not promote cell rupture when compared to untreated biomass. The hypothesis that may explain this phenomenon is that steam explosion and enzymes act on the same molecules of the cell structure, simply in different ways. Indeed, after steam explosion, some *Y. lipolytica* cells lose their cell wall under the action of high temperature and pressure. It is therefore likely that the enzyme acts on molecules or fragments of the wall that have already finished in solution after steam explosion. It is as if it were a "random" action, in which the enzyme does not necessarily act on the mannoproteins of cell walls that are still undamaged, but could act on fragments of them, without leading to any actual increase in breakdown or leakage of TAGs. On the contrary, the action on cells not pretreated in steam explosion is more effective and targeted, because wherever the enzyme binds, it will be a mannoprotein of an intact cell structure, which will thus be degraded and lead to a greater leakage of TAG.

3.3.6 Solvent extraction

Both the steam explosion hydrolysates and those obtained with the enzyme preparation that provided the best results were subjected to TAG extraction tests with solvent. This is because the tests performed led to cell rupture, releasing more TAG into the solution than the starting fermentation broth make them available for final separation that could be performed by green solvents. This concluding step was also the one selected by the partner BBEPP for the ultimate purification of the

broth post-homogenisation. Pursuing a parallel course facilitated a more meaningful comparison between the two processes.

In literature, the most widely used method for extracting lipids with solvents is the Folch method, which utilizes a mixture of methanol and chloroform, or the variant developed by Bligh and Dyer, which includes the addition of water (Breil et al., 2017). Although these methods represent the analytical standard, there is a growing trend towards choosing methods that have less impact on humans. Chloroform is a potentially carcinogenic agent. It can cause corrosive effects on the skin, eyes, and respiratory system through its vapors. In addition, prolonged exposure to chloroform can cause long-term damage to the liver, kidneys, and nervous system. Similarly, methanol can damage eyesight, even leading to blindness (Pila et al., 2022). Despite this, both substances continue to be used industrially for their properties. Chloroform, because it is immiscible in water, forms a two-phase mixture and promotes the transfer of lipids based on their polarity. Methanol, in contrast, is miscible in water and cannot do this. However, methanol acts as a precursor to the later separation with chloroform. It can break the hydrogen bonds of lipids and proteins thanks to its polarity. However, ethanol could also perform this task. Ethanol is less toxic than methanol and generally safer because it is more widely used and distributed in industry (Saini et al., 2021). In addition to ethanol, other solvents such as isopropanol, hexane, ethyl acetate, cymene, and limonene can also be used in lipid separation. Unfortunately, although these solvents are referred to as "green" and "biobased," and could represent a valid alternative to traditional methanol and chloroform, as they are less toxic to humans and the environment, the yields are lower than those obtained with the standard method. However, it is still worth continuing to search for ideal mixtures for effective separation (Breil et al., 2016**;** Imatoukene et al., 2020; Saini et al., 2021).

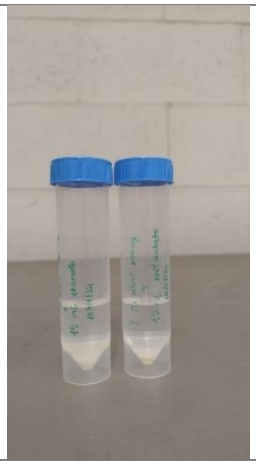
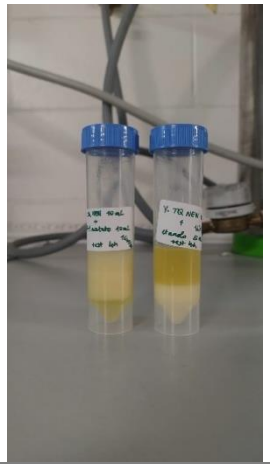
3.3.6.1 Ethanol and ethyl acetate

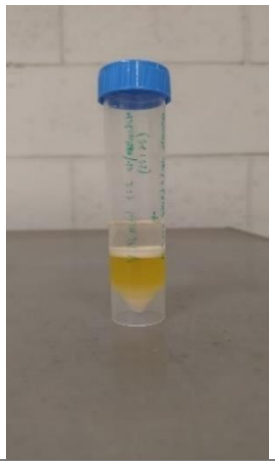
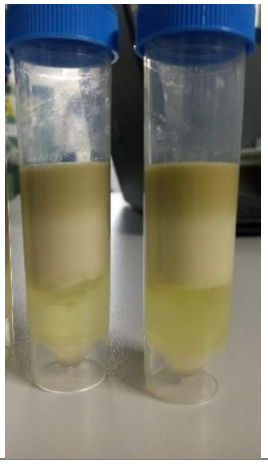
In this work, several small-scale (ml) solvent extraction tests were conducted. Table 3.6 summarizes all the most significant test conditions with ethanol (EtOH) and ethyl acetate (EtAc). After the reaction time had elapsed, each falcon tube was centrifuged at 3500 rpm for 10 minutes, and both the liquid and solid, where possible, diluted components were analyzed using the Chronolab spectrophotometric kit. The tests were performed on the post-microfiltration concentrate from test 5 of the steam explosion, referring to the protocols reported in the literature with minor modifications (Breil et al., 2016; Breil et al., 2017; Lin et al., 2004; Saini et al., 2021).

In the first case, pure EtOH or EtAc was used with a biomass/solvent ratio of 20:1 mg/ml. This ratio did not lead to visible phase separation, likely due to the small volumes, and would require unsustainable amounts of solvent if scaled up. Therefore, this approach was not effective.

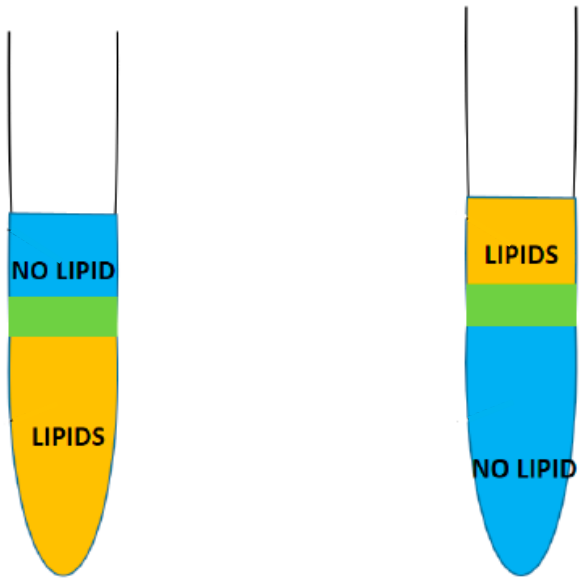
In the second case, a 2:3 v/v ratio between biomass and pure solvent was used. Although two distinct phases were observed, the TAGs were not transferred to the aqueous phase as expected, indicating this approach was also unsuccessful.

Table 3.6: Solvent extraction test with ethanol e ethyl acetate

Sample	Solvent	Methods	Comments	Picture	Ref.
300 mg	EtAc 15 ml EtOH 15ml	150 rpm 1h 30 °C	• Sample/Solvent ratio too high • No visible separation		(Breil et al., 2016)
10 ml	EtAc 15 ml EtOH 15ml	150 rpm 4h 30 °C	• Only 2 visible phases • Low TAG content in organic phase		(Saini et al., 2021)
2 g Conc.	EtAc+EtOH 2:1 (v/v) Or EtAc + EtOH 1:1 (v/v)	250 rpm 1h 30 °C	• Visible separation with liquid • Low TAG in organic phase		(Lin et al., 2004)
2 g powder			• The powder does not dissolve • No separation is noticeable		

10 ml	1) 10 ml mix EtAc + EtOH 3:1 (v/v) 2) 0.5 ml water + 1 ml EtAc	1) 250 rpm 10 min 30 °C 2) 250 rpm 10 min 30 °C	• Visible separation • Low TAG in organic phase		(Breil et al., 2017)
10 ml	1) 10 ml mix EtAc + EtOH 3:1 (v/v) 2) 0.5 ml KCl 0.8% + 1 ml EtAc	1) 250 rpm 10 min 30 °C 2) 250 rpm 10 min 30 °C	• 4 different phases identified • Salt facilitates visual separation of phases but not TAG		(Breil et al., 2017)

In the third case, a mixture of EtAc and EtOH with a ratio of 2:1 or 1:1 v/v was used on both the liquid and powder concentrates. Preparation involved further centrifugation, removal of the most viscous component, drying in an oven at 100°C for 40 hours, and then crushing into powder. Testing with the powder was halted due to negative results and incomplete interaction with the solvent. With the liquid concentrate, three distinct phases were identified. Thanks to the article by Breil et al. (2017), it was clear that the phase of interest containing the lipids should be the upper phase with ethyl acetate. In the classic Bligh and Dyer method, methanol binds with polar substances, such as sugars and proteins, separating them from the rest of the broth and transferring them to the upper phase (left image in Figure 3.11), while chloroform binds to lipids and transfers them to the lower phase. In the case of the green version of Bligh and Dyer, however, the lipids are transferred to the upper phase jointly with ethyl acetate, while the sugars are transferred to the lower phase along with ethanol (right image in Figure 3.11). Since the intermediate cell layer is very soft, with a high risk of “soiling” and “contaminating” the very thin upper phase with lipids at the time of sampling, thus falsifying the result in a positive direction, the Falcon tube was placed in a freezer at 0 °C. Given that the melting point of ethyl acetate is -83 °C, this resulted in a very compact intermediate layer of cells, while the ethyl acetate on the surface remained liquid, facilitating sampling. This technique was also used subsequently in other tests where this type of phase separation was observed.



Breil et al. "Bligh and Dyer" and Folch Methods for Solid–Liquid–Liquid Extraction of Lipids from Microorganisms. Comprehension of Solvatation Mechanisms and towards Substitution with Alternative Solvents, Int. J. Mol. Sci. (2017)

Figure 3.11: Comparison between classic Bligh and Dyer method and green Bligh and Dyer method

The upper ethyl acetate phase was expected to contain lipids, but the concentration of lipids there remained low (<1 g/l TAG).

In the fourth case, two consecutive steps were performed: a 3:1 v/v mixture of EtAc and EtOH was added in a 1:1 ratio with the biomass. The mixture was stirred for 10 minutes at 30 °C. In the next step, 0.5 ml of water and 1 ml of EtAc were added. After stirring for 10 minutes, the Falcon tube was centrifuged. According to the reference study (Breil et al., 2017), the addition of water would promote the formation of a two-phase system, but it did not facilitate lipid migration.

As for the fifth case, again in the same study (Breil et al., 2017), the use of salt (KCl) was proposed to promote, thanks to the cationic effect due to the addition of salt, the movement of lipids into the organic phase, while KCl remains in the liquid phase, to which it is more akin. The result was the formation of four phases. From bottom to top: a small accumulation of cell pellets, a straw-yellow liquid (probably a solution of ethanol, water, and sugars), another usual cell phase, and finally a darker, cloudy phase, which was very viscous and difficult to analyze with the spectrophotometric kit.

Overall, none of the tested approaches yielded a significant transfer of TAGs to the desired phase. The most plausible hypothesis is that, although the ethanol/ethyl acetate mixture is widely used in literature and proposed as a valid alternative to standard separation with methanol/chloroform, it presents some critical issues. First, the difference in polarity between the two solvents is sufficient to create visible phases, but it is not high enough to allow the migration of lipids in

one phase rather than the other. Ethanol is a polar solvent, but ethyl acetate is also a moderately polar solvent. For this reason, two new solvents, p-cymene and d-limonene, both nonpolar, were introduced in subsequent tests, used in combination with ethyl acetate and water.

3.3.6.2 P-Cymene and ethyl acetate

P-cymene, or 4-isopropyltoluene, is a terpene, an aromatic hydrocarbon widely used in the cosmetics and perfume industry, particularly for its thyme and cumin aroma. Recently, it has been studied as a solvent that could replace others such as n-hexane (Rather et al., 2023) DMA (Dimethylacetamide), THF (Tetrahydrofuran), and toluene (Ye & Thompson, 2025). In this study, it was used together with ethyl acetate and water for the extraction of TAGs from the fermentation broth of *Y. lipolytica* subjected to steam explosion and then microfiltered to be concentrated.

However, as no specific protocol was found in the literature, several attempts were made, first in the order of a few ml in Falcon tubes and then on a slightly larger scale in bottles, using the results (mostly visual) obtained from previous tests based on ethanol and ethyl acetate.

A mixture of ethyl acetate, p-cymene, and water (polar solvent/nonpolar solvent/water) was formulated in a ratio of 8:10:11 v/v/v (Saini et al., 2021), to which 200 g/l of KCl and the biomass were added in a ratio of 1:15 v/v with the solvent mixture. The mixture was vigorously stirred and left to agitate at 30 °C for 1 hour at 150 rpm. In the case of the Falcon test, the test tube was centrifuged at 3500 rpm for 10 minutes once the reaction was complete. In the case of the bottle, however, the phases were left to settle.

Figure 3.12 and Figure 3.13 show the appearance of both the test tube and the bottle.

As can be seen, there are four phases. From bottom to top: cell pellets and salt, the first, cloudier phase, emulsion, and the second, transparent phase. In addition, further bubbles can be seen on the surface of the Falcon tube, which are not visible in the bottle. However, analysis of the three liquid phases shows that the phase with the highest TAG content is the emulsion. Once again, there was no separation of lipids in either of the two solvents. Furthermore, as the emulsion is difficult to separate, it is also difficult to envisage the scalability of this method.

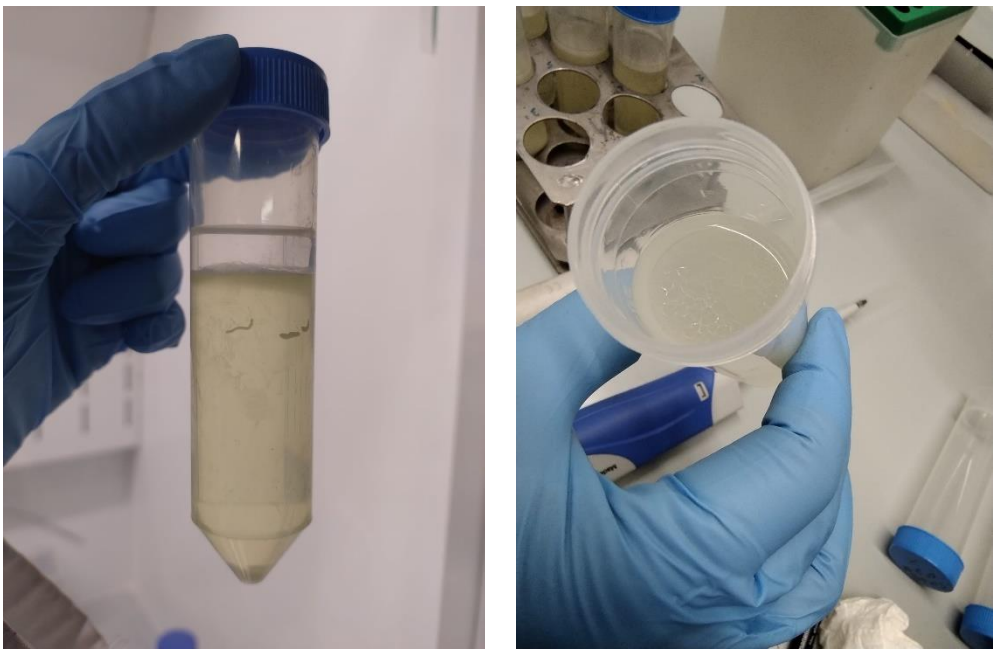


Figure 3.12: Solvent extraction with cymene in Falcon tube

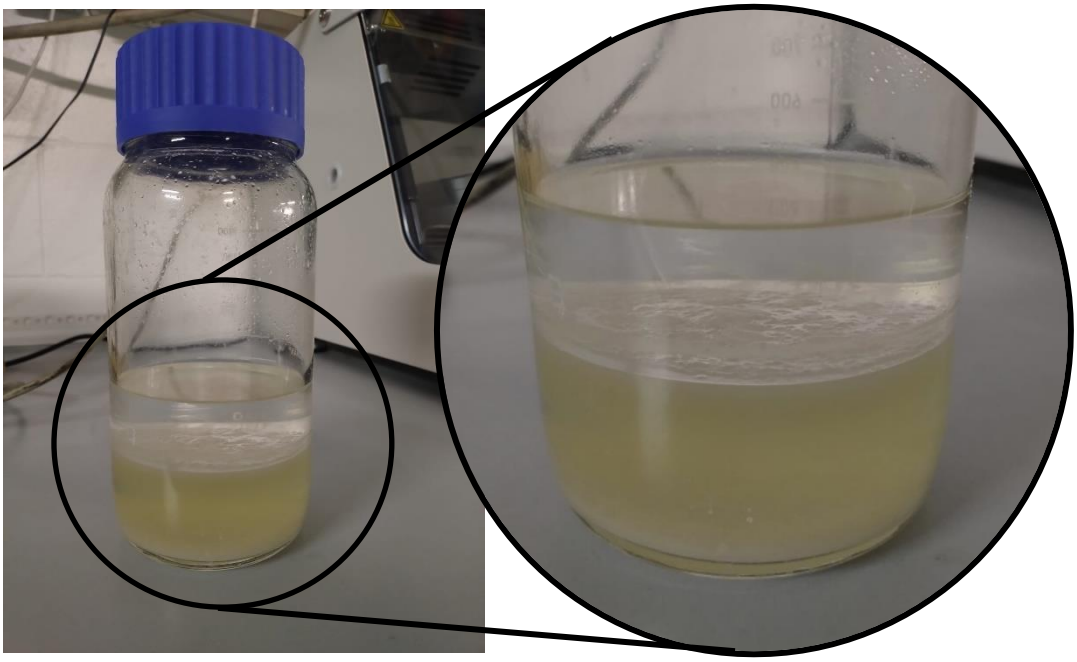


Figure 3.13: Solvent extraction with cymene in bottle

3.3.6.3 D-Limonene and ethyl acetate

D-limonene is also an aromatic hydrocarbon, a terpene with a typical orange scent, used in cosmetics and the food industry. It is also present in many cleaning products, including industrial ones, due to its degreasing properties. It is therefore an excellent potential solvent for lipids.

In this case, too, tests with d-limonene and ethyl acetate were first performed on a small scale in Falcon tubes, and then in 2 l bottles.

The previous protocol was followed, with a few minor modifications. In particular, the amount of salt was reduced, since in the previous test it had almost completely precipitated to the bottom, and the amount of biomass was increased in relation to the solvent, to try to obtain an intermediate phase that was easier to separate.

A mixture of ethyl acetate, limonene, and water (polar solvent/nonpolar solvent/water) was formulated in a ratio of 8:10:11 v/v/v (Saini et al., 2021), to which 75 g/l of NaCl and the biomass were added in a ratio of 1:3 v/v with the solvent mixture. The mixture was vigorously stirred and left to agitate at 30 °C for 1 hour at 150 rpm. In the case of the Falcon test, the test tube was centrifuged at 3500 rpm for 10 minutes once the reaction was complete. In the case of the bottle, however, the phases were allowed to settle. In addition, a bottle similar to the previous one was prepared, but without the addition of salt, to evaluate the actual benefits of adding salt. The bottle with salt was labeled "A," while the one without salt was labeled "B."

Figure 3.14 and Figure 3.15 show the appearance of both the test tube and the bottles.

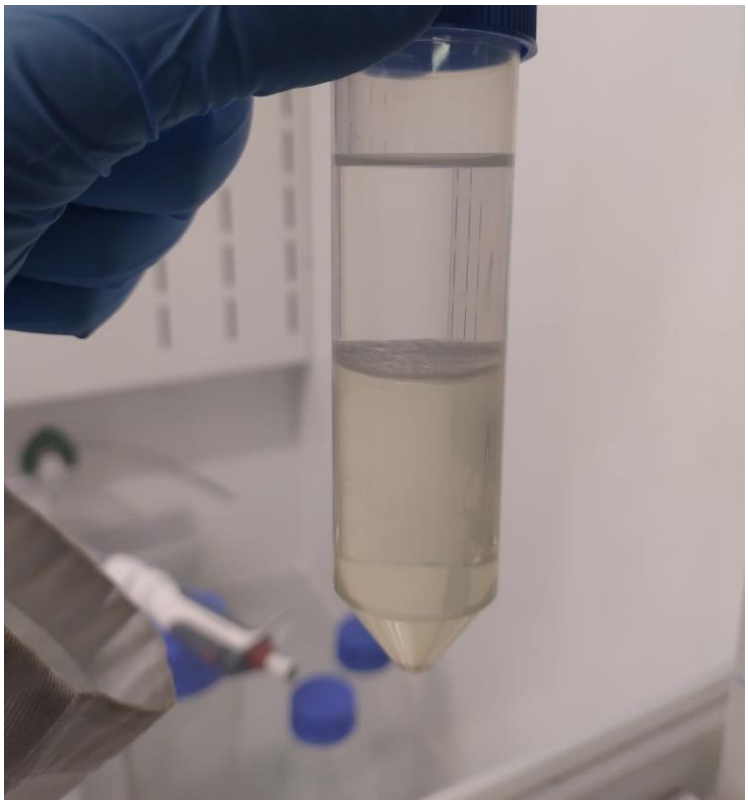


Figure 3.14: Solvent extraction with limonene in Falcon tube

As can be seen, the same four phases as in the Falcon test with cymene can also be identified in the Falcon test with limonene. However, the bottle test reveals macro differences that cannot be seen in Falcon.

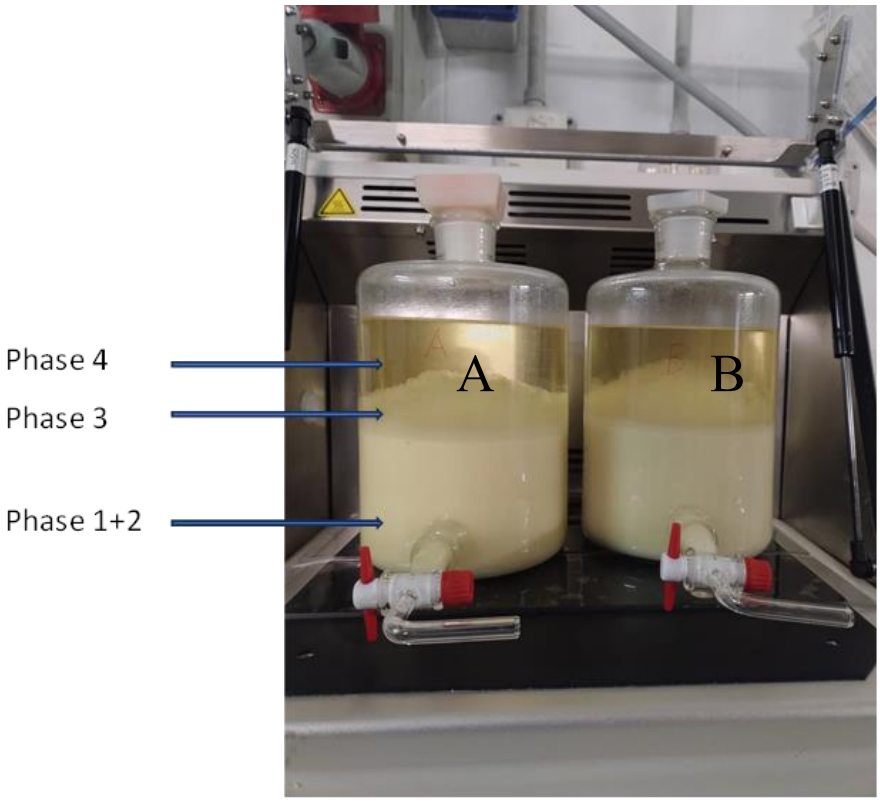


Figure 3.15: Solvent extraction with limonene in bottle

First, an intermediate phase with a very thick emulsion can be observed. Furthermore, the presence of salt leads to the formation of four phases that are more easily distinguishable visually in bottle A than in bottle B, once they have been left to settle. However, with the aid of the tap under the two bottles, it was only possible to recover three phases:

- the upper phase, called “phase 4,” which is very liquid and consists mainly of solvent;
- the middle phase, called “phase 3,” in which the emulsion is visible;
- the lower phase called “phase 1+2,” much more viscous than the others, composed of the cellular phase (and salt in the case of bottle A).

The lower phase is called “phase 1+2” because, although visually (especially in bottle A) two distinct phases can be seen, the boundary between the two becomes blurred and imperceptible when drawn off with a tap. In bottle A, the same salt that facilitates the visual distinction between the two phases makes it more difficult to physically separate them, which can only be done by centrifugation.

However, the three phases were all centrifuged, demonstrating that sampling with a tap led to contamination, especially in phases 1+ 2, and phase 3. Figure 3.16 shows the result of centrifuging these two specific phases for both bottle A and bottle B.

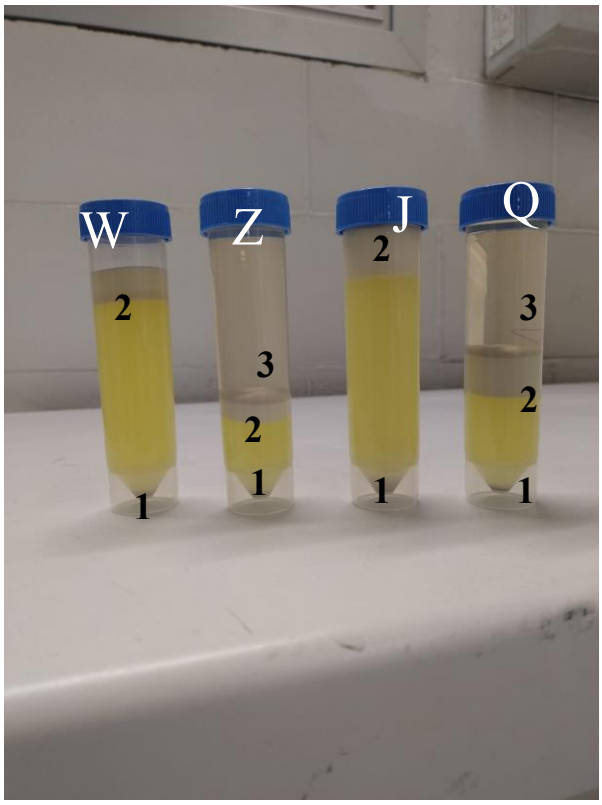


Figure 3.16: Result of centrifugation of phases 1+2 and phase 3. The Falcon tubes labeled "W" and "Z" refer to bottle A, while those labeled "J" and "Q" refer to bottle B

However, centrifuging phase 4 did not reveal any further "sub-phases." All phases present were further analyzed for each falcon, as was phase 4. The results are shown in Table 3.7 and Table 3.8.

Table 3.7: Results of phase analysis for bottle A

ID	Sample	Volume (l)	Concentration (g/l)	TAG (g)
W1	Bottle A phase 1+2	0.862	0.793	0.683
W2	Bottle A phase 1+2	0.189	81.2	15.4
Z1	Bottle A phase 3	0.029	0.683	0.020
Z2	Bottle A phase 3	0.022	101	2.18
Z3	Bottle A phase 3	0.014	2.58	0.037
	Bottle A phase 4	0.850	0.110	0.094

As can be seen, for bottle A, the phase with the highest TAG concentration is phase 3, the emulsion phase, particularly in subphase Z2, represented by the most solid and viscous component.

Table 3.8: Results of phase analysis for bottle B

ID	Sample	Volume (l)	Concentration (g/l)	TAG (g)
J1	Bottle B phase 1+2	0.773	0.552	0.427
J2	Bottle B phase 1+2	0.193	94.2	18.2
Q1	Bottle B phase 3	0.036	2.18	0.078
Q2	Bottle B phase 3	0.018	83.5	1.5
Q3	Bottle B phase 3	0.018	2.03	0.037
	Bottle B phase 4	0.744	0.290	0.216

For bottle B, on the other hand, the phase with the highest TAG concentration is the lower phase, 1+2, particularly subphase J2, once again the most viscous component. Again, there was no transfer of TAG to either of the two solvents, but the TAG remained in the "cell phase". The only difference between the two bottles is that in bottle A, the presence of salt may have favoured the migration of TAGs from the lower phase to the emulsion phase. In both bottles, however, the emulsion phase is very small (65 ml and 72 ml, respectively), resulting in 2.2 g and 1.6 g of TAGs, respectively. For this reason, it was decided to work on phases 1+2 for the next separation step, so that 15.7g and 18.6g of TAGs could potentially be extracted, respectively.

3.3.7 Distillation and filtration

The distillation protocol required that phases 1+2 of both bottles be treated separately with a rotary evaporator (Figure 3.17) at a temperature of 120°C, rotating at 15 rpm for 3.5 hours. In terms of future industrial implementation, vacuum evaporation could enable operation at lower temperatures and over shorter durations. Diathermic oil was used as the heating medium.

As for bottle A, 785 ml of solvent was evaporated, but only 33 ml of the remaining concentrate had an oily appearance. The rest had a granular appearance due to the presence of salt (Figure 3.18). Table 3.9 shows the results of the analyses on the distillate and concentrate. As expected, the concentrate contains the largest amount of TAG, recovering 10.7 g of TAG from the initial 14.9 g, with a concentration of 323 g/l. The rest of the TAG missing to satisfy the material balance was probably lost between the distillate and the rest of the granular-looking concentrate.

There are no results for bottle B because, due to an erroneous increase in temperature to 140 °C, phases 1+2 burned (Figure 3.19). The next steps, therefore, focused solely on bottle A.

All the concentrate from bottle A, including both the oily and granular phases, was filtered to extract as much oil as possible. To do this, a fruit press (Figure 3.20) was used.

Figure 3.17: Rotovapor

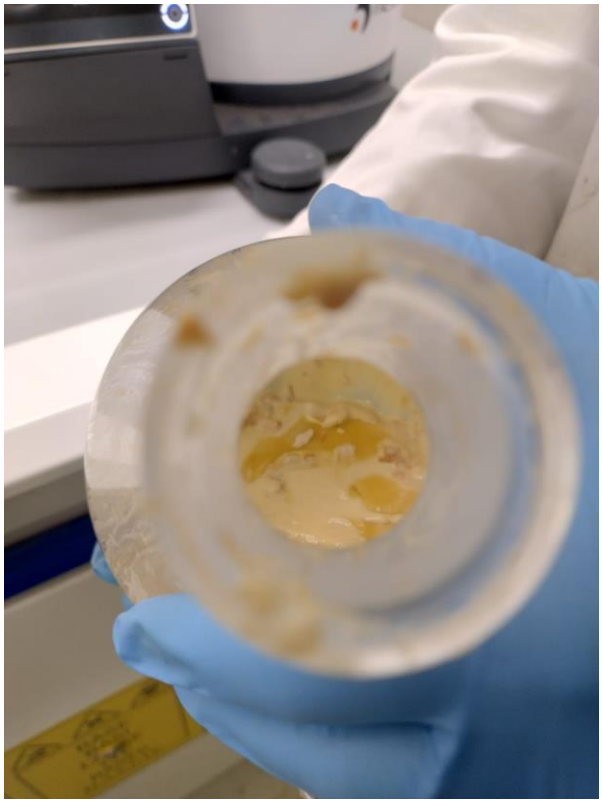


Figure 3.18: Bottle A after distillation

Table 3.9: Results of the distillation of bottle A

Sample	Volume (l)	Concentration (g/l)	TAG (g)
Phase I+II	0.975	15.3	14.9
Concentrate	0.033	323	10.7
Distillate	0.785	0.29	0.228

Figure 3.19: Bottlge B after distillation

This small press is essentially a filter press, consisting of a pneumatic rubber chamber that inflates when compressed air is introduced at 2.5 bar. The chamber pushes the product to be treated along the walls of the press, which are lined with a fabric filter bag. This operation yielded almost 2g of oily substance. (Figure 3.21). The lower separation yield was due to the instrument size being too big for the volume to be treated, which resulted in a partial dispersion of the materials. Nevertheless, it was useful for demonstrating the type of process applicable. Indeed, the filter bag appeared wet and greasy.

Thus, the lost oil probably remained stuck to the filter mesh. If we subtract the weight of the concentrate entering the filter press from the weight of the "cake" (Figure 3.22) remaining on the pneumatic surface, and consider the difference as liquid only, we can then apply the TAG concentration detected from the recovered oily substance. Potentially between 9g and 11g of TAG can be obtained.

Figure 3.20: Filter press

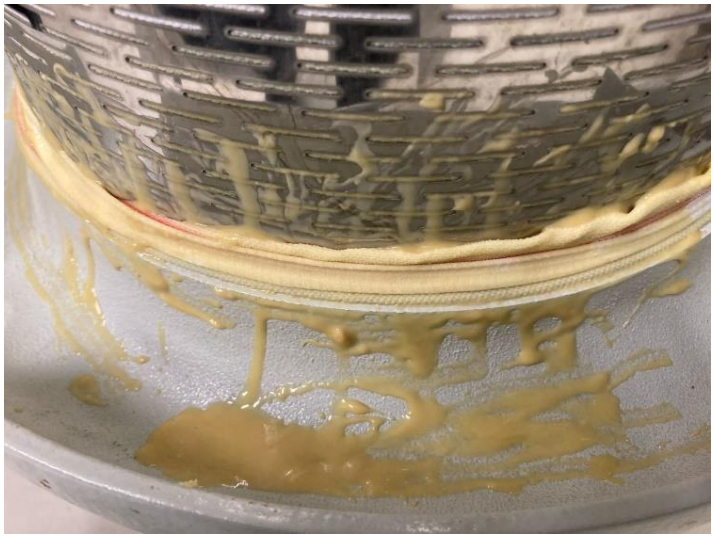


Figure 3.21: Extracted oil

Figure 3.22: Cake post filtration

This is theoretically the result that could be obtained with 100% separation efficiency (obviously impossible) and considering an oil density between 0.8 and 0.98 g/ml. These data are presented in Table 3.10.

Table 3.10: Recovered products

Sample	**Quantity (g)**
Post distillation concentrate	228
Post distillation and press concentrate	186.7
Recovered product	1.96
Recovered oil	0.42-0.51
Potentially recovered product	43.3
Potentially recovered oil	9-11

3.4 Material balances and general considerations

At the end of all these tests, it is essential to quantify the process's efficiency based on the quantity of oil extracted. Figure 3.23 shows the downstream process for obtaining TAGs, with detailed data on volume, quantity of TAGs extracted, and separation efficiency in Table 3.11, starting from untreated *Y. lipolytica*, through

pre- and post-steam explosion microfiltration, separation with solvent and filter press (last term in the table).

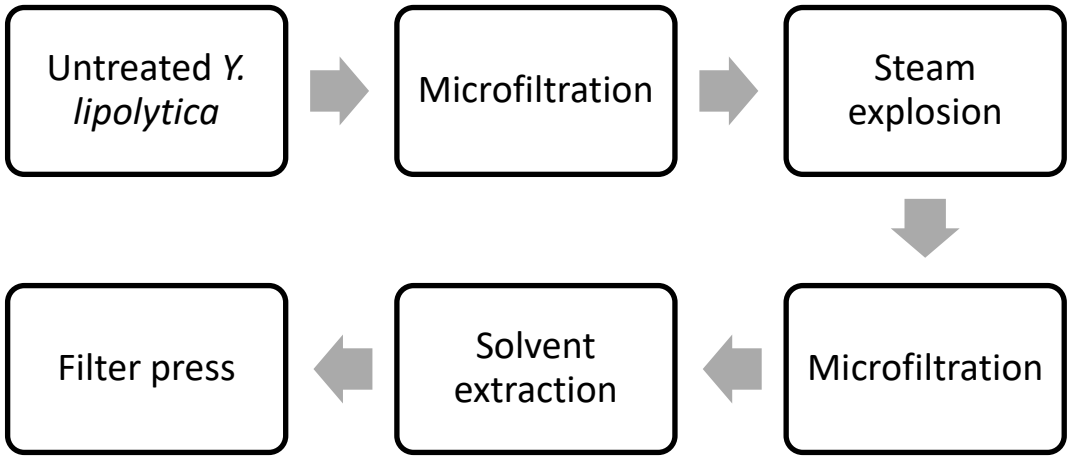


Figure 3.23: Downstream processing

Table 3.11: Percentage of TAG extraction from untreated Y. lipolytica

Sample	**Volume (l)**	**TAG quantity (g)**	**Extraction (%)**
Untreated *Y. lipolytica*	20	592	-
MF *Y. lipolytica*	4	352	59.5%
SE *Y. lipolytica*	28	697	118%
MF SE *Y. lipolytica*	7	564	95.3%
Solvent extraction	14.35	220	37.2%
Concentrate	0.486	157	26.5%
Oil	2-2.45	6.2-7.5	1-1.3%

The table is, however, the result of a calculation exercise: to give continuity to the process, it was decided to use the entire volume obtained in the previous step in the next one.

For each process step (steam explosion (SE), microfiltration (MF) before and after, solvent extraction, and filter press separation) the volume, TAGs amount, and extraction percentage compared to untreated *Y. lipolytica* are shown. While extraction reaches 95.3% after all post-steam explosion steps, this drops steeply during solvent extraction and becomes very low after filter press separation. This indicates that the primary issue is not extraction, but rather the separation and purification of TAGs from the fermentation broth. A recovery rate of 26% is in line with the industrial results of the BioSFerA project partner, BBEPP (BioSFerA Consortium, 2023), but the filter press filtration stage involves a significant loss, as TAGs are absorbed by the filter fabric. When comparing potential and actual

extractable oil, the separation efficiency is about 4.5%. BBEPP also faced adhesion loss in their chamber filter press, but with higher efficiency and a 19% TAG recovery.

In general, the final filtration step is the most critical because the material remains adhered to the walls. One solution that could lead to greater separation efficiency is the use of a filter with non-woven fabric, for example, which blocks solid particles and allows clean liquids to be obtained (Nowotec S.r.l., 2023).

In this work, the steam explosion extraction process was performed in its entirety, up to and including separation with solvent and beyond, due to volume availability. It would also be interesting to compare the extraction obtained with steam explosion and that obtained with enzymes. Indeed, at the end of enzymatic extraction with Alcalase, a TAG concentration of 164 g/l is obtained. If we imagine using the entire concentrated volume of *Y. lipolytica* post-microfiltration in enzymatic extraction, we obtain a quantity of TAG equal to 738 g in 4.5 l, thus an extraction efficiency of 125%. However, it is difficult to determine which of the two extraction techniques is the best overall at an industrial level. The main points to consider in this regard are summarized below:

- Steam explosion:
    - Equipment: complex infrastructure, with a system consisting of a high-pressure stainless-steel reactor, an expansion vessel, a steam generator, and the entire steam supply line.
    - Process: already implemented at an industrial level and easy to manage
    - Cost: high CAPEX but low OPEX, especially noteworthy when considering the potential for integrating the process into companies that already handle thermal waste (estimated at €0.29/kgTAG)
    - Reaction times: extremely short, in this specific case only 10 minutes of reaction time. Around 30 minutes if you consider all four phases (biomass loading, pressurization, maintenance of operating conditions, unloading/washing).

- Enzymatic extraction
    - Equipment: reactor with a temperature and pH monitoring and control system.
    - Process: It is well established at an industrial level, even though enzymes are highly sensitive to changes in pH and temperature (Jin et al., 2012)
    - Cost: high costs on a laboratory scale, which can be reduced by using a commercial enzyme preparation (estimated at $0.37–3.7/kg TAGs) (Pandey et al., 2016)

- Reaction times: It takes a lot longer than steam explosion. In this specific case, we are talking about 48 hours, but there is a possibility of optimising this to reduce the time to 24 hours.

In summary, enzymatic extraction with Alcalase does indeed result in a greater quantity of TAGs being extracted, as shown in Figure 3.10, but it has longer reaction times, higher operating costs, and there could be potential problems with the sensitivity of the enzymes to process changes. Steam explosion, on the other hand, has a slightly lower efficiency (however, we are still talking about extraction that is superior to what even analytical methods can achieve), but it is a process that is already implemented biotechnology process at industrial scale, uses no solvents other than water, and the costs are related to thermal energy.

3.5 Conclusion

This chapter explored how to exploit the potential of another group of waste, biogenic residues, for which the principal method of recovery is often thermal treatment, in addition to landfill. As part of the BioSferA project, of which this work is a central part, these residues were subjected to gasification to obtain syngas for fermentation. The syngas produced was not used directly as fuel, but as feedstock to produce acetate through anaerobic fermentation of a selected bacterial species. The acetate produced was used for the fermentation of *Yarrowia lipolytica*, a yeast engineered to increase TAG production. The TAGs were then extracted, purified, and hydroprocessed to produce biofuels for aircraft and ships. The aim of the project was to decarbonize the aviation and marine sectors, which are still heavily reliant on fossil fuels. Research into sustainable fuels is an essential step towards reducing emissions in these sectors, which are of growing importance to the population.

This work is part of the TAG extraction and purification phase, adopting an alternative method to homogenization for breaking down the yeast cell wall. The proposed process involves an initial reduction of excess water using a pilot microfiltration plant or a three-phase centrifuge. The fermentation broth was subjected to seven different steam explosion tests, varying the amount of biomass input, pressure/temperature, and any pre-treatments aimed at concentrating the starting biomass. The results showed that the best approach involves an initial concentration of the fermentation broth using a microfiltration membrane. The concentrate obtained was treated in a steam explosion plant at 150 °C, 5 bar for 10 minutes. The hydrolysate produced was then microfiltered again to remove excess water due to the steam explosion operations themselves and reduce the volume involved, also with a view to industrial scalability. This extraction process was compared with an enzymatic extraction process. Three different enzymes were tested, and the best was found to be the Alcalase enzyme preparation. The extraction process with Alcalase would provide even better results than that with steam explosion, as shown in Figure 3.10, but the industrial scalability of this process is

undermined by the cost of the enzymes and the use of a longer process than steam explosion. The post-steam explosion concentrate was then treated with different solvents to separate the extracted TAGs from the rest of the hydrolysed broth. Various tests showed that it is difficult to find a pair of green solvents that are as effective as the classic methanol and chloroform, the use of which has been avoided as they are toxic and dangerous to humans. The pair that led to greater TAG extraction in the emulsion or cellular phase was limonene and ethyl acetate, even in the variant with the addition of salt to promote TAG migration in a specific phase. The phase with the highest TAG content was then separated from the rest of the phases, and the remaining solvent was evaporated with a rotary evaporator. Finally, the concentrate obtained was filtered with a filter press. Unfortunately, this was the least effective step in the entire process, as the potentially extractable oily substance was lost during the filtration phase itself, leading to a recovery of only about 1% of the TAGs. If we stopped at the previous phase, however, solvent extraction and subsequent evaporation of the solvent itself led to a separation efficiency of 26.5%, in line with that obtained by the other project partner responsible for extracting TAGs, BBEPP.

In this work, therefore, the potential of alternative methods to traditional ones for breaking the cell wall of yeasts was explored, particularly engineered yeasts, which, as such, potentially have thicker walls that are more difficult to break. The results showed that both steam explosion and enzymatic extraction are valid methods for cell disruption and TAG extraction. In both cases, indeed, the TAG extraction percentage was even higher than that obtained with analytical methods. In the future, however, it will be important to evaluate more effective methods of TAG separation and purification to make the process sustainable on an industrial scale. Furthermore, the optimisation of yeast strains allows for the use of more concentrated fermentation broths, resulting in a reduction in the number of steps required for the downstream process.

Chapter 4

Conclusions and future developments

The studies conducted in this work are part of a broader context of waste management aimed at facilitating and maximizing the recovery of materials and energy from biogenic waste and residues. Indeed, one of the main challenges of the ecological transition is precisely the management of organic by-product flows to be integrated and valorized within the existing economy, moving towards a circular and bio-based economy: biodegradable plastics can be included in the organic waste management cycle after chemical or enzymatic pre-treatment and subsequent anaerobic digestion; anything that is not digested in the digester, such as plastic residues, shells, or even bioplastics that are not completely degraded, can be valorized through HTC and reused in the same anaerobic digestion process to boost biogas production and reduce the hydrogen sulfide content; additional woody residues that are difficult to digest can be used to produce syngas, which can be used as feedstock for two-stage fermentation and to produce TAGs to be hydroprocessed into drop-in biofuels for aircraft and ships.

The aim of this work is to demonstrate possible synergies between biotechnology and thermochemical processes. This approach can help reduce waste, optimize energy recovery, and promote the production of biofuels and biomolecules. The focus is on environmental and industrial sustainability.

Regarding the degradation of bioplastics, it has been demonstrated that a 24-hour enzymatic pretreatment with a mixture of amylase and esterase enzymes results in a 24% reduction in the weight of the bioplastic product. The pretreated pieces of bioplastic bags were then digested for 55 days in a pilot anaerobic digestion plant. After just 30 days of digestion, 67% degradation of the bag was achieved, and building blocks were recovered with concentrations of 130 mg/l of adipic acid, 13.6 mg/l of terephthalic acid, and 33.8 mg/l of 1,4-butanediol. At 55 days, the results were even better, with 97% degradation of the bag and concentrations of adipic acid, terephthalic acid, and 1,4-butanediol of 167 mg/l,

21.3 mg/l, and 76.6 mg/l, respectively. The separation of this material from the remaining organic waste is essential for industrial-scale pretreatment. This operation was performed manually in the laboratory, but obviously, at an industrial level, it must be mechanized. An appropriate sorter or separator is needed to effectively separate organic matter from bioplastics and minimize the amount of OFMSW that remains trapped in the plastic. If the size of the separated bioplastic items is not sufficient for pre-treatment, they must undergo a further size reduction phase using specially developed and dedicated machinery. The initial size reduction phase takes place when the waste is fed into the plant's feeding system and leads to shredding. An appropriate shredder must be used to perform this operation on an industrial scale. On the enzyme side, industrial scalability depends on: the unit cost of the enzyme (€/mg or €/ml), the amount of enzyme required (mg or µl per g of bioplastic treated), and the percentage of degradation per unit weight/volume of enzyme. An enzyme can produce high degradation yields but has a high cost, or vice versa. As the economic balance would not be favorable or, in any case, not convenient for large-scale implementation, from a process viewpoint, it would not be possible to pursue either option. To reduce the unit cost of the enzyme, solutions involving recirculation or immobilization of the enzyme itself could be envisaged, considering parameters such as the ideal recirculation percentage to ensure high process efficiency, the loss of efficiency of the enzyme itself after a certain number of cycles, and the cost of maintaining the immobilization support.

This work also addressed the complementary issue of non-biodegradable and non-recyclable plastics, which inevitably end up in organic waste streams. These materials, which derive from the selection and post-treatment of digestate, can be subjected to HTC, obtaining char, a liquid phase, and a small amount of gas. It has been shown that the aqueous phase of the HTC process can be fully integrated into anaerobic digestion up to 20% of the total volume, not hindering but rather promoting biogas production, if OFMSW is dosed in appropriate quantities. In combination with 10% w/w char, the amount of aqueous phase can increase up to 40% v/v. Indeed, if the char is correctly dosed to a large and active bacterial consortium, it increases biogas production and reduces the production of H_2S, a harmful by-product of anaerobic digestion. In addition, char can make higher aqueous phase concentrations (up to 40% v/v) accessible, provided it is used appropriately. In this way, materials initially considered non-recyclable have been reintroduced into the recovery cycle, contributing to the closure of the material balance and the reduction of final residues. The HTC process has therefore proved to be a bridge technology for the recovery of mixed and problematic flows, promoting integration between waste-to-energy and bioconversion. Industrially, an integrated HTC-anaerobic digestion line could be considered, possibly also including composting and upgrading to biomethane. In this hybrid plant model, the biogas produced and converted into biomethane could feed the HTC process itself, making the plant self-sufficient in terms of energy recovery and cogeneration.

Finally, the research focused on a biotechnological approach for the triglycerides production from engineered yeasts, using agricultural waste and lignocellulosic residues converted into fermentation syngas as a substrate. This

process allows the generation of jet fuel precursors, representing a valuable alternative for sectors that are difficult to electrify, such as aviation and shipping. The production of advanced biofuels from non-food resources thus meets a twofold need: to reduce dependence on petrochemicals and to valorize currently underutilized by-products. The work performed on the recovery and valorization of agricultural waste to produce triglycerides using engineered yeast represents an influential step towards the sustainable use of biogenic resources in high value-added applications. However, for the technology to mature towards real industrial applicability, further scientific and technological developments are needed on several levels. Indeed, in the proposed process, a microfiltration membrane is used to initially concentrate the fermentation broth. The concentrate was treated for 10 minutes in a steam explosion plant at 150 °C and 5 bar. Subsequently, the hydrolysate produced was microfiltered again to remove excess water generated by the steam explosion and reduce the volume involved, also to facilitate industrial scalability. A comparison between this extraction and the enzymatic extraction was provided. The Alcalase enzyme preparation offers an intriguing comparison. Indeed, the extraction process with Alcalase would provide better results than that with steam explosion. The costs of the enzymes and the longer process compared to steam explosion limit the industrial scalability of the process. To separate the extracted TAGs from the rest of the hydrolysed broth, the subsequent concentrate was treated with various solvents. The combination of limonene and ethyl acetate enhanced TAG extraction in the cell or emulsion phase. The salt variant also facilitated TAG migration. The phase with the highest TAG content was then separated from the other phases. Using a rotary evaporator, the residual solvent was evaporated. Finally, a filter press was used to filter the concentrate. Unfortunately, this was the least effective step in the entire process because the potentially extractable oily substance was lost during the filtration phase itself, yielding only about 1% of the TAGs recovered. However, as for the previous phase, solvent extraction and subsequent solvent evaporation achieved a separation efficiency of 26.5%. This confirms what was said earlier: scalability requires specific and more efficient oil separation equipment to make the entire process economically sustainable.

In conclusion, the full exploitation of biomass and organic waste is not only a sustainable management strategy but also a concrete opportunity to redefine the relationship between production, consumption, and the environment, transforming today's waste into tomorrow's resources. The challenge of ecological transition is not only played out in major energy policies, but also in the ability to restore value to the materials we discard. The path outlined demonstrates how the integration of heterogeneous bio-based processes can generate value from complex waste matrices, outlining a circular biorefinery model that can combine sustainability, innovation, and real industrial prospects. Bringing these processes from the laboratory to industry will not only close the waste cycle but also open up new, truly circular production chains capable of generating economic growth, employment, and a positive environmental impact on the territory.

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