UNIVERSITÀ
DEGLI STUDI
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Ph.D. DEGREE IN

Earth and Environmental Sciences and Technologies

Cycle XXXV

TITLE OF THE Ph.D. THESIS

Controlling dark fermentation of agro-industrial waste for valued organic acids production

Scientific Disciplinary Sector(s)

ICAR 03

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Final exam. Academic Year 2021/2022
Thesis defence: April 2023 Session


"Stefano Trudu gratefully acknowledges the Sardinian Regional Government for the financial support of his Ph.D. scholarship (P.O.R. Sardegna F.S.E. - Operational Programme of the Autonomous Region of Sardinia, European Social Fund 2014-2020 - Axis III Education and training, Thematic goal 10, Investment Priority 10ii), Specific goal 10.5".

Abstract

In recent years, our planet has been facing severe environmental degradation and climate change with consequences for the health of people and repercussions on the social and economic balance. Globally, the food sector alone produces more than a third of the global anthropogenic greenhouse gas (GHG) emissions, stemming from emissions of 11 to 19 billion tons per year estimated by the Intergovernmental Panel on Climate Change (IPCC). Approximately one-third of the food globally produced for human consumption is lost every year throughout the supply chain, from agricultural production to final household consumption. Organic waste contains valuable amounts of proteins, sugars, lipids, fibers, vitamins, and bioactive agents (antioxidants and antimicrobial agents, enzymes) worth recovering. Traditional waste management methods have raised concerns and limitations concerning the environmental compatibility or applicability of the recovered products. These methods are typically based on aerobic treatment and anaerobic digestion (AD) of the organic substrate, or a combination of them. From these traditional processes, it is possible to obtain compost and biogas that have a relatively low economic value.

In a context characterized by finite resources, the wastes represent both an environmental issue to be properly managed and a potential resource of secondary raw materials.

The negative effects on the environment and the current regulatory framework will make it imperative to shift from fossil-based to bio-based production methods in the near future. Organic wastes may represent a widely available and renewable source for the recovery of high-value products such as biochemicals, biofuels, and bioenergy.

In this respect, the so-called *waste biorefinery concept* represents the technical solution, as well as an even more sustainable evolution of the original biorefinery concept and, finally, the link between bio-economy and circular economy promoted by EU policies. Biorefinery is defined as a set of sustainable conversion processes from biomass to a spectrum of marketable products and energy. A waste biorefinery can be interpreted as an evolution of the biorefinery concept to include waste as an alternative to dedicated biomass.

For the implementation of a zero-waste biorefinery, a combination of treatment processes is required to obtain several valuable products. It is not possible to define a unique layout of the most suitable processes to be included in a biorefinery where the waste is the starting substrate, because the possible options available are related to the quantity, the specific local conditions, market trends and legislative constraints.

Nevertheless, the fundamental role that a bioprocess such as fermentation would play in a biorefinery scheme must be emphasized, thanks to its ability to hydrolyze, simplify and transform substrates into high-value products. While many studies have already addressed the issue of biohydrogen production from organic wastes (e.g., cheese whey, food waste), further efforts are still required to implement, through proper optimization of the operating parameters, economically and environmentally sustainable recovering processes of high-value chemicals.

In this context, the present Ph.D. thesis will pay attention to the possibility to produce valuable mixtures rich in organic acids through controlled dark fermentation (DF) of sheep cheese whey (CW). The main goal is the identification of the operating parameters which affect the reactions occurring during DF to engineer the process itself to produce specific organic acids (such as lactic, acetic, propionic, and butyric acid) useful in several applications. For this purpose, two specific applications were investigated: polyhydroxyalkanoates (PHA) production (**Part I**) and metal leaching (**Part II**).

The thesis, thus, consists of two parts and 9 chapters:

- **Chapter 1** provides a general overview of industrial production and applications of organic acids. The importance of dark fermentation as a tool for sustainable production and a description of the main parameters affecting the process, as well as the use of cheese whey as a promising substrate, are also discussed.

Part I

- **Chapter 2** summarizes the state of the art of PHA production by mixed microbial cultures from organic wastes. The chapter provides some fundamentals about the main stages involved: acidogenic dark fermentation, culture selection and enrichment, PHA accumulation, and PHA extraction.
- **Chapter 3** is an extract of the article "Silicone membrane contactor for selective volatile fatty acid and alcohol separation" published in the journal "Process Safety and Environmental Protection" in 2021 by Harish Ravishankar, Paolo Dessì, Stefano Trudu, Fabiano Asunis, Piet N.L. Lens, which focuses on the selective VFA recovery from the fermented CW.
- **Chapter 4** and **Chapter 5** present and discuss the results of the experimental activities carried out on PHA production using CW as a substrate. Specifically, a new configuration of PHA production, and the optimization of process parameters, are respectively discussed.

Part II

- **Chapter 6** introduces the field of resources sustainability with specific reference to the electrical and electronic equipment (EEE) production and end-of-life EEE management. Furthermore, it summarizes the main industrial technologies for metal valorization from waste from electrical and electronic equipment (WEEE).
- **Chapter 7** presents the strategy adopted to produce a bio-derived leaching solution through CW fermentation.
- **Chapter 8** explores the application aspects of fermented cheese whey as a selective leaching agent, aimed at the recovery of metals from WEEE samples.

- **Chapter 9** summarizes the main conclusions and the perspective.

Chapter 1

General introduction

In this chapter, a brief overview of the general knowledge about the industrial production and application of organic acids is given, highlighting the importance of dark fermentation as a tool for a sustainable production. In addition, a general overview of the dairy supply chain is provided, with a focus on the residues produced, their current management, innovative treatment, and valorization technologies.

1.1 Organic acids: industrial production and applications

About 90% of organic acids such as lactic, acetic, propionic, and butyric, are produced by fossil-based production processes. Each of them is a useful chemical or raw material widely used in food and beverage, cosmetic, agricultural, or pharmaceutical sectors (Asunis et al., 2022).

Lactic acid is a bulk chemical used in several sectors, such as in the chemical industry as an intermediate for lactate esters manufacturing. Lactic acid is widely used also in the food and beverage sector, as well as in the pharmaceutical and personal care sector. Global lactic acid production is expected to grow from 0.7 Mt in 2013 to 1.9 Mt in 2020 with a market size worth 8.7 billion $ by 2025 and a compound annual growth rate (CAGR) of 18.7% (Market Research Report, 2021). Interest in lactic acid has increased as it can be used to produce polylactic acid (PLA), an environmentally friendly and biodegradable polymer that is an optimal candidate to replace petroleum-derived plastics (Abdel-Rahman et al., 2013). Nowadays, most of the commercial lactic acid is produced by bacterial fermentation of carbohydrates from corn stover or cobs, sugarcane bagasse, molasses and wood processing waste (Cui et al., 2011; Laopaiboon et al., 2010; Wang et al., 2010).

The total global market for acetic, butyric and propionic acids is well consolidated. Acetic acid is an important building block widely used in chemical industries to manufacture plastics, synthetic fibers and pesticides and in food and beverage industries as a component of flavors, acidity regulators and preservatives. The acetic acid market demand is expected to reach 18.3 Mt by 2023,

with a CAGR of 4.27% (Atasoy et al., 2018). Propionic acid is mostly used in the form of calcium and sodium salts, and it could be used as a preservative for food (bread and other baked goods) and animal feed (directly or as its sodium salt). Moreover, propionic acid is a critical element in the manufacturing of vitamin E. Global demand is expected to reach 4.7 Mt by 2020, growing at a CAGR of 2.7%. Propionic acid is currently mainly synthesized by the petrochemical route, and hence its production is vulnerable to price fluctuations of propane and natural gas. Butyric acid is used in the animal feeding sector as a supplement and an antibiotic. It is largely recognized as an efficient energy source for animals, especially for swine and poultry. The total global demand for butyric acid is expected to reach 0.1 Mt by 2020, with a CAGR of 15.1%. The bio-based butyric acid production is growing due to it being approved as a food flavoring agent (taste and aroma additive) by the United States Food and Drug Administration (Atasoy et al., 2018).

As above-mentioned, organic acids can also be produced through biological fermentation processes, using pure cultures of microorganisms capable of guaranteeing high production yields and obtaining pure products that facilitate downstream purification processes. The main cost is associated with raw materials, which are usually refined and expensive sugars. The use of fermentation to obtain those products using low-cost substrates such as biowaste has been largely ignored in recent decades, but it is recently gaining more and more interest as a research topic (Agler et al., 2011). Volatile fatty acids (VFA) can be synthesized from organic waste streams, even in a heterogeneous mixture, from mixed microbial cultures (MMC) that perform hydrolysis and acidogenic fermentation processes such as dark fermentation (DF) (Asunis et al., 2022). The use of MMC needs a preliminary selection of the biomass, but eliminates the production of pure biomass, thereby reducing operating costs, facilitating process control and, in turn, potentially making waste derived-VFA production possible.

1.2 Dark fermentation

Fermentation is an anaerobic metabolic pathway that allows deriving energy from organic molecules (mostly carbohydrates) in absence of oxygen. Since oxygen is not available as the final electron acceptor, the same substrate is partly oxidized and partly reduced.

In DF, under appropriate conditions (absence of oxygen and light), microorganisms can convert organic substrates into gaseous products, mainly H_2 and CO_2, and into a mix of VFA and reduced end products, including alcohols (De Gioannis et al., 2017). DF applied to organic wastes (food

waste, agro-industrial waste) has been widely studied mainly focusing on H_2 production, whereas fewer studies have specifically targeted VFA production, although the latter accounts for 65%wt. of the degraded organic matter (Bastidas-Oyanedel et al., 2015). The production of organic acids poses additional challenges as the range of soluble products is much broader than the gaseous ones, and complex separation and purification are required in view of commercial use (Arslan et al., 2017). The fermentation of a complex substrate such as organic wastes involves the spontaneous onset of multiple metabolic pathways, particularly when the process relies on autochthonous microbial consortia, resulting in the generation of a wide range of products, including lactate, acetate, propionate, butyrate, ethanol, H_2 and CO_2 (Chen et al., 2013). The main metabolic pathways are summarized in **Table 1.1**.

Table 1.1 – Main metabolic pathways during fermentation for biobased products (Zhou et al., 2018).

Reaction	Typical Microbe	Conversion reactions
Glycolysis		glucose + $2NAD^+ \rightarrow 2CH_3COCOO^-$ + 2NADH + $4H^+$ +2ATP
Acetate pathway	*Clostridium pasteurianum*	glucose $\rightarrow 2CH_3COO^-$ + $4H_2$ + $2H^+$ + $2CO_2$
Butyrate pathway	*Clostridium butyricum*	glucose $\rightarrow CH_3(CH_2)_2COO^-$ + $2H_2$ + H^+ + $2CO_2$
Propionate pathway	*Clostridium butyricum*	glucose + $2H_2 \rightarrow 2C_2H_5COO^-$ + $2H^+$
Ethanol pathway		glucose $\rightarrow 2C_2H_5OH + 2CO_2$
Lactose hydrolysis	*Bacillus and Lactobacillus sp.*	lactose → glucose + galactose
Homolactic	*Streptococcus, Lactobacillus*	glucose $\rightarrow 2CH_3CH(OH)COO^-$ + $2H^+$
Heterolactic	*Leuconostac, Lactobacillus*	glucose $\rightarrow CH_3CH(OH)COO^-$ + $2H^+$ + CO_2 + CH_3COO^- glucose $\rightarrow CH_3CH(OH)COO^-$ + H^+ + CO_2 + C_2H_5OH

According to **Table 1.1**, acetic and butyric fermentation involves the production of H_2, while the propionic one its consumption. Two metabolic pathways of lactic fermentation can be identified: homolactic, which produces only lactic acid, and heterolactic which also produces acetic acid and ethanol. Although VFA mixtures are typically obtained during DF, selective VFA production might be achieved by promoting specific metabolic routes. In this respect, different yields and relative proportions between VFA are achievable by properly setting key operating parameters such as pH, temperature, hydraulic retention time (HRT) and organic loading rate (OLR) (Asunis et al., 2022). However, relatively little information is available on the influence of such parameters on VFA production, as most literature studies have targeted H_2 rather than VFA production, and other variables such as substrate composition, type of inoculum and applied pretreatment, reactor type and mode of operation must be considered.

1.2.1 Substrate

As mentioned above, the use of DF to produce organic acids can be carried out using low-cost substrates such as biowastes mainly composed of carbohydrates, proteins and lipids, which can affect both the amount and chemical composition of VFA produced (Strazzera et al., 2018). Lipids, due to their low solubility and slow biodegradation kinetics, represent the main concern during acidogenic reactions. Proteins can improve the fermentation process by providing nutrients for microbial growth. On the other hand, the hydrolysis of some proteins present in the wastes is considered a limiting phase. Carbohydrates, as opposed to lipids and proteins, are easily hydrolyzed into monomeric sugars that can be rapidly fermented into VFA (Shen et al., 2017). It has been reported that the use of more concentrated carbohydrate-rich substrates increases total acid production, compared to protein-rich substrates, due to the inhibition of microbial activity caused by free ammonium accumulation (Shen et al., 2017).

The variability of the data reported in the scientific literature in terms of concentration and distribution of VFA depends on the complexity, heterogeneity, geographic and seasonal variability of the composition of the organic wastes. This implies that the combined effect of substrate characteristics and operating conditions must be systematically studied to identify optimal conditions that maximize production yield and VFA composition (Atasoy et al., 2018; Lee et al., 2014).

1.2.2 Inoculum and pretreatment

The most common inocula used for DF are active sludges from municipal wastewater treatment plants and anaerobic sludges (Asunis et al., 2022).

The bacteria most involved in DF are the obligate anaerobes of *Clostridium sp.*, effective in converting a wide range of carbohydrates with high H_2 and organic acid yields, or the facultative anaerobes of *Escherichia coli* and *Enterobacteriaceae sp.*. To improve VFA production, a selection of fermentative bacteria from the inoculum should be performed, and methanogenic activity should be avoided by properly adopting operational parameters or applying pre-treatments. However, pre-treatment of the inoculum may affect the economic feasibility of the process and require careful consideration. While the use of pure crops and homogeneous/selected substrates makes it possible to produce specific industrial acids (Chen et al., 2013), the objective is much

more difficult in the case of MMC and heterogeneous residual substrates such as organic waste, where several indigenous organisms compete simultaneously for a complex substrate.

1.2.3 Operation mode

Regarding the operation mode, batch, fed-batch, semi-continuous and continuous modes can be adopted. According to Lee et al. (2014), the continuous mode might not be feasible for slow reactions, whereas the batch or semi-continuous operation mode seems to be more favorable for VFA production. The configuration of the reactor influences the hydrodynamics and, therefore, the substrate–microorganism contact and the liquid–gas mass transfer. The overhead gas pressure can lead to inhibitory effects, as a high H_2 partial pressure proved to favor the production of reduced compounds such as lactate, ethanol and propionate, which is associated with zero hydrogen production or even consumption (Zhou et al., 2018). The most used type of reactor is a continuous stirred tank reactor (CSTR) with no biomass recycling, where HRT and solid retention time (SRT) coincide. Other types of reactors, such as anaerobic leach bed reactors, up-flow anaerobic sludge blanket (UASB) and anaerobic sequencing batch reactor (ASBR) have been proposed too (Zhang et al., 2008; Zhou et al., 2018). These offer the possibility of decoupling HRT from SRT with possible improvements of the process.

1.2.4 Operating parameters

Among operational parameters, pH has the greatest impact on H_2 yield and VFA production. The best choice of pH value to optimize VFA production is strongly dependent on the composition of the substrate. Therefore, due to the heterogeneity of the organic waste, it is impossible to define an optimal pH to obtain a specific VFA, without referring to a particular substrate. In general, metabolic pathways involving the production of acetate and butyrate are favored in a pH range of 5 to 6, while a slightly lower pH would favor the production of butyrate at the expense of acetate (Infantes et al., 2011) and pH neutral or higher up to 8 promote propionate production (Cappai et al., 2014). Finally, it is important to note that despite the advantages of VFA production, adjusting the pH to weakly acidic or alkaline conditions by adding a large amount of chemicals could increase production costs and process complexity.

Temperature is also one of the most important operational parameters in DF, as it affects the rate of substrate hydrolysis and microbial growth. The temperature ranges, in which fermentation can be conducted, are mesophilic (25-45°C) and thermophilic (45-65°C). Under mesophilic conditions,

an increase of temperature is advantageous in terms of VFA concentration, yield and production rate. More specifically, it is possible to obtain a higher VFA production by increasing the temperature to 40-45°C, considered the optimal temperature for hydrolysis rates and most fermentation reactions (Arslan et al., 2017), while a further increase to 55°C has a negative effect due to thermal denaturation of essential proteins and enzymes.

HRT and OLR are also important factors to ensure stability and good yields of hydrogen and VFA production in the fermentation process. Several studies have focused on the determination of the optimal values of these two parameters, but the results are not always easily comparable, even with the same substrate used, due to the difference in other important parameters. HRT must be adequate to allow for hydrolysis and acidogenesis whose rate is crucial for heterogeneous and complex solid substrates such as organic wastes. Theoretically, since hydrolysis is commonly recognized as the limiting step of the process, the production of VFA is expected to increase with HRT. On the other hand, too long HRT would favor methanogens and, especially in view of a full-scale implementation, would reduce the mass rate of waste to be treated, requiring larger reactor volumes, and entailing higher capital costs.

OLR represents the mass of substrate fed to the reactor per unit of time and volume. Hence, the value of the OLR is influenced by the concentration of the substrate and the HRT. A positive effect of the OLR increase can be obtained by decreasing the HRT but remembering that too low HRT values would hinder the hydrolysis of the substrate.

1.3 Dairy residues as a promising substrate

Dairy residues, such as cheese whey (cheese-making process residue), are promising substrates for organic acid production through dark fermentation. Several reasons make the cheese whey particularly suitable. First, it is rich in carbohydrates (mainly lactose), as a carbon source to produce VFA, and proteins, as a source of nitrogen for microbial growth. Hence, fermentation may occur under optimal C/N ratios without the addition of external nutrients. Moreover, the microorganisms already present in the CW, such as *Lactobacillus*, can ferment lactose, allowing to avoid additional costs for the inoculum and pretreatments. And finally, dairy residues are managed with low, partial and sometimes unprofitable possibilities for reuse and recovery, representing a potential source of pollution in the event of inappropriate management.

The implementation of the biorefinery concept in the dairy production system with DF as the central process, could not only offer a modern solution to an environmental problem, but also contribute substantially to the economic balance of an entire productive sector, which is often in crisis.

1.3.1 Dairy industry

The dairy industry is the production chain in which milk and a variety of derived products are produced. The chain is divided into two sectors: (i) milk production, from the management of dairy herds until the delivery of the milk to the dairies; (ii) dairy processing, including the milk processing itself and the production of dairy products such as cheese, ricotta, cream, and butter.

Worldwide, the dairy industry is changing due to variations in milk prices, consumers demand, climatic conditions, and the spread of new food trends. World milk production reached almost 906 million tons in 2020, 2% more from 2019 and it is expected to increase by 9% up to 993 million tons in 2027 (Agricultural outlook, 2018). In 2020, the milk production in European farms reached 236 million tons, increasing by 1.6% from 2019, maintaining the second position in world milk production and the first in exports. The dairy sector plays a significant role in the European economy considering that it is the second biggest agricultural sector in terms of output value, representing 15% of the total EU agricultural income (The EU Dairy Sector, 2018).

Dairy is the first Italian food sector and represents more than 12% of the total turnover of the national food sector, the value of production exceeds 15.53 billion euros. Most of the national milk production is destined for processing into cheese; in fact, Italy is the largest producer and exporter of typical PDO (Protected Designations of Origin) cheeses in Europe. In 2020, 463,000 tons of cheese were exported, 98,000 tons of which under PDO, mostly in France, Germany and USA.

Large quantities of residues are produced during the cheese-making process: A part of them can be used to produce other dairy products such as ricotta. In other cases, treatments are required for avoiding landfilling and try to valorize them in other ways.

1.3.2 Residues produced from the dairy industry

The production chain begins with the collection of milk, which is possibly pasteurized (to eliminate any pathogenic germs) and in which specific microbial cultures (starter) are added. The process continues with the coagulation phase, where milk proteins and fats aggregate in a semi-solid gelatinous state to form the curd, which is appropriately separated from the liquid phase. The

residual liquid stream is called cheese whey. CW can be sweet or acidic, depending on the specific enzymes used to promote the coagulation phase. Subsequently, the curd is broken to facilitate the purging of the CW to be removed. After this phase, the cheese is cooked at temperatures ranging from 40 to 60ºC, followed by the extraction of the curd from the coagulation tanks and the pressing phase, in which it gives firmness and shape to the cheese and continues the release of whey. Finally, after salting (to prevent mold, accentuate the taste and form the crust), different ripening can take place, depending on the specific type of cheese to be produced.

The cheese-making industry produces three main types of waste: “cheese whey”, a residue of cheese production that can sometimes be used to make ricotta; “scotta” or second cheese whey, the residue of ricotta production; wastewater derived from washing of production lines, storage or dilution of whey (**Figure 1.1**).

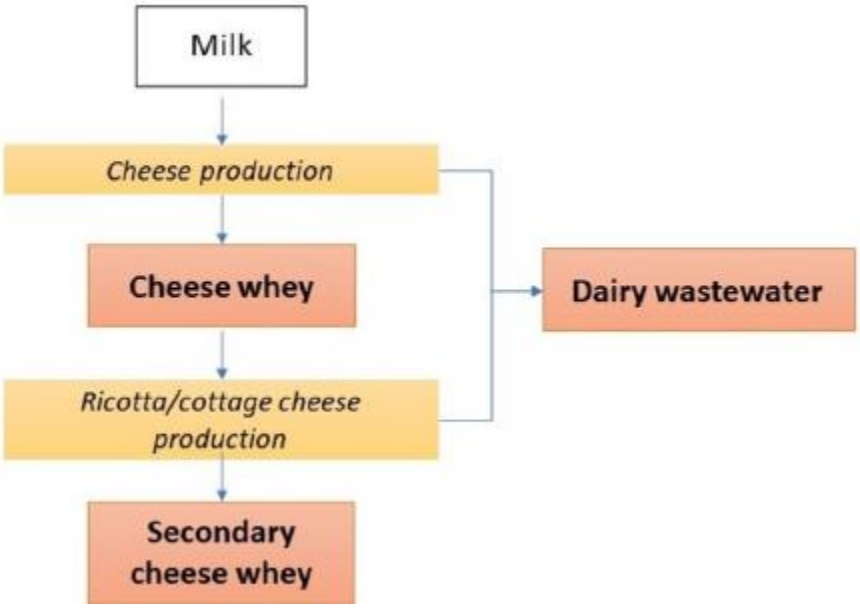

Figure 1.1 – Cheese-making process and residues produced (Asunis et al., 2022).

CW is the most important by-product of the cheese-making process with a high organic load and a specific production of 0.8-0.9 L per liter of processed milk, depending on the cheese yield and type of processed milk (Carvalho et al., 2013). Cheese whey is a green-yellowish liquid containing 55% of milk nutrients, of which 20% are proteins. It consists of 93% by volume of water, 70-72% of the total solids are lactose, 8-10% are proteins, 12-15% are minerals, then there are salts, traces of metals (such as zinc and lead), small amounts of lactic and citric acid, urea, uric acid and vitamins (Ryan & Walsh, 2016). **Table 1.2** summarizes the main chemical-physical characteristics

of CW. For each kg of cheese produced, about 9 liters of CW are generated, having a concentration of BOD_5 (5-days Biochemical Oxygen Demand) between 40 and 60 g/L and of COD (Chemical Oxygen Demand) between 50 and 80 g/L (Ryan & Walsh, 2016). As a comparison, domestic sewage arriving daily at a sewage treatment plant has from 110 mg/L to 400 mg/L BOD_5, while for COD the range is from 250 to 1000 mg/L.

After heating whey to a temperature of around 80ºC to produce ricotta cheese, the CW proteins coagulate and form a precipitate consisting essentially of lactalbumin and fat, lactose and mineral salts; the remaining liquid, enriched in salts and organic acids used as technological adjuvants, is called "scotta" or second cheese whey (SCW). The "scotta" differs from the CW qualitatively for the color (straw yellow in the case of CW and milky white in the case of the SCW). From the nutritional point of view, it is a poorer by-product than whey, in fact, it is characterized by a lactose content corresponding to about 4%wt. and a protein content much lower (0.1%wt.); it has relatively lower COD values (25 g/L) but still contains a relevant amount of suspended solids and fats, as well as a higher salt concentration (4.8 g/L against 3.5 g/L of whey).

The main sources of wastewater in dairy processing plants are: 1) losses of raw materials (especially milk) from equipment or pipelines and spills caused by overflows and malfunctions of plants; 2) materials used for cleaning and sanitizing. The polluting characteristics of dairy wastewater are similar to those of whey but with lower concentrations of the main pollutants as a result of dilution with washing water.

Table 1.2 - Cheese whey characterization (Carvalho et al., 2013).

Lactose	Proteins	Fats	Minerals	BOD_5	COD	TS	TSS	TVS	TN	TKN	$N\text{-}NH_4^+$	$N\text{-}NO_3^-$	TP	$P\text{-}PO_4$	Reference
–	–	–	–	–	74.5± 0.4	–	9.4±0.5	–	–	0.146	–	–	0.124	–	Erguè et al. (2001)
50	–	–	–	–	74.2	66.83	22.15	–	–	1.49	0.17	–	–	–	Ghaly and Kamal (2004)
–	–	–	–	–	73–86	–	20–22	–	0.9–1.2	–	0.06-0.15	7–10	0.42-0.54	0.34-0.43	Farizoglu et al. (2007)
49.2	–	–	–	–	102.1	70.9	–	–	1.76	–	–	–	–	–	Ferchichi et al., 2005
–	–	–	–	35.5–46.0	60.3-66.7	–	4.1–10.0	–	–	–	–	–	–	–	Blonskaja and Vaalu, (2006)
45.9±0.88	2.71±0.05	9.44±1.14	–	37.7±2.8	68.6±3.3	5.93±0.38	1.35±0.06	5.61±0.36	–	1.12±0.01	–	–	$0.5±1.8×10^{-3}$	–	Saddoud et al. (2007)
43.92	1.42	0.00	6.1		100	–	–	–	–	–	–	–	–	–	Yorgun et al. (2008)
–	–	0.99	–	29.5	73.4	–	7.2	–	–	–	–	–	–	–	Janczukowicz et al. (2008)
–	125±2[a]	0.9±0.5[b]	–	40±2.55	60±10	59±0.5	1.5±0.23	–	–	–	–	–	–	–	Gannoun et al. (2008)
42.6	–	–	–	–	86.3	–	6.9	–	0.2	–	–	–	–	–	Azbar et al. (2009a)
50–60	–	–	–	27–36	50–70	55–65	10–15	–	–	0.01–0.02	–	–	–	–	Ebrahimi et al. (2010)

[a] *as g kg^{-1}*

[b] *as (%)*

1.3.3 Cheese whey current management

The management of residues has always been a matter of concern for the dairy industry. These residues, primarily CW, are often considered as an undesirable by-product. In the past and in some cases still today, the most common CW disposal methods were the “informal” spilling of the broth into rivers or land spreading. Otherwise, it was used as food for animals. When the effluents from dairy waste are discharged into rivers, high amounts of biodegradable organic matter are rapidly consumed resulting in depletion of dissolved oxygen and increased eutrophication (Ahmad et al., 2019).

Hence, it is obvious that dairy bioresidues cannot be discharged directly into the environment without adequate treatment. The disposal of whey by discharge into water is now prohibited in most milk-producing nations by strict environmental legislation (Ryan & Walsh, 2016). Despite environmental concerns, a share of dairy effluents, including about 50% of the whey produced worldwide, is currently released into the receptor without treatment (Asunis et al., 2020).

The use of CW as animal feed seems to be a feasible option if the collecting farm is located close to the dairy manufacturing. Otherwise, the price for transportation would make it unaffordable. However, the use of CW as animal feed must be limited because the high content of lactose and minerals can cause health issues to the animals.

Land spreading may affect physical and chemical characteristics of the soil, leading to a decrease in crop yield and oxygen availability. Furthermore, the presence of nitrogen compounds such as ammonia or nitrate salts could also contaminate groundwater.

The current strategies for the dairy industry residues management are the recovery of proteins and lactose and dedicated aerobic and anaerobic treatments.

Proteins and lactose have functional properties that are essential for food applications. Proteins are recovered from whey by ultrafiltration, generating a whey permeate rich in lactose (80%wt. of CW lactose). Lactose from CW and the permeate is recovered by crystallization, and it is used widely within the food and confectionery industries, bakery industries and in the preparation of infant formula (Ryan & Walsh, 2016).

Aerobic treatment is the most used in the dairy industry even if it has reduced efficiency with respect to anaerobic processes; it usually consists of trickling filters, aerated lagoons, and activated sludge processes. The low performance is mainly due to rapid acidification (caused by poor water buffer capacity) and filamentous growth (high lactose level). Trickling filters usually produce high-quality final effluents, but their use is limited to high-strength effluents (more than 0.3 $kgBOD_5/m^3$), due to the problem of strong fouling (Ahmad et al., 2019). SBR system is preferred because of its loading capabilities and flexibility. Activated sludge is also a typical process in the dairy industry, often combined with aerobic sewage stabilization (filter presses and centrifuges) for the use of sludge as fertilizer; however, from an energy point of view, it is not economically viable, due to the high organic load of dairy effluents and the consequent large amount of oxygen required for aeration and the excess of produced sludge (Asunis et al., 2020).

Anaerobic digestion, in which organic substrates are converted into biogas, is a well-established process to exploit the energy content of CW (De Gioannis et al., 2017). However due to its high organic load and low alkalinity, the fermentation of lactose can generate an accumulation of volatile fatty acids, leading to consequent acidification and inhibition of methanogenic activity, affecting the yield of gasification and the stability of the process (De Gioannis et al., 2014). One of the options to mitigate acidification is the addition of external alkalis (e.g., lime, bicarbonate, or hydroxide) or an appropriate dilution, but both strategies would increase operating costs and volumes to be treated. A more sustainable alternative is the co-digestion with substrates with a

high buffer capacity, such as sewage sludge (Carrieri et al., 1993), manure (Vivekanand et al., 2018), poultry manure (Gelegenis et al., 2007) and cattle slurry (Comino et al., 2012) or fish silage (Vivekanand et al., 2018). On the other hand, the use of a two-stage configuration, in which the physical separation of the biochemical reactions of acidification and methanogenesis takes place could be a viable solution; in the first stage, hydrolysis and fermentation phases are conducted, while the second stage is dedicated to methanogenesis phase. The advantages of this configuration allow to have a greater production of biogas, with the possibility of recovering hydrogen from the first stage and methane from the second (De Gioannis et al., 2017), have greater process stability, and lower risks of inhibition; However, the size of the plant would increase, as well as the investment and operating costs.

1.3.4 CW valorization in a waste biorefinery approach

The potential associated with CW is gradually emerging, especially for biotechnological processes. Innovative treatments are a priority to deal with the high organic load of raw CW and CW valorization is advantageous for the environment and for a sustainable bioeconomy (Asunis et al., 2020). In this paragraph, an overview of the most promising technologies for CW valorization are presented, mainly techniques and processes that can be adapted to the approach of the waste biorefinery. In **Figure 1.3** an example of integrated processes for dairy residues is proposed, in which DF is the main process for the production of high-value products that are fundamental for other applications.

Dark fermentation

DF has a central role as a promising option for CW valorization due to its high carbohydrate content, which can be converted to organic acids (lactic and VFA), bioethanol and hydrogen (Akhlaghi et al., 2019; Asunis et al., 2019; De Gioannis et al., 2014). Organic acids are products with a high value on the market for chemicals, and their production could represent an extra source of income for the dairy industry.

In the absence of CW pre-treatments and external inoculum, DF of CW mainly involves three steps: (i) lactose hydrolysis into glucose and galactose; (ii) conversion of monomeric sugars into lactate by homolactic microorganisms; (iii) conversion of lactate into H_2 and VFA by fermentative microorganisms, such as *Clostridium* (**Figure 1.2**). DF for lactic acid production is conducted by bacteria such as *Lactobacillus, Lactococcus, Streptococcus, Bacillus* and *Enterococcus* (Ryan &

Walsh, 2016). Lactose is converted into lactate with a theoretical yield of 4 mol/mol$_{Lactose}$ (homolactic fermentation). If ethanol and acetic acid are produced in this process, the lactic acid production is halved (heterolactic fermentation). Since lactic acid bacteria have limited potential to biosynthesize aminoacids, the presence of a nitrogen source is crucial for their growth (Prazeres et al., 2012). Due to its high protein content, raw CW can be used for lactate production, although enzymatic hydrolysis of lactose might be necessary.

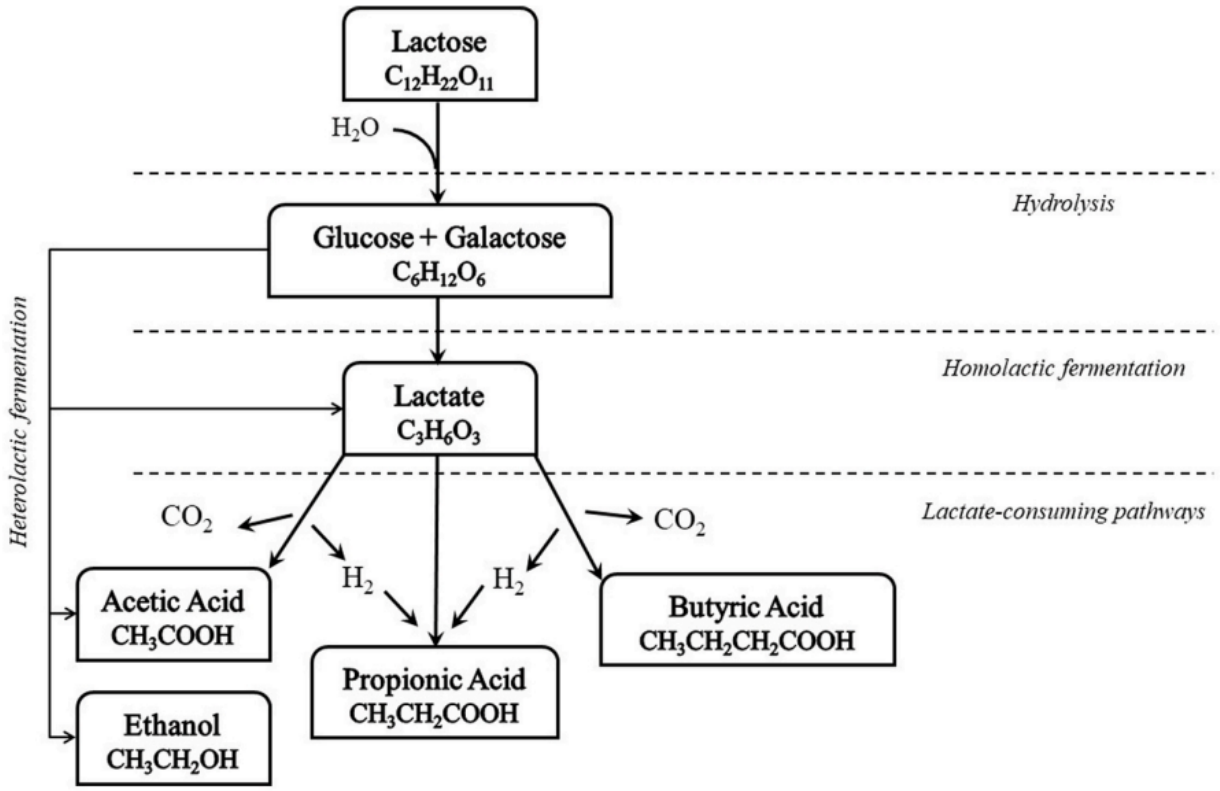

Figure 1.2 – Fermentation pathways from CW indigenous microorganisms (Asunis et al., 2020).

Biopolymers

Lactic acid and VFA produced through CW fermentation can be used for biopolymer production such as polylactic acid (PLA) and polyhydroxyalkanoates (PHA). PLA is the largest biodegradable/compostable plastic produced worldwide and it is used in food packaging, textile, agriculture, electronics, transportation and in the biomedical field. PHA are bio-based and biodegradable polymers produced from organic substrates by various microorganisms, which accumulate polymers inside the cell for energy storage purposes. The production of PHA from CW will be discussed in detail in **Part 1** of this thesis.

Bioelectrochemical systems

Energy from the organic part of CW can be recovered through BES (bioelectrochemical systems), as electricity in MFC (microbial fuel cell), or for the H_2 synthesis in MEC (microbial electrolysis cell) (Logan et al., 2006; Rago et al., 2017). In MFC, specific microorganisms, namely exoelectrogens, oxidize the organic substrate and transfer the electrons to an anode electrode. Electrons then flow to a cathode electrode through an external circuit, producing electric power, and combine with an electron acceptor, such as oxygen, closing the circuit (Logan et al., 2006). In MEC, the protons resulting from substrate oxidation are the final electron acceptors, producing H_2, if enough energy is provided as input current to drive the reaction (Rago et al., 2017).

Hydrogenotrophic biomethanation

The hydrogen-based economy is an interesting and innovative technology, but it remains still inapplicable due to a series of technical and physical limitations that characterize the use of H_2 as an energy carrier. The main advantage of using hydrogen instead of methane is that it does not emit CO_2, since currently its higher conversion efficiency is contrasted by higher production, transport and plant costs; on the contrary, methane is certainly more practical and convenient than hydrogen for the following reasons (Rosato, 2021):

- CO_2 emissions from methane combustion do not alter the atmospheric carbon balance because they come from short-life biomass and not from fossil fuels.
- The energy density of biomethane (expressed in MJ/m^3) is greater than that of hydrogen although the latter has a PCI higher than methane (120 MJ/kg vs. 50 MJ/kg.), because hydrogen has a very low density (about 0.090 kg/Nm^3 vs. 0.702 kg/Nm^3 of methane).
- Methane does not brittle metals, so ordinary steel can be used to build tanks and pipelines.
- The greater dangerousness of hydrogen would involve greater costs plant compared to methane to ensure the safety of the user, indeed the range of methane explosivity is limited (between 5% and 15% by volume), while that of hydrogen is between 4% and 75% by volume.

For these reasons, in the perspective of a dairy biorefinery, it is possible to use the H_2 and CO_2 obtained during the DF to produce CH_4, thus achieving a dual purpose: to reduce CO_2 emissions and have an energy carrier easily usable.

Methane synthesis from a mixture of hydrogen and carbon dioxide can be both chemical and biological. Regardless of the nature of the process, the required amount of H_2 is at least four times the volume of CO_2 to be converted: $4H_2 + CO_2 = CH_4 + 2H_2O$

The chemical process is an industrial process that requires complex and expensive plants, profitable only on very large scales, while the biological one is a process that already takes place in all the anaerobic digesters by a particular group of microorganisms called *Archaea hydrogenotrophs*.

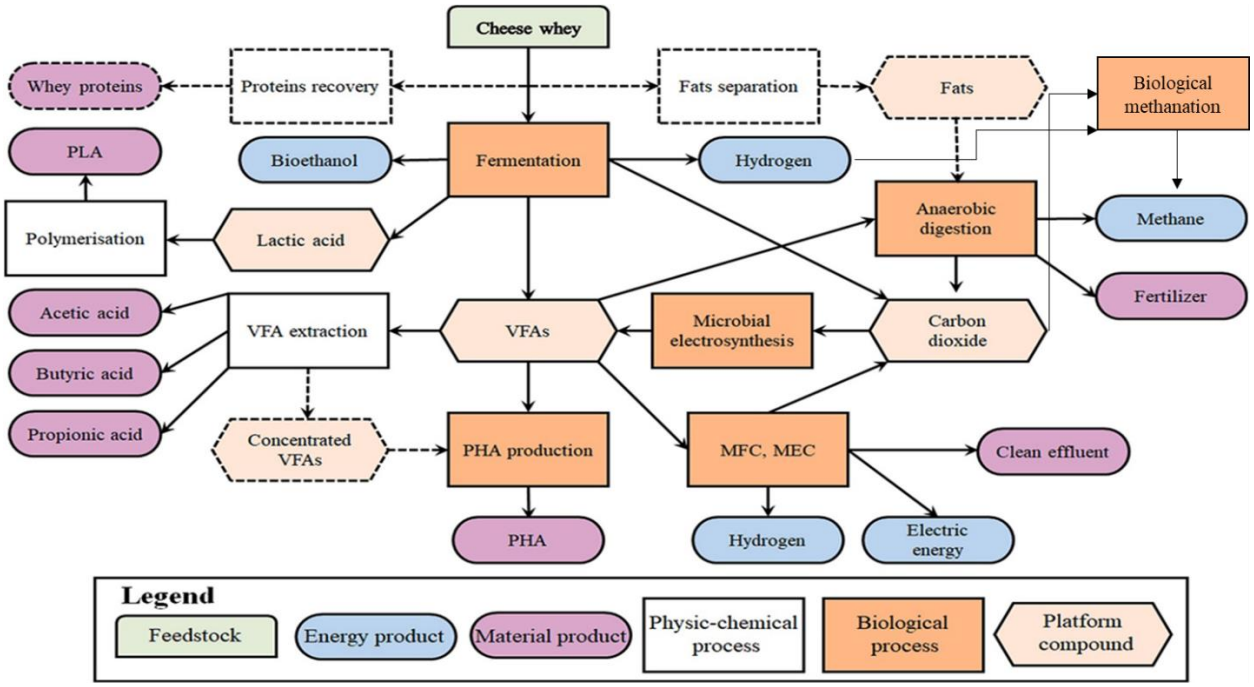

Figure 1.3 – Schematic representation of cheese whey valorization in a biorefinery approach for biochemical and bioenergy production (Asunis et al., 2020).

1.4 Scope and novelty of the PhD thesis

In the described context, this work is addressed to implement new strategies of biological and chemical processes for the valorization of dairy-industry waste in order to provide high value secondary products of interest for the market. Specifically, the activity is focused on the identification of the operating parameters which affect the DF process towards a sustainable production of specific organic acids useful in several applications.

For this purpose, two specific applications are identified: PHA production (**Part I**) and metal leaching (**Part II**), with the view to investigate the two challenging fields of bioplastics production and critical metals recovery from Hi-Tech scraps, for facing the concerns of environmental pollution and resource scarcity.

In **Part I**, a novel configuration of PHA production, involving a VFA extraction phase, is studied with the view to improve productivity and selectivity of the production process. In parallel, an investigation on the operating parameters (DF and accumulation stage) involved in the traditional process configuration is studied with the purpose to improve their effects on the final outcomes.
In **Part II**, a new strategy to produce a leaching mixture through a low cost and low chemicals demanding dark fermentation of CW is tested and its efficiency in metal leaching for metal recovery from scraps is studied on a selection of WEEE.

Figure 1.4 summarizes the scope and structure of the present thesis.

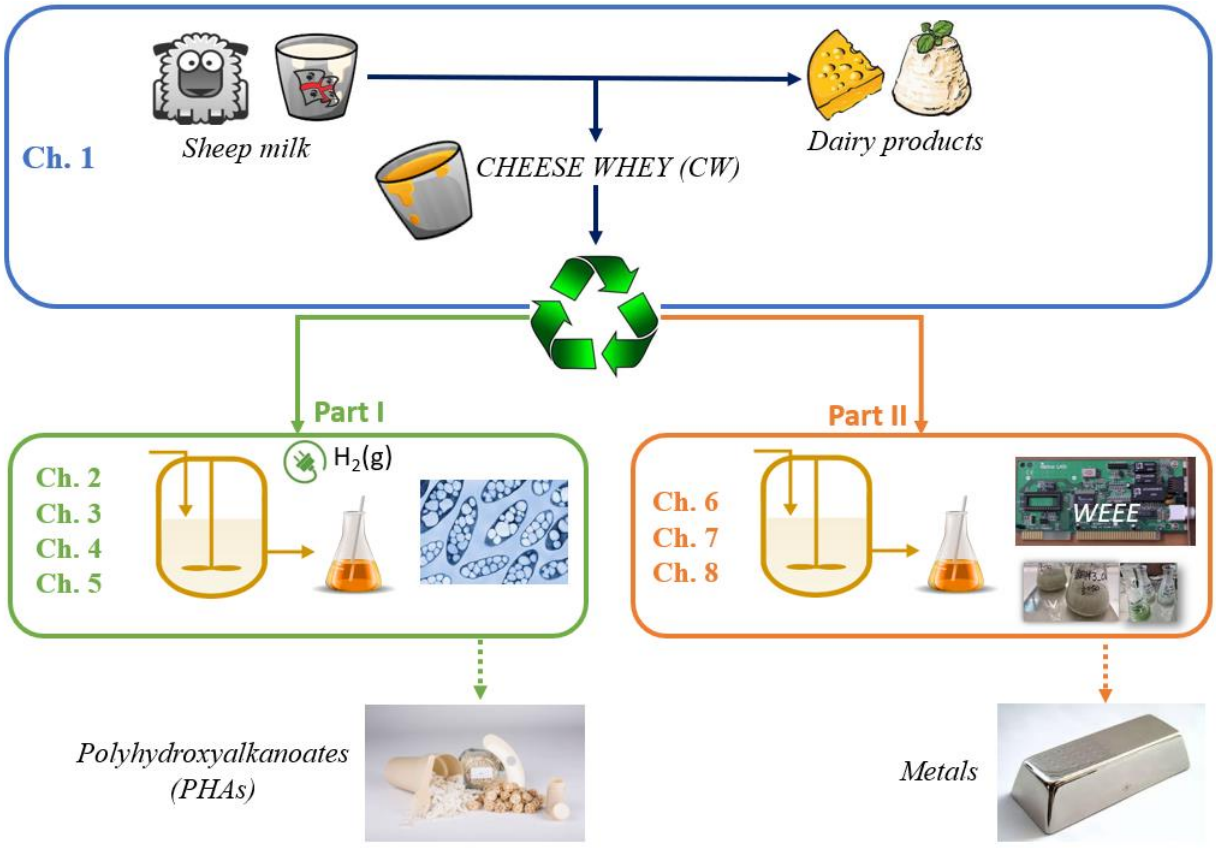

Figure 1.4 - Scope and structure of the thesis.

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PART I

Polyhydroxyalkanoates production from organic waste

Microorganisms are able to synthesize a wide range of polymers as intracellular storage materials. Polyhydroxyalkanoates (PHA) have emerged as a promising alternative to replace synthetic petroleum-based plastics thanks to their biodegradable and biocompatible nature (Ganesh Saratale et al., 2021). However, the consolidation on the market for PHA has not attained its possible peak in production and commercialization, mainly due to low yields and productivity, complexity in purification, and high production costs. Feedstock could account for about 40% of the total production cost (Amaro et al., 2019). The use of biowaste or a low-cost byproduct as a starting substrate could overcome these problems and make the PHA competitive with fossil-based plastics (Amaro et al., 2019). Nowadays, there is a growing interest in developing processes for PHA production from biowaste, promoted by rapid changes in legislation and environmental sustainability perspectives. For instance, the new circular bioeconomy package proposed by the EU for 2030 includes the conversion of biowaste into biofuels and bio-based products as a core strategy for a cleaner and more competitive Europe (European Commission, 2020).

Moreover, combining the use of a biowaste carbon source with mixed microbial cultures (MMC), which do not require sterile conditions and expensive pure feedstocks, is appealing for lowering production costs and making the process more sustainable, both economically and environmentally.

Chapter 2

Polyhydroxyalkanoates

This chapter gives an overview of the current state of the art of PHA production by mixed microbial cultures from organic wastes. Useful information about the opportunities and drawbacks associated with sustainable PHA production using waste fluxes are discussed. In addition, the chapter summarizes the advances in the research on fermentation conditions and metabolic approaches for the enhancement of microbial growth and PHA production.

Figure 2.1 - PHA inclusions inside some cell microorganisms (Türk, 2014).

2.1 Polyhydroxyalkanoates production and biosynthesis

Polyhydroxyalkanoates are polyesters synthesized in the form of polymer inclusions by microorganisms under nutrient-limiting conditions as carbon and energy source reserves (**Figure 2.1**). Nowadays, more than 90 microbial species are known to produce PHA and about 150 PHA monomers have been identified (Kumar et al., 2019). The type and structure of the synthesized PHA is determined by the producing microorganisms and their growing conditions, as well as the carbon source provided. In **Figure 2.2** the general chemical structure of PHA is presented.

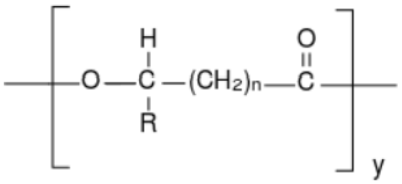

Figure 2.2 – General chemical structure of PHA.

PHA can be classified according to the length of their chain in short chain length (SCL) with 3-5 carbon atoms, medium chain length (MCL) with 5-15 C atoms, and long chain length (LCL) with more than 14 C atoms. The most common are poly(3-hydroxybutyrate), poly(3-hydroxyvalerate), and poly(3-hydroxybutyrate-co-3-hydroxyvalerate), usually reported as PHB, PHV and P(3HB-co-3HV), respectively (Pala-Ozkok et al., 2022). The properties of PHA are strongly dependent on the polymer's monomer composition. SCL PHA are characterized by a high degree of crystalline structure, thermostable, stiff, and brittle properties whereas, MCL PHA have a low crystalline structure and elastomeric characteristics with low melting temperature. PHA are used for many applications in various fields that include engineering, agriculture, food, chemistry, pharmacy, and medicine (Silva et al., 2021). They are more suitable for food packaging but currently they are much costlier as compared to other biopolymers such as PLA (7-12 €/kg vs 2-3 €/kg) and this has restricted their application to the production of expensive goods in the medical and pharmaceutical domain (Cinelli et al., 2019). Industrial production raises serious concerns as PHA are mainly produced by utilizing pure sugars, edible vegetable oils, food crops, etc. thus directly competing with food supply production. In addition, the expenses for carbon sources count 50% of the overall production, approximately (Ganesh Saratale et al., 2021). In recent decades the research in the production of these biopolymers has increased, thanks to the possibility of producing PHA from renewable sources, such as dairy residues (Asunis et al., 2022), olive mill wastewater (Campanari et al., 2014; Dionisi et al., 2005), fruit waste (Silva et al., 2022), and sugar molasses (Albuquerque et al., 2010).

The PHA biosynthesis pathway is closely linked to different metabolic pathways with which it shares different intermediates, especially acetyl-CoA (Lu et al., 2009; Tan et al., 2014). Under nutrient balanced conditions, the ratio between the carbon source and the essential nutrients, such as nitrogen- and phosphorus-based compounds, is suitable to sustain the active growth of microorganisms in non-limiting conditions. Conversely, under nutrient unbalanced conditions, i.e., when an essential nutrient such as nitrogen and phosphorus is limited in presence of an excess of

carbon, coenzyme A levels are non-inhibitory allowing acetyl-CoA to be directed towards PHA synthetic pathways for PHA accumulation (Jung & Lee, 2001). This metabolic regulation strategy, in turn, enables PHA-accumulating microbes to maximize nutrient resources in their adaptation to environmental conditions. PHA biosynthesis can occur from different carbon sources, such as sugar or VFA, and thus, through different metabolic pathways. The acetyl-CoA remains the crucial intermediate for both sugars and VFA as a carbon source. In the first case, glucose is metabolized to produce pyruvate which is then converted to acetyl-CoA, while in the second case, VFA can be catabolized into acetyl-CoA by the enzymatic activity of the β-oxidation pathway (Lu et al., 2009). The type of VFA influences the PHA synthesis pathway involved that in turn determines the type of obtained PHA monomers. For example, VFA with odd versus even chain lengths influences the balance of hydroxybutyrate (3HB) and hydroxyvalerate (3HV) in the PHA copolymer. While even VFA such as acetate and butyrate are activated directly to acetyl-CoA, odd VFA such as propionate and valerate are activated to propionyl-CoA (Pardelha et al., 2014).

2.2 PHA production by mixed microbial cultures

According to Ganesh Saratale et al., (2021), waste streams as substrate for PHA production are not adequate in terms of biomass productivity. Hence, an optimization of various operational parameters and improvement in fermentation technology are required to enhance microbial growth and consequently PHA production. Among the waste streams, cheese whey is one of the most promising (Asunis et al., 2020).

The conventional PHA production process by using MMC and cheese whey as the substrate consists of three different stages (**Figure 2.3**): (i) acidogenic fermentation, in which the complex organic matrix is converted into a mixture of metabolites, such as organic acids (OA), that are precursors for PHA biosynthesis; (ii) culture selection and enrichment, in which PHA-storing microorganisms are enriched from an aerobic mixed culture; (iii) PHA accumulation stage, where the selected culture is fed with the PHA-precursors to reach its maximum PHA storage capacity.

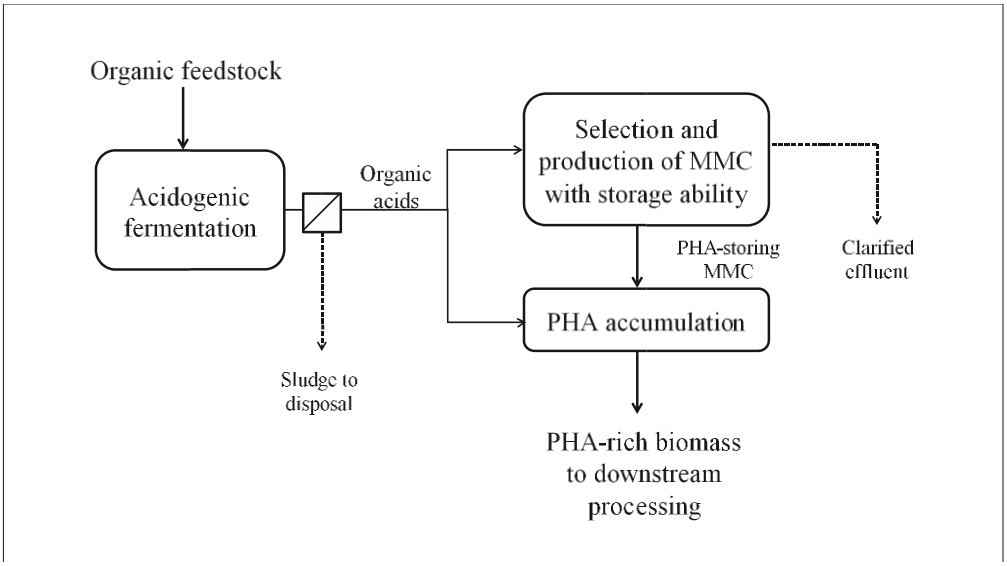

Figure 2.3 Three-stage production process (Valentino et al., 2017).

2.2.1 Acidogenic fermentation

Stage I consists in an acidogenic dark fermentation in which complex organic substrates, rich in carbohydrates, are converted to effluent rich in VFA, which are considered PHA precursors. Stage I is used only when a waste carbon source is used, and it is not required when the feedstock used is already rich in readily biodegradable organic carbon. In those cases, it has been shown that the substrate could be used directly for both Stage II and Stage III (Pala-Ozkok et al., 2022). Another case in which Stage I is not required is when the selected feedstock is a synthetic mixture of VFA. VFA, such as acetic, propionic, butiryc, valeric and caproic acid are recommended for MMC PHA production since they are readily made available and efficiently converted into PHA (Valentino et al., 2017).

Parameters such as HRT, SRT, pH, temperature and OLR affect the type of VFA produced during fermentation (Valentino et al., 2016) and, therefore, also the polymer composition obtainable in the following stages. Methanogenic activity during fermentation should be inhibited by imposing low pH (Reis et al., 2011), low SRT, low temperatures (Valentino et al., 2014; Beccari et al., 2009) or their combination.

Acidogenic fermentation is usually carried out in continuous flow in order to have steady acclimatized conditions and reach a high conversion rate and yields. Typical reactor configurations include CSTR, UASB, biofilms reactors, and packed-bed biofilm reactors.

2.2.2 Culture selection and enrichment

Stage II has the goal of promoting conditions for the survival and growth of PHA-accumulating bacteria. In the case of MMC processes, Stage II is required for the selection and enrichment of a strain capable of PHA production from a mixed consortium used as inoculum such as activated sludge (AS) from wastewater treatment plant (WWTP). Culture enrichment is possible by applying the so-called feast/famine regime, which consists of alternating periods of presence (feast) and absence (famine) of the carbon source under fully aerobic conditions (Valentino et al., 2017).

In the feast phase, when the organic substance is abundant, competitive mechanisms are created between the different bacterial species present and those that can quickly assimilate carbon are advantaged. From this point of view, the accumulation of PHA is a rapid assimilation mechanism that favors bacterial species able to accomplish it.

With this strategy, PHA-accumulating bacteria are the only ones able to survive in the next phase of famine, when there is no substrate because the accumulated PHA will be reused as a source of energy and for growth. The production processes of PHA with MMC can be classified based on the strategy used for the selection and enrichment of PHA-accumulating biomass. The applied strategy determines environmental stress in conditions of carbon excess and nutrient deficiency. The most applied strategies to promote the growth of bacteria capable of accumulating PHA are:

- Anaerobic dynamic feeding (ADF): short periods with the presence of substrate (feast) and long periods of absence (famine) are cycling alternated in aerobic conditions. The duration of the feast and famine phases is determined by the ability of the bacteria to quickly assimilate (in the form of PHA) the substrate provided (feast phase) and to withstand long periods without substrate (famine phase) thanks to the use of the PHA accumulated in the feast phase, as a source of carbon. A ratio of the duration of these phases (called F/F) less than 0.2 indicates the presence of PHA accumulating biomass (Valentino et al., 2019). The use of this regime determines the selection and growth of PHA-accumulating biomass that has a competitive advantage over other non-accumulating microorganisms, which do not survive this type of regime (Albuquerque et al., 2007; M. a. M. Reis et al., 2003).
- Uncoupled C and N (FF&UnCN): periods with the presence of carbon and absence of nitrogen (feast) and absence of carbon with the presence of nitrogen (famine), are cycling alternated in aerobic conditions. In the feast phase, no nitrogen is provided, while as soon

as the famine phase begins (when the carbonaceous substrate has been stored in the form of PHA) nitrogen is provided to ensure survival and stimulate the growth of the PHA-accumulating biomass (Oliveira et al., 2017).

- Anaerobic/aerobic (An/Ae): it exploits the capacity of polyphosphate-accumulating bacteria (PAO) of accumulating carbon in the form of PHA in the absence of external electron acceptors (anaerobic phase), and then consume them in the next aerobic phase (Serafim et al., 2008).
- Aerobic/anoxic (Ae/Anox): cyclic alternation of aerobic periods with the presence of substrate (feast) and anoxic in the absence of substrate (famine). In the anoxic period, denitrification can also occur with the use of stored PHA as a carbon source (Basset et al., 2016).
- Permanent feast: this strategy is based on the use of mixed photosynthetic bacteria (PMC: phototrophic mixed culture) in the continuous presence of substrate but in the absence of electron acceptors (O_2). Light can be present continuously or intermittently. The ability of phototrophic anoxygenic bacteria is to use external carbon to produce ATP using internal mechanisms to oxidize reduced molecules typical of metabolism (NADH, NADPH). One of these mechanisms is the accumulation of PHA which requires the reduction of its precursors during the formation of the polymer (Fradinho et al., 2016).

Most of the studies performed within the last 10 years have been based on ADF enrichment (Kourmentza et al., 2017). The enrichment strategy plays a central role in obtaining a selection of microorganisms with high PHA storage capacity. Also, the performance of the biomass enrichment depends on several parameters, including the effect of HRT, SRT, OLR, pH, temperature, nitrogen concentration, dissolved oxygen concentration (DO), cycle length, influent concentration, F/F ratio, and food/microbe ratio (Valentino et al., 2017).

The profile of dissolved oxygen is an indication of carbon utilization in the reactor. When the organic substrate is supplied, the concentration of dissolved oxygen in the reactor decreases; when the organic carbon is ending, the dissolved oxygen starts to increase up to an asymptotic value, indicating the end of the feast phase and the beginning of the famine phase.

For the optimal selection of biomass, the F/F ratio should be 0.2 or in any case not exceeding 0.33 (Valentino et al., 2015). This has also been demonstrated by (Reis et al. (2011) who state that for

low values of the F/F ratio there is good storage of PHA due to the adaptation of biomass to cyclic conditions; while for high values of F/F ratio, the selection mechanism is lacking, resulting in the development of both accumulating biomass and biomass that cannot accumulate.

OLR is generally set in a range between 0.3 and 1.2 g_{COD}/L*d (Beccari et al., 1998; Beun et al., 2002; Martins et al., 2003; Serafim et al., 2008).

Valentino et al. (2015) report that the mechanism of accumulation of PHA is disadvantaged by too low SRT and combining high OLR and low HRT (on the order of 1 day) corresponds to a better selection of biomass with good storage capacity and therefore with a higher yield and rate of polymer production.

Regarding process configuration, a continuous system has been proposed where instead of an SBR, the feast and the famine phases were operated in separate CSTR (Albuquerque et al., 2010).

Finally, nutrients are crucial in the selection phase to support the growth of biomass, with a ratio of C:N:P = 100:10:1 (Duque et al., 2014).

2.2.3 PHA Accumulation

The accumulation phase is the final step in the production of PHA. This phase consists of continuous conditions of feast with the objective of achieving maximum PHA production with the pre-selected culture. In this case, the intake of nutrients such as nitrogen and phosphorus should be avoided or limited compared to the organic substance provided, to minimize the growth phenomena of biomass. Nutrient deficiency leads to higher PHA accumulation levels, as demonstrated in the studies of (Johnson et al., 2009) and (Serafim et al., 2008). The best results were obtained with N/COD ratios between 2 and 15 mg_N/g_{COD} and P/COD between 0.5 and 3mg_P/g_{COD} (Valentino et al., 2014).

This phase can be carried out in batch or fed-batch with single or multiple substrate inputs (Valentino et al., 2017), but in some cases also in the same reactor of Stage II.

The most adopted feed strategy is the pulse-wise feeding strategy. This strategy consists of a repeated pulse feed until the maximum storage capacity of the culture is reached. Adopting this strategy, a PHA content in the cell up to 65%wt. can be reached (Duque et al., 2014). Higher is the PHA content and more PHA per unit of biomass is produced, and the extraction is more

straightforward, thus reducing the costs. PHA yields strongly varied from 0.02 to 0.9 $g_{PHA}/g_{substrate}$ (Rodriguez-Perez et al., 2018). Interestingly, around 60 % of the studies reviewed by (Rodriguez-Perez et al., 2018) reported values lower than 0.5 $g_{PHA}/g_{substrate}$. Pilot-scale studies usually report lower yields with PHA content of 24-35%wt. and yields lower than 0.4 $g_{PHA}/g_{substrate}$.

2.3 Downstream processes

At the end of the three phases a biomass rich in PHA is obtained, with production yields that can reach even 40-60%wt. of PHA on the weight of volatile suspended solids (VSS) (Colombo et al., 2017; Duque et al., 2014).

The downstream treatment has an important impact on the costs, the final properties of the biopolymer, and the overall environmental sustainability of the process as the recovery methods are usually based on the use of halogenated solvents, which are toxic and hazardous (Fiorese et al., 2009; Silva et al., 2021).

The general downstream processing of PHA involves subjecting the biomass recovered from the cultivation broth at the end of the production process to procedures for polymer extraction from the cells, and its subsequent purification, depending on the intended area of application (**Figure 2.4**).

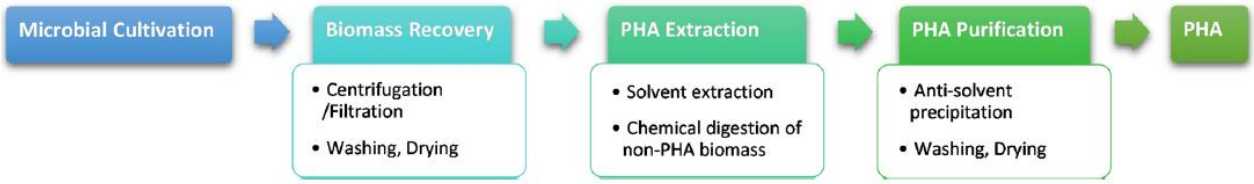

Figure 2.4 – General downstream processing of PHA from (Silva et al., 2021)

In order to obtain a satisfactory result in terms of the economic value of the extracted product, it is essential to choose the most appropriate extraction method depending on the subsequent use of PHA.

The extraction methods most commonly applied today are:

- Solvent extraction: is a technique used when a high purity of the extracted PHA is required; the method consists of pre-treatment, precipitation, centrifugation or filtration phases. In the pre-treatment phase, it is ensured that, as a result of cell lysis, PHAs may be available in the liquid phase of an appropriate organic solvent, like chloroform, methyl ether or methylene chloride (Rameshwari & Meenakshisundaram, 2014). Due to the addition of substances in which PHA are not soluble, such as methanol and ethanol, the precipitation phase occurs; finally, with the filtration or centrifugation phase, PHA are permanently separated to the liquid phase.
- Digestion of non-PHA cell mass: the digestion consists in a physical, enzymatic or chemical treatment followed by a separation step to separate PHA granules from the solubilized cell. Chemical digestion is carried out using surfactants, alkalis or acids (typically sodium hypochlorite). Enzymatic digestion is a more expensive and complex procedure and needs a chemical pre-treatment, and enzymatic hydrolysis of the cell wall compounds and proteins to use surfactants.
- Alternative solvents: the search for greener solvents or innovative techniques to extract and purify PHA, has gained increasing attention from the scientific community. Hence, alternative low toxicity solvents, like propylene carbonate, have been evaluated for their efficiency in PHA extraction. For medium chain length PHA there is a larger number of solvents that can be used for the extraction procedure since those polymers are soluble also in solvents, such as acetone, hexane-ethyl acetate and methyl tert-butyl ether (Fiorese et al., 2009; Jiang et al., 2006).
- Alternative techniques: among the most environmentally sustainable approaches are to note innovative processes such as ultrasound-assisted extraction, dissolved air floatation, non-ionic surfactants, and supercritical fluids.

For all these approaches, bacterial cells are first collected by centrifugation or filtration of the broth, the cell-free supernatant is usually treated or discarded, while the biomass pellet is used for PHA extraction. The dry biomass is often pre-treated with methanol for the removal of lipids and coloring compounds, which contributes to increasing the purity of the extracted biopolymer (Silva et al., 2021).

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Chapter 3

Silicone membrane for selective volatile fatty acid extraction

In this chapter will be presented a summary of the article "Silicone membrane contactor for selective volatile fatty acid and alcohol separation" published in the journal "Process Safety and Environmental Protection 148", 2021 – Harish Ravishankar, Paolo Dessì, Stefano Trudu, Fabiano Asunis, Piet N.L. Lens (doi.org/10.1016/j.psep.2020.09.052).

This chapter is based on the extraction of VFA by a commercial non-porous tubular silicone membrane known to be permeable to organic substances. Silicone is also known to be a cheap and low-risk material for "fouling". The process is mainly based on the concentration gradient that is established between the silicone tubular membrane inside which the fermentate flows (called "feed") and the distilled water in which the membrane is immersed (called "draw"), as shown in **Table 3.1**.

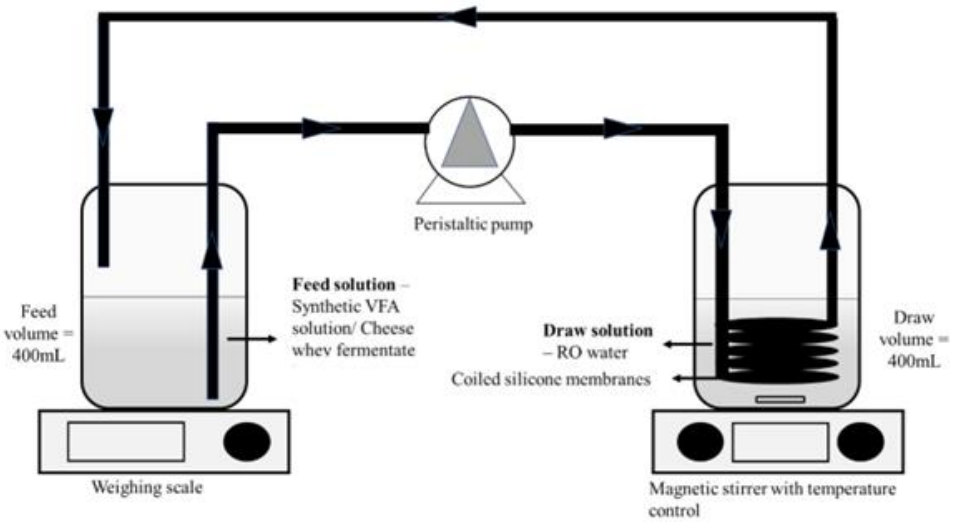

Figure 3.1 – Schematic experimental set-up (Ravishankar et al., 2021).

3.1 Introduction

VFA can be used in the chemical or pharmaceutical industry, but also as feedstocks for bioprocesses such as PHA production (Asunis et al., 2022). VFA can be synthesized from organic waste (see Chapter 4), even in a heterogeneous mixture, from MMC that perform acidogenic fermentation processes such as DF (Bastidas-Oyanedel et al., 2015). Separation and recovery of these valuable products is still a major bottleneck due to low concentrations and the complex physiochemical nature of the fermentate and digestate (Zacharof & Lovitt, 2013). Moreover, the recovery of organic acids is typically 30–40% of the processing cost (Bekatorou et al., 2016), therefore economically viable recovery processes need to be developed. Several separation methods have been reported in the literature, i.e. solvent extraction, adsorption, and membrane processes, including electrodialysis, reverse osmosis/nano-filtration and membrane extraction (Atasoy et al., 2018), each having its own benefits and drawbacks.

Membrane processes are well documented in the literature and used for VFA separation (Aktij et al., 2020). Membrane systems usually involve application of high pressure (e.g. nanofiltration and reverse osmosis) or an electric field (e.g. electrodialysis) across a semi-permeable or ion exchange membrane, respectively (Aghapour Aktij et al., 2020). However, both processes are energy intensive and still require considerable research to make them cost-effective. Another type of membrane based VFA separation process is the vapor permeation membrane contactor that works on vapor pressure difference and concentration gradient (Aydin et al., 2018).

Typically, the use of sodium hydroxide allows the recovery of the VFA in the form of sodium salt in the aqueous phase. Then, the use of a mineral acid in the downstream process is required to achieve the recovery of the products in the free acid form. This last step requires the use of another chemical and produce a mineral salt that will need to be disposed of in most of the cases (Outram and Zhang, 2018). The use of silicone membrane and water as extractant has recently been proposed as a cost-effective solution (Outram and Zhang, 2018). Silicone is known to be permeable to organic substances and resistant to fouling problems. The use of water as an extractant avoids the use of expensive and non-environmentally friendly chemicals, making the process solvent-free.

This article investigated the innovative application of silicone membranes for VFA separation from synthetic solution and fermented cheese whey, with water as the extractant. The VFA

extraction through the silicone membranes and their recovery, flux, mass transfer coefficient, and separation factor were examined at different temperatures (20, 30 and 40 °C) and pH (3 and 5).

3.2 Materials and methods

3.2.1 Synthetic solution and fermented cheese whey

A synthetic solution containing an equal amount of acetic, propionic, butyric, valeric and caproic acid (5 g/L each) was prepared. Equal concentrations were chosen to avoid concentration-related changes in flux and separation factor. Fermented cheese whey (fCW), rich in VFA, was obtained after fermentation of cheese whey (obtained from the dairy industry Dairygold, Mitchelstown, Ireland) at 35 °C and pH 5 for 7–8 days (Dessì et al., 2020). Preliminary analysis of the VFA content showed the predominance of butyric and acetic acid with an average concentration of 4.6 and 4.0 g/L, respectively.

3.2.2 Experimental set-up

A system consisting of two beakers (feed and draw), a silicone tube membrane (peroxide cross-linked, with an internal diameter of 3 mm and external diameter of 5 mm, and 2 m length, VWR Ltd), a peristaltic pump (Masterflex) and a system of non-permeable tubes (Masterflex L/S Tygon E-Lab E-3603) connecting the feed and the draw was used for the experiments (**Figure 3.1**). The feed beaker contained 400 mL of synthetic solution or fermented cheese whey, whereas the draw beaker contained 400 mL of deionized water. The peristaltic pump was operated at 55 mL/min. The draw solution was stirred at 150 rpm by a magnetic stirrer with temperature control, inside which the silicone membrane was immersed for extraction tests. The experiments were performed at pH 3 and 5 at three different extraction temperatures (20, 30 and 40 °C). The pH values chosen were below and slightly above the pKa of the acids. Before the start of each experiment, if necessary, the feed pH was adjusted using H_2SO_4 or NaOH.

3.2.3 Analytical methods

The pH and conductivity for the draw and feed solution were monitored using a pH and conductivity probes (AB200). The change in mass of the feed was monitored using a weighing scale (Ohaus Scout® SKX). VFA concentrations were measured using a Varian 450 gas chromatograph (GC) equipped with a flame ionization detector and an SGE BP-21 column (30 m

long, internal diameter 0.25 mm and film thickness 0.25 µm). Prior to GC analysis, fermented cheese whey samples were centrifuged at 11,000 rpm for 6 min (Eppendorf non-IVD Centrifuge 5430 G) and the supernatant was filtered (0.2 µm) and diluted appropriately.

3.2.4 Calculations

The following parameters were calculated based on the equations from (Outram & Zhang, 2018) and (Aydin et al., 2018).

The flux (J) of individual VFA was calculated using equation 1:

$$J = \frac{1}{A}\frac{\Delta m}{\Delta t} \quad Eq\ (1)$$

where Δm is the mass of VFA permeated through the membrane (g), A is the membrane surface area (m^2) and Δt is the time (h).

The overall mass transfer coefficient, K, was estimated using equations 2 and 3:

$$J_i = AK\left(C_{i,D} - C^*_{i,D}\right) \quad Eq\ (2)$$

$$ln\left(\frac{C_{i,Dt} - C^*_{i,D}}{C_{i,D0} - C^*_{i,D}}\right) = \frac{AKt}{V_F} \quad Eq\ (3)$$

where $C_{i,D0}$ is the initial concentration at t= 0, $C_{i,Dt}$ is the concentration at time, $C^*_{i,D}$ denotes the equilibrium concentration, V_f is the initial volume of the VFA solution, t is time (h) and A is the surface area (m^2). The values were calculated using the draw VFA concentration.

The membrane separation factor (β_{VFA}) was estimated using equation 4:

$$\beta_{VFA/Water} = \frac{\frac{VFA\ weight\ fraction\ in\ permeate}{VFA\ weight\ fraction\ in\ the\ feed}}{\frac{Water\ weight\ fraction\ in\ the\ permeate}{Water\ weight\ fraction\ in\ the\ feed}} \quad Eq\ (4)$$

The water weight fraction in the permeate and feed is calculated using equation 5:

$$W_{water,i} = \frac{\rho_i \text{ x } V_i - (\sum m_{VFA,i})}{\rho_i \text{ x } V_i} \quad Eq\ (5)$$

where ρi is the density of the solution (g/L), V_i is the volume of the solution (L) and $m_{VFA,i}$ is the mass of individual VFA (g).

The recovery (%wt.) was calculated using equation 6:

$$Recovery\ = \left(\frac{Concentration\ of\ VFA\ in\ draw\ solution\ \ (t_n)}{Concentration\ of\ VFA\ \ in\ feed\ solution\ (t_0)}\right) * 100 \quad Eq\ (6)$$

where t_0 and t_n represent the start and the end time (h) of experiment.

3.3 Results and discussion

3.3.1 VFA recovery from synthetic solution

Electrical conductivity and pH were monitored for the feed and draw solution, which in this experiment was an indicator of the VFA migration. The pH of the draw solution showed a sharp decrease during the initial 2 h (for all conditions), indicating a rapid diffusion of VFA across the silicone membrane (**Figure 3.2**a,b). The conductivity profiles of the feed solutions (**Figure 3.2**c,d)

decreased with time, while that of the draw solution increased, further confirming the VFA migration.

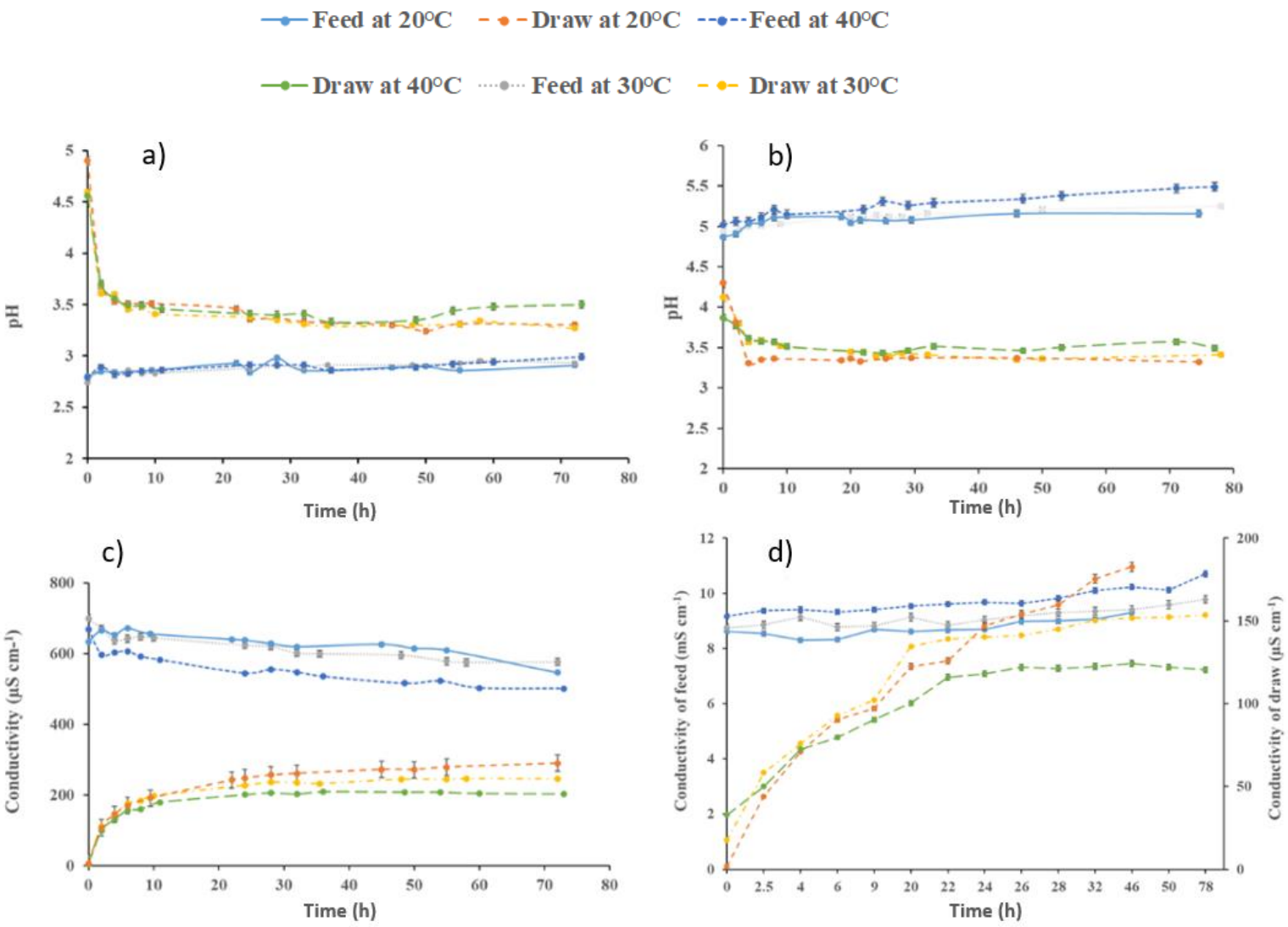

Figure 3.2 - pH profiles of the draw and feed solutions at different temperatures and pH: **a)** pH 3, **b)** pH 5; Conductivity profiles of the draw and feed solutions at different temperatures and pH: **c)** pH 3, **d)** pH 5.

Figure 3.3 shows the concentration profile of VFA in the draw solution over time. The VFA concentration increased over time, except for caproic acid, which initially increased and then decreased for all conditions. This suggests the possibility of evaporative loss or the formation of

an immiscible layer of the draw solution due to the low solubility as opposed to other VFA in water (Khor et al., 2017). Nonetheless, the rapid increase in caproic acid concentration in a short time can be used for its selective extraction from a VFA mixture even at such high initial concentrations.

Based on the initial VFA concentration in the feed and final VFA concentration in the draw solution, the recovery of individual VFA was calculated for the experimental conditions (**Figure 3.4**). The increase in temperature considerably decreased the VFA mass transfer from the synthetic VFA solution and hence reduced the VFA recovery at both pH values investigated. At pH 3, the recovery trend after 70 h was valeric > caproic > butyric > propionic > acetic acid. However, caproic acid recovery could be enhanced when extracting at a shorter extraction time of 32, 24 and 11 h at 20, 30 and 40 ◦C, respectively, where its concentration was higher as compared to 70 h. A maximum recovery of 5, 15, 29, 45 and 38 %wt. was obtained at pH 3 and 20 ◦C for acetic, propionic, butyric, valeric and caproic acid, respectively. At pH 5, the VFA recovery followed a similar trend as observed at pH 3. Since the extraction process through silicone is driven by a concentration gradient, a maximum of half the initial concentration in the feed solution can be theoretically obtained in the draw solution. The recovery of VFA was lower than the theoretical equilibrium concentration of 2.5 g/L, which could possibly be due to the adsorption of VFA on the silicone membrane due to its high hydrophobicity. A similar observation of concentrations below the predicted equilibrium concentration is reported for a synthetic VFA solution and was attributed to the distribution of VFA in the membranes (Outram & Zhang, 2018).

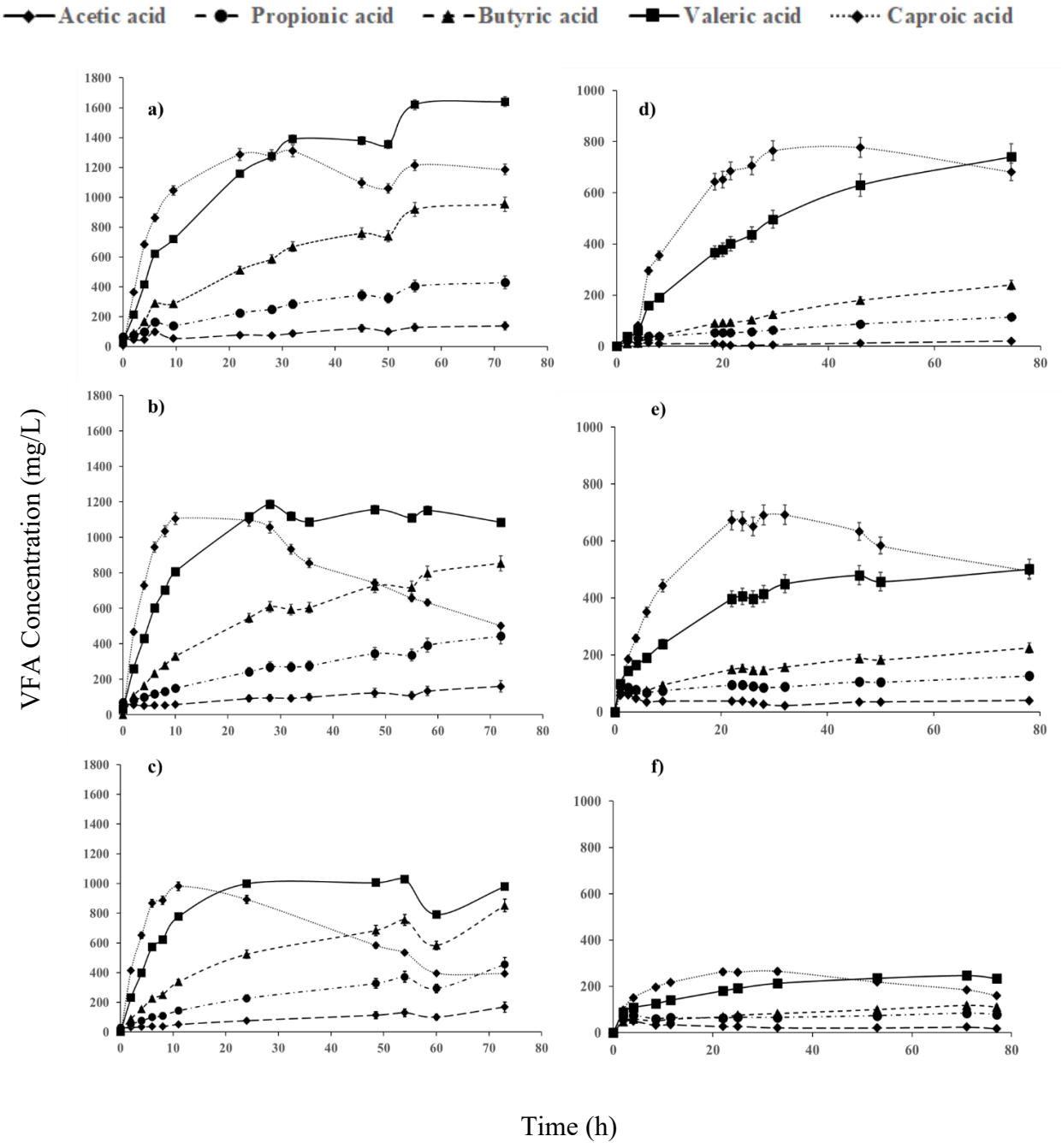

Figure 3.3 - Draw solution VFA concentrations with synthetic VFA mixture as feed at pH 3 a) 20°C, b) 30°C, c) 40°C, and pH 5 d) 20°C, e) 30°C and f) 40°C.

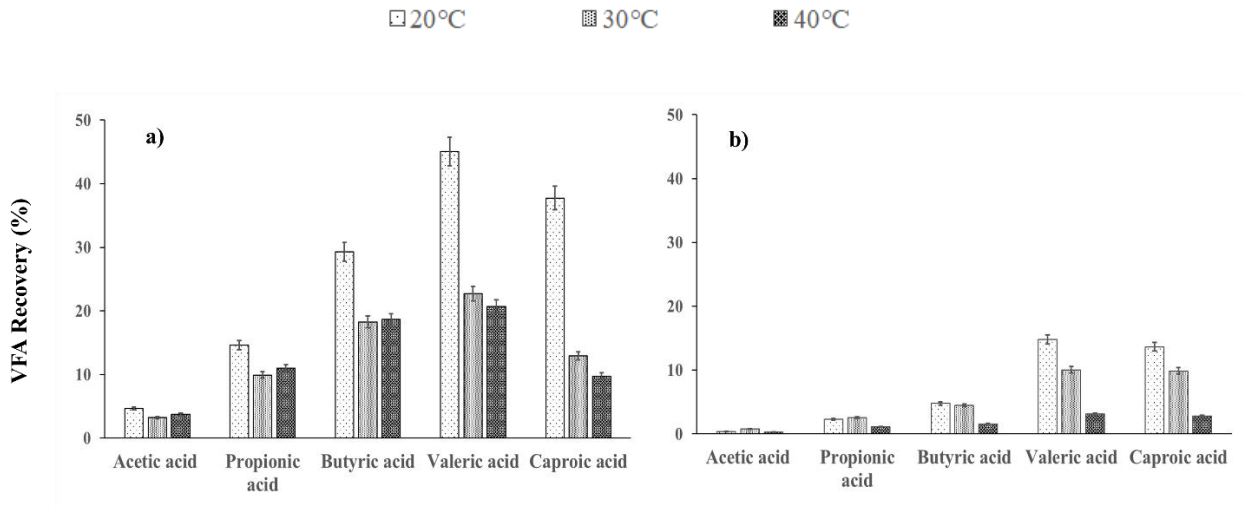

Figure 3.4 - VFA recovery through the silicone membrane from synthetic feed at a) pH 3, b) pH 5.

3.3.2 VFA recovery from fermented cheese whey

For all experimental conditions tested for VFA extraction from fermented cheese whey, butyric acid concentrations in the draw solution increased faster than the acetic acid concentration (**Figure 3.5**). A substantially higher VFA extraction was achieved at pH 3 than 5, and at both pH values, a higher temperature favored VFA extraction. This effect of temperature on VFA extraction through the membrane can be understood through the relationship between penetration and temperature established by Van't Hoff–Arrhenius relationship (Eq. 7):

$$P = P_0 \exp(\frac{-E_p}{RT}) \qquad \text{Eq. (7)}$$

where P is the penetration, Po is a pre-exponential factor, R is the molar gas constant, T is the temperature and Ep is the apparent activation energy of permeation required for VFA into the membrane and the opening between the polymeric chains of the membrane to allow the VFA to diffuse (Han et al., 2001).

The maximum recovery efficiency for acetic and butyric acid after 70 h operation at 40 °C amounted to, respectively, 7 and 21.5%wt. for pH 3, and 3.5 and 7%wt. for pH 5 (**Figure 3.6**). The lower removal efficiency was observed compared to the synthetic VFA solution can be attributed to the presence of the solids and ions present in the fermented (Aydin et al., 2018). For example, the presence of calcium and phosphate ions in the fermented.

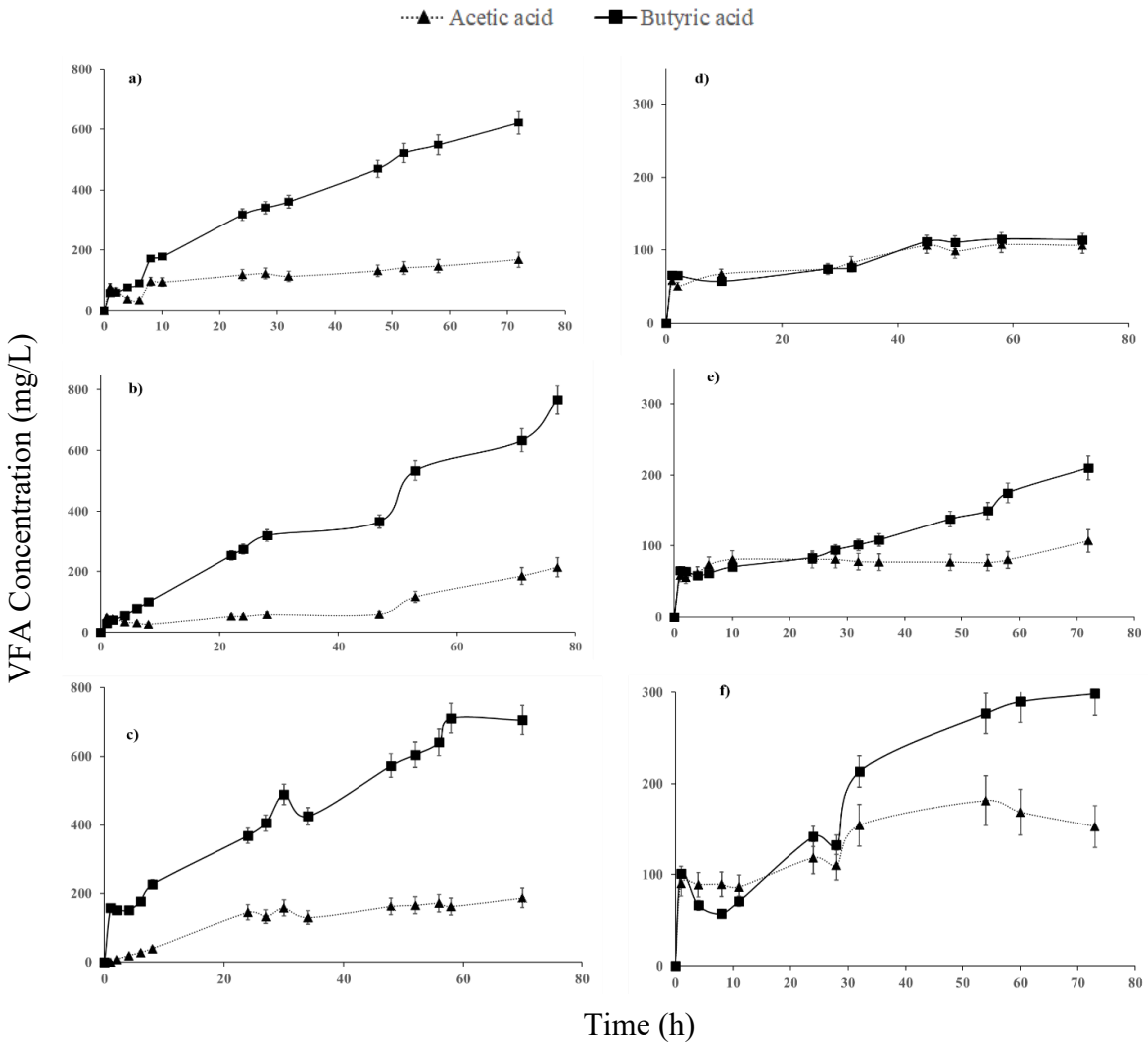

Figure 3.5 - Draw solution VFA concentrations with fermented cheese whey as feed at pH 3 a) 20°C, b) 30°C, c) 40°C and pH 5 d) 20°C, e) 30°C and f) 40°C.

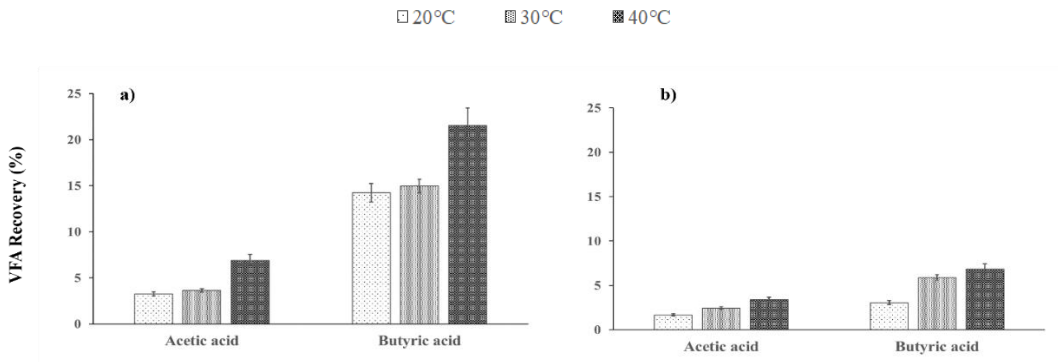

Figure 3.6 - VFA recovery by the silicone membrane from fermented cheese whey at a) pH 3 and b) pH 5.

3.3.3 Flux, mass transfer coefficient and separation factor

Temperature and pH affected the mass transfer through the silicone membrane. The flux values of the fatty acids generally decreased with an increase in extraction temperature. This was a result of the higher net vapor pressure in the draw side, resulting in resistance to diffusion and hence reduction in the flux. From the tests with the synthetic solution valeric acid showed the highest overall flux after 70 h of operation (0.70±0.07 g/m^2*h) at pH 3 and 20 °C, followed by caproic acid (0.52±0.06 g/m^2*h) (**Table 3.1**). However, at the first 32 h of operation at pH 3 and 20 °C, caproic acid was extracted with a maximum flux of 1.3±0.02 g/m^2*h. From fCW at pH 3, the maximum flux obtained for butyric and acetic acid was 0.28±0.02 g/m^2*h (at 30°C) and 0.09±0.00 g/m^2*h (at 40°C), respectively.

The overall mass transfer coefficients of VFA extracted at different temperatures and pH (**Table 3.2**) were calculated considering the maximum concentration obtained in the draw solution as the equilibrium concentration. The overall mass transfer coefficients of VFA followed the order of the carbon chain length (caproic > valeric > butyric > propionic > acetic acid) for both pH values and for both solutions investigated. The longer chain acids, indeed, have a higher affinity to the silicone membrane due to their higher hydrophobicity (Yesil et al., 2014). The coefficients were lower at pH 5 as compared to pH 3, although the effect of temperature on the overall mass transfer coefficients was not observed. Overall, the coefficients obtained from fCW are similar to those obtained for the synthetic VFA solution for the most conditions tested, confirming the net driving force for VFA separation is the free acid concentration.

The separation factor is defined as the ability of a membrane to separate a target compound and is a crucial parameter when selecting membranes. The separation factor calculated in the present work shows the selectivity of VFA over water. The non-porous silicone membrane used in this study does not support water transfer and the separation of VFA depends solely on the concentration gradient that acts as the driving force. Valeric and caproic acid had a higher separation factor from the synthetic VFA solution as compared to other VFA at both pH 3 and 5 (**Table 3.3**). As the separation factors were calculated based on the final concentrations of VFA in the draw solution, caproic acid due to its low solubility showed a lower separation factor compared to valeric and butyric acid at pH 3 and 30 as well as 40°C. However, the separation factor for caproic acid increased at pH 5, which is due to the lower separation of acetic, butyric and propionic acid at pH 5 as opposed to pH. The separation factor increased for acetic, propionic and butyric acid with increase in temperature at both pH values investigated. High separation factor values (>1) indicate better selectivity, suggesting the suitability of the membrane. For separation of volatile organic carbons (VOC), such as acetic acid, ethylene glycol and dimethyl acetamide from water, the separation factor typically ranges from 1-5 for silicone membranes. An increase in separation factor beyond 5 provides very little additional benefits for VFA separation. When fCW was used as the feed, butyric acid had a higher separation than acetic acid at both pH 3 and 5. The increase in temperature had a negligible effect on the separation factor of both acids, indicating butyric acid had a better selectivity over acetic acid regardless of the temperature.

Table 3.1 - Flux of VFA across a silicone membrane for different feed composition and temperatures.

	Flux ($g/m^2/h$)											
VFA	**Synthetic VFA solution**						**Fermented cheese whey**					
	pH 3			**pH 5**			**pH 3**			**pH 5**		
	20°C	**30°C**	**40°C**	**20°C**	**30°C**	**40°C**	**20°C**	**30°C**	**40°C**	**20°C**	**30°C**	**40°C**
Acetic acid	0.04±0.00	0.04±0.00	0.03±0.02	0.01±0.00	0.02 ±0.02	0.01±0.01	0.04±0.02	0.06±0.04	0.09±0.00	0.02±0.00	0.02±0.00	0.03±0.00
Propionic acid	0.16±0.01	0.16±0.02	0.10±0.04	0.05±0.00	0.06±0.05	0.04±0.00	-	-	-	-	-	-
Butyric acid	0.40±0.03	0.38±0.03	0.20±0.08	0.10±0.00	0.09 ±0.00	0.05±0.00	0.25±0.08	0.28±0.02	0.25±0.02	0.02±0.00	0.07±0.00	0.09±0.00
Valeric acid	0.70±0.07	0.47±0.07	0.23±0.10	0.32±0.03	0.20 ±0.00	0.12±0.16	-	-	-	-	-	-
Caproic acid	0.52±0.06	0.22±0.06	0.09±0.04	0.30±0.06	0.20 ±0.02	0.10±0.02	-	-	-	-	-	-

Table 3.2 - Mass transfer coefficient values of VFA through a silicone membrane with synthetic and fermented cheese whey as the feed.

VFA	Mass transfer coefficient (µm/s)											
	Synthetic VFA solution						Fermented cheese whey					
	pH 3			pH 5			pH 3			pH 5		
	20°C	30°C	40°C	20°C	30°C	40°C	20°C	30°C	40°C	20°C	30°C	40°C
Acetic acid	0.12±0.02	0.06±0.02	0.10±0.00	0.02±0.00	0.04±0.00	0.06±0.04	0.06±0.04	0.03±0.01	0.09±0.04	0.12±0.09	0.12±0.08	0.14±0.01
Propionic acid	0.15±0.01	0.08±0.03	0.13±0.00	0.05±0.03	0.04±0.01	0.08±0.07	-	-	-	-	-	-
Butyric acid	0.17±0.01	0.13±0.06	0.16±0.00	0.07±0.02	0.06±0.01	0.18±0.06	0.14±0.02	0.13±0.02	0.08±0.01	0.17±0.03	0.07±0.01	0.20±0.04
Valeric acid	0.23±0.02	0.33±0.20	0.30±0.07	0.11±0.05	0.16±0.08	0.16±0.07	-	-	-	-	-	-
Caproic acid	0.49±0.17	0.92±0.76	0.73±0.49	0.35±0.23	0.46±0.35	0.55±0.32	-	-	-	-	-	-

Table 3.3 - Separation factor of VFA from synthetic solutions and fermented cheese whey.

Synthetic VFA solution						
VFA	**pH 3**			**pH 5**		
	20°C	**30°C**	**40°C**	**20°C**	**30°C**	**40°C**
Acetic acid	0.17±0.01	0.25±0.01	0.28±0.01	0.06±0.00	0.13±0.00	0.16±0.00
Propionic acid	0.53±0.02	0.74±0.01	0.83±0.01	0.38±0.00	0.47±0.00	0.62±0.00
Butyric acid	1.06±0.00	1.34±0.00	1.41±0.00	0.70± 0.00	0.80±0.00	0.85±0.00
Valeric acid	1.63±0.02	1.64±0.02	1.56±0.02	1.55±0.00	1.64±0.00	1.71±0.00
Caproic acid	1.37±0.00	0.94±0.00	0.74±0.01	1.78±0.01	1.87±0.01	1.58±0.00
Fermented cheese whey						
VFA	**pH 3**			**pH 5**		
	20°C	**30°C**	**40°C**	**20°C**	**30°C**	**40°C**
Acetic acid	0.39±0.01	0.40±0.01	0.38±0.01	0.77±0.01	0.65±0.01	0.68±0.01
Butyric acid	1.68±0.01	1.68±0.01	1.68±0.01	1.32±0.02	1.33±0.02	1.28±0.02

3.4 Conclusion

The applicability of silicone membranes for VFA extraction from synthetic solution and fermented cheese whey using water as an extractant was demonstrated. Acetic and butyric acid extraction from fCW (pH 3; 40 ◦C) was achieved with 7% and 21.5% recovery efficiency, respectively, showcasing that longer chain VFA had better selectivity through silicone membrane.

Further studies are required with the aim of optimization of the extraction system and better understanding of the different affinity of VFA for the silicone at different operational condition (pH, temperature, cross flows).

In addition, the integration of the extraction method described for the PHA production from CW can have several positive aspects:

• Modulation of the composition of VFA, PHA precursors;

• Improve PHA storage using a nutrient-poor substrate during the PHA accumulation stage.

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Chapter 4

Implementing silicone membrane extraction in polyhydroxyalkanoates production from biowaste

In the present chapter, VFA extraction by using silicone membranes for improved PHA production from sheep CW was investigated. To this aim, in the present study batch, dark fermentation tests were performed on raw CW without any addition of biomass inoculum nor any pre-treatment of the substrate at an operating pH of 6. The DF effluent, rich in VFA was characterized and used for (i) selection and enrichment of a PHA-storing MMC and (ii) submitted to a VFA extraction process made by silicone membranes. The selected biomass was then submitted to PHA accumulation tests using the extracted VFA as a feed.

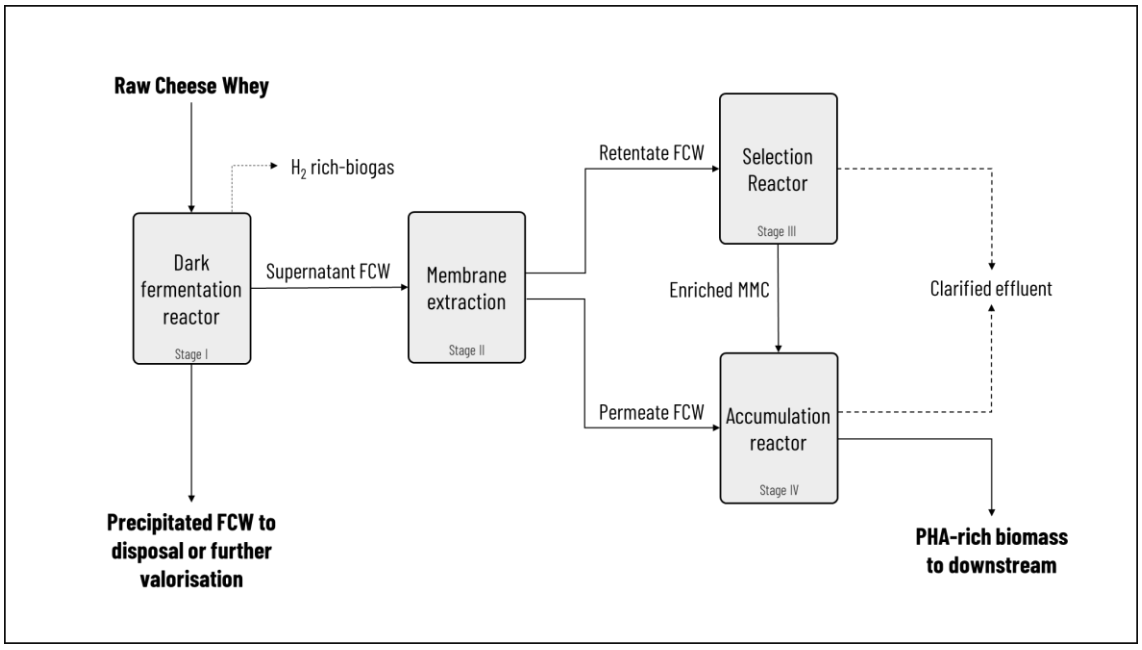

Figure 4.1 – Experimental set-up of the four-stage process proposed for PHA production by mixed microbial cultures (MMC) using raw sheep cheese whey (CW) as substrate.

4.1 Introduction

The use of environmentally-friendly products increased the interest in renewable resources as an alternative to petrochemical products. PHA are examples of such promising products, as they are biodegradable polymers with numerous potential applications. PHA production approach consists of using an open MMC and cheap feedstocks (waste or industry by-products feedstock). The PHA process generally comprises three stages: (1) acidogenic fermentation stage (conversion of organic carbon into fermentation products); (2) culture selection stage (enrichment in PHA-storing organisms through a feast and famine regime); and (3) PHA accumulation stage (PHA production up to the culture's maximum capacity).

The PHA production depends not only on the process's operational parameters but is strongly affected by the substrate characteristics. Indeed, the potential challenge of PHA production from biowaste can be identified in an insufficient acidification degree, an excessive amount of nutrients (nitrogen and phosphorous in particular), high suspended solids, high salinity and the presence of unknown contaminants (Estévez-Alonso et al., 2021). Nitrogen-deficient substrates, such as fruit waste (Matos et al., 2021a; Silva et al., 2022), and olive oil mill wastewater (Campanari et al., 2017), are usually preferred since the PHA-storage ability of selected microorganisms is enhanced under nutrient-limiting conditions and abundance of carbon. However, the development of a mixed culture capable of PHA storing still required a certain amount of nutrients during the selection stage, making necessary their supplementation (Oliveira et al., 2018a). Focusing on dairy industry effluents, successful PHA production has been reported using whey-based substrates (Asunis et al., 2022; Colombo et al., 2016, 2019; Duque et al., 2014b; Gouveia et al., 2017; Oliveira et al., 2018a). Nitrogen derived from whey proteins and other components can be metabolized efficiently during the selection stage, although it is still debated whether a further addition of nutrients is required to achieve the highest PHA yields (Oliveira et al., 2018a).

When treating nutrient-rich substrates, it is vital to avoid high amounts of nutrients entering the accumulation stage. A possible strategy is to extract and concentrate the PHA precursors after the fermentation stage while retaining the nutrients. Volatile fatty acids (VFA) extraction is an emerging research topic resulting from the growing interest in VFA for a wide range of applications, including PHA production. Only a few studies report a detailed study in which VFA extraction was applied to the PHA production process.

Recently, (Outram & Zhang, 2018) reported a low-cost method for VFA extraction from fermented effluents, based on silicone membrane and distilled water as extractant. Such method was then integrated to fermented cheese whey ((Ravishankar et al., 2020), see Chapter 3). Adding VFA

extraction to the PHA production chain can help to optimise the C/N ratio between the selection and accumulation stage.

In this study, for the first time, an innovative 4-stage process for PHA production from nutrient-rich ovine cheese whey is proposed, in which a selective VFA extraction step through silicone membrane is added to the conventional three-step process.

4.2 Materials and methods

4.2.1 Substrate and inoculum

The substrate used for the multistage process was raw sheep CW produced in a local dairy which processes around 20 Mtonnes of milk per year (Argiolas Formaggi, Sardinia). The sampled CW was homogenized in a 2 L bottle and frozen at -15°C (Asunis et al., 2019). The CW was thawed at room temperature for 12 hours when needed. Table 1 reports the CW characterisation, as analysed before the experimental trials. Sheep CW is both a carbon- and nitrogen-rich residue (27.9 and 1.9 g/L, respectively). Most of the carbon (up to 66% of the TOC) is constituted by easily biodegradable carbohydrates in the form of lactose while the nitrogen mostly derives from the whey proteins (estimated up to 90% of the TN). Whey proteins are also a source of slowly biodegradable carbon (around 17% of TOC).

Fermentation tests were carried out without the addition of any external inoculum and exploiting the natural CW microflora as source of microorganisms (Asunis et al., 2022). The inoculum adopted for the culture selection of a PHA-producer MMC was sampled from the aeration tank of a municipal wastewater treatment plant located in Cagliari (Italy). The initial volatile suspended solid content was around 7 gVSS/L.

4.2.2 Experimental set-up

To produce PHA from CW, a four-stage experimental set-up (**Figure 4.1**) was employed, which included: a dark fermentation (DF) batch reactor for CW acidification (Stage I); a silicone membrane VFA extraction unit (Stage II); a sequencing batch reactor (SBR) for the selection and enrichment of MMC (Stage III); and a fed-batch reactor for the accumulation of PHA (Stage IV).

4.2.2.1 Stage I: acidogenic fermentation

Batch DF tests were carried out under controlled conditions (39°C, pH 6, 200 rpm of agitation, 6 days) using raw CW as a substrate (Asunis et al., 2019). The fermentation was followed by a decanting step of around 12 hours, where the fermented cheese whey (fCW) was clarified to remove the coarse solid part to facilitate the following stages. Overall, five 2 L DF batch runs were performed

and the fCW obtained from the different batches (8 L in total) was then mixed and frozen at -15°C into 2 L bottles to be used in Stage II.

4.2.2.2 Stage II: silicone membrane extraction

The experimental set-up for the extraction stage (**Figure 4.2**) was adapted from the previous study ((Ravishankar et al., 2020), see Chapter 3).

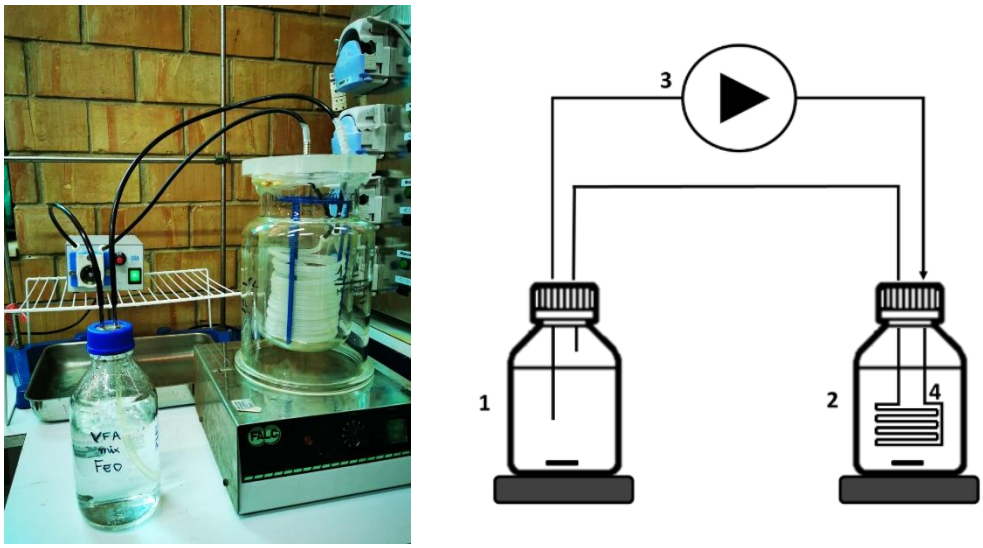

Figure 4.2 – Silicone membrane set-up adopted for the present study.

Briefly, one glass bottle (fed tank) was filled with 1.7 L of fCW and connected to a silicone membrane coil (internal diameter 3.2 mm, wall thickness 1.6 mm, length 4.9 m) submerged into a second bottle containing 1.7 L distilled water (draw tank). The fCW was recirculated at 200 mL/min flow rate using a peristaltic pump (SP 311/12, VELP Scientifica, Italy). The draw bottle was mixed using a magnetic stirrer. Prior to the extraction experiment, the fCW pH was corrected to around 3 with H_2SO_4 to maximize the fraction of VFA in the undissociated form that can migrate through the membrane. The experiment was performed at room temperature (25 °C). The extraction was performed in four 13 d batches.

The extraction stage resulted in two different effluents (8 L each) with different chemical characteristics: (i) a retentate fCW (rfCW), containing both carbon (VFA and lactic acid) and nitrogen (whey protein, ammonia) in a suitable ratio for the selection of a PHA-producing MMC, and (ii) a permeate fCW containing exclusively VFA, optimal for the PHA accumulation stage.

4.2.2.3 Stage III: culture selection and enrichment

The selection stage was carried out in an SBR (Diachrom biotechnology, Switzerland; 4 L working volume) inoculated with fresh activated sludge and operated at 25°C with HRT and SRT of 1 and 4 days, respectively. The operating pH was uncontrolled during the selection and spanning between 8-

9. The applied organic loading rate (OLR) was 0.62 ± 0.05 g_C/L*d (equivalent to 2.1 g_{COD}/L*d). The fCW was supplemented by allylthiourea (20 mg/L) to inhibit nitrification (Colombo et al., 2016).
The SBR cycle length was 12 h, consisting of four discrete phases: (i) influent filling (4 min), (ii) aeration (671 min), (iii) biomass purge (1 min), settling (40 min), and (iv) withdrawal of the clarified supernatant (4 min). Aeration was provided at 200 NL/h flow rate. Aeration and pumping were automatically controlled by an Arduino-based software developed within the research group. The dissolved oxygen concentration was measured by a polarographic probe (InPro 6800, Mettler Toledo) and used to identify the length of the feast phase and thus calculate the feast to famine (F/F) ratio in order to easily assess the performance of the feast and famine regime.

4.2.2.4 Stage IV: PHA accumulation

The maximum PHA storage capacity of the selected MMC was assessed in a fed-batch mode in a 1 L reactor. The accumulation tests were performed after observing a stable F/F regime (days 24-36, equivalent to 6-9 SRT). The biomass was collected from the SBR reactor at the end of the famine phase. The reactor was operated at room temperature (25°C), oxygen was supplied by sparging air and pH was uncontrolled. Three different substrates were tested in duplicate: permeate fCW (only VFA as C source); rfCW and fCW. The reactor was fed manually every two hours to ensure carbon excess, for a total of 8 hours (Rossi et al., 2021).

4.2.3 Analytical methods

Concentration of total solids (TS), volatile solids (VS), total suspended solids (TSS) and volatile suspended solids (VSS), according to the Standard Methods for the Examination of Water and Wastewater (APHA/AWWA, 1995). Total organic carbon (TOC) concentrations were measured using a Shimadzu TOC analyzer equipped with modules for the analysis of both liquid and solid samples. Soluble carbohydrates (sCarbo) were analyzed using the colorimetric phenol sulphuric acid method using glucose as the standard (DuBois et al., 1956). The soluble protein (sProte) content was determined spectrophotometrically at 750 nm by the alkaline copper method as described by (Lowry et al., 1951), using bovine serum albumin (BSA) as the standard. All the spectrophotometric analyses were performed with a HITACHI U-200 spectrophotometer. Lactic acid was determined by spectrophotometric method using nitrate solution ($FeNO_3$ $9H_2O$) at 0.2% as a method adopted by Borshchevskaya, et al. (2016). The sample concentration was measured by reading to the Hitachi U-2000 spectrophotometer at 490 nm wavelength. The concentration of VFA, was determined using a gas chromatograph with flame-ionization detection (model 7890B, Agilent Technology) equipped

with a capillary column (HP-FFAP, 25 m, inner diameter 0.32 mm, Agilent Technology). The samples were filtered using a 0.45 µm membrane and then acidified with concentrated H_3PO_4 (pH < 3). The injection volume was 0.6 µL. The temperatures of the injector and the detector were 230 °C and 300 °C, respectively. The oven temperature was initially set at 60 °C (3-min holding time), followed by a ramp of 10 °C/min up to 160 °C. Helium gas (1.6 mL/min, splitless) was used as the carrier gas.

The elemental analysis of the sample was carried out with a CHN analyzer (CHN 628, Leco, USA).

PHA were determined by GC using a method adapted from (Serafim et al., 2004). Lyophilized biomass (around 4 mg) was incubated for methanolysis in a 20% vol. H_2SO_4 in methanol solution (1 mL) and extracted with chloroform (1 mL). The mixture was then digested at 100 °C for 3.5 h. After the digestion step, the organic phase (methylated monomers dissolved in chloroform) was extracted and injected (1 µL) into a GC equipped with a flame ionization detector (model 7890B, Agilent Technology, USA) and a capillary column (HP-FFAP, 25 m, inner diameter 0.32 mm, Agilent Technology, USA) using helium as carrier gas at constant pressure (14.5 psi). The temperatures of the injector and the detector were 280 ºC and 230 ºC, respectively. The oven temperature was initially set at 40 °C, followed by a ramp of 20 °C/min until 100 °C, then 3 °C/min until 175 and 20 °C until a final temperature of 220 °C (4 minutes holding time). The 3-hydroxybutyrate (HB) and 3-hydroxyvalerate (HV) concentrations were calibrated using a commercial polymer PHBV (88%/12%) (Sigma-Aldrich, CAS number 80181-31-3). Benzoic acid (50 mg/L) was used as an internal standard and was added prior to the methanolysis step.

4.2.4 Performance parameters calculation

For the acidogenic fermentation stage, the fermentation yield ($Y_{OA/CW}$ or $Y_{VFA/OA}$) was calculated as the ratio between the organic acids (OA) produced (considered as the sum of lactic acid and VFA concentrations) and the initial TOC content, both expressed on a carbon basis.

For the extraction stage, a recovery yield R_e was defined as the amount of VFA extracted through the silicone membrane divided by the initial amount (Eq. 1):

$$R_e(\%C) = gC_{VFA}^{Draw\ tank} / gC_{VFA}^{Fed\ tank} \cdot 100 \qquad \text{Eq. 1}$$

The PHA content of the biomass was determined on a mass basis and expressed as dry weight (%wt.) as a percentage of the measured VSS (Eq. 2):

$$PHA = g_{PHA}/g_{VSS} \cdot 100 \qquad \text{Eq. 2}$$

while the concentration of active biomass (X_a) was estimated as the difference between the concentration of VSS and PHA. A biomass carbon content of 44.2 $mmol_{C\text{-}X}/g_X$ was assumed on the basis of the chemical formula $C_5H_7NO_2$ (Valentino et al., 2014).

The feast-to-famine (F/F) ratio was calculated as the ratio between the length in the hour of the two phases.

Specific substrate uptake rates ($-q_{TOC}$, $-q_{OA}$ and $-q_{PROTE}$), PHA production rate (q_{PHA}) and maximum specific growth rates (μ_{max}) were calculated from the slope of the linear regression (at least $R^2>0.80$, (Asunis et al., 2022; Matos et al., 2021b)) of TOC, OA, proteins, PHA and X_a specific concentrations, respectively, over time.

The PHA storage yields ($Y_{PHA/TOC}$ or $Y_{PHA/OA}$) were calculated by dividing the q_{PHA} by the $-q_{TOC}$ or $-q_{OA}$. Biomass growth yield ($Y_{X/TOC}$) was calculated by dividing μ_{max} by $-q_{TOC}$.

The overall PHA production yield ($Y_{PHA/CW}$) was calculated from a carbon mass balance. Key assumptions are based on the work of (Valentino et al., 2019).

4.3 Results and discussion

4.3.1 Acidogenic fermentation of raw cheese whey

The acidogenic fermentation stage aims to convert the organic compounds contained in raw CW (mainly carbohydrates) into a pool of OA, which are PHA precursors. Batch dark fermentation of CW typically involves two-steps: a conversion of carbohydrates into lactic acid (homolactic pathway), followed by a conversion of lactic acid into a mixture of VFA, mostly acetic, propionic and butyric acid (**Figure 4.3**). Such a pathway was observed in previous studies on CW (Asunis et al., 2019) and tequila vinasses (García-Depraect et al., 2020) fermentation.

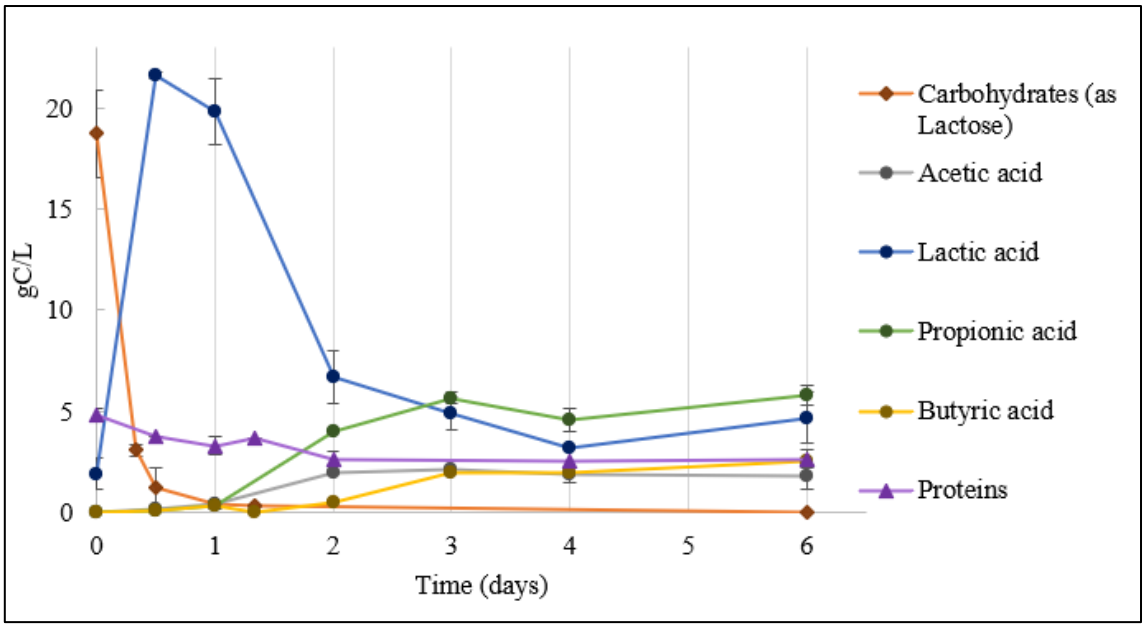

Figure 4.3 - Time evolution of carbohydrates (as lactose), OA (lactic, acetic, propionic and butyric acid) and proteins during batch dark fermentation tests carried out with CW. The concentration is expressed on carbon basis (g_C/L).

The fermentation yields ($Y_{OA/CW}$ and $Y_{VFA/OA}$) were 0.71 $g_{C\text{-}OA}/g_{C\text{-}CW}$ and 0.51 $g_{C\text{-}VFA}/g_{C\text{-}CW}$, respectively, meaning that 71%wt. of the initial CW carbon was converted into OA, 51%wt. of whose were VFA. The remaining fraction was lactic acid, found at 11.7 g/L concentration in the fermentate, suggesting an incomplete conversion of lactic acid into VFA. The obtained fermentation yields are in the medium-high range of values reported by other studies aiming at PHA production, including CW (0.4).80 g_C/g_C,(Colombo et al., 2016, 2019; Duque et al., 2014a; Oliveira et al., 2018b)) fruit waste (0.74 g_{COD}/g_{COD}) and similar agroindustrial wastes (Sabapathy et al., 2020). The main characterization parameters of fCW are reported in **Table 4.1**. The fCW was composed of lactic, acetic, propionic and butyric acid in the proportion of 33/12/38/17 (as $\%g_{C\text{-}OA}$).

While carbohydrates were fully consumed (over 99%) during the first fermentation step (24 hours, homolactic pathway), whey proteins were only consumed by 60% (from 10.9 to 4.5 g/L), with 28% degradation in the first 24 hours concomitantly with carbohydrate fermentation to lactic acid.
In general, results from the DF stage confirmed the inherent potential of CW to product high concentration PHA precursors (around 32 g_{OA}/L). Highly concentrated OA streams are crucial to minimize capital and operational costs of fermentation reactors in the PHA production process (Matos et al., 2021a).

Table 4.1 - Characterisation of the substrates used and produced in the present study.

Parameter	Unit of measure	Raw CW	Fermented CW (fCW)	Retentate fCW (rfCW)	Permeate fCW
Substrate for		Dark Fermentation	S/L separation and extraction	Culture selection	PHA Accumulation
pH	-	6.13 ± 0.49	6.00 ± 0.00	2.91 ± 0.26	3.05 ± 0.06
Total organic carbon (TOC)	g_C/L	27.92 ± 2.22	20.29 ± 2.75	15.38 ± 1.86	2.63 ± 0.50
Dissolved organic carbon (DOC)	g_C/L	25.44 ± 2.14	17.12 ± 2.69	15.38 ± 1.86	2.63 ± 0.50
Total carbohydrates	g_C/L	42.04 ± 4.83	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Total proteins	g_C/L	10.55 ± 0.56	4.85 ± 1.87	5.41 ± 0.02	0.0 ± 0.0
Lactic acid	g_C/L	3.87 ± 0.16	11.72 ± 3.10	13.80 ± 3.10	0.0 ± 0.0
Total VFA	g_C/L	0.0 ±0.0	21.04 ± 0.84	12.80 ± 1.41	5.25 ± 0.37
OA composition La/Ac/Pr/Bu	C% basis	100/0/0/0	33/12/38/17	45/12/9/31	0/6/54/40
Total Nitrogen (TN)	g_C/L	1.90 ± 0.08	1.57 ± 0.06	1.35 ± 0.12	0.0 ± 0.0
Ammonia	g_C/L	0.06 ± 0.01	0.04 ± 0.01	0.05 ± 0.03	0.0 ± 0.0
TOC/TN	g_C/g_N	14.7	12.9	11.4	∞

4.3.2 VFA extraction from fermented cheese whey through silicone membrane

Since VFA can migrate through the silicone membrane only in undissociated form (Dessì et al., 2020; Ravishankar et al., 2020), the pH of the clarified fCW was corrected to around 3 by adding H_2SO_4 before the VFA extraction step (pK_a of VFA is around 4.7-4.8). Regarding the presence of residual lactic acid in the fCW and in the perspective of the following stages, it is worth mentioning that: (i) lactic acid is supposed to not cross the silicone membrane in the proposed extraction stage (Dessì et al., 2020); (ii) lactic acid is a PHA precursor, though butyric acid is a more energetically favorable substrate for PHA production (Kourmentza & Kornaros, 2016).

The extraction stage generated two different flows: a permeate (permeate fCW) and a retentate (rfCW). As expected, only VFA were detected in the permeate fCW while lactic acid and other compounds were retained in rfCW. **Figure 4.4** shows the VFA concentration in the permeate fCW over time. After 14 days, the total VFA concentration was 2.6 $g_{C\text{-}VFA}$/L (5.25 g_{VFA}/L), equal to 30% and 18% of the initial VFA and OA content, respectively (expressed on a C basis). The highest recovery was obtained for butyric acid (46%), indicating that its concentration was close to the equilibrium between the two solutions and thus close to the maximum theoretically achievable recovery of 50% (since the extraction is a concentration-driven process). Lower recovery yields were

observed for propionic (28%) and acetic acid (10%). The differences in terms of extraction recovery are due to different affinity to the silicone membrane. Specifically, butyric acid migrates faster than shorter chain acids through the silicone membrane due to its higher hydrophobicity as already described in chapter 3.

The permeate fCW contains only carbon in the form of VFA (**Table 4.1**), making it a promising medium for the PHA accumulation stage, during which it is necessary to ensure nutrient limiting conditions. Furthermore, the different diffusion rates of VFA through the silicone membranes open possibilities for fine-tuning the composition of the permeate by tailoring the membrane thickness and the contact time. Different VFA pools result in the production of different types of PHA, which can be adapted to the application of interest (Carvalheira et al., 2022). On the contrary, the rfCW still contains the nutrients (nitrogen derived from whey proteins and ammonia) necessary for harvesting MMC capable of PHA storage and is thus ideal for the selection stage (Asunis et al., 2022).

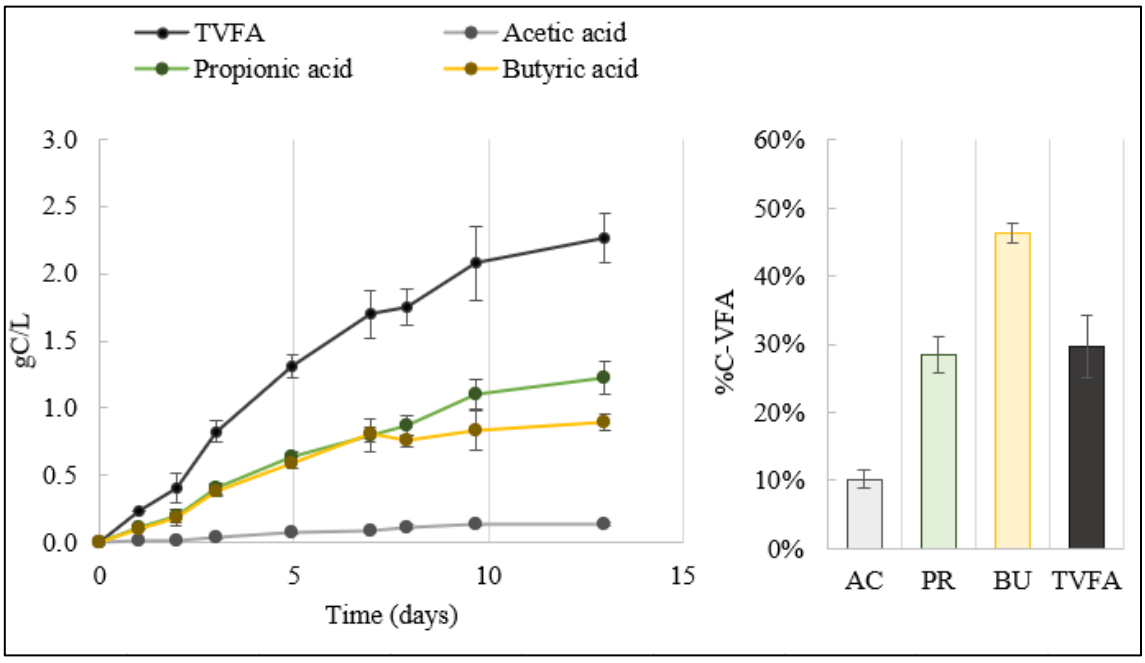

Figure 4.4 - VFA extraction from the fCW, acidified to pH 3.0, via silicone membrane at room temperature: VFA concentration over time on a carbon basis (left); percentage of VFAs recovered with respect to the initial concentrations in the fCW (right).

4.3.3 Culture selection and enrichment with retentate fCW

The selection of PHA-storing MMC was performed using rfCW and by applying the so-called feast and famine regime in SBR reactor for 36 days (around 9 SRTs). The nutrient-rich rfCW had a C/N ratio of around 13 g_C/g_N, which is in the optimum range for selecting PHA accumulating microorganisms (Silva et al., 2017). A feast and famine (F/F) ratio of 0.17 ± 0.1 was observed

(**Appendix A1 - Figure A1.4.1**), in line with the values generally recognised to induce the selection of microorganisms with a good PHA-storage capacity (Dionisi et al., 2006; Valentino et al., 2017). The average biomass concentration, after an acclimation period, was 1.39 ± 0.29 g_{VSS}/L.

Around 15% of the carbon content of the retentate fCW used as substrate in the selection stage was in the form of whey proteins (**Table 4.1**), which likely sustained the growth of PHA-accumulating microorganisms. This is confirmed by the observed protein consumption of 58%, mainly during the famine phase, for a total specific consumption of 2 $mg_{C\text{-}PROTE}/g_{C\text{-}X}$ h. It has been recently reported that the presence of proteins can be beneficial for the enrichment process if they are below 30% as COD balance (Roibás-Rozas et al., 2021), whereas higher concentrations can cause the competitive growth of undesired, non-PHA accumulating microorganisms (Argiz et al., 2020).

The SBR effluent has a concentration of around 0.1 g_C/L, mostly constituted by residual whey proteins (around 230 mg/L).

4.4.4 PHA accumulation stage

The storage response of the selected culture was evaluated in fed-batch accumulation tests with different substrates, namely permeate fCW, retentate fCW and raw fCW.

Table 4.2 summarizes the main performance parameters obtained for the different substrates. The highest PHA storage response was obtained with the nutrients-free permeate fCW. The maximum PHA content and PHA storage yield of 37%wt. and 0.53 $g_{C\text{-}PHA}/g_{C\text{-}TOC}$ were more than double than those obtained with fCW and rfCW (0.25 and 0.20 $g_{C\text{-}PHA}/g_{C\text{-}TOC}$). Clearly, the PHA-synthesis was favored by the absence of nutrients in the permeate fCW, resulting in fully nutrient limiting condition and an excess of carbon in VFA form which favored PHA accumulation. When using the nutrients-containing substrates fCW and rfCW, growth and storage response occurred simultaneously, decreasing the carbon available for storage. In fact, the highest specific growth rate was 90 $mg_{C\text{-}X}/mg_{C\text{-}Xi}$ h observed with the rfCW, which had the lowest C/N ratio, around two-times and three-times higher than the specific growth rates obtained with the fCW (46 $mg_{C\text{-}X}\ mg_{C\text{-}Xi}\ h^{-1}$) and permeate fCW (26 $mg_{C\text{-}X}/mg_{C\text{-}Xi}$ h), respectively.

The PHA obtained was always a copolymer of 3-HB and 3-HV monomers, with a ratio strongly dependent on the substrate. The HV content varied between 46% with the permeate fCW and 69% with the rfCW.

Table 4.2 - Main parameters and performances obtained in the selection and accumulation stage of the proposed bioprocess. Note that the selection stage was performed using retentate fCW as substrate.

Parameters	Unit of measure	fCW	Retentate fCW	Permeate fCW
Selection and enrichment stage				
Feast length	h/h	n.a.	0.17 ± 0.01	n.a.
Biomass	g_{VSS}/L	n.a.	1.39 ± 0.29	n.a.
X_a*	g/L	n.a.	1.31 ± 0.27	n.a.
PHA content*	%wt	n.a.	14 ± 0	n.a.
HV fraction*	%wt	n.a.	59 ± 1	n.a.
-q_{TOC}*	$mg_{C\text{-}TOC}/g_{C\text{-}X}$ h	n.a.	71 ± 36	n.a.
-q_{OA}*	$mg_{C\text{-}OA}/g_{C\text{-}X}$ h	n.a.	69 ± 36	n.a.
q_{PHA}*	$mg_{C\text{-}PHA}/g_{C\text{-}X}$ h	n.a.	32 ± 6	n.a.
-q_{PROTE}	$mg_{C\text{-}PROTE}$ /$g_{C\text{-}X}$ h	n.a.	2 ± 0	n.a.
$Y_{PHA/TOC}$*	$g_{C\text{-}PHA}/g_{C\text{-}TOC}$	n.a.	0.45	n.a.
$Y_{PHA/OA}$*	$g_{C\text{-}PHA}/g_{C\text{-}OA}$	n.a.	0.46	n.a.
PHA Accumulation stage				
Duration test	h	8	8	8
PHA content	%wt	28 ± 3	23 ± 0	37 ± 1
HV fraction**	%wt	59 ± 8	69 ± 5	46 ± 4
-q_{TOC}	$mg_{C\text{-}TOC}/g_{C\text{-}X}$ h	353 ± 99	368 ± 20	209 ± 20
-q_{OA}	$mg_{C\text{-}OA}/g_{C\text{-}X}$ h	325 ± 106	230 ± 121	202 ± 12
q_{PHA}	$mg_{C\text{-}PHA}/g_{C\text{-}X}$ h	84 ± 6	74 ± 9	110 ± 12
-q_{PROTE}	$g_{C\text{-}PROTE}/g_{C\text{-}X}$ h	28 ± 6	45 ± 9	2 ± 1
μ_{max}	1/h	46 ± 20	90 ± 0	26 ± 6
$Y_{PHA/TOC}$	$g_{C\text{-}PHA}/g_{C\text{-}TOC}$	0.23	0.16	0.52
$Y_{PHA/OA}$	$g_{C\text{-}PHA}/g_{C\text{-}OA}$	0.26	0.26	0.54
$Y_{X/TOC}$	$g_{C\text{-}X}/g_{C\text{-}TOC}$	0.25	0.26	0.13

n.a. not applicable

* calculated at the end of the feast phase

** measured at the end of the test

4.5 Conclusions

The present study proposed for the first time the use of a silicone membrane extraction for the PHA production from sheep cheese whey. CW is characterized by a high protein content that can limit the PHA accumulation process, which should take place under nutrient limiting conditions. The addition of a membrane extraction stage to the classic 3-stage process allowed the improvement of the process performance. The best results in terms of PHA production were obtained with permeate fCW (VFA-rich, nutrient-free solution). In fact, the maximum content of PHA was 37%wt. and 28%wt. using permeate fCW and fCW, respectively. The main reason for this behavior is the limitation of the biomass growth process in favor of storage mechanisms.

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Chapter 5

Cheese whey fermentation and PHA production

This chapter presents the experimental activity carried out during the abroad period at "Faculdade de Ciências - Tecnologia da Universidade Nova de Lisboa". The approach followed consisted of the use of fermented cheese whey in the third stage of an MMC PHA-production process. The specific goals of the work were: 1) Assess acidogenic fermentation of Cheese Whey in a CSRT using different operational conditions (OLR, HRT) for the production of a VFA-rich fermented stream. 2) Acclimatization of a PHA-storing MMC in an SBR. 3) Assess PHA production using different feeding strategies.

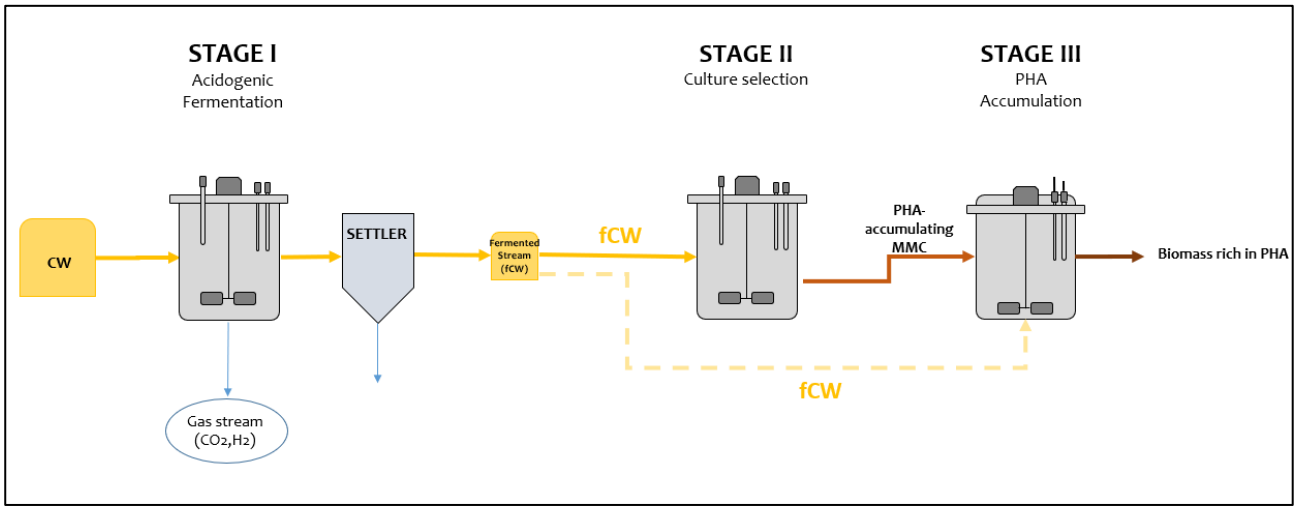

Figure 5.1 – Experimental set-up of the three-stage process for PHA production by MMC.

5.1 Introduction

In recent years, several researchers have applied the 3-stage process to produce PHA using MMC and CW as the substrate (Albuquerque et al., 2010; Asunis et al., 2022; Carvalheira et al., 2022; Colombo et al., 2017). However, the complexity of the effects of process parameters on the microbial metabolic networks is a drawback that makes the task of optimizing the process challenging. Regarding the dark fermentation stage, the effect of micro-aeration contamination on the microorganism activity and the product formation has been evaluated. Another aspect that needs to be considered is the control of the parameters involved in the process that heavily depend on the reactor configuration (Dionisi et al., 2006). Many studies have been carried out in batch mode due to the simplicity of conduction. However, the need to develop large-scale processes requires the study and design of reactors for the

continuous production of products. The most used configuration is the continuous stirred tank reactor (CSTR). In the case of CSTR, the main operating parameters to be considered are temperature, pH, HRT and OLR. The temperature, which can be mesophilic (25-40 °C) or thermophilic (40-65 °C) influences the rate of hydrolysis of the substrate and the rate of production of VFA. pH influences the yield and spectrum of VFA produced. The type and concentration of VFA produced at a given pH seem to be the same regardless of the starting conditions (Gouveia et al., 2017).
The HRT and the OLR, parameters studied in this work, are among the most important factors to ensure stability and good yields of VFA production in the fermentation process. Several studies have focused on determining the optimal values of these two parameters in in cheese whey dark fermentation processes, but the results are not always easily comparable given the differences in other equally important parameters.

Regarding the accumulation stage, new feeding strategies are studied to obtain the maximum storage capacity of the culture. The most adopted feed strategy is the pulse-wise feeding strategy, which in the present paper will be compared with a continuous feeding strategy.

5.2 Materials and methods

5.2.1 Feedstock and inocolum

Powder cheese whey supplied by Lactogal (Portugal) was used as a substrate for acidogenic fermentation. The characterization parameters for CW are reported in

Table 5.1. Although the CW is rich in nitrogen, which mostly derives from the whey proteins, a supplementation of NH_4Cl was added to the reactor (C:N of 100:0.5). The CSTR was carried out with the addition of an external inoculum of anaerobic granular sludge (around 1.5 L), from an anaerobic digestion power plant, considered a suitable biomass source due to the presence of bacteria capable of enhancing the fermentation. The granules were kept for 24 h under anaerobic conditions in the reactor before starting to feed CW to allow their acclimatization.

The SBR was inoculated with sediments collected from Rio Tejo (Porto Brandão, Caparica). The enriched PHA accumulating organisms from SBR were used as inoculum for the accumulation tests.

Table 5.1 – Powder cheese whey characterization.

Parameter	g/g_{CW} (% wt.)
Lactose	78.4
Total COD	1.04
Soluble COD	0.95
Soluble TOC	-
Total protein	11.66
Soluble protein	10.22
Total N	15.8
Soluble N (NH_4^+)	0.28
Soluble P	3.55

5.2.2 Experimental set-up

The production of PHA from CW by MMC was performed in a three-stage production process that consist of: (i) a continuous stirred tank reactor (CSTR) for acidogenic fermentation, (ii) a sequencing batch reactor (SBR) for culture selection and (iii) a fed-batch or continuous reactor for the PHA accumulation (**Figure 5.1**).

5.2.2.1 Acidogenic fermentation

Acidogenic fermentation was performed for 299 days in a 5L-CSTR reactor (steel, process control) equipped with mechanical stirring (150 rpm). Feed was provided by peristaltic pumps connected to the concentrated CW and water to dilute online at the desired organic loading rate (OLR). The CSTR effluent was clarified by a settler. Both CSTR and settler were connected to the gas line equipped with a gas flowmeter (BPC μFlow).

The reactor was connected to an automatic system to keep the temperature at 30 ± 1 °C and a controlled pH of 4.5 ± 0.1 through the addition of NaOH (1-2 M) and HCl (1 M). The initial hydraulic retention time (HRT) and OLR of 1 d and 5 g_{COD}/L^*d were used, respectively. The OLR was subsequently increased stepwise up to 20 g_{COD}/L^*d. Different stressed conditions were tested during the fermentation operation varying the operative parameters such as HRT, OLR and presence of micro aeration.

Micro-aeration (flow 3-90 mL/min) was provided by a peristaltic pump with a diffuser for fine bubbles and allowed the presence of small amounts of O_2 in the fermentation mix. ORP and dissolved oxygen were continuously monitored.

Fermented cheese whey (fCW) was collected from CSTR under stable operation conditions, characterized and frozen at -20 °C into 2 L bottle to be used for selecting and enriching a PHA-accumulation culture stage.

5.2.2.2 Culture selection

The culture selection stage was carried out in a sequencing batch reactor (SBR) of 2 L working volume, inoculated with sediments collected from Rio Tejo (Porto Brandão, Caparica). The SBR cycle length was 8 h and consisted of 4 steps: (i) feeding (5 min), (ii) aeration (438 min), (iii) settling (30 min), and (iv) supernatant withdrawal (7 min). The reactor was operated in a temperature-controlled room (19 ± 1 °C) and controlled pH of 8.40 ± 0.5 with an HRT of 16 h, a SRT of 3 days, and an OLR of 60 $C_{mmol\text{-}VFA}$/L*d. The SRT was kept at 3 days by purging 222 mL of mixed liquor at the end of each aeration phase (end of famine). Air was provided through fine bubbles diffusor and the aeration rates were adjusted in order to guarantee not-limiting DO conditions in the reactor. The fCW used as feedstock was diluted with a mineral solution containing the concentrations of the following components (mg/L): ATU (10); EDTA-2Na (50); $MgSO_4$ (50); $CaCl_2$ (50); $FeCl_3 \cdot 6H_2O$ (1.5); H_3BO_4 (0.15); $CuSO_4 \cdot 5H_2O$ (0.03); KI (0.03); $MnCl_2 \cdot 4H_2O$ (0.12); $Na_2MoO_4 \cdot 2H_2O$ (0.06); $ZnSO_4 \cdot 7H_2O$ (0.12); and $CoCl_2 \cdot 6H_2O$ (0.15) (Huang et al., 2018). The SBR was operated under a feast and famine regime with uncoupled carbon and nitrogen availability to obtain a selective pressure for PHA-accumulating organisms. The applied C:N:P (mol basis) ratio was 100:5:2. The N supplementation was fed to the reactor after the end feast, instead, P was given during the carbon feed. When stable enriched PHA accumulating microorganisms were obtained, the accumulation tests were performed.

5.2.2.3 PHA-accumulation tests

PHA-accumulation tests of the selected culture were performed in a 2 L reactor using 500 mL of selected biomass collected from the SBR at the end of the famine phase as the inoculum.

The accumulation procedure in a fed-batch reactor consisted of a pulse-wise feeding strategy of fermented cheese whey (fCW) under nitrogen-limiting conditions using the same controlled conditions of pH, temperature, aeration, and stirring that were used for the culture selection. The same food-to-microorganism (F/M) ratio found in the selection stage was kept for the test.

The PHA-accumulation test using a continuous feeding strategy was conducted in the same 2 L reactor, initially fed with 60 mL of fCW substrate and then fed with a flow rate of 2.1 mL/min. This value was decided based on the VFA consumption per time observed during the first pulse of the pulse-wise strategy test.

5.2.3 Analytical methods

Fermentation performance was periodically evaluated by analyzing the amount and composition of the biogas and the composition of the effluent produced.

The biogas composition was determined by a gas chromatograph equipped with a TCD detector and 50 m CP-Molsieve 5A and 25 m PoraBOND Q columns. Argon was used as the mobile phase (flow rate of 5 mL/min), and the injection port and detector temperatures were 120 °C and 70 °C, respectively.

The effluent parameters were measured as follows: total solids (TS), volatile solids (VS), total suspended solids (TSS) and volatile suspended solids (VSS), according to the Standard Methods for the Examination of Water and Wastewater (APHA/AWWA, 1995). Chemical oxygen demand (COD) was determined with a Hach Lange kit (LCK 914; Hach-Lange, Germany). Total soluble organic carbon (TOC) concentration through Shimazu TOC analyzer. Lactose, lactic acid, VFA, and ethanol by high-performance liquid chromatography (HPLC) as described by Oliveira et al. (2018, 2017), with a Chromaster VWR Hitachi instrument equipped with both RI and UV (wavelength 210 nm) detectors, a Bio-Rad 125–0129 pre-column and an Aminex HPX-87H (Bio-Rad) column (0.01 M H_2SO_4 eluent, flow rate 0.6 mL/min and column temperature 60 °C).

PHA content and composition were quantified through gas chromatography with a Bruker 430-GC instrument, as described by Lanham et al., (2013).

5.2.4 Calculations

The acidification degree (Y_{FP}, %) and $FP_{productivity}$ were calculated according to the equation (1) and (2), respectively:

$$Y_{FP} = \frac{FP}{COD_{IN}} \cdot 100 \qquad \text{(Eq. 1)}$$

$$FP_{productivity} = \frac{FP}{HRT} \qquad \text{(Eq. 2)}$$

Where FP (fermentation products) is the concentration of VFA and ethanol produced (g_{COD}/L) and COD_{IN} is the total COD fed in the reactor (g_{COD}/L).

Lactose consumption was calculated according to equation (3), as the ratio between the concentration of lactose ($Lactose_{EFFL.}$) found in the effluent and the initial concentration of lactose ($Lactose_{IN}$):

$$Lactose_{consumption} = \frac{Lactose_{EFFL.}}{Lactose_{IN.}} \qquad \text{(Eq. 3)}$$

The F/F ratio was calculated as the ratio between the period lengths of feast and famine phases of the SBR cycle.

The PHA content of the biomass was determined on a mass basis and expressed as a percentage of the measured VSS (%wt., gPHA/gVSS), as expressed in equation 4:

$$PHA\ (\%wt.) = g_{PHA}/g_{VSS} \cdot 100 \qquad \text{(Eq. 4)}$$

while the concentration of active biomass (X_a) was estimated as the difference between the concentration of VSS and PHA. For determining cell growth, the generic chemical formula $C_5H_7NO_2$, was used.

Specific substrate uptake rates ($-q_{FP}$, $C_{mol\text{-}VFA}/C_{mol\text{-}xi}$*h) and PHA production rate (q_{PHA}, $C_{mol\text{-}PHA}/C_{mol\text{-}xi}$*h) were calculated from the slope of the linear regression of the obtained data (Matos et al., 2021b)) plotted over time.

The PHA storage yield ($Y_{PHA/FP}$) was calculated by dividing the q_{PHA} by the $-q_{FP}$.

Volumetric PHA productivity (gPHA/L*d) was determined according to the amount of PHA produced per unit accumulation reactor volume and per unit time.

Specific PHA productivity ($gPHA/g_{Xi}$*d) was determined according to the amount of PHA produced per initial biomass and per unit time.

5.3 Results and discussion

5.3.1 Stage I: Acidogenic fermentation

The CSTR was operated for 281 days under different operating conditions. **Table 5.*2*** summarized all the conditions adopted. The initial conditions were: OLR of 5 g_{COD}/(L*d), pH of 4.5, a temperature of 30 ℃, HRT of 1 day, and strictly anaerobic conditions.

Table 5.2 - Summary of all operational conditions adopted during this work

	Days	**HRT d**	**OLR g/L*d**	**Micro-aeration mL/min**
A	0-21	1	5	0
B	21-57	1	10	0
C	57-70	1	Δ10-20	0
D	70-146	1	20	0
E	146-208	1	20	Δ 0-90
F	211-225	2	20	12
G	225-251	1	20	0
H	251-268	2	20	12
I	268-281	4	20	12

Effect of OLR

The main goal of the first part of the experimentation (from phase A to phase D) was to maintain stability and good performance of the reactor (in terms of production of liquid and gaseous

metabolites) by gradually increasing the OLR from 5 up to 20 g_{COD}/(L*d). The gas production detected during phase A (OLR = 5 g_{COD}/L*d) was very low as was the production of liquid metabolites. By increasing OLR up to 10 g_{COD}/L*d (phase B), an increase in VFA concentration up to 182.0 C_{mmol}/L (**Figure 5.*2***,

Table 5.3) as well as in gas production (**Figure 5.*2*** was observed. Soluble metabolites were mainly composed of acetic acid (43%), followed by butyric (35%), and small quantities of valeric, caproic and propionic acid were measured as well. Ethanol (Et.OH) was detected also (9%), whereas lactose and lactic acid were not detected, demonstrating a complete conversion of lactose to lactic acid and of lactic acid to VFA. On the other hand, gas production was characterized by an increasing percentage of methane in the outflow (up to 35% vol.) and a negligible presence of H_2 (**Figure 5.1**).
After reaching steady conditions, i.e.: after approximately 35 days, the OLR was gradually increased (phase C) to 20 g_{COD}/L*d (phase D). An increase in VFA concentration up to 341.6 C_{mmol}/L was observed during phase D, with a clear prevalence of butyric acid (46%) over the other acids produced, compared to what was observed in the previous phase B. Lee et al., (2014) found that the applied OLR has a significant influence on the distribution of the VFA during CW DF. Increasing the OLR from 4 to 24 g_{COD}/L*d, the percentage of acetate dropped from 53% to 22%. In another study on starchy wastewater (Yu, 2001), increasing the OLR from 10 to 26 g_{COD}/L*d, butyric acid became the main acid produced. In the present study, the increase of the OLR up to 20 g_{COD}/L*d had a beneficial effect also on gas production in terms of volume and composition. The percentage of methane in the produced gas decreased to zero from the 126th day onwards. Correspondingly, the percentage of H_2 increased over time to an average value of 35% vol. Increasing the OLR therefore also had an inhibiting effect on methanogens thus favoring the metabolic activity of hydrogen-producing bacteria. The fermented CW (fCW) was collected during days 120-126 to use as feedstock for the culture selection and accumulation tests.

Effect of micro-aeration

Once the target of reaching the OLR 20 g_{COD}/(L*d) was achieved, the CSTR was subjected to different stressed conditions, starting from the presence of micro-aeration provided by peristaltic pump and diffuser to fine bubble at the flow from 3 to 90 mL/min (phase E). No significant variation was observed in the total VFA concentration at 3 mL/min and 12 mL/min of microaeration, although a slight increase in the percentage of butyric acid in the mixture was assessed (phase D: 46% vs. phase E: 55 % C_{mol} basis). Only when the air flow was increased up to 36 mL/min (day 187) a deterioration of the reactor performance was observed, in terms of both gas production and liquid metabolites.
Yien et al. (2016) found a positive effect of limited aeration on VFA production: low oxygen presence leads to a high VFA fermentation yield and an increased presence of facultative acidogens (such as *Firmicutes*, *Proteobacteria*, etc.). Moreover, microaeration would affect the VFA composition by inhibiting caproate production, whereas no clear effect on butyrate is observed. In some cases, the presence of O_2 can increase the production of ethanol or lactate which are electron donors for chain elongation (Baleeiro et al., 2021; Lambrecht et al., 2019).

Effect of HRT

The effect of changing HRT was assessed during phases F (HRT = 2 d) and I (HRT = 4 d) by keeping constant the microaeration flow at 12 mL/min. Total VFA production increased by increasing HRT up to 2 d to 526 C_{mmol}/L (vs. 349.8 C_{mmol}/L, phase E). The increase in HRT also influenced the composition of the volatile acids produced, with an increase in the presence of acetic acid (48%) compared to the previous phase (phase E, 31%). On the other hand, an incomplete lactose conversion (94%) and a decrease in the fermentation degree (49%) and productivity (9.8 $gVFA_{COD}/L_{reactor}$ day) were observed. A further increase of HRT up to 4 d increased VFA production, as expected, but was detrimental for the substrate conversion: lactose consumption dropped to 76% and the fermentation degree to 36%. No relevant changes in the composition of the VFA pool were observed following the increase in HRT.

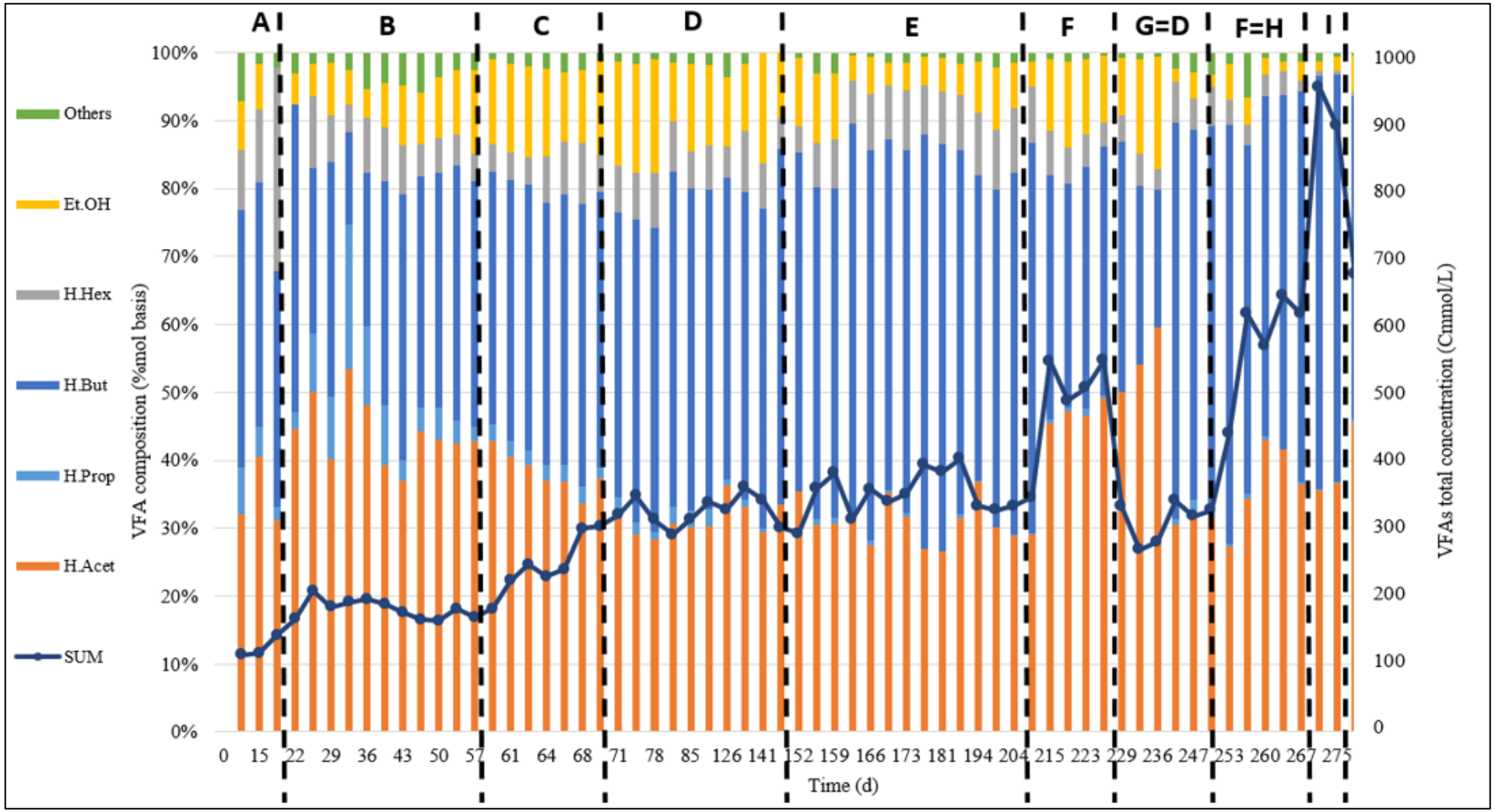

Figure 5.2 – Composition and concentration of the VFA produced under different operating conditions.

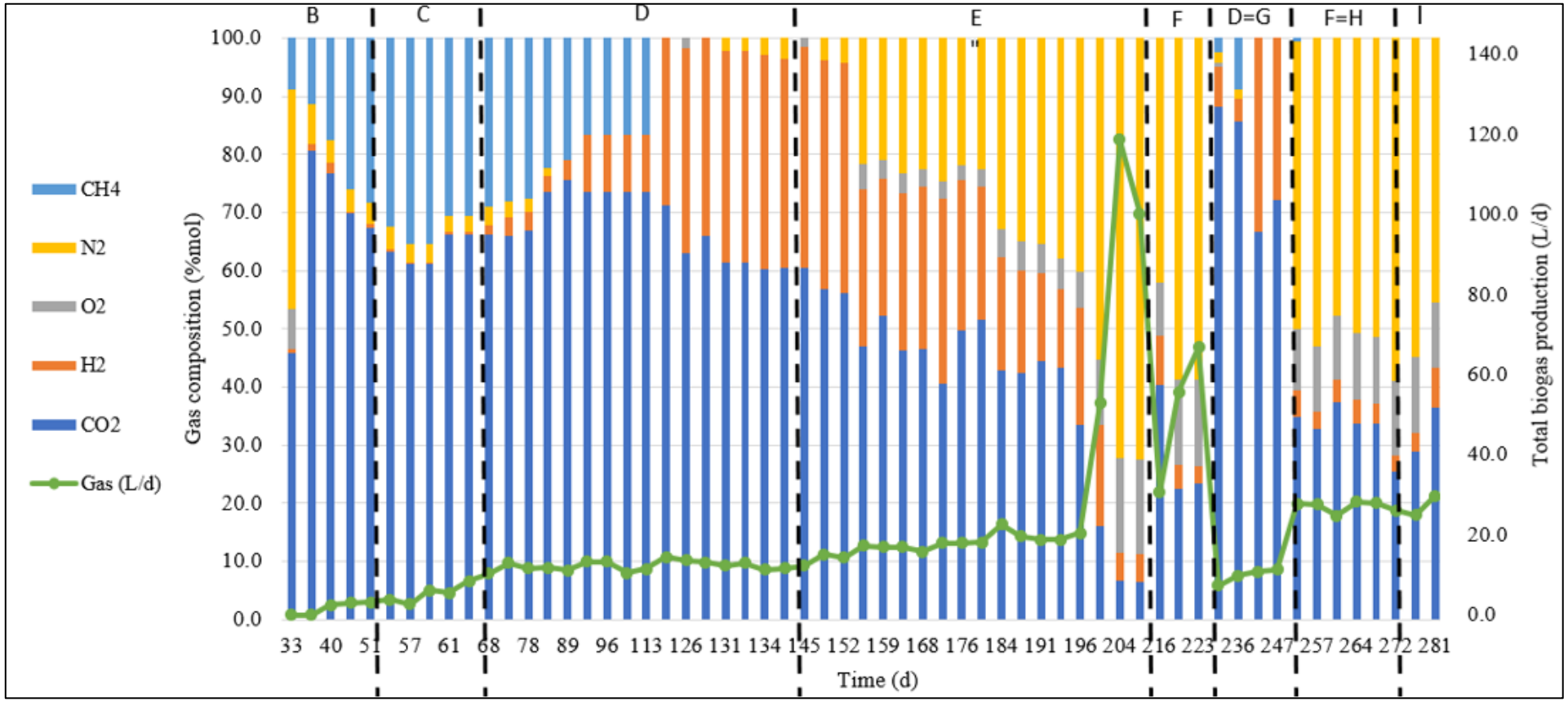

Figure 5.3 - Composition and concentration of the biogas produced under different operating conditions.

Table 5.3 - Performance of the CSTR under different operating condition.

	Average concentration (C_{mmol}/L)	Composition (% C_{mol} basis)						Fermentation degree (g_{VFA_COD}/g_{COD_feed})	FP productivity ($g_{VFA\_COD}/(L_{reactor}*d)$)	Lactose consumption ($g_{lact\_effl.}/g_{lact\_feed}$)
		H.Acet	H.Prop	H.But	H.Hex	EtOH	Others			
B	182.0 ± 6.2	43 ± 4	2 ± 4	35 ± 7	6 ± 2	9 ± 2	2 ± 1	69 ± 1	6.4 ± 0.4	100
D	341.6 ± 13.5	32 ± 3	1 ± 1	46 ± 1	7 ± 2	12 ± 3	2 ± 1	66 ± 3	13.2 ± 0.6	100
E	349.8 ± 22.8	31 ± 2	1 ± 0	55 ± 4	8 ± 1	4 ± 3	1 ± 1	67 ± 5	13.4 ± 1.0	99 ± 1
F	526.7 ± 28.6	48 ± 2	1 ± 1	36 ± 1	4 ± 1	11 ± 1	1 ± 1	49 ± 2	9.8 ± 0.5	94 ± 1
G	328.6 ± 10.8	31 ± 1	1 ± 1	56 ± 2	5 ± 1	3 ± 1	3 ± 1	62 ±2	12.4 ± 0.4	100
H	624.2 ± 15.1	39 ± 4	0 ± 0	53 ± 3	3 ± 1	3 ± 1	2 ± 1	57 ± 4	11.3 ± 0.7	100
I	920 ± 40.6	41 ± 0	0 ± 0	52 ± 3	1 ± 0	4 ± 1	2 ± 1	36 ± 2	7.3 ± 0.4	76

5.3.2 Stage II: Culture selection

The selection of PHA-accumulating MMC was performed in an SBR reactor using a conventional anaerobic dynamic feeding strategy, called feast and famine regime, and the uncoupled carbon and nitrogen feeding strategy. An external source of N was added at the end of the feast phase to provide a nutrient supplementation and guarantee better selection performance with lower F/F ratios (Carvalho et al., 2022). An F/F ratio < 0.2 was used as a useful performance indicator for the selection of microorganisms with a good PHA-storage capacity (Dionisi et al., 2006; Valentino et al., 2017; Asunis et al., 2022).

After a good selection of the biomass was achieved (i.e.: F/F = 0.08 ± 0.02), a complete monitoring cycle (8 h) was performed (**Figure 5.4** and **Figure 5.5)** in terms of DO profile, VFA consumption and PHA production over time. As the fCW was fed to the reactor (A), cells started consuming VFA and storing carbon in the form of PHA (feast phase). The selected biomass showed a preference towards butyric acid and hexanoic acid which are consumed within the first 30 minutes (B). Only when these acids are depleted, biomass begins to use up acetic acid (C). When the VFA have been completely consumed, an increase in dissolved oxygen is observed, indicating that PHA production has stopped (end of feast). After 1 h of feeding, nitrogen, in the form of ammonia, was added to the reactor, allowing cell growth (D). During the famine phase (E) only MO that stored PHA had available carbon for their survival and cell growth. Indeed, during this phase, a decrease in the content of PHA is observed. To support the fact that during the feast phase the selected biomass accumulated PHA, two samples of biomass during the famine phase and immediately after the end of the feast phase were taken. The samples were filled with 10 µL of nile blue and put in a thermostatic bath at 55 °C for 20 minutes. Then they were observed under the microscope with a 100X objective using UV light. The nile blue staining is used in this process to identify if and how much the cells are accumulating PHA. Nile blue is a compound that under UV light lets bacteria with internal PHA reserves shine. As can be seen in **Figure 5.6**, it is clear, from the higher quantity of shining biomass at the end feast, how the selection step allowed to enrich the culture with PHA-storing bacteria.

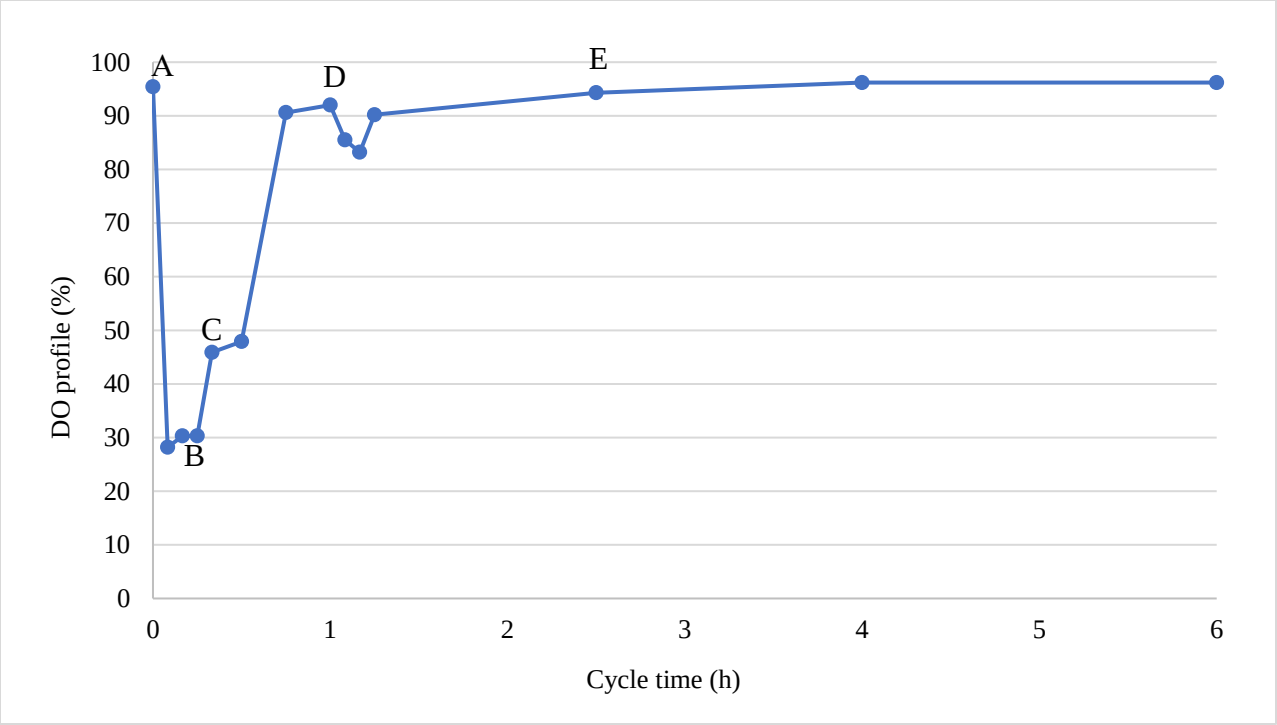

Figure 5.4 – DO profile during an 8-hour cycle.

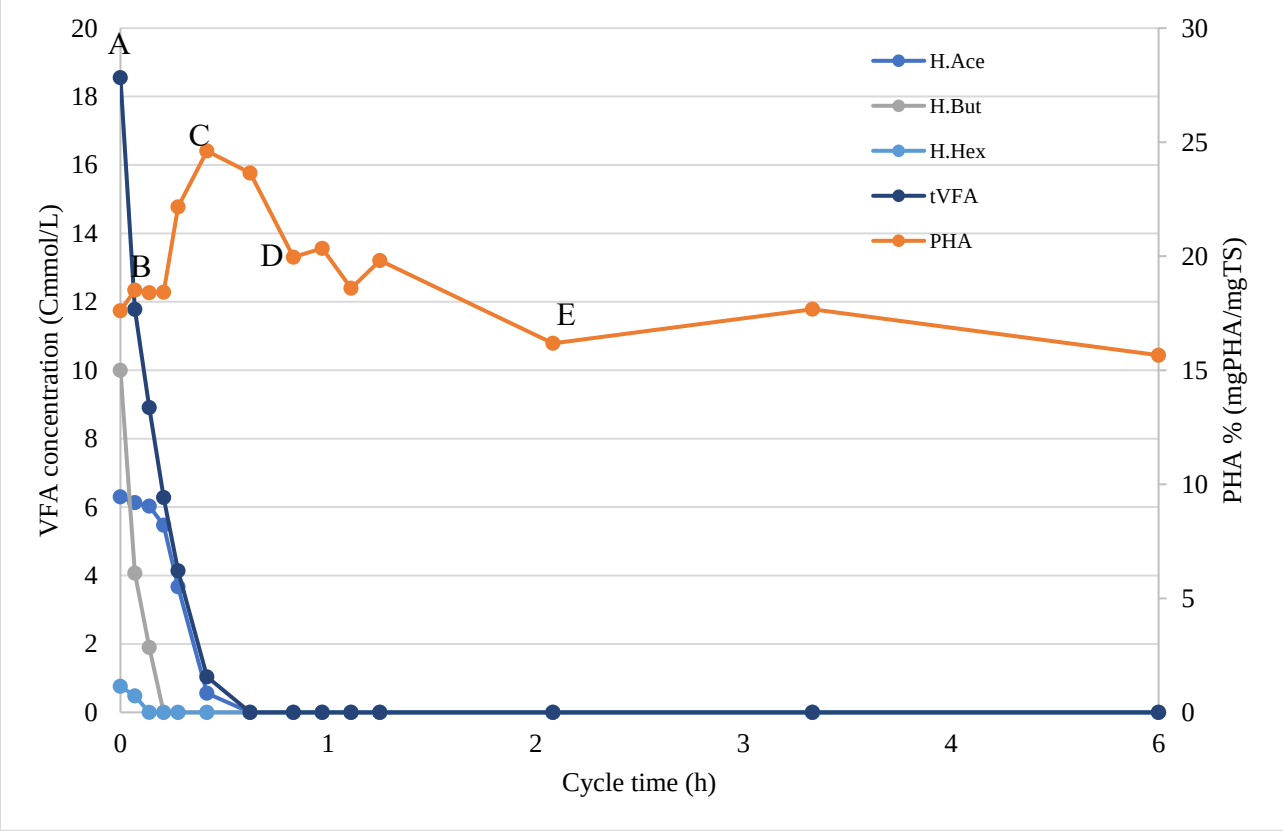

Figure 5.5 – VFA consumption and PHA accumulation during an 8-hour cycle

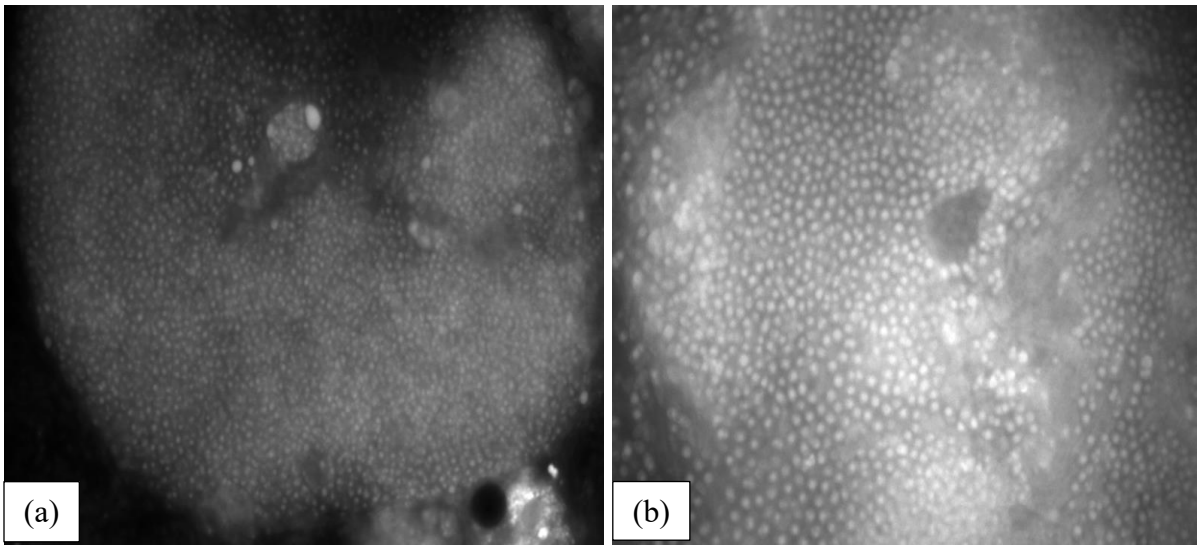

Figure 5.6 - Intracellular PHA granules identified by using Nile Blue staining under the microscope with a 100x. Sample of the selected culture (a) at the end of the famine phase, (b) at the end of the feast phase.

5.3.3 PHA accumulation

The accumulation tests were carried out using two different feeding strategies: pulse-wise and continuous feeding. The first accumulation test was conducted in a fed-batch reactor where pulses of fCW were given using the same food-to-microorganism ratio (F/M) found in the selection reactor. The reactor was fed with 7 pulses, reaching a maximum PHA content of 54.8 %wt. (mg_{PHA}/mg_{TS}) with a polymer composition of 93:3:4 %wt. (HB/HV/HHx) (**Table 5.4**). The obtained polymer is composed predominantly of HB, as expected, the FCW being mainly composed of HB precursors such as acetic and butyric acid.

The accumulation test was conducted by adopting a continuous feeding strategy, thus providing always available VFA for the selected culture. The maximum PHA content (51.0 %wt.) and the polymer composition at end of the test (HB/HV/HHx = 93:4:3 %wt.) were similar compared to those obtained adopting a pulse-wise strategy.

Productivity and VFA consumption have shown different behavior (**Figure 5.7**a) and b). In the first accumulation test it is observed that the consumption of H.Hex and H.But is faster than those of H.Acet, which is consumed later. In the second one (b) there is a continuous consumption of H.Hex and H.But, while H.Acet tends to accumulate.

Therefore, with the second strategy, it was possible to provide a higher amount of VFA over time (192.6 vs. 100.6 C_{mmol}, **Table 5.5)**. In Appendix 1, **Figure A1.5.1** the VSS, PHA and biomass profiles over time per are shown.

Table 5.4 – Performance parameters obtained for the PHA-accumulation stage under different feeding strategies.

		Fed-batch	**Continuous**
VFA Profile (HAc/HBut/HCap/Others)	% Cmol basis	34/56/6/4	34/56/6/4
Biomass concentration	g_{VSS}/L	2.7	2.95
PHA initial content	% mgPHA/mgTS	20.8	15.1
PHA_{max} content	% mgPHA/mgTS	52.9	51.0
PHA composition (HB/HV/HHx)	% Cmol basis	93/3/4	93/4/3
-qVFA	$Cmmol_{VFA}/(Cmmol_x*h)$	0.39 ± 0.04	-
qPHA	$Cmmol_{PHA}/(Cmmol_x*h)$	0.21 ± 0.05	-
$Y_{PHA/VFA}$	Cmmol-$_{PHA}$/Cmmol-$_{VFA}$	55.3	-
Productivity	$g_{PHA}/(L*h)$	0.34	0.52
Specific productivity	$mg_{C\text{-}TOC}/(g_{C\text{-}X}*h)$	0.16	0.21

Table 5.5 – VFA consumption under different feeding strategies.

Feeding strategy	**H.Acet**	**H.But**	**H.Hex**	**VFA**
	C_{mmol}	C_{mmol}	C_{mmol}	C_{mmol}
Pulse-wise	41.1	46.8	6.2	100.6
Continuous	45.4	127.2	13.1	192.6

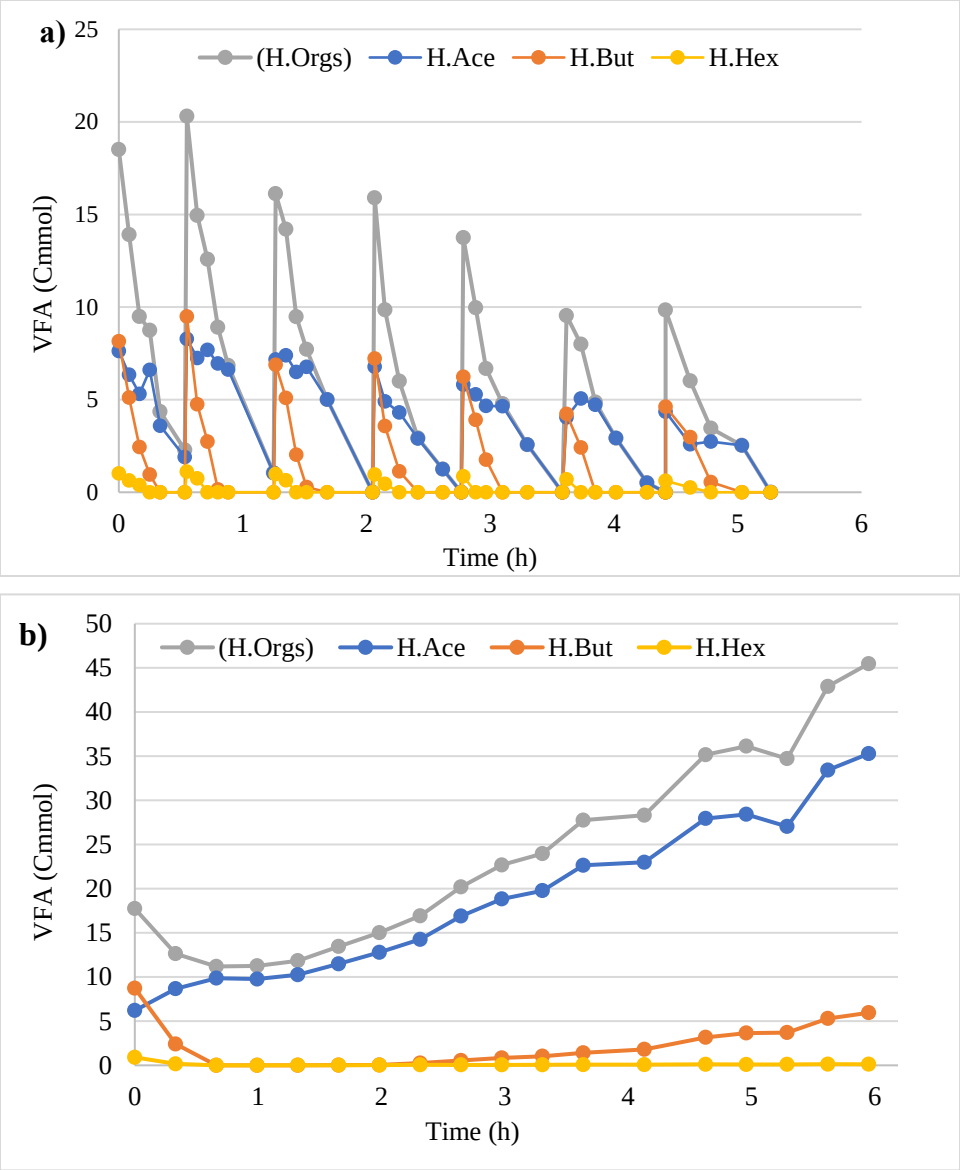

Figure 5.7 - VFA profile over time using: (a) pulse-wise strategy; (b) continuous strategy.

5.4 Conclusions

The feasibility of producing PHA by controlling the operating conditions of acidogenic fermentation was demonstrated. The changes in OLR allowed to reach a good fermentation degree and to increase the butyric acid concentration at the expense of acetic acid. No significant variation was observed in the total VFA concentration when microaeration was provided at flow rate of 3 mL/min and 12 mL/min, although a slight increase in the percentage of butyric acid in the mixture was assessed. Further increasing of aeration flow led to deterioration of the reactor performance,

in terms of gas production and liquid metabolites. The increase in HRT also influenced the composition of the volatile acids produced, with an increase in the presence of acetic acid (48%). On the other hand, lower performance parameters were observed, in terms of fermentation degree (49%) and productivity (9.8 $gVFA_{COD}/L_{reactor}$ day).

In the accumulation stage, even though the continuous feeding strategy allowed to provide higher amount of VFA per time, similar values were obtained compared to those obtained adopting a pulse-wise strategy, in terms of PHA content and polymer composition.

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Part II

Bio-derived leaching solution for metal recovery from end-of-life WEEE

Electrical and electronic equipment (EEE) is increasingly part of our daily life. In recent decades, they have largely contributed to improving the quality of life, providing various benefits and opportunities in a variety of sectors: among them, energy, transport, health, safety, and education (Vaccari et al., 2019). Today, digitization represents one of the most relevant goals of modern society. At the same time, EEE production requires high amounts of resources. Metals, including base, noble and "rare earth metals", play an important role in increasing new technologies level. Consequently, high and growing quantities of metals are required for Hi-Tech production, with a specific reference to electronic devices. Owing to the geological reserves and geopolitical constraints, some metals are facing serious supply risks. These metals are called "critical metals" because they combine high industrial importance and high risk of shortage. The supply risk may be due to the limited reserves available and economically accessible, as well as to geo-political concerns.

At the end of the life cycle of the EEE, they became WEEE (Waste from Electrical and Electronic Equipment), one of the major waste streams of our times (with an annual growth higher than 5%). The correct recovery of WEEE makes it possible to obtain significant benefits from an environmental and economic point of view: on the one hand, the dispersion of toxic and harmful substances into the environment is avoided, on the other hand, large quantities of secondary raw materials (steel, iron, copper, aluminum, plastics) are produced saving energy and limiting greenhouse gases emissions (Ollio, 2011).

Chapter 6

Waste from Electrical and Electronic Equipment

In this framework, this chapter summarizes the importance of the valorization of metals from waste, their impact on the environment if they are not properly managed, and the main recycling technologies used to recover metals with particular attention to WEEE (Waste from electrical and electronic equipment), today considered “urban mines” due to the high amount and quality of contained metals.

6.1 What is WEEE?

Waste from Electric and Electronic Equipment represents electrical and electronic equipment that is no longer used by consumers or that has reached its end-of-life (EoL). Computers, mobile phones, fridges, and cookers are examples of this category of waste (Birloaga & Vegliò, 2018).

In recent decades, a huge increase in the consumption of high-tech products has been the result of rapid technological progress (Izatt et al., 2014; Otieno and Omwenga, 2015).

The first directive regarding WEEE promulged by European Union (2002/96/EC) grouped WEEE into 10 primary categories, classified per product type and legislative relevance. Furthermore, according to the EU Directive, each EU State is required to develop strategies to achieve the collection and recycling targets set out in the Directive and to collect separately domestic WEEE at an annual rate of 4 kg per capita (European Union, 2003b). Responsibility principles for EEE producers and polluters in the WEEE management chain were adopted. Later, Directive 2012/19/EU, still maintaining the aim and approach, revised the 2002/96/EC grouping WEEE into 6 new categories and updating collection targets to the more challenging 65% of WEEE placed on the market in the previous three years or the 85% of WEEE generated by the year.

The production of EEE, and consequently of WEEE, is associated with technological innovation and the growth of demand in electronics. Driven by technological innovation and people’s demand for electronics, the replacement rate of electronic devices is getting faster. Therefore, the annual worldwide production of WEEE is constantly growing (Liu et al., 2021).

The real amount of WEEE produced annually is very complex to estimate due to the presence of unofficial disposal routes and of obsolete or not-longer-working equipment that remains inside homes (Baldé et al., 2015; Bigum et al., 2012).

An estimation of the amount produced globally is reported in "The Global e-waste monitor 2020" (Baldé et al., 2015). The data reported show an increase of WEEE from 44.4 million tons in 2014 to 53.6 million tons (Mt) in 2019. This data is expected to grow up to 74.7 Mt in 2030. Among the continents, Europe was found to be the largest producer of WEEE per capita (16.2 kg/inh*year), out of a total production of 12 Mt. On the other side it is also the most virtuous in WEEE management with the 42.5% of WEEE adequately collected and recycled. In Italy and Sardinia, data on total and per capita collection have been published by the WEEE Coordination Centre. In 2021, 385,258 t of WEEE were collected in Italy and properly disposed of (+5.3% compared to 2020), corresponding to a 6.46 kg per capita collection primarily by the northern regions. In Sardinia in 2021 the annual and per capita productions were 16,000 tons and 9.93 kg/inh, respectively.

6.2 Metal content and composition

In addition to the environmental concerns related to its management, WEEE has also economic and social implications. This is due to the relatively high amount of base, precious and rare elements contained in it that can be considered important secondary raw materials.

WEEE is a heterogeneous mixture of metals, multi-element alloys, and polymetallic structures, as well as plastics, metalloids & semiconductors, and glass fibers, the percentage of which varies depending on the equipment considered (Cui & Zhang, 2008b). The complexity of WEEE tends to increase with technological development: modern devices can involve up to 60 elements present as mixtures of metals and non- and semi-metals (Bloodworth, 2014). This complexity, related to the key role each species plays in the device, is even increased by the intimate connection between the materials mentioned above, which makes them extremely difficult to manage and treat. A relevant example is represented by the printed circuit boards (PCBs), essential parts of EEE, which are one of the most intriguing and rich sources of potential secondary raw materials due to their unique qualitative and quantitative metallic composition. The exact metal composition of this waste stream is difficult to specify due to the variability in EEE configurations, but rough numbers can be estimated. As shown in **Figure 6.1**, PCBs contain base metals (iron, aluminum, tin, lead,

nickel, zinc, etc.) but also noble metals such as gold, silver, palladium, and copper in proportions many times higher than their primary minerals, so much so they earn the name of "urban mines".

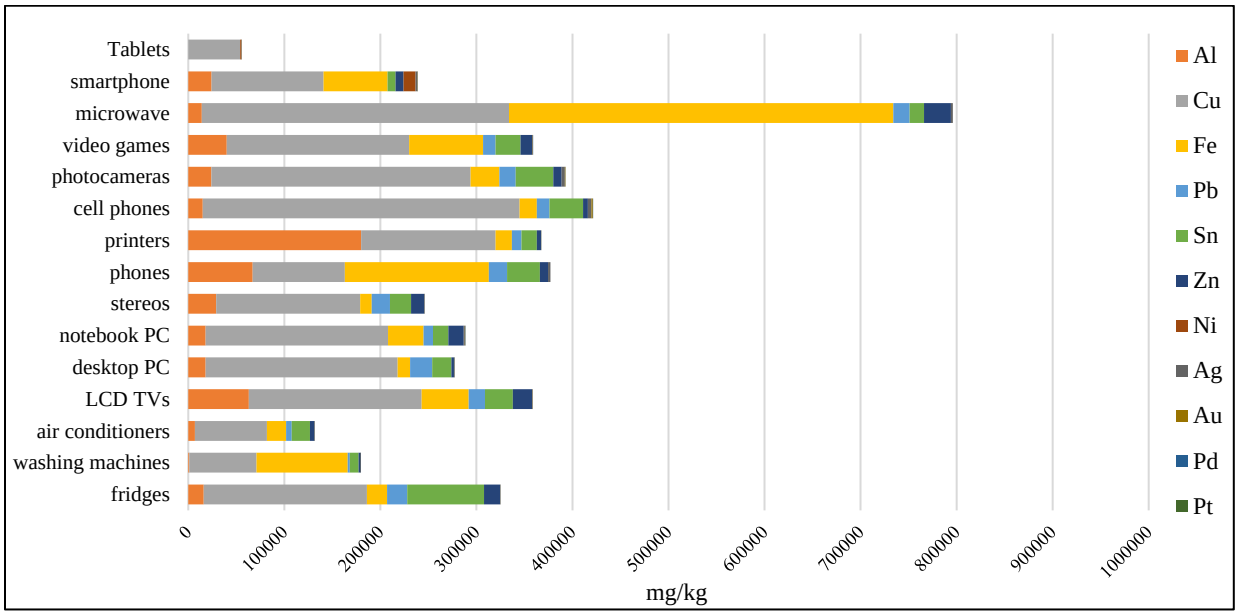

Figure 6.1 – Average metal composition in the most common printed circuit boards.

Among them, several metals are listed by the European Commission as critical (e.g. rare earths, indium, platinum group metals, indium, lithium, etc.) (European commission, 2010). Critical metal content in electrical and electronic equipment is of high economic importance due to the high risk related to their supply chain. These metals are used to produce microprocessors, printed circuit boards, cathode ray tubes, liquid crystal displays, LEDs and permanent magnets, among others. The recovery of these metals represents a real challenge, a recovery that would allow them to feed the supply chain and thus reduce the environmental impacts related to their extraction from mines (Serpe et al., 2019, Batnasan et al., 2019). Sustainable approaches should address the preservation of the natural resources of the elements in order to ensure their availability to future generations. For these reasons, the best solution would be the reintegration of WEEE into the economic cycle through the recovery of metals and the plastic fraction. Given their critical metal content, recovery of critical metals from WEEE is a primary objective. It is necessary to increase the efficiency of the recycling of secondary resources, to reduce the pressure on the extraction from ores. WEEE could be an important resource of metals in the transition to a circular economy.

6.3 Technologies for metal recovery from WEEE

Most industrial metal recovery processes from WEEE start from a disassembling phase to make the equipment suitable for a subsequent treatment. In particular, the parts that can be sent for reuse are removed manually, as well as the components containing dangerous substances; if deemed economically viable, some additional items are also dismantled (Andersson et al., 2019) and undergo a mechanical pre-treatment in which WEEE size is reduced and the metal values are released. Then the different fractions of materials (ferrous, non-ferrous, plastics, glass, etc.) are separated using various sorting operations such as magnet and eddy-current separation and density baths (Andersson et al., 2019). Depending on the technology used, this step often has a significant impact on the costs of the entire recovery process (Tanskanen, 2013).

Furthermore, concerning the enhancement of the greatest part of material values, more intimate processes can be approached. This typically consists of pyrometallurgical and/or hydrometallurgical processes.

6.3.1 Pyrometallurgical processes

Pyrometallurgy involves a set of processes that typically include "Smelting" as the initial step, in which waste materials are treated at very high temperatures and in an oxidizing environment inside a furnace. The plastic components are then incinerated, and the metals are melted. Among them, base metals (such as iron, lead and zinc) precipitate in the form of oxides, thus leaving a copper alloy rich in precious metals. This alloy is then used as an anode in a subsequent step of copper electrorefining that releases gold, silver, platinum and palladium in the form of anodic mud. The latter is then typically enhanced by hydrometallurgical treatments followed by electrolytic refinement. Because of its positive aspects, such as high efficiency and the existence of plants already in operation with these technologies, pyrometallurgy has been one of the most used treatment techniques for the recovery of metals from WEEE in the metallurgical industries worldwide in the last twenty years: Rönnskår Smelter and Umicore are examples of this. However, copper smelters are required to adapt their processing cycles to treat complex materials such as WEEE, allowing them to process only small quantities of WEEE at a time. The main disadvantages of pyrometallurgical processes are the high energy expenditure, high operating and maintenance costs, production of dangerous gaseous by-products resulting from the degradation of plastics during the process, and the lack of selectivity in the recovery of the most precious

metals. The latter issue is typically faced by combining a pyrometallurgical step with a hydrometallurgical treatment.

6.3.2 Hydrometallurgical processing

Hydrometallurgical processes are based on chemical treatments involving leaching agents for metal dissolution, followed by separation, purification, and recovery phases. These treatments typically use aqueous leaching agents such as strong acids (e.g. sulfuric, nitric, and hydrochloric) and/or complexing agents in oxidizing environment, which are often highly hazardous for operators and the environment in conventional treatments. The hydrometallurgical approach is generally more repeatable, selective, and controllable than the pyrometallurgical one, as well as requiring less energy expenditure and limiting the emission of toxic gaseous pollutants resulting from the thermal degradation of organic compounds. Moreover, it requires fewer investment costs, the reason why it is used in developing countries for the recovery of metals, with concerns related to the unappropriated level of precaution in operations. On the other hand, metal dissolution and separation require a large consumption of chemical reagents (some of which are particularly dangerous), thereby generating high volumes of by-products and highly contaminated wastewater. For these reasons, over the years, there has been an increasing focus on the use of more environmentally sustainable leaching agents (such as thiourea and thiosulphate) or alternative

processes, such as electrocatalytic or bio-metallurgical processes, as summarized in **Figure 6.*2*** (Tuncuk et al., 2012b).

Scrap Type	Leaching reagent	Recycled Metals	References
Scrap integrated circuits	(i) Thiourea – ferric sulfate leaching	(i) Au (69.36%), Ag (100%), Cu (100%)	Lee et al. (2010)
	(ii) Aqua regia leaching	(ii) Au (100%), Ag (88.51%), Cu(100%)	
	(iii) NH_3–$(NH_4)_2S_2O_8$ leaching	(iii) Au (12.76%), Ag (100%), Cu (88.87%)	
	(iv) H_2SO_4 leaching	(iv) Au (6.05%), Ag (90.37%), Cu (100%)	
Scrap TV Boards	$H_2SO_4 + H_2O_2$	Cu (>98%)	Deveci et al. (2010)
PCB	Column bioleaching (*Sulfobacillus thermosulfidooxidans* and *Thermoplasma acidophilim*)	Zn (80%), Al (64%), Cu (86%), Ni (74%)	Ilyas et al. (2010)
PCB	After thermal pre-treatment; HCl leaching	Cu (98%)	Havlik et al. (2010)
PCB	Lime Sulfur Synthetic Solution (LSSS) method $Na_2S_2O_3$–$CuSO_4$–NH_4OH leaching	Au (92%), Ag (90%)	Li and Huang (2010)
PCB	$HCl + HNO_3$	Ag (98%), Pd (93%), Au (97%)	Park and Fray (2009a)
PCB	(i) H_2SO_4	(i) Sn (<0.01%), Cu (<0.01%)	Castro and Martins (2009)
	(ii) H_2SO_4 + HCl	(ii) Sn (96.3%), Cu (29.8%)	
	(iii) HCl	(iii) Sn (98.2%), Cu (20%)	
	(iv) $HCl + HNO_3$	(iv) Sn (85.8%), Cu (34.3%)	
PCB	Bioleaching (*At. ferrooxidans*)	Cu (>99%)	Yang et al. (2009)
PCB	Tri-*n*-butyl phosphate (TBP), Cyanex 272, Cyanex 301	Zn, Ni (99%)	Park and Fray (2009b)
PCB	Bioleaching (*S. thermosulfidooxidans*)	Ni (81%), Cu (89%), Al (79%), Zn (83%)	Ilyas et al. (2007)
Computer PCB	HNO_3/HCl leaching	Au	Sheng and Etsell (2007)
	Cu(II)–NH_3–$(NH_4)_2SO_4$ leaching	Cu (90%)	Koyama et al. (2006)
PCB	Electrowinning		
E-scrap	HNO_3/HCl leaching	Au, Cu	Madenoglu (2005)
Mobile phones PCB	(i) For Pd recycling; HCl/NaCl with HNO_3/H_2O_2 leaching	(i) Pd (93–95%)	Quinet et al. (2005)
	(ii) For Au, Ag recycling; cyanide/thiourea leaching	(ii) Au, Ag (>95%)	
Computer PCB	Bioleaching (*At. ferrooxidans*)	37–82% Cu	Choi et al. (2004)
Non-mounted PCB	HNO_3 leaching	Cu, Ni (>90%)	Kinoshita et al. (2003)
	Solvent extraction		
PCB	(i) H_2SO_4/H_2O_2 leaching	(i) Cu, Fe, Zn, Ni, Al (>95%)	Oh et al. (2003)
	(ii) $CuSO_4$–NH_4OH–$(NH_4)_2S_2O_3$ leaching	(ii) Au, Ag (>95%)	
PCB	HNO_3 leaching	Cu, Pb (>95%)	Mecucci and Scott (2002)
	Electrowinning		
	Bioleaching		Brandl et al. (2001)
PCB dust	(i) Bacteria (*At. ferrooxidans*)	(i) Cu, Ni, Zn (>90%)	
	(ii) Fungi (*A. niger*)	(ii) Cu and Sn (65%); Al, Ni, Pb, Zn (95%)	
Wasted electronic parts	KI/I_2 and NaCl/hypochlorite leaching	Au (88%), Ag (65%)	Shibata and Matsumoto (1999)
	Solvent extraction		

Figure 6.2 – Summary of experimental work carried out on different material/reagent for metal recovery from e-waste.

However, their application at industrial scale is often hindered, for example by the low process speed and the relatively lower profitability compared to pyrometallurgical processes. In addition, mechanical treatments of electronic waste, addressed to increase the efficiency of the following chemical processes, are time-consuming and costly, as well as physical separation is not completely selective so it can result in the loss of a certain amount of valued metals (e.g., up to 20% of precious metals (PM)). Anyway, considering the importance that WEEE is assuming over time and regulations on sustainable WEEE management wide spreading worldwide, companies are paying more attention to the impacts that recovery processes could have on the environment. In this framework, a hydrometallurgical method recently developed at the University of Cagliari adopts a selective approach by using eco-friendly leaching agents specifically tailored to the chemical properties of the different classes of metals contained in WEEE.

Among them, bio-derived organic acids have been selected for the leaching of metals with a low oxidation potential as a greener alternative to strong inorganic acids, thanks to their powerful complexing capabilities which impel metal reactivity.

6.3.3 The selective hydrometallurgical process of the University of Cagliari

A selective and sustainable hydrometallurgical method has been described by (Serpe et al., 2015), aimed at the selective recovery of the different types of metals present in WEEE, exploiting the action of leaching agents with low impact on the environment and human health.

The proposed process, validated on a real sample consisting of the non-ferrous metallic fraction obtained from the mechanical comminution and separation of mixed printed circuits boards and small EEE, involves three main steps:

- **Step 1** (24 - 48 h): **Dissolution of base metals** with 3M $C_6H_8O_7$ (citric acid) aqueous solutions at a reflux temperature of about 100 ºC (citric acid is a widely available and cheap weak biobased acid). Under these conditions, the citric acid solution is selective towards low-reduction potential metals, which account for about 20% of the mass of the WEEE sample used. Once leaching is complete, dissolved metals can be recovered from the solution in the form of citrate salts or by using appropriate precipitating reagents.
- **step 2** (~ 48 h): **Recovery of Copper and Silver** from the residual solid of Step 1 using a buffer solution of ammonia (NH_3, 33%) and ammonium sulfate $(NH_4)_2SO_4$ in an oxidizing environment by hydrogen peroxide (H_2O_2) or an alkaline solution of IO_3^- and I^-, prepared by I_2 disproportion into NaOH(aq).
    - using the IO_3^-/I^- mixture, silver precipitation occurs during the leaching process;
    - using H_2O_2, selective AgX precipitation is caused by the addition of halide salts to the ammonia solution.

  Copper is recovered by chemical (cementation) or electrochemical (electrowinning) reduction; the residual solid containing AgI is filtered off and washed using an aqueous solution of $Na_2S_2O_3$ (0.5 g in 50 mL of H_2O dist.), in order to dissolve AgI(s). Silver metal recovery is obtained by electrowinning of the latter solution;
- **step 3**: **Recovery of gold** by treating the solid residue resulting from the previous two phases. This is treated with an aqueous solution of KI and I_2 in a molar ratio of 5.3:1. Gold dissolves forming the complex $[AuI_2]^-$. After leaching, lasting between 30 and 60 minutes,

H_2O_2 and HCl are added to the solution to oxidize the iodide and recover it as I_2. Gold, on the other hand, is recovered from the solution by electrodeposition.

Experiments performed on different scale and materials confirmed the efficiency and selectivity of the method achieving gold, silver and copper recovery yields higher than 70% and up to 99%, depending on the preliminary comminution treatments undergone by the material.

With the aim of improving the use of resources and limiting the environmental impact, a key further step forward that will be explored in this thesis is to replace commercial reagents (i.e., citric acid used in the process described above), with leaching mixtures obtainable from organic waste fermentation.

6.4 Bio-derived organic acids solutions

Currently, commercial production of organic acids relies primarily on chemical conversions of precursors from petroleum processing. Nevertheless, low-cost biological production from organic by-products and waste streams could play a pivotal role as a sustainable alternative that pursues circular bio-economy goals. Moreover, the use of these renewable and widely available substrates stimulates the transition toward innovative and environmentally friendly strategies for waste valorization, besides the more conventional ones.

The fermentative processes of agro-industrial waste containing high concentrations of sugars are to be considered excellent precursors to produce organic acids, promising in terms of their use as leaching agents. From the fermentation of the cheese whey, it is possible to obtain different leaching mixtures rich in organic acids (lactic, acetic, propionic and butyric acid). Depending on the operating conditions used during fermentation, the latter can be oriented toward the production of specific acids.

In the present Ph.D. research project, we aimed to produce bio-derived, sustainable and low-cost leaching mixtures for the selective metal dissolution, capable of replacing the use of commercial reagents and exploiting a dairy waste that currently is a waste to treat. Furthermore, the direct application of such leaching mixtures in the dissolution of metals for their recovery from secondary raw materials is studied.

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Chapter 7

Lactic acid production through cheese whey fermentation

This chapter presents a description of the fermentation process of sheep cheese whey to produce a leaching mixture with the following characteristics: the highest lactic acid concentration, the lowest residual carbohydrate concentration and pH 4. In **Figure 7.1** a schematic representation of the treatment is shown.

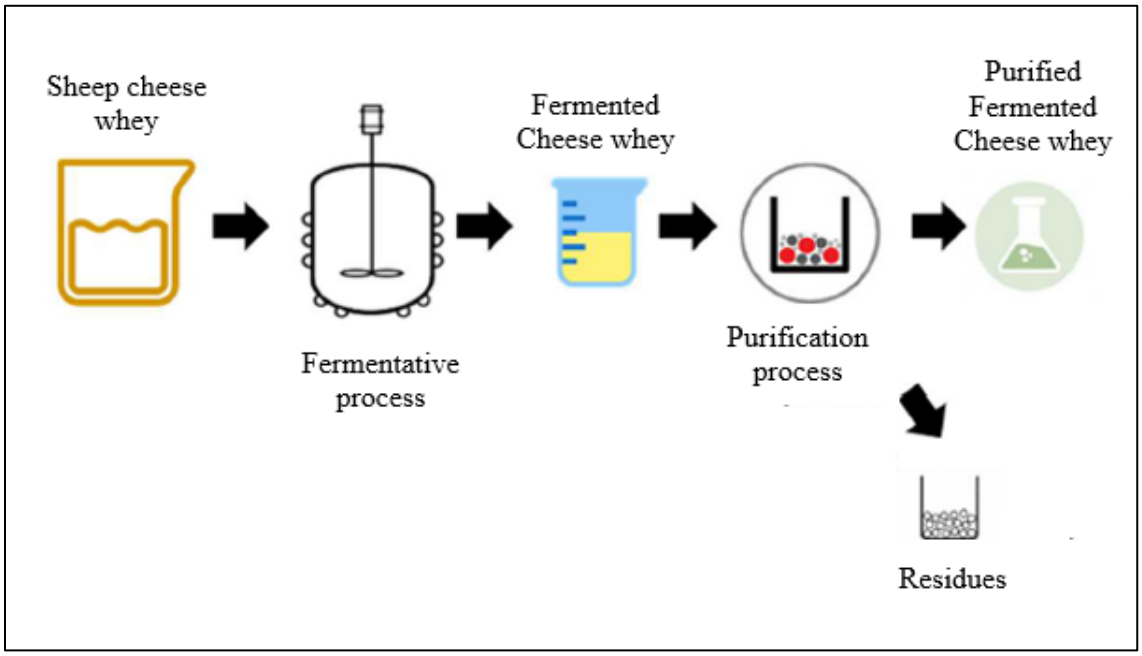

Figure 7.1 – Schematic representation of the cheese whey fermentation and purification adopted.

7.1 Introduction

Lactic acid, or 2-hydroxypropanoic acid, is a platform chemical with a wide range of applications in the food (e.g., beverage), chemical (e.g., metal complexing agent), cosmetic (e.g., skin rejuvenation agent), medical and pharmaceutical industries (e.g., controlled drug delivery system) (Abedi & Hashemi, 2020; Alves de Oliveira et al., 2018; Upadhyaya et al., 2014). Recently, interest in lactic acid has increased as it can be used to produce polylactic acid (PLA), an environmentally-friendly and biodegradable polymer which is an optimal candidate to replace

petroleum-derived plastics (Bernardo et al., 2016). As evidence of this, the global lactic acid market increased from 1,220 kilotons in 2016 to 1,960 kilotons in 2025, with a CAGR of 18.7% (Nwamba et al., 2021).

Lactic acid can be obtained by either chemical or biological processes. The main drawback of the chemical process (i.e.: lactonitrile hydrolysis with strong acids) is the production of a racemic mixture of D- and L-lactic acid, which cannot be used for industrial applications where pure lactic acid isomers are required (Nwamba et al., 2021). The cost of raw materials and production of process waste further limit the use of the chemical route (Bernardo et al., 2016).

Biological processes lead to the production of pure lactic acid (D- or L-lactic acid), under mild operating conditions, with the additional advantage of using renewable resources (Nwamba et al., 2021). Several fermentative microorganisms, including fungi, yeast, autochthonous bacteria and metabolically engineered bacteria can produce lactic acid and can be used for industrial purposes. Among bacteria, lactic acid bacteria (LAB), particularly *Lactobacillus*, are the most frequently used in industrial applications (Abdel-Rahman et al., 2013). Based on the end product of their fermentation, LAB can be classified into 2 groups: homofermentative and heterofermentative LAB. The former only produce lactic acid, while heterofermentative LAB produce a mixture of lactic, acetic acid and ethanol (Hofvendahl e Hahn-Hagerdal, 2000). LAB are widely used for the fermentation of several lignocellulosic substrates such as corn stover (Wang et al., 2010), hardwood pulp (Hama et al., 2015), rice (Lu et al., 2009), Curcuma longa biomass (Nguyen et al., 2013), cottonseed and corncob (Bai et al., 2016). However, the complexity of the lignocellulosic structure limits the biodegradability of the substrate, requiring intensive pre-treatments before the fermentation process. Moreover, the fermentative production of lactic acid accounts for 30–68% of the total production costs for the purchase of conventional substrates (Guilherme et al., 2012). Among the most promising non-lignocellulosic substrates are agro-industrial wastes, such as cheese whey (CW), due to the significant residual carbohydrate concentration. CW is the primary by-product of the dairy industry and contains lactose, proteins, fats, water-soluble vitamins, mineral salts, and other essential nutrients for microbial growth. Therefore, it is a promising and suitable raw material for lactic acid production (Panesar et al., 2007). Biochemically, cheese whey fermentation involves a step in which lactose can be hydrolyzed into glucose and galactose, then homofermentative LAB can produce four moles of lactic acid (Juodeikiene et al., 2016; Li & Cui, 2010) by the following reaction:

$C_{12}H_{22}O_{11}$ (Lactose) + H_2O → $C_6H_{12}O_6$ (Glucose) + $C_6H_{12}O_6$ (Galactose) → $4C_3H_6O_3$ (Lactic acid)

A pH range of 5-7 has been identified as optimal to provide a suitable reaction environment for Lactobacillus survival (Asunis et al., 2019). However, during lactic fermentation, the pH value tends spontaneously to the pK_a value of lactic acid (3.83), which has an inhibitory effect on cell metabolism and thus on lactic acid production. In general, LAB cannot grow below pH 4 (Hayek & Ibrahim, 2013). More resistant bacterial strains can be obtained with genetic engineering (Abedi & Hashemi, 2020) or with specific pre-treatments such as UV and nitrosoguanidine addition (Abedi & Hashemi, 2020). It is evident that the use of specific pre-treatments and inocula increases the complexity and costs of the fermentation process. On the other hand, a strong base (e.g.: NaOH or KOH) could be dosed to counteract the lowering of the pH. Another relevant point is that, although lactic fermentation in CW is spontaneous, it evolves towards H_2-fermentation since lactic acid is an ideal substrate for hydrogen-producing bacteria (HPB) (Asunis et al., 2019).
Therefore, it is necessary to find a sustainable method that inhibits HPB (and therefore the associated consumption of lactic acid), while ensuring maximization of the conversion yield of lactose into lactic acid. Maintaining a low pH (i.e. pH = 5.5) does not seem to inhibit HPB, while a too low pH (<5) limits the complete conversion of lactose to lactic acid (Asunis et al., 2019).

In this work, an innovative approach to obtaining lactic acid through CW fermentation without the use of specific external inocula and limiting the dosage of pH conditioners is presented. CW fermentation is carried out in two phases:

- Phase I: operating pH is continuously controlled to 6;
- Phase II: operating pH is not anymore controlled, and the fermentative lactic acid production continues until substrate depletion or until pH value becomes limiting for the bacteria activity.

The extension of Phase I is crucial for the efficient conversion of carbohydrates to lactic acid, while an adequate duration of Phase II is necessary to achieve complete carbohydrates conversion. The shift between phase I and II, i.e., the interruption of the pH control, cannot be identified simply by the elapsed fermentation time since the start of the batch test (Verma et al., 2023). Reaction time is a parameter strongly linked to the type of cheese whey (origin of the milk: sheep, goat, cow, buffalo, etc.), its storage (refrigeration, freezing, freeze-drying) and the chemical, physical

and biological characteristics of the specific sample of CW (pH, nutrient composition, initial carbohydrate content and bacterial culture). On the other hand, the evolution of the concentration of lactic acid cannot be a robust parameter of the progress of the fermentation process, being linked to the initial concentration of lactose in CW. In the present work, a quantitative parameter based on the concentration of lactic acid and of residual carbohydrates, subject of a recently submitted patent (Italian patent N. 102022000007502, 2022), is presented and applied on a set of experimental data.

7.2 Materials and methods

7.2.1 Substrate

Samples of fresh raw sheep cheese whey (CW) were collected at a medium-size dairy industry in southern Sardinia (Italy) which processes ovine milk to produce Pecorino cheese. CW was collected immediately after the cheese production process, transported to the laboratory in about 1 hour, divided into 2 L-bottles and stored at -15 °C until use to prevent biodegradation. The required amount of sample was thawed at room temperature (25-30 °C) for about 12 hours before starting each fermentation test. The thawed cheese whey was used as feed material in batch fermentation experiments without any additional external inoculum. The choice to store the CW samples frozen was based on evidence from previous studies that freezing at -18°C followed by thawing at 25 °C in 2 L containers did not significantly alter the total microorganism count (Asunis et al., 2019). The main characterization parameters for post-thaw CW are reported in **Table 7.1**.

Table 7.1 – Main characterization parameters of CW (average value ± standard deviation)

Parameter	Unit of measure	CW
pH	-	6.5 ± 0.2
Total solids, TS	%	6.8 ± 0.5
Volatile solids, VS	%	5.7 ± 0.9
Total carbohydrates, tCarbo	g/L	51.4 ± 7.5
Soluble carbohydrates, sCarbo	g/L	46.3 ± 3.8
Lactic acid, HLa	g/L	2.9 ± 0.5
Total proteins, tProte	g/L	12.1 ± 1.5
Soluble proteins, sProte	g/L	8.4 ± 1.7
Total organic carbon, TOC	g/L	29.4 ± 3.1
Soluble organic carbon, sTOC	g/L	25.2 ± 1.9
Soluble ammonia nitrogen, NH_4^+	mg/L	162.9 ± 11.3

7.2.2 Experimental set-up

One-stage *lactate-driven* dark fermentation (LD-DF) test at a constant pH of 6, as it provides the most favourable conditions for lactic acid production (Asunis et al., 2019), was performed on sheep cheese whey. CW were sampled from the reactor at 2-hour intervals for the first 12 hours of fermentation and 1 hour for the following 12 hours for a total of 9 aliquots (**Figure 7.2**). Each aliquot underwent uncontrolled-pH fermentation test in a sealed flask in an orbital shaker-incubator. The ratio of the molar concentration of lactic acid to carbohydrate (α_j, Eq. 1) was calculated for each aliquot j, with j= 1,…, 9.

$$\alpha_j = \frac{[\mathrm{HLa}]_j}{[\mathrm{sCarbo}]_j} \quad (1)$$

where: $[HLa]_j$ and $[sCArbo]_j$ are the molar concentration of lactic acid and soluble carbohydrates calculated for each aliquot j, with j= 1,…, 9, respectively.

The criterium adopted to identify the optimal value of the α_j parameter was the co-occurrence of i) an almost total consumption of the initial carbohydrates and ii) a final pH ≤ 4.

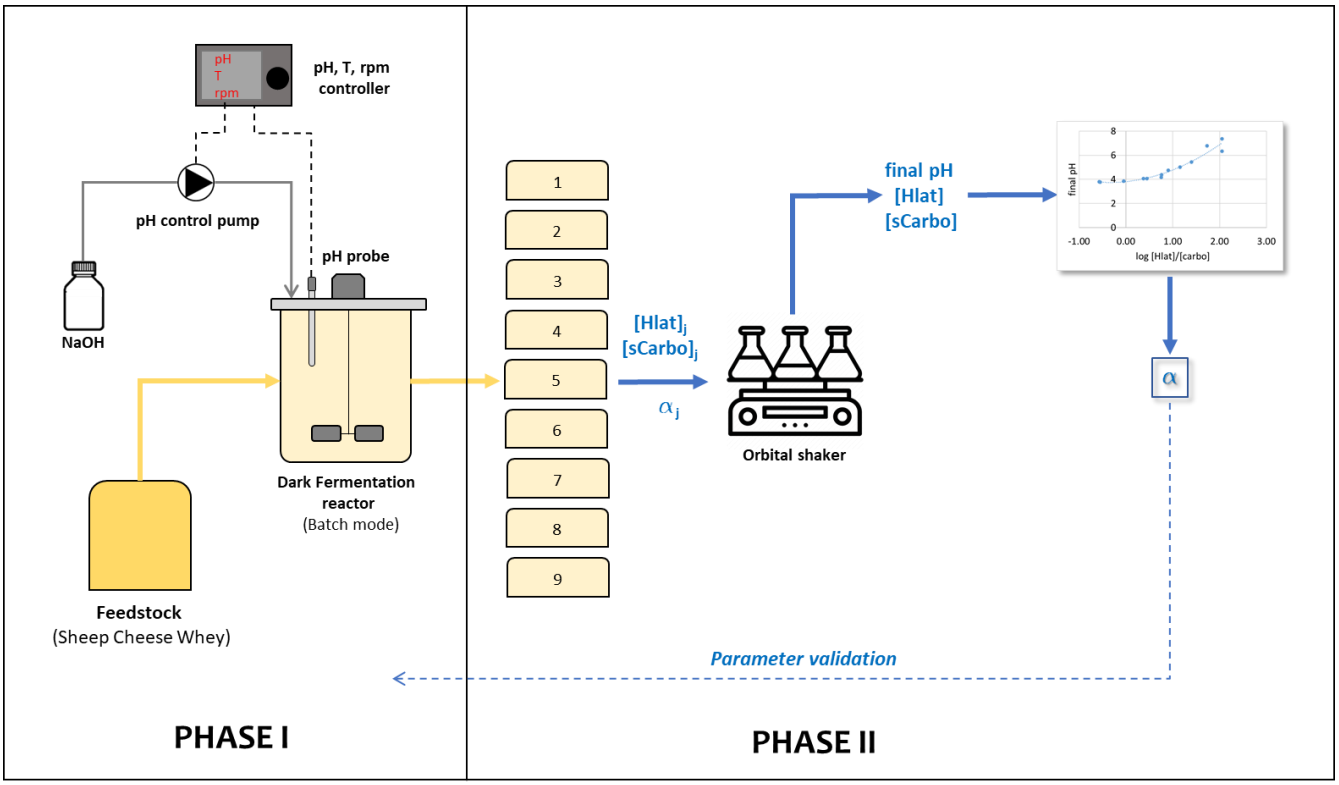

Figure 7.2 - Experimental set-up used to obtain the optimal value of the α parameter. Phase I: operating pH is continuously controlled to 6; Phase II: operating pH is not yet controlled.

To validate the obtained optimal value of the α parameter, six two-stage LD-DF tests, in which the first phase was carried out at an operating pH of 6, (Phase I) and a subsequent phase in which the pH was not controlled (Phase II), were performed. The shift from Phase I to Phase II occurred according to the value of the α_j parameter determined in the previous experimental phase.

All the batch dark fermentation tests were carried out at 39 ± 1°C using a glass reactor (BIOFLO 110 - New Brunswick Scientific; BioCommand Lite software; working volume = 1.8 L). The reactor was equipped with a mechanical stirrer (150 rpm) and automatic pH control software that continuously controlled the addition of a 5M NaOH solution. The reactor was covered with a black plastic film to prevent photo-fermentative reactions and preliminary purged with N_2 gas to ensure anaerobic conditions. No external inoculum was added considering that the cheese whey biomass can sustain the fermentation process.

Scale-up of the process has been studied by conducting a fermentation test on 16 L of CW. As for the validation tests of parameter α, the fermentation test was carried out in a batch inside a glass reactor with a working volume of 30 L and conducted under mechanical stirring and a temperature

of 39 ºC. Phase I was conducted at pH 6 and the shift from Phase I to Phase II occurred according to the value of the α_j.

The fermented CW (fCW) was treated with ethanol in a volume ratio of 3:5, mixed for 1 h (Reax 20, Heidolph) and centrifuged (Du Pont Instruments Sorvall SS-3 Automatic Centrifuge) for 15 minutes at 15000 rpm. The centrifuged residue was removed, and ethanol was recovered from the solution by Rotavapor (BUCHI Labortechnik AG, R-3 HB).

The resulting purified fermented CW (pfCW) and the solid residue, mainly composed of exhausted biomass and protein, were stored at -15 °C in 2-L bottles prior to use in further valorization processes.

7.2.3 Analytical methods

Process performance during Phases I and II was evaluated by monitoring concentrations of total (tTOC) and soluble (sTOC) organic carbon, soluble carbohydrates (sCarbo), total (tProte) and soluble proteins (sProte), and lactic acid (HLa). The tTOC and sTOC concentrations were measured using a Shimadzu TOC analyzer equipped with modules for the analysis of both liquid and solid samples. sCarbo were analyzed using the colorimetric phenol sulphuric acid method using glucose as the standard (DuBois et al., 1956). The sProte content was determined spectrophotometrically at 750 nm by the alkaline copper method as described by (Lowry et al., 1951), using bovine serum albumin (BSA) as the standard. All the spectrophotometric analyses were performed with a HITACHI U-200 spectrophotometer. The concentration of HLa was analyzed using a Jasco high-pressure liquid chromatography System equipped with UV/Vis Detector UV-4075, Column Oven CO-4061, RHPLC Pump PU-4180 and an Acclaim Organic Acid column. All analyses were conducted with isocratic elution (H_2PO_4 1N at 0.8 mL/min), and the oven temperature was set at 60 °C.

7.2.4 Statistical analysis

All analyses were run in triplicate and results are presented as average values of the replicates and the associated standard deviation.

7.2.5 Calculations

The carbohydrates and lactic acid molar fraction (χ_{Carbo}, χ_{HLa}) were calculated according to Equations (2) and (3).

$$\chi_{Carbo} = \frac{[sCarbo]}{[sCarbo]+[HLa]} \quad (2)$$

$$\chi_{HLa} = \frac{[HLa]}{[sCarbo]+[HLa]} \quad (3)$$

where [sCarbo] and [HLa] are the molar concentration of soluble carbohydrates and lactic acid, respectively.

The ratio of the molar concentration of lactic acid to carbohydrate (α) at the end of Phase I was calculated according to Equation (4)

$$\alpha = \frac{[HLa]_{phaseI}}{[sCarbo]_{phaseI}} \quad (4)$$

In order to estimate the fermentation performance, carbohydrate consumption (Eq. 5) and lactic acid production yields (Eq. 6) were calculated:

$$Carbo_{consumpt} = 100 - \frac{sCarbo_{Phase\ II}}{sCarbo_{IN}} \quad (5)$$

where $sCarbo_{IN}$ and $sCarbo_{PhaseII}$ are the initial carbohydrates and the carbohydrates at the end of the Phase II, expressed by mass;

$$Y_{HLa} = \frac{C_{HLa-Phase\ II}}{C_{sCarbo-IN}} * 100 \quad (6)$$

where $C_{sCarbo\text{-}IN}$ and $C_{Hla\text{-}PhaseII}$ are the initial carbohydrates and the lactic acid at the end of Phase II, expressed as mass of carbon.

In order to estimate the performance of the purification treatment, protein, carbohydrates and lactic acid removal and ethanol recovery were calculated as in equations (7) and (8), respectively.

$$Removal_{Prote,Carbo,HLa} = 100 - \frac{Prote,Carbo,HLa_{pfSCW}}{Prote,Carbo,HLa_{Phase\ II}} \quad (7)$$

where $(Prote, Carbo, HLa)_{PhaseII}$ and $(Prote, Carbo, HLa)_{pfCW}$ are the mass of protein, carbohydrates and lactic acid in CW collected at the end of Phase II, and after the purification treatment, respectively.

$$EtOH_{recovery} = \frac{EtOH_{recovered}}{EtOH_{in}} \quad (8)$$

where $EtOH_{recovered}$ and $EtOH_{in}$ are the ethanol mass recovered from the solution by rotavapor and the ethanol mass initially added for the purification treatment, respectively.

7.3 Results and discussion

7.3.1 Parameter selection

The fermentation process can be oriented in different ways, depending on the products to be obtained. In this study, fermentation is aimed at the production of lactic acid, by pursuing an innovative two-stage approach to maximize carbohydrate conversion and reduce the use of external reagents to control operating pH. **Figure 7.3** shows the trends of substrate degradation during the Phase II of the fermentation tests. Each point corresponds to an aliquot sampled from the DF reactor; concentrations of lactic acid and soluble carbohydrates were determined at the end of the Phase II, i.e.: when no pH variation was registered for at least 24 hours.

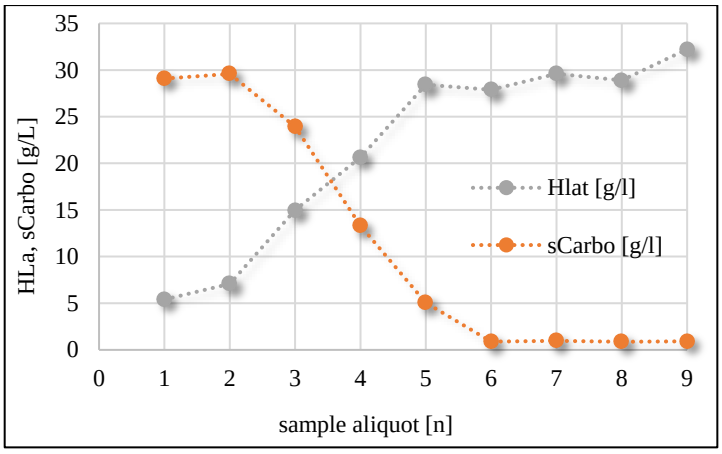

Figure 7.3 – Evolution of soluble carbohydrates and lactic acid during fermentation tests as a function of the sample aliquot.

Figure 7.4 shows values of the molar fraction of lactic acid χ_{HLa} and soluble carbohydrates χ_{Carbo}, during the one-stage *lactate-driven* dark fermentation (LD-DF) test, for each aliquot. The value of the final pH achieved at the end of Phase II (i.e., when operating pH is not yet controlled and the

fermentative lactic acid production continues until substrate depletion or until pH value becomes limiting for the bacteria) was also reported for each aliquot, *j*. The data were plotted against the corresponding values of the molar fraction of lactic acid to soluble carbohydrates (α_j) calculated for each aliquot, *j*.

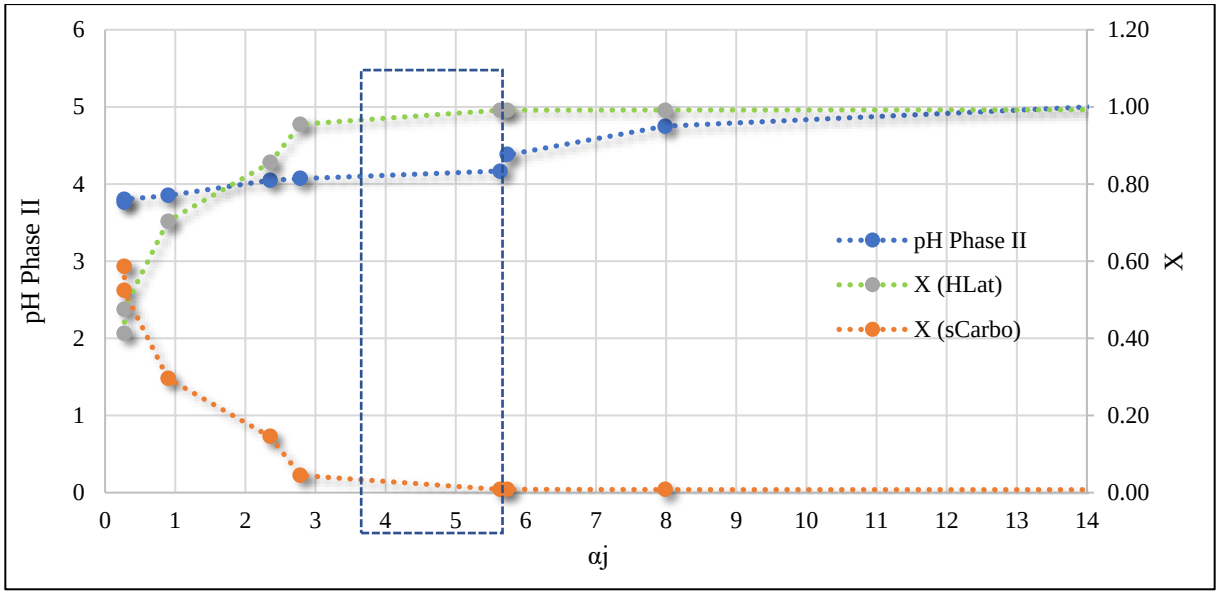

Figure 7.4 - Trends of the molar fraction of lactic acid and soluble carbohydrates during Phase I of one-stage lactate-driven dark fermentation test, final pH achieved at the end of Phase II, corresponding α_j value.

Given that the reaction time is not a reliable parameter to identify the shift moment between Phase I (pH controlled) and Phase II (pH uncontrolled), which allows to maximize the conversion yield of lactose into lactic acid and minimizes the addition of external reagents, it was decided to use the parameter α_j calculated as described in Eq. 1. From **Figure 7.4** it can be extrapolated that for values of $\alpha_j < 4$ the molar fraction of residual carbohydrates is > 0.04 (and thus the molar fraction of lactic acid is < 0.96) and the pH is < 4. Therefore, adopting values of α_j <4 exposes the risk of not fully converting carbohydrates into lactic acid. Moreover, for values of α_j <3 the molar fraction of residual carbohydrates rises rapidly. For values of $\alpha_j > 6$, the molar fraction of residual carbohydrates is even lower, equal to 0.01, but the pH tends to rise to values > 4.2. Adopting values of α_j >6, therefore, implies a dosage of a basic reagent which is not necessary to achieve in any case an almost complete conversion of the lactose into lactic acid. Finally, the best compromise between the maximum production of lactic acid, the minimum content of carbohydrates and the minimum pH is reached with a value of α between 4 and 6.

7.3.2 Parameter validation

To confirm the robustness of the parameter obtained, 5 batch fermentation tests were carried out, varying the ratio α as follows (see **Table 7.2** for details):

- CWDF_HLa_1, 2 and 3: $\alpha > 6$;
- CWDF_HLa_4: $\alpha = 4$;
- CWDF_HLa_5: $\alpha < 4$.

Table 7.2 – Experimental conditions adopted in the DF tests.

Test	t_0			**Phase I**				**Phase II**		
	sCarbo [g/L]	HLa [g/L]	Initial pH	Time [h]	sCarbo [g/L]	HLa [g/L]	α	sCarbo [g/L]	HLa [g/L]	Final pH
CWDF_HLa_1	45.0 ± 0.8	2.8 ± 0.1	6.5	17.3	11.1 ± 0.5	26.7 ± 0.3	9.2	3.3 ± 0.1	36.7 ± 0.4	4.2
CWDF_HLa_2	48.0 ± 1.0	2.6 ± 0.1	6.3	18.5	6.6 ± 0.6	30.5 ± 0.2	17.7	1.3 ± 0.1	36.3 ± 0.3	4.6
CWDF_HLa_3	46.3 ± 0.2	3.1 ± 0.2	6.8	14.5	7.7 ± 0.1	28.7 ± 0.2	14.2	1.5 ± 0.1	39.3 ± 0.5	4.7
CWDF_HLa_4	**47.1 ± 0.2**	**2.3 ± 0.1**	**6.7**	**13.0**	**17.9 ± 0.1**	**18.7± 0.1**	**4.0**	**6.3 ± 0.3**	**36.1 ± 0.1**	**4.0**
CWDF_HLa_5	51.5 ± 1.0	3.0 ± 0.2	6.4	11.8	25.4 ± 0.9	24.2 ± 0.1	3.6	10.3 ± 0.1	27.4 ± 0.1	3.9

The tests CWDF_HLa_1, 2 and 3 showed that if $\alpha > 6$, a low residual carbohydrate content (1.3 – 3.3 g/L) with a carbohydrate consumption of 92.6 - 97.2 % (**Table 7.3**), which are converted to lactic acid (36.3 – 39.3 g/L) with a yield in the range of 73.6 to 85.8 %wt, were observed.

On the contrary, if $\alpha < 4$ (CWDF_HLa_5), a higher residual concentration of unconverted carbohydrates (10.3 g/L, $Carbo_{consumpt}$ = 80% wt), with a final lower HLa concentration (27.4 g/L, Y_{HLa} = 49.9%) were obtained. In this operating condition, lactic fermentation of carbohydrates leads to a low pH (3.8-4.0) before all carbohydrates have been completely converted, with the consequent inhibition of the fermentative activity of bacteria.

When $\alpha = 4$ (CWDF_HLa_4), a residual concentration of unconverted carbohydrates of 6.3 g/L, with $Carbo_{consumpt}$ = 86.7% wt and a final lower HLa concentration (36.1 g/L (Y_{HLa} = 75.2%) were obtained.

Table 7.3 – Performance data for the DF tests.

Test	$\mathbf{Carbo_{consumpt}}$	$\mathbf{Y_{HLa}}$
	[%wt]	[%wt]
CWDF_HLa_1	92.6	73.6
CWDF_HLa_2	97.2	76.7
CWDF_HLa_3	96.9	85.8
CWDF_HLa_4	86.7	75.2
CWDF_HLa_5	80.0	49.9

7.3.3 Purification of the fermented cheese whey

Fermented substrates require purification treatment to recover lactic acid, as other suspended and soluble substances such as proteins, lipids and residual unconverted carbohydrates could interfere with the subsequent utilization.

Precipitation (i.e., the formation of a solid phase by changing the conditions of a liquid solution) is widely used on laboratory scale and in industrial processes to concentrate and/or purify proteins. The first examples of protein precipitation are the works of Lewith and Hofmeister (Hofmeister, 1888; Lewith, 1887). In these cases, precipitation was promoted by adding salts to protein-containing aqueous solutions. Protein precipitation can also be achieved by using other precipitant agents such as organic solvents (Pinheiro et al., 2016).

To optimize protein recovery by solvent precipitation, it is critical to first understand the factors that control protein solubility in non-aqueous solvents. In aqueous solution, proteins adopt a structure which exposes hydrophilic regions to the surrounding aqueous solution, allowing formation of a hydration layer that shields protein-protein interactions. Disruption of this hydration layer causes protein precipitation (Arakawa & Timasheff, 1985). Organic solvents, having reduced dielectric strength, increase the attractive force between opposingly charged ions (Green & Hughes, 1955). For a heterogeneously charged protein surface, the positive charges of one protein can combine with the negative charges of another, leading to aggregation of the sample in organic solvent (Crowell et al., 2013; Scopes, 1993).

The fermented cheese whey was treated with ethanol in a volume ratio of 3:5, and then was mixed and centrifuged. The centrifuged organic matter was removed, and ethanol was recovered from the solution by rotavapor. After purification, the pfCW was quantified and characterized, demonstrating that the treatments carried out slightly affected the concentration of dissolved lactic acid and most of the organic matter was removed, particularly protein (**Table 7.4**).

Table 7.4 – Performance of the purification treatment

Removal$_{Prote}$ [% wt.]	**Removal$_{Carbo}$** [% wt.]	**Removal$_{HLa}$** [% wt.]	**EtOH$_{recovery}$** [% vol.]
79.4 ± 2.9	7.3 ± 3.0	14.9 ± 5.6	83.9 ± 8.0

The application of this technique avoiding the acidification step is possible because the pH of fCW is close to the pKa of the lactic acid. This avoids the costs of reagents to reach lower pH and disposal of inorganic salts precipitated (e.g., gypsum).

7.3.4 Scale up

The controlled fermentation of around 20 L of CW was performed in a 30 L working volume reactor (**Figure 7.5**) in order to produce an amount of leaching mixture enough for carrying out all the tests on samples selected in Chapter 8.

Phase I was conducted at pH 6 controlled by dosing NaOH and was interrupted when a value of α = 5.2 was reached (value within the optimal range as shown in **Figure 7.4**). During phase II, thanks to the natural bacterial activity it has been possible to lower the pH to 3.85 and to achieve a high lactic acid concentration of approximately 35 g/L. The final concentration of non-reacted residual carbohydrates was very low, equal to 4.8 g/L. Concerning the fermentation performances, carbohydrate consumption of 90 %wt., and lactic acid production yields of 67 %wt., was obtained. Before being used as leaching solution, the fCW was subjected to a purification pretreatment with ethanol. The purified fermented cheese whey (pfCW), whose characteristics are reported in **Table 7.5**, was used for leaching tests as described in Chapter 8.

Table 7.5 – Purification performances and characterization of fCW and pfCW of the scale up assay.

Parameter	**Unit of measure**	**fCW**	**pfCW**	**Removal %wt.**
Volume	L	16.6	15.8	

HLa	g/L	34.7	35.3	3
Carbo	g/L	4.8	4.8	5
Prote	g/L	5.2	0.8	85
pH	-	3.85	-	

Figure 7.5 – Fermentation reactor (30 L working volume).

7.4 Conclusions

The present work demonstrated the possibility to turn CW into a valuable mixture rich in lactic acid by adopting an appropriately optimized two-steps dark fermentation. The selected parameter α, based on the ratio between the concentration of lactic acid and that of residual carbohydrate estimated, demonstrated to be a sensitive parameter to control the lactic fermentation. In order to obtain a fCW that respects the characteristics required by the subsequent leaching process (high concentrations of lactic acid, low concentrations of carbohydrates and pH 4) by limiting the use of chemicals as well as keeping low the costs for treatment, it was established that the best value of this parameter is between 4 and 6. Moreover, scale-up experiments demonstrated that a larger scale does not affect the efficiency of the process and allowed to prepare enough pfCW for the tests to be carried out in Chapter 8.

The use of ethanol for the purification treatment is particularly advantageous for its simplicity of application, the possibility of recycling the anti-solvent and the possible enhancement of the protein component of the treated cheese whey.

In order to make the proposed approach even more sustainable and circular, the residual product of the purification step (mainly composed of proteins and biomass) can be turned into a further resource by anaerobic digestion or by hydrothermal carbonization (HTC).

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Chapter 8

Metals recovery from WEEE

This chapter presents a systematic study of process parameters using a lactic acid synthetic solution (at a known concentration and pH) on samples of printed circuit boards and small IT devices. The use of the bio-derived leaching mixture obtained in Chapter 7 was evaluated on the same samples under the most promising operating conditions identified. Preliminary tests on the applicability of the leaching mixture to samples of non-processed electronic boards are also conducted to assess the degree of efficiency of the process on metals exposed.

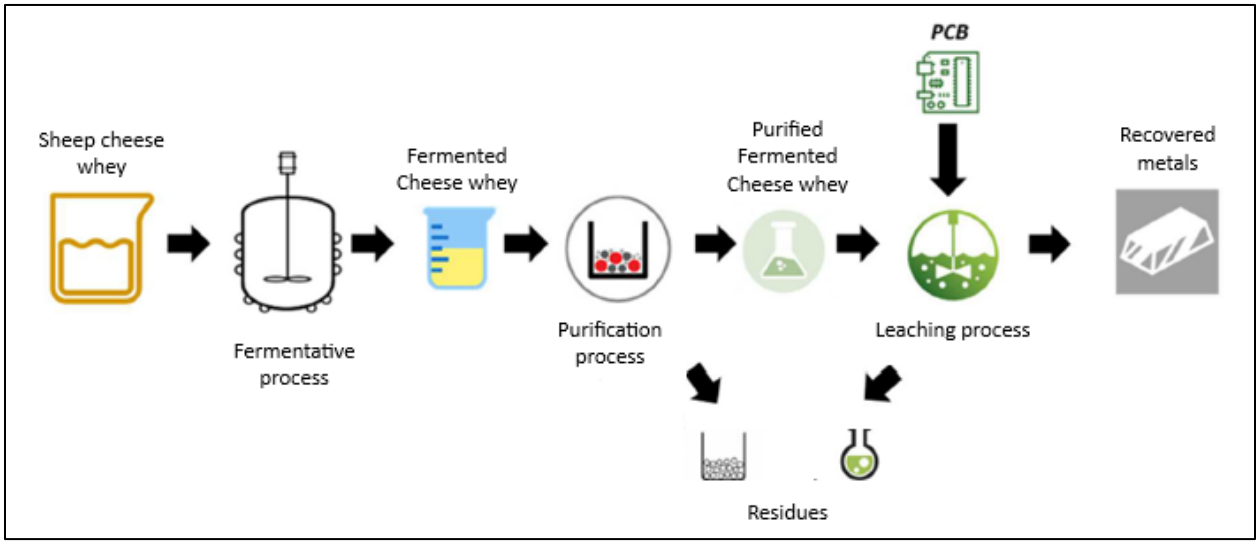


Figure 8.1 - Schematic representation of the proposed process.

8.1 Introduction

Nowadays, the wide spreading of worldwide regulations on sustainable waste management stimulates the recycling of materials and their use as secondary sources giving a new life to waste in order to preserve natural reserves. This reduces, in turn, the waste production and the contamination caused by the dispersion of even toxic materials in the environment. WEEE (Waste of Electrical and Electronic Equipment) is one of the most important waste streams to which attention is paid, with a worldwide production of 53.6 Mt in 2019 and a flow rate that grows every year by more than 5% (Forti et al., 2020). The heterogeneous composition of WEEE consists of a

wide variety of materials including ferrous and non-ferrous metals, plastics, glasses, and others (Kandra et al., 2021). Among WEEE, PCBs (printed circuit boards) receive special attention due to their relevant content of metals, both qualitatively and quantitatively. Indeed, they can contain up to 40 %wt. of metals, a large part of those considered "critical" at a global level because of the high industrial interest combined with the risks in the supply. Among them, base metals (and semi-metals) such as Fe, Pb, Zn, Al, Sn, In, Ga, Ta, Si, and precious and noble metals (NMs) like Cu, Ag, Pd, Au, are present. Thanks to their high economic value, the recycling of NMs makes a significant contribution to the economic sustainability and profitability of the entire recovery process, even if NMs can be contained in these scraps in very small quantities (i.e. 0.01-0.1 %wt. of Au, Ag and Pd) (Rigoldi et al., 2019). Despite the associated economical, industrial, and environmental interest, metals recovery from WEEE is still at an early stage. This is mainly due to the difficult application of conventional industrial processes inherited from ore mining (Worrell & Reuter, 2014). The most diffused industrial practices are based on pyrolysis and electrolysis, typically performed by well-established smelters. Although pyrometallurgical methods are very efficient, they are not fully selective and raise both economic and environmental issues. Hydrometallurgical methods are also applied, typically based on the use of strong acids such as nitric, able to leach base metals as well as copper, silver and palladium, and *aqua regia* (1:3 HNO_3:HCl) for gold, palladium and platinum. Strong acids, as well as other leaching agents widely industrially used for NMs leaching and recovery such as cyanides, ensure high efficiency through low energy demanding processes. On the other hand, these agents are harmful, often producing toxic gaseous by-products and large amounts of wastewater. Non-selective leaching methods require a subsequent metal separation phase applied on the leachate that typically involves selective extraction (liquid/liquid, liquid/solid) of the metal of interest from the multimetal leaching mixture. Finally, the recovery of the metal from the solution can be performed by chemical reduction (cementation) or electrochemistry (electrowinning).

With the view to develop new, more sustainable processes for metals recovery from secondary sources, large research efforts are devoted to exploit the potential of hydrometallurgy (which is more controllable and predictable, less energy-demanding and potentially causes fewer emissions than pyrometallurgy), by finding leaching agents characterized by a higher selectivity and a low environmental impact (Tuncuk et al., 2012; Cui & Zhang, 2008). In this context, wide attention has been devoted to the use of weak organic acids (OA) for base metals dissolution and recovery,

in spite of strong inorganic acids. Indeed, in line with the circular bioeconomy, OA can be produced from renewable sources, may work as selective lixiviants towards metals with low reduction potential and do not produce harmful gaseous emissions. In the modern biorefinery view, organic wastes and by-products may be seen as promising renewable sources for green energy and biochemicals production. These sources can contribute to lowering the use of fossil fuels and raw materials for chemicals production, thereby decreasing the global greenhouse emissions (Stephen & Periyasamy, 2018).

A recent hydrometallurgical approach based on the use of safe and recyclable reagents, among them OA, has been studied at University of Cagliari (Serpe et al., 2015). A three-step process, addressed to selectively dissolve and recover the various metals present in WEEE, was developed. In the first phase, a 3M aqueous citric acid solution under reflux temperature dissolves Sn, Zn, Pb, Ni and other base metals in 24-48h. The citric acid solution has shown to be selective towards low reduction potential metals ($E°<0$), which account for about 20% of the mass of the WEEE sample used. At the end of the first leaching phase, dissolved metals can be recovered from the solution in the form of citrate salts or by using suitable precipitating agents. During the second phase, the solid residue is treated with a buffer solution of ammonia and ammonium sulfate in a 2:1 molar ratio, under oxidizing conditions, to recover Cu and Ag by chemical or electrochemical reduction. In the last phase, gold is dissolved with an aqueous solution of KI and I_2 (molar ratio 5.3:1) in about 1h under room conditions and then recovered from the solution by electrodeposition. After metals recovery, the residual leaching solution may be easily treated for ammonia and I_2 reagents recycling (Rigoldi et al., 2019). The good results obtained by using citric acid as lixiviant for base metals stimulated further investigation on the use of other bioderived organic acids for metals dissolution from a variety of secondary sources. Preliminary results obtained by our research group in the framework of the selective cobalt dissolution from “hard metals” waste (WC-Co mixtures) distinguish OA in two main categories depending on the capability to provide the oxidizing species in the leaching process: OA like citric acid, where the metal oxidation is carried out by H^+ (Class 1 OA) and other OA, typically characterized by higher pK_a but good complexing properties of the deprotonated ligand, where oxidation is played by an external oxidant like O_2 (Class 2 OA) (Amadou, 2020). As an example of a Class 2 bioderived OA, aqueous lactic acid solutions demonstrated to work efficiently on cobalt dissolution even under very mild conditions (0.5M, from room to refluxing temperature, $2<pH<4$, aerobic conditions). It is worthy to note that diluted

lactic acids solutions are easy-to-handle and eco-friendly. Furthermore, over 90% of the commercial lactic acid is produced through fermentation processes. The main feedstock are hexose sugars and agro-industrial by-products like corn syrups, molasses, beet extracts, whey, and other kinds of starches (Dusselier et al., 2013).

This chapter evaluates the efficiency and selectivity of the bioderived leaching mixture, produced via a new patented controlled cheese whey fermentation process, towards metal-containing electronic waste. Specifically, in the present study, we checked the influence of operating temperature, lixiviant concentration, and liquid-to-solid ratio on the efficiency and selectivity of base and critical metals dissolution from different WEEE samples.

8.2 Materials and methods

8.2.1 WEEE samples

The test specimens used for this experimentation are:

- Samples pre-treated mechanically in the following form and composition:

Sample 1 (**Table 8.1**, **Figure 8.2**). A fine (average particle size: 0.4 mm) non-ferrous metal fraction of comminuted PCBs and small electronic equipment (e.g., mobile phones, notebooks, etc.), as provided by a company that treats WEEE, by applying a mechanical shredding and separation process. The metal content of the shredded WEEE sample has been determined through ICP-AES analysis after treatment in a Milestone Ethos 1 Microwave digester (Serpe et al., 2015).

Table 8.1 – Average metal content of **Sample 1.**

Metal	**Composition (%wt.)**
Cu	79
Sn	10
Pb	7
Zn	2
Ni	0.5
Ag	0.06
Au	0.01
Others (Al, Cr, Mn, Fe, Co)	1.43

Figure 8.2 – Sample 1, non-ferrous metal fraction of WEEE.

Sample 2 (**Table 8.2**, **Figure 8.3**). Triturated RAM (gold fingers) obtained by a milling treatment, containing the ferrous and non-ferrous metal fraction, and composite vitreous-plastic material. A quantitative characterization of Sample 2 is reported in **Table 8.2**, obtained as the average of the analysis of individual samples treated in the course of this work, summing the metal content of all fractions obtained from the separation and recovery phases (leaching solutions and residual solids). This characterization was then used to calculate the leaching yields of the specific test.

Table 8.2 also compares the composition of the sample obtained as described above with a previous systematic characterization as reported in (Serpe et al., 2019).

Table 8.2 – Average metal content of **Sample 2.**

Metal	**[1]Composition (%wt.)**	**[2]Composition (%wt.)**
Cu	15 (±1)	15 (±1)
Sn	1.9 (±0.3)	3.9 (±1)
Pb	0.8 (±0.5)	0.6 (±0.1)
Ni	4.2 (±0.7)	2.9 (±0.4)
Al	1.4 (±0.2)	1.4 (±0.5)
Fe	7 (±3)	4 (±0.4)
Ag	0.04 (±0.01)	-

[1]Composition from (Serpe et al., 2019)
[2]Composition obtained from this work

Figure 8.3 – Sample 2, shredded RAM boards.

- Non-pre-processed samples:

Sample 3 (**Figure 8.4**). RAM boards without any kind of preliminary treatment.

Figure 8.4 – RAM boards used as **Sample 3.**

8.2.2 Leaching solutions

Leaching tests were conducted, preliminarily, using lactic acid solutions at 0.5-3 M concentration obtained by diluting the commercial reagent (FU-BP E 270, 88-90.5%) with deionized water and adapting the pH value to 4 by the addition of a 2.5 M NaOH solution (pellets, Sigma-Aldrich). Subsequently, tests were carried out using purified fermented sheep cheese whey (pfCW) obtained as described in Chapter 7 in two different runs for the two kinds of samples.

Table 8.3 summarizes the characterization of the pfCW used for treating Sample 1 (pfCW1) and Sample 2 (pfCW2).

Table 8.3 – Characterization of $pfCW_1$ and $pfCW_2$ used for treating Sample 1 and Sample 2, respectively.

Parameter	**Unit of measure**	**$pfCW_1$**	**$pfCW_2$**
HLa	mol/L	0.5	0.4
Carbo	g/L	n.a.	4,8
Prote	g/L	1.9	0,8
pH	-	4	3.85

n.a. not available

8.2.3 Leaching tests

Leaching tests were performed using lactic acid solutions varying different operational parameters (reaction time, temperature, leaching concentration, oxidizing conditions, and liquid-to-solid ratio - L/S) depending on the sample treated. Then, the best conditions have been applied using pfCW. All the experiments on Sample 1 and 2 were carried out in triples, both with HLa and with pfCW, to the variation of the experimental conditions as summarized in **Table 8.4**.

8.2.3.1 Tests using synthetic lactic acid solutions

Sample 1

The experiments were carried out using 0.5-1 g of Sample 1 (L/S = 200 mL/g) at different leaching concentrations (0.5-3M) and pH 4, reaction time (8-24h), temperatures (room: 20-25 °C, and reflux: 100 °C), and oxidizing conditions (forced aeration, addition of H_2O_2). At fixed times, an aliquot (2 mL) of the solution was sampled and centrifuged at 10,000 rpm for 10 minutes (Dupont Instruments Sorvall SS-3 Automatic Centrifuge). Hence, the clean solution was analyzed for dissolved metals determination. At the end of each reaction, the solid residue was separated from the solution by filtration under vacuum through a glass microfiber filter (Whatman, 0.8 μm).

Sample 2

Leaching tests were conducted with different set-up depending on the process temperature.
Tests at room temperature on sample 2 were carried out by reacting an appropriate quantity of material (0.5-4g) with the synthetic leaching solution at 0.5M and pH 4, in a 250 mL open flask under constant magnetic stirring.
The tests at higher temperature were conducted by reacting Sample 2 and the leaching solution inside a closed Teflon vessel for 6 hours under the action of microwaves. The runs were carried out by 6 cycles at 100°C for 10 + 50 minutes under a microwave power up to 500 W and followed by 20 minutes of cooling.

Preliminary test on sample 3

RAM boards (Sample 3) were treated whole, without undergoing pre-treatments. In the described preliminary tests, RAM boards were reacted with a synthetic HLa 0,5 M solution at room temperature. The leaching was performed into capped plastic bottles kept under continue rotative mixing (Rotax 6.8, Velp Scientifica) at 8 r.p.m.
Two main experiments were carried out, both involving two boards merely fragmented and immersed in 130 mL of leaching solution:

- **Test 1**: total duration 21 days, with an intermediate (after 9 days) and final sampling of the solution;
- **Test 2 (in double)**: total duration 13 days, with daily opening of the vessel to restore the oxygen content of the mixture and contextual intermediate sampling (after 2, 3, 6, 8, 10 and 13 days) of the solution for analysis.

These experiments were addressed to assess the type and amount (mg) of leached metals under the mild reported conditions (by ICP-OES measurements on the leachate), as well as the look of the boards, under metallographic optical microscopy, before and after leaching.
At the end of the described treatments, the treated samples were furtherly leached for 7 days with HLa in an open beaker under room conditions and mechanical stirring, in the presence of H_2O_2 (35%). This leaching was addressed to dissolve higher reduction potential metals such as Ni and Cu. Specifically, 1 mL of H_2O_2 was added once a day for 2 days. Then, the solution was filtered of and renewed. To this new solution a total of 1 mL of H_2O_2 was added one shot and left reacting for 1 day; 1 mL was added dropwise during the 2nd day, and finally 1 mL of H_2O_2 during the last

day. Intermediate samples were collected at various times of the test (1 sample after 2, 4 and 7 days) for analysis.

8.2.3.2 Tests using pfCW

A multistep leaching experiment was carried out on 1 g of Sample 1 using $pfCW_1$ with the characterization reported in **Table 8.3**. The following conditions were adopted for each step:

1) r.T., 200 mL $pfCW_1$ (L/S = 200 mL/g), 8 h → Solution 1 (S1)

2) Δ, 200 mL $pfCW_1$, 8 h → Solution 2 (S2)

3) r.T., 200 mL $pfCW_1$, H_2O_2 (1,5 mL), 48 h → Solution 3 (S3)

4) r.T., 50 mL $pfCW_1$, H_2O_2 (0,5 mL), 8 h → Solution 4 (S4)

Leaching tests with $pfCW_2$ on Sample 2 were performed at 0.4 M concentration and pH 3.85 with L/S equal to 50 mL/g, both at room temperature and 100 ºC. As described for tests using a synthetic solution, the tests at room temperature were carried out in open flask under constant magnetic stirring and tests at higher temperature were conducted inside a closed Teflon vessel for 6 hours under the action of microwaves. The runs carried out by 6 cycles at 100°C for 10 + 50 minutes under a microwave power up to 500 W and followed by 20 minutes of cooling.

The leaching solution was renewed three times.

8.2.4 Samples characterization

Liquid and solid fractions were analyzed by ICP-AES (Perkin Elmer Optima DV 7000) towards an appropriate calibration plot after acid digestion to provide the metal composition of the sample under examination. This composition was then used as a reference to calculate the percentage leaching yields corresponding to each sample.

Digestion conditions: a) final and intermediate sample aliquots were digested by 0.5 mL HNO_3 65%, 0.5 mL H_2SO_4 98% at room temperature for 24 hours; b) solid residues were digested under microwaves with an acidic mixture (5 ml HNO_3 + 3 ml H_2O or 3 ml HNO_3 + 3 ml HF (40%) + 3 ml H_2O) and the following procedure: two steps of 10 and 20 minutes, 1000 W and 200 °C (220 °C for tests with HF).

8.2.5 Summary of all tests

Table 8.4 - Summary of the conditions used for tests with the synthetic solution and with pfCW at pH 4.

Leaching solution	Sample	Conc	L/S (mL/g)	T (°C)	Time (h)
Synthetic	S1	0.5	200	r.T.*	24
Synthetic	S1	0.5,1,3	200	r.T.*	8
Synthetic	S1	0.5	200	r.T.*	24
Synthetic	S2	0.5	200,100, 50, 25	r.T.*	48
Synthetic	S2	0.5	50	100**	12
Synthetic	S3	0.5	-	r.T.*	312
Synthetic***	S3	0.5	-	r.T.*	168
$pfCW_1$	S1	0.5	200	r.T.	88
$pfCW_2$	S2	0.5	50	r.T.*	72
$pfCW_2$	S2	0.5	50	100**	24

*r.T. = room temperature (20-25 °C);
**Temperature of 100 °C under microwave (MW);
***Addition of 5 mL H_2O_2.

8.3 Results and discussion

Leaching tests were performed first using a synthetic lactic acid solution on the test specimens described in the 8.2.1 section and varying several operating parameters. The progress of the reaction was monitored over time, carrying out periodic sampling in order to evaluate the leaching profile of the different metals and the selectivity of the process on the real samples. Based on the results obtained, the conditions regarded as optimal were used to apply leaching tests using the pfCW.

8.3.1 Synthetic lactic acid solution

8.3.1.1 Sample 1

Reaction time and temperature

Sample 1 (0.5 g) was reacted with 100 mL of a 0.5M lactic acid solution at pH 4, under magnetic bar stirring for 24h at room (r.T.) and reflux (Δ) temperature. **Figure 8.5** and **Figure 8.6** report the metals concentration (ppm) of the leachate over time and the efficiency of the leaching process in 24 h (dissolution yield %wt.).

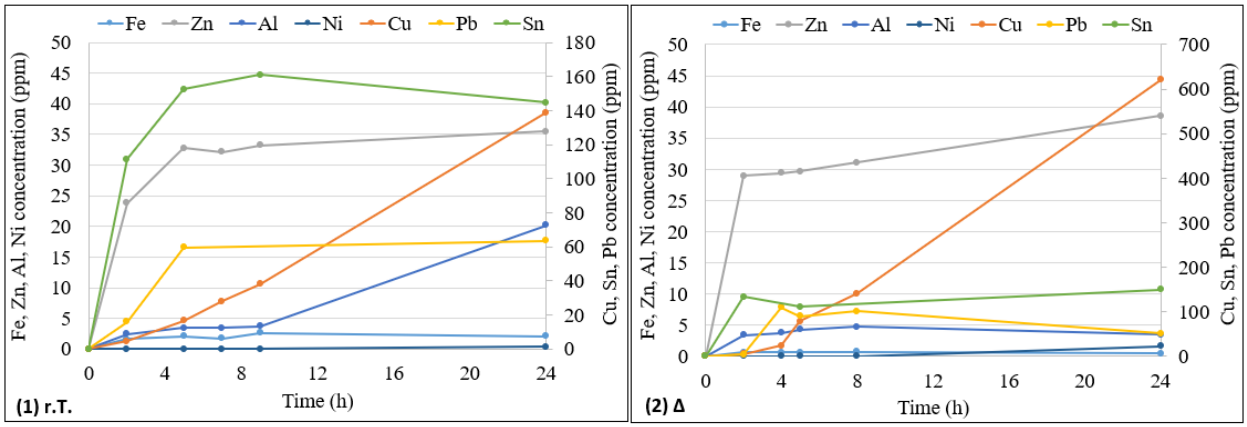


Figure 8.5 - 24 h metal leaching profiles with 0.5M lactic acid solution at pH 4 on Sample 1 (L/S = 200 mL/g) at (1) room and (2) reflux temperature.

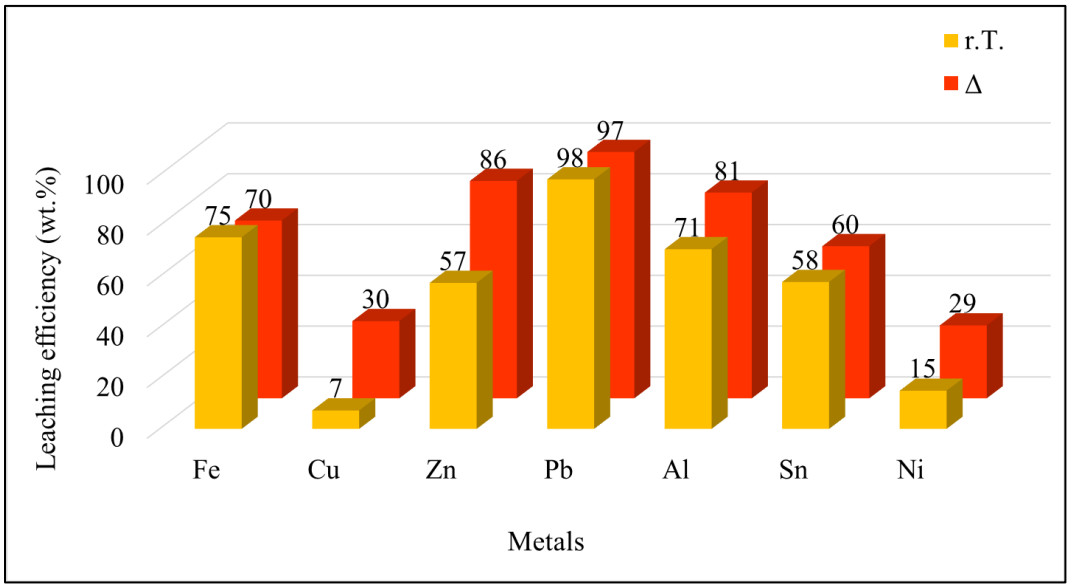


Figure 8.6 - Leaching efficiency (wt.%) for each metal after 24h of reaction of Sample 1 with 0.5M lactic acid solution (L/S = 200 mL/g) at pH 4 at room and reflux temperature.

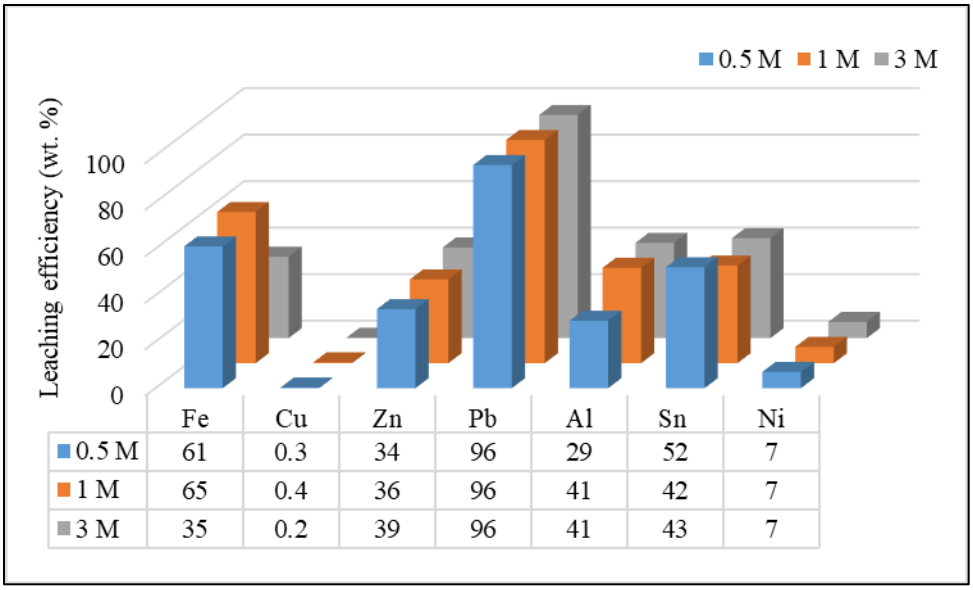
As shown, in both cases base metals promptly react with the leaching solution, reaching the highest leaching yields within 8 h. As an exception, Ni is less prone to react and its concentration in solution increases slowly over time. Pb is the metal that dissolves with the highest efficiency: just within 4 h it dissolves almost completely (98 and 97%wt. at r.T. and Δ, respectively). High and

almost comparable yields were found for Fe (70 and 75%wt.). Sn dissolves quickly (within 4 h) but the leaching yield is not quantitative, reaching around 60%wt. at both temperature conditions. This evidence suggests potential superficial passivation phenomena and/or precipitation of poorly soluble products. In terms of base metal leaching, the increasing temperature seems to affect significantly mainly Zn (57 and vs. 86%wt.), Al (71 and vs. 81%wt.) and Ni (15 and vs. 29%wt.) dissolution. Negligible effects were found for the other base metals. Considering NMs, only copper can be partially leached by the solution. Specifically, at r.T. the reaction seems very selective towards base metals, leaving NMs almost unreacted over time. The increase in the temperature allows copper to slightly dissolve reaching a yield of 30%wt. in 24 h, with significant growth after 8 h leaching.

In summary, comparing the different operating temperatures, for Ni, Al and Zn a significant increase of the leaching efficiency at reflux, is observed. The heating does not appear to produce any evident efficiency gain for the other base metals. Furthermore, the refluxing solution seems to be able, in longer times, to dissolve copper as well. On the one hand, this opens the way for the possible use of lactic acid solutions for copper leaching and recovery. On the other hand, the refluxing lactic acid solution demonstrated lower selectivity with respect to the r.T. one.

Concentration

In order to evaluate the effect of the leaching agent concentration on the dissolution efficiency, experiments on test specimen 1 (1g) were conducted at 0.5, 1, and 3 M at room temperature. 8 h leaching tests were carried out because longer times did not seem to significantly improve the yields while lower the selectivity over base metals.



	Fe	Cu	Zn	Pb	Al	Sn	Ni
0.5 M	61	0.3	34	96	29	52	7
1 M	65	0.4	36	96	41	42	7
3 M	35	0.2	39	96	41	43	7

Figure 8.7 – Leaching efficiency (wt.%) for each metal after 24h of reaction of Sample 1 with 0.5M lactic acid solution (L/S = 200 mL/g) at pH 4 at room temperature and different concentrations.

As shown in **Figure 8.7**, the leaching efficiency of the metals does not seem to benefit significantly from an increase of lixiviant concentration.

Oxidation conditions

With the view to improve Ni dissolution efficiency and promote the dissolution of NMs like Cu, experiments addressed to highlight the role of the oxidizing species in the leaching efficiency, were performed.

Specifically, forced aeration and H_2O_2 addition (2 mL, 35%wt.) were tested on a mixture of 1g of Sample 1 and 200 mL of lactic acid solution (0.5M) under r.T. and stirring for 24h. **Figure 8.8** summarized the obtained results with respect to Ni and Cu leaching efficiency.

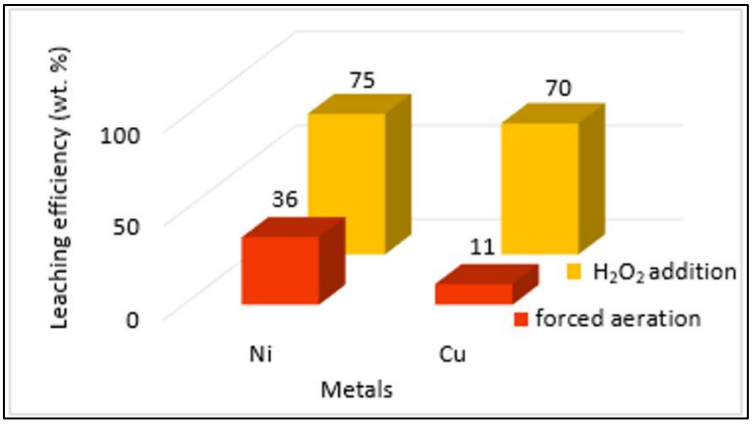


Figure 8.8 - Leaching efficiency (wt.%) for Ni and Cu after 24h of reaction of Sample 1 with 0.5M lactic acid solution (L/S = 200 mL/g) at pH 4 under forced aeration and H_2O_2 addition.

Comparing the results obtained under the new oxidizing conditions to the ones in open flask (able to ensure the amount of O_2 required by the reaction), Ni and Cu dissolution efficiency increases (from 15 to 36%wt. and from 7 to 11%wt., respectively under forced aeration and to 75 and 70%wt., respectively in the presence of H_2O_2).

The obtained results open the way to a two-step process where lactic acid solutions may work as selective leaching agents for base metals (1st step) and for copper and nickel (2nd step - H_2O_2 addition) recovery in appropriate operative conditions: 1) room conditions in an open flask; 2) room conditions and H_2O_2 addition, respectively.

8.3.1.2 Sample 2

Liquid-to-solid (L/S) ratio

Leaching tests with synthetic HLa on Sample 2 were conducted for 24 h at room temperature and adopting a L/S of 200 mL/g, with the aim of studying the behavior of this composite sample, containing both the metal and the plastic-glass fraction, in the conditions already used on Sample 1 (deprived of the plastic-glass fraction, magnetic metals and aluminum). During the test described above, it was observed that the lower specific weight of Sample 2 compared to Sample 1 (heavy because it consists entirely of metals) make the stirring easier and more efficient during the test with more reproducible experimental results.

With the aim to optimize the experimental conditions to meet plant and economic needs, Sample 2 has been treated with HLa 0,5 M solutions at pH 4 under room conditions by decreasing L/S between the leaching solution and the treated material. The L/S ratios investigated were, in

addition to 200 previously considered, 100, 50 and 25. In **Figure 8.9** leaching profiles are compared under the different test conditions.

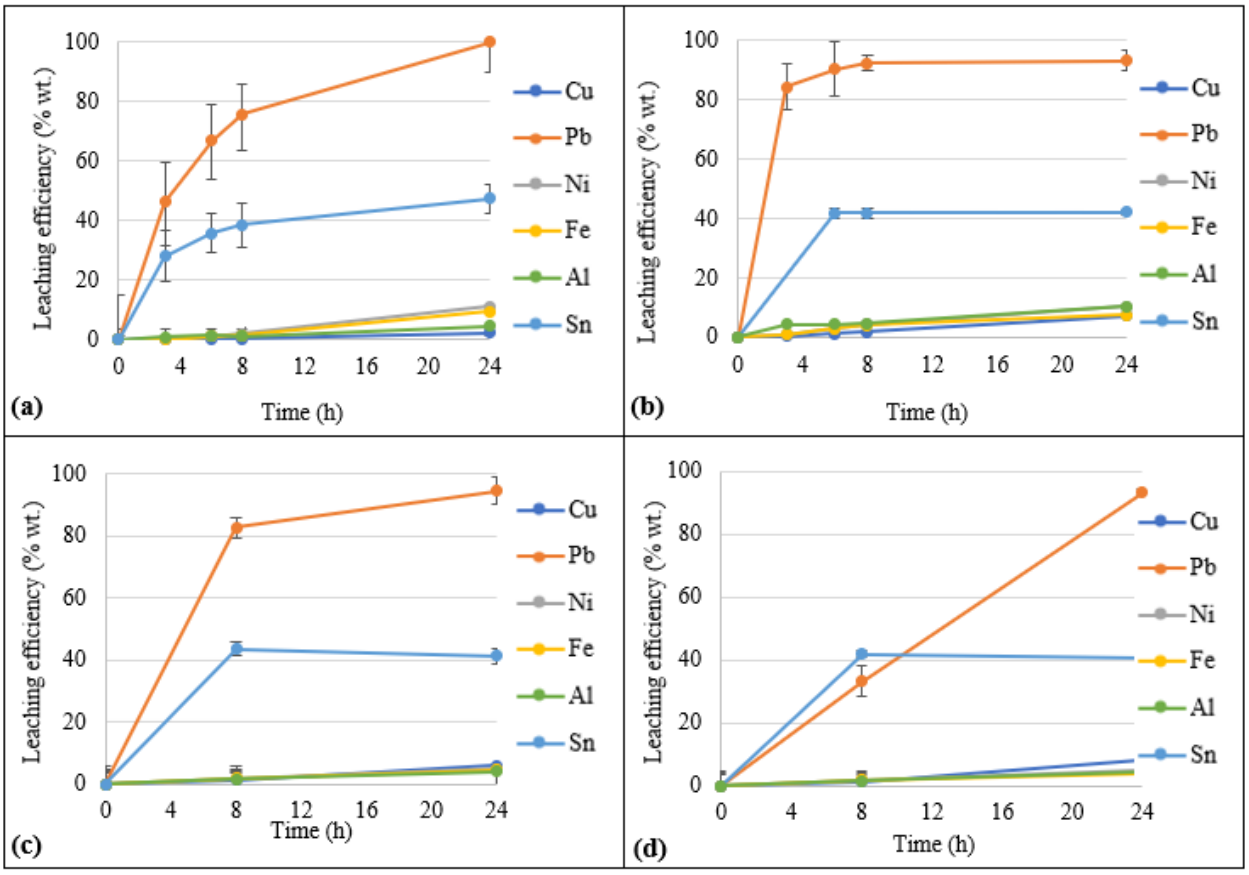


Figure 8.9 - Metal leaching profiles at L/S 200 (a), 100 (b), 50 (c) and 25 (d) with synthetic lactic acid solution. L/S expressed as mL/g.

From **Figure 8.9**, it is evident that leaching with lactic acid solution at pH 4 is effective in the selective dissolution of base metals, with efficiency against Pb, as seen for Sample 1. The leaching trend of Sn is also reproducible: the curve has a strong slope in the first fraction of the process followed by a lightly increasing trend up to a value of 40-50%wt. of dissolution over 24 hours. The reactivity of Cu and Al is poor, as well as that of Ni and Fe, thus favoring the selectivity of the process.

To maximize the efficiency of the process by limiting the volume of solution used (limiting the volume required for reactors), L/S 50 mL/g was selected as the one with the best cost/benefit ratio. By repeating the tests for a further 24 hours and renewing the solution, a slight increasing trend was observed for Cu, Fe and Ni, while the Sn passivation phenomena seem confirmed because no further dissolution is observed (**Appendix A2 - Figure A1.8.**). For comparison, tests adopting L/S

of 200 and 100 mL/g were also conducted on Sample 1. The results confirm an almost complete dissolution of Pb and a partial or limited dissolution for Cu, Al and Sn (**Appendix A2 - Figure A.8.2**).

Temperature

Leaching tests varying temperatures (room and 100 ºC) were conducted on Sample 2, using the synthetic HLa solution and L/S 50 mL/g, to verify the effect of temperature on the efficiency and selectivity of the process.

The tests at room temperature were conducted for 24 hours in an open flask on a plate with magnetic stirring. The tests at 100 ºC were carried out in a capped vessel in a microwave oven, for 6 hours. In both cases, at the end of the first leaching phase, the solution was renewed, and the process repeated under the same conditions.

In **Figure 8.10**, the results obtained at room temperature and at 100 ºC were compared.

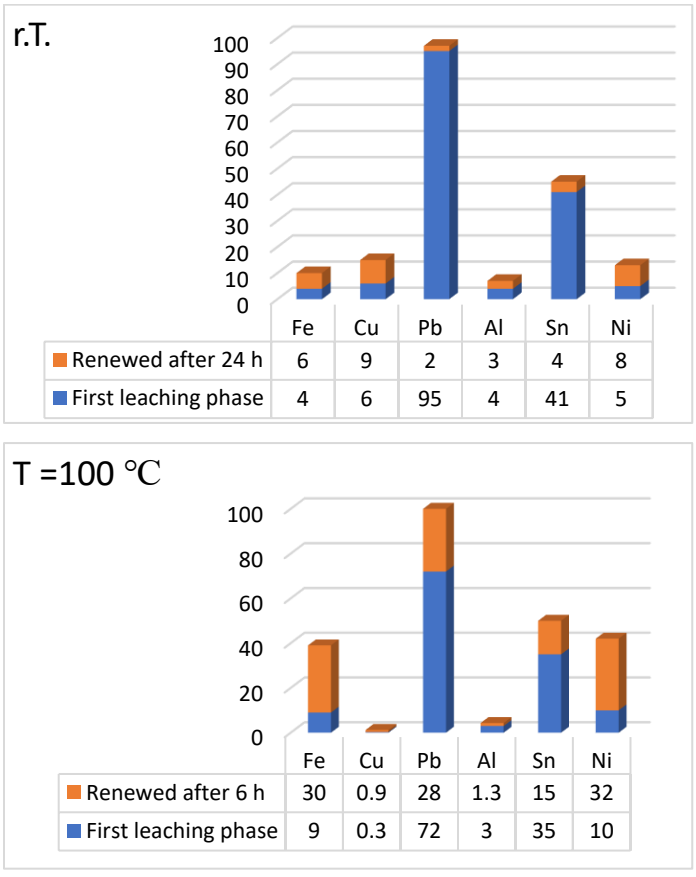


Figure 8.10 – Leaching results using synthetic lactic acid solution (0.5 M, pH 4, L/S = 50 mL/g) at room temperature (r.T.) and at 100 ºC renewing the solution after 24 and 6 hours, respectively.

As can be seen from the graphs, the higher leaching temperature with microwaves allows to maximize the dissolution efficiency of base metals such as Pb, Sn, Fe and Ni, even increasing the selectivity (Cu and Al remain essentially unreacted), although in the first leaching fraction the results at two different temperatures are comparable.

8.3.1.3 Sample 3

Preliminary leaching tests were carried out on whole RAM boards, as detailed in the Materials and Methods section par. 8.2.3.1, in order to assess the action of the lixiviant on the surface metals. The qualitative Test 1 carried out on Sample 3 at room temperature for a total of 21 days, showed that the reaction into closed bottle led to an evident leaching of the exposed base metals as well as the detachment of unreacted metals and components (**Figure 8.11**). Among the unreacted materials, thin layers of gold, MLCC (Multilayer Ceramic Capacitors) and chips, detached following the dissolution of the underlying metal layers and/or the solder alloy, were found. Specifically, the ICP-OES analysis of the leachate pointed out a gradual but significant dissolution of Sn, which reached values of 220 mg Sn leached within 13 days of treatment (on 2 RAM boards it represents around the 20wt.% of the whole Sn expected).

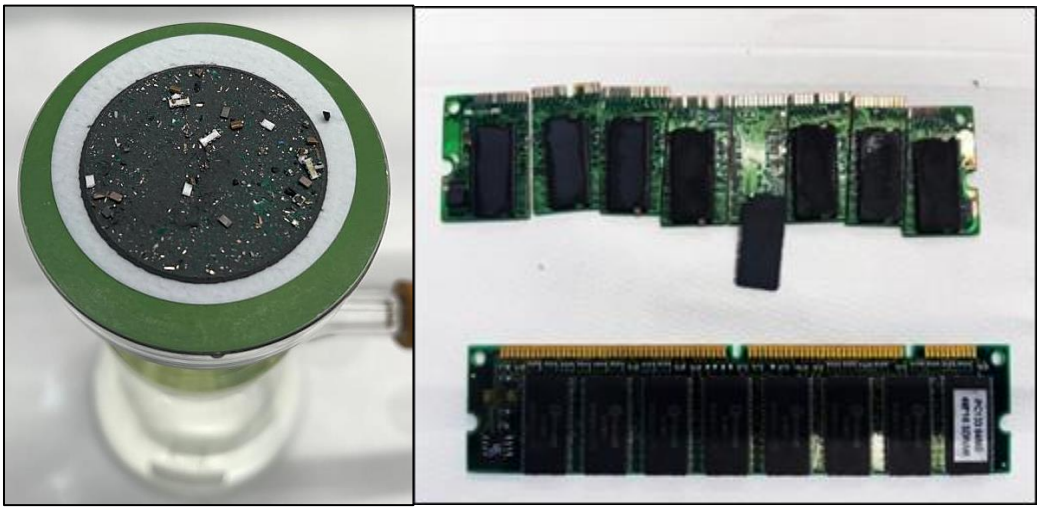


Figure 8.11 – Residual solid recovered filtering the final solution (left); detached chips and appearance of the board before and after treatment (right).

A further, more quantitative experiment was hence carried out in order to draw the metal leaching profile in the time. In this case, the oxygen content was restored in the leaching environment by opening periodically the vessel with the view to favor the oxidation reaction and to increase the leaching efficiency. As shown in **Figure 8.12** Similarly to the previously described experiment, Sn

was found as the predominant metal in the leaching solution, maybe because among the base metals exposed it was the most abundant being the main constituent of the solder alloy. On the other side, the effect of the oxygen restoration in the vessel seemed to significantly improve the Sn dissolution yield obtaining 650 mg Sn dissolved in 13 days which represents around the 50wt.% of the whole Sn expected.

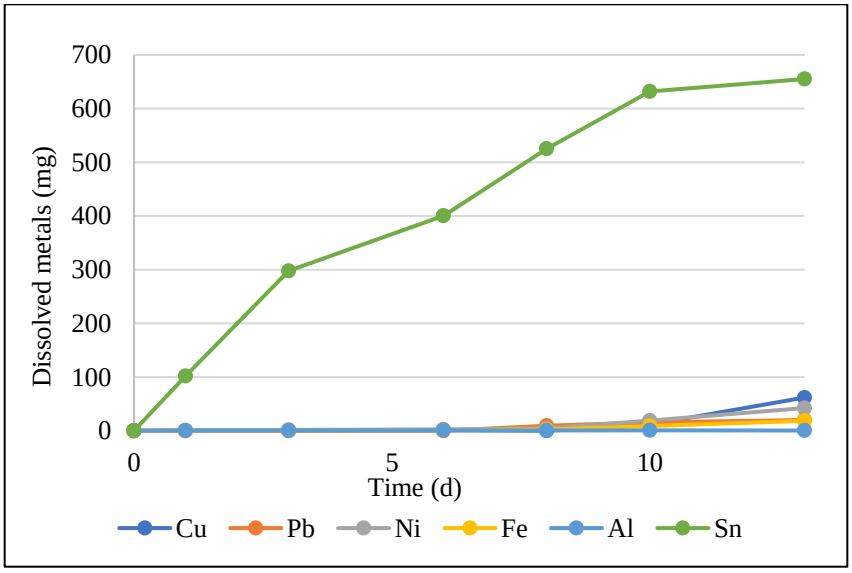


Figure 8.12 – Metals leaching profile during Test 2 ([HLa] = 0.5 M, pH 4) carried out on Sample 3.

Besides Sn also other base metals dissolved but detectable amounts were found just after 9-10 days of treatment.

In order to leach higher reduction potential exposed metals, aliquots of H_2O_2 solution were added in a subsequent step. As expected, the presence of H_2O_2 in the leaching environment promotes base metals supplementary dissolution. Moreover, the reactivity of metals such as Cu (which is characterized by a positive reduction potential) was enhanced by the concomitant presence of a complexing agent (the lactate ion) and a strong oxidant agent. **Figure 8.13** shows the RAM boards during the treatment with H_2O_2 and **Figure 8.14** shows the leaching profile obtained.

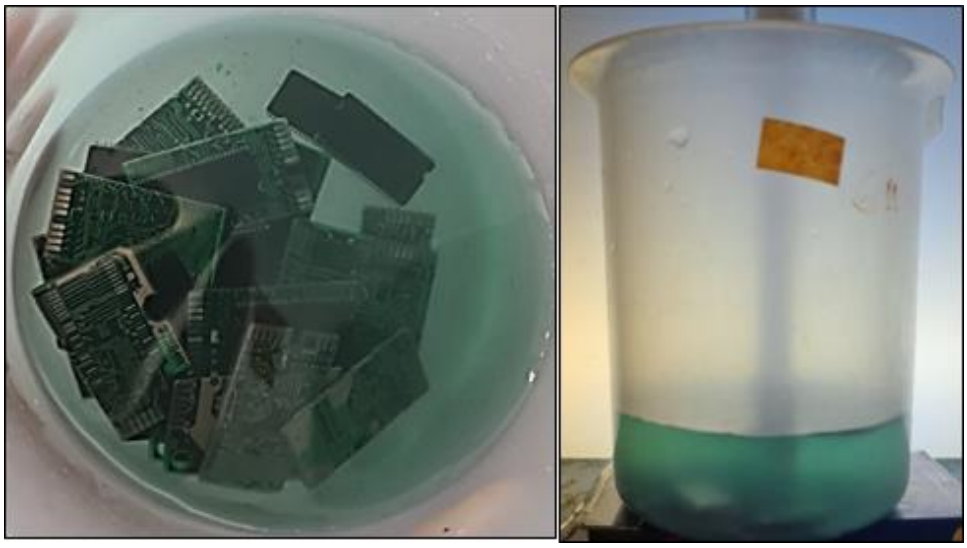


Figure 8.13 – Sample 3 treatment in an open beaker under room temperature and mechanical stirring, in the presence of H_2O_2.

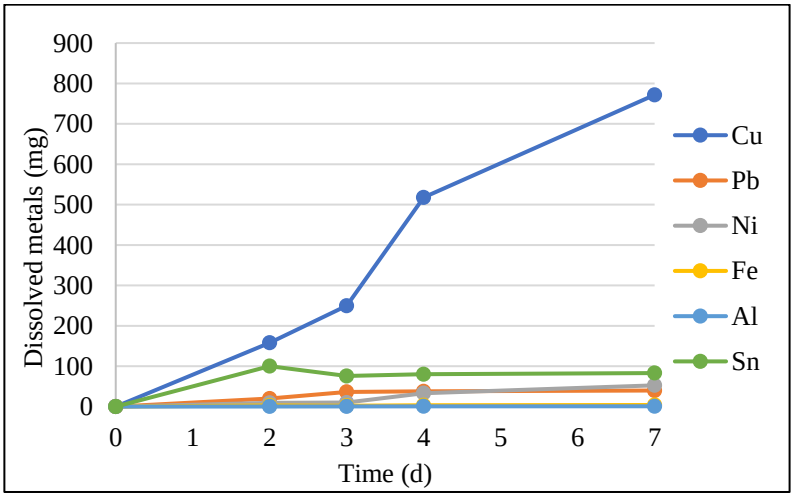


Figure 8.14 - Metals leaching profile with HLa 0.5 M, pH 4 and with H_2O_2 conducted on Sample 3.

Before and after this last treatment, optical micrographs of portions of the boards were collected and reported in **Figure 8.15** which clearly highlights the gold removal during the treatment.

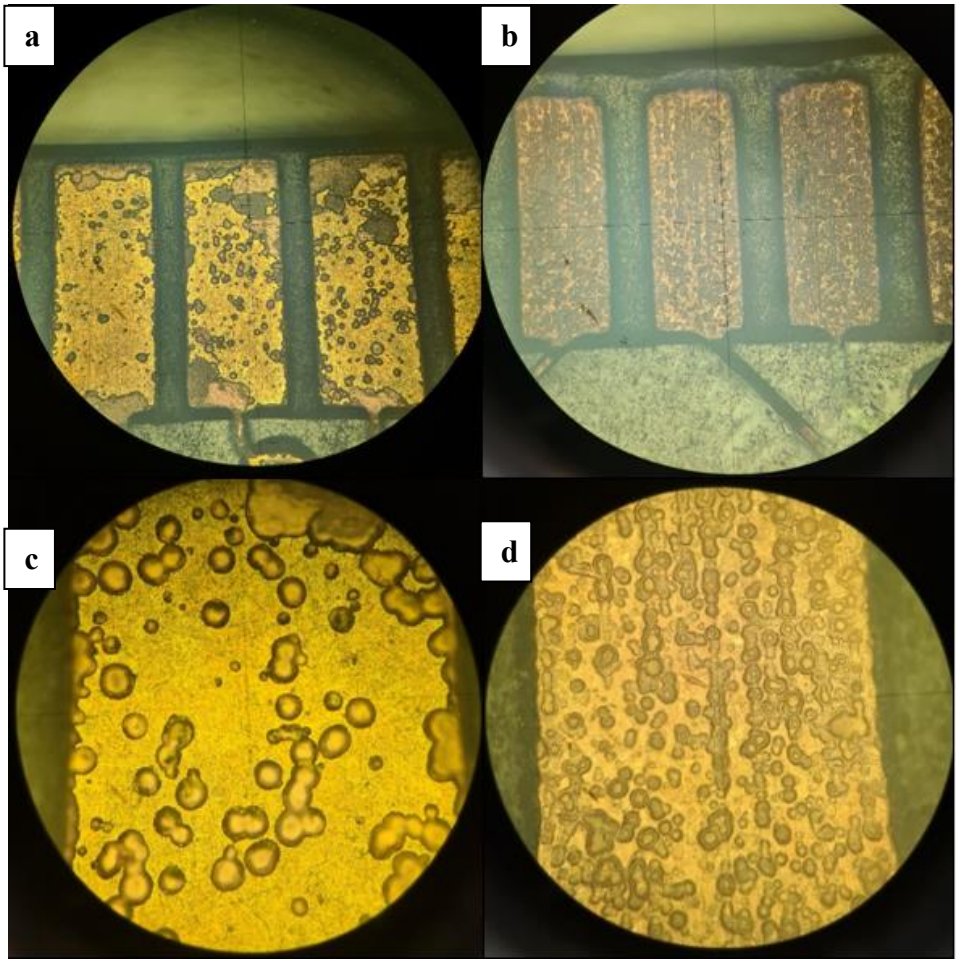


Figure 8.15 - Optical metallographic microscope analysis of the RAM boards before and after the treatment with H_2O_2: before, 5x(a) and 10x (c), and after 5x(b) and 10x (d).

These preliminary experiments suggest a good leaching capability of lactic acid solutions on the boards. Further experiments are needed to better investigate the efficiency and selectivity of the approach even in comparison with other leaching methods proposed in the literature. It is worth noting that the studied approach seems valued in order to obtain a non-manual removal of the electronic components from the boards, as well as a concentration of solid noble metals, specifically gold, which remain unreacted on the bottom of the vessel.

8.3.2 Leaching tests using pfCW

As summarized in **Table 8.4**, leaching tests using the pfCW, under the conditions used with the synthetic HLa solution, have been carried out on Sample 1 and Sample 2 powders which differ significantly by composition.

8.3.2.1 Sample 1

As reported in paragraph 8.2.1, Sample 1 is the non-ferrous metal fraction of comminuted PCBs and small electronic equipment, subjected to separative processes of removal of plastic-glass materials, ferrous metals, and aluminum.

In order to evaluate the behavior of different metals, comparing pfCW with respect to synthetic HLa solution, a multistep experiment was carried out on 1 g of Sample 1 using $pfCW_1$ ([HLa] = 0.5M) as detailed below:

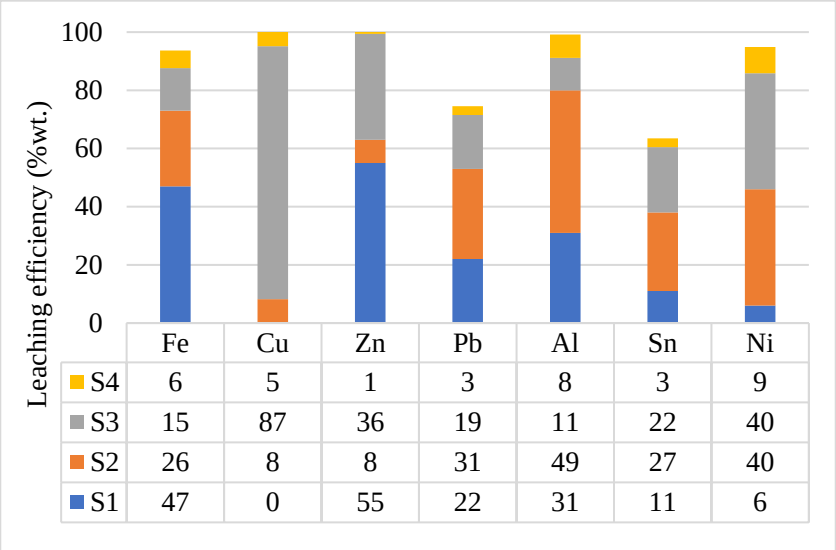
1) r.T., 200 mL $pfCW_1$ (L/S = 200 mL/g), 8 h → Solution 1 (S1)

2) Δ, 200 mL $pfCW_1$, 8 h → Solution 2 (S2)

3) r.T., 200 mL $pfCW_1$, H_2O_2 (1,5 mL), 48 h → Solution 3 (S3)

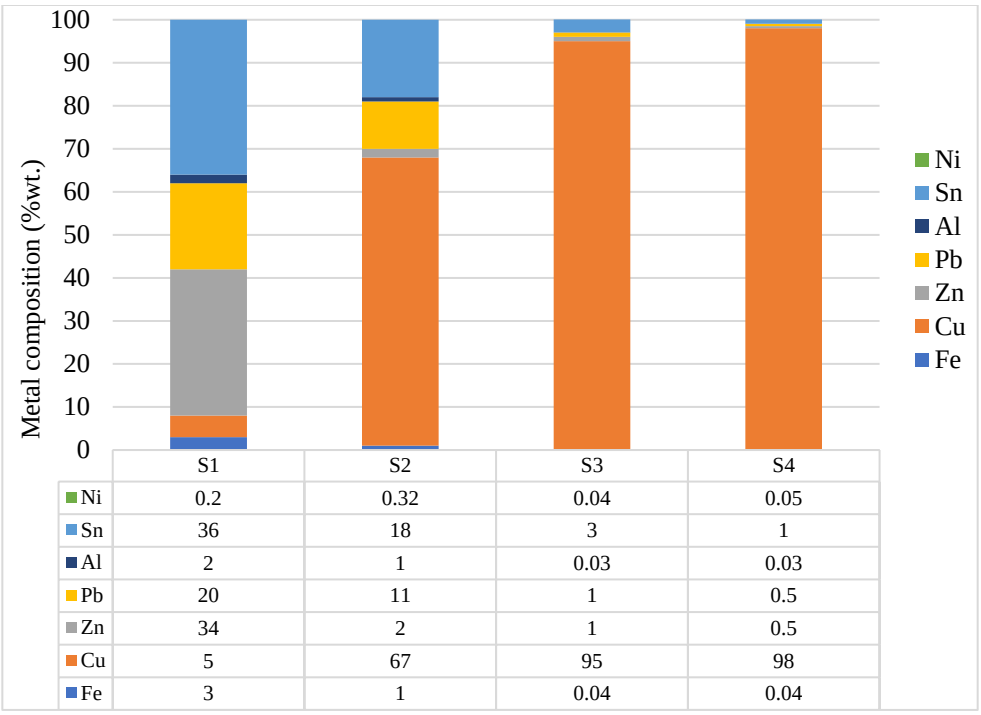
4) r.T., 50 mL $pfCW_1$, H_2O_2 (0,5 mL), 8 h → Solution 4 (S4)

Figure 8.16 and **Figure 8.17** show the leached metal (%wt.) between leaching solutions of the multistep test, and the metal composition (%wt.) of each solution, respectively.



	Fe	Cu	Zn	Pb	Al	Sn	Ni
S4	6	5	1	3	8	3	9
S3	15	87	36	19	11	22	40
S2	26	8	8	31	49	27	40
S1	47	0	55	22	31	11	6

Figure 8.16 - Leaching efficiency of the multistep test using pfCW1 (HLa = [0.5 M]) on Sample 1. Conditions: 1) r.T., 200 mL $pfCW_1$ (L/S = 200 mL/g), 8 h; 2) Δ, 200 mL $pfCW_1$, 8 h; 3) r.T., 200 mL $pfCW_1$, H_2O_2 (1,5 mL), 48 h; 4) r.T., 50 mL $pfCW_1$, H_2O_2 (0,5 mL), 8 h.



	S1	S2	S3	S4
Ni	0.2	0.32	0.04	0.05
Sn	36	18	3	1
Al	2	1	0.03	0.03
Pb	20	11	1	0.5
Zn	34	2	1	0.5
Cu	5	67	95	98
Fe	3	1	0.04	0.04

Figure 8.17 – Metal composition of each solution (S1, S2, S3, S4) of the multistep test using pfCW1 (HLa = [0.5 M]) on Sample 1. Conditions: 1) r.T., 200 mL $pfCW_1$ (L/S = 200 mL/g), 8 h; 2) Δ, 200 mL $pfCW_1$, 8 h; 3) r.T., 200 mL $pfCW_1$, H_2O_2 (1,5 mL), 48 h; 4) r.T., 50 mL $pfCW_1$, H_2O_2 (0,5 mL), 8 h.

As shown, pfCW alone (Step 1 and 2) confirms the effectiveness and selectivity towards the base metals while the presence of H_2O_2 allows to reach good dissolution yields also for Cu and Ni. **Table 8.5** provides a comparison of the leaching efficiencies of different metals by treating 1 g Sample 1 with the synthetic HLa solution and $pfCW_1$ after 8 h at room temperature and further 8 hours at reflux temperature.

Table 8.5 – Sum of the leaching efficiencies of Sample 1 treated for 8 h at r.T. and 8 hours at reflux temperature, using synthetic HLa solution and $pfCW_1$ with the same [Lat] = 0.5 M, L/S = 200.

	Leaching efficiency (%wt.)	
	Synthetic HLa solution	$pfCW_1$
Fe	86	74
Cu	5	7
Zn	38	60
Pb	97	48
Al	53	83
Sn	52	32

Ni	24	44

As can be seen from the table, while the quantity of leached copper remains almost unchanged on varying the leaching solution (similar selectivity), the relative quantities of leached base metals slightly change. Specifically, Zn, Al and Ni are mostly leached from the pfCW, while Sn, Pb and Fe from the commercial lactic acid solution. The lower amount of lead in the pfCW leachate is reasonably attributed to the formation of low solubility salts, due to the presence of a significant amount of chloride salts in the mixture.

The further aspect of interest is the increased dissolution efficiency of the copper upon the addition of H_2O_2. As observed using commercial lactic acid, also using pfCW it is possible to efficiently bring copper into solution up to almost quantitatively even only at room temperature. This result, together with the approximately quantitative leaching of the nickel, is of absolute importance since, in addition to the replacement of the citric acid used for the original process in Phase 1, there is also possibility of replacing the ammonia treatment used for the Phase 2.

8.3.2.2 Sample 2

Sample 2 (characterization reported in **Table 8.2**) contains the plastic-glass fraction, magnetic metals and aluminum that were not present in Sample 1. Leaching tests using this composite sample were performed using $pfCW_2$ to study the difference connected with the compositional characteristics of the test samples.

Figure 8.18 shows a comparison of the metal leaching efficiency of the $pfCW_2$ with respect to the synthetical HLa solution, with [HLa] = 0.5M under L/S = 50mL/g. The solution in both tests was renewed once.

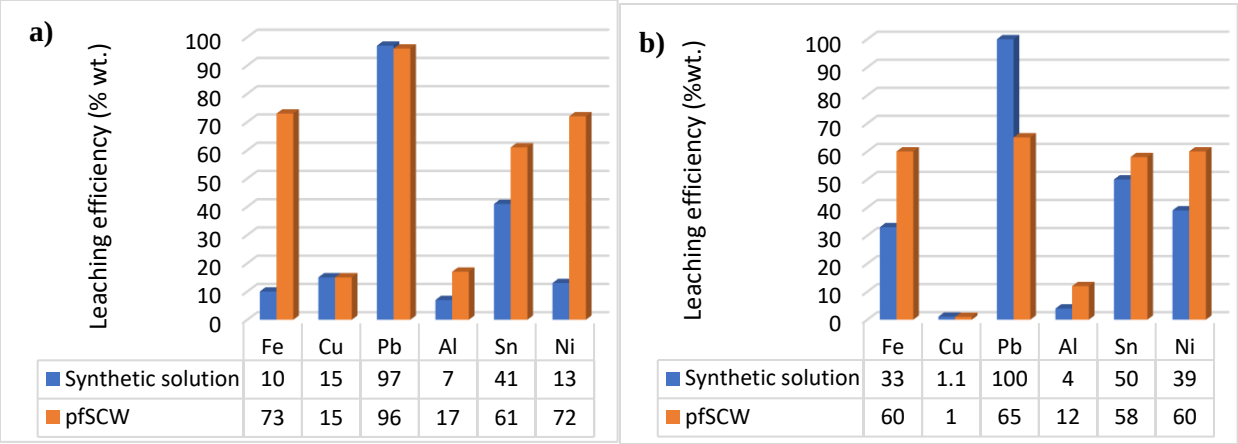


Figure 8.18 – Leaching efficiency comparison using synthetic solution and pfCW$_2$ on aliquots of Sample 2, at room temperature (a) and 100 °C - MW (b) after 48 and 12 hours, respectively (solution renewed after 24 and 6 hours).

From the graphs we can see that the use of pfCW$_2$ is as efficient and selective as the HLa synthetic solution, with the significant difference in the high leaching capacity shown against Ni and Fe. This represents a considerable advantage in the use of pfCW$_2$, as it allows to remove most of the interfering metals with the subsequent phase of dissolution and recovery of Cu and Ag. Moreover, this different behavior suggests the presence of the complexing action of some other chemical species, not yet identified, present in the pfCW$_2$ and not in the commercial lactic acid solution, that can favor the leaching of these metals generally less reactive. The repeated observation that the Sn reaches leaching yields not exceeding 50-60%wt. suggests that this metal species undergoes passivation phenomena, as for the Al that is not leached, if not a minimum percentage under any of the conditions used. On the other side, as expected due to the presence of halide salts, mainly chlorides, into solution, the amount of Pb measured in the leachate is lower than expected. Further investigations of these aspects are therefore requested and stimulating.

The leaching tests with pfCW$_2$ have also been continued, renewing the solution for a further 2 times. From the graph (c) in **Figure 8.19**, it is observed that within 24 hours it is surprisingly possible to bring in solution more than 90%wt. of Fe and almost 100%wt. of Ni. The dissolution yield of Al grows slightly as well as that of Cu, losing in selectivity.

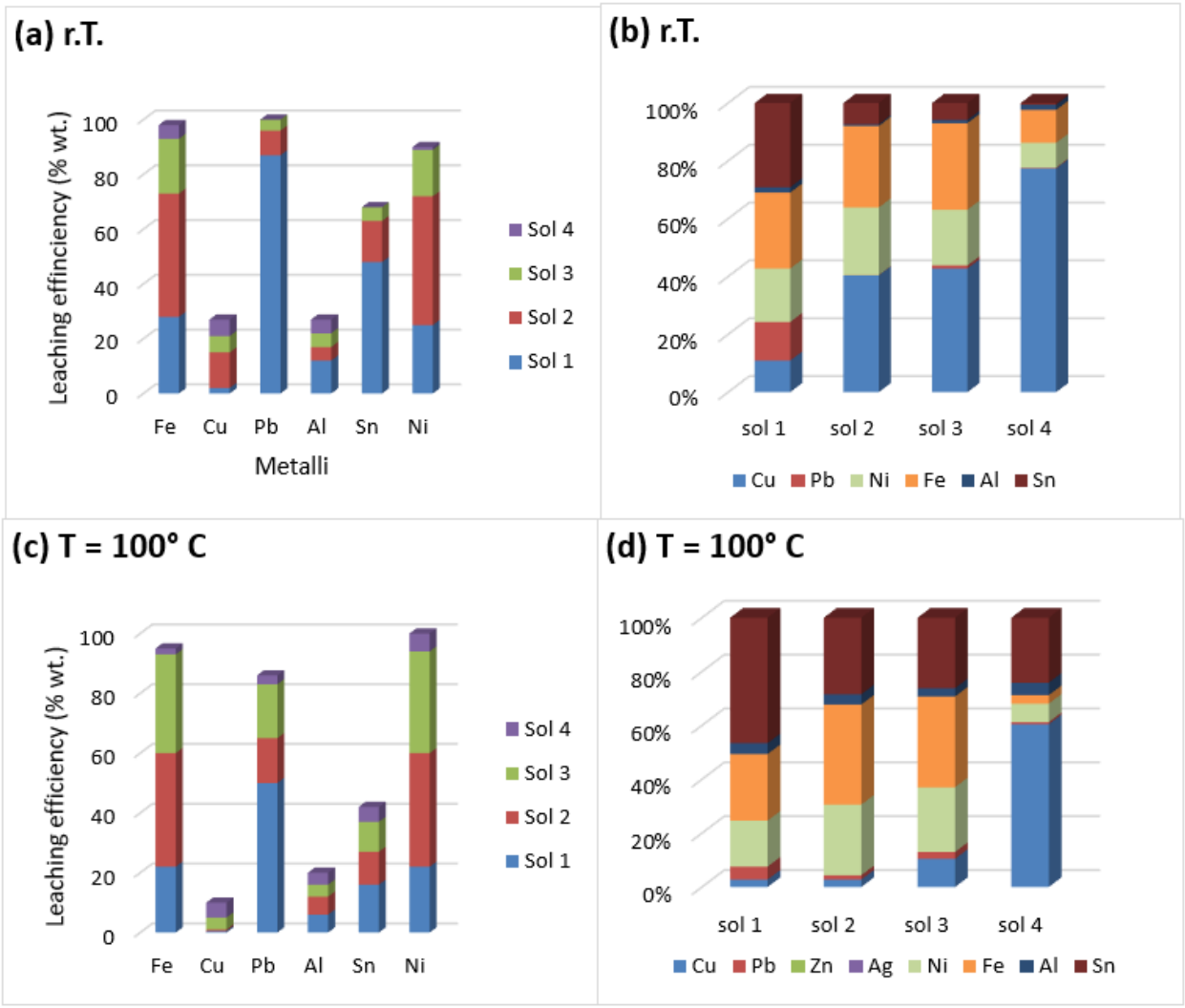


Figure 8.19 – Leaching efficiency (% wt.) at room temperature (a) and 100 °C – MW (c) and metal composition (%) of the solutions at room temperature (b) and 100 °C – MW (d)

8.4 Conclusions

In this work, the direct application of fermented cheese whey as a low cost and bio-derived leaching mixture for metals recovery from WEEE has been evaluated. Considerations connected with the compositional characteristics and refinement degree of the test samples were considered, as well as optimized specific process parameters (reaction time, temperature, leaching solution Concentration and L/S).

Based on the experiments, the main conclusions are:

- pfCW can be produced with optimal concentration and pH characteristics for the hydrometallurgical treatment of metal containing composite materials;
- pfCW demonstrates efficiency and selectivity in the dissolution of base metals, with particular reactivity demonstrated towards Pb, Ni and Fe both at room and reflux temperature;
- pfCW can be a valid alternative to commercial lactic acid as well as to citric acid solutions previously proposed for the Phase 1 of the WEEE metals recovery method;
- pfCW, in the presence of a small amount of low cost and environmentally benign H_2O_2, demonstrated to be active towards Cu present in the solid residue of the first leaching phase, providing a further selective step to the metal recovery process as an alternative to the buffer ammonia solutions.

Related to the composition and the degree of refinement of the test specimen, the experimental results show comparable efficiency and selectivity, with the vitreous-plastic materials contained in Sample 2 not hampering the efficacy of the process, rather adding a more favorable average density for mixing. This relevant result makes robust the process which demonstrated to be applied on a variety of samples varying in composition as well as physical pre-treatments.

Finally, the higher dissolution rate of Ni and Fe using pfCW may represent an added value in dissolving metals in non-processed RAM boards. Experiments in this direction are planned for a better set up of the process.

The phases of selective dissolution described must then be considered in the broader approach aimed at the complete valorization of the described *urban mines*, that includes the phases of selective dissolution of silver, gold and other noble metals and the recovery of metals from the leaching solutions.

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Chapter 9

Conclusions and perspectives

The present study pointed out the possible implementation of CW valorization processes to support the dairy supply chain and promote a circular bioeconomy model. Cheese whey is confirmed to be an outstanding substrate for high value-added mixture production through integrated biochemical processes. In particular, the organic acids production from sheep cheese whey led us to emphasize the pivotal role that dark fermentation plays in a dairy valorization process scheme, mainly related to its ability to hydrolyze and simplify the organic substance and convert it to marketable products.

From the experimental work carried out during the PhD, we demonstrated fCW is a crucial feedstock for challenging industrial applications. Specifically, we discussed its potential use in:

PHA production (Part I), where we showed that, besides the production of a green energy carrier such as H_2(g) during fermentation:

- an additional stage of VFA extraction in the conventional 3-stages PHA production configuration showed to improve the process performance. A 4-stages PHA production process – *1) acidogenic fermentation;* ***2) VFA extraction****; 3) culture selection; 4) PHA accumulation* – provides the best configuration found for high productivity;
- the use of silicone membranes for VFA extraction allows increasing selectivity (membrane permeability increases with the length of the VFA chain) which affects the following polymerization stage in terms of final PHA composition;
- the acidogenic fermentation stage was tested in both batch and continuous configuration, where the former achieved the highest conversion rate while the latter the highest VFA productivity;
- against, concerning the PHA accumulation step, the change in the feeding strategy (continuous *vs* pulse-wise) did not highlight significant improvements in both PHA content and productivity.

Metal leaching from electronic waste materials (Part II), where we showed that:

- a powerful leaching mixture able to dissolve base metals under mild conditions was obtained by controlled DF;

- the innovative CW fermentation approach, carried out in two phases related to the pH control of the process, led to mixtures rich in lactic acid without the use of specific external inoculum and limiting the dosage of pH conditioners;
- the good quality of the mixture was confirmed also upscaling this strategy by treating 16 L CW in one batch, and a bio-derived leaching mixture with optimal lactic acid concentration, residual carbohydrates and pH was produced;
- the direct application of purified fermented cheese whey (pfCW) as a leaching mixture for metals recovery from WEEE demonstrated good efficiency and selectivity, still towards Ni and Fe, and can be considered a valid alternative to commercial organic acid solutions in the dissolution of base metals;
- noteworthy, in the presence of small amount of H_2O_2, pfCW showed to be active toward positive reduction potential Cu;
- related to the composition and the degree of refinement of the test specimen, the experimental results demonstrated to be applied on a variety of samples varying in composition as well as physical pre-treatments.

9.1 Perspectives

Based on the experimental part and on the main conclusions, the Technology Readiness Level (TRL) of the controlled fermentation of CW as well as PART I and II applications is assessed at 4: Technology Validated in Lab (see **Figure 9.1**).

In the perspectives, several studies are planned in order to increase the TRL making the different parts of this work more prone to be technologically transferred.

Figure 9.1 – Technology Readiness Level

Specifically, some aspects should be carefully taken into account, paying attention to the technical-economic and environmental sustainability of the processes:

For PHA production

- Since the 4-stage PHA production process has never been performed before on sheep cheese whey, the results obtained can be improved. A promising future step could be the optimization of the extraction process, varying the geometric parameters of the membrane and/or the flow, in order to improve the selectivity of VFA and to maximize their recovery (up to 50%wt.), while reducing the time required for extraction. Operationally, a richer solution of PHA precursors could improve the accumulation stage and PHA productivity.

For metal leaching and recovery

- To extend the material recycling cycle to the different metal and non metal fractions contained in WEEE will help to make the whole process profitable;
- Further investigations on the optimal recycling configuration involving pfCW related to the typology of metal-containing scrap, are necessary for improving efficiency and selectivity not just in the leaching phase but also in the metal recovery step;

- Recycling rates of the leaching agent and, the corresponding amount and quality of wastewater production, should be carefully assessed;

  Finally, for both kinds of applications of repowered CW here described, the energy expenditure of the processes with the related amount of CO_2 production, and the potential internal renewable energy sources (e.g. exploiting H_2 from fermentation) will be carefully assessed on a robust pilot scale, in order to provide a solid database for future technical-industrial feasibility studies, as well as impact assessment through Life Cycle Assessment (LCA).

Appendix

Appendix A1 – Figures

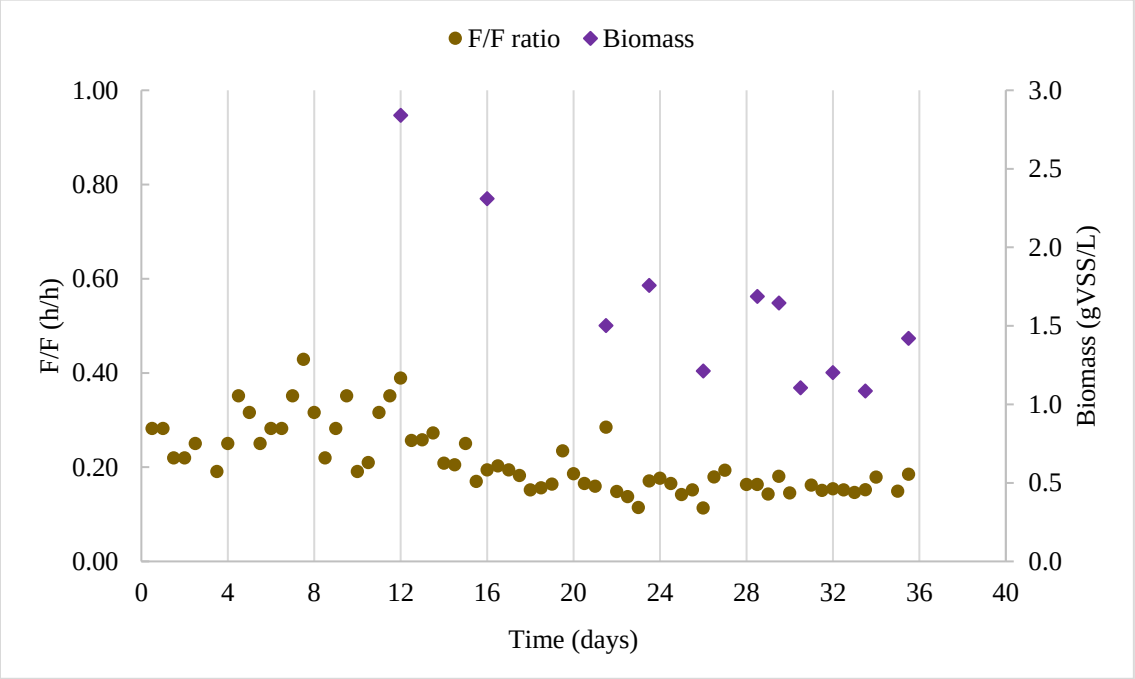


Figure A1.4.1 - Feast and famine ratio (F/F) and biomass concentration overtime during the selection stage using the retentate FCW (rFCW) as substrate. Note that during the first 3 SRT (till 24th cycle, acclimation period) biomass concentration was higher than 3 g_{VSS}/L and thus they were not displayed.

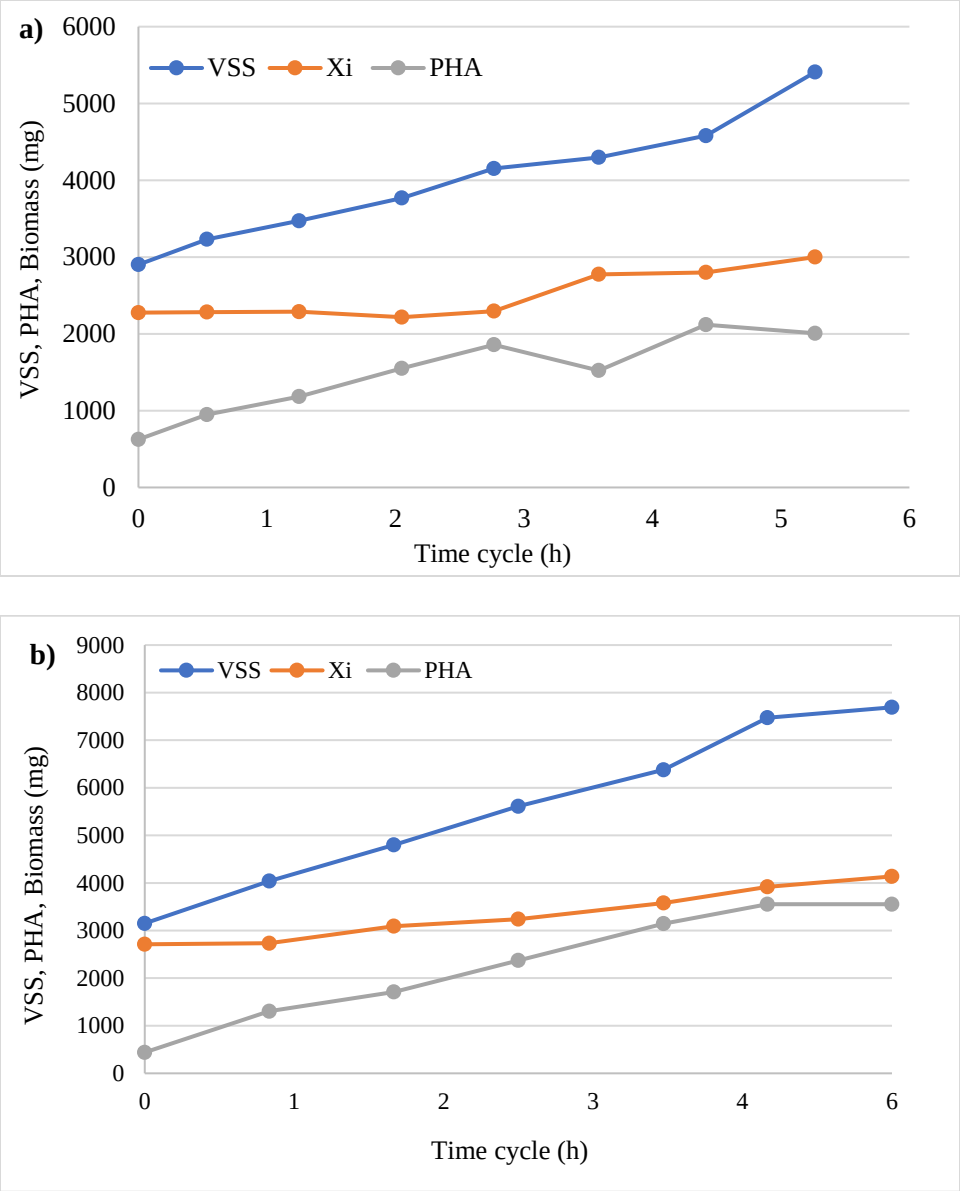
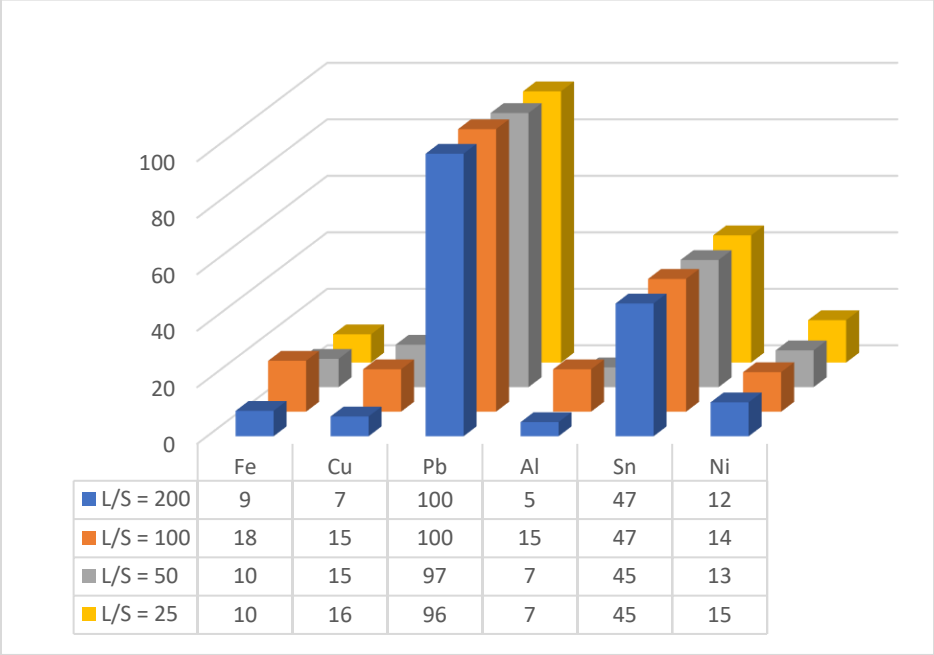
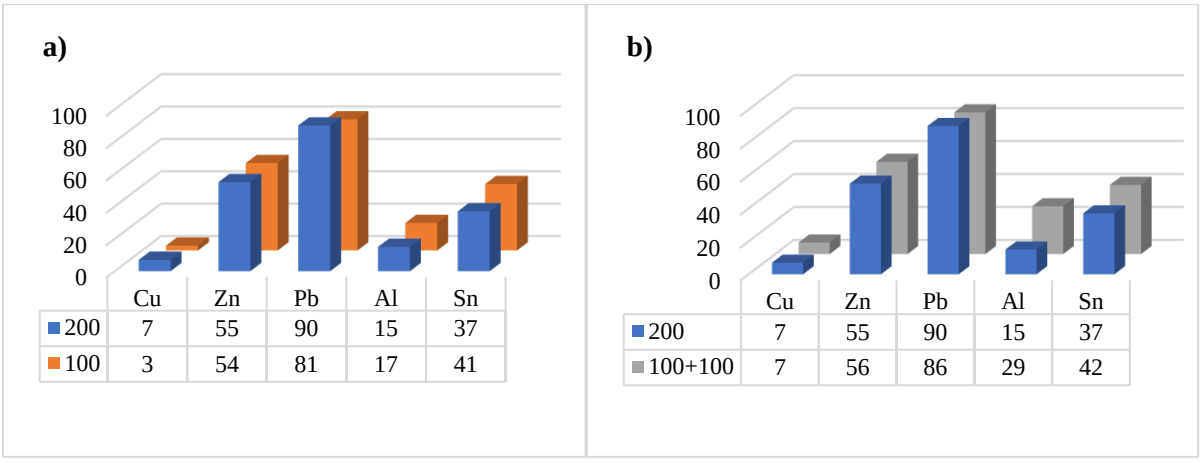


Figure A1.5.1 – VSS, PHA and biomass profile during: (a) pulse-wise accumulation test; (b) continuous accumulation test.



	Fe	Cu	Pb	Al	Sn	Ni
L/S = 200	9	7	100	5	47	12
L/S = 100	18	15	100	15	47	14
L/S = 50	10	15	97	7	45	13
L/S = 25	10	16	96	7	45	15

Figure A1.8.1 – Leaching efficiency on Sample 2 at different L/S at the end of the test (during test with L/S 100 the solution was renewed after 24 h.



	Cu	Zn	Pb	Al	Sn
200	7	55	90	15	37
100	3	54	81	17	41

	Cu	Zn	Pb	Al	Sn
200	7	55	90	15	37
100+100	7	56	86	29	42

Figure A1.8.2 – Leaching efficiency on Sample 1 after 24 h (a) and after 48 h (b) (during test with L/S 100 the solution was renewed after 24 h.