UNIONE EUROPEA
Fondo Sociale Europeo

Ministero dell'Università e della Ricerca

PON Ricerca e Innovazione 2014 - 2020

REACT EU

Università Ca'Foscari Venezia

PhD Program

in Environmental Sciences

37° Cycle

Research Thesis

Exploiting tannery sludge as a renewable resource for platform chemicals production in a combined biological process

SSD: ING-IND/25

PhD Coordinator
Prof. Dr. Enrico Bertuzzo

Supervisor
Prof. Dr. Francesco Valentino

PhD Candidate
Giulia Adele Tuci
969573

La borsa di dottorato è stata cofinanziata con risorse del
Programma Operativo Nazionale Ricerca e Innovazione 2014-2020, risorse FSE REACT-EU
Azione IV.4 “Dottorati e contratti di ricerca su tematiche dell’innovazione”
e Azione IV.5 “Dottorati su tematiche Green”

Table of contents

Figures

Tables

Abbreviations

AD: anaerobic digestion
AF: anaerobic fermentation
ALA5: 5-aminolevulinic acid
ANOVA: analysis of variance
ASV: amplicon sequence variants
BES: bioelectrochemical system
BMP: biochemical methane potential
BOD: biological oxygen demand
CA: chronoamperometry
CBB: calvin-Bassham-Benson
CE: coulombic efficiency
CETP: common effluent treatment plant
CH: chabazite (zeolite)
CHP: combined heat and power unit
CL: clinoptilolite (zeolite)
COD: chemical oxygen demand
COD_{SOL}: soluble COD
CSTR: continuously stirred tank reactor
CV: cyclic voltammetry
EC: energetic consumption
FID: flame ionization detector
GC: gas chromatography
GHG: greenhouse gases
HB: hydroxybutyrate
HRT: hydraulic retention time
HV: hydroxyvalerate
ICP-MS: inductively coupled plasma-mass spectrometry
ISE: ion selective electrode
LOQ: limit of quantification
MCFA: medium chain fatty acids
NE: percentage of utilized nitrogen
NMDS: non-metric multidimensional scaling
MW: microwave
mw: molar weight
OD600: optical density at 600 nm
OFMSW: organic fraction of municipal solid waste
OLR: organic loading rate
OM: organic matter
P: productivity
PHA: polyhydroxyalkanoates
PNSB: purple non-sulfur bacteria
q: rate
REF: reference electrode
rH_2: theoretical hydrogen production rate
rpm: revolutions per minute
SCFA: short chain fatty acids
SCP: single cell proteins
SGP: specific gas production
SHE: standard hydrogen electrode
SRA: sequence read archive
SRT: sludge retention time
S/I: substrate inoculum ratio
TCD: thermal conductivity detector
TH: thermally hydrolyzed
TIC: total inorganic carbon
TKN: total kjeldahl nitrogen
TN: total nitrogen
TOC: total organic carbon
TP: total phosphorous
TS: total solids
UHPLC: ultra-high-performance liquid chromatography
VFA: volatile fatty acids
VS: volatile solids
VSS: volatile suspended solids
WE: working electrode
WW: wastewater
WWTP: wastewater treatment plant
Y_F: fermentation yield

INTRODUCTION AND AIM OF THE THESIS

This thesis explores the development of a hypothetical biorefinery designed to valorize tannery sludge, a very abundant and challenging toxic waste characterized by a high chromium content, with an estimated production of 1 million tonnes globally every year (EU leather industry, 2020), of which around 25.5 kilotonnes only in Italy (UNIC, 2023). This waste is currently landfilled due to its toxic nature, posing significant disposal issues and environmental risks. While its toxicity has historically limited the investigation of potential uses, this research paves the way for new valorization possibilities by focusing on integrating multiple technologies within a biorefinery to optimize resource recovery, reduce landfilled waste volume, and mitigate environmental impacts.

Biorefinery concepts were initially established to transform biomass, mainly from agriculture, forestry, and the food sector, into fuels and high-value bioproducts, aiming to lessen dependence on fossil fuels. Recently, biorefinery research has expanded to focus on biological sludge streams, especially sewage sludge, which provides a consistent and reliable feedstock from wastewater treatment facilities (Cecconet & Capodaglio, 2022). Biorefineries utilizing sewage sludge have investigated various conversion methods, including anaerobic digestion and thermochemical and biochemical extractions of valuable substances like volatile fatty acids (VFAs), polyhydroxyalkanoates (PHAs), and enzymes. Nevertheless, other types of industrial sludges, such as tannery sludge, remain largely unexamined, despite their regular generation and substantial organic content. Within this framework, the current study introduces an innovative biorefinery approach focused on tannery sludge, thereby broadening the application of circular bioeconomy strategies to an underutilized waste stream with great potential for valorisation.

As outlined in Figure 1, the process begins with the pretreatment of tannery sludge, which facilitates its further utilization. Pretreatment is not essential, but it significantly improves the biological valorization process, as the sludge's complex and recalcitrant nature requires initial processing to enhance the hydrolysis step and, consequently, the whole process's efficiency. This work evaluated various pretreatment methods, each aimed at improving substrate accessibility, accelerating microbial activity, and ultimately boosting the yield of valuable by-products while also considering cost-effectiveness.

After pretreatment, the first step involves anaerobic fermentation, which covers only the initial stages of anaerobic digestion, aiming to produce VFAs rather than biogas, which will be the focus of the next step. VFAs are valuable due to their high value and versatility across various sectors; they can be utilized in several industries and serve as precursors for biofuels, bioplastics, and other industrial products. Along with the VFAs and solid residue, this process also produces biogas that consists mainly of CO_2 and a smaller percentage of methane. After this initial step, the process is divided into two distinct streams: (1) a liquid fraction rich in VFAs, to be directed towards VFA extraction, and (2) a solid-rich fraction.

The solid-rich fraction undergoes a second pretreatment before being processed in an anaerobic digestion (AD) unit. Here, the sludge is further degraded, producing biogas with a high methane content (CH_4) and carbon dioxide (CO_2). The methane can be captured and used either to generate energy, supporting the biorefinery's energy demands, or sold as renewable energy, creating an additional revenue stream and reducing reliance on fossil fuels. Additionally, the anaerobic digestion step further minimizes the sludge's total solids (TS) content, leading to an overall reduction of up to 50–60% compared to the current scenario, which is essential for minimizing landfill disposal.

The biorefinery does not end with the treatment of the sludge itself, incorporating an innovative approach to manage one of the process's primary byproducts, CO_2, produced during the fermentation and digestion phases. Instead of releasing this greenhouse gas into the atmosphere, the CO_2 is redirected into a bioelectrochemical system (BES) integrated with the purple non-sulfur bacterium (PNSB) *Rhodopseudomonas palustris*. This system uses CO_2 to grow biomass, which is subsequently harvested as single-cell proteins (SCP). SCP, an emerging alternative protein source, have the potential to serve as a sustainable feed additive for livestock and aquaculture, according to the added-value compounds of this bacterium (carotenoids, bacteriochlorophylls, among others). This approach can offer environmental advantages compared to conventional protein sources (soybean meal as an example) related mainly to land and resource use. Notably, the BES operates in a low-cost setup without requiring external organic carbon sources or light, further enhancing the sustainability of the process. The final residual sludge is dried and prepared for disposal.

A preliminary techno-economic assessment of the proposed biorefinery concept, indicates promising feasibility, as will be further discussed throughout this thesis. The integrated process not only reduces sludge sent to landfill by over 50%, thus lowering its environmental burden and landfill requirement, but also generates valuable products that can generate revenue. Economically, the system reduces overall sludge disposal costs and, through VFA valorisation, achieves an estimated annual saving of approximately 2.67 million € compared to the current landfilling scenario. While these findings derive from a preliminary evaluation and more aspects need to be considered for a full techno-economic assessment, they suggest that this proposed scenario could represent a sustainable and cost-effective alternative for sludge management in the tannery sector.

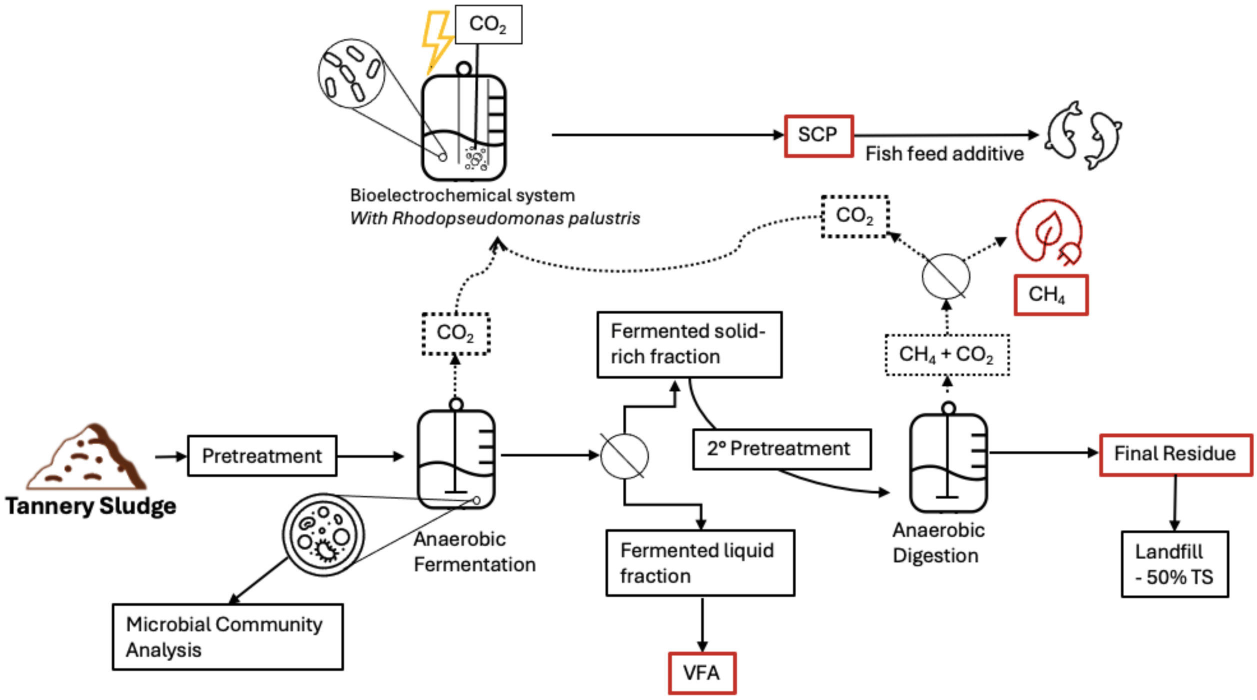


Figure 1: The presented hypothetical biorefinery.

This thesis is structured in 7 chapters, which follow the different steps of this hypothetical biorefinery, starting from the substrate itself. The chapters explore in detail the different biotechnologies involved and the steps taken to maximize process efficiency. Out of these chapters, one is dedicated to the investigation of the microbial community found in the semi-continuous reactors, which was analyzed thanks to the cooperation with the water research institute of the CNR (CNR-IRSA).

Overall, this research focuses on two main objectives. The first and foremost is to evaluate the feasibility of anaerobic fermentation (AF) and anaerobic digestion on tannery sludge, a substrate that is abundant, highly recalcitrant, and has received very limited attention in the scientific literature. The second objective is to demonstrate the feasibility of this proposed biorefinery setup to valorize tannery sludge into valuable by-products, such as VFAs, methane, and SCP. By integrating various biotechnological processes, this system seeks to achieve substantial waste reduction while generating products with economic and environmental value. This approach aims not only to contribute to more sustainable waste management practices but also to create opportunities for further innovations in the valorization of this hazardous industrial waste.

CHAPTER 1: The substrate

The selected substrate for this research is tannery sludge, one of the main and most challenging waste streams in the tanning industry, a sector with a critical role in the global economy. In 2022, the leather goods market was valued at over two hundred thousand billion dollars, with further growth expected on a global scale (Bielak et al., 2023). Leather is a unique, durable, and flexible material derived from the processing of animal hides and skins, typically a byproduct of the food industry (Dixit et al., 2015). However, the tanning industry is known for its negative environmental impact, primarily due to its significant water demand, high volumes of solid, liquid, and gaseous waste, and the use of various hazardous chemicals that pose risks to humans, ecosystems, and the environment (*e.g.,* hexavalent chromium). Moreover, the sustainability of this industry is also affected by a significant contribution to deforestation and greenhouse gas emissions associated with intensive animal farming. The Italian Tanners' Association (UNIC, 2022) estimated that for one square meter of leather, more than one hundred litres of water and two kilograms of chemicals are consumed. In particular, many of the leather manufacturing procedures are defined as 'wet processes', requiring water. Water is necessary for different purposes: it acts as a solvent for the hide-chemical reactions, softens the skin, and removes the residual chemicals. Overall, according to the type of tanning technology and the desired final product, one ton of raw hide consumes approximately 15 to 50 m^3 of water, about 500 kg of chemical products, and about 9.3 to 42 GJ of energy. The outputs of the tanning process include from 200 to 250 kg of finished leather, 15-50 m^3 of wastewater, 450 to 730 kg of untanned, tanned, and finished solid waste, between 1 and 10 kg of organic solvent emissions, and about 500 kg of sludge with a solid fraction of almost 40% (Black et al., 2013). For simplicity, the waste residues of the leather manufacturing process can be grouped into three categories: wastewater, gaseous emission, and solid waste, which includes sludge. An estimation of the volume production and composition of leather industry waste is represented in Figure 1.1.

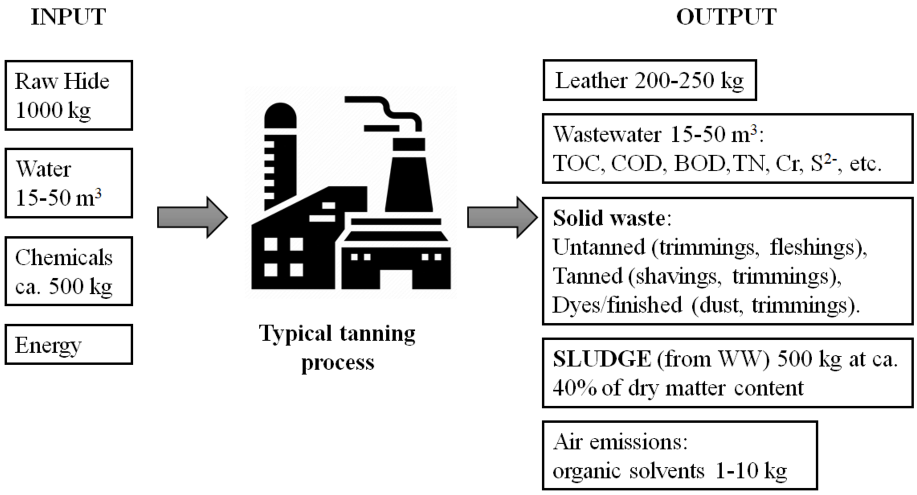


Figure 1.1: Typical inputs and outputs of a tanning process.

Overall, several residues are produced by the tanning industry, but this research focuses on tannery sludge due to its abundance and high organic content, which makes it a promising substrate for AD. Tanning sludge is primarily generated from the treatment of the wastewater generated during the tanning process, in particular from primary and secondary sedimentation tanks following physical-chemical and biological wastewater treatments, respectively. The primary sludge mainly consists of a portion of settleable suspended solids that can be easily degraded, such as collagen and proteins, alongside another fraction of solids that are difficult to degrade, including fibres and some non-degradable inorganic materials like metal salts and carbonates. Secondary sludge includes both the settleable solids lost during primary sedimentation and the substances produced from biological treatments, including biomass residues, dissolved and suspended biodegradable matter, and non-biodegradable matter attached to the biomass.

Traditional tannery sludge treatments primarily aim to reduce volume, organic matter content, and the number of disease-causing microorganisms. Figure 1.2 presents mainstream technologies and mechanisms for managing tannery sludge. Primary, secondary, and tertiary sludge from the respective wastewater treatment stages are first mixed in a homogenization tank to ensure uniform particle size and distribution of chemical composition. The thickening process aims to reduce the sludge volume by lowering water content through physical treatments such as settling, flotation, or centrifugation. Conditioning can be chemical, involving the dosing of inorganic coagulants and/or organic flocculants, or thermal, where the sludge is heated under controlled conditions. This process aims to increase both the particle sedimentation rate and the permeability of the sludge. The stabilisation process aims to reduce the odour and putrescence of the sludge caused by the organisms' decays and to decrease the level of pathogenic organisms. It can be achieved either chemically, for example, with lime, or biologically with anaerobic treatments. The final conventional step of the sludge treatments in a common effluent treatment plant (CETP) is the dewatering process. The dehydration of tanning sludge aims to minimize the volume and weight of the sludge by partial separation of the liquid component. This process can be carried out mechanically by filtration or centrifugation, thermally, or in very limited cases, using natural methods like drying beds. The resulting product has a semi-solid consistency and can reach 90% solid content. At the end of the tannery sludge management process, the recovered liquid is recycled within the plant, and the sludge is disposed of in landfills according to the regulations.

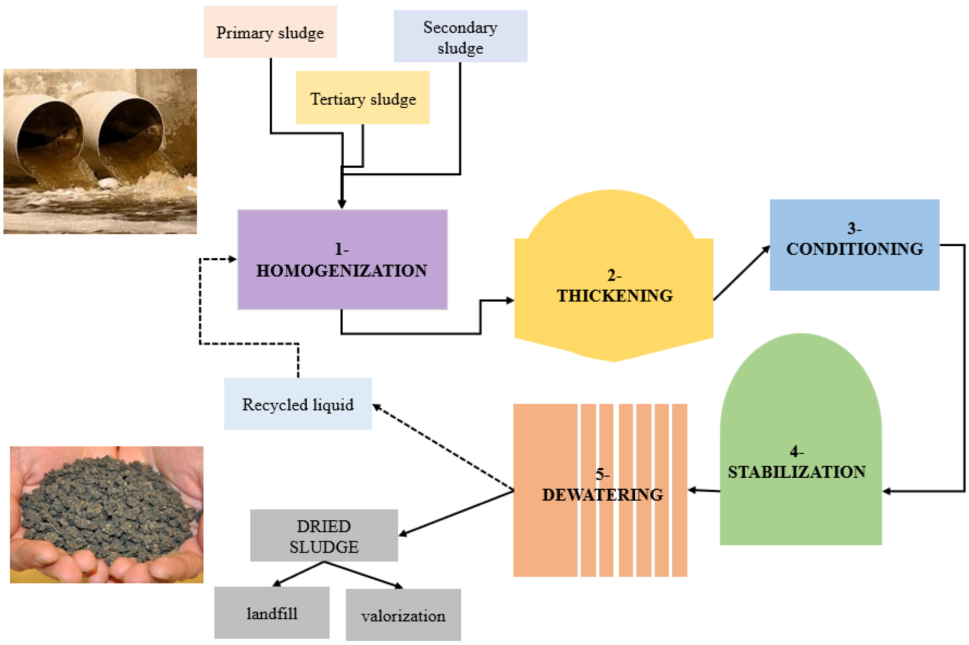


Figure 1.2: Example of tanning sludge treatment in CETP.

Overall, the chemical and physical properties of tannery sludge are affected by the technologies used in treatment plants and primarily by the nature of the tanning process, making it quite variable. The composition of the dry sludge used in this study was the following: 830 ± 14 g TS/kg total solids, 590 ± 4 g VS/kg volatile solids, a chemical oxygen demand (COD) of 793 ± 18 g COD/kg TS, total phosphorous and nitrogen (as TKN) of 7.9 ± 0.4 g P/kg TS and 32.8 ± 0.9 g N/kg TS, respectively, and total chromium content of 19155 ± 310 mg Cr/kg TS. It is estimated that about 90% of global leather industries use trivalent chromium sulphate as a tanning agent due to its greater effect on the mechanical properties of the final product (Guo et al., 2022). However, only around 70% of the chromium added during leather processing actually reacts with the skins, while the rest is discharged and remains in the wastewater effluent (Mannucci et al., 2010; Gottardo et al., 2023). Primary physicochemical treatments can effectively remove the trivalent chromium dissolved in the effluent. Consequently, primary tannery sludge is typically rich in precipitated chromium mixed with inorganic and organic compounds, making it a hazardous waste; the concentration of trivalent chromium has been measured at approximately 2.5% (w/w) (Moktadir et al., 2023) and around 500 mg/kg (Moktadir and Rahman, 2022). The risks associated with such high levels of Cr (III) include high toxicity, potential accumulation in plants, soil, freshwater, and the food chain. This toxicity is also linked to the potential oxidation from trivalent to hexavalent chromium species (Cr VI), a well-known hazardous chemical. Besides the chromium content, tannery sludge is also rich in other hazardous materials that include sulphides, metals like Calcium (Ca), Magnesium (Mg), Nickel (Ni), Zinc (Zn), Iron (Fe), and pathogens.

Overall, the amount and the chemical composition of the tanning sludge depend on different variables, such as the tanning technologies used, the type of processed leather (bovine, ovine, etc.), and the wastewater and sludge management applied in treatment plants. As for wastewater contamination, hazardous organic matter and heavy metals, particularly chromium, are among the primary concerns in sludge management (Wystalska and Sobik-Szołtysek, 2019). Globally, tannery sludge is currently landfilled due to its limited treatment options and legislation requirements.

The challenges of a sustainable management of tannery sludge involve multiple levels. Its environmental and socio-sanitary impacts are linked to the presence of hazardous chemicals, the production of unpleasant odours, and the issue of high organic matter content discarded in landfills (Moktadir et al., 2020). From an economic perspective, the dewatering and disposal of tannery sludge account for nearly 40% of the total costs of wastewater treatment plants in Italy (Polizzi et al., 2018), which will likely rise soon due to the lack of new landfill sites. Overall, there is a clear need for innovative valorisation techniques to minimize the great environmental and economic impacts associated with this abundant waste.

CHAPTER 2: Anaerobic Bio-processes

Many efforts have been made toward the sustainable valorization of tannery sludge; however, most of the proposed technologies remain at the laboratory scale (Tuci et al., 2022). Among the various options explored, anaerobic digestion emerges as a very promising treatment method for wastewater (WW) and sludge. This process not only allows to produce value-added products, such as VFAs and biogas, but also helps mitigate the environmental impacts of this waste by reducing its volume, thereby offering a sustainable solution for waste management and renewable energy production (Mannucci et al., 2010; Mpofu et al., 2021). Furthermore, the fermentation products, including VFAs, can serve as a carbon source for further biotechnological applications, such as biopolymer production, biological nutrient recovery, and chain elongation processes (Vidal-Antich et al., 2021).

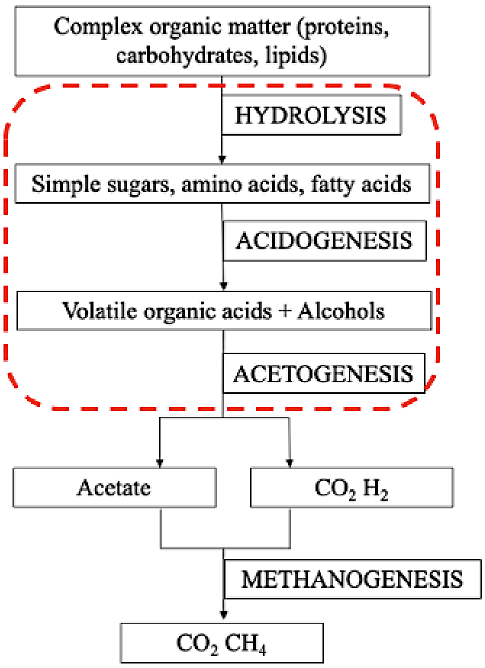


Figure 2.1: The main steps of AF (within the red box) and AD, which includes the whole process.

2.1 Anaerobic digestion (AD) in the recent literature review: general concepts

AD is a well-established technology for the treatment of various organic wastes, including municipal, agricultural, and industrial residues (Valentino et al., 2021; De Baere & Mattheeuws, 2013). This process offers several key benefits, such as stabilizing organic waste, reducing total solids, and recovering valuable bio-based products. AD is conducted by a complex community of anaerobic bacteria and archaea that, in the absence of oxygen, break down complex organic matter into simpler, value-added products like VFAs, hydrogen, and methane (Li et al., 2011). The AD process consists of four primary stages, as shown in Figure 2.1: hydrolysis, acidogenesis, acetogenesis, and methanogenesis, with the first three collectively referred to as anaerobic fermentation (Meegoda et al., 2018). The first stage of the fermentation process is hydrolysis, during which hydrolytic bacteria secrete extracellular enzymes (e.g., amylases, lipases, or proteases) that convert polysaccharides into monosaccharides, proteins into amino acids, and fats into polyols and fatty acids (Worwąg & Kwarciak-Kozłowska, 2019). This phase is often the rate-limiting step, especially when working with more complex substrates that have intricate floc structures and rigid cell walls; in these cases, pretreatments are crucial to enhance the efficiency of this phase and, consequently, the entire process (Xiao et al., 2021). In the second acidogenesis stage, acidogenic bacteria convert the hydrolyzed products into VFAs and other reduced molecules (*e.g.,* alcohols; Meegoda et al., 2018). This phase is relatively robust, as the bacteria involved, including *Clostridium*, *Escherichia*, and *Pseudomonas*, are less sensitive to changes in pH or temperature (Worwąg & Kwarciak-Kozłowska, 2019).

Then acetogenesis takes place, where acetogenic bacteria from the *Syntrophobacter* and *Syntrophomonas* genera convert VFAs and other intermediates into acetic acid, hydrogen, and carbon dioxide (Gerardi, 2003). The process is completed with the stage of methanogenesis when obligate anaerobes of the *Methanobacterum, Methanococcus,* and *Methanogenium* genera utilize the acetic acid, hydrogen, and CO_2 to produce methane and additional CO_2 (Srisowmeya et al., 2020). Methanogenesis typically occurs under different optimal conditions than the preceding stages, which is why some authors advocate for separating the acidogenic and methanogenic phases into two-stage systems (Micolucci et al., 2020; Valentino et al., 2021). This approach allows for the establishment of operational conditions that minimize the initial lag phase and promote microbiota specialization in each reactor, enabling the attainment of the desired products at every stage (Srisowmeya et al., 2020).

Despite the potential of AD for resource recovery and waste reduction, implementing this technology for complex waste streams like tannery sludge can pose challenges. Factors including high protein content, the presence of chromium and other toxic substances, the formation of sludge granules, and a slow degradation rate can hinder the hydrolysis phase and complicate downstream processing (Mannucci et al., 2010). However, implementing targeted and effective pretreatments has the potential to mitigate these obstacles, enhancing process efficiency and overall feasibility.

In the following sections, key parameters influencing the efficiency and outcomes of AD will be explored, including temperature, pH, and pretreatment methods. Selecting these parameters is crucial for optimizing the process and maximizing the valuable bio-products obtained.

2.1.1 pH

The pH is one of the most important factors that significantly influence the efficiency of hydrolysis and acidification (Fang et al., 2020). Acidogenic microorganisms, responsible for producing VFAs, thrive within specific pH ranges, typically between 5.0 and 11.0, depending on the substrate used (Lee et al., 2014). Acidic conditions (pH below 3) and alkaline conditions (pH above 12) are lethal for most of these microorganisms (Liu et al., 2012), making it essential to control pH within an appropriate range. The optimal pH for VFA production varies depending on the type of substrate. For example, in wastewater digestion, acidic to neutral pH values are preferred, while alkaline conditions are favored for sludge (Lee et al., 2014). Maintaining alkaline conditions also helps inhibit methanogenesis, preventing VFAs from being converted into methane and thus promoting their accumulation (Lee et al., 2014). However, as VFAs accumulate, they can lower the pH, which needs to be balanced to maintain process stability. Additionally, Liu and colleagues (2012) observed that hydrolysis of sludge improves in alkaline conditions due to the ionization of carboxyl groups in carbohydrates and proteins, which enhances their breakdown.

Several studies have investigated the relationship between pH and VFA production. Garcia-Aguirre and colleagues (2017) found that the highest VFA yield (8.3 g COD/L) from municipal solid waste was achieved under alkaline conditions at mesophilic temperatures. Similarly, Cheah et al. (2019) noted that increasing the pH from 6.0 to 7.0 in food waste digestion enhanced VFA production, especially propionic and valeric acids. Valentino et al. (2021) further confirmed that slightly higher pH values (around 6.6) in food waste digestion resulted in greater VFA concentrations compared to lower pH levels (5.0–5.5).

The pH has also been shown to impact the composition of the VFAs produced. For instance, Wu et al. (2023) reported that alkaline pH conditions promoted the production of acetic and propionic acids when sludge was used as the substrate. However, when food waste was the substrate, the primary fermentation products were butyric and acetic acids. Overall, the pH significantly influences both the yield and composition of VFAs, with slightly alkaline conditions being ideal for working with sludge.

2.1.2 Temperature

Temperature directly impacts the growth of microorganisms, enzymatic activity, and the hydrolysis of organic matter into soluble substances (Zhou et al., 2018). As the temperature rises, these biological processes generally become more active, with faster hydrolysis and acidification (Rubal et al., 2012). For instance, Hao and Wang (2015) observed a tenfold increase in VFA production under thermophilic conditions compared to mesophilic conditions. However, there are contrasting results regarding thermophilic efficiency. While thermophilic conditions can enhance hydrolysis, it does not always ensure higher VFA yields. In some cases, mesophilic conditions may be more favorable for VFA production, as illustrated by Worwąg and Kwarciak-Kozłowska (2019), where sludge fermentation at 55°C produced 40% fewer VFAs compared to 37°C. This demonstrates that although thermophilic digestion is faster, it may not always be the most productive option in terms of VFA yield. Moreover, this increased productivity comes with trade-offs, as microorganisms in thermophilic conditions (50–60 °C) are less resilient to environmental changes, which may affect the stability of the process (Kim et al., 2006). Valentino et al. (2021) studied the production of VFAs and biogas from a mixture of the organic fraction of municipal solid waste and waste-activated sludge under mesophilic (37 °C) and thermophilic (55 °C) conditions. The results showed similar VFA production under both conditions; however, while thermophilic conditions yielded higher VFA levels, the process was less stable due to frequent fluctuations, which made it more difficult to control. Soomro et al. (2020) investigated a broader temperature range (25–52 °C) using different substrate types, including mixed waste, food waste, and paper fractions. The highest VFA yield (21.5 g COD/L) was obtained at 42 °C for mixed waste, while food waste yielded the best results at 37 °C (15.1 g COD/L) and paper waste at 52 °C (10.2 g COD/L). These findings suggest that the optimal temperature for VFA production depends heavily on the specific properties of the substrate, including its composition and complexity.

Research highlights that temperature not only affects the VFA yield, but also the composition of VFAs produced. Jiang et al. (2013) found that butyrate predominated at 55 °C, while acetate and propionate were the main products at 35 °C when food waste was used as the substrate.

From an energy perspective, mesophilic conditions are more cost-effective as they require less energy input. Thus, while higher temperatures can enhance hydrolysis rates and boost VFA production, the associated energy costs and process instability must be carefully considered.

Furthermore, temperature can influence the choice of the organic loading rate (OLR) and the hydraulic retention time (HRT), key parameters for tests with larger volumes. Namely, higher temperatures are typically linked to faster biological processes, which can consequently be associated with higher OLRs and shorter HRTs. However, while an increase in OLR can enhance VFA production by providing more substrate, an excessively high OLR can compromise reactor stability, for example, due to rapid decreases in pH resulting from increased VFA production (Vázquez-Fernández et al., 2022). A high HRT value can positively influence VFA production since a longer HRT provides microorganisms with more time to react with the waste (Lee et al., 2014). However, it's important to note that an excessively prolonged HRT may lead to stagnant VFA production (Lee et al., 2014). Additionally, reactors with a higher HRT would require a proportionally larger volume to treat the same amount of waste compared to those with shorter HRTs, resulting in increased costs.

2.1.3 Total Solids

Anaerobic digestion is typically categorized into wet digestion (TS < 15%) and dry digestion (TS > 15%) (Karthikeyan et al., 2013). Wet digestion promotes better homogeneity and provides microorganisms with improved access to the substrate, while also diluting inhibitory products (Pommier et al., 2007). Motte et al. (2013) observed a 30% decrease in conversion efficiency when transitioning from wet (10% TS) to dry conditions (33% TS). Conversely, a high TS content allows for the use of a smaller reactor, reducing initial costs and energy consumption (Motte et al., 2013), while also releasing more nutrients and allowing bacteria to generate more VFAs (Liu et al., 2018a). Nonetheless, high TS concentrations can also hinder the process by reducing mass transfer, lowering settling rates, and increasing the substrate's viscosity, which negatively impacts microbial activity and metabolic pathways (Battista et al., 2018). Overall, as explained by Liu et al. (2018b), VFA production increases in tandem with the increasing TS content up to a certain level, after which a higher TS content inhibits further degradation, resulting in a lower conversion efficiency. Therefore, there is an optimal TS range for efficient VFA production, beyond which the advantages are outweighed by process inefficiencies caused by increased viscosity and reduced microbial activity. This threshold depends on the rheology and mass transfer of the system (Liu et al., 2018a). In continuous systems, the quantity of substrate expressed in terms of chemical oxygen demand (COD), total solids, or volatile solids fed into the fermenter per day per unit of working volume is referred to as the OLR (Varghese et al., 2022). A higher OLR means a greater rate of organic matter being introduced into the system, which can lead to increased production rates of VFAs; however, high OLRs may cause similar issues to those associated with high TS content. Furthermore, balancing OLR with other parameters such as HRT, temperature, and pH is crucial.

2.1.4 Pretreatments

Hydrolysis in AD represents the main rate-limiting step of the whole process; therefore, enhancing this step is crucial for complex substrates like tannery sludge, which has a high concentration of proteins and organic macromolecules (Zhai et al., 2020). Pretreatments can be mechanical, chemical, thermal, or enzymatic, and aim to break down organic substances, thereby increasing the soluble COD and making the organic substances available to microorganisms (Liu et al., 2017). Mechanical pretreatments, such as grinding and ultrasonic disintegration, are commonly used to break down large organic particles into smaller, more readily degradable pieces. This mechanical breakdown increases the surface area available for microbial activity, thus speeding up the hydrolysis phase (Zhou et al., 2018).

Thermal pretreatment, which involves heating sludge to temperatures between 60 °C and 180 °C, is an effective method for enhancing the solubilization of organic compounds. Climent et al. (2007) demonstrated that thermal treatment could release a significant amount of organic material from sludge, with the most substantial solubilization occurring after a 60-minute treatment at 90 °C. However, the efficiency of thermal pretreatment tends to decrease at lower temperatures. Chemical pretreatments, which use acids, alkalis, or oxidizing agents such as hydrogen peroxide and ozone, are also commonly applied to degrade complex organic matter. These chemical agents facilitate the breakdown of proteins, lipids, and other recalcitrant materials into more soluble compounds, enhancing the overall biodegradability of the substrate. For example, acid and alkali treatments have been found to be particularly effective for the solubilization of lignocellulosic materials, which are common in organic waste streams (Lee et al., 2014). Therefore, the choice of the pretreatment method should rely on a thorough evaluation of both the operational objectives and the economic feasibility of the chosen technology. In large-scale operations, where energy and operational costs are significant concerns, lower-energy options or a combination of methods may offer the best balance between performance and sustainability.

In this thesis, the investigated pretreatments were of the thermal and chemical types, which yielded promising results in the literature. Namely, one explored pretreatment combines microwave irradiation with hydrogen peroxide (MW-H_2O_2), which has been shown to increase soluble chemical oxygen demand (COD_{SOL}) by more than 30 times (Xiao et al., 2012). Microwaves accelerate hydrolysis by rapidly and uniformly heating sludge, making it more energy-efficient than conventional thermal methods. Hydrogen peroxide is a mild oxidant that produces reactive radicals (OH•), destroying cellular structures and simplifying complex organic matter into more soluble substances (Eskicioglu et al., 2008). However, excessive dosages of H_2O_2 can inhibit microbial activity, making dosage optimization critical. The other chosen pretreatment is a simpler thermal pretreatment, aiming to assess whether the same efficiency can be achieved with a more straightforward process, thus reducing costs.

2.2 The Products of AF: Volatile Fatty Acids (VFAs)

Among the products of AD and AF, namely hydrogen, biogas, and VFAs, the latter are the main focus of this research. VFAs are a class of linear aliphatic mono-carboxylate compounds that are categorized based on the length of their aliphatic carbon tails, which give them unique chemical properties. While VFAs is a broader term, this thesis will also refer to them as short-chain fatty acids (SCFAs) to specify those with carbon chains ranging from two to five carbon atoms, namely, acetic acid (C_2), propionic acid (C_3), butyric acid (C_4), and valeric acid (C_5), which are the primary focus in the context of AF. However, medium-chain fatty acids (MCFAs), which contain between six and twelve carbon atoms, such as caproic acid (C_6), can also be produced in smaller quantities. Chemically, VFAs are part of the carboxylic acid family, which is defined by the presence of a carboxyl group (-COOH), where a carbon atom is double-bonded to an oxygen atom and single-bonded to a hydroxyl group. This functional group makes VFAs polar compounds with strong hydrophilic properties. Despite their high boiling points, VFAs can be separated from aqueous solutions through steam distillation due to their volatility, as the combined vapor pressures of water and the acids allow co-evaporation at temperatures below their individual boiling points (Worwąg & Kwarciak-Kozłowska, 2019). VFAs have many applications across a variety of industries. In the chemical sector, they serve as essential building blocks and precursors for chemicals such as esters, ketones, aldehydes, and alcohols (Valentino et al., 2021). They are also utilized in the food, textile, pharmaceutical, and bioenergy sectors. For instance, they are employed as preservatives and flavoring agents in food production, serve as intermediates in the synthesis of pharmaceuticals, and act as feedstock in the production of biofuels (Baumann & Westermann, 2016). They also have applications in the production of polyhydroxyalkanoates (PHAs), a biodegradable plastic synthesized and accumulated by some microorganisms as carbon and energy reserves usually under nutrient-limited conditions (Tan et al., 2014). The use of VFAs in the production of PHAs has attracted considerable interest because of the increasing demand for sustainable, biodegradable plastics. PHAs have thermoplastic properties similar to traditional plastics, but in contrast to petrochemical-based plastics, they are fully biodegradable.

The market demand for VFAs is continuously growing, driven by their wide range of applications. Currently, VFAs are mainly synthesized chemically, either from non-renewable fossil fuels such as crude oil (Volker et al., 2014) or through the fermentation of pure sugars like glucose and xylose (Zhu & Yang, 2004). While these processes allow high yields and low by-products, they present sustainability challenges. Fossil fuel-based production methods contribute to greenhouse gas emissions and climate change, while sugar fermentation raises ethical concerns regarding the use of

food crops for chemical synthesis (Zigová et al., 1999). In addition, the market price of VFAs tends to rise with the number of carbon atoms in the molecule, with longer-chain fatty acids being more valuable due to their wider industrial applications (Zhou et al., 2018; Ramos-Suarez et al., 2021).
Thus, the increasing demand for sustainable alternatives has led to a growing interest in producing VFAs through biological processes like AF. This approach offers a valuable alternative to fossil-based technology by utilizing organic waste materials and reducing the environmental impact of the production.

2.3 The application of AD on tannery sludge

While anaerobic digestion has been widely applied to various organic waste streams, its application to tannery sludge is very limited and presents challenges and opportunities. Anaerobic digestion has been proven to be an eco-friendly technology able to deal with tannery wastes by reducing their volume, preventing and reducing pollution, and recovering biogas as a source of renewable energy. To date, some studies have been conducted on the treatment of tannery wastewater (Achouri et al., 2021) and tannery solid waste (Polizzi et al., 2018), but relatively less effort has been made toward the application of this technology on tannery sludge. This specific sludge is rich in refractory compounds, which can hinder microbial activity, particularly during the hydrolysis phase. In particular, the nature and amount of the different compounds that can be found in tannery sludge are difficult to identify, both because the composition can vary widely according to the tanning process employed, and because the specific formulations of some chemicals used (such as dyes) are often patented. Therefore, effective implementation of AD for this substrate requires careful selection and optimization of key parameters, with a specific attention to pretreatments to improve this rate-limiting step.

While the literature provides several examples of studies with similar substrates, the limited research on tannery sludge makes it crucial to try and explore different working conditions to make the process both feasible and efficient.

CHAPTER 3: Explorative AF batch tests

This chapter examines the first step of this research aiming to evaluate the potential for VFA production from tannery sludge, as this substrate is sparsely investigated in the literature and, therefore, limited information is available regarding its viability for anaerobic fermentation. For this purpose, batch tests were conducted to determine the productivity and optimal conditions for maximum VFA production, focusing on various parameters, including TS content, temperature, and pretreatments.

This initial work lays the foundation for all the subsequent research conducted in this thesis.

This chapter reports a published paper in the journal Processes (MDPI) titled "***Microwave-Hydrogen Peroxide Assisted Anaerobic Treatment as an Effective Method for Short-Chain Fatty Acids Production from Tannery Sludge***" (https://doi.org/10.3390/pr10112167) written by Giulia Adele Tuci, Francesco Valentino, Edoardo Bonato, Paolo Pavan and Marco Gottardo.

Figure 3.1: The bottles used for the batch tests.

3.1 Introduction

The concepts of Circular Economy and sustainable growth are the main focuses of the European Union's 2030 strategy to meet the Paris agreement requirements (ec.europa.eu). The plan is especially concerned with the improvement of the life cycle of products and the reduction of waste across all sectors. Consequently, resource recovery from wasted materials has gained great importance, especially if such materials are renewable and characterized by high organic content.

In this context of waste production, the tannery sector merits remarkable consideration, as Italy is one of the leading countries in this industry, with a value of production of 3.5 billion euros in 2020 (UNIC, 2020). In 2020 the production of finished leather products in Italy was around 97 million square meters; also, according to the Italian tannery industry sustainability report of 2020, Italian tanneries generate an average of 1.65 kg of waste per square meter of leather produced, with 20.8% of it being sludge. The tanning process is made of different phases both mechanical and chemical and involves the use of chromium salts as tanning agents (Abreu & Toffoli, 2009; Alibardi & Cossu, 2016a), resulting in wastewaters characterized by high amounts of this compound, as well as organic and inorganic substances (Basegio et al., 2022; Liu et al., 2019; Mpofu et al., 2020, 2021). The wastewater produced is commonly treated by centralized industrial wastewater treatment plants, where the physical-chemical treatments and biological treatments produce the so-called tannery sludge as a waste product. The management of this sludge is one of the main issues of the tannery industry, as it is a waste with high environmental impact due to the high presence of chromium and therefore it must be properly disposed of (Silva et al., 2010; Zhai et al., 2020). This sludge is classified as a special non-hazardous residue according to Italian legislation (Ronchi decree, 1997), and its current destination is a second-class type B controlled landfill (D.Lgs. 04/06).

Therefore, at present times, all the sludge from the tannery industry is disposed of in landfills (Alibardi & Cossu, 2016a), wasting all the high-quality organic material that characterizes this waste (Pietrelli et al., 2019). Hence, its potential for the exploitation of new valorization routes is still almost totally unexplored. Previous studies provided some examples of alternative uses of this sludge as a ceramic pigment (Abreu & Toffoli, 2009) building material (Basegio et al., 2002), biochar (Skrzypczak et al., 2022), and more, but the main management method remains landfilling.

In terms of biological waste valorization, the anaerobic digestion process is established at full-scale for the treatment of different kinds of organic waste (Valentino et al., 2021). More recently, within the options of anaerobic waste treatment, many laboratory and pilot scale studies have shown the high potential for valuable resource recovery, such as short-chain fatty acids (SCFAs), through acidogenic fermentation process (Naresh Kumar et al., 2022). SCFAs are products of great commercial interest, as they can be further utilized as building blocks for the chemical industry or as precursors of reduced chemicals and derivatives (Valentino et al., 2021).

Several factors affect the productivity of SCFAs from the acidogenic fermentation of organic waste, namely the pH, temperature and total solids (TS), as explained in chapter 2. Such factors have extensively been analyzed in previous studies on different substrates with sometimes contrasting results, that strongly depend on the specific substrate's characteristics.

With a specific reference to tannery sludge, the application of the anaerobic fermentation to extract the SCFAs has not been thoroughly explored yet. To the best authors' knowledge, there is only one literature study where tannery sludge utilization is related to SCFA production (Zhai et al., 2020) in this research, the authors produced biochar from tannery sludge and bamboo, which was then added to the tannery sludge to improve the production of SCFAs during the anaerobic fermentation.

As underlined by Zhai and colleagues, the tannery sludge is rich in proteins and macromolecular organic matter, so a mild oxidative treatment was considered a viable option to support the hydrolysis of the organic compound and improve the SCFAs production performance. Additionally, partial oxidation is desirable as it reduces the concentration of S species (Horn et al., 2022) which are abundant in tannery sludge. Hydrogen peroxide (H_2O_2) is an oxidizing agent that is similar to oxygen in effect but is significantly stronger. Hydrogen peroxide can oxidize organic compounds directly or indirectly through OH free radicals produced by the breakdown of oxygen–oxygen single bond (Woodard & Curran, 2006). From a literature review, it was found that the use of H_2O_2 combined with thermal treatment has a higher potential and can be preferred to the simple oxidation, as both treatments can disrupt the sludge floc structure and cause microbial cell rupture, hence solubilizing particulate organics (Tyagi et al., 2011; Ambrose et al., 2020).

Moreover, hybrid pretreatments make use of the advantages of the synergism between individual techniques, while also overcoming the drawbacks of individual treatments (Ambrose et al., 2020).

Microwave (MW) irradiation was chosen for the thermal pretreatment, as it allows for rapid uniform heating and energy efficiency, while still providing sludge solubilization and the enhancement of the sludge anaerobic digestion, compared to the more costly traditional heating alternatives (Liu et al., 2017; Özön & Erdinçler, 2019).

The purpose of this work is to provide a viable alternative to the wasteful disposal of tannery sludge in landfills and use it instead to produce SCFAs, in line with the EU strategy. A comprehensive acidogenic fermentation batch tests assay has been developed and discussed in this study, where the effect of the temperature, pH, total solids (TSs) concentration, and the combined MW-H_2O_2 pretreatment has been investigated on SCFA production (yield and concentration) and distribution.

3.2 Materials and methods

3.2.1 Substrate collection and characteristics

The sludge used for the experiments was a mixture of primary and secondary sludge, obtained from the wastewater treatment plant (WWTP) of Montebello Vicentino (northeast Italy). This WWTP treats about 10000 m^3/d of industrial wastewater produced by 23 tannery plants. Tannery wastewaters are subjected to primary treatment and sedimentation (accomplished inside both tanneries and WWTP), and secondary biological treatment (anoxic/aerobic process) used for biochemical oxygen demand (BOD) and nitrogen removal from the primary clarified effluent.

Regarding the chemical and physical parameters, the tannery sludge showed an average dry matter content of 830 ± 14 g TS/kg (Total Solids) and 590 ± 4 g VS/kg (Volatile Solids). The chemical oxygen demand (COD) was 793 ± 18 g COD/kg TS; total phosphorus and nitrogen (as TKN) were respectively 7.9 ± 0.4 g P/kg TS and 32.8 ± 0.9 g N/kg TS.

3.2.2 Batch tests' rationale

Batch tests were performed aiming to investigate the effects of different combinations of initial pH, temperature, combined MW-H_2O_2 treatment, and total solids (TS) concentration on SCFAs production and distribution from tannery sludge fermentation.

Acidogenic fermentation experiments were conducted under both mesophilic (M, 40 ± 1°C) and thermophilic (T, 55 ± 1°C) environments, starting from a different TS concentration: namely 80 g TS/L or 8.0% w/w (M8 and T8) and 120 g TS/L or 12% w/w (M12 and T12). Also, a separate series of tests was performed at 80 g TS/L, with MW-H_2O_2 pretreated sludge (M8-P and T8-P) (Table 3.1).

*Table 3.1: Summary of the operating conditions investigated in the batch tests and the assigned names; *H_2O_2 at 35% w/w.*

Operating Conditions	Acidogenic Fermentation Batch Test					
	M8	M12	M8-P	T8	T12	T8-P
Temperature (°C)	40	40	40	55	55	55
Solids' content (g TS/L)	80	120	80	80	120	80
Range of initial pH	5 - 11	5 - 11	5 - 11	5 - 11	5 - 11	5 – 11
MW-H_2O_2 pretreatment *(80-90°C, 600 W, 10 min; 0.2 g H_2O_2*/ g TS)*	no	no	yes	no	no	yes

Each test was prepared by diluting the dried sludge with tap water to reach the required TS concentrations, which have been chosen based on the performances of a gravity sludge thickening. For each of the conditions, four different initial pH values (5.0, 7.0, 9.0, and 11.0) were tested. Sodium hydroxide (NaOH) and sulfuric acid (H_2SO_4) were added to reach the initial pH values. The MW-H_2O_2 pretreatment was performed based on the strategy proposed by (Ambrose et al., 2020; Liu et al., 2017, 2018c) with some adaptations. Namely, after sludge dilution and pH adjusting, the bottles were heated to 80°C with a microwave oven set at 600W for 10 minutes; the treatment was carried out with intermittent breaks every 1.30 min to avoid water loss by evaporation and to allow manual mixing of the sludge (Ambrose et al., 2020). After the sludge was allowed to cool to room temperature, the H_2O_2 was added at the chosen dosage of 0.2 g H_2O_2/ g TS, using H_2O_2 at 35%.
The bottles were left for 40 minutes to rest allowing the H_2O_2 to react and then were heated again with the MW at 90 °C, to avoid reaching boiling temperature, with the same method described before.

3.2.3 Batch tests' preparation and monitoring

Each test was performed in duplicate, in 250 mL glass bottles sealed with a cap with a silicon plug. As anaerobic inoculum, 50 mL of mesophilic anaerobic digestate from a full-scale digester located in Treviso (northeast Italy) was used (35 g VS/L). Batch fermentation tests were monitored until the plateau in SCFAs production was reached at around three weeks, during which the sludge was manually mixed several times a day. Liquid samples (5.0 mL) were collected for SCFAs analysis, pH measurements, $N\text{-}NH_4^+$, $P\text{-}PO_4^{3-}$ and soluble COD. Hexavalent Chromium [Cr(VI)] was also analyzed in the liquid phase at the end of MW-H_2O_2 pretreatment and at the end of each test to quantify any possible [Cr(VI)] release. All the bottles were never opened as the samplings were performed through a syringe injected into the plug.

The pH was monitored but not adjusted as the aim of this research is to eventually perform these fermentations on continuous mode and on larger scale and the constant adjustment of the pH could not be sustainable in view of process scale-up.

3.2.4 Analytical methods and calculations

Analyses were conducted according to Standard Methods (Apha Awwa, 2005) for the TKN, $N\text{-}NH_4^+$ total phosphorus, $P\text{-}PO_4^{3-}$, VS, TS, [Cr(VI)], and COD. SCFAs were determined using an Agilent 6890 N gas chromatograph equipped with a flame ionization detector (FID) (T = 250 °C). Samples were analyzed through an Agilent J&W DB-FFAP fused silica capillary column (15 m length, 0.53 mm i.D. 0.5 mm film) using hydrogen as carrier. The inlet was working in split mode, with a split ratio of 20:1. The instrument was programmed with a ramp temperature from 80 °C to 100°C (10 °C/min). Before GC analyses, samples were centrifuged at 4.500 rpm for five minutes and the supernatant was filtered at 0.2 mm using acetate cellulose syringe filters (Whatman).

Acidogenic fermentation performances were evaluated through the quantification of the SCFAs concentration, the ratio between the SCFAs and the soluble COD (both on a COD basin), and the fermentation yield (Y_F), calculated by the ratio between the produced SCFAs and the initial VS of the feedstock (VS_0). The $N\text{-}NH_4^+$ release (%) in the liquid fraction with respect to the initial TKN was calculated according to the following equation:

$N\text{-}NH_4^+$ release (%) = $[N\text{-}NH_4^+]_t/TKN_0$.

3.3 Results and discussion

3.3.1 Effect of initial pH on the tannery sludge fermentation

Regardless of the initial value, the pH of each test converged to around 7.0 after approximately 4 days and then settled. For this reason, no significant influence of the pH on the acidification performances was observed. This was due to the observed capacity of the tannery sludge to act as a buffer, resulting in the maintenance of the pH to around 7, even with relatively high concentrations of SCFAs. In fact, the characteristic of this sludge is the presence of lime, which acts as a pH buffer, due to the release of OH^- ions from the residual $Ca(OH)_2$ present in the sludge. Lime is abundantly used in the tannery process during the “Liming” operation, which consists in the utilization of lime ($Ca(OH)_2$) and Na_2S

to remove hairs and flesh from the skins and split up the fiber bundles in the raw hides. Moreover, lime can be used to correct the pH of the wastewater before the primary sedimentation treatment. The quantification of the alkalinity confirmed the good buffering capacity of the fermented streams (Tables 3.S1 and 3.S2); based on the whole conducted experimental plan, the alkalinity in the final effluent was in the range of 2.46–2.89 g $CaCO_3$/L, higher than the values reported in the literature for other substrates and related to the buffered fermented streams where the pH was controlled by the digestate recirculation (Valentino et al., 2021).
This characteristic is very noteworthy for future large-scale applications, allowing the process to self-regulate and continue without external intervention to correct the pH. On the contrary, other substrates, such as food waste, are very sensitive to high SCFA concentration as it can cause the pH values to decrease, resulting in toxic conditions for the fermentative bacteria in the reactor (Franke-Whittle et al., 2014; Zhang et al., 2022). In this paper, the results from all the different pH are reported. It is more appropriate to refer to the initial pH, which has been set from 5.0 to 11.0. However, the presence of lime drove the pH around neutrality in less than 5 days (around one-sixth of the total length of the test). Considering this behavior, a net influence of this parameter on the results has not been observed.

3.3.2 Production of SCFAs over time

As shown in the graphs (Fig. 3.2 A-B-C) all the tests in mesophilic conditions show similar behavior of a fast initial SCFAs increase until a plateau in concentration was reached.
The plateau was reached after approximately 10 days, for the series M8, and after approximately 15 days, for the series M12, proving that, from a qualitative point of view, the initial TSs affected the acidification process. The applied pretreatment (series M8-P) prolonged the time for the plateau achievement (15 days, approximately) compared to tests conducted under the same initial TS level (M8). The required time for the maximum SCFA concentration is a measurement of the total exploitation potential of this sludge in terms of the organic matter acidification and future development in the continuous process, by choosing the most appropriate HRT.

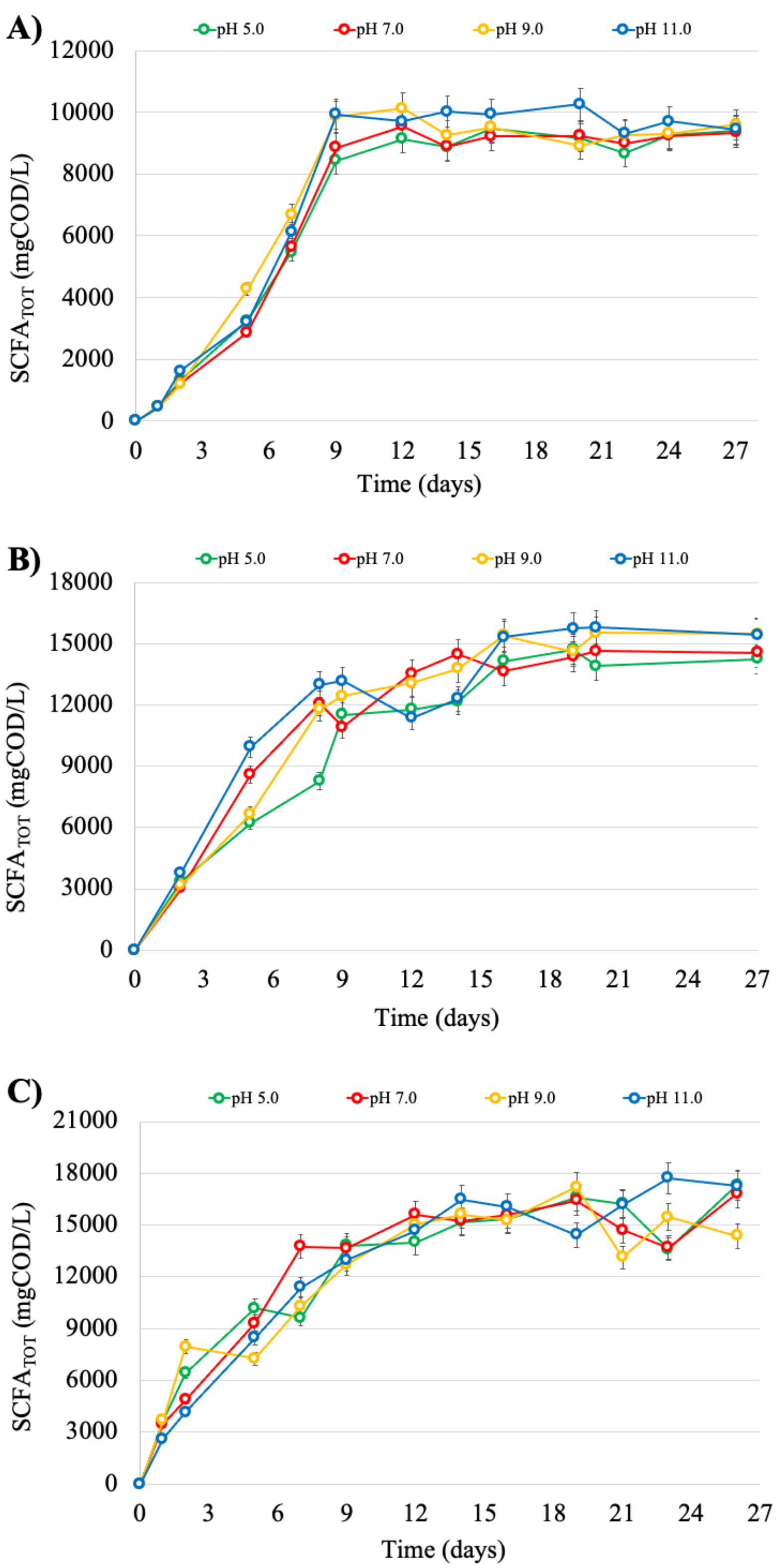


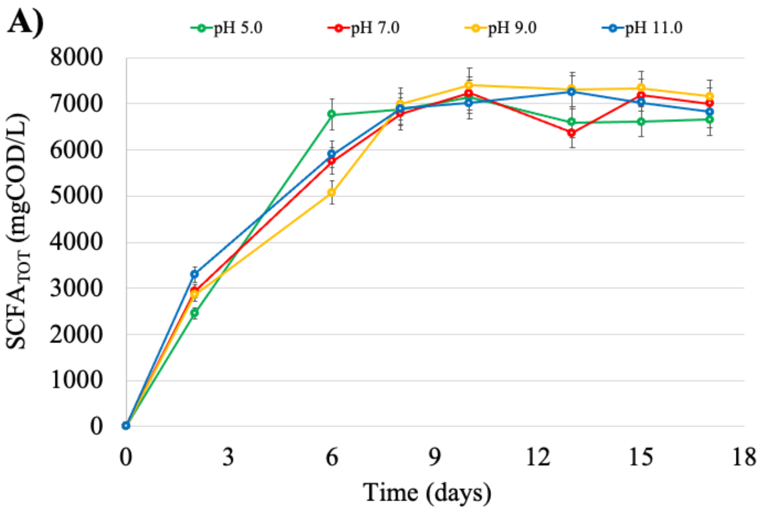
Figure 3.2: SCFAs concentration trends (mgCOD/L) in the mesophilic batch test M8 (A); mesophilic batch test M12 (B); mesophilic batch test M8-P (C). All three series under initial pH of 5.0, 7.0, 9.0, and 11.0.

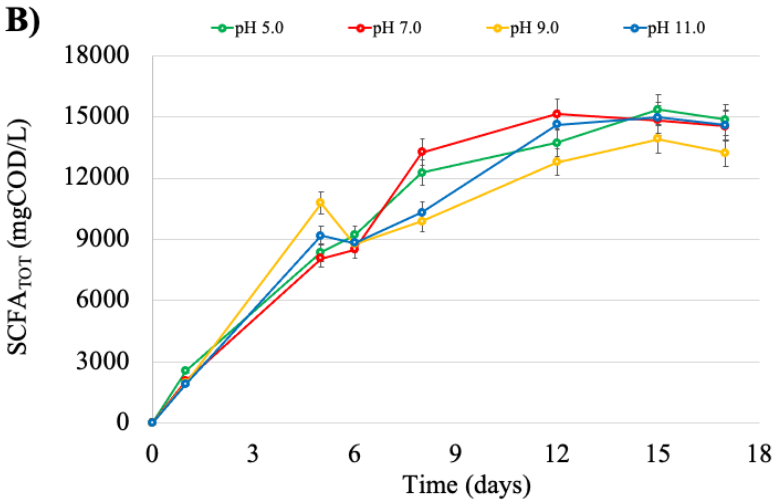
As for the maximum concentration of SCFAs achieved, the series conducted with the pretreated sludge (M8-P) showed the highest values, ranging between 16.8 and 17.7 g COD_{SCFA}/L, demonstrating how the partial oxidation of the organic matter coupled with the microwave treatment, can substantially boost the acidification performances. In fact, H_2O_2 is a strong oxidizing agent (E_0 =1.78 V) and therefore it can oxidize some organic compounds directly.

For instance, H_2O_2 attacks the double bond of an alkene, producing a hydroperoxide; then, the alcohols and the ketones are produced from the hydroperoxide. In addition, H_2O_2 can also produce OH free radicals by the breakage of the oxygen-oxygen single bond. The free radicals are extremely reactive; they can attack organic molecules and, therefore, produce another free radical by the chain reaction mechanisms. In the H_2O_2 pretreatment, the tannery sludge was also subjected to the MW to support the mechanism cited above. Hence, such a pretreatment was aimed to reduce the molecular complexity (for example, by breaking the double bonds) of organic compounds and thus support the following biological process.

In addition, the maximum SCFA concentration depended on the initial TS level, being in the range of 9.5–10.25 g COD_{SCFA}/L and 14.6–15.8 g COD_{SCFA}/L in the series M8 and M12, respectively. In practice, the applied pretreatment almost doubled the SCFA production (M8 vs. M8-P); moreover, the inhibition phenomenon can be excluded since the increase of the TS level in M12 led to an increase in the SCFA concentration, compared to the M8 series. In fact, the tannery sludge is generally rich in protein and macromolecular organic matter, whose decomposition leads to the intense ammonium inhibition for the anaerobic bacteria and a low hydrolysis rate of the organic matter (Zhai et al., 2020).

As regards the thermophilic series, the behavior of T8 and T12 was analogous to the mesophilic conditions, even though the SCFAs plateau was reached earlier, in approximately 8 and 12 days for the T8 and T12, respectively, due to the faster kinetic favored by a higher temperature (Figure 3.3 A, B); Vidal-Antich et al., 2021). Furthermore, in this case, the initial TS level affected the required time to fully exploit the acidification process, in terms of the SCFAs production. By using the pretreated sludge (T8-P series), the SCFAs trend was completely different from the other thermophilic and mesophilic series (Figure 3.3 C). The T8-P series was characterized by a continuous increase in the production of SCFAs for the whole duration of the experiment, with only a slight slowdown during the final days of the experiment. This can be caused by a synergetic effect of a higher operation temperature (55 °C) and the pre-oxidation of the sludge, as the solubilization of the organic matter is favored by both, triggering an almost continuous fermentation mechanism. From the point of view of this waste valorization under a continuous process operation, this condition could be tricky for the choice of the right HRT, since a value of more than 20 days may strongly affect the economic feasibility of the process.





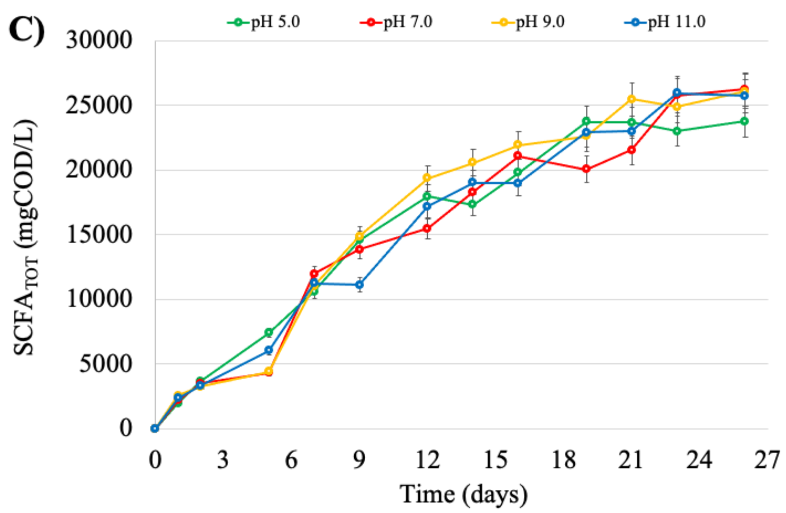


Figure 3.3: SCFAs concentration trends (mgCOD/L) in the thermophilic batch test T8 (A); thermophilic batch test T12 (B); thermophilic batch test T8-P (C). All three series under initial pH of 5.0, 7.0, 9.0, and 11.0.

The thermophilic series T8 and T12 gave a lower peak of SCFAs concentration compared to the corresponding mesophilic series M8 and M12. The range of maximum SCFAs level was 7.1-7.4 and 13.9-15.3 g COD_{SCFA}/L for the series T8 and T12, respectively. Furthermore, in this case, the inhibition phenomenon on the acidogenic culture could be excluded.

Moreover, the thermophilic temperature did not give any benefit, in terms of SCFAs extraction. Given the different trend observed in T8-P series, compared to T8 and T12, and the highest SCFAs concentrations obtained (23.7-26.2 g COD_{SCFA}/L), the synergistic effect of thermophilic temperature and MW-H_2O_2 pretreatment, substantially improved the organic matter acidification, even though the required time for such performance could be difficult to transfer for the design of a continuous fermentation process.

Apart from possible improvement for the T8-P condition, mainly addressed to reach the SCFAs plateau in a shorter time, mesophilic conditions offered a good compromise for the tannery sludge acidification process, with the maximum SCFAs concentration between 16.8 and 17.7 g COD_{SCFA}/L, achieved approximately, in two weeks of anaerobic condition.

Since the literature is scarcely furnished with data related to SCFAs from tannery sludge, a reasonable comparison can be assessed with municipal sludge-related experiments. Different authors highlighted that SCFA concentration can be higher under mesophilic conditions and with a longer retention time, with an optimal pH for the acidogenic fermentation between 7.0 and 8.0 (Niero et al., 2021; Zhang et al., 2019a); under the thermophilic condition, the sludge acidogenesis is usually less performing, showing that the available COD_{SOL} remained not converted into SCFAs. Hydrolysis has also been identified as an important step, applied as the hyperthermophilic treatment (70 °C, 24–48 h) for the solubilization of the organics and the increase of their biodegradability (Niero et al., 2021; Zhang et al., 2019a); in addition, increasing the initial pH had an overall positive effect on the hydrolysis (Zhang et al., 2022). In the case of the tannery sludge described here, no considerations can be made about the pH effect, since the presence of lime, a particular characteristic of this sludge, acted as a buffer and maintained the pH around 7.0 for the whole duration of the experiments.

The maximal SCFA concentration obtained is comparable or higher than the values reported in the literature for the municipal sludge (primary, secondary, or mixture), where typical maximum concentrations range between 6 - 10 g COD_{SCFA}/L (Niero et al., 2021; Zhang et al., 2019a; Morgan-Sagastume et al., 2015).

3.3.3 SCFAs/COD_{SOL} ratio

The degradation of the organics usually leads to a progressive increase in their solubilization and, in turn, in the COD_{SOL} level. If this parameter is not sustained by a parallel acidification process, the SCFAs/COD_{SOL} ratio remains low, while an effective conversion of the organic matter into SCFAs generally leads to an increase in this ratio (Morgan-Sagastume et al., 2015), highlighting an effective acidification process.

Hence, in the acidogenic fermentation process, it is important to have a good balance between the organic matter solubilization and acidification processes. Moreover, the parameter SCFAs/COD_{SOL} ratio is an important indication of the technical feasibility of the waste fermentation process, as it indicates the concentration of SCFAs in the fermentation broth and a higher concentration greatly simplifies the downstream operations to separate the SCFA.

To the best of the authors' knowledge, the literature does not furnish data on this parameter related to tannery sludge utilization; hence, this work has started to cover such a gap in the knowledge. Figure 3.4 A shows the SCFAs/COD_{SOL} ratio in the mesophilic series, calculated at the maximum SCFA concentration achieved (in most cases, at the end of the experiment). All three conditions showed similar results, with a range of variability of 0.70–74 COD/COD in M8, 0.72–0.75 COD/COD in M12, and 0.70–0.76 COD/COD in M8-P (average data). Given the standard deviation within such pretreatment did not have a substantial impact on the SCFAs/COD ratio.

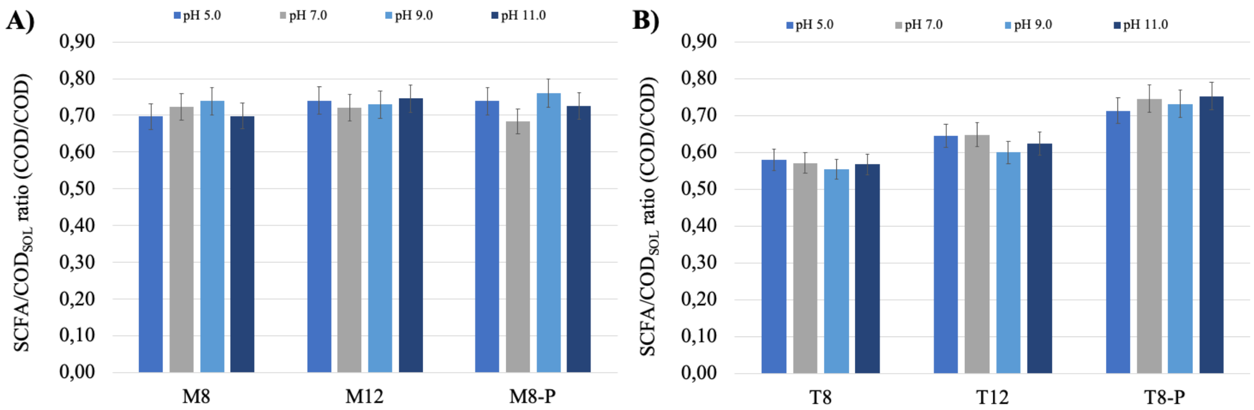


Figure 3.4: SCFAs/COD$_{SOL}$ ratio (COD/COD) of the mesophilic batch series M8, M12, and M8-P under initial pH 5.0, 7.0, 9.0, and 11.0 (A); SCFAs/COD$_{SOL}$ ratio (COD/COD) of the thermophilic batch series T8, T12, and T8-P under initial pH 5.0, 7.0, 9.0, and 11.0 (B).

Figure 3.4 B shows the results for the same parameter, obtained in the thermophilic tests. The T8 series was the only condition showing values below 0.60 COD/COD, within the narrow range of 0.55–0.58 COD/COD and even lower compared to the corresponding M8 series. The T12 showed a slight increase compared to the T8 series (0.60–0.65 COD/COD), but still lower than the results obtained in M12. Only in the T8-P series did the SCFAs/COD$_{SOL}$ reach above 0.70 COD/COD, and comparable to the values obtained in the mesophilic M8-P series (0.71–0.75 COD/COD). Based on these considerations, it is reasonable to assume that the MW-H_2O_2 pretreatment brought substantial benefits to the thermophilic acidification of the COD$_{SOL}$, which remained unconverted in a higher percentage under both T8 and T12 series. Overall, the mesophilic condition appeared preferable for the COD$_{SOL}$ conversion into SCFAs, independently from the initial TS level; however, the MW-H_2O_2 pretreatment made the two applied temperature regimes equivalent, in terms of the SCFAs/COD$_{SOL}$ ratio. Even though these conversion performances can be improved, these results demonstrated that more than 70% of the organic matter contained in the tannery sludge can be easily converted into SCFAs. Other carbon sources, such as the organic fraction of municipal solid waste (OFMSW) and/or different food by-products, are characterized by a high content of putrescible matter; compared to tannery sludge, these carbon sources can be enriched in SCFAs up to 90% of the soluble COD (Gottardo et al., 2022). Moreover, the acidogenic fermentation of the municipal sewage sludge is generally characterized by similar results, compared to this work (0.51–0.69 COD/COD; Presti et al., 2021), showing discontinuous improvements when the fermentation process is conducted on thermally hydrolyzed sludge (0.46–0.75 COD/COD; Zhang et al., 2019a; Liu et al., 2018d).

3.3.4 Fermentation yield (Y_F)

The importance of the Y_F is related to the quantity of SCFAs potentially recovered from a waste carbon source, in terms of the mass balance assessment (not seen in this study) in a full-scale scenario. Hence, the quantification of this parameter is crucial for the further economic evaluation of the unborn tannery-based biorefinery value chain. Figure 3.5 A, B shows the Y_F in all of the performed series.

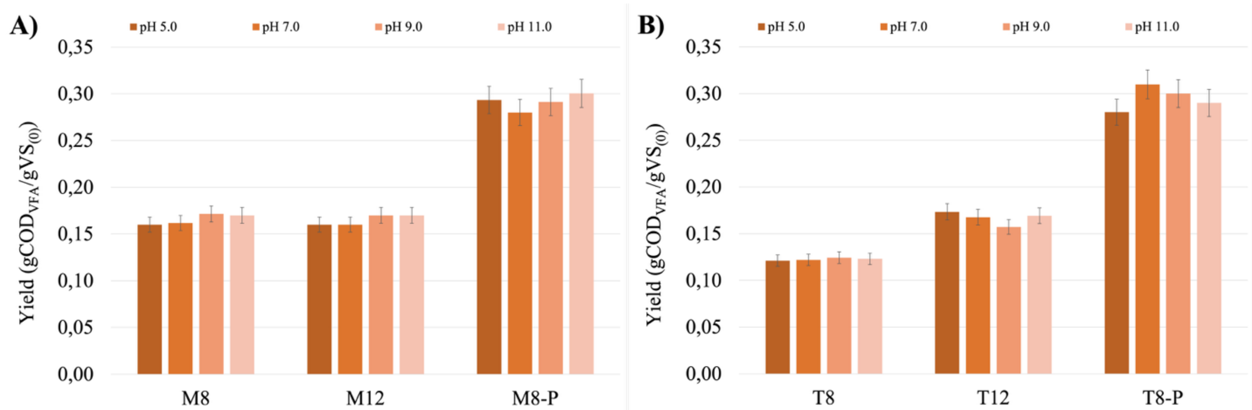


Figure 3.5: Fermentation yield values (Y_F; g COD_{SCFA}/g VS_0) in the mesophilic batch series M8, M12, and M8-P (A); fermentation yield values (Y_F; g COD_{SCFA}/g VS_0) in the thermophilic batch series T8, T12, and T8-P (B).

Under mesophilic conditions, the Y_F was not affected by the increase in VSs, being in the range 0.16-0.17 g COD_{SCFA}/g VS_0 in the series M8, and 0.16-0.17 g COD_{SCFA}/g VS_0 in the series M12. On the contrary, the thermophilic series T8 and T12 showed different performances: 0.12 g COD_{SCFA}/g VS_0 and 0.16-0.17 g COD_{SCFA}/g VS_0 for T8 and T12 series, respectively. A higher solids concentration and thermophilic environment (T12) probably boosted the solids solubilization and their conversion into SCFAs, compared to the less favorable T8 condition, where the initial VS amount was lower. The Y_F of non-pretreated tannery sludge was found to be comparable to or higher than that of municipal sewage sludge (0.06 g COD_{SCFA}/g VS_0) or primary/sewage sludge mixture (0.11 g COD_{SCFA}/g VS_0) in mesophilic fermentation (Morgan-Sagastume et al., 2014). However, other mesophilic investigations on sewage sludge reported higher yields, compared to those reported here for the non-pretreated tannery sludge (0.21-0.33 g COD_{SCFA}/g VS_0; Morgan-Sagastume et al., 2015). The chosen pretreatment greatly improved the Y_F for both the mesophilic and thermophilic series, showing a range of 0.28–0.30 g COD_{SCFA}/g VS_0 and 0.28–0.31 g COD_{SCFA}/g VS_0, respectively in M8-P and T8-P. This showed that the combined pretreatment, as well as the H_2O_2 dosage, was undoubtedly useful to increase the tannery sludge fermentability. Under all of the conditions investigated, these numbers represent the optimal SCFA yields, even higher than the yields obtained in the mesophilic fermentation of the thermally hydrolyzed (TH) municipal sludge (0.22 g COD_{SCFA}/g VS_0), where the authors stated that the advantage of the TH-sludge in the COD solubilization overcame its disadvantage of a low biodegradability (Zhang et al., 2019a). Overall, these values are in line (and even higher) with the yields from other experiments dealing with municipal sewage sludge, usually considered suitable for fermentation or AD systems, revealing that tannery sludge can be employed for this process as well with promising results for further continuous trials and future scale-ups of the technology.

3.3.5 Nutrients' release and quantification

The ammonium and phosphate concentrations in the liquid fraction were measured to estimate the nutrient release and their potential recovery. The release of this macro-nutrient was affected only by the application of the combined MW-H_2O_2 pretreatment; no effects were quantified by the temperature, pH, or the initial TS level.

In summary, the nutrients' release in the mesophilic conditions led to final ammonium and phosphate concentrations of 781 ± 11 (M8), 1181 ± 38 (M12), 1605 ± 44 (M8-P) mg N-NH_4^+/L, and 1.16 ± 0.04 (M8), 2.00 ± 0.03 (M12), 2.40 ± 0.08 (M8-P) mg P-PO_4^{3-}/L (average data calculated by considering the tests at the different initial pH).

Similarly, in the thermophilic tests, the final ammonium and phosphate concentrations were 789 ± 19 (T8), 1216 ± 29 (T12), 1586 ± 40 (T8-P) mg N-NH_4^+/L, and 2.3 ± 0.2 (T8), 2.7 ± 0.1 (T12), 2.8 ± 0.1 (T8-P) mg P-PO_4^{3-}/L.

Considering the N-NH_4^+ release, all the tests conducted with non-pretreated sludge allowed for a release of around 30% of the TKN into the liquid fraction; in particular, the ammonium released in M8 and M12 were 29.6% and 29.8%, respectively; in the T8 and T12 series, it was equal to 30.0 and 30.9%. These results proved that the maximum release of nitrogen from this substrate was roughly 30%, regardless of the TS content and the temperature applied. M8-P and T8-P proved, once again, that the MW-H_2O_2 pretreatment can improve this process overall, as the NH_4-N release values reached 61.5% for the M8-P and 73.8% for the T8-P. Definitively lower performances were obtained for the phosphorous release, which showed values remarkably lower than the ammonium release, and always below 1.0%, regardless of the TS level, temperature, or the pretreatment application.

Based on these results, the application of a pretreatment must be considered for a series of benefits which cannot be differently reached. Both the ammonium release (and potential recovery), as well as the SCFA production, have been positively affected by the MW-H_2O_2 pretreatment and future investigations into a continuous reactor, will be also addressed the TS and VS reduction (which in turn affects the tannery sludge disposal cost).

3.3.6 SCFAs composition

As for the other parameters, independent from the tested conditions (temperature, VS level, MW-H_2O_2 pretreatment), no difference between the different initial pH, has been observed for the SCFA spectrum. The following Figures 3.6 and 3.7 represent the average SCFA profile corresponding to the maximum yield for each series. The mesophilic series M8 and M12 showed quite similar values (Figure 3.6), with a dominance of acetic (37–41% COD_{Ac}/COD_{SCFA}, respectively, in the M8 and M12 series) and butyric acid (22% COD_{But}/COD_{SCFA}, in both series), followed by isovaleric (16–14% COD_{Isov}/COD_{SCFA}), propionic (12–13% COD_{Pr}/COD_{SCFA}) and a lower amount of isobutyric (8–7% COD_{Isob}/COD_{SCFA}) and valeric (5–3% COD_{Val}/COD_{SCFA}) acid. The MW-H_2O_2 pretreatment caused a shift in the composition, towards acetic acid, making up 52% of the total, at the expense of the other SCFAs, which all decreased in the M8-P series. Apparently, in addition to the increase in biodegradability, the MW- H_2O_2 pretreatment moved the metabolic mesophilic fermentation pathways more forward to the last fermentation products (e.g., acetic acids).

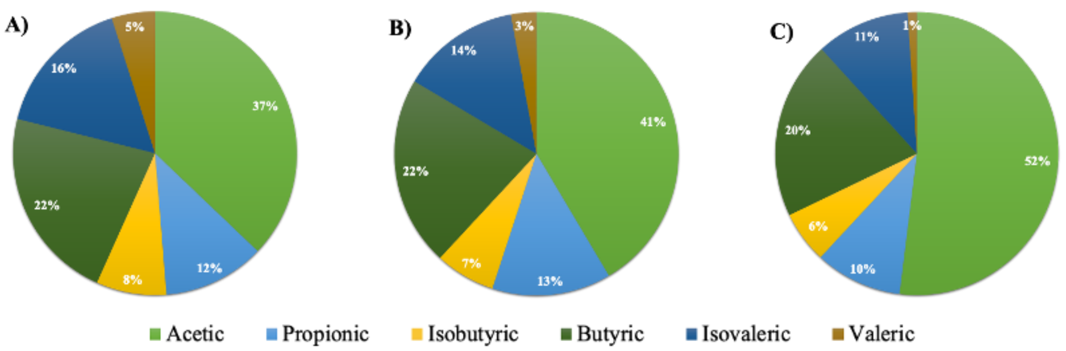


Figure 3.6: Composition in terms of COD_{SOL} percentage of the SCFAs obtained from the mesophilic batch series M8 (A), M12 (B), and M8-P (C) as the average of all the initial pH.

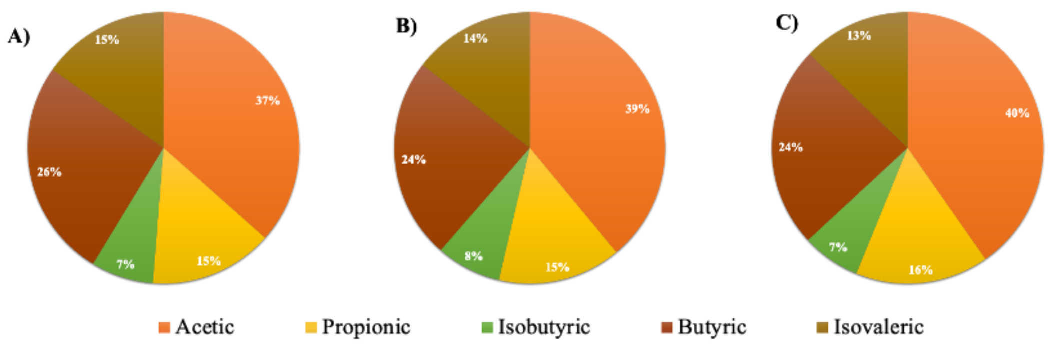


Figure 3.7: Composition in terms of COD_{SOL} percentage of the SCFAs obtained from the thermophilic batch series T8 (A), T12 (B), and T8-P (C) as the average of all the initial pH.

Moreover, in the thermophilic series, neither the TS nor the pretreatment changed the SCFAs composition (Fig. 3.7). The dominating acids for T8, T12, and T8-P remained acetic (37, 39, and 40% COD_{Ac}/COD_{SCFA} respectively), and butyric (26, 24 and 24% COD_{But}/COD_{SCFA}), followed by isovaleric (15, 14 and 13% COD_{Isov}/COD_{SCFA}), propionic (15, 15 and 16% COD_{Pr}/COD_{SCFA}) and a lower amount of isobutyric (7, 8 and 7% COD_{Isob}/COD_{SCFA}). Compared to the mesophilic trials, the higher temperature applied in T8, T12, and T8-P, annulled the MW-H_2O_2 impact on SCFA distribution observed in the M8-P series. Noticeably, valeric acid completely disappeared, compared to the corresponding mesophilic series. This was the only substantial change, caused by the two applied temperature regimes. Compared to the composition of the sewage sludge fermentation liquids, the tannery sludge could generate more acetic acid (up to 50% COD_{Ac}/COD_{SCFA}) under the specific condition of the MW-H_2O_2 pretreatment, followed by the mesophilic acidification process. The SCFAs distribution observed in the other tested conditions was quite similar to those distributions described in the literature, from batch sewage sludge acidification without pH-control strategy, thermally and not-thermally pretreated: acetic (30-40% COD_{Ac}/COD_{SCFA}) and butyric (20-30% COD_{But}/COD_{SCFA}) acids are generally dominating, with a lower amount of propionic (15-22% COD_{Pr}/COD_{SCFA}), isobutyric, valeric and isovaleric were roughly present between 10-15% COD/COD_{SCFA} (Zhang et al., 2019a; Morgan-Sagastume et al., 2014, 2015). Valeric acid was also reported with a higher percentage under the mesophilic (26-27% COD_{Val}/COD_{SCFA}) and thermophilic (21-24% COD_{Val}/COD_{SCFA}) sewage sludge fermentation process (Zhang et al., 2019a).

3.3.7 Perspective of tannery sludge utilization in a new biorefinery value-chain

Due to its high chromium ion content (40–80 g/kg), tannery sludge is considered hazardous waste and its management represents a big challenge for the leather industry (Jin et al., 2018). However, the recent strategies in the European Union are focused to set the transition toward a circular economy to maintain the value of products/materials/resources for the longest time possible, together with the objective of waste minimization. The development of a biorefinery represents a method to approach resource valorization, aiming to use available renewable feedstock to provide high-value marketable products while minimizing the energy consumption and waste generation. In this sense, the potential for microbial fermentation and the recovery of SCFAs from tannery sludge has never been investigated in detail, and this study furnishes a deep evaluation. The two tables (Tables 3.S1 and 3.S2) summarize all the results obtained in this work from the mesophilic and thermophilic experiments. Within a future and desirable biorefinery scenario for the tannery waste valorization, apart from the possible improvements of the Y_F, further efforts must be devoted to continuous experiments and the scaled-up approach. The results obtained in this study demonstrated the feasibility to recover a significant amount of SCFAs in the liquid phase, with no risk related to the Cr(VI) release. The Cr(VI) concentration quantified at the end of the MW-H_2O_2 pretreatment was below the LOQ of 0.03 mg/L; the same results were obtained at the end of each fermentation experiment, demonstrating that no Cr(VI) release occurred for the whole tests' length, under the two investigated temperatures. Given the high content of the macromolecular organic matters, the consideration of a multi-purpose biorefinery approach is reasonable and it could allow to recover, not only bio-based chemicals (such as SCFAs), but also interesting biofuel, such as biohythane (Gottardo et al., 2022). However, in addition to other biorefinery scenarios developed at a pilot or semi-demonstrative scale on urban and agri-food waste (Righetti et al., 2020; Moretto et al., 2020a), the chromium issue in the tannery waste biorefinery, must be considered its distribution among the generated fluxes and fate in the final bio-products, if any. In fact, it is noteworthy that producing biobased molecules instead of biofuels increases the economic added value, contributing to the development of a real and effective bioeconomy scenario (Uçkun Kiran et al., 2015).

3.4 Conclusion

This study aimed at evaluating the performance of tannery sludge in an anaerobic fermentation process to obtain SCFAs, and to assess the most appropriate conditions in terms of the pH, operating temperature, VS content, and oxidizing and heating pretreatments. The batch test trials revealed that the combined MW-H_2O_2 pretreatment associated with the mesophilic or thermophilic conditions gave comparable results, in terms of yield (being 0.28–0.30 and 0.28–0.31 g COD_{SCFA}/g VS_0 in the mesophilic and thermophilic series, respectively) and the SCFAs/COD_{SOL} ratio (the SCFA amount counted for more than 70% of the soluble COD). The SCFAs production, over time, showed maximum values in the range of 16,800–17,700 mg COD_{SCFA}/L (achieved in approximately two weeks) and 23,700–26,200 mg COD_{SCFA}/L (approximately achieved in three weeks) respectively for the mesophilic and thermophilic trials. Lower performances were related to the absence of the pretreatment (especially for the Y_F) and no substantial effect was found to be linked to the different initial VS levels.

The performed tests revealed a particular characteristic of tannery sludge, which was its capacity to act as a buffer, resulting in the maintenance of a neutral pH even with high concentrations of SCFAs, meaning that the tannery sludge fermentation does not need the use of chemicals for the pH control and the fermentative bacteria are far from being at risk of becoming inhibited by sudden pH drops.

Overall, this work represents the first and deep evaluation of microbial SCFA production from tannery sludge and future efforts will be dedicated to the investigation of the pretreatment and continuous process configuration, as a part of the mandatory action for the maximization of the resource recovery from this abundant and polluting waste, which is currently wasted completely, with dangerous environmental consequences and high economic expenses for the producers.

Supplementary materials (Chapter 3)

Table 3.S1: Summary of the main results (average data) obtained in the mesophilic (M8, M12, M8-P) anaerobic fermentation of tannery sludge.

Parameter	Unit	Series M8				Series M12				Series M8-P			
		pH 5.0	pH 7.0	pH 9.0	pH 11.0	pH 5.0	pH 7.0	pH 9.0	pH 11.0	pH 5.0	pH 7.0	pH 9.0	pH 11.0
SCFAs	g COD/L	9.49	9.35	9.59	10.3	14.7	14.6	15.5	15.8	17.3	16.8	17.1	17.7
SCFAs/COD_{SOL}	COD/COD	0.72	0.72	0.74	0.70	0.74	0.72	0.73	0.75	0.75	0.70	0.76	0.73
Y_F	$gCOD_{SCFA}/gVS_0$	0.16	0.16	0.17	0.17	0.17	0.17	0.18	0.18	0.29	0.28	0.29	0.30
Acetic acid	g COD/L	4.73	4.73	4.52	4.23	6.22	6.13	6.78	6.05	9.06	7.95	9.86	8.51
Propionic acid	g COD/L	1.33	1.25	1.07	1.21	1.79	2.08	2.18	2.18	1.88	2.00	1.28	1.81
Isobutyric acid	g COD/L	0.57	0.88	0.82	0.71	0.99	0.99	1.04	1.14	0.93	1.00	0.99	1.09
Butyric acid	g COD/L	1.71	1.38	1.87	2.23	3.59	2.93	3.23	3.40	3.36	3.39	3.09	4.30
Isovaleric acid	g COD/L	0.50	0.89	1.00	1.48	1.94	2.02	2.11	2.15	1.83	1.91	1.81	1.81
Valeric acid	g COD/L	0.65	0.21	0.33	0.35	0.21	0.5	0.18	0.89	0.25	0.17	0.17	0.21
COD_{SOL}	g COD/L	13.1	13.0	12.5	14.7	19.9	20.3	21.3	21.2	23.0	23.8	22.6	24.4
Alkalinity	g $CaCO_3$/L	2.50	2.48	2.54	2.50	2.49	2.46	2.46	2.52	2.58	2.63	2.64	2.63
$N\text{-}NH_4^+$	mg/L	770	785	776	795	1132	1204	1167	1221	1620	1610	1638	1550
$P\text{-}PO_4^{3-}$	mg/L	0.93	1.17	0.99	1.56	1.77	2.34	1.88	2.02	2.62	2.55	2.16	2.26
Chromium VI	mg/L	< 0.03	< 0.03	< 0.03	< 0.03	< 0.03	< 0.03	< 0.03	< 0.03	< 0.03	< 0.03	< 0.03	< 0.03

Table 3.S2: Summary of the main results (average data) obtained in the thermophilic (T8, T12, T8-P) anaerobic fermentation of tannery sludge.

Parameter	**Unit**	**Series T8**				**Series T12**				**Series T8-P**			
		pH 5.0	pH 7.0	pH 9.0	pH 11.0	pH 5.0	pH 7.0	pH 9.0	pH 11.0	pH 5.0	pH 7.0	pH 9.0	pH 11.0
SCFAs	g COD/L	7.15	7.19	7.34	7.26	15.35	14.84	13.92	14.97	23.76	26.20	26.09	25.74
SCFAs/COD_{SOL}	COD/COD	0.58	0.57	0.55	0.57	0.64	0.65	0.60	0.62	0.71	0.74	0.73	0.75
Y_F	$gCOD_{SCFA}/gVS_0$	0.12	0.12	0.12	0.12	0.17	0.17	0.16	0.17	0.28	0.31	0.30	0.29
Acetic acid	g COD/L	2.91	2.80	2.41	2.45	5.90	5.89	5.48	5.74	12.25	10.34	10.86	10.46
Propionic acid	g COD/L	1.06	1.09	1.06	1.04	2.02	2.15	2.46	2.02	3.38	4.30	4.01	3.45
Isobutyric acid	g COD/L	0.49	0.52	0.60	0.55	1.22	1.14	1.07	1.15	1.34	1.98	2.05	1.59
Butyric acid	g COD/L	1.73	1.71	2.01	2.10	3.90	3.48	2.92	3.96	4.23	6.34	5.65	6.97
Isovaleric acid	g COD/L	0.95	1.08	1.26	1.13	2.31	2.19	2.00	2.10	2.57	3.26	3.51	3.27
Valeric acid	g COD/L	-	-	-	-	-	-	-	-	-	-	-	-
COD_{SOL}	g COD/L	12.4	12.6	13.2	12.8	23.8	22.9	23.2	24.0	33.3	35.2	35.7	34.2
Alkalinity	g $CaCO_3$/L	2.70	2.72	2.72	2.70	2.77	2.74	2.76	2.74	2.89	2.93	2.87	2.86
$N\text{-}NH_4^+$	mg/L	880	725	810	741	1268	1284	1218	1131	1432	1569	1665	1679
$P\text{-}PO_4^{3-}$	mg/L	2.2	2.2	2.6	22	2.8	2.8	2.8	2.5	2.6	2.7	2.9	2.8
Chromium VI	mg/L	< 0.03	< 0.03	< 0.03	< 0.03	< 0.03	< 0.03	< 0.03	< 0.03	< 0.03	< 0.03	< 0.03	< 0.03

CHAPTER 4: Process upscaling and bio-methanation tests

Once the potential for VFA production from tannery sludge has been confirmed, the next step is upscaling the fermentation process, which is the focus of this second article. Additionally, this study includes an assessment of chromium behavior, a mass-energy and economic balance to evaluate the process's feasibility, and biomethanation tests to assess the potential for biogas production from tannery sludge.

This chapter is from a published paper in the journal Biochemical Engineering Journal (Elsevier) titled "***Minimizing tannery sludge in landfilling through a mixed microbial culture approach: Effect of oxidizing pretreatment, temperature and hydraulic retention time on process performances and chromium fate***" (https://doi.org/10.1016/j.bej.2023.109073) written by Giulia Adele Tuci, Francesco Valentino, Aditi Chitharanjan Parmar, Paolo Pavan, Marco Gottardo.

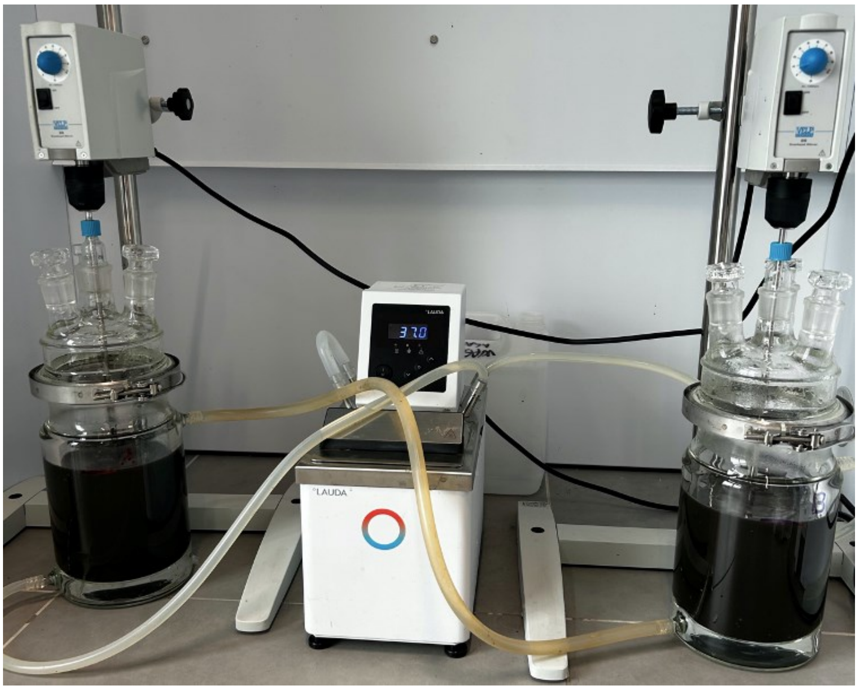


Figure 4.1: The sCSTR setup.

4.1 Introduction

The tannery industry has an important role in many countries where it has contributed significantly to employment and economic expansion (Zhao et al., 2022). However, this industry is still known for its high environmental impact related to the large water consumption and production of wastewater, which is characterized by a high content of chemical compounds, such as dyes, chromium salts and solvents (Zhao et al., 2022). In particular, chromium Cr(III) sulfate is widely used as a tanning agent as it imparts thermal stability, chemical resistance, and endurance to the finished leather products (Oruko et al., 2020). Cr(III) can also exist in the effluents in its hexavalent state [Cr(VI)], which is more toxic, mobile, and soluble (Hashem et al., 2022).

According to the UNIC sustainability report of 2021, Italy's tannery industry accounts for 23% of the global value of leather and is regarded as the industry's global leader (UNIC, 2021). Consequently, a very significant amount of wastewater, and therefore of sludge is produced, which is disposed of in second-class type B controlled landfills due to its toxic nature, with significant environmental and economic impacts, as previously explained.

Nowadays, anaerobic digestion (AD) is the only fully established technology that allows the production of biogas and fertilizers from organic waste while reducing their mass or solids' content. Tannery sludge could be a great candidate for similar microbial technology, however with some challenges. It has been observed that methanogenesis can be negatively impacted by inhibitors deriving from the tanning process, such as hydrogen sulfide (H_2S), ammonia (NH_3), and toxic metals (Mpofu et al., 2021). Additionally, Achouri et al. (2021) observed that the main limitation in treating solid-rich tannery effluent with AD was the particularly low hydrolysis rate, probably due to the presence of high-molecular-weight substances.

One of the most promising methods to increase the rate of the hydrolysis stage is the use of hydrogen peroxide (H_2O_2) to oxidize the high-molecular-weight compounds (Liu et al., 2018c). This process is achieved through the oxidizing action of the OH free radicals on the OM, which are formed by the breakdown of oxygen-oxygen single bonds (Ambrose et al., 2020). As a consequence, the biodegradability of the OM increases due to the creation of lower-molecular-weight and bioavailable-to-microorganisms compounds (Liu et al., 2018c). This pretreatment has been previously employed with promising results due to its effectiveness, low costs, as well as easy availability, conduction, and harmless by-products generation (Ambrose et al., 2020; Liu et al., 2017).

Considering the no more sustainable disposal of tannery sludge, exclusively destined for a landfill, this study is aimed at its exploitation for the production of SCFAs, as value-added products and marketable platform chemicals through anaerobic fermentation (AF) process (Varghese et al., 2022).

The biogas recovery from any residual flux is necessary for a further reduction of the sludge, and in turn, for the minimization of the landfilling. In this study, a thorough analysis of tannery sludge as a substrate for microbial anaerobic processes has been performed. A first assessment of batch hydrolysis tests was aimed at evaluating the ideal H_2O_2 dosage to promote sludge biodegradability, quantified by the hydrolysis constant (*k*h) (Angelidaki et al., 2009). In addition to its effect on the OM, H_2O_2 has the potential of removing the sulfur species in the sludge (H_2S), freeing the elemental S that could also be eventually recovered (Horn et al., 2021). According to the chosen H_2O_2 dosage, a round of acidification batch tests was then focused on the effect of the temperature (T) on the SCFAs production, utilizing the pre-oxidized sludge previously obtained. Based on these assessments, two semi-continuous fermentation tests were performed at 4 and 8 days as hydraulic retention times (HRT) and at mesophilic T (40 °C) to compare SCFA production (concentration and yield) and composition. Moreover, Cr fate was discussed through its quantification in the semi-continuous fermentation effluents. Biochemical methane potential (BMP) batch tests were performed to simulate the AD on the solid-rich AF residue, to quantify the biogas production and to define the whole tannery sludge reduction through the final mass-energy balance. The latter was applied on the hypothetical two-steps anaerobic treatment (AF+AD) to quantify the mass reduction of landfilled sludge and the potential economic income from the produced SCFAs.

4.2 Materials and methods

4.2.1 Rationale of the approach

The designed flowchart consisted of a) hydrolysis potential assessment of the tannery sludge to increase its biodegradability; b) AF step for SCFAs production and fermentation yield maximization; c) solid/liquid separation, to obtain a SCFAs-rich liquid stream and a solid-rich fraction to be used in the subsequent AD step; d) AD for the production of biogas from the solid-rich fraction; e) a final drying step for the quantification of the solids abatement. Real data coming from hydrolysis tests, AF and AD steps have been discussed together with some assumptions to give a first idea about the technical and economic viability of the technology value chain through a detailed mass-energy balance as discussed below.

4.2.2 Substrate's characteristics and inoculum

A mixture of primary and secondary tannery sludge coming from the wastewater treatment plant (WWTP) of Montebello Vicentino (northeast Italy) was used as a carbon source. The WWTP treats different wastewaters produced by more than 20 tannery plants (about 10000 m^3/d), both with primary treatment (alkaline flocculation) and secondary biological treatment (anoxic/aerobic process). The tannery sludge was firstly subjected to a dewatering and drying step; its chemical and physical characteristics were the following: total and volatile solids content of 830 ± 14 g TS/kg and 590 ± 4 g VS/kg respectively; chemical oxygen demand (COD) 793 ± 18 g COD/kg TS; total phosphorus (TP) and nitrogen (as TKN) were 7.9 ± 0.4 g P/kg TS and 32.8 ± 0.9 g N/kg TS, respectively; total chromium (Cr) was equal to 19155 ± 310 mg Cr/ kg TS.

4.2.3 Experimental setup and parameters of the hydrolysis tests

The hydrolysis tests were set up in 250 mL glass bottles sealed with a cap with a silicon plug, with 180 mL working volume, and each test was performed in duplicate. As anaerobic inoculum, 50 mL of mesophilic anaerobic digestate from a full-scale digester located in Treviso (northeast Italy) was used (35 g VS/L) and was previously incubated at 40 ◦C for one week to reach the endogenous methane production. Similarly, 50 mL of thermophilic inoculum from a parallel laboratory- scale digester was utilized for the thermophilic (55 °C) experiments. The tests were performed at neutral pH. Tannery sludge was added following the methodology proposed by Angelidaki et al. (2009); in particular, the substrate to inoculum (S/I) ratio was set at 0.3 (VS basin) for all the experiments. For the oxidative pretreatment of the sludge, four different H_2O_2 dosages were chosen: 0.1, 0.2, 0.4, 0.6 g H_2O_2/g TS (using a H_2O_2 solution at 35% w/w (Liu et al., 2017)). Since tannery sludge is rich in complex and slowly biodegradable substances, it has been treated with H_2O_2 to boost its hydrolysis. The oxidizing pretreatment was carried out in open bottles (250 mL) maintained under mixing for 2 h. The hydrolysis tests have been also conducted on the untreated tannery sludge (0.0 g H_2O_2/g TS). In each test, the biogas production was daily monitored; the data have been elaborated by the model described elsewhere (Angelidaki et al. 2009) to monitor the sludge's hydrolysis rate, which was quantitatively represented by the hydrolysis constant kh (d^{-1}), as explained in Mata-Alvarez et al. (1990). Namely, this constant represents the absolute value of the slope of the line obtained by applying a first-order kinetic model to the initial biogas production data, thus reflecting the speed of substrate hydrolysis.

4.2.4 Experimental setup and parameters of the batch acidogenic fermentation tests

The AF batch tests were conducted at different T, to investigate its effect on SCFAs production. According to the hydrolysis tests, a single H_2O_2 dosage was chosen for the oxidizing pretreatment (0.4 g H_2O_2/g TS), which was as previously explained for the hydrolysis tests.

A fixed aliquot of dried tannery sludge was used as inoculum to fix a S/I ratio of 8.0 w/w (VS basin) in 200 mL working volume (Tuci et al., 2022). All the bottles were sealed with a silicon plug-equipped cap and placed in a different water bath at five different temperatures: 15, 25, 30, 40, and 50 °C. Each test was conducted in triplicate. The bottles were manually mixed twice a day and a fixed-volume aliquot (around 10 mL) was regularly sampled to measure pH, COD_{SOL}, and SCFAs concentration. In particular, the SCFAs concentration was monitored until the plateau was reached, as indication of the maximal SCFAs production in batch.

4.2.5 Semi-continuous acidogenic fermentation

The AF semi-continuous tests, meaning that the reactors were operated on a fill-and-draw basis, allowing for better operational stability and experiment duration compared to batch tests, were conducted according to the best performances obtained in the hydrolysis and AF batch tests. The dosage of 0.4 g H_2O_2/g TS for the hydrolysis and the T of 40 °C were chosen.

Two parallel semi-continuous stirred tank reactors (sCSTR) of 1.5 L working volume were inoculated with tannery sludge, diluted with tap water at 20 g VS/L. The two sCSTR were maintained at the fixed T with water recirculation into an external thermostatic jacket and stirred with a mechanical impeller at 100 rpm. A pre-established mesophilic lag phase of one week was chosen for the inoculum's acclimation (only stirred); afterwards, both reactors were fed once per day with H_2O_2 pretreated tannery sludge, and maintained at two different hydraulic retention times (HRT) of 4.0 and 8.0 days (Run 1 and Run 2 respectively). The HRTs were equal to sludge retention time (SRT) since no settling phase was performed. The two HRTs were chosen based on the observed SCFAs trends in the AF batch tests; in particular the required times to achieve at least 75% and 90% (roughly) of the maximum SCFA production were considered. A fixed aliquot (according to the chosen HRT) was withdrawn for the effluent characterization, in terms of COD_{SOL}, SCFAs, $N\text{-}NH^+_4$, pH, and Cr. Regarding the SCFAs production, in addition to their concentration, the fermentation yield (Y_{SCFA}^{CSTR}) and the ratio between the SCFAs concentration and the COD_{SOL} (COD_{SCFA}/ COD_{SOL}) were monitored. Total Cr was analyzed in the liquid phase at the end of the H_2O_2 pretreatment and regularly in the effluents to quantify any possible Cr release. The pH was naturally maintained neutral by the sludge itself which has a pH buffer characteristic due to the presence of lime ($CaCO_3$) (Tuci et al., 2022).

4.2.6 Biochemical methane potential (BMP) tests

The BMP tests were set up in duplicate as indicated in the methodology of the hydrolysis tests. Similarly, 50 mL of mesophilic anaerobic digestate from the Treviso full-scale digester was used (35 g VS/L) as inoculum after one week of incubation (40 °C) to reach the endogenous methane production. The tests were performed at neutral pH, by using the solid-rich fraction of the fermentation effluent taken from Run 2 as substrate. The S/I ratio was set at 0.3 (VS basin). To investigate the effect of the oxidizing treatment on biogas production, parallel BMP tests were also conducted on hydrolyzed solid-rich fraction, which was treated with H_2O_2 (0.2 and 0.4 g H_2O_2/g TS) in open bottles (250 mL), maintained under mixing for 2 h before being inoculated. Biogas production and CH_4 content was daily monitored to quantify the maximum specific gas production (SGP; m^3/kg VS) achievable and the hypothetical HRT to be considered in the scaled-up continuous AD process, according to the model described elsewhere (Jensen et al., 2011).

4.2.7 Analytical methods

The analyses were carried out according to the Standard Methods for the quantification of total solids (TS), volatile solids (VS), soluble COD (COD_{SOL}), ammonia, total nitrogen (as TKN), phosphorous (TP), total Cr and Cr(VI) (Apha Awwa, 2005).

Ammonium ions were measured on filtered samples (0.2 µm porosity) using the Nessler spectrophotometric method (Varian DMS 90 UV-Visible Spectrophotometer; absorbance at the wavelength of 420 nm). The CODSOL analyses were also carried out on filtered samples (0.2 µm porosity) by using specific kits (Merck-VWR). For TKN and TP, a small aliquot of dried sludge (0.1 g) was firstly subjected to an acidic digestion with in a top-louder microwave (Speedwave XPERT, Berghof); the following spectrophotometric methods for ammonia and orthophosphate were adopted for the quantification (Apha Awwa, 2005).

Total Cr analysis was performed on samples previously basified, centrifuged, and filtered with a 0.2 µm porosity filters; the quantification was carried out with an inductively coupled plasma-mass spectrometry (ICP-MS) NexION 350X (Perkin Elmer) coupled with the autosampler seaFAST (ESI). The Cr(VI) analyses were also performed on filtered samples (0.2 µm porosity) with the direct spectrophotometric determination Cr(VI) by diphenylcarbazide (Apha Awwa, 2005); the absorption measurements were carried out at 540 nm wavelength.

The SCFAs were analyzed using an Agilent 6890 N gas chromatograph (GC) equipped with a flame ionization detector (FID) at a temperature of 250 ◦C. An Agilent J&W DB-FFAP fused silica capillary column (DB-FFAP; 15 m length, 0.53 mm ID, 0.5 µm film thickness) was utilized as a stationary phase. Hydrogen was utilized as carrier. The inlet was set in split mode, with a split ratio of 20:1. The instrument was designed to operate with ramp temperature, from 80 °C to 100 °C, at 10 °C per minute.

Before GC analysis, each sample was centrifuged for 10 min at 4500 rpm and the supernatant was filtered through 0.2 μm acetate- cellulose filter porosity (Whatman).
CH_4 analyses were performed with the same GC reported above, which was also equipped with HP-PLOT MOLESIEVETM column (30 m x 0.53 m ID x 25 μm film thickness) and with a thermal conductivity detector (TCD) at 250 °C. The injector was maintained at 120 °C and 70 kPa. Gas samples (200 μL) were taken with a gas-type syringe; the analyses were conducted at 40 °C (oven temperature) for 8 min, by using Argon as gas carrier.

4.2.8 Calculation and statistical analysis

The degradation kinetic of the hydrolysis tests was evaluated in terms of the hydrolysis rate (*k*h; d^{-1}), which was calculated according to the linear regression of the logarithmic equation of generated gas vs time, according to the first order kinetic model described elsewhere (Angelidaki et al., 2009).
In the acidogenic fermentation batch tests, the acidification performances were evaluated by the ratio between the SCFAs and the COD_{SOL}, and the fermentation yield (Y_{SCFA}^{batch}), which was calculated considering the produced SCFAs over time (“t”) and VS_0 as the initial VS concentration in the batch tests:

(1) $Y_{SCFA}^{batch} = [COD_{SCFA}]_t / VS_0$

In the sCSTR experiments, the ratio between the SCFAs and the COD , as well as the fermentation yield (Y_{SCFA}^{CSTR}) and rate (q_{SCFA}^{CSTR}), ammonia release, and SCFAs volumetric productivity (P_{SCFA}^{CSTR}) were also quantified according to the following equations:

(2) $Y_{SCFA}^{CSTR} = [COD_{SCFA}]_t / VS_0$;
(3) $q_{SCFA}^{CSTR} = [COD_{SCFA}]_t / (VS_0 \times HRT)$;
(4) $N\text{-}NH_4^+$ release (%) = $[N\text{-}NH_4^+]_t / TKN_0$;
(5) $P_{SCFA}^{CSTR} = [COD_{SCFA}]_t / HRT$.

In the calculations of yield (2) and rate (3) the VS_0 is used as a reference because it represents the total amount of biodegradable organic matter available in the substrate , thus allowing the quantification of SCFA production in relation to the actual fermentable fraction of the substrate.

The BMP tests were assessed with regards to the SGP (m^3_{biogas}/kg VS), which was analyzed as a function of the modeled HRT to be adopted in the AD process, according to the methodology described in Jensen et al. (2011). Namely, the SGP was modeled using equation 6:

$$(6)\ SGP(t) = SGP\infty * \left(\frac{d^{-1} * t}{1 + d^{-1} * t}\right)$$

Where SGP∞ represents the maximum specific gas production obtainable from the substrate, and d^{-1} the kinetic coefficient obtained from the linearization of early-stage biogas data. This parameter was also used to estimate the digestion efficiency at each HRT, expressed as the percentage of theoretical potential (SGP∞) achieved.

The parameters characterizing the performances of AF batch and semicontinuous reactors were reported as mean values (with standard deviation). The *t*-test, the analysis of variance (ANOVA) and Tukey HSD post hoc tests were conducted to detect the results' significance. Shapiro–Wilk test was carried out to see if the data were normally distributed; Bartlett test and F-test were used to determine the homoscedasticity between the several data groups. All the statistical analyses were performed with R open-source program (The R Foundation for Statistical Computing, version 4.0.3).

The parameters for the mass and energy balance were set considering a production of 37000 tons/year of tannery sludge, which was the total amount from the two tannery WWTPs (Medio Chiampo S.p.a. and Acque del Chiampo S.p.a.). Reference parameters and boundary conditions are given in a previous work (Valentino et al., 2019), and are reported in Table 4.4. For the economic feasibility assessment, the disposal cost of the dried sludge was assumed to be 250 €/ton. The electrical energy required (0.237 €/kWh) to recover SCFAs from fermentation broth was considered 0.8 kWh/m^3, with an efficiency of 70% and the average SCFA selling price of 2000 €/ton (Tharani et al., 2021). For tap water and H_2O_2, a price of 0.55 €/m^3 and 0.30 €/kg were respectively considered.

4.3 Results and discussion

4.3.1 Hydrolysis tests

The hydrolysis rate, represented by the *k*h, is a pivotal information about the substrate biodegradability and its utilization as SCFA source. This parameter was analyzed for four different dosages of H_2O_2 (Fig. 4.2). Only one example described the benefit of using H_2O_2 on tannery sludge for the production of SCFAs (Tuci et al., 2022); however, the adopted pretreatment was combined with microwave application, and it foresaw a single H_2O_2 dosage (0.2 g H_2O_2/g TS). In addition, no investigation on the sludge biodegradability and *k*h data was performed. Given the poor literature information, a wider range of H_2O_2 has been explored here, together with a quantitative evaluation of the *k*h values.

For the mesophilic conditions (40 °C), the *k*h of the untreated tannery sludge was 0.177 ± 0.04 d^{-1}; for the pretreated oxidized sludge, the *k*h values were 0.189 ± 0.03, 0.187 ± 0.03, 0.197 ± 0.04, and 0.196 ± 0.03 d^{-1} at increasing H_2O_2 dosage respectively. Compared to untreated sludge, a remarkable improvement in the tannery sludge biodegradability was obtained at the dosage of 0.4 g H_2O_2/g TS. This appeared the ideal condition in a perspective of reagents and cost-saving approach, considering the comparable result obtained at the highest H_2O_2 dosage.

In the thermophilic tests (55 °C), the *k*h values did not show a significative increase: they were equal to 0.182 ± 0.04 d^{-1} (no pre-treatment), 0.189 ± 0.04 d^{-1} (0.1 g H_2O_2 /g TS), 0.197 ± 0.04 d^{-1} (0.2 g H_2O_2/g TS), 0.206 ± 0.04 d (0.4 and 0.6 g H_2O_2/g TS). According to the *k*h data, the increase of the performances appeared relatively limited, and potentially not favorable for a cost-saving approach. However, the data from the thermophilic conditions also confirmed the dosage of 0.4 g H_2O_2/g TS as the best option and it was chosen in the following batch AF tests. In addition, since a clear indication from the optimal T was not reached in terms of hydrolysis rate, the following batch AF tests were conducted under a wider T range.

The *k*h values obtained here were higher than mesophilic *k*h values related to primary or secondary municipal sludge (0.110 – 0.170 d^{-1}), indicating the good biodegradability of tannery sludge also without any pretreatment (Abubakar et al., 2017). Other organic sources not linked to the activities of the water utilities also showed similar *k*h values: 0.250 d^{-1}, 0.095 d^{-1}, and 0.121 d^{-1} for kitchen waste, paper, and garden waste, respectively (Trzcinski et al., 2012). Generally, all the *k*h values were evaluated in perspective of biogas production during the established AD process; to the best authors' knowledge, this is the first work where the quantification of the *k*h was performed as additional information for the AF process.

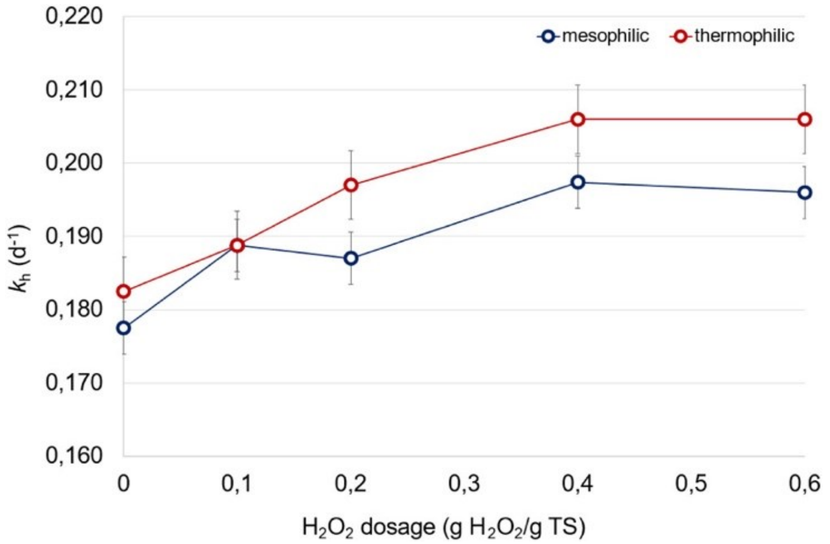


Figure 4.2: Hydrolysis rate (kh) of tannery sludge at different H_2O_2 dosages and temperature.

4.3.2 Production of SCFAs in the batch tests

The following Fig. 4.3 a shows the trend of the total SCFAs concentration over time in batch AF. Independently from the adopted T, the production of the SCFAs started from the first day, without any lag-phase period or inhibition phenomena. At the lowest T (15 °C), the SCFAs trend showed the lowest initial kinetic, according to the SCFAs profile in the first 4 days. A remarkable improvement in the initial kinetics and in the SCFAs concentration was observed at higher T. The series with intermediate T (25 and 30 °C) were characterized by similar performances: the SCFAs concentration heavily increased over the first 4–5 days, with a modest increase occurring after that. The series conducted with higher T (40 and 50 °C) showed a comparable trend, even though the SCFAs concentration reached values above 14.0 g COD_{SCFA}/ L. This fact indicated that the chosen H_2O_2 dosage, together with T > 30 °C boosted the acidification of the OM, which would otherwise remain only scarcely hydrolyzed. In fact, since the waste produced by the leather industry (proteins, oils, dyes, hair, lime, tannins, etc.) is commonly conveyed to centralized WWTP (Rossi et al., 2023), the final sludge is quite rich in proteins. This can limit the SCFAs production if the OM is not efficiently hydrolyzed or properly balanced by carbohydrates or starch-based by-products addition (Strazzera et al., 2021). Given the increase of the COD_{SOL}, generally caused by an hydrolysis step, it becomes crucial to measure the effectiveness of the acidification process through a comparison between the SCFAs and COD_{SOL} values (Varghese et al., 2022).

As depicted in Figure 4.3 b, both the SCFAs/COD ratio and yield (Y_{SCFA}^{batch}) had the same trend as a function of the T. The low T did not ensure an effective acidification performance since almost 50% of the solubilized COD remained unconverted. The final SCFAs concentration was 9.5 ± 0.3 g

COD/L. The conversion yield was also the lowest achieved (0.16 ± 0.01 g COD_{SCFA}/g VS_0). Higher T improved both parameters: the SCFAs/COD_{SOL} ratio was equal to 0.61 ± 0.04, 0.63 ± 0.04, 0.73 ± 0.04, 0.72 ± 0.04 COD/COD at 25, 30, 40 and 50 °C respectively (ANOVA test, Tukey HSD post hoc test, α = 0.05); the Y_{SCFA}^{batch} increased up to 0.22 ± 0.02 and 0.23 ± 0.02 g COD_{SCFA}/g VS_0 at 20 and 30 °C, and up to 0.28 ± 0.03 g COD_{SCFA}/g VS_0 at 40 and 50 °C (ANOVA test, Tukey HSD post hoc test, α = 0.05). In practice, the series with the highest T were similar in terms of acidification performances; hence, the T value of 40 °C was chosen as the preferred one for the following semi-continuous process in a perspective of a cost and energy-saving approach. Both SCFAs/COD ratio and Y_{SCFA}^{batch} can be considered satisfactory and in line with values obtained in previous studies conducted with raw and thermally pretreated sewage sludge, a comparable substrate produced in water utility sector: 0.44 – 0.82 COD/COD and 0.15 – 0.29 g COD_{SCFA} /g VS_0 for SCFAs/COD_{SOL} ratio and Y_{SCFA}^{batch}, respectively (Gottardo et al., 2022; Lorini et al., 2022). In addition, sewage sludge fermentation liquids are generally characterized by lower SCFA concentration (up to 11 g COD/L; Lorini et al., 2022) compared to the values obtained in this study (roughly up to 16 g COD/L). This was probably due to the presence of a certain amount of primary treatment derived tannery sludge, more putrescible and fermentable compared to the sludge coming from the biological wastewater treatment.

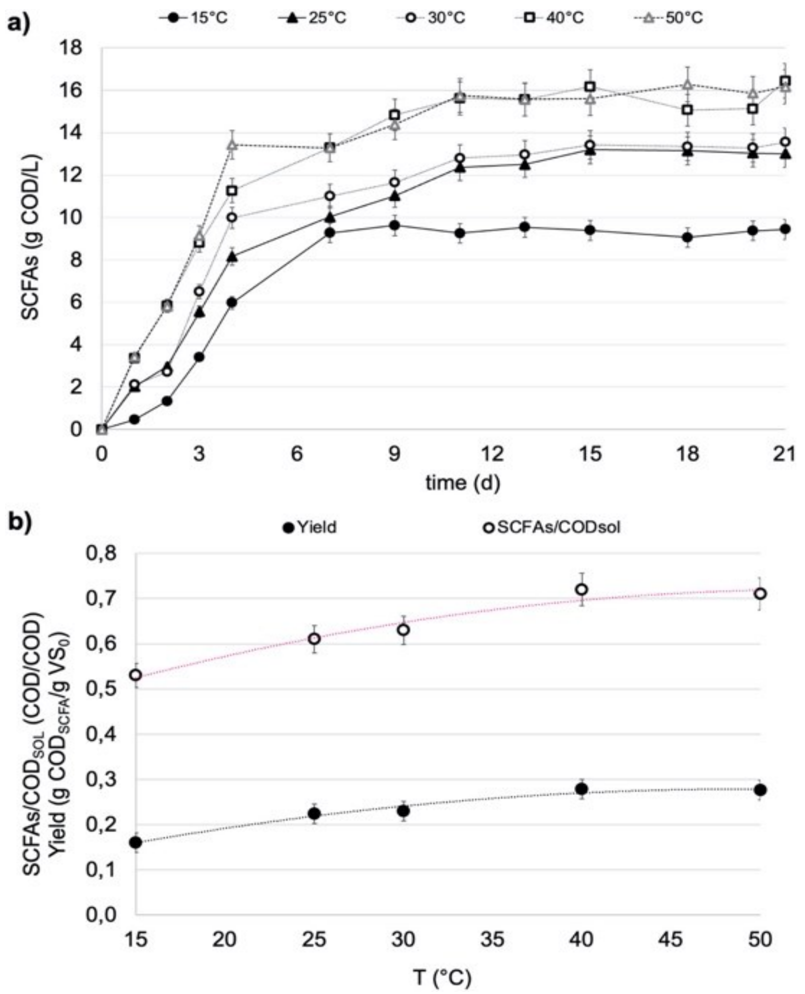


Figure 4.3: Evolution of produced SCFAs in the batch acidogenic fermentation tests (a); trend of the Y_{SCFA}^{batch} *and SCFA/COD ratio at different temperatures (b).*

4.3.3 Production of SCFAs in the semi-continuous CSTR

Based on the results from the AF batch tests, the two HRTs of 4 and 8 days were selected for the mesophilic semi-continuous fermentation tests. The HRT values were chosen according to the trend of SCFAs concentration observed in the AF batch tests (paragraph 4.3.2). The two reactors were both characterized by a short acclimation time to reach a steady-state production of SCFAs, namely around 8–9 days in Run 1 (HRT 4 days) and 13 days in Run 2 (HRT 8 days) (Fig. 4.4 a-b). Afterward, the production was maintained stable for the whole duration of the experiment, probably favored by the stability of the pH. The latter was naturally maintained between 6.5 and 7.5, confirming the already observed characteristic of tannery sludge to act as a pH buffer (Tuci et al., 2022). Once the steady state was reached, the COD_{SOL} were 18.2 ± 0.2 g/L and 21.8 ± 0.5 g/L in Run 1 and Run 2 respectively (*t*-test, $\alpha = 0.05$). The difference between these two values was quite contained and, even if higher

HRT allowed for higher OM solubilization, the oxidizing pre-treatment already had the most impact on the COD_{SOL} values.

The process conducted at higher HRT (Run 2) was characterized by higher SCFAs average values (16.0 ± 1 g COD_{SCFA}/L) compared to Run 1 (12.8 ± 0.6 g COD_{SCFA}/L) (*t*-test, $\alpha = 0.05$), both referring to the pseudo-steady state, but the SCFAs/COD_{SOL} ratio were both satisfying (~0.70 COD/COD), demonstrating the effectiveness of the oxidizing pretreatment to favor the acidification in a semicontinuous process.

Although Run 2 performed better in terms of SCFAs concentration than Run 1, its rate and productivity were lower than values in Run 1. This fact agreed with the culture adaptation at lower HRTs, which typically exert selective pressure on the kinetically faster microorganisms that can survive at high-rate processes (Gottardo et al., 2022). The specific fermentation rate, in the pseudo-steady state, in Run 1 was 63.1 ± 0.6 mg COD_{SCFA}/(g VS d), roughly 50% higher than rate in Run 2 (40 ± 1 mg COD_{SCFA}/(g VS d)); similarly, the SCFAs productivity was 2.58 ± 0.12 and 1.63 ± 0.12 g COD_{SCFA}/(L d) in Run 1 and Run 2 respectively (*t*-test, $\alpha = 0.05$). On the contrary, the fermentation yield (Y_{SCFA}^{CSTR}) showed the opposite behavior: 0.32 ± 0.01 vs 0.25 ± 0.01 g COD_{SCFA}/g VS_0 in Run 2 and Run 1, respectively (*t*-test, $\alpha = 0.05$).

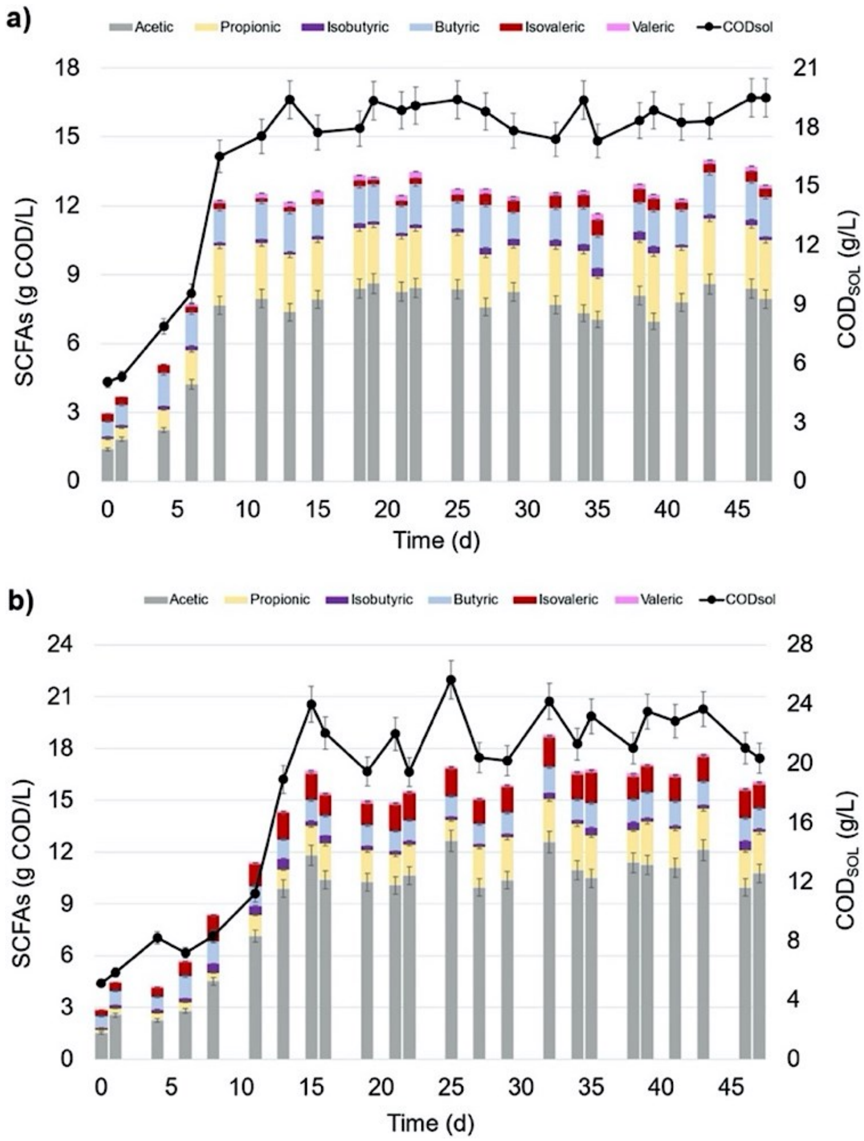


Figure 4.4: Trends of the COD_{SOL} and SCFAs concentrations at 4.0 days (a) and 8.0 days (b) as HRT.

Lower HRT favored the selection of microorganisms' consortia with a high rate but a lower efficiency in the conversion of OM, which required a longer time for efficient conversion into SCFAs. Given the importance of the Y_{SCFA}^{CSTR} its implication in the mass-balance assessment and in the SCFA overall economy of the approach, higher HRT has to be considered in the value chain in order to minimize the amount of unconverted OM. On the other hand, high HRT means high reactor's volume, which in turn affects the energy balance (discussed below). The literature furnished very limited information about the exploitation of tannery sludge as SCFA source. A forerunner article evaluated the combined microwave-H_2O_2 pretreatment on SCFA production only in batch tests (Tuci et al., 2022). The authors reported a maximum SCFA concentration of 26 g COD_{SCFA}/L but the long time required was unsustainable for the technology implementation in a continuous mode. Compared to sewage sludge (also produced within WWTPs), tannery sludge appeared a more profitable source in terms of SCFA production, being partially derived from primary treatments and, in turn, consisting of a more fermentable OM (Ali et al., 2021). A largely adopted practice consisting of the thermal hydrolysis gave lower performances in terms of SCFAs production and OM conversion compared to the tannery sludge: Castro-Fernandez et al. (2023) obtained 8.0 ± 0.9 g COD_{SCFA}/L from acidified thermally pretreated sewage sludge, with a yield of 0.26 g COD_{SCFA}/g VS_0; Zhang et al. (2019) obtained 7.57 and 9.63 g COD_{SCFA}/L in the acidification process of raw and thermally pretreated sewage sludge respectively, with a conversion yield in the range 0.15–0.22 g COD_{SCFA}/g VS_0 in mesophilic conditions. Two recent works reported a range of SCFAs concentration and Y_{SCFA}^{CSTR} in the range 7.3–8.4 g COD_{SCFA}/L and 0.30–0.38 g COD_{SCFA}/g VS_0 respectively, after thermophilic AF of thermally treated sewage sludge (Gottardo et al., 2022; Lorini et al., 2022). Due to the utilization of raw sewage sludge (no pretreatment), Presti et al. (2021) obtained a low SCFAs concentration (< 3.0 g COD_{SCFA}/L) and a yield of 0.38 g COD_{SCFA}/g VSS_0 (volatile suspended solids). This yield was exclusively calculated on the VSS, and it was certainly lower than fermentation yields obtained with pretreated municipal and tannery sludge.

4.3.4 SCFAs composition

The composition of the SCFAs in the effluent remained relatively constant once it reached the steady phase and it was comparable for both HRTs. A remarkable dominance of acetic acid was obtained, probably favored by the utilization of a pretreated sludge, accounting for approximately 62.1 ± 0.6% and 67 ± 1% (COD basis) of the total SCFAs in Run 1 and Run 2 respectively. Propionic acid was next, accounting for 19.7 ± 0.4% (Run 1) and 13.2 ± 0.6% (Run 2); butyric acid was less than 10% in both runs. The other acids were present in lower amounts. The low amount of butyric acid was not surprising as protein-rich substrates like tannery sludge, usually produce less of this acid compared with carbohydrate-rich substrates (Alibardi & Cossu, 2016b). Given that acetic and propionic acids covered 80% of the total COD_{SCFA} (roughly), the SCFAs spectrum was not heavily fragmented, facilitating the downstream processes necessary to concentrate and/or separate the SCFAs in the fermentation broth (Aktij et al., 2020). The downstream steps are necessary to improve the marketability of the SCFAs, at least for applications that require purified products such as pharmaceuticals' manufacturing, plasticizers, production of esters or conversion into fine chemicals (Atasoy et al., 2018). Even though many options have been reviewed (among others extraction and distillation, adsorption, membrane-based technologies), this aspect still needs more investigation and efforts to improve the cost-effectiveness of the technologies.

4.3.5 Fate of the chromium in the semi-continuous process

The presence of Cr was evaluated in the liquid fraction of the oxidized tannery sludge, which was then utilized as carbon source for the two sCSTR. The Cr was partially released after the oxidative pre-treatment, having a concentration of 119 ± 11 mg/L (total Cr); no Cr (VI) was observed, indicating that the H_2O_2 pretreatment was not sufficient to oxidize the released Cr or the Cr initially associated to the sludge. Apart from the stringent regulation on potable waters and the related Cr limit (0.05 mg/L for total Cr; EU Directive 2020/2184), this value (119 mg/L) is rather higher than the maximum discharge limit to the aquatic environment in Europe, which is equal to 5 mg/L for total Cr (Vaiopoulou & Gikas, 2020).

The trend of the Cr is depicted in Figure 4.5, for both investigated HRTs. The peak of the maximum Cr concentration was found at different times (4 and 8 days), according to the adopted HRT. However, after the period of one HRT was covered, the Cr concentration started to progressively decrease until its disappearance in the liquid effluent. This occurred approximately after 20 and 40 days of operation in Run 1 and Run 2 respectively (roughly 5 HRTs in both runs). The literature reported that OM (both colloidal and soluble) can play a significant role in the distribution of Cr, especially in its most common state of Cr(III) (Hao et al., 2022). The leather industry is one of the most important contributors of OM-Cr(III) complexes, usually utilized to obtain the required color, strength or softness of the leather. The OM-Cr(III) structures are usually characterized by high complexation affinity and stability over a wide pH range (Wang et al., 2018). On the other hand, these complexes can be not completely fixed, entering the wastewater, and remaining in the final sludge. Given the presence of Cr in the liquid phase of H_2O_2-treated tannery sludge, the maximum Cr concentration should have been equal to that of oxidized sludge (119 mg/L) in both reactors if no removal phenomena had occurred. The observed decrease in Cr concentration can be explained hypothesizing a two-steps mechanism: (i) the anaerobic consortium was responsible for the OM degradation (and its conversion into SCFAs) and the destruction of the OM-Cr(III) complexes; (ii) the free Cr was presumably removed via precipitation as $Cr(OH)_3$, due to the neutral pH of the fermentation broth and the lime presence (source of OH^-) in the tannery sludge. In support of this interpretation, the Pourbaix diagram shows how Cr is present in the form of hydroxides for pH greater than 3.5 and, in addition, negative redox potential (typical for anaerobic processes) causes the formation of $Cr(OH)_3$ as the most frequent precipitate under neutral pH (Shaker & Albishri, 2014). Independently from the fermentation rate of the two consortia, a complete Cr removal was obtained approximately in 5 HRTs. Before this time, a post-treatment on the fermentation broth could be eventually necessary to remove the Cr and to utilize the SCFA-rich stream for the targeted applications. The Cr removal from industrial wastewater is mainly based on chemical, physic, or combined approach such as chemical precipitation, adsorption, Fenton and Fenton-like oxidation, advanced oxidation processes, and electro-coagulation with post-oxidation (Hao et al., 2022).

These results were useful for supporting two important outcomes: a) the utilization of tannery sludge fermentation liquid did not any have environmental-safety issues related to the Cr initially present in the sludge (at least under the tested conditions); b) the produced SCFAs did not act as organic ligands (in the form of OM-Cr(III)) and they did not contribute to the Cr presence in the liquid phase. Hence, even though SCFAs can have an important role in governing the Cr mobility in the environment, showing a certain potential for complexation with Cr (Duan et al., 2013), the investigated working conditions allowed to valorize the tannery sludge without concerns about the Cr speciation and fate.

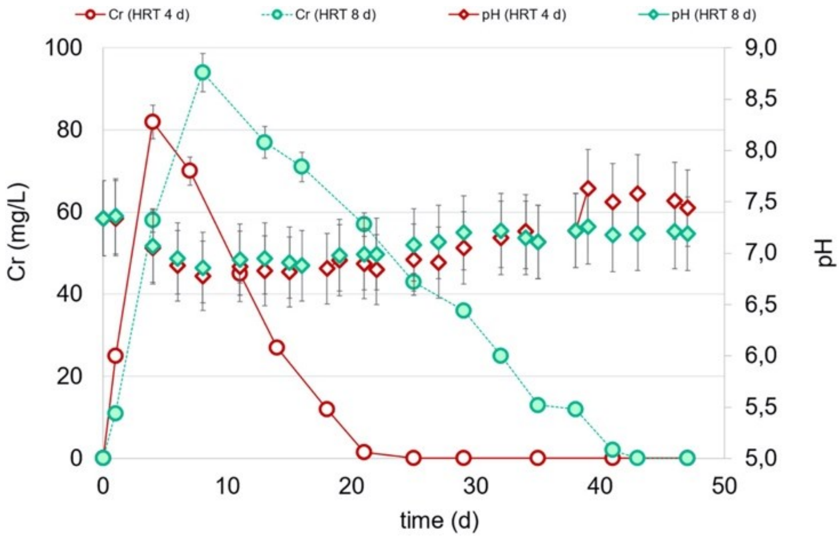


Figure 4.5: Trends of the Cr and pH in the two semi-continuous reactors at different HRT.

4.3.6 BMP tests of the solid-rich pre-fermented tannery sludge

The BMP tests were implemented to assess the potential biogas production from the solid-rich pre-fermented fraction. The importance of this step is related to the possibility of further reducing the sludge's mass and to recover energy from the residue part coming from the AF step. In vision of a full-scale process, the energy generation should be at least sufficient to sustain the thermal energy requirement of AF and AD steps, both conducted at controlled T. The cumulative biogas production and the modeled Specific Gas Production (SGP) of each mesophilic BMP test are reported in Figure 4.6 a-b. The volumetric biogas production was 0.21 ± 0.01, 0.36 ± 0.02, and 0.40 ± 0.02 mL after 30 days in the tests conducted with 0.0, 0.2, and 0.4 g H_2O_2/g TS as dosage, respectively. The data showed again a positive effect of the oxidizing treatment and a progressive increase in the performance with the increasing H_2O_2 dosage. The best performance was obtained at the highest H_2O_2 dosage, which gave a maximum SGP of 0.52 m^3/kg VS (in 30 days). To the best authors' knowledge, the literature does not furnish data about the biogas production potential of treated or untreated tannery sludge (given its landfilling as the only disposal practice) and the H_2O_2 utilization has never been discussed as a potential pretreatment agent to improve sludge biodegradability and biogas production. A recent study described how biogas production can be increased when waste from the leather industry (shavings and sludge) is mixed with other sources (agricultural waste) due to the improved nutrient balance (Simioni et al., 2022). Leather shavings were also subjected to mesophilic anaerobic digestion with tannery sludge (Agustini et al., 2018), showing a remarkable benefit in biogas production when tanning agents were present (chromium among others).

The modeled SGP provided some noteworthy information. With 20 days as HRT, the untreated solid-rich fraction was characterized by an SGP of 0.25 m^3/kg VS. Considering the same HRT, the dosage of 0.2 and 0.4 g H_2O_2/g TS increased the SGP up to 0.43 and 0.48 m^3/kg VS respectively, doubling the biogas production compared to the solid-rich fraction utilized without the post hydrolysis. The obtained SGP corresponded to 80% (roughly) of the maximum biogas achievable; if HRT > 20 d had been chosen, this percentage would be increased due to the prolonged biodegradation of the OM, concomitant with alkalinity and biogas production (Liu et al., 2018a). On the other hand, higher HRTs brings to the increase of reactor's volume and associated costs. The choice of the HRT is a matter of compromise between the biogas potentially obtainable and the volumetric limitation to be established through a detailed mass-energy balance, such as the one in paragraph 4.3.7. The CH_4 content was approximately similar in all the text (independently from the H_2O_2 dosage), being in the range 51–56 % v/v in the whole length of the BMP tests. These results are in line with the available literature SGP values and CH_4 content of tannery sludge (up to 0.20 m^3/kg VS and 50–60 % v/v respectively), which was not subjected to any pretreatment step (Akyol et al., 2015). Not pretreated secondary tannery sludge (with lower biodegradability compared to primary sludge) exhibited a biogas production in the range 0.11– 0.22 m^3/kg VS (~55% CH_4 v/v) (Mpofu et al., 2020). Compared to sludge utilization, mixture of tannery and slaughterhouse wastewater produced more biogas (0.23–0.36 m^3/kg VS as SGP) but with considerably lower CH_4 level (20–45 % v/v) (Mpofu et al., 2021).

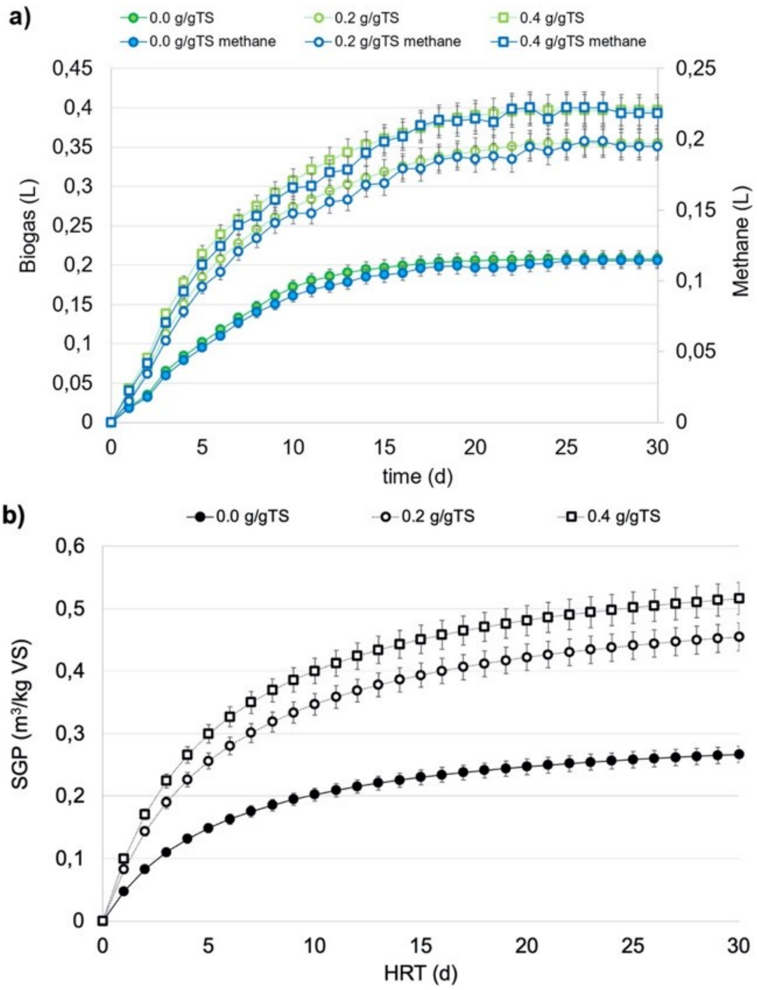


Figure 4.6: Evolution of produced biogas (a) and theoretical SGP values (b) modelled in the three experiments at different H_2O_2 dosages under mesophilic conditions.

4.3.7 Mass-energy balance and economic consideration

The mass-energy balance was performed starting from a production of 37,000 ton/y of tannery sludge; according to its features reported in paragraph 4.2.2, the TS and VS flow rates are 99,065 kg TS/d and 70,419 kg VS/d (currently disposed of in landfills). The design choices used for the presented calculations are summarized in Table 4.1.

Table 4.1: Summary of the design choices made and the motivations behind them.

Design choice	**Motivation**
H_2O_2 dosage of 0.4 g/g TS	Better performance between tested dosages
HRT = 8 d	Better acidification yield
T = 37 °C	Good performance with lower energy consumption

In the proposed approach (Figure 4.7), this sludge is firstly subjected to a pre-hydrolysis step where water tap water (1,308,089 kg/d) and H_2O_2 (39,625 kg/d; at 0.4 g H_2O_2/kg TS) are added, while also being heated to 37 °C, which is the T maintained for the subsequent AF process.

After the oxidizing pretreatment, the sludge flow rate is 1,467,070 kg/d (roughly at 7% w/v of solids' content), entering a CSTR with a volume of 11,737 m^3 (HRT 8 d). In this stage, the VS flow rate is reduced to 58,236 kg VS/d, due to a small aliquot of generated gas (SGP 0.1m^3/kg VS; Micolucci et al., 2020). The resulting SCFAs-rich effluent (8.00 ton SCFAs/d, due to the P_{SCFA}^{CSTR} of 1.63 g COD /(L d); Run 2, Table 4.2) is then treated through a solid/liquid separation unit, obtaining two streams: the liquid fraction (1,142,392 L/d; employed to extract the SCFAs), and the solid-rich residue (roughly at 25% w/v of TS content). The latter has to be diluted and treated (post-hydrolysis at 0.4 g H_2O_2/kg TS dosage) before entering the AD step. The flow rate of this residue is 324,678 kg/d, which becomes 1,088,150 L/d after dilution (~7% w/v TS content). The volume and the operating conditions of the CSTR anaerobic digester are established according to the BMP results (SGP 0.48 m^3/kg VS; Volume 21,763 m^3, HRT 20 days, 37 °C). The consequent amount of produced biogas is 26,115 m^3/d, corresponding to a VS content of 29,368 kg/d (based on the average composition of 55%− 45% v/v of CH_4-CO_2 respectively). The residual digestate was also subjected to another liquid/solid separation before being dried up to a TS content of 83% w/v (approximately similar to the initial value). Overall, the final amount of dried sludge to be disposed of in the landfill is 56,996 kg/d, corresponding to less than 50% of the tannery sludge in input.

In the energy balance, the option of employing the biogas in a boiler was chosen as the final drying step (290,906 MJ/d); the utilization of a combined heat and power unit (CHP) to valorize the AD-derived biogas does not allow to obtain a positive thermal balance. Since the boiler has an efficiency of 90%, the production of 540,875 MJ/d of thermal energy has been estimated. The required thermal energy for the whole process is around 498,694 MJ/d, according to the chosen mesophilic conditions for the AF and AD (energy and cost-saving approach), and the final drying unit. Hence, the thermal balance was characterized by a net energy production of 42,181 MJ/d, completely covering the needs of the process without additional costs. It is noteworthy that almost 60% of the required thermal energy is necessary for the final drying step.

In terms of the economic feasibility of the whole value chain, the current disposal cost (37,000 tons/y in landfilling) sustained by the tannery industries is around 9,250,000 €/y. With the implementation of this flowchart, the main costs would be represented by the utilization of the H_2O_2 (6,704,702 €/y), tap water (347,666 €/y), and electricity (67,117 €/y), while the disposal costs would drop to 4,417,162 €/y. The profit instead would derive from the sale of the SCFAs which would be around 4,957,982

€/y, in other words an economic saving of 2,671,335 €/y compared to the current scenario. Table 4.3 summarizes all the energy and economic-related items discussed above. In addition to the economic factors, this process has different environmental benefits in terms of greenhouse gases (GHG) emissions. In particular, fossil fuel energy is totally excluded since CH_4 is produced from the sludge itself and there is no additional CO_2 production from external C-source; also, unintentionally CH_4 release (from old landfills) is surely reduced due to the obtained sludge reduction.

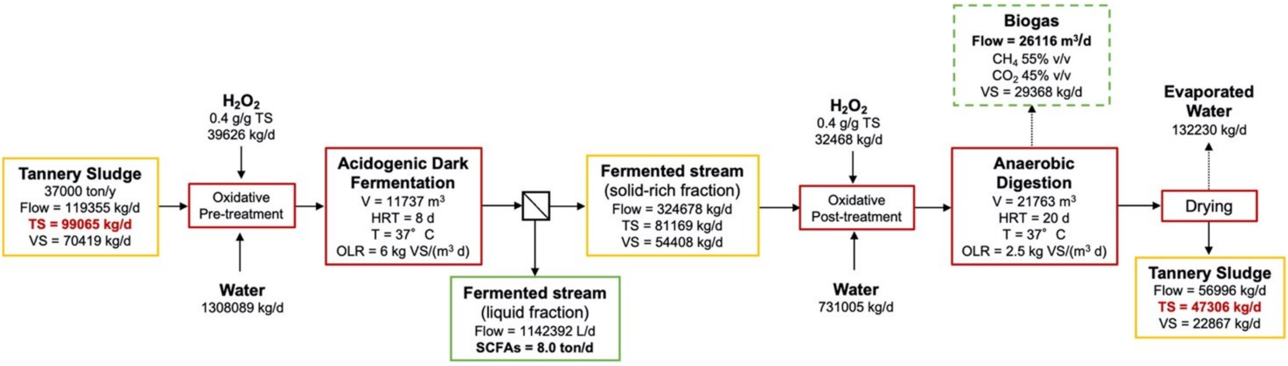


Figure 4.7: Mass balance of the whole value chain in perspective of tannery sludge utilization.

Table 4.2: Summary of the effluent's characteristics and reactors' performances under batch and semi-continuous acidogenic fermentation conditions.

Parameter	**Unit**	**Batch** ***(0.4 g H_2O_2/g TS)***					**sCSTR** ***(0.4 g H_2O_2/g TS; 40°C)***	
		T (°C)					HRT (days)	
		15	25	30	40	50	4.0	8.0
COD_{SOL}	g/L	17.9 ± 0.2	20.6 ± 0.3	20.4 ± 0.7	22.3 ± 0.5	22.7 ± 0.1	18.2 ± 0.2	21.8 ± 0.5
COD_{SCFA}	g/L	9.5 ± 0.3	13.2 ± 0.4	13.5 ± 0.5	16.4 ± 0.7	16.3 ± 0.3	12.7 ± 0.1	16.0 ± 0.3
COD_{SCFA}/COD_{SOL}	g/g	0.53 ± 0.3	0.64 ± 0.5	0.66 ± 0.4	0.73 ± 0.2	0.72 ± 0.1	0.69 ± 0.01	0.73 ± 0.01
Acetic acid	g COD/L	4.2 ± 0.1	5.8 ± 0.6	6.1 ± 0.5	7.8 ± 0.3	7.7 ± 0.3	7.93 ± 0.11	10.66 ± 0.21
Propionic acid	g COD/L	1.6 ± 0.3	2.2 ± 0.3	2.1 ± 0.4	2.7 ± 0.2	3.2 ± 0.2	2.51 ± 0.06	2.11 ± 0.09
Butyric acid	g COD/L	1.9 ± 0.5	2.8 ± 0.3	2.9 ± 0.1	3.0 ± 0.3	2.7 ± 0.3	1.54 ± 0.05	1.25 ± 0.03
Valeric acid	g COD/L	1.0 ± 0.3	1.6 ± 0.6	1.6 ± 0.2	1.8 ± 0.4	1.7 ± 0.4	0.21 ± 0.01	0.15 ± 0.01
Isovaleric acid	g COD/L	0.9 ± 0.3	0.8 ± 0.2	0.7 ± 0.3	1.1 ± 0.2	1.0 ± 0.2	0.36 ± 0.03	1.47 ± 0.03

Y_{SCFA}^{batch}	g COD_{SCFA}/(g VS_0)	0.16 ± 0.01	0.22 ± 0.03	0.23 ± 0.01	0.28 ± 0.03	0.28 ± 0.02	-	-
q_{SCFA}^{CSTR}	mg COD_{SCFA}/(g VS d)	-	-	-	-	-	63.1 ± 0.6	40 ± 1
Y_{SCFA}^{CSTR}	g COD_{SCFA}/(g VS_0)	-	-	-	-	-	0.25 ± 0.01	0.32 ± 0.01
P_{SCFA}^{CSTR}	g COD_{SCFA}/(L d)	-	-	-	-	-	2.58 ± 0.02	1.63 ± 0.03
N-NH_4^+ release	mgN-NH_4^+/g TKN	-	-	-	-	-	42 ± 1 %	53 ± 2 %

Table 4.3: Energy and economy-related items in the new scenario of tannery sludge valorization.

Parameter		**Unit**	**New scenario**	**Current scenario**
Tannery sludge production		ton/y	37,000	37,000
SCFAs production		ton/y	2,879	-
CH_4 production*		m^3/y	5,170,890	-
Required Energy	Acidogenic Fermentation	MJ/d	119,449	-
	Anaerobic Digestion	MJ/d	88,340	-
	Drying	MJ/d	290,906	-
Produced Energy		MJ/d	540,875	-
Cost**	Disposal	€/y	4,417,162	9,250,000
	Tap Water	€/y	347,666	-
	H_2O_2	€/y	6,704,702	-
	Electrical Energy	€/y	67,117	-
Revenue		€/y	4,957,982	-

*from SGP 0.48 m^3/(kg VS) and CH_4 content of 55% v/v.

**The electrical energy is related to the SCFAs extraction and recovery; the disposal is related to the tannery sludge.

Table 4.4: Reference parameters and boundary conditions for energy balance.

Parameter	Unit	Value
Boundary Conditions		
Operative temperature anaerobic processes	°C	40
Water temperature	°C	20
Air temperature	°C	20
Ground temperature	°C	25
Heat Transfer Coefficient		
Outer concrete reactor wall	$W/(m^2\ °C)$	0.7
Inner concrete reactor wall	$W/(m^2\ °C)$	1.2
Floor	$W/(m^2\ °C)$	2.85

4.4 Conclusions

A first-of-kind approach for the tannery sludge valorization and related outcomes has been proposed in this study. Tannery sludge was effectively utilized as a renewable carbon source to produce SCFAs as added-value products along with biogas. Hydrolysis and AF batch tests highlighted the optimal dosage of H_2O_2 (0.4 g H_2O_2/g TS) for pre-treatment and T (40 °C) for acidification. The following mesophilic experiments conducted in the sCSTR at an HRT of 8 days allowed to obtain a SCFA-rich stream at a content of 16.0 g COD_{SCFA}/L, dominated by acetic acid (67% COD basin), and corresponding to an acidification yield of 0.32 g COD_{SCFA}/g VS_0. Environmental or toxicity issues concerning the Cr presence in the fermentation broth were not significant, since Cr was not transferred from the sludge to the liquid matrix, despite its partial release in the oxidizing pretreatment.
Given the quantified biogas production (0.48 m^3/kg VS) from the solid-rich residue, separated after the AF step, the proposed multi-steps process has the potential to strongly reduce the tannery sludge of more than 50% without the need of external fossil fuel sources for the energy requirements. As a consequence, the landfilling, currently the only disposal practice, can be significantly downsized with a yearly economic saving higher than 2,600,000 € for the tannery industries and associated water utilities, compared to the existing state. Even though a new direction for tannery sludge management has been indicated in this study, a margin of improvement is still possible, especially on the hydrolysis step (needed amount of the oxidizing agent and associated cost), and on the acidification yield (roughly 30% of the VS has been converted into SCFAs). Different pretreatment (including thermal) or even H_2O_2-combined ones addressed to increase the VS solubilization and to decrease the H_2O_2 dosage should be deeply investigated along with other reactor's configuration, such as decoupling HRT and SRT to increase the SCFAs yield and productivity.

CHAPTER 5: AF process enhancement with zeolite addition

One further step has been to enhance the efficiency of the AF process, both through changes in the process itself and by incorporating other materials. In particular, this chapter focuses on the response of the AF process when zeolites, a highly promising material that is well known for its absorption capabilities for heavy metals, are added.
This chapter is from the published paper in the journal Environmental Research (Elsevier) titled "***Tannery sludge valorization through zeolite-assisted anaerobic process for short-chain fatty acids (SCFAs) production***" (https://doi.org/10.1016/j.envres.2023.118046) written by Giulia Adele Tuci, Francesco Valentino, Paolo Pavan, Marco Gottardo.

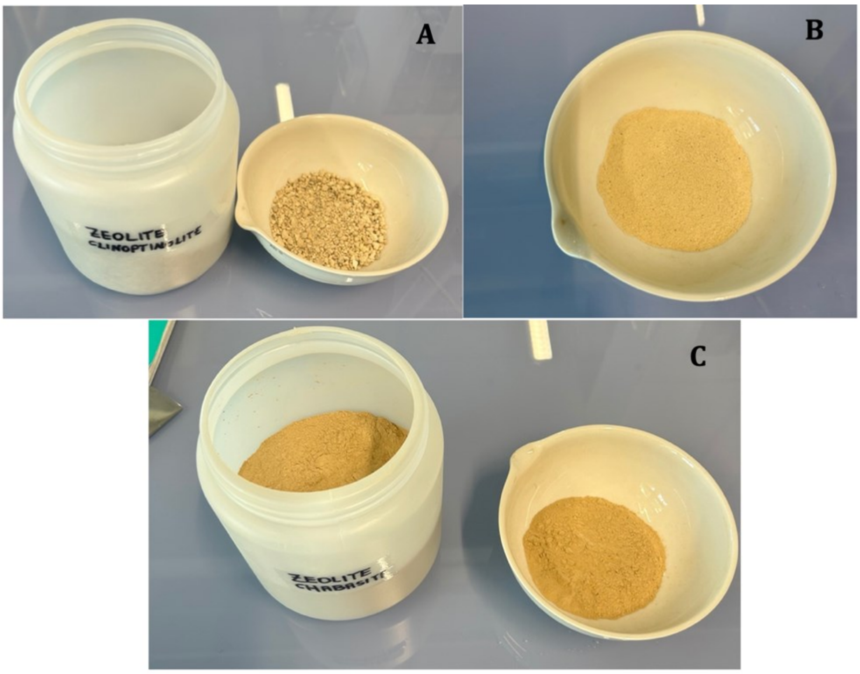


Figure 5.1: Zeolites. A: clinoptilolite, B: pulverized clinoptilolite, C: chabazite.

5.1 Introduction

Tanning is the process that transforms raw hides into leather. The aim is to create a product that is resistant to microbial degradation, thermal stress, and humidity using a sequence of mechanical and chemical processes (Alibardi & Cossu, 2016a). This practice in most cases worldwide (90%) employs trivalent chromium sulfate (Cr(III)) as a tanning agent, due to its higher effectiveness at increasing the mechanical strength of the final product (Abreu & Toffoli, 2009), impacting, on the other hand, on the toxicity of the resulting waste. The wastewater from tanneries is collected and then treated in wastewater treatment plants (WWTP), leaving tannery sludge as the main resulting waste. This sludge is characterized by the presence of Chromium as well as various chemical compounds such as sulfides, organic pollutants, several metals, and pathogenic microorganisms (Zhao et al., 2022), and consequently the Italian legislation (UNIC, 2021) requires it to be disposed of in second-class type B controlled landfill (D. Lgs. 04/06) exclusively. Therefore, new valorization routes to exploit its potential are still almost unexplored.

Landfilling however is a very environmentally impactful practice, consequently, different ways to reduce the impact and volume of this waste need to be investigated.

Anaerobic digestion (AD) is a well-established method to produce biogas from organic waste through four stages (hydrolysis, acidogenesis, acetogenesis, and methanogenesis). In the initial stages before methanation, the process is referred to as anaerobic fermentation, employed to produce other added-value products, such as short-chain fatty acids (SCFAs), while maintaining a common objective with anaerobic digestion: the generation of high-value products and the reduction of final waste volume. This microbial technology proves particularly appealing for managing tannery sludge, an ideal candidate due to its abundance and significant environmental impact (Tuci et al., 2023). The reduction of tannery sludge volume is consequently highly attractive, making anaerobic digestion and fermentation valuable solutions for addressing both environmental concerns and waste disposal costs. The first stage, the hydrolysis of the organic matter, is key as it directly affects the efficiency of the following steps. With solid-rich waste, such as tannery sludge, due to high-molecular-weight substances, hydrolysis faces limitations that can lead to reduced acidification yield and an excessively fragmented SCFA composition (Achouri et al., 2021; Zhang et al., 2019a).

Consequently, to increase the solubilization of the organic matter a mild thermal pretreatment was chosen due to the positive effects on similar substrates (Zhen et al., 2017) and its increased effectiveness on proteins (Li & Noike, 1992), which are prevalent in this sludge. Moreover, the use of thermal pretreatment instead of external chemical agents was selected as a potential method to reduce costs and promote eco-friendliness when considering the scale-up of this technology.

Tannery sludge contains both high levels of ammonia (32.8 ± 0.9 g N/kg TS), (Tuci et al., 2023) and Cr (19155 ± 310 mg Cr/kg TS), which can be inhibitory to the anaerobic digestion process, as they can disrupt microbial activity, leading to reduced efficiency and process instability (Montalvo et al., 2020). To mitigate this issue zeolites have been selected due to their ability to absorb these compounds (Montalvo et al., 2012), thus helping mitigate these issues and promoting a more favorable environment for anaerobic microorganisms, which can ultimately lead to increased process efficiency.

Zeolites are crystalline aluminosilicates and hydrates that possess tiny molecular-sized pores allowing molecules of a specific size to pass through. They are primarily composed of elements such as aluminum (Al), oxygen (O), and various metals like titanium (Ti), tin (Sn), zinc (Zn), and others (Montalvo et al., 2020), and their structure consists of a repetition of SiO and AlO tetrahedra bonded together by shared oxygens (Kesraoui-Ouki et al., 1994).
This material is of great interest not only due to its non-toxicity but also to its easy availability, low cost, and excellent physical and chemical properties (Rodríguez-Iznaga et al., 2022). Zeolites are characterized by two main aspects, namely their ability to absorb and lose water and other particles of the appropriate dimension, and their ion exchange property (Montalvo et al., 2012). In particular, the ability of zeolites to effectively absorb Cr_3^+ and NH^+_4 ions is due to their net negative charge which allows for a strong affinity for transition metal cations and other positively charged ions (Barros et al., 2004).
In this research chabazite and clinoptilolite were selected due to their promising absorption properties for ammonia and heavy metals (Montalvo et al., 2012) and their easy availability.
This study aims to analyze tannery sludge as a substrate for AD, focusing on the combined effect that these two types of chosen zeolite and the mild thermal pretreatment have on the different fermentation parameters, namely the ammonia, soluble chemical oxygen demand (COD_{SOL}), SCFAs production, and Cr release in the fermentation broth. The operating parameters for semi-continuous fermentation tests were determined based on previous tests from this research group (Tuci et al., 2023), including two hydraulic retention times (HRT; 4–8 days). The fermentation parameters were compared to the "blank" condition without the addition of zeolite to assess the impact of this material.

5.2 Materials and methods

5.2.1 Substrate's characteristics and inoculum

The carbon source for this study was a blend of primary and secondary tannery sludge collected from the wastewater treatment plant (WWTP) located in Montebello Vicentino, in the northeastern region of Italy, where wastewater originating from over 20 tannery plants is treated. The treatment process involves both primary treatments, utilizing alkaline flocculation, and secondary biological treatments through an anoxic/aerobic process. After these steps, the primary and secondary tannery sludge are mixed and undergo a dewatering and drying procedure before landfilling (Alibardi & Cossu, 2016a). The resulting dried sludge is characterized by the following chemical and physical characteristics: 830 ± 14 g of total solids (TS)/kg and 590 ± 4 g volatile solids (VS)/kg; COD of 793 ± 18 g COD/kg TS; total phosphorus (TP) and total Kjeldahl nitrogen (TKN) are 8 ± 1 g P/kg TS and 32 ± 1 g N/kg

TS, respectively. The total chromium content is 19155 ± 310 mg Cr/kg TS, and the protein content is 20 % (w/w), in line with those found in Kuligowski et al. (2023).

5.2.2 Zeolite

The natural zeolites employed in this research were clinoptilolite (CL) and chabazite (CH).

The Clinoptilolite was characterized by a 70%–80% clinoptilolite content, and water retention of 33.7%. Since 90% of the material had a granulometry >1 mm it was grounded with a mortar and pestle before usage.

The Chabazite used was made of 64% chabazite, with a water retention of 37.8%, for what concerns the granulometry, 98% of the tested mineral material measured less than 0.15 mm, and was consequently used as-is. For the determination of metal content in each mineral matrix, the samples were initially disintegrated through acid digestion. The digested samples were analyzed using an ICP-MS NexION 350X (PerkinElmer) coupled with the seaFAST autosampler (ESI). For confirmation, major elements (Al, Si, Fe, Mg, K, Na, Ca) were analyzed using an ICP-EOS Optima 5300 DV (PerkinElmer).

The results are presented in Table 5.1. Based on the values presented in the table, it was possible to determine the Si/Al molar ratio of the tested zeolitic rocks. This ratio is of extreme interest as it proportionally influences the chemical and physical properties of zeolites (Jiang et al., 2018; Li et al., 2017). As the Si/Al ratio increases, the zeolite surface becomes more hydrophobic; conversely, as the ratio decreases, the zeolite surface becomes more hydrophilic (Jiang et al., 2018; Wang et al., 2019). In general, zeolites can be categorized based on the Si/Al ratio into low-silica zeolites (Si/Al molar ratio between 1 and 3), medium-silica zeolites (Si/Al molar ratio between 3 and 10), and high-silica zeolites (Si/Al molar ratio greater than 10) (Csicsery, 1984). Based on the data in Table 5.1 and it was found that all tested zeolitic materials had a Si/Al molar ratio of approximately 3.3 for Chabazite and 4.3 for Clinoptilolite.

Table 5.1: Values in milligrams per gram (mg/g) for the main elements in the respective minerals.

Element (mg/g)	Clinoptilolite (CL)	Chabazite (CH)
Aluminum	64.5	80.3
Manganese	0.88	1.34
Silicon	294	253
Sodium	13.06	4.35
Magnesium	7.81	6.71
Potassium	13.24	38.86
Calcium	20.21	45.83
Iron	20.04	25.55

5.2.3 Semi-continuous acidogenic fermentation

The chosen dosage of zeolite was 0.26 g/gVS, as it yielded the most SCFAs in Silva et al. (2021) and the best heavy metal absorption in Zorpas et al. (2002). Moreover, a mild thermal pretreatment was chosen for the sludge, which was performed by heating diluted tannery sludge and tap water (S/I ratio of 7.0 w/w VS basis) for 20 h at the temperature of 70 °C while being continuously stirred. This protocol was adapted from Gottardo et al. (2022). The thermally pretreated sludge was then used as feedstock for all the semi-continuous tests. Overall, three rounds of two parallel semi- continuous stirred tank reactors (sCSTR) with a working volume of 1.5 L were inoculated with the tannery sludge at a concentration of 70 g TS/L after dilution with tap water. The sCSTRs were maintained under thermophilic conditions (50 ± 2 °C) with water recirculation into an external thermostatic jacket. After a pre-established lag phase of one week, the reactors were fed once per day according to the chosen HRTs (4.0 and 8.0 days). The working conditions for all runs are summarized in Table 5.2. In particular, reactors R-Ia and R-Ib were simply fed with thermally pretreated tannery sludge, while the zeolite-amended runs were fed the same amount of thermally pretreated tannery sludge with the addition of the two types of zeolite with a concentration of 13 g/L (corresponding to 0.26 g/gVS; Chabazite for R-IIa and R-IIb, and Clinoptilolite for R-IIIa and R-IIIb). A fixed aliquot was withdrawn daily from the reactors according to the chosen HRT and was used for the effluent characterization, in terms of COD_{SOL}, SCFAs, $N–NH^{+}_{4}$, pH, and Cr. The pH was maintained around 7 by the sludge itself which has a pH buffer characteristic due to the presence of lime ($CaCO_3$) (Tuci et al., 2022). The biogas production during the fermentation was measured utilizing a gas counter attached to the bioreactors (Idea bioprocess SRLS technology).

Table 5.2: Operating conditions of the six runs of acidogenic fermentation of thermally pretreated tannery sludge, with dosages of Clinoptilolite (CL) and Chabazite (CH).

CSTR Run	HRT (d)	OLR (g VS/l d)	CL (mg/l)	CH (mg/l)
R-Ia	4	12.6	0	0
R-Ib	8	6.05	0	0
R-IIa	4	12.6	0	13 g/L
R-IIb	8	6.05	0	13 g/L
R-IIIa	4	12.6	13 g/L	0
R-IIIb	8	6.05	13 g/L	0

5.2.4 Analytical methods

The analyses were conducted following the Standard Methods (Apha Awwa, 2012) for quantifying various parameters, including TS, VS, CODSOL, ammonia, TKN, and both forms of chromium (Cr(III) and Cr (VI)). To ensure accurate measurements, the samples were subjected to centrifugation and filtered using 0.2 μm porosity filters before the ana- lyses, except for TS, VS, and TKN samples. The concentration of NH^{+}_{4} was determined using an ion-selective electrode (ISE, HI4101) with a PTFE membrane permeable to NH_3 gas. The quantification of crude protein amounts was performed according to the TKN and then multiplying the result by the conversion factor of 6.25 (ISO 937:1978(en)). Total Cr quantification was carried out with an inductively coupled plasma-mass spectrometry (ICP-MS) NexION 350X (PerkinElmer) coupled with the autosampler seaFAST (ESI). The Cr(VI) analyses were performed with the direct spectrophotometric determination Cr(VI) by diphenylcarbazide (Apha Awwa, 2012); the absorption measurements were carried out at 540 nm wavelength.

For the analysis of short-chain fatty acids (SCFAs), an Agilent 6890 N gas chromatograph (GC) equipped with a flame ionization detector (FID) was employed, maintaining a temperature of 250 °C. The stationary phase utilized was an Agilent J&W DB-FFAP fused silica capillary column (DB-FFAP; 15 m length, 0.53 mm ID, 0.5 mm film). Hydrogen was used as the carrier gas with a split ratio of 20:1, and the inlet was set in split mode. The GC instrument operated using a ramp temperature, starting from 80 °C to 100 °C, and increasing at a rate of 10 °C per minute. Before GC analysis, each sample underwent centrifugation at 4500 rpm for 10 min, and the supernatant was filtered through a 0.2 μm acetate-cellulose filter porosity (Whatman).

The biogas produced during the fermentation was analyzed for the CO_2 percentage through a portable infrared gas analyzer GA2000TM (Geotechnical InstrumentsTM).

5.2.5 Calculation and statistical analysis

In the sCSTR experiments, the acidification performances were evaluated by the following parameters: the ratio between the SCFAs and the COD_{SOL}, the fermentation yield (Y_{SCFA}^{CSTR}) according to equation (1), and rate (q_{SCFA}^{CSTR}) according to equation (2). Ammonia release, which was calculated according to equation (3), and SCFAs volumetric productivity (P_{SCFA}^{CSTR}; Simonetti et al., 2021), as per equation (4), were also quantified.

(1) $Y_{SCFA}^{CSTR} = [COD_{SCFA}]_t / VS_0;$

(2) $q_{SCFA}^{CSTR} = [COD_{SCFA}]_t / (VS_0 \times HRT);$

(3) $N\text{-}NH_4^+$ release (%) = $[N\text{-}NH_4^+]_t / TKN_0;$

(4) $P_{SCFA}^{CSTR} = [COD_{SCFA}]_t / HRT.$

The analysis of the BMP tests with regards to the specific gas production (SGP) (m^3_{biogas}/kg VS), was assessed as a function of the modeled HRT to be adopted in the AD process, according to the methodology described in (Angelidaki et al., 2009).

The *t*-test, the analysis of variance (ANOVA), and Tukey HSD post hoc tests were conducted to detect the results' significance. Shapiro–Wilk test was employed to assess if the data were normally distributed; Bartlett test and F-test were used to determine the homoscedasticity between the data groups. All the statistical analyses were performed with R open-source program (The R Foundation for Statistical Computing, version 4.0.3).

The parameters for the mass balance consider a production of 37000 tons/year of tannery sludge, the total amount generated from the two tannery WWTPs in the considered area (Medio Chiampo S.p.a. and Acque del Chiampo S.p.a.). Reference parameters and boundary conditions are given in a previous work (Valentino et al., 2019).

5.3 Results and discussion

5.3.1 Production of SCFAs in the semi-continuous CSTR

Overall, this study involves six experimental runs, wherein each run featured two parallel reactors with distinct HRTs of 4 (a) and 8 (b) days. The study explored three conditions: no zeolite added (R-Ia and b), chabazite added (R-IIa and b), and clinoptilolite added (R–III a and b).

The HRT values of 4 and 8 days were chosen according to the trend of SCFA concentration observed in a previous experiment on this substrate (Tuci et al., 2022), and were also selected bearing in mind that high HRTs are linked to larger reactor volumes. Therefore, opting for a lower HRT value has also been influenced by a cost-effective strategy for potential full-scale deployment.

The acclimation time required to reach a steady SCFA production was quite short and homogeneous for all the tests, namely 9 and 12 days for R-Ia and R-Ib, respectively, 9 days for both R-IIa and R-IIb; and 7 and 11 days for R-IIIa and R-IIIb, respectively. This is likely due to the fermentative bacteria already present in the dried sludge and the stability of the pH, which was naturally maintained between 6.8 and 7.8 in all runs, due to the buffering capacity of tannery sludge (Tuci et al., 2022).

Once the steady state was reached the parameters all remained quite stable, except for the SCFA composition in some tests.

For what regards the COD_{SOL} values were significantly different between R–I and the tests with added zeolites (R–II and R–III), especially for the higher HRT adopted which led to a higher solubilization of the organic matter (OM) (Table 5.3). Namely tests R-Ia, R-IIa, and R-IIIa showed values of 23.1 ± 0.4, 19.4 ± 0.2, and 20.4 ± 0.4 g/L, respectively. According to the doubled HRT, the COD_{SOL} in R-Ib, R-IIb, and R- IIIb was equal to 28.2 ± 0.5, 22.1 ± 0.3, and 23.0 ± 0.2 g/L, respectively. It is apparent for both HRTs that the runs with added zeolite showed lower COD_{SOL} values, this is explained by the already mentioned retention capability of the zeolites, which consequently absorbed a portion of the soluble OM. The absorption of the OM, and particularly proteins, by the zeolites has already been observed in the literature (Montalvo et al., 2012; Tang et al., 2023). Moreover, the COD removal has been observed to be slightly higher for higher particle dimensions, which might explain why the clinoptilolite, which was pulverized through a mortar, was less effective than chabazite (Fernandez et al., 2008).

These values also highlight the effectiveness of the mild thermal pretreatment in solubilizing the OM, as shown in Zhang et al. (2019) where the thermally hydrolyzed sewage sludge had a COD_{SOL} almost doubled compared to raw sludge.

The HRT had a strong impact on SCFA production, as shown in Fig. 5.2. The average SCFA values generally increased when higher HRT was adopted, being equal to 16671, 17026, and 14582 mgCOD/L in R-Ia, R- IIa, and R-IIIa respectively; by increasing the HRT to 8.0 days, the average SCFAs values showed a statistically significant increase, reaching values of 20260, 20203, 17110 mgCOD/L in R-Ib, R-IIb and R- IIIb respectively (ANOVA test, Tukey HSD post hoc test, $\alpha = 0.05$). The tests without zeolite addition were overall the best for productivity, while especially for the lower HRT the production in test R–III was significantly lower than the other two runs.

The lower content of SCFAs in the tests with the zeolites might be due to their absorption ability which has been observed (Nordell et al., 2013; Tang et al., 2023) to also include SCFAs. Namely in

Nordell et al. (2013) the amount of volatile fatty acids produced from slaughterhouse waste was reduced by more than 3 times with the addition of zeolites, especially concerning acetic acid.

However, the tests without zeolite addition (R-Ia and b) yielded a higher amount of SCFAs than their mesophilic equivalents in Tuci et al. (2023), which reached a mean total value of SCFAs of 12761 mgCOD/L for the HRT of 4 days, and 16194 mgCOD/L for the higher HRT, this is likely due to the higher effectiveness of the thermal pretreatment which allowed for a higher COD solubilization, as already discussed.

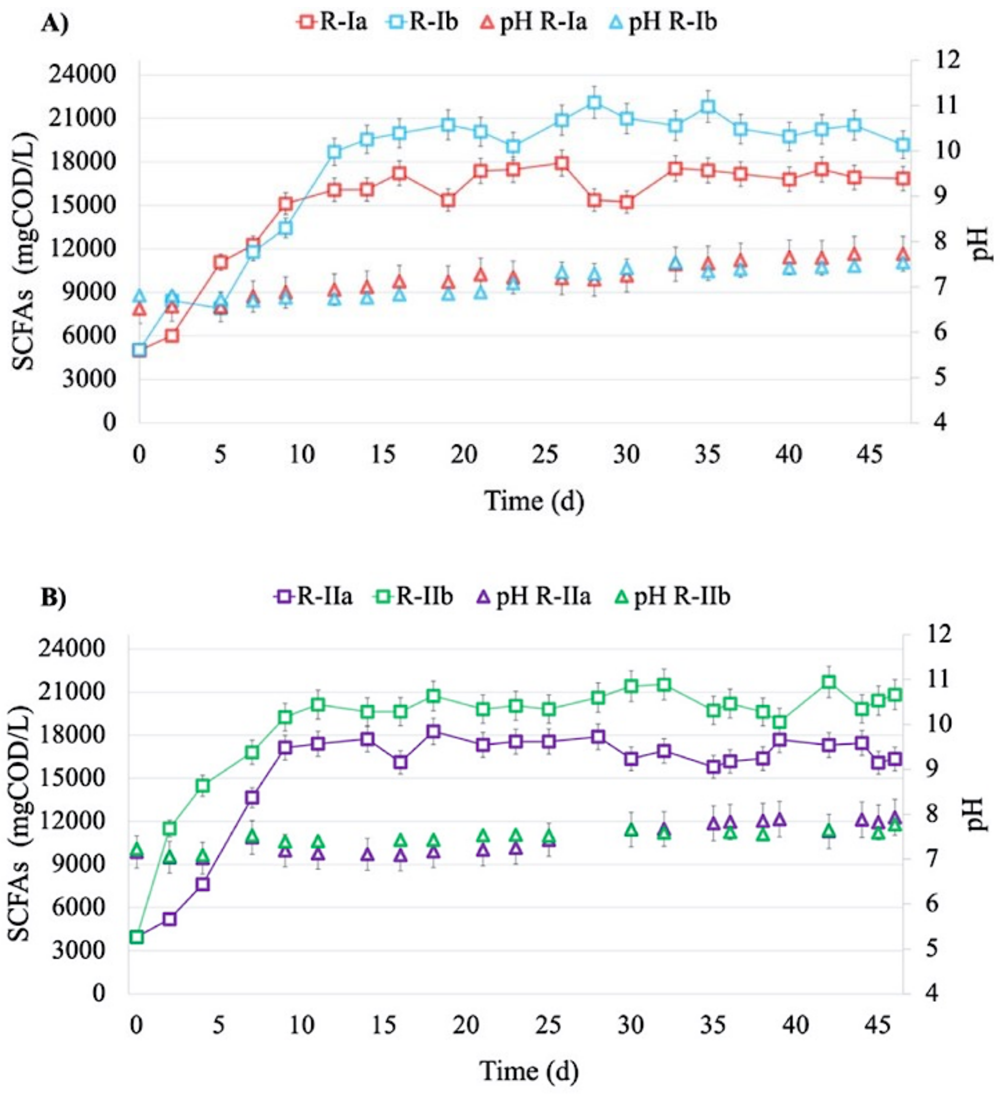
Regarding the COD_{SOL} utilization (% COD/COD), both R–I and R–III had similar SCFAs/COD_{SOL} ratios (~0.70 COD/COD), showing a satisfying result in line with our previous experiments (Tuci et al., 2023), and better performing than similar tests on comparable substrates, such as Zhang et al. (2019) where the thermally pretreated sludge obtained a value of 0.52 COD/COD. Conversely, test R–II was characterized by a higher ratio of 0.87 COD/COD (R-IIa) and 0.92 COD/COD (R-IIb), showing a significantly improved acidification efficiency aided by the addition of Chabazite, resulting in more than 90% of the dissolved COD being converted into SCFAs.

The fermentation yield is a crucial parameter as it is connected to the potential recovery of SCFAs from the carbon source, particularly concerning the mass balance evaluation, within a real-world operational setting.

For all tests, the yield was higher for the higher HRTs, while the rate showed the opposite behavior. This supports the idea that lower HRTs encourage the selection of microorganisms with faster kinetics, suitable for high-rate processes, but less efficient in the conversion of OM. Moreover, in all the runs these parameters were quite homogeneous, namely the rate ($mgCOD_{SCFA}$/(gVSd)) was 83 ± 1, 84 ± 1, and 72 ± 1 for runs R-Ia, R-IIa and R-IIIa respectively; and 50.2 ± 0.6, 50.0 ± 0.4, and 42.0 ± 0.3 for R-Ib, R-IIb, and R-IIIb respectively. The yield (g COD_{SCFA}/g VS_0) was 0.33 ± 0.01, 0.34 ± 0.01, 0.29 ± 0.01 for R-Ia, R- IIa and R-IIIa respectively, and 0.40 ± 0.01, 0.40 ± 0.01, and 0.34 ± 0.01 R-Ib, R-IIb and R-IIIb. Confirming that zeolites did not exert a noticeable influence on acid production and the related parameters. Overall, while R–I and R–II showed homogeneous yield and rate values, R–III performed worse for all these parameters, in line with the lower SCFA production.

While the data on tannery sludge's performance is very scarce in the literature, a comparison with sewage sludge reveals higher profitability of tannery sludge to produce SCFAs. Zhang et al. (2019) achieved yields of 0.19 and 0.17 g COD_{SCFA}/g VS_0 for thermally pretreated sludge and raw sludge, respectively. Meanwhile, Gottardo et al. (2022) obtained a yield of 0.22 with an HRT of 4 days using the same pretreatment method as this study but with sewage sludge as substrate, and an HRT of 6 days. Castro-Fernandez et al. (2023), obtained 8.0 ± 0.9 g COD_{SCFA}/L from acidified thermally pretreated sewage sludge, achieving a yield of 0.26 g COD_{SCFA}/g VS_0.

Overall, this data confirms that tannery sludge is a promising substrate for SCFA production, while zeolites did not have a significant impact at the dosage employed in this study.



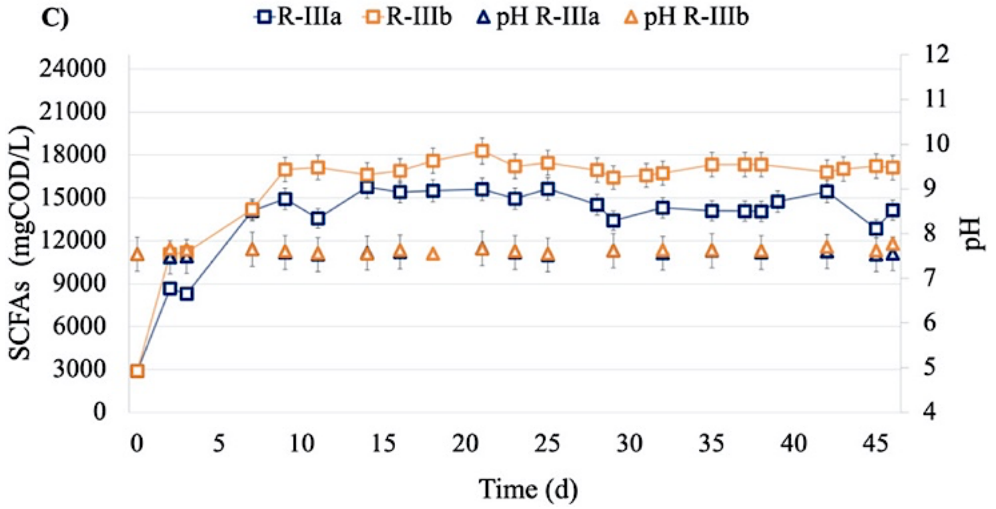


Figure 5.2: Trends of the SCFAs and pH in the semi-continuous reactors without zeolite addition (A), with CH (B), and with CL (C).

5.3.2 SCFAs composition

For what concerns the SCFAs composition in the effluent, a peculiar behavior was observed, as for tests R-IIIa, R–III–b, and R-IIb it remained relatively constant once it reached the steady phase; however, for the other runs (R-Ia and R-Ib; R-IIa) it changed after 26–33 days, in all cases shifting towards a higher propionic acid production at the expense of acetic acid, clearly delineating the process into two periods, P1 and P2, characterized by different compositions.

The tests without zeolite addition both showed this shift (Fig. 5.3), namely in test R-Ia, between days 9–30 (P1) the composition was characterized by high proportions of propionic (24%) and acetic (54%) acids, with relatively low levels of other SCFAs. However, in the later period (days 33–47; P2), propionic acid substantially increased (40%) at the expense of acetic acid (38%). R-Ib showed a similar behavior, even if slightly less prominent. In both periods (P1: days 12–23 and P2: days 26–47), acetic acid remained dominant (from 67 to 51%), but propionic acid increased in the later stage (from 14 to 25%). Similarly, with the lower HRT, the test with zeolite chabazite (R-IIa) showed a shift from P1 to P2 around day 30 (Fig. 5.4), again involving propionic and acetic acids shifting from 58 to 33% of acetic and 15–30% of propionic. However, this was not observed when the HRT was higher, as in R-IIb propionic accounted for 18% of the total and acetic for 54% for the whole duration of the test. Differently, both runs with clinoptilolite (Fig. 5.5) showed a more consistent composition without any shifts. Namely R-IIIa with propionic acid making up 29% of the total and acetic 37%, consistent with the final composition of the other tests; and R-IIIb with similar contributions of the two acids making up 34% and 36% of the total (for propionic and acetic, respectively). It is worth noting that the shifts in SCFA composition were all coupled with a shift in pH between the first and second periods. Namely, in the runs that showed a change in SCFA composition, the pH shifted towards higher values, which have been shown to promote propionic acid production from fermentative processes (Da Ros et al., 2020). The pH values in R-Ia changed from 7.0±0.2 in P1 to 7.5 ± 0.2 in P2, and in R-Ib from 6.9± 0.0 to 7.4 ± 0.0. Similarly, R-IIa experienced a shift from a pH of 7.2 ± 0.2 to 7.8 ± 0.1. In contrast, runs with clinoptilolite maintained a consistent pH value of 7.6 ± 0.1 throughout the entire test duration, and were also characterized by the absence of any shifts in SCFA composition. This shift in pH and composition is prevalent in the runs without absorbents, while the configuration of the zeolite-amended reactors, characterized by a hybrid configuration with suspended-absorbed biomass, could reduce or even prevent this shift. It has been previously observed that zeolites can act as a pH regulator, probably due to their ion exchange capacity (Castellar et al., 1998). However, chabazite coupled with a low HRT process did not manage to influence the pH nor the SCFAs distribution, which remains homologous to the suspended biomass tests (R–I), probably due to the reduced retention time, while at higher HRTs it does allow for a higher consistency of the process. Clinoptilolite instead was able to maintain stability throughout the test suggesting that this zeolite could reduce pH variations in the reactor, thus potentially promoting more consistent SCFA production over time, which can have positive implications for the downstream processes necessary to extract the SCFAs from the fermentation broth (Aktij et al., 2020).

Another possible explanation for these variations could be linked to the zeolites' influence on microbial activity and fermentation processes. Zeolites might create a favorable environment for specific microbial communities that produce different SCFAs. Moreover, the adsorption capabilities of zeolites may impact the accessibility of organic compounds required for microbial fermentation. However, these hypotheses are mere speculations, and further investigation into the microbial communities and the interactions with substrates within these reactor systems could provide important insights into this shift in SCFA composition.

When the test reached the end, propionic and acetic made up more than 60% of the total, proving that the nature of the substrate has the most significant impact on the degradation products. Moreover, it has been shown that higher temperature increases the amount of shorter chain acids produced, like acetic and propionic (Zhang et al., 2019a).

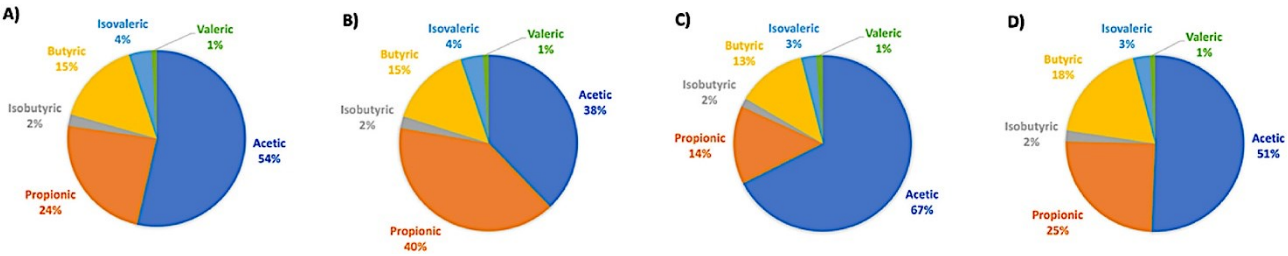


Figure 5.3: Composition in terms of the COD_{SOL} percentage of the SCFAs obtained from the thermophilic CSTR of R-Ia P1 (A) and P2 (B), and R-Ib P1 (C) and P2 (D).

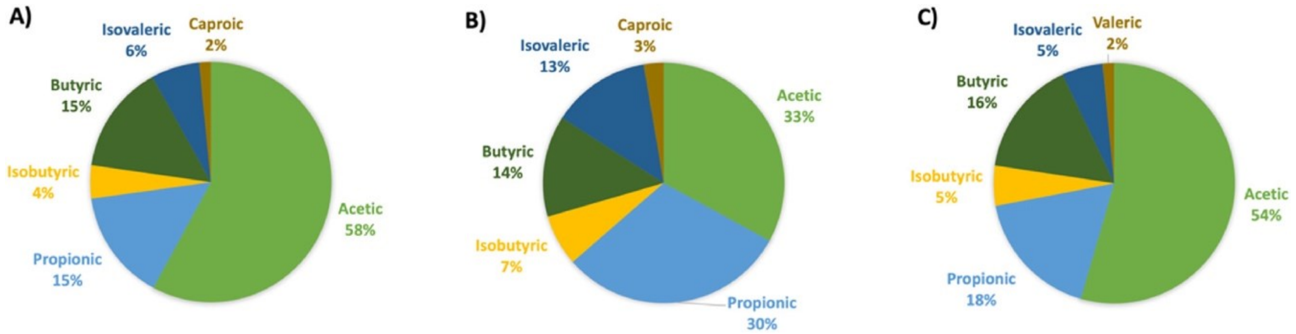


Figure 5.4: Composition in terms of the COD_{SOL} percentage of the SCFAs obtained from the thermophilic CSTR of R-IIa P1 (A) and P2 (B), and R-IIb (C).

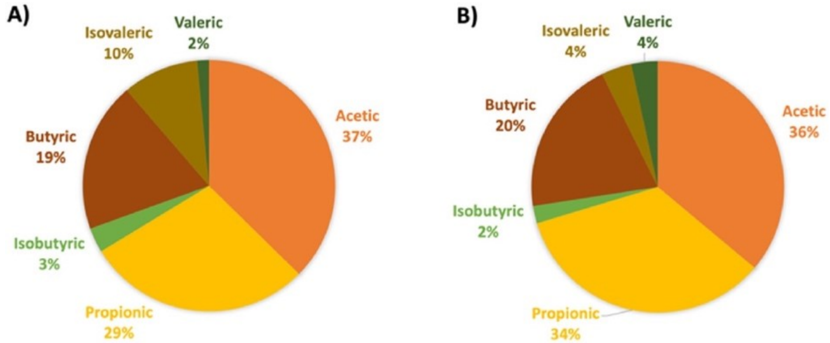


Figure 5.5: Composition in terms of the COD_{SOL} percentage of the SCFAs obtained from the thermophilic CSTR of R-IIIa (A) and R-IIIb (B).

5.3.3 Effect of zeolite addition on ammonia release

For what concerns the ammonia, the steady periods were characterized by very similar concentrations between tests R–I and R–II for both HRTs (1127 ± 23 mg/L and 1098 ± 45 mg/L for HRT 4, and 1078 ± 33 mg/L, and 1291 ± 45 mg/L for HRT 8, respectively), while R–III reached significantly higher values of 1478 ± 47 mg/L and 1518 ± 47 mg/L for R-IIIa and R-IIIb, respectively. These values correspond to an ammonia release percentage of over 60% for test R–III, compared to around 55-45% for the other tests (Table 5.3). These results are comparable with Tang et al. (2023), who found similar ammonia concentrations in comparable tests using waste-activated sludge as a substrate and zeolites. Tang and coauthors explained that zeolite addition seemed to have improved the solubilization of the organic matter, releasing more ammonia in the liquid fraction.

From these tests, it appears that ammonia was not absorbed by the zeolites, as its concentration remained homogeneous between all runs. This result is in contrast with the literature where zeolites proved effective in NH_4^+ absorption (Montalvo et al., 2012), as in Wang et al. (2015) where the NH_4^+ content in the test with 10% clinoptilolite was around half of the test without zeolite. This discrepancy can likely be attributed to the substantial presence of cations in tannery sludge, particularly calcium ions (Ca^+). These calcium ions primarily originate from the liming processes involved in tanning and from primary wastewater treatments. Additionally, sodium ions (Na^+) are introduced into the sludge due to the use of various chemicals during the tanning process (Sawalha et al., 2020). Studies have shown that sodium ions, in particular, play a significant role in the cation exchange process essential for the absorption of NH_4^+ by zeolites (Rodríguez-Iznaga et al., 2022), and have been observed to decrease from the liquid medium after the addition of zeolites (Wang et al., 2015). It has been hypothesized that these cations, along with chromium (Cr) and OM-Cr(III) structures, may interact with and potentially diminish the capacity for NH_4^+ absorption.

Moreover, clinoptilolite was shown to be less effective than chabazite at absorbing ammonia from a solution with a similar concentration as this study (Montegut et al., 2016), which is confirmed by the higher ammonia concentration in R–III. Overall, it can be hypothesized that, while zeolites increased the solubilization of the organic matter, releasing NH_4^+, only chabazite managed to absorb some of the released ammonia while clinoptilolite did not, resulting in the observed higher concentration in R–III.

Overall, it is important to note that the observed concentration of NH_4^+ in this research did not seem to have any inhibitory effect on the fermentation.

5.3.4 Fate of the chromium in the semi-continuous process

The presence of Cr was analyzed in the liquid fraction, both in the pretreated sludge and in the reactors, and it is shown in Fig. 5.6.

As expected, the mild thermal pretreatment did not lead to the formation of Chromium (VI), which was not observed, consequently, the discussed results only regard Cr(III).

This aligns with our previous research, where stronger oxidative conditions similarly did not oxidize Cr(III) bound to the organic matrix (Tuci et al., 2023).

Moreover, in the mentioned previous study, a mild oxidizing pretreatment allowed the release of around 119 ± 11 mg/L of Cr, and throughout the mesophilic fermentation, its content in the liquid fraction reached 0. On the contrary, the mild thermal pretreatment adopted in this study caused the Cr in the liquid fraction to be almost 3 times higher (335 mg/L). The trend of Cr concentration is presented in Fig. 5.6. After an initial peak after around 1 HRT, the concentration progressively decreased for all tests. However, in the tests without zeolite addition (R–I) after around 20 days for R-Ia, and 35 days for R-Ib, the decreasing trend stopped and the Cr content remained stable with values around 129 mg/ L for the lower HRT and 123 for the higher, meaning around 60% reduction. On the other hand, the tests with the zeolite continued decreasing until no Cr was found in the liquid effluent, namely after 28 days for both tests with HRT of 4 days, and 39 and 42 days for chabazite and clinoptilolite respectively, in the HRT = 8 d. While the reduction of Cr content in R–I was probably related to OM- Cr complex degradation and subsequent precipitation as $Cr(OH)_3$, favored by the neutral pH and the lime presence in the sludge, as explained in Tuci et al. (2023), with such high concentration these mechanisms could not completely remove it. This highlights the absorption capacity of both zeolites for Cr, managing to absorb a significant amount of this toxic metal, allowing for the utilization of the SCFA-rich effluent without further treatment for Cr removal.

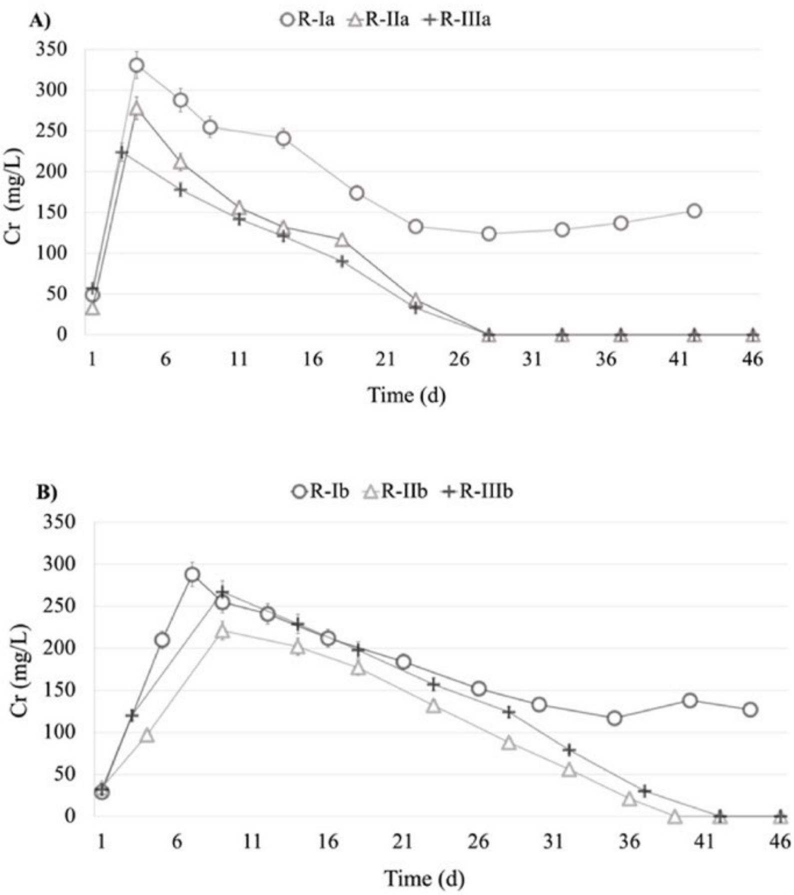


Figure 5.6: Trends of the Cr in the two semi-continuous reactors at HRT = 4 d (A), and at HRT = 8 d (B).

5.3.5 Mass balance assessment

The mass balance was conducted using parameters detailed in Tuci et al. (2023). Namely, it considers 37,000 tons per year of tannery sludge, which is the average production in the cluster of northeast Italy. The related TS and VS flow rates are 99,065 kg TS/d and 70,419 kg VS/d. To optimize waste reduction for the minimization of the landfilling practice, the process was split into two phases, as shown in Fig. 5.7. The first phase involves mild thermal pretreatment (20 h at 70 °C) followed by anaerobic fermentation in a CSTR at 55 °C to produce SCFAs. In the second phase, the solid-rich effluent from the first step is conveyed into an anaerobic digestor at 55 ◦C for biogas production. This mass balance relied on data from reactor R-IIb, as it yielded the most SCFAs and biogas compared to the other zeolite-amended tests.

Initially, the sludge flow rate is 119,355 kg/d with 7% w/v solids' content, to which 183,09 kg/d of zeolite are added, bringing the TS to 117,374 kgTS/d, processed in an 11,737 m^3 CSTR with an HRT of 8 days. After the first phase of acidogenic fermentation, the VS flow rate was reduced to 48,541 kg VS/d, thanks to the generated biogas (SGP of 0.19 m^3/kg VS; 99% CO2, 1% not identifiable).

Additionally, the process resulted in a SCFAs-rich effluent (1,107,233 m^3/d) containing 9.69 tons of SCFAs per day (considering the mean SCFAs production from R-IIb of 20 g COD/L). After a first solid/liquid separation, the SCFA-rich effluent is separated for acid extraction, while the solid-rich residue undergoes the second phase of anaerobic digestion. The flow rate entering the anaerobic digester is now 914,541 m^3/d after dilution (approximately 10% w/v TS content). The CSTR anaerobic digester (18,291 m^3) operated with the following conditions: HRT of 20 days, and a temperature of 55 °C. The SGP of 0.60 m^3/kg VS_{FED} was adopted (Figure 5.S1). This phase generates 27,436 m^3/d of biogas, equivalent to 28,304 kg VS/ d (considering a composition of 58% CH_4 and 42% CO_2 by volume). The final step of the process involves a necessary step preceding sludge drying to achieve an 83% w/v TS content, typically performed before landfill disposal. Ultimately, the amount in TS of dried sludge (with the added zeolites) destined for landfill is 58,247 kg TS/d, representing a 41% reduction compared to the initial dried tannery sludge input. This reduction is noteworthy as it would on the one hand reduce the landfilled sludge and consequently the environmental impact of this waste, and on the other hand provide some added-value products from a wasted material, in line with the circular economy principles.

However, from an economic perspective it has to be taken into account that the zeolites need to be purchased for a price that for natural zeolites ranges around 200-500 cent/kg (Europomice SRL). Consequently, the addition of this material also has an economic impact on the process that for the proposed scenario would be around 9000 €/d. Consequently, the choice of including zeolites has to keep this aspect into consideration, whether the priority of the process is to have a liquid fraction free from Cr or to maximize cost effectiveness, through a specific economic assessment that was not included in this study.

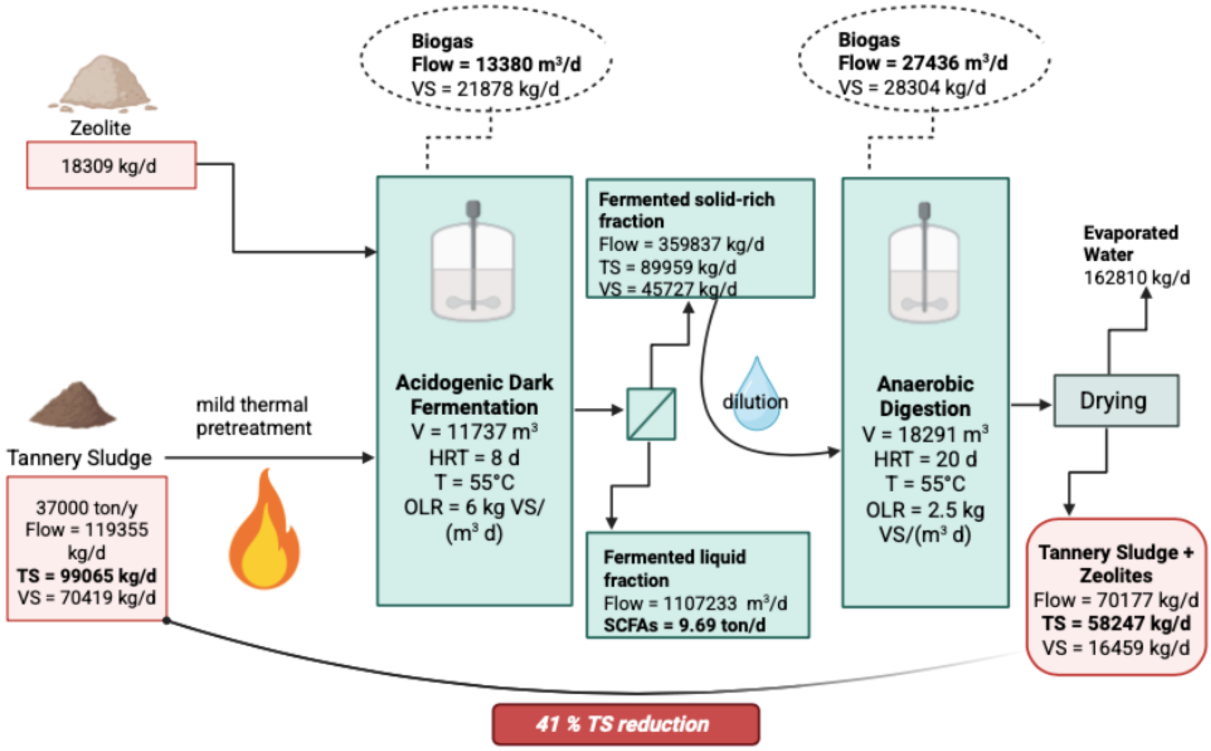


Figure 5.7: Mass balance visual representation.

Table 5.3: Summary of the effluent's characteristics and reactors' performances under semi-continuous acidogenic fermentation conditions. P1 and P2 refer to the two phases in the runs R-Ia, R-Ib, and R-IIa, as explained in paragraph 5.3.2.

			sCSTR *(mild thermal pretreatment; 50°C)*					
			Name					
Parameter		**Unit**	R-Ia (HRT = 4 d, no zeolite)	R-Ib (HRT = 8 d, no zeolite)	R-IIa (HRT = 4 d, CH)	R-IIb (HRT = 8 d, CH)	R-IIIa (HRT = 4 d, CL)	R-IIIb (HRT = 8 d, CL)
COD_{SOL}		g/L	23.1 ± 0.4	28.2 ± 0.5	19.4 ± 0.2	22.1 ± 0.3	20.4 ± 0.4	23.0 ± 0.2
COD_{SCFA}		g/L	16.7 ± 0.2	20.3 ± 0.2	17.03 ± 0.17	20.2 ± 0.2	14.6 ± 0.2	17.0 ± 0.1
COD_{SCFA}/COD_{SOL}		g/g	0.72 ± 0.01	0.72 ± 0.01	0.87 ± 0.01	0.92 ± 0.01	0.71 ± 0.01	0.75 ± 0.01
Acetic acid	P1	g COD/L	8.7 ± 0.2	13.3 ± 0.4	10.0 ± 0.1	11.0 ± 0.2	5.5 ± 0.2	6.23 ± 0.09
	P2		6.5 ± 0.3	10.4 ± 0.3	5.5 ± 0.4			
Propionic acid	P1	g COD/L	3.9 ± 0.2	2.8 ± 0.3	2.6 ± 0.1	3.5 ± 0.2	4.1 ± 0.1	5.78 ± 0.11
	P2		6.8 ± 0.2	5.1 ± 0.2	5.1 ± 0.1			
Butyric acid	P1	g COD/L	2.5 ± 0.2	2.5 ± 0.2	2.5 ± 0.1	3.1 ± 0.1	2.8 ± 0.1	3.14 ± 0.09
	P2		2.5 ± 0.1	3.8 ± 0.2	2.3 ± 0.1			
Isovaleric acid	P1	g COD/L	0.6 ± 0.1	0.5 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.4 ± 0.1	0.68 ± 0.03
	P2		0.7 ± 0.1	0.6 ± 0.1	2.2 ± 0.2			
q_{SCFA}^{CSTR}		mg COD_{SCFA}/(g VS d)	83 ± 1	50.2 ± 0.6	84 ± 1	50.0 ± 0.4	72 ± 1	42.0 ± 0.3
Y_{SCFA}^{CSTR}		g COD_{SCFA}/(g VS_0)	0.33 ± 0.01	0.40 ± 0.01	0.34 ± 0.01	0.40 ± 0.01	0.29 ± 0.01	0.34 ± 0.01
P_{SCFA}^{CSTR}		g COD_{SCFA}/(L d)	3.18 ± 0.04	2.00 ± 0.02	3.14 ± 0.07	1.96 ± 0.02	2.65 ± 0.04	1.55 ± 0.01
$N\text{-}NH_4^+$ release		mgN-NH_4^+/g TKN	48 ± 1 %	46 ± 1 %	47 ± 2 %	55 ± 2 %	63 ± 2 %	64 ± 2 %

5.4 Conclusions

In this study, the production of SCFAs from thermally pretreated tannery sludge within sCSTRs was explored, with a focus on the influence of zeolite additions. Notably, the ammonia concentration did not benefit from zeolite addition, with R–I and R–II being quite homogeneous and R–III showing a higher ammonia release. Conversely, the soluble Chemical Oxygen Demand (COD_{SOL}) values consistently differed between runs with and without zeolites, with zeolites effectively absorbing organic matter and contributing to lower COD_{SOL} values. The highest SCFA production was observed in tests without zeolite addition, particularly with the higher HRT, reaching levels of 16014 mg/L, corresponding to an acidification yield of 0.4 g COD_{SCFA}/g VS_0. Moreover, the addition of chabazite allowed for a substantial improvement in acidification efficiency, reaching a SCFA to COD_{SOL} ratio of 0.92 COD/ COD.

Throughout the experiments, SCFA composition displayed temporal variations, with some tests shifting towards higher propionic acid production towards the end of the runs. Additionally, zeolites exhibited a remarkable capacity to absorb chromium from the fermentation broth, achieving complete removal over time, whereas tests without zeolites only reached a 60% reduction in Cr concentration. The mass balance of the anaerobic fermentation-digestion reactors revealed that this process could reduce the mass of the landfilled sludge by 41%, thus reducing the environmental impact while also obtaining biogas and SCFAs as added-value products.

These findings offer valuable insights for optimizing SCFA production from tannery sludge and highlight the potential of zeolites in heavy metal removal within waste treatment processes. Further research is needed to explore the microbial dynamics and substrate interactions, particularly those influencing the SCFA composition.

Supplementary materials (Chapter 5)

Biochemical Methane Potential (BMP) test

The BMP tests were set up in 250 mL glass bottles sealed with a cap with a silicon plug, with 180 mL working volume, and each test was performed in duplicate. Namely, 50 mL of thermophilic anaerobic digestate from the Treviso full-scale digester, with a concentration of 35 g VS/L, was used as an inoculum after one week of incubation at 50 °C to achieve endogenous methane production. The solid-rich fraction from the fermentation effluent was employed as substrate. The S/I ratio was set at 0.3 (VS basin). Daily monitoring of biogas production and CH_4 content was performed to quantify the biogas composition and the maximum specific gas production (SGP; m^3/kg VS) achievable and the hypothetical HRT to be considered in the scaled-up continuous AD process, according to Angelidaki et al., (2009). The biogas produced from the Biochemical Methane Potential (BMP) tests was quantified by a flow meter (Ritter CompanyTM),

The percentage of CH_4 and H_2 in the biogas were determined by a gas chromatograph GC Agilent Technology 6890 NTM equipped with a column HP-PLOT MOLESIEVETM (30 m x 0.53 m ID x 25 µm thickness of the film), using a thermal conductivity detector (TCD) at 250 °C. The injector was maintained at 120 °C and 70 kPa. Gas samples (200 µL) were taken using a gas-type syringe; the analyses were conducted at a constant T of 40 °C for 8 min with Argon as gas carrier.

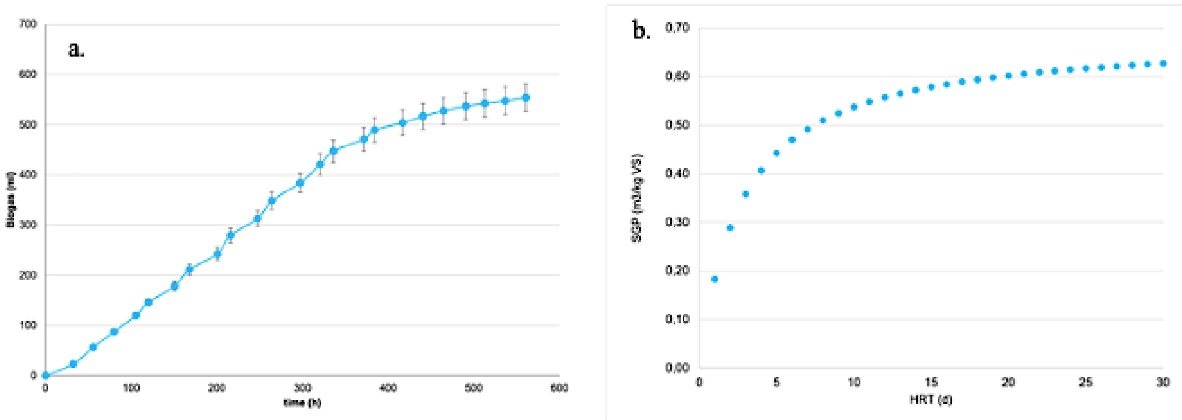


Figure 5.S1: Evolution of produced biogas (a) and theoretical SGP values (b) modeled in the BMP tests at thermophilic conditions.

As shown in Figure 5.S1, with 20 days as modeled HRT, this residue was characterized by an SGP of 0.6 m^3/kg VS_{FED}, corresponding to 88 % of the maximum SGP achievable. This result was used for the mass balance assessment.

CHAPTER 6: Microbial community evolution in the AF bio-processes

All the AF experiments presented on tannery sludge have been carried out without the addition of a specific inoculum; thus, the microbial community involved in the fermentation was naturally present in the substrate and was selected throughout the process. The composition of the microbial community can significantly impact the process, affecting stability and the efficiency of VFA production. Therefore, analyzing its composition and variations is essential for truly understanding and optimizing the process.

This additional step was made possible through cooperation with the Water Research Institute, National Research Council of Italy, CNR-IRSA in Rome, where samples of microorganisms taken from some of the previously discussed semi-continuous experiments were analyzed and presented in the article reported in this chapter.

Through an examination of microbial diversity, activity, functional redundancy, and metabolic potential, this study offers insights into the role of specific microbial consortia in degrading organic matter and synthesizing VFAs, while dealing with the presence of Cr. These findings pave the way for targeted strategies that improve process efficiency via microbial selection and process control.

This chapter reports the article currently under review, titled "***Microbial-driven valorization of tannery sludge for fatty acids production: unraveling microbiome activity and functional redundancy***" written by Barbara Tonanzi, Alessio Massimi, Francesco Valentino, Giulia Adele Tuci, Marco Gottardo, Simona Rossetti, and Simona Crognale.

6.1 Introduction

The leather tanning industry, a pivotal component of the global fashion and footwear sectors, is inseparably connected to extensive environmental challenges. The generation of substantial quantities of tannery sludge, a complex matrix of organic matter, toxic inorganic compounds, and microorganisms, poses a difficult threat to ecosystems due to its high organic load, heavy metal content, and recalcitrant nature (Abreu & Toffoli, 2009; Ali et al., 2015; Alibardi & Cossu, 2016a). Due to the high content of organic and inorganic substances, often hazardous to the environment, these sludges pose a significant challenge for industrial waste management. Traditional waste management practices, such as landfilling and incineration, while seemingly expedient, intensify environmental issues by contributing to soil and water pollution, greenhouse gas emissions, and resource depletion (Alibardi & Cossu, 2016a).

In particular, the Italian leather industry generates a significant amount of tannery sludge as a byproduct of leather processing. As outlined in the UNIC sustainability report of 2021, Italy's tannery sector is a global leader, accounting for 23% of the total global leather value (UNIC, 2021). Italian legislation, particularly Legislative Decree 152/2006, strictly regulates the disposal of tannery sludge, requiring companies in the sector to adopt suitable technologies and treatment processes to minimize environmental impact. To reduce environmental impact and valorize these wastes, recent years have seen a growing interest in innovative solutions such as anaerobic digestion, co-composting, and biofuel production (Augustini et al., 2020; Zhai et al., 2020). These technologies allow for the transformation of tannery sludge into energy resources and useful bio-based materials, thus promoting a circular economy in the leather sector. In the pursuit of sustainable and circular economy principles, the valorization of tannery sludge emerges as a requiring imperative (Chojnacka et al., 2021; Moktadir et al., 2023). By transforming this waste into valuable products, the leather industry can mitigate its environmental impact while generating economic benefits. Microbial-based technologies represent a promising alternative for achieving this goal. Employing the metabolic capabilities of microorganisms, these technologies can effectively degrade complex organic matter, remove contaminants, and produce high-value products (Ameen, 2023; Angenent et al., 2016; Ashraf et al., 2018; Reyes-Romero et al., 2021; Saxena et al., 2020).

Fermentation and anaerobic digestion of tannery sludge align with the principles of circular economy. By transforming this waste into valuable bioproducts, these processes contribute to a more sustainable and resource-efficient leather industry. The anaerobic fermentation of tannery sludge produces short-chain fatty acids (SCFAs), which can be further converted into biofuels, bioplastics, or used as a nutrient source for algae cultivation (Han et al., 2019; Wang & Yin, 2022; Wu et al., 2019). This approach not only reroutes waste from landfills but also creates a circular pathway that recovers resources from a previously discarded material, thereby minimizing environmental impact and promoting economic growth. Pre-treatment of sludge prior to anaerobic process is a critical step to enhance microbial accessibility and overall process efficiency (Braguglia et al., 2012; Gagliano et al., 2015; Gallipoli et al., 2014; Gianico et al., 2013; Khadaroo et al., 2019; Tonanzi et al., 2021). Physical, chemical, and biological pre-treatments can disrupt the complex organic matrix of sludge, increasing the surface area available for microbial attack and solubilizing recalcitrant organic compounds (Ariunbaatar et al., 2014; Chang et al., 2020; Gottardo et al., 2022; Wang & Yin, 2022). For instance, thermal hydrolysis has been shown to be effective in disrupting the sludge structure and increasing volatile solids solubilization (Gagliano et al., 2015; Gherghel et al., 2019; Gianico et al., 2015; Jeong et al., 2019). Furthermore, the combination of physical and chemical pre-treatments can synergistically enhance the performance of anaerobic digestion processes (Khadaroo et al., 2019; Li et al., 2023a). Recent improvements in biotechnology have shown the potential of microbial communities to address the complexities of tannery sludge. For instance, the application of anaerobic fermentation technology and oxidative or thermal pretreatment has shown high aptitude in minimizing landfilling by producing SCFAs, reducing organic matter content and solubilizing toxic compounds such as chromium, highly abundant in this waste (Abate et al., 2021; Tuci et al., 2024, 2023; Zhai et al., 2020; Zhu et al., 2024). These studies highlighted the potential of microbial-based approaches to transform tannery sludge from waste into a resource opportunity. Despite the key role of microorganisms in such process, the taxonomic diversity and functional potentialities of microbial communities involved in the production of fatty acids from tannery sludge have not been investigated so far. To effectively enhance biotreatment processes and product recovery from tannery sludge, a comprehensive characterization of the microbial community and its performance is necessary. For the first time, this study aims to characterize microbial communities and their evolution during anaerobic fermentation of tannery sludge in semi-continuous processes to produce SCFAs. The insights gained will contribute to the development of innovative bioprocesses for a more sustainable and circular leather industry.

6.2 Materials and methods

6.2.1 Experimental set up

Microbiological analyses were performed on samples coming from SCFAs-producing semi-continuous reactors described in Tuci et al. (Tuci et al., 2024, 2023) (Table 1). These studies utilized a mixture of primary and secondary tannery sludge obtained from the Montebello Vicentino wastewater treatment plant (WWTP) in northeast Italy. The WWTP processes wastewater from over 20 tanneries, employing alkaline flocculation and anoxic/aerobic treatment. Four different semi-continuous stirred tank reactors (sCSTRs) of 1.5 L working volume were operated to assess anaerobic fermentation (AF) of tannery sludge subjected to hydrogen peroxide (H_2O_2) or thermal pretreatment (Run Ox and Th, respectively). The reactors were inoculated with tannery sludge, maintained at 40°C and 50°C, respectively, and daily fed with pre-treated sludge. A hydraulic retention time (HRT) of 4 and 8 days and organic loading rate (OLR) of 12.6 and 6.05 gVS/ Ld were tested in parallel (Table 6.1). Reactor performance was evaluated by monitoring SCFAs production, chemical oxygen demand (COD), ammonium, pH, and chromium concentration. SCFAs composition, COD, and other chemical parameters, including fermentation yield (Y), were analysed according to the methods outlined in Tuci et al. (2023).

Table 6.1: Operational parameters of anaerobic reactors.

Run	HRT (d)	OLR (gVS/Ld)	Substrate Pretreatment	Temperature (°C)	Reference
Ox_4	4	12.6	0.4 g H_2O_2/ g TS	40	Tuci et al. 2023
Ox_8	8	6.05	0.4 g H_2O_2/ g TS	40	Tuci et al. 2023
Th_4	4	12.6	70°C, 20 hours	50	Tuci et al. 2024
Th_8	8	6.05	70°C, 20 hours	50	Tuci et al. 2024

HRT: hydraulic retention time; TS: total solids; VS: volatile solids

6.2.2 In situ detection methods (FISH and CARD-FISH)

Biomass samples were collected from the anaerobic reactors and promptly fixed in a formaldehyde-ethanol solution (2% and 48% vol/vol, respectively) before storage at -20°C. Prior to analysis, the fixed biomass was disrupted by vortexing with glass beads. FISH and CARD-FISH analyses were performed using the EUBmix oligonucleotide probe set (equimolar concentrations of EUB338, EUB338-II, and EUB338-III) (Matturro et al., 2013; Nielsen et al., 2009; Tonanzi et al., 2018). Probe details and conditions are reported in probeBase (http://www.microbial-ecology.net/probebase/). Following hybridization, total cells were detected with DAPI using Vectashield Mounting Medium (Vector Labs, Italy). Epifluorescence microscopy was used to count fluorescent cells in at least 10 randomly selected microscopic fields per sample. Mean and standard deviation values were calculated using Microsoft Excel. The FISH/CARD-FISH ratio for each sample was determined by dividing the average bacterial abundance estimated by FISH analysis to the one obtained by CARD-FISH analysis, as previously reported in Tonanzi et al. (2018).

6.2.3 Sample collection and processing for DNA extraction

Several anaerobic samples were collected from each reactor throughout the experimental period. For DNA extraction, duplicate 2 mL aliquots were centrifuged as reported in Tonanzi et al. (2018), and the resulting pellets were stored at -20°C. The DNeasy PowerSoil Pro kit (QIAGEN, Antwerp, Belgium) was used for DNA extraction according to the manufacturer's instructions.

6.2.4 16S rRNA gene amplicon sequencing and data analysis

To characterize the microbial community structure within the reactors, 16S rRNA gene amplicon sequencing was performed using Illumina technology as previously described (Crognale et al., 2019). Briefly, the V1-V3 region of the bacterial 16S rRNA gene was amplified using primers 27F (5′-

AGAGTTTGATCCTGGCTCAG-3′) and 534R (5′-ATTACCGCGGCTGCTGG-3′). Subsequent sequence data processing and analysis were conducted using QIIME2 (v. 2017.12), including demultiplexing, DADA2 quality control, and taxonomic assignment of amplicon sequence variants (ASVs) (Callahan et al., 2016; Quast et al., 2013). A total of 167080 reads were obtained by high-throughput sequencing of the V1-V3 region of the bacterial 16S rRNA gene, that resolved into 4701 ASVs. The dataset is available through the Sequence Read Archive (SRA) under accession PRJNA1178871.

6.2.5 Statistical analysis

A non-metric multidimensional scaling (NMDS) ordination plot, based on the Bray-Curtis matrix, was performed in order to graphically synthesize the dissimilarity between samples. Process parameters and microbial community data were overlaid onto the NMDS plot using a vector-fitting procedure. Vector length indicated the correlation between NMDS axes and variables, facilitating the identification of factors driving sample variation. Process data and values of major bacterial taxa revealed by 16S rRNA gene high-throughput sequencing (only genera ≥1% of total reads were considered) were normalized by log(X + 1). Statistical analyses were performed by using PAST software package (Palaeontological STatistics, ver. 4.04) (Hammer et al., 2001).

6.3 Results and discussion

6.3.1 Impact of microbial activity on SCFAs production efficiency

As reported in the previous works of Tuci et al. (2024, 2023), four semi-continuous reactors (Table 6.1) were operated at different HRT (4 and 8 days), sludge pretreatment (oxidative and thermal), OLR (6.05 and 12.6 gVS/Ld), and temperature (40 e 50°C). Fermentative performance and stability of the process were monitored via SCFAs production and their profiles. Figure 6.2 shows an NMDS ordination plot based on Bray-Curtis distances of log-transformed SCFA concentrations, highlighting clear differentiation between runs conducted using H_2O_2 pretreated sludge in mesophilic conditions (Ox_4 and Ox_8) and thermal pretreated sludge in thermophilic conditions (Th_4 and Th_8). In particular samples closer together share more similar characteristics, while arrows indicate the direction and strength of correlation of each SCFA or parameter with the specific condition. For each test, a SCFAs/COD ratio of approximately 70% (on a COD basis) was observed, despite the sludge pretreatment, temperature and OLR difference experimented (Table 6.1). Notably, the SCFAs profiles obtained from the mesophilic reactors Ox_4 and Ox_8 remained remarkably stable once they reached steady-state operation, as shown in figure 6.1.

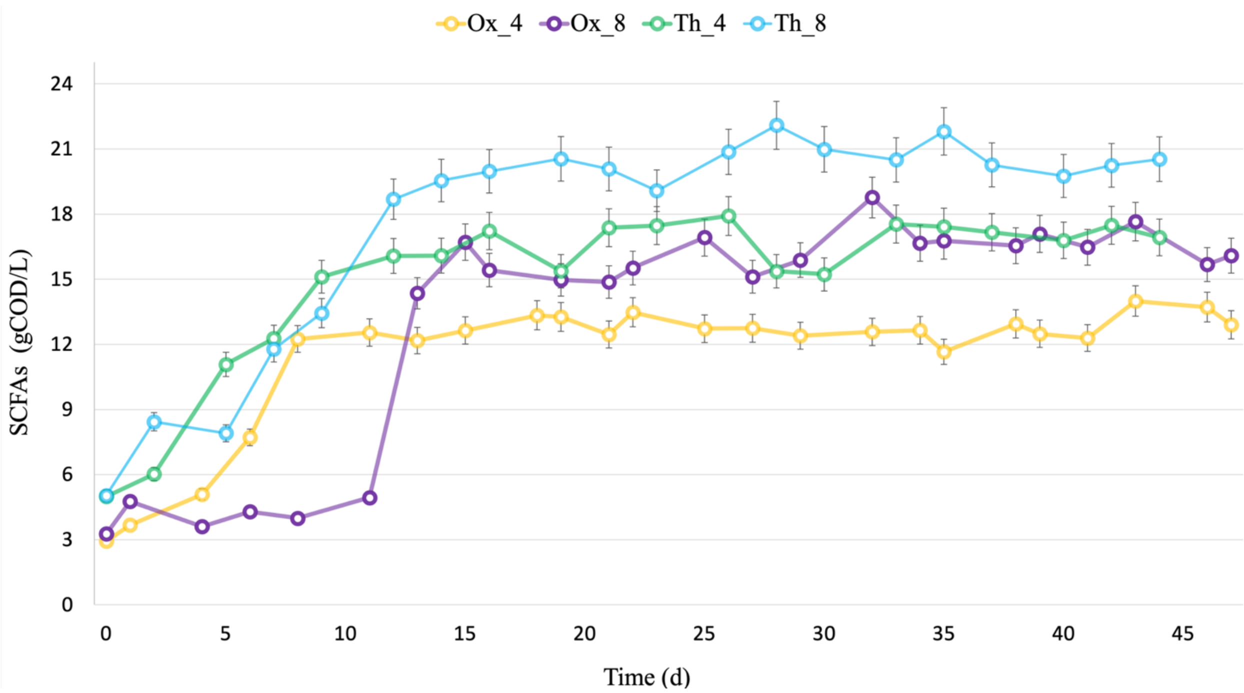


Figure 6.1: SCFA production over time of the 4 conditions.

Acetic acid was the main SCFA accounting for approximately 62% and 67% of the total SCFAs (on a COD basis) in runs Ox_4 and Ox_8, respectively. Propionic acid was the second most abundant SCFA, comprising 13% in Ox_4 and 19.7% in Ox_8, followed by butyric acid constituting less than 10% of the total SCFAs in both reactors (Tuci et al., 2023). In Th systems, the analysis of the SCFAs profile revealed that acetic acid was the predominant component during the initial 30 and 23 days of experiments in Th_4 and Th_8, respectively, accounting for approximately 54% and 67% of the total acids (Tuci et al., 2024).

Propionic acid followed as the second most abundant acid, with a percentage of 24% and 14%, respectively. In the subsequent phase, a significant increase in propionic acid, equal to approximately 67% and 78.6% compared to the previous data, was observed, at the expense of acetic acid, in both experiments. This change was correlated with the increase in pH of the system (Da Ros et al., 2020; Tuci et al., 2024). The fermentation products obtained in the Ox systems exhibited a relatively higher concentration of acetate and valerate acids in comparison to the Th systems, where butyrate and propionate were observed at higher proportions (Figure 6.2).

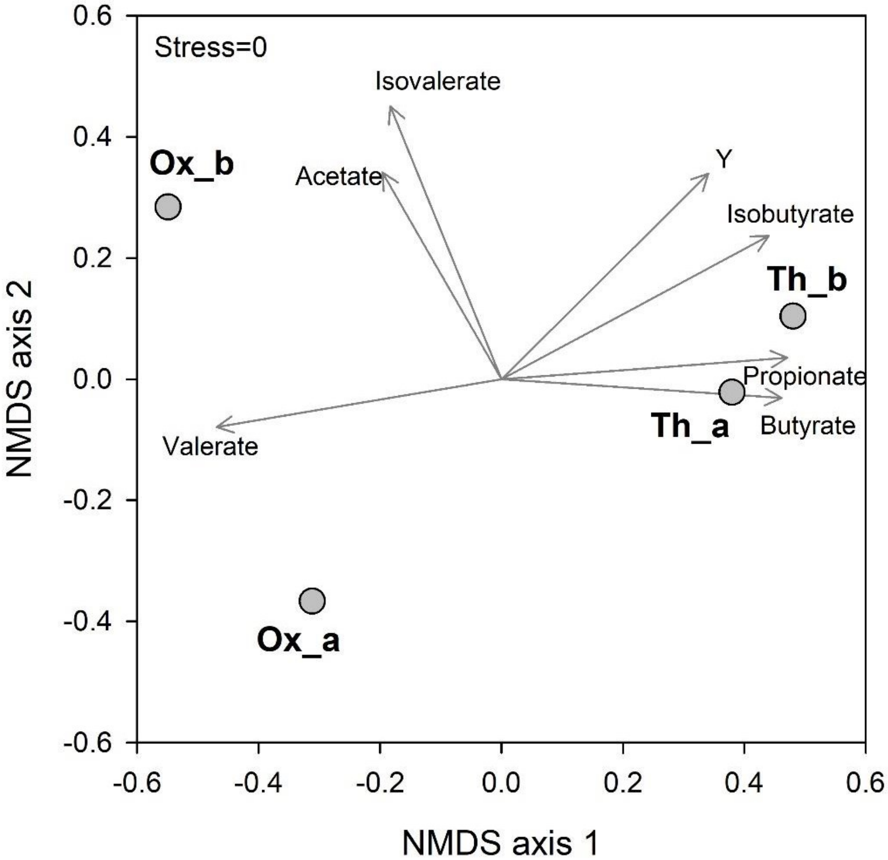


Figure 6.2: NMDS ordination plots, based on Bray-Curtis distance matrixes of log(X+1) transformed data. The vector length is proportional to the correlation between the NMDS axes and each parameter.

The acidification performances were evaluated also by the fermentation yield (Y), a ratio between the produced SCFAs over time, and VS at the initial concentration in the test. Data showed that Y was higher in Ox_8 with respect to Ox_4 (0.32 ± 0.01 and 0.25 ± 0.01 g COD_{SCFA}/g VS) suggesting that a higher HRT associated with a lower OLR promoted a higher conversion rate of organic matter in SCFAs. This evidence was also confirmed by the evaluation of the FISH/CARD-FISH ratio. As previously reported in Tonanzi et al. (2018), this parameter can be used to evaluate the dynamics of microorganisms in biological process. In fact, FISH analysis detects physiologically active microorganisms in complex populations through the detection of cells with high ribosome content (>103 ribosome per cell), while CARD-FISH analysis can be used to evaluate the total microbial community including the microbial components with low activity (few ribosomes per cell).

The FISH/CARD-FISH ratio estimated for bacteria over the operation is reported in Figure 6.3, where values are expressed as: percentage of hybridized cells count to total counts of DAPI-stained cells; ratio determined by dividing the average bacterial abundance estimated with FISH analysis by that

obtained with CARD-FISH analysis; ratio between the produced SCFAs over time and VS at the initial concentration in the tests. This parameter permits to explicate the fraction of active bacteria out of total members belonging to this domain. In experiment Ox_4, a FISH/CARD-FISH ratio of 79.6% was observed, while in Ox_8, with a longer HRT, this ratio increased to 89.1% (Figure 6.3). These findings were statistically correlated with the estimated Y in these processes (p-value < 0.05), supporting the observed similar trends. A higher Y was observed in runs performed using thermally pretreated tannery sludge and thermophilic conditions compared to tests Ox_4 and Ox_8 (Figure 6.3). In particular, with HRT of 8 days (run Th_8) was imposed, the Y value reached 0.4 ± 0.01 g COD_{SCFA}/g VS. In this case as well, the detected FISH/CARD FISH ratio aligned with the trend of the Y, reaching a maximum of 95% in the Th_8 test (Figure 6.2). This high ratio corresponded to the bacterial elevated metabolic activity, which is consistent with the best yields achieved in this study.
The data indicated that higher HRTs correlated with increased biomass activity, leading to improved conversion of organic matter to SCFAs, despite the lower OLR experienced in run Ox_8 and Th_8. Additionally, elevated temperatures enhanced bacterial activity, resulting in higher yields (Y), particularly at longer HRTs.

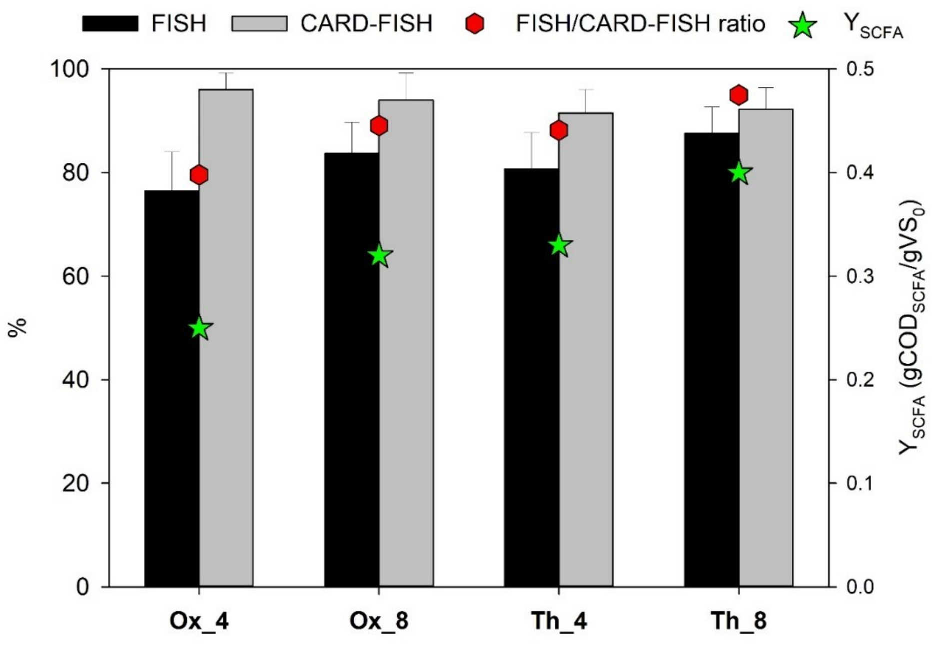


Figure 6.3: Bacterial abundance estimated by FISH and CARD-FISH analysis, FISH/CARD-FISH ratio and fermentation yield (Y).

6.3.2 Bacterial taxonomic diversity

The bacterial community was analyzed via 16S rRNA gene sequencing in samples collected from all experiments both at the beginning of the process and at steady state (t0 and tf, respectively). Tannery sludge was used as inoculum in all runs after one week of acclimatation. Sequencing analyses showed a massive microbial component attributable to the *Firmicutes* phylum (96-98% in Ox runs, and 77-81% in Th runs) at the initial time of all trials (t0, Figure 6.S1). In particular, most of the sequences belonged to the *Clostridiales* order. The almost exclusive presence of these microorganisms can be explained mainly by the nature of the sludge used as inoculum, rich in organic matter and in particular proteins. Several studies have shown that members of the *Clostridiales* order were able to utilize protein and glucose to produce SCFAs (Zhai et al., 2020; Zhang et al., 2019b). Furthermore, the phylum *Firmicutes* and in particular the class *Clostridia*, have been often linked to chromium contaminated environments (Branco et al., 2005; Yu et al., 2016). Indeed, the tannery sludge used in this study reported a total chromium (Cr) amount equal to 19.15 ± 0.3 g Cr/kg TS (Tuci et al., 2024, 2023), and this evidence may explain the high presence of this taxon at the beginning of the operations.

The bacterial composition observed in the runs using H_2O_2-pretreated sludge at both short (Ox_4) and long (Ox_8) HRTs during the steady state was similar. Almost all sequences belong to *Actinobacteria* (34% and 42%, for Ox_4 and Ox_8, respectively), *Bacteroidetes* (36% and 37%), and *Firmicutes* (28% and 21%), with a small portion (less than 2% in both experiments) attributable to the *Proteobacteria* phylum (Figure 6.S1).

Sequences affiliated with genus *Proteiniphilum* (*Bacteroidetes* phylum) represented 34 and 35% of the total reads in Ox_4 and Ox_8 tests, respectively (Figure 6.4). *Proteiniphilum sp.* is well known for its proteolytic metabolism aimed at producing SCFAs such as mainly acetate and propionate (Hahnke et al., 2016). The selective enrichment of this microorganism during the fermentation process was primarily influenced by the substrate composition. Its ability to efficiently degrade recalcitrant COD and rapidly adapt to nitrogen-rich organic matter, like proteins and collagen found in tannery sludge, facilitated its dominance within the microbial community (Zhu et al., 2024). In addition to the previously identified microorganisms, the reactors contained other microorganisms with similar metabolic functions (Dong et al., 2011; Yin et al., 2021). Notably, sequences belonging to genera *Proteiniborus*, *Proteiniclasticum* and *Clostridium sensu stricto 13* (*Firmicutes*, *Clostridia*) comprised between about 3 and 5% of the total reads obtained in both sequencing runs (Figure 6.4). The microbial community selected inside the Ox fermenters also highlighted the presence of genus *Corynebacterium 1* (*Actinobacteria*), which covered specifically 22% and 16% of the total reads in

Ox_4 and Ox_8, respectively (Figure 6.4). This microorganism has been extensively studied in tannery sludge and connected processes, and in particular in relation to its resistance to Cr (Ameen, 2023; Baba et al., 2020; Viti et al., 2003). Moreover, species belonging to *Corynebacteriumare* chemoorganotrophs with a fermentative metabolism (Tauch & Sandbote, 2014).

Regarding the experiments conducted under thermophilic conditions with the feed sludge pretreated at high temperature (Th_4 and Th_8), most of the found sequences belong to the phylum *Coprothermobacteraeota* (62% and 51%, for Th_4 and Th_8, respectively), followed by the phylum *Firmicutes* (31% and 44%). In these experiments, sequences attributable to *Proteobacteria* (less than 1%) and to *Tenericutes* (1% and 3%) were also found (Figure 6.S1). Reads affiliated with genus *Coprothermobacter sp.* represented the most sequences obtained in these runs (61.6% and 51% in Th_4 and 8, respectively) (Figure 6.4). This microorganism was isolated for the first time in tannery sludge, and is a fermentative proteolytic bacterium (Gagliano et al., 2015; Kersters et al., 1994; Pavan et al., 2019). Furthermore, the genus D8A-2 (*Firmicutes*, *Clostridia*) was found to comprise between 5.4% and 12.3% of the total sequences in Th_4 and Th_8, respectively (Figure 6.4). This taxon has been previously reported in thermophilic anaerobic systems characterized by high levels of SCFAs, where it is thought to play a role in syntrophic interactions, especially in the presence of elevated ammonia concentrations (Lee et al., 2019; Li et al., 2022a). The prominent presence of *Clostridia D8A- 2* in these systems can once again be attributed to its ability to metabolize proteins. Indeed, the HRT in Th_8 facilitates the degradation of proteins, leading to a higher production of ammonia compared to Th_4, which creates a more favorable environment for the growth of this microorganism. In addition, the Th reactors contained other fermentative bacteria, such as *Desulfitibacter* (2.3% and 6.9%, respectively) and *Desulfotomaculum* (4.1 and 6.4% in Th_4 and 8). Certainly, *Desulfotomaculum* genus is of particular interest since it is known to employ metals such as Cr as terminal electron acceptors, and is capable of oxidizing H_2 while reducing CO_2 to acetate (Tebo & Obraztsova, 1998). As reported in previous studies, *Desulfitibacter* and *Desulfotomaculum spp.* are associated with the oxidation of sulfur in anaerobic digestion processes (Nielsen et al., 2006; Tebo & Obraztsova, 1998). As a consequence, the presence of this type of metabolic activity in the herein studied systems could also reduce the toxic effects of sulfides and heavy metal (*e.g.*, Cr) on other microorganisms, as previously demonstrated by Zhu et al. (2024). Moreover, *Tepidimicrobium* genus was retrieved in both Th runs (2.4% and 3.4% of the total reads in Th_4 and 8, respectively; Figure 6.4). The presence of this microorganism in processes involving the use of tannery sludge is well documented (Kong et al., 2019). This taxon had a strong adaptability and is usually observed in high organic-containing sewage sludges where it uses both carbohydrates and peptide compounds to produce acetate, ethanol, butyrate, hydrogen and carbon dioxide (Kong et al., 2019; Niu et al., 2009).

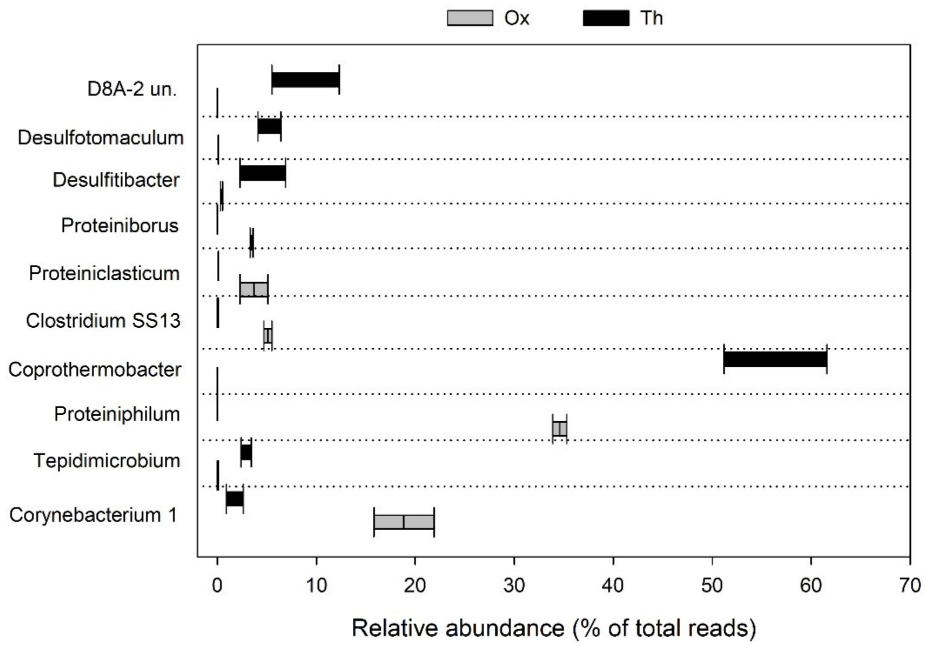


Figure 6.4: Box plot showing the relative abundance (% of total reads) of the 10 most abundant bacterial genera in Ox and Th runs.

To elucidate the connection between the biological data generated from the analysis of the various reactors and the key process parameters, a vector-fitting procedure was employed on the NMDS ordination plot based on chemical dissimilarity, where the stress value (i.e., <0.2) suggests an accurate representation of the dissimilarity among samples, and the relative abundance of the main genera (≥1% in at least one sample) is projected onto the NMDS ordination synthesising the microbiological dissimilarity (Figure 6.2 and 6.5). As expected, microbial community analysis revealed differences between the two clusters. The genera *Brevibacterium*, *Clostridium sensu stricto 13*, *Proteiniborus*, *Proteiniphilum* and *Coriobacteriales* unknown (vectors 19, 20, 21, 22 and 23, respectively) were significantly related to Ox runs. Conversely, *Tepidimicrobium, Desulfotomaculum, Ruminococcaceae* unknown, *Iziplasmatales, Coprothermobacter* and *Clostridia D8A-2* (vectors 1, 2, 3, 4, 5 and 6, respectively) were significantly correlated with Th systems (Figure 6.5). The NMDS analysis confirmed the separation of Ox and Th systems based on their microbial composition and SCFAs profiles. The distinct microbial communities observed in Ox and Th fermentations mainly resulted from the different incubation temperatures used in the studied processes. Temperature significantly influenced microbial growth and activity, affecting enzyme kinetics, membrane fluidity, and cellular stability. As expected, higher temperature promoted thermophilic or thermotolerant microorganisms, while lower temperature favored mesophilic organisms. These temperature-driven differences can have implications for fermentation, affecting product yields and substrate conversion rates. While microbial taxa diverged among the different tested reactors due to temperature, the overall functional outcome remained consistent. Indeed, different microbial communities selected in Ox and Th tests exhibited the same proteolytic metabolism responsible for the observed SCFAs production, highlighting the occurrence of redundancy.

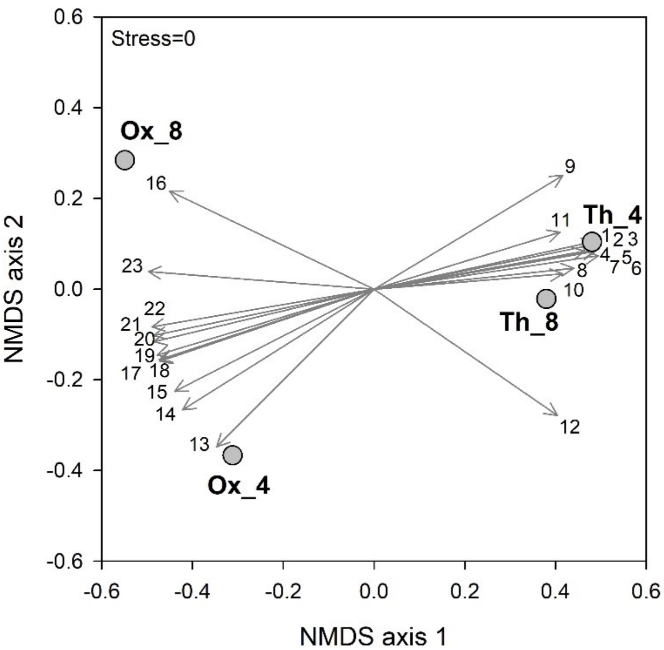


Figure 6.5: NMDS ordination plots, based on Bray-Curtis distance matrixes of log(X+1) transformed data. The vector length is proportional to the correlation between the NMDS axes and each parameter.

6.4 Conclusions

For the first time in this study, the microbial community activity and composition responsible for the SCFAs production from pretreated tannery sludge was in-depth explored. Overall, oxidative and thermal pretreatment of tannery sludge led to high SCFAs production, especially in systems fed with a low OLR (6.05 gVS/Ld) and operated with a high HRT (8 d). Moreover, the process temperature emerged as a critical factor in enhancing organic matter conversion to fatty acids. This parameter, together with the used protein-rich substrate, strongly influenced the selection of specific microorganisms responsible for the entire process.

In response to the different operating conditions tested, a different core microbiome was selected, although always composed of proteolytic microorganisms (e.g., *Proteiniphilum, Proteiniclasticum,* *Proteiniborus*). The presence of taxonomically different bacteria with the same metabolic potentialities strongly suggested that highly performing SCFAs microbial producers could be strategically selected to optimize fatty acid production. By exploiting the metabolic capabilities of these specialized microorganisms, it may be possible to significantly improve the efficiency and yield of the SCFAs synthesis processes.

Supplementary Materials (Chapter 6)

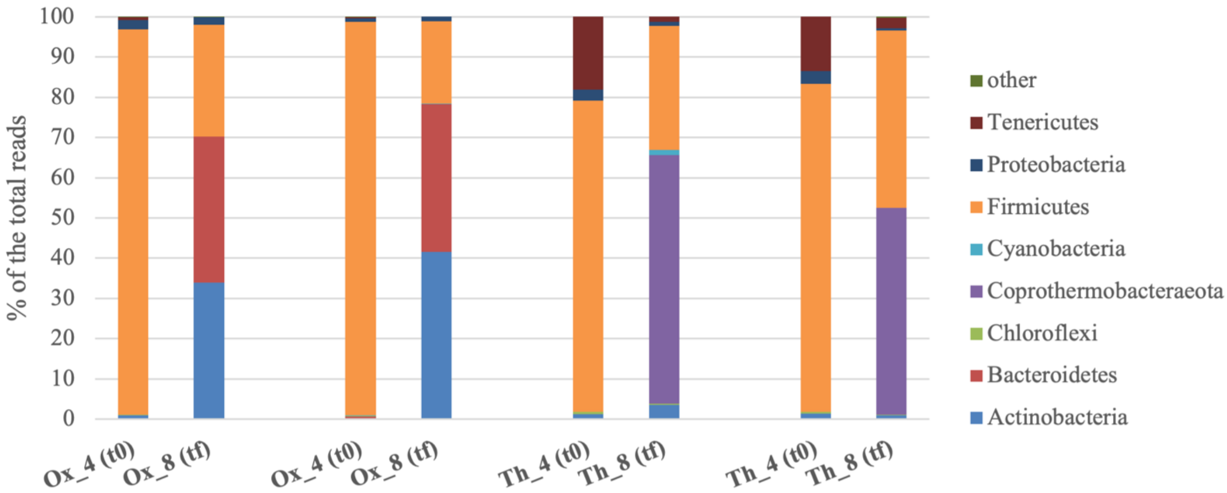


Figure 6.S1: Bacterial community composition at phylum level at the beginning (t0) and the end (tf) of the tests.

CHAPTER 7: CO_2 capture via dark-bioelectrochemical growth of *Rhodopseudomonas palustris*

In the proposed biorefinery scenario, after completing the steps of AF and AD, two key waste streams are also generated: the residual sludge and the biogenic CO_2.

The environmental impact of releasing CO_2 into the atmosphere is well-documented (IPCC, 2023), and with growing concerns over climate change, the need to mitigate carbon emissions has never been more urgent. Carbon capture and utilization technologies are gaining attention for their potential to tackle this issue by converting CO_2 into valuable products or sequestering it for long-term carbon storage sinks. This biorefinery model proposes an innovative approach to repurpose CO_2, thereby minimizing waste and maximizing revenue. The next section is focused on the bioelectrochemical system (BES) designed to convert CO_2 into a valuable product: single-cell proteins (SCP).

The development of this BES was carried on during my visiting period at the Leibniz Institute for Natural Product Research and Infection Biology Hans Knöll Institute in Jena, Germany, where I worked in the bio pilot plant group led by Prof. Dr. Miriam Agler-Rosenbaum.

These BES experiments are discussed in the following paper, which is still a work in progress as these results still need to be confirmed by additional tests before submission. The working title of this paper is "***Cultivation of Rhodopseudomonas palustris in a bioelectrochemical system in the dark as a carbon sink*".**

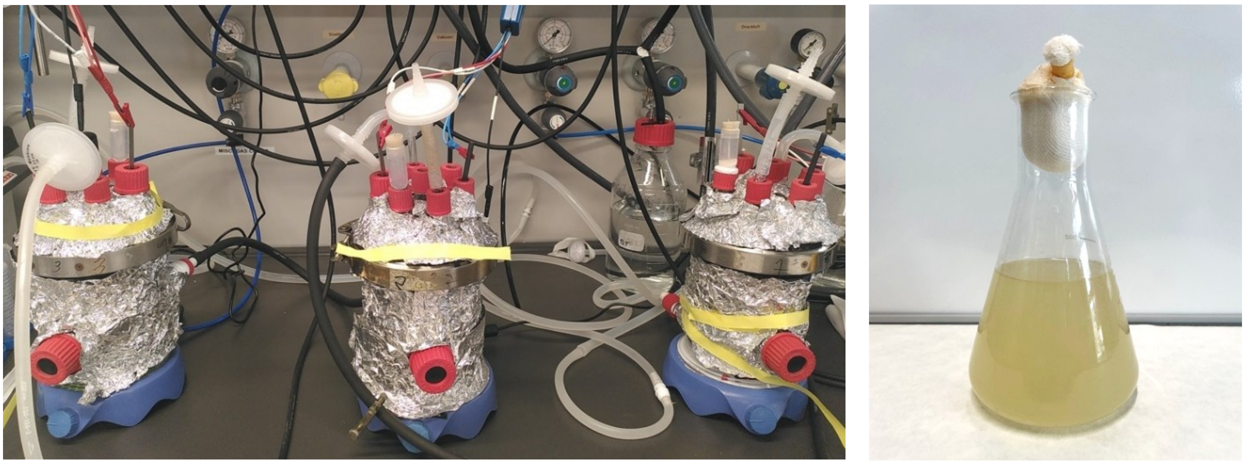


Figure 7.1: The bioelectrochemical reactors and the inoculum of R. palustris.

7.1 Introduction

The greenhouse effect and global warming are pressing environmental concerns, with CO_2 being the largest contributor, accounting for over 68% of the total greenhouse gas emissions in the atmosphere (Wang et al., 2024). Various methods exist for carbon sequestration; however, biological methods offer distinct advantages over physical and chemical ones, such as high safety, low energy consumption, no secondary pollution (Wang et al., 2024), and the possibility of carbon sequestration directly from flue gas or other CO_2-rich exhaust gases (Spanoghe et al., 2021).

In this context, *Rhodopseudomonas palustris*, a photosynthetic Gram-negative purple non-sulfur bacterium (PNSB) from the family *Bradyhizobiaceae,* emerges as a great candidate for this technology due to its high adaptability. Namely, this bacterium is able to grow in various conditions, being able to use light, inorganic, and organic compounds as carbon and energy sources under both anaerobic and aerobic conditions (Madigan & Jung, 2009). Most important, *R. palustris* can fix CO_2 through the Calvin-Bassham-Benson (CBB) cycle, contributing to cell metabolism (Li et al., 2022b). In general, PNSB have a wide range of applications, including the treatment of wastewaters and gas streams, soil remediation, extraction of valuable chemicals, polyhydroxyalkanoates (PHAs) production, and use as fertilizers (Capson-Tojo et al., 2020).

In particular, PHAs are biodegradable, water-insoluble polymers stored in bacterial cells (Raza et al., 2018). They are typically accumulated as intracellular carbon storage granules under unfavorable growth conditions, such as nutrient limitation (Rajvanshi et al., 2023), and are gaining a great deal of attention for their potential to make bioplastics with mechanical properties comparable to conventional plastics (Mannina et al., 2020).

Among PHAs, poly(3-hydroxybutyrate) (PHB) is the most well-known, consisting of repeating 3HB units (Sudesh et al., 2000). However, PHB is brittle and has a low extension to break, significantly limiting its range of applications (Wallen & Davis, 1972). In contrast, the incorporation of 3-hydroxyvalerate (3HV) into the polymer structure alters its thermomechanical properties, enhancing flexibility and reducing brittleness, making it a more versatile and commercially desirable bioplastic (Braunegg, 2002; Brown et al., 2022), thus making this co-polymer five to six times more expensive than the PHB homopolymer (Jacquel et al., 2008). Beyond its use as a biopolymer, PHA also has potential applications in single-cell protein (SCP) production, a protein-rich microbial biomass with potential as a supplement in animal food due to its high protein, vitamin, and lipid content (Muhammad Saeed et al., 2016). SCP production requires minimal land and resources, making it an efficient and environmentally sustainable protein alternative (Pesante et al., 2022). Recent research focused on employing microorganisms capable of producing PHAs as part of SCP, as the presence of PHA in SCP has been shown to promote growth (Rodriguez-Estrada et al., 2021) and boost the immune system, offering increased resistance to pathogens (Laranja et al., 2014; Suguna et al., 2014). Moreover, this approach eliminates the need for costly PHA extraction processes while still making use of this polymer.

R. palustris has been extensively studied and grown in a wide array of conditions; however, in this research, the use of a bioelectrochemical system (BES) for the growth of this bacterium on CO_2 as the only C source was explored. The main positive aspect of this setup is the possibility of direct conversion of CO_2 into valuable products using electric energy as the primary energy source. This eliminates the need for organic carbon inputs, aligning with circular economy principles and reducing reliance on biomass resources while simultaneously acting as a carbon sink.

Moreover, to reduce even further the resources needed for this process, the BES was set in the dark. In fact, previous studies have successfully investigated BES setups for this purpose (Soundararajan et al., 2019; Doud & Angenent, 2014), but they always included light.

Additionally, this study investigates the ability of *R. palustris* to produce PHA, a pathway known to be active in *R. palustris* under light conditions (Ranaivoarisoa et al., 2019; Montiel-Corona & Buitrón, 2022; Cerruti et al., 2020), but not yet studied in the dark, as traditionally, most studies on *R. palustris* focus on its phototrophic growth, neglecting its diverse possible lifestyles (Tec-Campos et al., 2023). However, this approach has significant drawbacks related to the additional energy and costs associated with the artificial illumination required, the difficulties in delivering the light to all the biomass equally, and the need for appropriate light and dark cycles (Capson-Tojo et al., 2020; Cerruti et al., 2020). Some sparse studies have shown that other *Rhodopseudomonas* species can carry out chemoautotrophic growth in the dark with CO_2 fixation by oxidizing H_2 and reducing O_2 (Siefert & Pfennig, 1979). However, these studies relied on H_2 supplied externally, which presents drawbacks such as low gas solubility, safety concerns, and increased costs. To overcome these limitations, this research implements a BES to facilitate the *in-situ* formation of H_2, serving as the electron donor. Additionally, *R. palustris* has demonstrated the ability to uptake electrons directly from electrodes (Guzman et al., 2019; Sun et al., 2023), providing a potential extra direct source of energy.

Moreover, in this research, a single-chamber BES was developed for several reasons. Firstly, the single-chamber design enhances scalability, making the process more suitable for industrial applications (Jadhav et al., 2022). Secondly, the *in-situ* production of oxygen from the anode can further support the growth of *R. palustris*. This dual approach of maximizing growth and minimizing resource allocation aligns with the principles of a biorefinery, aiming for efficiency and sustainability (Harnisch et al., 2024).

To evaluate the optimal conditions for the growth of *R. palustris*, two runs of experiments were conducted. The first run utilized a potentiostatic mode to perform an initial assessment of the BES and the most appropriate electric current to be employed for maximized biomass growth. Following this, a galvanostatic mode was employed with the reference electrode present but not connected, simulating a two-electrode setup, which is characterized by higher simplicity, stability, and easier scalability.

7.2 Materials and Methods

7.2.1 Culture preparation

Rhodopseudomonas palustris was pre-grown in the dark in 30 mL of R8AH medium (2.50 g L^{-1} Malic acid, 1 g L^{-1} yeast extract, 1.25 g L^{-1} $(NH_4)_2SO_4$, 0.2 g L^{-1} $MgSO_4$ x $7H_2O$, 0.07 g L^{-1} $CaCl_2$ x $2H_2O$, 0.01 g L^{-1} Fe(III) citrate, 0.02 g L^{-1} Na_2-EDTA, 0.60 g L^{-1} KH_2PO_4, 0.90 g L^{-1} K_2HPO_4, 1 mL L^{-1} trace elements solution and 7.50 mL L^{-1} vitamin solution) at 28 °C in a 300 mL shake flask maintained at 180 rpm.

The pre-culture was used as an inoculum for the reactors, and the amount of inoculum added was chosen to achieve a final cell density of OD600 = 0.1 in each reactor.

7.2.2 Bioelectrochemical reactors set up

Single-chamber reactors (in triplicates) were implemented in this study to investigate both potentiostatic and galvanostatic setups. The runs lasted 24 days, but the calculations considered 21 days to account for initial adaptation and fluctuations.

Single chamber 0.75 L glass reactor manufactured by Gaßner Glastechnik GmbH were used. The reactors were filled to 500 mL with the same R8AH medium without the yeast extract and malic acid, thus removing the organic carbon sources. To maintain a neutral pH, 1.68 g/L of $NaHCO_3$ were added, both as a pH buffer and an additional C source.

The reactors received a constant influx of gas, composed of 20% CO_2 and 80% Nitrogen, at an approximate volume flow of 0.1 L min^{-1}. This gas composition was used in order to provide both CO_2 as a C source, and Nitrogen both as a measure to prevent the formation of an explosive atmosphere and as a possible source of N, while the percentages were those of the gas mix available. All gas connections were made with oxygen-tight Tygon® tubing (VWR, Radnor, USA), with attached 0.2 µm PTFE vent filters to avoid contamination (Merck, Darmstadt, Germany). The reactors were kept under constant stirring at 100 rpm, and at a temperature of 28 °C with water recirculation into an external thermostatic jacket.

The cathode was made of a "POCO EDM-3" graphite rod with a graphite block (Novotec, Reuver, Netherlands) glued at the end of it with Leit C carbon-based conductive glue, giving a final surface of 156 cm^2. A Titan MOX (Ir/MMO) 150x40 mm (Jentner Plating Technology GmbH) was used as the anode, while the reference electrodes were in-house made $Ag/AgCl_{sat\ KCl}$ electrodes. The reactors were operated using a potentiostat (VMP3; BioLogic, Seyssinet-Pariset, France).

All WE potentials reported in this study refer to values against Ag/AgCl reference electrode (*ca.* +197 mV vs. standard hydrogen electrode SHE).

Chronoamperometry (CA) was applied throughout the operation, supplemented with cyclic voltammetry (CV) every 48h.

The galvanostatic condition was set imposing an electric current of -6.5 mA. This value was chosen after analyzing the data of the potentiostatic run.

7.2.3 Analytical methods

The concentrations of organic acids and alcohols in the culture supernatant (centrifugation at 10000 rpm for 3 min) were determined using an UHPLC system (Jasco, Tokio, Japan) equipped with DAD, RI (Jasco RI-930) and UV (210 nm) detectors. The column Aminex HPX-87H, 300 mm × 7.8 mm, 9 µm (Bio-Rad Laboratories, Hercules, USA) was used in combination with the precolumn Kromasil 100 C18, 40 mm × 4 mm, 5 µm (Dr Maisch GmbH, Ammerbuch-Entringen, Germany). The samples were diluted 1:10 with the mobile phase (0.005 M H_2SO_4), and 50 µL were used for injection. The column was maintained at 50°C and an isocratic flow rate of 0.5 mL min^{-1} was applied. The autosampler was flushed with bidistilled water.

To measure bacterial growth in suspension, the optical density at 600 nm was determined with a BioPhotometer (Eppendorf, Hamburg, Germany), and the pH was measured with a pH meter for small volumes (Mettler Toledo, Columbus, USA).

At the end of each experiment, the biomass in the reactors was collected. The liquid phase was centrifuged at 10000 rpm at 4°C for 20 min; then the supernatant was analyzed with the UHPLC, and the centrifuged was dried in an oven set at 100°C. For calculations, the dried biomass was weighted and approximated to a molecular formula of $C_5H_7NO_2$ (mw 113 g/mol).

PHA analyses were performed by mixing 5.0 mL of mixed liquor with 1.0 mL of NaClO solution (5% active Cl_2) then analysed according to the procedure described in Moretto et al. (2020b). The PHA was extracted, hydrolysed, and finally esterified to 3-hydroxyacyl methyl esters and quantified by GC method. The abundance of 3-hydroxybutyric (3HB) and 3-hydroxyvaleric (3HV) monomers were quantified using P(3HB-*co*-3HV) Sigma-Aldrich standard polymer (5 wt % 3HV).

For the analysis of pigments, thin-layer chromatography was performed according to the method described in Zeb and Murkovic (2010), trying the different extraction methods proposed in the paper. The spectrophotometric analysis was achieved with a CLARIOSTAR spectrophotometer (BMG Labtech).

Inorganic and organic carbon were measured by TOC (Total Organic Carbon Analyzer-Analytik Jena, Germany). The same instrument, with an additional module attached, was used for Total Nitrogen analysis.

The 5-aminolevulinic acid (ALA5) analysis was performed according to the method described in Yu et al. (2022).

7.2.4 Calculations

Coulombic efficiencies (CE) were calculated according to the following equation:

$$CE\ (\%) = \frac{F * n * z}{Q} * 100$$

Where F = Faraday constant (96 485 mC meq^{-1}); n = moles of product (mmol); z = number of electrons needed to obtain the reduced product starting from CO_2 (meq/mmol) and Q = delivered charge (mC).

Product	**n = m/mw [mmol]**	**z [meq/mmol]**
PHB $[COCH_2CH(CH_3)O]_n$	m_{PHB}/86 mg/mmol	18
PHV $[COCH_2CH(CH_2CH_3)O]_n$	m_{PHH}/118 mg/mmol	24
SCP $[C_5H_7NO_2]$	m_{SCP}/113 mg/mmol	20

In which m_{SCP} is the amount of biomass produced minus the amount of PHB and PHV inside.

$$m_{SCP} = m_{biomass} - m_{PHB} - m_{PHV}$$

The theoretical hydrogen production rate ($r_{theo}H_2$) was calculated as follows.

$$r_{H_2}(\frac{mmol}{d}) = \frac{Q(mC)}{F\left(\frac{mC}{meq}\right)2(\frac{meq}{mmol})d(d)}$$

In which Q are cumulative equivalents of charge delivered, 2 are the electrons needed to produce a molecule of H_2, and d are the days passed during the experimental period.

The energetic consumptions for each process were evaluated as follows.

$$EC(Wh) = 24|\Delta V|i$$

In which 24 are the hours in a day (h/d), ΔV (V) and i (A) represent the average potential difference and the average electric current registered during the experimental period, respectively.
The costs for energy considered is 0.237 €/kWh, as in Tuci et al. (2023).
For what regards the N utilization, considering the 1.25 g/L of $(NH_4)_2SO_4$ present in the media and the molecular formula for SCP of $C_5H_7NO_2$, the percentage of utilized nitrogen is:

$$NE\ (\%) = \frac{n_{SCP} * mw_{((NH_4)_2SO_4)}}{V_{rea} * [(NH_4)_2SO_4]} * 100$$

In which n_{SCP} are the moles of SCP produced, and V_{rea}, $[(NH_4)_2SO_4]$ and $mw_{((NH_4)_2SO_4)}$ are the volume of the reactor (L), the concentration of ammonium sulfate in the media (mg/L), and the molecular weight of ammonium sulfate (g/mol), respectively.

7.3 Results and Discussion

7.3.1 Overview of growth and hypothetical pathways

The selected experimental conditions were optimized for temperature and pH, ensuring no additional stress factors were added, as the primary objective was to determine whether the bacteria could grow without light, using inorganic carbon and hydrogen as the only energy and carbon sources, in the presence of oxygen (all the results presented correspond to the mean of the triplicates).
In typical light-exposed conditions, *Rhodopseudomonas palustris* employs the Calvin cycle to fix CO_2 and produce biomass through photosynthesis. However, in a BES without light, a previous study (Sun et al., 2022) has hypothesized that *R. palustris* may still be able to activate the Calvin cycle using electrons derived from alternative sources to light.
In this BES, a single-chamber design was used, which allowed for O_2 accumulation, produced at the anode via water oxidation ($2\ H_2O \rightarrow O_2 + 4\ H^+ + 4\ e^-$). The presence of O_2 likely boosted growth by enabling aerobic respiration and enhancing ATP generation.
This energy supported the Calvin cycle and other growth-related processes alongside electron uptake from H_2, thus mimicking a photosynthesis-like mechanism in the dark. It has been previously demonstrated that *R. palustris* can grow in the dark (Madigan et al., 1982) and can perform chemoautotrophy in the dark by fixing CO_2 in a co-culture (Liu et al., 2021). However, its capability of growing in a BES in the dark in pure culture with no organic carbon has not been verified before this study, making this a critical first step in understanding the potential of *R. palustris* in BESs.

After establishing bacterial survival and growth, secondary analyses focused on metabolite production, including a wide array of products typically produced by this microorganism, such as organic acids, ethanol, ALA5, and PHA. The aim was to assess whether these metabolites, commonly produced in light-driven systems, could also be synthesized in the BES.

For what regards the key resources provided for bacterial growth, the inorganic carbon was supplied both by continuous CO_2 flow and through the growth medium, which contained 1.68 g/L of $NaHCO_3$ as a pH buffer and carbon source. At the same time, O_2 and H_2 were produced *in situ* by the electrodes. Lastly, the Nitrogen was provided by the growth medium as $(NH_4)_2SO_4$ and was also present in the gas influx. The atmospheric nitrogen was provided both as a measure to prevent the formation of an explosive atmosphere and considering that *R. palustris* has been shown to fix atmospheric nitrogen (N_2) using specialized nitrogenases (Brown et al., 2022).

7.3.2 Growth under potentiostatic conditions

The first experimental run employed a potentiostatic setup, where the cathode was held at a fixed potential of -1.1 V vs. REF. This potential was selected to sustain electrolytic hydrogen production at a rate sufficient for bacterial growth and was calculated based on the Nernst equation, accounting for possible losses. During this run, CA was used to obtain data about the current and, consequently, hydrogen production.

Moreover, in this study, both H_2 and the cathode were available as potential electron donors, as the chosen potential was enough to allow for *in situ* hydrogen production, and *R. palustris* has been previously shown to be able to uptake electrons directly from the cathode in different growth conditions. Thus, to investigate the bacteria's preference for electron uptake, CV (performed between -1.1 and 0 V) was employed to monitor potential biofilm formation on the electrode surface, which would indicate direct electron uptake. However, the CV data (Figure 7.3) showed no clear evidence of significant biofilm formation, leading to the hypothesis that H_2, rather than direct electron uptake, was the primary electron source under these growth conditions.

CA data confirmed continuous H_2 and O_2 production during the experiment, showing a mean current of -4.8 ± 0.1 mA, which corresponds to a theoretical H_2 production (rH_2) of 2.1 mmol H_2/day.

As shown in Figure 7.2, growth data (measured via OD600) showed a steady increase, with a maximum mean OD600 of 0.55. A lag phase of two days was observed, suggesting rapid adaptation of the biomass to the BES conditions. The growth also appears quite consistent throughout the experiment, with just a slight decrease towards the end of the test, thus never reaching a plateau, which would suggest that the conditions and resources for growth were maintained throughout the test. The dry biomass measured at the end of the experiment was 120 mg.

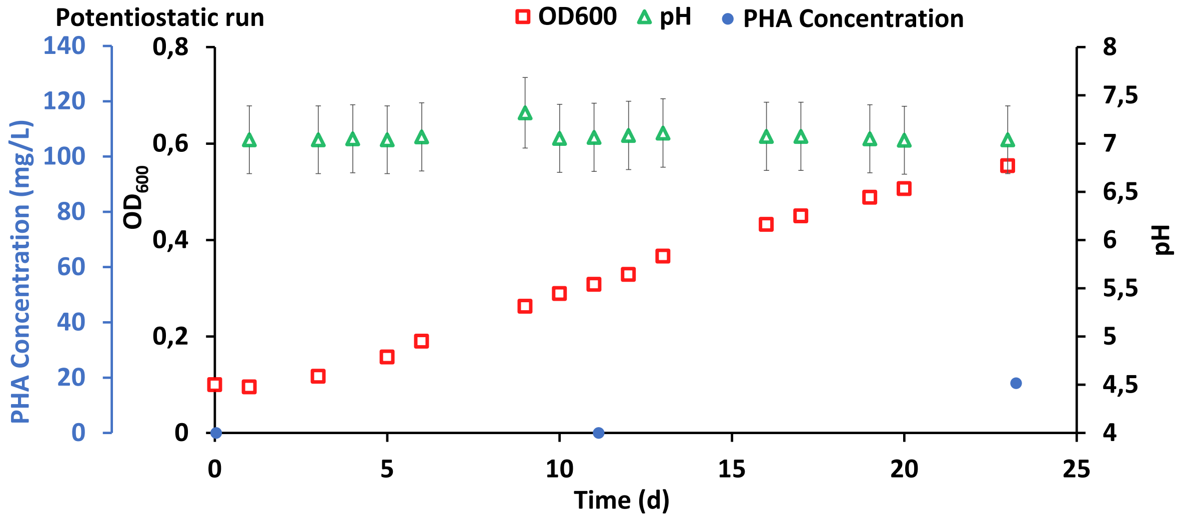


Figure 7.2: Growth (as OD600), pH and PHA production of the potentiostatic run.

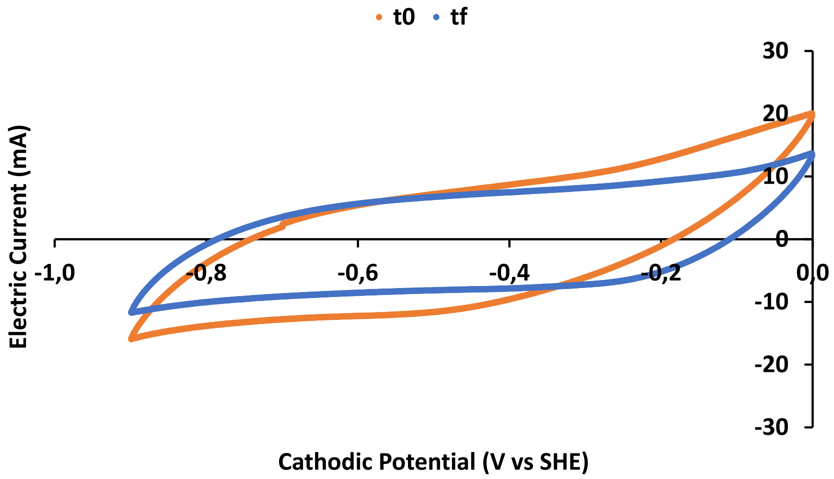


Figure 7.3: CV from the beginning (t0) and the end (tf) of the potentiostatic run.

Figure 7.2 also shows PHA production. In the potentiostatic run, being characterized by a less constant current, the resources for bacterial growth were less consistent, and consequently, the growth was slowed down, only attaining its maximum OD600 values at the end of the run, thus not reaching a clear stationary phase before the end of the experiment. Since PHA accumulation typically occurs during periods characterized by a lack of biomass growth (Mukhopadhyay et al., 2005), it was unsurprising that only a small amount of PHA was produced by the end of the experiment. The accumulated PHA reached a modest concentration of 18 mg/L and was composed entirely of 3HB.

This suggests that the metabolic priority under these conditions was biomass growth rather than polymer storage.

As previously introduced, PHA is typically stored when growth conditions are unfavorable, usually due to nutrient depletion. However, in this case, nitrogen analysis highlights that although an initial decrease in its concentration was recorded (Fig. 7.4), it was never low enough to become a limiting factor. This was also confirmed by the NE% of 19.2% obtained from this setup. This also would suggest that no nitrogen fixation was performed by the bacteria.

Moreover, as shown in Figure 7.4, while the TN was expected to decrease in tandem with the biomass growth, only an initial decrease was observed followed by a rather stable behavior, further investigations need to be performed to clarify this result.

Figure 7.4 also shows the total organic and inorganic carbon. The TOC is very low, as expected, and is probably related to some residues of the inoculum growth medium and the presence of dead cell fragments. In contrast, the TIC remains consistently high throughout the run, both due to the $NaHCO_3$ added to the medium and the continuously fluxed CO_2.

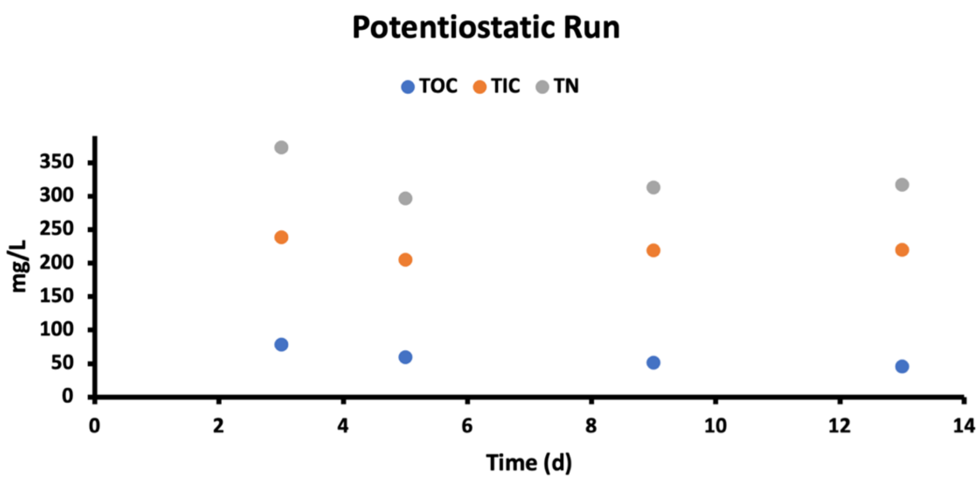


Figure 7.4: Total organic carbon, total inorganic carbon and total nitrogen of the potentiostatic run expressed in mgC/L and mgN/L.

From the rH_2, the total biomass produced, and the PHA, it was possible to calculate the coulombic efficiencies (CE) for these products. The CE_{SCP} (calculated as final dry weight – PHA weight) was 22.27%, while the CE_{PHA} was only 2.14%. Overall, the cumulative CE was then 24.41%.

These results are explained by the very small amount of PHA produced in these runs. Consequently, the energy in the system was either lost or went into biomass growth.

The energetic consumption results indicated a very low energy cost of 0.00154 € for the entire experiment. Given that this should be the maximum contribution to be sustained aside from the basic growth medium, it highlights the remarkably low expenses linked to running this experimental setup. Overall, this initial run aimed at understanding the BES with *R. palustris* and observing the possible changes in the system, with the intent of shifting to a more stable condition, the galvanostatic setting, which allows for more consistent current, thus more stable growth conditions.

7.3.3 Growth under galvanostatic conditions

Based on the results from the potentiostatic run, the chosen current for this run was set at -6.5 mA, corresponding to a theoretical hydrogen production of 2.91 mmol H_2/day.

While the final OD600 reached in this run was similar to that observed under potentiostatic conditions (OD600 = 0.62), this result was reached around 10 days sooner, likely due to the consistent current supply, resulting in steadier hydrogen and oxygen production. The dry biomass measured at the end of the experiment was 130 mg.

Overall, as shown in Figure 7.5, the growth showed a different behavior from the potentiostatic run, with two distinct phases: the first phase (from days 0 to 13) focused primarily on biomass growth, with no detectable PHA. However, after day 13, the maximum OD600 was reached, the growth plateaued, and PHA production began. By the end of the experiment, a more significant amount of PHA had accumulated, with a concentration of 119 mg/L.

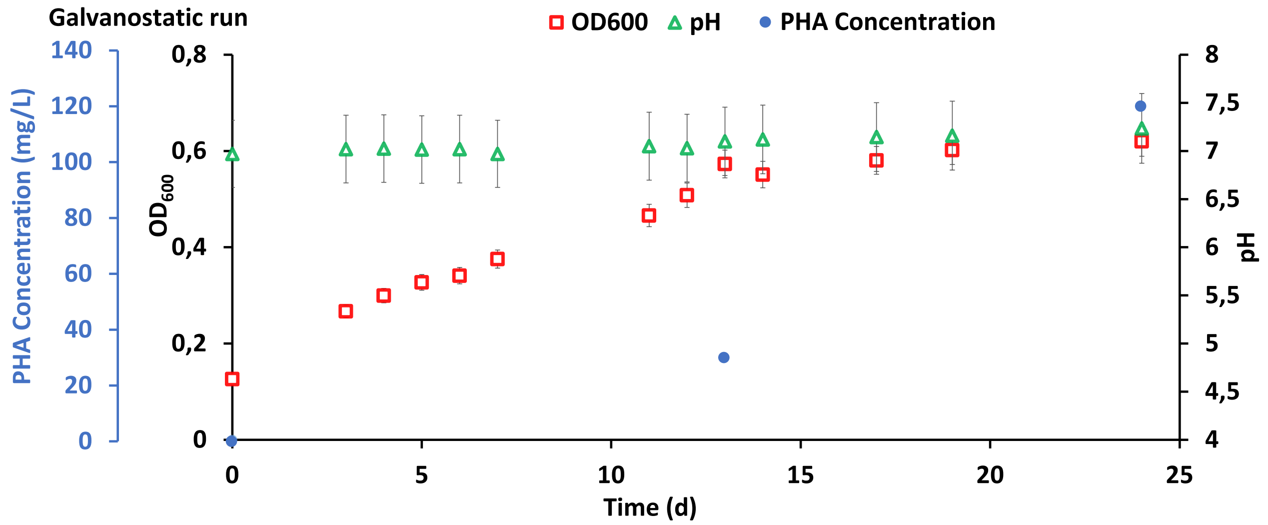


Figure 7.5: Growth (as OD600), pH, and PHA production of the galvanostatic run.

Similarly to the other setup, the nitrogen concentration in the medium was likely not the trigger for PHA production, as its concentration never reached values below 300 mgN/L (as a mean of the triplicates), as shown in Figure 7.6. Again, this was confirmed by the NE% calculation, which highlighted that only 12.7% of the total N present in the medium was used for bacterial growth, suggesting that, like in the potentiostatic run, no nitrogen fixation was performed.

The same unexpected behavior of TN concentration was observed, as there was no significant decrease throughout the tests. The reason for this behavior requires further investigation, as previously stated. Moreover, the TOC and TIC results showcase the same trends observed and discussed for the previous setup.

Overall, these results seem to confirm that N isn't the limiting factor for growth. Consequently, the reason why the OD of 0.6 appears to be the upper limit for the growth of *R. palustris* in this setup may be related to the challenge of growing on inorganic carbon in the dark, possibly causing the bacteria to stop growing beyond this limit and instead start accumulating PHA.

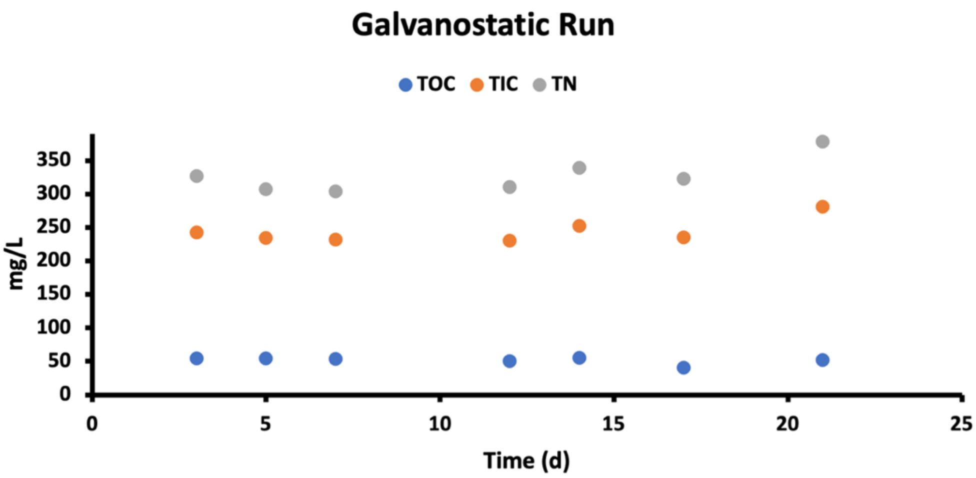


Figure 7.6: Total organic carbon, total inorganic carbon and total nitrogen for the potentiostatic run expressed in mgC/L and mgN/L.

For what regards the CE for this setup, as expected, the CE_{SCP} was much lower than that of the potentiostatic run, since a more significant energy amount went into PHA production. Namely, the CE_{SCP} was 10.3%, while the CE_{PHA} was 10.0%. Overall reaching a cumulative CE of 20.3%, comparable to the potentiostatic setup, as shown in Figure 7.7. Moreover, it is worth noticing that, like the potentiostatic run, the energetic cost for this experiment was equal to 0.00182 €.

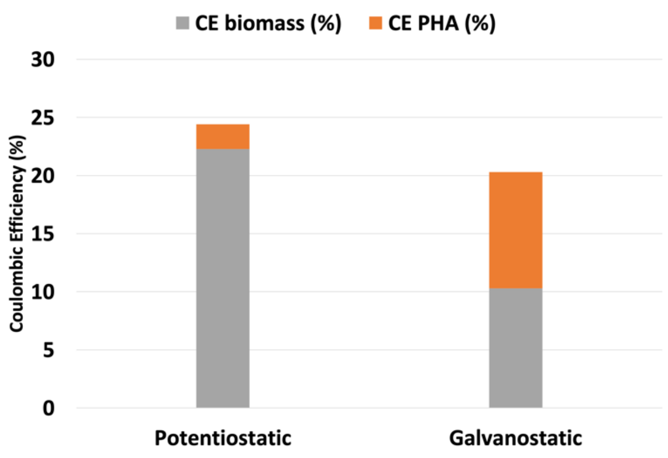


Figure 7.7: Visual representation of the respective CEs for the two setups.

A significant aspect of this setup was the composition of the produced PHA, which included not only 3HB but also a substantial proportion of 3HV, amounting to 75 wt%. This is an unusual result, as typical PHA compositions from PNSB grown with organic carbon sources tend to show lower 3HV percentages, usually well below 25% (Fradinho et al.; 2019, Dan et al., 2023; Mukhopadhyay et al., 2005).

This higher proportion of 3HV observed might have different explanations: one reason for this uncommon behavior could stem from the significant and constant amount of reducing power provided by this setup, which could encourage the production of 3HV, the more reduced co-polymer. Moreover, 3HV is typically produced from odd-numbered carbon sources, which might potentially include CO_2.

Furthermore, it has been extensively reported in the literature (Corona et al., 2017) that, in PNSB, the pathways for H_2 and PHB production compete for electrons and reducing power, which reduces the efficiency of PHB formation. However, in the setup described in this paper, the continuous production of H_2 by the BES may help overcome this issue, thus enhancing PHB production.

However, further studies are needed to confirm these hypotheses and explore the underlying mechanisms. Moreover, at this stage, it is difficult to directly compare these results with other studies, as this experiment is the first to investigate PHA production in this setup. The absence of a model or data for assessing the impact of these unique conditions on polymer composition highlights the need for further research in this area.

While both potentiostatic and galvanostatic settings demonstrated successful *R. palustris* growth in the dark, the galvanostatic setup showed more predictable hydrogen production and growth rates, making it a more promising approach for future BES applications.

7.3.4 Metabolites and pigment production

The liquid fraction from both runs was analyzed to identify key metabolites usually produced by *R. palustris*, namely acetic acid, formic acid, fumaric acid, lactic acid, succinic acid, and malic acid. However, none of these compounds were detected.

The absence of these metabolites could be attributed to the organism's prioritization of biomass growth over secondary metabolite production in response to the unique conditions of the BES.

Moreover, ALA5 was also analyzed and was not found in any of the samples.

Another parameter of interest was the color of the biomass. Under light-deprived conditions, *R. palustris* biomass exhibited a light pink color, much different than its usual bright red. This prompted an analysis of carotenoid production, as these pigments are light-sensitive, and the dark conditions might have inhibited their production. The results from thin-layer chromatography did not show the presence of carotenoids, and this was confirmed by spectrophotometric analysis. As observed in Figure 7.8, the region of the spectrum where carotenoids typically absorb light (between 348 and 442 nm, Zeb & Murkovic, 2010) showed no peaks.

These results are consistent with previous findings by Madigan and colleagues (1982), who reported that *R. palustris* can cease carotenoid production after multiple subcultures in dark conditions. Carotenoids may also have roles in oxidative stress protection (Li et al., 2023b), but under the conditions of this experiment, where oxygen production at the anode was limited, this function may not have been required.

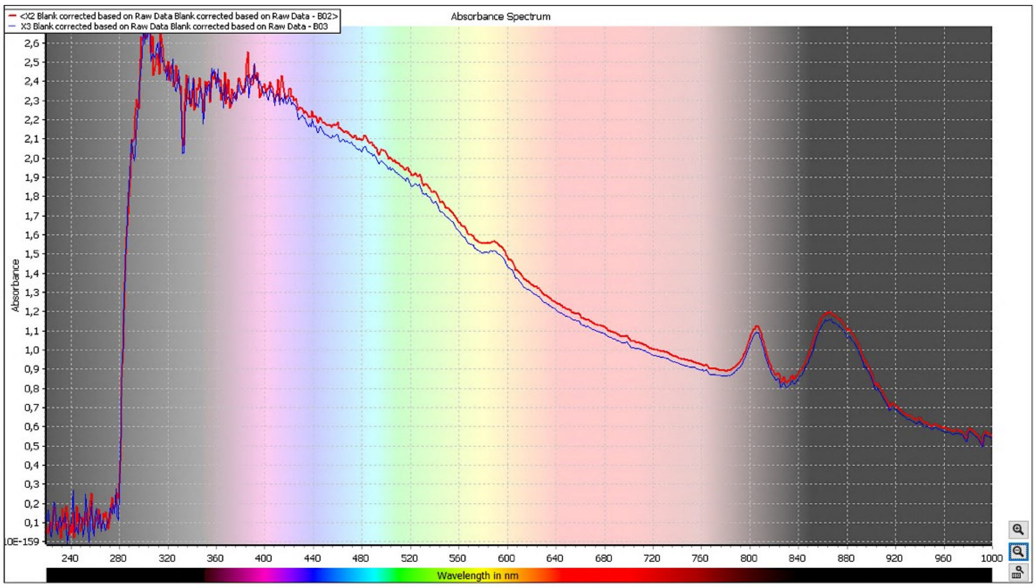


Figure 7.8: Absorbance spectrum of R. palustris grown in the dark in the BES.

7.4 Conclusions

In this study, *R. palustris* was grown on CO_2 in a BES under dark conditions. The most successful condition was the galvanostatic setting, which allowed for a maximum growth of 0.62, as OD600, and the production of PHA of 119 mg/L. No additional metabolites were detected, and the pigmentation of the bacteria was significantly reduced, as carotenoid production was likely shut down.

While the growth rate and maximum biomass achieved were modest, it is important to note that these conditions are significantly different from optimal ones for *R. palustris*, particularly due to the lack of light and organic carbon (for heterotrophic metabolism). Nonetheless, the experiment demonstrated that *R. palustris* can successfully grow using only inorganic carbon, oxygen, and hydrogen in the dark while also producing PHA.

Moreover, the PHA produced under galvanostatic conditions was mainly (75%) composed of 3HV, a very rare occurrence possibly due to the significant reducing power in the reactor, due to the implemented BES. This could represent a highly innovative technique for obtaining a substantial amount of 3HV with minimal resource allocation while also acting as a CO_2 sink.

However, the trigger for PHA production still needs to be identified, as N limitation, which typically prompts PHA production, did not occur in this setup as N levels remained quite consistent throughout the whole run for both setups.

Overall, both conditions showed a CE of around 20% indicating that 4 out of 5 electrons were lost, probably as H_2. The similar CE for both setups, also linked to the similar maximum OD600 reached, could highlight an upper growth limit for *R. palustris* in a BES, with yet unknown causes. And would suggest that, if this upper limit is confirmed, the key to improving the CE could lie in electric current reduction more than growth optimization.

Future studies should focus on examining the possible underlying mechanisms that may trigger PHA production. Moreover, this process has the potential to be eventually integrated as a part of a biorefinery, uptaking CO_2 directly from bio-processes (anaerobic fermentation and/or anaerobic digestion) to be used for the production of added-value products.

FINAL DISCUSSIONS AND FUTURE PESPECTIVES

This thesis investigated the technical feasibility with some economic considerations of a biorefinery approach for the valorization of tannery sludge, an abundant yet biologically recalcitrant waste stream. This work aimed to convert this waste into valuable products, including VFAs, methane, and SCP, by integrating biotechnologies to optimize resource recovery, reduce waste volume, and mitigate environmental impacts.

An initial literature review highlighted the lack of prior research concerning the application of tannery sludge for biotechnological purposes, primarily due to its complex composition and high chromium content. Consequently, the first objective of this study was to assess the feasibility of AD of tannery sludge.

Despite of the high Cr content (around 3 wt%), the possibility of using tannery sludge as a substrate for AD was confirmed and the optimal conditions to maximize VFA production, in terms of temperature and initial solids load, were assessed. Additionally, the efficiency of an H_2O_2-MW pretreatment to solubilize organic matter, thereby enhancing the fermentation process, was quantified. The best overall acidification performance was evaluated in terms of acidification yield (0.31 g COD_{VFA}/g VS_0) and a maximum VFA concentration (26 g COD_{VFA}/L). The study also assessed VFA composition, showing that pretreated tannery sludge generally is a source of high selective VFA spectrum, as acetic acid made up 50% of the total acids produced (COD_{Ac}/COD_{VFA}). Moreover, the results from this first study highlighted this substrate's pH buffering capabilities, allowing the process to remain at neutral pH without the need for external chemical additions, even with consistent VFA production, thus promoting overall process stability and reducing the costs associated with external pH correction. This was linked to the high content of $CaCO_3$ added during the tanning process.

The following study was focused on a more thorough assessment of the optimal H_2O_2 dosage in the pretreatment stage. A dosage of 0.4 g H_2O_2/g TS and mesophilic temperature (40 °C) were the most suitable conditions to promote sludge biodegradability and VFA production in batch tests. Moreover, a scale-up of the process was carried out, employing continuously stirred tank reactors with a volume of 1.5 L to investigate the most appropriate temperature and HRT in such set-up. Among the two investigated HRTs (4 and 8 days), the highest VFA concentration (16.0 g COD_{VFA}/L) and acidification yield (0.32 g COD_{VFA}/g VS_0) were obtained at 8.0 days as HRT. Additionally, this study included an analysis of chromium behavior, revealing that it was initially released (up to 119 mg/L) into the liquid phase but was completely removed in less than 5 HRTs (probably due to the adsorption

on the biological flocks), allowing for the utilization of fermentation liquid without environmental safety concerns.
In addition to VFAs, the process was designed to achieve methane recovery through a subsequent AD step using the solid-rich residue from AF as substrate. This step enhances the economic and environmental sustainability of the process, as methane can serve as a renewable energy source, either to be sold for revenue or to fuel the process itself. This residue showed its potential as a biogas source, achieving a specific gas production (SGP) of 0.48 m^3/kg VS. Finally, the overall performance of the AF and AD was assessed in terms of waste reduction and environmental benefits. Compared to conventional disposal methods, the proposed system achieved a significant reduction in total solids (roughly 50%), minimizing landfill requirements and the associated environmental impacts. A potential economic benefit close to 2,700,000 €/y compared to the current management practices was finally evaluated.
An additional step forward was taken with a third article that investigated the performance of AF in thermophilic conditions in six CSTR runs and a mild thermal pre-treatment. Moreover, two of these runs incorporated zeolites (chabazite in run II, and clinoptilolite in run III), chosen due to their high potential in heavy metal removal. Results highlighted zeolites' impact on chromium concentration and the SCFA/COD_{SOL} ratio. Namely, chromium release in the liquid fraction, due to thermal pretreatment, reached 335 mg/L, a much higher concentration compared to the previous tests. Consequently, complete removal proved challenging in the tests without zeolite, while in zeolite-amended runs, complete removal was achieved, confirming the materials' heavy metal absorption capacity. SCFA concentrations reached > 20 g COD/L, with acidification efficiency varying from 0.70 to 0.92 COD/COD, with chabazite addition. These findings underscore zeolites' potential in heavy metal absorption and acidification process enhancement, paving the way for applications with tannery sludge.
To have a more thorough understanding of the process, the microbial community of most of the semi-continuous tests was analyzed, thanks to the cooperation with the CNR-IRSA. The bacterial community in pretreated tannery sludge was shaped by its high protein content and chromium contamination. Initially, *Firmicutes* (predominantly from the *Clostridiales* order) dominated across all runs, likely due to their resilience in environments rich in proteins and heavy metals. However, upon reaching a steady state, mesophilic conditions (with H_2O_2 pretreatment) favored a shift toward *Actinobacteria*, *Bacteroidetes*, and *Firmicutes*, with *Corynebacterium 1* standing out for its chromium resistance and its well-documented presence in tannery sludge. Among fermentative bacteria, *Proteiniphilum* was the most abundant, thanks to its capacity to degrade recalcitrant COD

and efficiently metabolize nitrogen-rich organic matter such as proteins and collagen, both abundant in tannery sludge.

Under thermophilic conditions (with thermal pretreatment), the bacterial community was dominated by *Coprothermobacteraeota*, a phylum specialized in proteolytic metabolism at high temperatures. In particular, *Coprothermobacter sp.* was the most abundant genus in these runs, another well-documented genus in tannery sludge. The chromium-associated genus *Desulfotomaculum* was also detected, known to utilize metals like chromium as terminal electron acceptors, potentially mitigating its toxic effects on other microorganisms.

Despite taxonomic differences between conditions, all microbial communities exhibited a strong proteolytic metabolism, indicating functional redundancy and a high degree of adaptation to the tannery sludge environment.

In addition to sludge valorization, this thesis also explored an innovative approach for biogenic CO_2 capture. In particular, CO_2 was used as the sole carbon source for the growth of *Rhodopseudomonas palustris* in a BES set in a novel dark condition, aiming to obtain microbial biomass and intracellular PHA. The results from this study show the feasibility of this challenging setup. The most successful condition was the galvanostatic setting, which allowed for a maximum growth of 0.62, measured as optical density (OD) at a wavelength of 600 nm, and a production of PHA of 119 mg/L. The PHA composition in this setting was made of 75% 3HV, generally not observed in the literature with both chemotrophic and phototrophic biomasses. This result might be explained by the significant amount of reducing power provided by this setup, which could encourage the production of more 3HV, the more reduced co-polymer unit. This could represent a highly innovative technique for obtaining a substantial amount of 3HV with minimal resource allocation while also acting as a CO_2 sink.

While these results still need to be confirmed and the underlying mechanisms understood, this experiment demonstrated that *R. palustris* can successfully grow using only inorganic carbon, oxygen, and hydrogen in the dark, while also producing PHA. Moreover, this result highlights the added value of integrating CO_2 fixation into waste treatment strategies, further closing the resource loop.

The findings of this research demonstrate that integrating multiple biotechnological processes can significantly improve the sustainability of tannery sludge management. Compared to landfilling, the only current disposal method, the proposed system not only reduces waste volume by at least half but also generates valuable by-products with economic potential, overall diminishing both the environmental impact of a very polluting industry and the economic strain on the industry itself for the disposal of such an abundant yet hazardous waste.

While this thesis presents a comprehensive biorefinery process, the maximum scale of the experiments described was semi-continuous with reactors of only a few liters. The ultimate goal, however, is to achieve industrial-scale implementation, which requires further research and development. The next step would be the implementation of pilot plants to assess whether the same productivity and parameters can be maintained at a larger scale. Additionally, future research should focus on process optimization, for example, by exploring other pretreatment methods or additives.

A major challenge that remains is the presence of chromium in the final sludge, which consequently still requires the sludge's disposal in landfills. Although chromium removal and recovery strategies have been proposed in the literature (Pantazopoulou & Zouboulis, 2020; Ma et al., 2017), they still present several drawbacks mainly concerning chemical investments and the risk of secondary waste production with hazardous properties. Consequently, implementing a chromium removal technique within the biorefinery could facilitate an even further reduction of landfilled sludge and a possible recovery of chromium, thus properly closing the loop around this waste and minimizing its environmental impact.

From a microbiological perspective, in this thesis an initial step in understanding the microbial communities involved in tannery sludge bioconversion was presented. Future research should delve deeper into the functional roles of key microbial species, their interactions, and their resilience to varying process conditions. Harnessing this knowledge could lead to targeted microbial engineering or bioaugmentation strategies to further enhance process stability and performance.

Moreover, as BESs were only explored in the final part of my PhD, the investigation in this direction is still in its early stages, and the results presented in this thesis only make up a very small preliminary knowledge of the possibilities of this technology combined with *Rhodopseudomonas palustris*. Future studies should explore their full capabilities, optimizing operational conditions and assessing their scalability for real-world applications.

Overall, this study contributes to the broader effort of developing circular economy solutions for tanning industrial waste streams. By demonstrating that tannery sludge can be successfully converted into bio-based products, it opens new pathways for sustainable waste management and resource recovery. Although challenges remain, the results obtained in this work provide a solid foundation for further advancements in the field. The stakeholders for this proposed biorefinery are mainly WWTP operators, as they are directly involved in the management and disposal of tannery sludge and could greatly benefit from more sustainable and cost-effective alternatives.

BIBLIOGRAPHY

2030 Climate & Energy Framework. Available online: https://ec.europa.eu/clima/policies/strategies/2030_en (accessed on 26 May 2022).

Abate, T. A., Desta, A. F., & Love, N. G. (2021). Evaluating tannery wastewater treatment performance based on physicochemical and microbiological characteristics: An Ethiopian case study. Water Environment Research, 93(5), 658-669. https://doi.org/10.1002/wer.1364

Abreu, M. A. D., & Toffoli, S. M. (2009). Characterization of a chromium-rich tannery waste and its potential use in ceramics. *Ceramics International*, *35*(6), 2225-2234. https://doi.org/10.1016/j.ceramint.2008.12.011

Abubakar, U. A., Ajibike, M. A., & Adie, D. B. (2017). Hydrolysis rates of domestic wastewater sludge using biochemical methane potential tests. *Nigerian Journal of Technology*, *36*(4), 1315-1323. https://doi.org/10.4314/njt.v36i4.43.

Achouri, O., Panico, A., Bencheikh-Lehocine, M., Derbal, K., Arias, D., Iasimone, F., ... & Pirozzi, F. (2021). Role of H_2O_2 dosage on methane production from tannery wastewater: experimental and kinetic study. *Journal of Water Process Engineering*, *43*, 102313.https://doi.org/10.1016/j.jwpe.2021.102313.

Agustini, C. B., da Costa, M., & Gutterres, M. (2020). Biogas from tannery solid waste anaerobic digestion is driven by the association of the bacterial order bacteroidales and archaeal family methanosaetaceae. *Applied biochemistry and biotechnology*, *192*(2), 482-493. https://doi.org/10.1007/s12010-020-03326-6

Agustini, C. B., Spier, F., da Costa, M., & Gutterres, M. (2018). Biogas production for anaerobic co-digestion of tannery solid wastes under presence and absence of the tanning agent. *Resources, Conservation and Recycling*, *130*, 51-59. https://doi.org/10.1016/j. resconrec.2017.11.018.

Aktij, S. A., Zirehpour, A., Mollahosseini, A., Taherzadeh, M. J., Tiraferri, A., & Rahimpour, A. (2020). Feasibility of membrane processes for the recovery and purification of bio-based volatile fatty acids: A comprehensive review. *Journal of Industrial and Engineering Chemistry*, *81*, 24-40. https://doi.org/10.1016/j.jiec.2019.09.009.

Akyol, Ç., Demirel, B., & Onay, T. T. (2015). Recovery of methane from tannery sludge: the effect of inoculum to substrate ratio and solids content. *Journal of Material Cycles and Waste Management*, *17*, 808-815. https://doi.org/10.1007/s10163-014-0306-2.

Ali, P., Zalivina, N., Le, T., Riffat, R., Ergas, S., Wett, B., ... & De Clippeleir, H. (2021). Primary sludge fermentate as carbon source for mainstream partial denitrification–anammox (PdNA). *Water Environment Research*, *93*(7), 1044-1059. https://doi.org/10.1002/wer.1492.

Ali, Z., Malik, R. N., Shinwari, Z. K., & Qadir, A. (2015). Enrichment, risk assessment, and statistical apportionment of heavy metals in tannery-affected areas. *International Journal of Environmental Science and Technology*, *12*, 537-550. https://doi.org/10.1007/s13762-013-0428-4

Alibardi, L., & Cossu, R. (2016a). Pre-treatment of tannery sludge for sustainable landfilling. *Waste management*, *52*, 202-211. https://doi.org/10.1016/j.wasman.2016.04.008

Alibardi, L. & Cossu, R. (2016b). Effects of carbohydrate, protein and lipid content of organic waste on hydrogen production and fermentation products, *Waste management*, 47, 69–77, https://doi.org/10.1016/j.wasman.2015.07.049.

Ambrose, H. W., Chin, C. T. L., Hong, E., Philip, L., Suraishkumar, G. K., Sen, T. K., & Khiadani, M. (2020). Effect of hybrid (microwave-H_2O_2) feed sludge pretreatment on single and two-stage anaerobic digestion efficiency of real mixed sewage sludge. *Process Safety and Environmental Protection*, *136*, 194-202. https://doi.org/10.1016/j.psep.2020.01.032

Ameen, F. (2023). Improving Tannery Wastewater Treatments Using an Additional Microbial Treatment with a Bacterial–Fungal Consortium. *Biology*, *12*(12), 1507.https://doi.org/doi.org/10.3390/biology12121507

Angelidaki, I., Alves, M., Bolzonella, D., Borzacconi, L., Campos, J. L., Guwy, A. J., ... & Van Lier, J. B. (2009). Defining the biomethane potential (BMP) of solid organic wastes and energy crops: a proposed protocol for batch assays. *Water science and technology*, *59*(5), 927-934.https://doi.org/10.2166/wst.2009.040.

Angenent, L. T., Richter, H., Buckel, W., Spirito, C. M., Steinbusch, K. J., Plugge, C. M., ... & Hamelers, H. V. (2016). Chain elongation with reactor microbiomes: open-culture biotechnology to produce biochemicals. *Environmental science & technology*, *50*(6), 2796-2810. https://doi.org/10.1021/acs.est.5b04847

Apha Awwa, W.E.F. (2005). Standard Methods for the Examination of Water and Wastewater. American Public Health Association, American Water Works Association, and Water Environment Federation.

Apha Awwa, W.E.F. (2012). Standard Methods for the Examination of Water and Wastewater. American Public Health Association, American Water Works Association, and Water Environment Federation.

Ariunbaatar, J., Panico, A., Esposito, G., Pirozzi, F., & Lens, P. N. (2014). Pretreatment methods to enhance anaerobic digestion of organic solid waste. *Applied energy*, *123*, 143-156. https://doi.org/10.1016/j.apenergy.2014.02.035

Ashraf, S., Naveed, M., Afzal, M., Ashraf, S., Rehman, K., Hussain, A., & Zahir, Z. A. (2018). Bioremediation of tannery effluent by Cr-and salt-tolerant bacterial strains. *Environmental monitoring and assessment*, *190*, 1-11.

Atasoy, M., Owusu-Agyeman, I., Plaza, E., & Cetecioglu, Z. (2018). Bio-based volatile fatty acid production and recovery from waste streams: Current status and future challenges. *Bioresource technology*, *268*, 773-786. https://doi.org/10.1016/j. biortech.2018.07.042.

Baba, A., Garba, S. T., & Bello, H. S. (2020). Bioremediation potential of immobilized Corynebacterium kutsceri in the treatment of tannery industrial effluent from Challawa Industrial Estate, Kano State, Nigeria. *Journal of the Turkish Chemical Society Section A: Chemistry*, *7*(2), 335-350.

Barros, M. A., Silva, E. A., Arroyo, P. A., Tavares, C. R. G., Schneider, R. M., Suszek, M., & Sousa-Aguiar, E. F. (2004). Removal of Cr (III) in the fixed bed column and batch reactors using as adsorbent zeolite NaX. *Chemical Engineering Science*, *59*(24), 5959-5966. https://doi. org/10.1016/j.ces.2004.07.040.

Basegio, T., Berutti, F., Bernardes, A., & Bergmann, C. P. (2002). Environmental and technical aspects of the utilisation of tannery sludge as a raw material for clay products. *Journal of the European ceramic Society*, *22*(13), 2251-2259. https://doi.org/10.1016/S0955-2219(02)00024-9

Battista, F., Almendros, M. G., Rousset, R., Boivineau, S., & Bouillon, P. A. (2018). Enzymatic hydrolysis at high dry matter content: The influence of the substrates' physical properties and of loading strategies on mixing and energetic consumption. *Bioresource technology*, *250*, 191-196. https://doi.org/10.1016/j.biortech.2017.11.049

Baumann, I., & Westermann, P. (2016). Microbial Production of Short Chain Fatty Acids from Lignocellulosic Biomass: Current Processes and Market. BioMed Research International. Hindawi Limited. https://doi.org/10.1155/2016/8469357.

Bielak, E., Sawoszczuk, T., & Syguła-Cholewińska, J. (2023). Application of Chromatographic and Microbiological Analyses to Identify and Assess the Durability of Antimicrobial Properties of Innovative Materials for the Footwear Industry─ Leather Modified with Origanum vulgare Oil. *Industrial & Engineering Chemistry Research*, *62*(14), 5864-5876. https://doi.org/10.1021/acs.iecr.2c04527

Black, M., Canova, M., Rydin, S., Scalet, B. M., Roudier, S., & Sancho, L. D. (2013). Best available techniques (BAT) reference document for the tanning of hides and skins. *Eur Comm Database*, *46*, 2013.

Braguglia, C. M., Gianico, A., & Mininni, G. (2012). Comparison between ozone and ultrasound disintegration on sludge anaerobic digestion. *Journal of Environmental Management*, *95*, S139-S143. https://doi.org/10.1016/j.jenvman.2010.07.030

Branco, R., Chung, A. P., Veríssimo, A., & Morais, P. V. (2005). Impact of chromium-contaminated wastewaters on the microbial community of a river. *FEMS microbiology ecology*, *54*(1), 35-46. https://doi.org/10.1016/j.femsec.2005.02.014

Braunegg, G. (2002). Sustainable poly (hydroxyalkanoate)(PHA) production. In *Degradable Polymers: Principles and Applications*(pp. 235-293). Dordrecht: Springer Netherlands. https://doi.org/10.1007/978-94-017-1217-0_8

Brown, B., Wilkins, M., & Saha, R. (2022). Rhodopseudomonas palustris: A biotechnology chassis. *Biotechnology Advances*, *60*, 108001. https://doi.org/10.1016/j.biotechadv.2022.108001

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature methods*, *13*(7), 581-583. https://doi.org/10.1038/nmeth.3869.DADA2

Calvin, K., Dasgupta, D., Krinner, G., Mukherji, A., Thorne, P. W., Trisos, C., ... & Hauser, M. (2023). IPCC, 2023: Climate Change 2023: Synthesis Report, Summary for Policymakers. Contribution of Working Groups I, II and III to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change [Core Writing Team, H. Lee and J. Romero (eds.)]. *IPCC*, Geneva, Switzerland, 1-34.

Capson-Tojo, G., Batstone, D. J., Grassino, M., Vlaeminck, S. E., Puyol, D., Verstraete, W., ... & Hülsen, T. (2020). Purple phototrophic bacteria for resource recovery: challenges and opportunities. *Biotechnology advances*, *43*, 107567. https://doi.org/10.1016/j.biotechadv.2020.107567

Castellar, M. R., Aires-Barros, M. R., Cabral, J. M., & Iborra, J. L. (1998). Effect of zeolite addition on ethanol production from glucose by Saccharomyces bayanus. *Journal of Chemical Technology & Biotechnology: International Research in Process, Environmental AND Clean Technology*, *73*(4), 377-384. https://doi.org/10.1002/(SICI)1097-4660 (199812)73:4<377::AID-JCTB973>3.0.CO;2-I

Castro-Fernandez, A., Taboada-Santos, A., Balboa, S., & Lema, J. M. (2023). Thermal hydrolysis pre-treatment has no positive influence on volatile fatty acids production from sewage sludge. *Bioresource Technology*, *376*, 128839. https://doi.org/10.1016/j.biortech.2023.128839

Cecconet, D., & Capodaglio, A. G. (2022). Sewage sludge biorefinery for circular economy. *Sustainability*, *14*(22), 14841. https://doi.org/10.3390/su142214841

Cerruti, M., Ouboter, H. T., Chasna, V., van Loosdrecht, M. C., Picioreanu, C., & Weissbrodt, D. G. (2020). Effects of light/dark diel cycles on the photoorganoheterotrophic metabolism of Rhodopseudomonas palustris for differential electron allocation to PHAs and H2. *bioRxiv*, 2020-08. https://doi.org/10.1101/2020.08.19.258533

Chang, Z., Long, G., Zhou, J. L., & Ma, C. (2020). Valorization of sewage sludge in the fabrication of construction and building materials: A review. *Resources, Conservation and Recycling*, *154*, 104606. https://doi.org/10.1016/j.resconrec.2019.104606

Cheah, Y. K., Dosta, J., & Mata-Álvarez, J. (2019). Enhancement of volatile fatty acids production from food waste by mature compost addition. Molecules, 24(16). https://doi.org/10.3390/molecules24162986.

Chojnacka, K., Skrzypczak, D., Mikula, K., Witek-Krowiak, A., Izydorczyk, G., Kuligowski, K., ... & Kułażyński, M. (2021). Progress in sustainable technologies of leather wastes valorization as solutions for the circular economy. *Journal of Cleaner Production*, *313*, 127902. https://doi.org/10.1016/j.jclepro.2021.127902

Climent, M., Ferrer, I., del Mar Baeza, M., Artola, A., Vázquez, F., & Font, X. (2007). Effects of thermal and mechanical pretreatments of secondary sludge on biogas production under thermophilic conditions. *Chemical Engineering Journal*, *133*(1-3), 335-342. https://doi.org/10.1016/j.cej.2007.02.020.

Corona, V. M., Le Borgne, S., Revah, S., & Morales, M. (2017). Effect of light-dark cycles on hydrogen and poly-β-hydroxybutyrate production by a photoheterotrophic culture and Rhodobacter capsulatus using a dark fermentation effluent as substrate. *Bioresource technology*, *226*, 238-246. http://dx.doi.org/10.1016/j.biortech.2016.12.021

Crognale, S., Casentini, B., Amalfitano, S., Fazi, S., Petruccioli, M., & Rossetti, S. (2019). Biological As (III) oxidation in biofilters by using native groundwater microorganisms. *Science of the Total Environment*, *651*, 93-102. https://doi.org/10.1016/j.scitotenv.2018.09.176

Csicsery, S. M. (1984). Shape-selective catalysis in zeolites. *Zeolites*, *4*(3), 202-213.

Da Ros, C., Conca, V., Eusebi, A. L., Frison, N., & Fatone, F. (2020). Sieving of municipal wastewater and recovery of bio-based volatile fatty acids at pilot scale. *Water research*, *174*, 115633. https://doi.org/10.1016/j.watres.2020.115633

Dan, T., Jing, H., Shen, T., Zhu, J., & Liu, Y. (2023). Performance of production of polyhydroxyalkanoates from food waste fermentation with Rhodopseudomonas palustris. *Bioresource Technology*, *385*, 129165. https://doi.org/10.1016/j.biortech.2023.129165

De Baere, L., & Mattheeuws, B. (2013). Anaerobic digestion of the organic fraction of municipal solid waste in Europe-Status, experience and prospects. ISTANBUL3WCONGRESS 2013, 38.

Dixit, S., Yadav, A., Dwivedi, P. D., & Das, M. (2015). Toxic hazards of leather industry and technologies to combat threat: a review. *Journal of Cleaner Production*, *87*, 39-49. https://doi.org/10.1016/j.jclepro.2014.10.017.

Dong, Y., Butler, E. C., Philp, R. P., & Krumholz, L. R. (2011). Impacts of microbial community composition on isotope fractionation during reductive dechlorination of tetrachloroethylene. *Biodegradation*, *22*, 431-444. https://doi.org/10.1007/s10532-010-9416-2

Doud, D. F., & Angenent, L. T. (2014). Toward electrosynthesis with uncoupled extracellular electron uptake and metabolic growth: enhancing current uptake with Rhodopseudomonas palustris. *Environmental science & technology letters*, *1*(9), 351-355. dx.doi.org/10.1021/ez500244n

Duan, L., Jiang, W., Song, Y., Xia, S., & Hermanowicz, S. W. (2013). The characteristics of extracellular polymeric substances and soluble microbial products in moving bed biofilm reactor-membrane bioreactor. *Bioresource technology*, *148*, 436-442. https://doi.org/10.1016/j.biortech.2013.08.147.

Eskicioglu, C., Prorot, A., Marin, J., Droste, R. L., & Kennedy, K. J. (2008). Synergetic pretreatment of sewage sludge by microwave irradiation in presence of H2O2 for enhanced anaerobic digestion. *Water Research*, 42(18), 4674–4682. https://doi.org/10.1016/j.watres.2008.08.010.

European Leather Industry. (2020). *Social and Environmental Report.*

Fang, W., Zhang, X., Zhang, P., Wan, J., Guo, H., Ghasimi, D. S., Morera, X. C., & Zhang, T. (2020). Overview of key operation factors and strategies for improving fermentative volatile fatty acid production and product regulation from sewage sludge. *Journal of Environmental Sciences*, *87*, 93–111. https://doi.org/10.1016/j.jes.2019.05.027

Fernández, N., Montalvo, S., Borja, R., Guerrero, L., Sánchez, E., Cortés, I., ... & Raposo, F. (2008). Performance evaluation of an anaerobic fluidized bed reactor with natural zeolite as support material when treating high-strength distillery wastewater. *Renewable Energy*, *33*(11), 2458-2466. https://doi.org/10.1016/J.RENENE.2008.02.002.

Fradinho, J. C., Oehmen, A., & Reis, M. A. M. (2019). Improving polyhydroxyalkanoates production in phototrophic mixed cultures by optimizing accumulator reactor operating conditions. *International Journal of Biological Macromolecules*, *126*, 1085-1092. https://doi.org/10.1016/j.ijbiomac.2018.12.270

Franke-Whittle, I. H., Walter, A., Ebner, C., & Insam, H. (2014). Investigation into the effect of high concentrations of volatile fatty acids in anaerobic digestion on methanogenic communities. *Waste management*, *34*(11), 2080-2089. https://doi.org/10.1016/j.wasman.2014.07.020

Gagliano, M. C., Braguglia, C. M., Gianico, A., Mininni, G., Nakamura, K., & Rossetti, S. (2015). Thermophilic anaerobic digestion of thermal pretreated sludge: role of microbial community structure and correlation with process performances. *water research*, *68*, 498-509. https://doi.org/10.1016/j.watres.2014.10.031

Gallipoli, A., Gianico, A., Gagliano, M. C., & Braguglia, C. M. (2014). Potential of high-frequency ultrasounds to improve sludge anaerobic conversion and surfactants removal at different food/inoculum ratio. *Bioresource technology*, *159*, 207-214. https://doi.org/10.1016/j.biortech.2014.02.084

Garcia-Aguirre, J., Aymerich, E., González-Mtnez. de Goñi, J., & Esteban-Gutiérrez, M. (2017). Selective VFA production potential from organic waste streams: Assessing temperature and pH influence. *Bioresource Technology*, 244, 1081–1088. https://doi.org/10.1016/j.biortech.2017.07.187.

Gerardi, M. H. (2003). *The microbiology of anaerobic digesters*. John Wiley & Sons.

Gherghel, A., Teodosiu, C., & De Gisi, S. (2019). A review on wastewater sludge valorisation and its challenges in the context of circular economy. *Journal of cleaner production*, *228*, 244-263. https://doi.org/10.1016/j.jclepro.2019.04.240

Gianico, A., Braguglia, C. M., Cesarini, R., & Mininni, G. (2013). Reduced temperature hydrolysis at 134 C before thermophilic anaerobic digestion of waste activated sludge at increasing organic load. *Bioresource Technology*, *143*, 96-103. https://doi.org/10.1016/j.biortech.2013.05.069

Gianico, A., Braguglia, C. M., Gallipoli, A., & Mininni, G. (2015). Innovative two-stage mesophilic/thermophilic anaerobic degradation of sonicated sludge: performances and energy balance. *Environmental Science and Pollution Research*, *22*, 7248-7256. https://doi.org/10.1007/s11356-014-3123-1

Gottardo, M., Bolzonella, D., Tuci, G. A., Valentino, F., Majone, M., Pavan, P., & Battista, F. (2022). Producing volatile fatty acids and polyhydroxyalkanoates from foods by-products and waste: A review. *Bioresource Technology*, *361*, 127716. https://doi.org/10.1016/j.biortech.2022.127716

Gottardo, M., Crognale, S., Tonanzi, B., Rossetti, S., D'Annibale, L., Dosta, J., & Valentino, F. (2024). Volatile fatty acid production from hydrolyzed sewage sludge: effect of hydraulic retention time and insight into thermophilic microbial community. *Biomass Conversion and Biorefinery*, *14*(13), 14921-14932. https://doi.org/10.1007/s13399-022-03659- 8.

Gottardo, M., Tuci, G. A., Parmar, A. C., Pavan, P., & Valentino, F. (2023). A combined treatment of aerobic activated sludge and powdered activated carbon: Pilot-scale study of per/polyfluoroalkyls (PFASs), organic matters, chromium, and color removal from tannery wastewaters. *Journal of Water Process Engineering*, 55, 104165. https://doi.org/10.1016/j.jwpe.2023.104165

Guo, S. S., Yu, C. Y., Zhao, X. Y., Wang, J. Y., Su, M., Yang, X. Y., & Yang, J. Y. (2022). The chromium migration risk from tannery sludge into shallow soil and groundwater: Influence factors, modeling, and microbial response. *Journal of Cleaner Production*, 374, 133776. https://doi.org/10.1016/j.jclepro.2022.133776

Guzman, M. S., Rengasamy, K., Binkley, M. M., Jones, C., Ranaivoarisoa, T. O., Singh, R., ... & Bose, A. (2019). Phototrophic extracellular electron uptake is linked to carbon dioxide fixation in the bacterium Rhodopseudomonas palustris. *Nature communications*, *10*(1), 1355. https://doi.org/10.1038/s41467-019-09377-6

Hahnke, S., Langer, T., Koeck, D. E., & Klocke, M. (2016). Description of Proteiniphilum saccharofermentans sp. nov., Petrimonas mucosa sp. nov. and Fermentimonas caenicola gen. nov., sp. nov., isolated from mesophilic laboratory-scale biogas reactors, and emended description of the genus Proteiniphilum. *International journal of systematic and evolutionary microbiology*, *66*(3), 1466-1475. https://doi.org/10.1099/ijsem.0.000902

Hammer, Ø., & Harper, D. A. (2001). Past: paleontological statistics software package for education and data analysis. *Palaeontologia electronica*, *4*(1), 1. https://doi.org/10.1016/j.bcp.2008.05.025

Han, W., He, P., Shao, L., & Lü, F. (2019). Road to full bioconversion of biowaste to biochemicals centering on chain elongation: A mini review. *Journal of Environmental Sciences*, *86*, 50-64. https://doi.org/10.1016/j.jes.2019.05.018

Hao, J. and Wang, H. (2015) 'Volatile fatty acids productions by mesophilic and thermophilic sludge fermentation: Biological responses to fermentation temperature', *Bioresource Technology*, 175, pp. 367–373. Available at: https://doi.org/10.1016/j.biortech.2014.10.106.

Hao, Y., Ma, H., Wang, Q., Zhu, C., & He, A. (2022). Complexation behaviour and removal of organic-Cr (III) complexes from the environment: A review. *Ecotoxicology and Environmental Safety*, *240*, 113676. https://doi.org/10.1016/j.ecoenv.2022.113676.

Harnisch, F., Deutzmann, J. S., Boto, S. T., & Rosenbaum, M. A. (2024). Microbial electrosynthesis: opportunities for microbial pure cultures. *Trends in Biotechnology*. https://doi.org/10.1016/j.tibtech.2024.02.004

Hashem, M. A., Payel, S., Mim, S., Hasan, M. A., Nur-A-Tomal, M. S., Rahman, M. A., & Sarker, M. I. (2022). Chromium adsorption on surface activated biochar made from tannery liming sludge: a waste-to-wealth approach. *Water Science and Engineering*, *15*(4), 328-336.https://doi.org/10.1016/j.wse.2022.09.001.

Horn, E. J., Oyekola, O. O., Welz, P. J., & van Hille, R. P. (2021). Biological desulfurization of tannery effluent using hybrid linear flow channel reactors. *Water*, *14*(1), 32. https://doi.org/10.3390/w14010032

ISO 937:1978(en), Meat and meat products — Determination of nitrogen content (Reference method), Available online: https://www.iso. org/obp/ui/#iso:std:iso:937:ed-1:v1:en (accessed in December 2023).

Jacquel, N., Lo, C. W., Wei, Y. H., Wu, H. S., & Wang, S. S. (2008). Isolation and purification of bacterial poly (3-hydroxyalkanoates). *Biochemical engineering journal*, *39*(1), 15-27. https://doi.org/10.1016/j.bej.2007.11.029

Jadhav, D. A., Park, S. G., Pandit, S., Yang, E., Abdelkareem, M. A., Jang, J. K., & Chae, K. J. (2022). Scalability of microbial electrochemical technologies: Applications and challenges. *Bioresource Technology*, *345*, 126498. https://doi.org/10.1016/j.biortech.2021.126498

Jensen, P. D., Ge, H., & Batstone, D. J. (2011). Assessing the role of biochemical methane potential tests in determining anaerobic degradability rate and extent. *Water science and technology*, *64*(4), 880-886.https://doi.org/10.2166/wst.2011.662.

Jeong, S. Y., Chang, S. W., Ngo, H. H., Guo, W., Nghiem, L. D., Banu, J. R., ... & Nguyen, D. D. (2019). Influence of thermal hydrolysis pretreatment on physicochemical properties and anaerobic biodegradability of waste activated sludge with different solids content. *Waste Management*, *85*, 214-221. https://doi.org/10.1016/j.wasman.2018.12.026

Jiang, J., Zhang, Y., Li, K., Wang, Q., Gong, C., & Li, M. (2013). Volatile fatty acids production from food waste: effects of pH, temperature, and organic loading rate. *Bioresource technology*, *143*, 525-530. https://doi.org/10.1016/j.biortech.2013.06.025.

Jiang, N., Shang, R., Heijman, S. G., & Rietveld, L. C. (2018). High-silica zeolites for adsorption of organic micro-pollutants in water treatment: A review. *Water research*, *144*, 145-161. https://doi.org/10.1016/J.WATRES.2018.07.017.

Jin, M., Lian, F., Xia, R., & Wang, Z. (2018). Formulation and durability of a geopolymer based on metakaolin/tannery sludge. *Waste Management*, *79*, 717-728. https://doi.org/10.1016/j.wasman.2018.08.039

Karthikeyan, O. P., & Visvanathan, C. (2013). Bio-energy recovery from high-solid organic substrates by dry anaerobic bio-conversion processes: A review. *Reviews in Environmental Science and Biotechnology*. https://doi.org/10.1007/s11157-012-9304-9.

Kersters, I., Maestrojuan, G. M., Torck, U., Vancanneyt, M., Kersters, K., & Verstraete, W. (1994). Isolation of Coprothermobacter proteolyticus from an anaerobic digest and further characterization of the species. *Systematic and applied microbiology*, *17*(2), 289-295. https://doi.org/10.1016/S0723-2020(11)80021-4

Kesraoui-Ouki, S., Cheeseman, C. R., & Perry, R. (1994). Natural zeolite utilisation in pollution control: A review of applications to metals' effluents. *Journal of Chemical Technology & Biotechnology: International Research in Process, Environmental AND Clean Technology*, *59*(2), 121-126. https://doi.org/10.1002/JCTB.280590202.

Khadaroo, S. N., Poh, P. E., Gouwanda, D., & Grassia, P. (2019). Applicability of various pretreatment techniques to enhance the anaerobic digestion of Palm oil Mill effluent (POME): A review. *Journal of Environmental Chemical Engineering*, *7*(5), 103310. https://doi.org/10.1016/j.jece.2019.103310

Kong, X., Li, C., Wang, P., Huang, G., Li, Z., & Han, Z. (2019). Soil pollution characteristics and microbial responses in a vertical profile with long-term tannery sludge contamination in Hebei, China. *International Journal of Environmental Research and Public Health*, *16*(4), 563. https://doi.org/10.3390/ijerph16040563

Kuligowski, K., Cenian, A., Konkol, I., Świerczek, L., Chojnacka, K., Izydorczyk, G., ... & Bandrów, P. (2023). Application of leather waste fractions and their biochars as organic fertilisers for ryegrass growth: Agri-environmental aspects and plants response modelling. *Energies*, *16*(9), 3883. https://doi.org/10.3390/en16093883.

Kumar, A. N., Sarkar, O., Chandrasekhar, K., Raj, T., Narisetty, V., Mohan, S. V., ... & Kim, S. H. (2022). Upgrading the value of anaerobic fermentation via renewable chemicals production: a sustainable integration for circular bioeconomy. *Science of the Total Environment*, *806*, 150312. https://doi.org/10.1016/j.scitotenv.2021.150312

Laranja, J. L. Q., Ludevese-Pascual, G. L., Amar, E. C., Sorgeloos, P., Bossier, P., & De Schryver, P. (2014). Poly-β-hydroxybutyrate (PHB) accumulating Bacillus spp. improve the survival, growth and robustness of Penaeus monodon () postlarvae. *Veterinary microbiology*, *173*(3-4), 310-317.

Lee, J., Koo, T., Yulisa, A., & Hwang, S. (2019). Magnetite as an enhancer in methanogenic degradation of volatile fatty acids under ammonia-stressed condition. *Journal of environmental management*, *241*, 418-426. https://doi.org/10.1016/j.jenvman.2019.04.038

Lee, W. S., Chua, A. S. M., Yeoh, H. K., & Ngoh, G. C. (2014). A review of the production and applications of waste-derived volatile fatty acids. *Chemical Engineering Journal*, *235*, 83-99. https://doi.org/10.1016/j.cej.2013.09.002

Li, C., Hao, L., Lü, F., Duan, H., Zhang, H., & He, P. (2022a). Syntrophic acetate-oxidizing microbial consortia enriched from full-scale mesophilic food waste anaerobic digesters showing high biodiversity and functional redundancy. *Msystems*, *7*(5), e00339-22.

Li, M., Ning, P., Sun, Y., Luo, J., & Yang, J. (2022b). Characteristics and application of *Rhodopseudomonas palustris* as a microbial cell factory. *Frontiers in bioengineering and biotechnology*, *10*, 897003. https://doi.org/10.3389/fbioe.2022.897003

Li, X., Liu, H., Zhang, Z., Zhou, T., & Wang, Q. (2023a). Sulfite pretreatment enhances the medium-chain fatty acids production from waste activated sludge anaerobic fermentation. *Science of The Total Environment*, *871*, 162080. https://doi.org/10.1016/j.scitotenv.2023.162080

Li, M., Zhu, T., Yang, R., Wang, Z., Liu, M., & Yang, J. (2023b). Carotenoids synthesis affects the salt tolerance mechanism of *Rhodopseudomonas palustris*. *Frontiers in Microbiology*, *14*, 1292937. https://doi.org/10.3389/fmicb.2023.1292937

Li, Y. Y., & Noike, T. (1992). Upgrading of anaerobic digestion of waste activated sludge by thermal pretreatment. *Water Science and Technology*, *26*(3-4), 857-866. https://doi.org/10.2166/ WST.1992.0466.

Li, Y., Li, L., & Yu, J. (2017). Applications of zeolites in sustainable chemistry. *Chem*, *3*(6), 928-949. https://doi.org/10.1016/j.chempr.2017.10.009.

Li, Y., Park, S. Y., & Zhu, J. (2011). Solid-state anaerobic digestion for methane production from organic waste. Renewable and Sustainable Energy Reviews. Elsevier Ltd. https://doi.org/10.1016/j.rser.2010.07.042.

Liu, H., Han, P., Liu, H., Zhou, G., Fu, B., & Zheng, Z. (2018a). Full-scale production of VFAs from sewage sludge by anaerobic alkaline fermentation to improve biological nutrients removal in domestic wastewater. *Bioresource technology*, *260*, 105-114. https://doi.org/10.1016/j.biortech.2018.03.105

Liu, H., Wang, J., Liu, X., Fu, B., Chen, J., & Yu, H. Q. (2012). Acidogenic fermentation of proteinaceous sewage sludge: effect of pH. *Water research*, *46*(3), 799-807. https://doi.org/10.1016/j.watres.2011.11.047.

Liu, H., Wang, Y., Zhang, H., Huang, G., Yang, Q., & Wang, Y. (2019). Synchronous detoxification and reduction treatment of tannery sludge using Cr (VI) resistant bacterial strains. *Science of the total environment*, *687*, 34-40. https://doi.org/10.1016/j.scitotenv.2019.06.093

Liu, J., Jia, R., Wang, Y., Wei, Y., Zhang, J., Wang, R., & Cai, X. (2017). Does residual H_2O_2 result in inhibitory effect on enhanced anaerobic digestion of sludge pretreated by microwave- H_2O_2 pretreatment process?. *Environmental Science and Pollution Research*, *24*, 9016-9025. https://doi.org/10.1007/s11356-015-5704-z

Liu, J., Yang, M., Zhang, J., Zheng, J., Xu, H., Wang, Y., & Wei, Y. (2018b). A comprehensive insight into the effects of microwave-H_2O_2 pretreatment on concentrated sewage sludge anaerobic digestion based on semi-continuous operation. *Bioresource technology*, *256*, 118-127. https://doi.org/10.1016/j.biortech.2018.01.126

Liu, N., Jiang, J., Yan, F., Xu, Y., Yang, M., Gao, Y., ... & Zou, Q. (2018c). Optimization of simultaneous production of volatile fatty acids and bio-hydrogen from food waste using response surface methodology. *RSC advances*, *8*(19), 10457-10464. https://doi.org/10.1039/c7ra13268a.

Liu, X., Huang, L., Rensing, C., Ye, J., Nealson, K. H., & Zhou, S. (2021). Syntrophic interspecies electron transfer drives carbon fixation and growth by Rhodopseudomonas palustris under dark, anoxic conditions. *Science Advances*, *7*(27), eabh1852. https://doi.org/10.1126/sciadv.abh1852

Liu, X., Khalid, H., Amin, F. R., Ma, X., Li, X., Chen, C., & Liu, G. (2018d). Effects of hydraulic retention time on anaerobic digestion performance of food waste to produce methane as a biofuel. *Environmental Technology & Innovation*, *11*, 348-357. https://doi. org/10.1016/j.eti.2018.06.004.

Lorini, L., Munarin, G., Salvatori, G., Alfano, S., Pavan, P., Majone, M., & Valentino, F. (2022). Sewage sludge as carbon source for polyhydroxyalkanoates: a holistic approach at pilot scale level. *Journal of Cleaner Production*, *354*, 131728. https://doi.org/10.1016/j. jclepro.2022.131728.

Ma, H., Zhou, J., Hua, L., Cheng, F., Zhou, L., & Qiao, X. (2017). Chromium recovery from tannery sludge by bioleaching and its reuse in tanning process. *Journal of Cleaner Production*, *142*, 2752-2760. https://doi.org/10.1016/j.jclepro.2016.10.193.

Madigan, M., Cox, J. C., & Gest, H. (1982). Photopigments in Rhodopseudomonas capsulata cells grown anaerobically in darkness. *Journal of Bacteriology*, *150*(3), 1422-1429.

Madigan, M. T., & Jung, D. O. (2009). An overview of purple bacteria: systematics, physiology, and habitats. *The purple phototrophic bacteria*, 1-15.

Mannina, G., Presti, D., Montiel-Jarillo, G., Carrera, J., & Suárez-Ojeda, M. E. (2020). Recovery of polyhydroxyalkanoates (PHAs) from wastewater: a review. *Bioresource technology*, *297*, 122478. https://doi.org/10.1016/j.biortech.2019.122478

Mannucci, A., Munz, G., Mori, G., & Lubello, C. (2010). Anaerobic treatment of vegetable tannery wastewaters: a review. *Desalination*, *264*(1-2), 1-8. https://doi.org/10.1016/j.desal.2010.07.021

Mata-Alvarez, J., Cecchi, F., Pavan, P., & Llabres, P. (1990). The performances of digesters treating the organic fraction of municipal solid wastes differently sorted. *Biological wastes*, *33*(3), 181-199.

Matturro, B., Tandoi, V., & Rossetti, S. (2013). Different activity levels of Dehalococcoides mccartyi revealed by FISH and CARD-FISH under non-steady and pseudo-steady state conditions. *New Biotechnology*, *30*(6), 756-762. https://doi.org/10.1016/j.nbt.2013.07.003

Meegoda, J. N., Li, B., Patel, K., & Wang, L. B. (2018). A review of the processes, parameters, and optimization of anaerobic digestion. *International journal of environmental research and public health*, *15*(10), 2224. https://doi.org/10.3390/ijerph15102224.

Micolucci, F., Gottardo, M., Bolzonella, D., Pavan, P., Majone, M., & Valentino, F. (2020). Pilot-scale multi-purposes approach for volatile fatty acid production, hydrogen and methane from an automatic controlled anaerobic process. *Journal of Cleaner Production*, *277*, 124297. https://doi.org/10.1016/j.jclepro.2020.124297.

Moktadir, M. A., & Rahman, M. M. (2022). Energy production from leather solid wastes by anaerobic digestion: A critical review. *Renewable and Sustainable Energy Reviews*, *161*, 112378. https://doi.org/10.1016/j.rser.2022.112378.

Moktadir, M. A., Ahmadi, H. B., Sultana, R., Liou, J. J., & Rezaei, J. (2020). Circular economy practices in the leather industry: A practical step towards sustainable development. *Journal of Cleaner Production*, *251*, 119737. https://doi.org/10.1016/j.jclepro.2019.119737.

Moktadir, M. A., Ren, J., & Zhou, J. (2023). A systematic review on tannery sludge to energy route: Current practices, impacts, strategies, and future directions. *Science of The Total Environment*, *901*, 166244. https://doi.org/10.1016/j.scitotenv.2023.166244

Montalvo, S., Guerrero, L., Borja, R., Sánchez, E., Milán, Z., Cortés, I., & De La La Rubia, M. A. (2012). Application of natural zeolites in anaerobic digestion processes: A review. *Applied Clay Science*, *58*, 125-133. https://doi.org/10.1016/j.clay.2012.01.013.

Montalvo, S., Huiliñir, C., Borja, R., Sánchez, E., & Herrmann, C. J. B. T. (2020). Application of zeolites for biological treatment processes of solid wastes and wastewaters–a review. *Bioresource technology*, *301*, 122808. https://doi.org/10.1016/j. biortech.2020.122808.

Montégut, G., Michelin, L., Brendlé, J., Lebeau, B., & Patarin, J. (2016). Ammonium and potassium removal from swine liquid manure using clinoptilolite, chabazite and faujasite zeolites. *Journal of environmental management*, *167*, 147-155. https://doi.org/10.1016/j. jenvman.2015.11.027.

Montiel-Corona, V., & Buitrón, G. (2022). Polyhydroxybutyrate production in one-stage by purple phototrophic bacteria: influence of alkaline pH, ethanol, and C/N ratios. *Biochemical Engineering Journal*, *189*, 108715. https://doi.org/10.1016/j.bej.2022.108715

Moretto, G., Lorini, L., Pavan, P., Crognale, S., Tonanzi, B., Rossetti, S., ... & Valentino, F. (2020a). Biopolymers from urban organic waste: influence of the solid retention time to cycle length ratio in the enrichment of a mixed microbial culture (MMC). *ACS Sustainable Chemistry & Engineering*, *8*(38), 14531-14539. https://doi.org/10.1021/acssuschemeng.0c04980

Moretto, G., Russo, I., Bolzonella, D., Pavan, P., Majone, M., & Valentino, F. (2020b). An urban biorefinery for food waste and biological sludge conversion into polyhydroxyalkanoates and biogas. *Water Research*, *170*, 115371. https://doi.org/10.1016/j.watres.2019.115371

Morgan-Sagastume, F., Hjort, M., Cirne, D., Gérardin, F., Lacroix, S., Gaval, G., ... & Werker, A. (2015). Integrated production of polyhydroxyalkanoates (PHAs) with municipal wastewater and sludge treatment at pilot scale. *Bioresource technology*, *181*, 78-89. https://doi.org/10.1016/j.biortech.2015.01.046

Morgan-Sagastume, F., Valentino, F., Hjort, M., Cirne, D., Karabegovic, L., Gerardin, F., ... & Werker, A. (2014). Polyhydroxyalkanoate (PHA) production from sludge and municipal wastewater treatment. *Water Science and Technology*, *69*(1), 177-184. https://doi.org/10.2166/wst.2013.643

Motte, J. C., Trably, E., Escudié, R., Hamelin, J., Steyer, J. P., Bernet, N., ... & Dumas, C. (2013). Total solids content: A key parameter of metabolic pathways in dry anaerobic digestion. *Biotechnology for Biofuels*, 6(1). https://doi.org/10.1186/1754-6834-6-164.

Mpofu, A. B., Kibangou, V. A., Kaira, W. M., Oyekola, O. O., & Welz, P. J. (2021). Anaerobic Co-digestion of tannery and slaughterhouse wastewater for solids reduction and resource recovery: Effect of sulfate concentration and inoculum to substrate ratio. *Energies*, *14*(9), 2491. https://doi.org/10.3390/en14092491

Mpofu, A. B., Welz, P. J., & Oyekola, O. O. (2020). Anaerobic digestion of secondary tannery sludge: optimisation of initial pH and temperature and evaluation of kinetics. *Waste and Biomass Valorization*, *11*(3), 873-885. https://doi.org/10.1007/s12649-018-00564-y

Muhammad Saeed, M. S., Iqra Yasmin, I. Y., Murtaza, M. A., Iqra Fatima, I. F., & Shehwar Saeed, S. S. (2016). Single cell proteins: a novel value added food product.

Mukhopadhyay, M., Patra, A., & Paul, A. K. (2005). Production of poly (3-hydroxybutyrate) and poly (3-hydroxybutyrate-co-3-hydroxyvalerate) by Rhodopseudomonas palustris SP5212. *World Journal of Microbiology and Biotechnology*, *21*, 765-769. https://doi.org/10.1007/s11274-004-5565-y

Nielsen, M. B., Kjeldsen, K. U., & Ingvorsen, K. (2006). Desulfitibacter alkalitolerans gen. nov., sp. nov., an anaerobic, alkalitolerant, sulfite-reducing bacterium isolated from a district heating plant. *International journal of systematic and evolutionary microbiology*, *56*(12), 2831-2836. https://doi.org/10.1099/ijs.0.64356-0

Nielsen, P. H., Kragelund, C., Seviour, R. J., & Nielsen, J. L. (2009). Identity and ecophysiology of filamentous bacteria in activated sludge. *FEMS microbiology reviews*, *33*(6), 969-998. https://doi.org/10.1111/j.1574-6976.2009.00186.x

Niero, L., Morgan-Sagastume, F., & Lagerkvist, A. (2021). Accelerating acidogenic fermentation of sewage sludge with ash addition. *Journal of Environmental Chemical Engineering*, *9*(6), 106564. https://doi.org/10.1016/j.jece.2021.106564

Niu, L., Song, L., Liu, X., & Dong, X. (2009). Tepidimicrobium xylanilyticum sp. nov., an anaerobic xylanolytic bacterium, and emended description of the genus Tepidimicrobium. *International Journal of Systematic and Evolutionary Microbiology*, *59*(11), 2698-2701. https://doi.org/10.1099/ijs.0.005124-0

Nordell, E., Hansson, A.B., & Karlsson, M. (2013). Zeolites relieves inhibitory stress from high concentrations of long chain fatty acids. *Waste Management*, 33, 2659–2663. https://doi.org/10.1016/j.wasman.2013.08.009.

Oruko, R. O., Selvarajan, R., Ogola, H. J. O., Edokpayi, J. N., & Odiyo, J. O. (2020). Contemporary and future direction of chromium tanning and management in sub Saharan Africa tanneries. *Process Safety and Environmental Protection*, *133*, 369-386. https://doi.org/10.1016/j. psep.2019.11.013.

Özön, E., & Erdinçler, A. (2019). Effects of microwave, H_2O_2/MW and H_2O_2/heat pre-treatments on the methane production from wastewater sludges: experimental and modeling approach. *Environmental Science and Pollution Research*, *26*, 35411-35421. https://doi.org/10.1007/s11356-019-05190-2

Pantazopoulou, E., & Zouboulis, A. (2020). Chromium recovery from tannery sludge and its ash, based on hydrometallurgical methods. *Waste Management & Research*, *38*(1), 19-26. https://doi.org/10.1177/0734242X19866903.

Pavan, M.E., Pavan, E.E., Kämpfer, P., Pettinari, M.J., & López, N.I. (2019). Coprothermobacterota, in: Bergey's Manual of Systematics of Archaea and Bacteria. John Wiley & Sons, Inc. https://doi.org/10.1002/9781118960608.pbm00036

Pesante, G., Zuliani, A., Cannone, E., Greco, F., Tesoriero, C., Vettori, A., & Frison, N. (2022). Biological conversion of agricultural residues into microbial proteins for aquaculture using PHA-producing mixed microbial cultures. *Journal of Cleaner Production*, *378*, 134554. https://doi.org/10.1016/j.jclepro.2022.134554.

Pietrelli, L., Ippolito, N. M., Reverberi, A. P., & Vocciante, M. (2019). Heavy Metals Removal and Recovery from Hazardous Leather Sludge. *CET Journal-Chemical Engineering Transactions*, *76*.

Polizzi, C., Alatriste-Mondragón, F., & Munz, G. (2018). The role of organic load and ammonia inhibition in anaerobic digestion of tannery fleshing. *Water Resources and Industry*, *19*, 25-34. https://doi.org/10.1016/j.wri.2017.12.001

Pommier, S., Chenu, D., Quintard, M., & Lefebvre, X. (2007). A logistic model for the prediction of the influence of water on the solid waste methanization in landfills. *Biotechnology and Bioengineering*, 97(3), 473–482. https://doi.org/10.1002/bit.21241.

Presti, D., Cosenza, A., Capri, F. C., Gallo, G., Alduina, R., & Mannina, G. (2021). Influence of volatile solids and pH for the production of volatile fatty acids: batch fermentation tests using sewage sludge. *Bioresource Technology*, *342*, 125853. https://doi.org/10.1016/j.biortech.2021.125853

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., ... & Glöckner, F. O. (2012). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic acids research*, *41*(D1), D590-D596. https://doi.org/10.1093/nar/gks1219

Rajvanshi, J., Sogani, M., Kumar, A., Arora, S., Syed, Z., Sonu, K., ... & Kalra, A. (2023). Perceiving biobased plastics as an alternative and innovative solution to combat plastic pollution for a circular economy. *Science of The Total Environment*, *874*, 162441. http://dx.doi.org/10.1016/j.scitotenv.2023.162441

Ramos-Suarez, M., Zhang, Y., & Outram, V. (2021). Current perspectives on acidogenic fermentation to produce volatile fatty acids from waste. *Reviews in Environmental Science and Bio/Technology*, *20*(2), 439–478. https://doi.org/10.1007/s11157-021-09566-0

Ranaivoarisoa, T. O., Singh, R., Rengasamy, K., Guzman, M. S., & Bose, A. (2019). Towards sustainable bioplastic production using the photoautotrophic bacterium Rhodopseudomonas palustris TIE-1. *Journal of Industrial Microbiology and Biotechnology*, *46*(9-10), 1401-1417. https://doi.org/10.1007/s10295-019-02165-7

Raza, Z. A., Abid, S., & Banat, I. M. (2018). Polyhydroxyalkanoates: Characteristics, production, recent developments and applications. *International Biodeterioration & Biodegradation*, *126*, 45-56. https://doi.org/10.1016/j.ibiod.2017.10.001

Reyes-Romero, B., Gutiérrez-López, A. N., Hernández-Altamirano, R., Mena-Cervantes, V. Y., Ruiz-Baca, E., Neri-Torres, E. E., ... & Vazquez-Arenas, J. (2021). Removal of concentrated Cr (III) from real tannery wastewater using abiotic and anaerobic processes with native microbial consortia. *Journal of Environmental Chemical Engineering*, *9*(1), 104626. https://doi.org/10.1016/j.jece.2020.104626

Righetti, E., Nortilli, S., Fatone, F., Frison, N., & Bolzonella, D. (2020). A multiproduct biorefinery approach for the production of hydrogen, methane and volatile fatty acids from agricultural waste. *Waste and Biomass Valorization*, *11*, 5239-5246. https://doi.org/10.1007/s12649-020-01023-3

Rodríguez-Iznaga, I., Shelyapina, M. G., & Petranovskii, V. (2022). Ion exchange in natural clinoptilolite: aspects related to its structure and applications. *Minerals*, *12*(12), 1628. https://doi.org/10.3390/MIN12121628, 1628 12, 1628.

Rodriguez-Estrada, U., Tachibana, L., Carla Dias, D., Ben-Hamed, S., Sampaio-Goncalves, G., Rosa Sussel, F., & Ranzani-Paiva, M. J. (2021). Dietary pure polyhydroxyalkanoate effects on growth, nutrient utilization, apparent digestibility, and hematology in Nile tilapia. *North American Journal of Aquaculture*, *83*(4), 240-254. https://doi.org/10.1002/naaq.10183

Ronchi Decree. (1997). Available online: https://www.parlamento.it/parlam/leggi/deleghe/97022dl.htm (accessed in March 2022).

Rossi, D., Cappello, M., Antognoli, M., Brunazzi, E., & Seggiani, M. (2023). Pyrolyzed tannery sludge as adsorbent of volatile organic compounds from tannery air emissions. *Chemical Engineering Journal*, *454*, 140320. https://doi.org/10.1016/j.cej.2022.140320.

Rubal, J. S., Torre, J. a. C., & González, I. (2012). Influence of temperature, agitation, sludge concentration and solids retention time on primary sludge fermentation. *International Journal of Chemical Engineering*, *2012*, 1–8. https://doi.org/10.1155/2012/861467

Sawalha, H., Al-Jabari, M., Elhamouz, A., Abusafa, A., & Rene, E. R. (2020). Tannery wastewater treatment and resource recovery options. In *Waste biorefinery* (pp. 679-705). Elsevier. https://doi.org/ 10.1016/B978-0-12-818228-4.00025-3.

Saxena, G., Purchase, D., Mulla, S. I., & Bharagava, R. N. (2020). Degradation and detoxification of leather tannery effluent by a newly developed bacterial consortium GS-TE1310 for environmental safety. *Journal of Water Process Engineering*, *38*, 101592. https://doi.org/10.1016/j.jwpe.2020.101592

Shaker, M. A. (2014). Dynamics and thermodynamics of toxic metals adsorption onto soil-extracted humic acid. *Chemosphere*, *111*, 587-595. https://doi.org/10.1016/j.chemosphere.2014.04.088.

Siefert, E., & Pfennig, N. (1979). Chemoautotrophic growth of Rhodopseudomonas species with hydrogen and chemotrophic utilization of methanol and formate. *Archives of Microbiology*, *122*, 177-182.

Silva, J. D., Leal, T. T., Araújo, A. S., Araujo, R. M., Gomes, R. L., Melo, W. J., & Singh, R. P. (2010). Effect of different tannery sludge compost amendment rates on growth, biomass accumulation and yield responses of Capsicum plants. *Waste Management*, *30*(10), 1976-1980. https://doi.org/10.1016/j.wasman.2010.03.011

Silva, R. M., Abreu, A. A., Salvador, A. F., Alves, M. M., Neves, I. C., & Pereira, M. A. (2021). Zeolite addition to improve biohydrogen production from dark fermentation of C5/C6-sugars and Sargassum sp. biomass. *Scientific reports*, *11*(1), 16350. https://doi.org/10.1038/ s41598-021-95615-1.

Simioni, T., Agustini, C. B., Dettmer, A., & Gutterres, M. (2022). Anaerobic co-digestion of tannery wastes and untreated/pretreated oat straw. *BioEnergy Research*, *15*(1), 589-601. https://doi.org/10.1007/s12155-021-10285-1.

Simonetti, S., Martin, C. F., & Dionisi, D. (2021). Anaerobic fermentation for the production of short chain organic acids: Product concentration, yield and productivity in batch experiments at high feed concentration. *Journal of Environmental Chemical Engineering*, *9*(5), 106311. https://doi.org/ 10.1016/j.jece.2021.106311.

Skrzypczak, D., Szopa, D., Mikula, K., Izydorczyk, G., Baśladyńska, S., Hoppe, V., ... & Witek–Krowiak, A. (2022). Tannery waste-derived biochar as a carrier of micronutrients essential to plants. *Chemosphere*, *294*, 133720. https://doi.org/10.1016/j.chemosphere.2022.133720

Soomro, A. F., Abbasi, I. A., Ni, Z., Ying, L., & Liu, J. (2020). Influence of temperature on enhancement of volatile fatty acids fermentation from organic fraction of municipal solid waste: Synergism between food and paper components. *Bioresource Technology*, *304*, 122980. https://doi.org/10.1016/j.biortech.2020.122980

Soundararajan, M., Ledbetter, R., Kusuma, P., Zhen, S., Ludden, P., Bugbee, B., ... & Seefeldt, L. C. (2019). Phototrophic N2 and CO2 fixation using a Rhodopseudomonas palustris-H2 mediated electrochemical system with infrared photons. *Frontiers in microbiology*, *10*, 1817. https://doi.org/10.3389/fmicb.2019.01817

Spanoghe, J., Vermeir, P., & Vlaeminck, S. E. (2021). Microbial food from light, carbon dioxide and hydrogen gas: kinetic, stoichiometric and nutritional potential of three purple bacteria. *Bioresource Technology*, *337*, 125364. https://doi.org/10.1016/j.biortech.2021.125364

Srisowmeya, G., Chakravarthy, M., & Devi, G. N. (2020). Critical considerations in two-stage anaerobic digestion of food waste–A review. *Renewable and Sustainable Energy Reviews*, *119*, 109587. https://doi.org/10.1016/j.rser.2019.109587.

Strazzera, G., Battista, F., Andreolli, M., Menini, M., Bolzonella, D., & Lampis, S. (2021). Influence of different household food wastes fractions on volatile fatty acids production by anaerobic fermentation. *Bioresource technology*, *335*, 125289. https://doi.org/10.1016/j.biortech.2021.125289.

Sudesh, K., Abe, H., & Doi, Y. (2000). Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters. *Progress in polymer science*, *25*(10), 1503-1555. https://doi.org/10.1016/S0079-6700(00)00035-6

Suguna, P., Binuramesh, C., Abirami, P., Saranya, V., Poornima, K., Rajeswari, V., & Shenbagarathai, R. (2014). Immunostimulation by poly-β hydroxybutyrate–hydroxyvalerate (PHB–HV) from Bacillus thuringiensis in Oreochromis mossambicus. *Fish & shellfish immunology*, *36*(1), 90-97. https://doi.org/10.1016/j.fsi.2013.10.012.

Sun, C., Yu, Q., Zhao, Z., & Zhang, Y. (2022). Extracellular electron uptake for CO_2 fixation by Rhodopseudomonas palustris during electro-cultivation in darkness. *Science of The Total Environment*, *849*, 157864. http://dx.doi.org/10.1016/j.scitotenv.2022.157864

Sun, C., Yu, Q., Zhao, Z., & Zhang, Y. (2023). Enhancing photosynthetic CO_2 fixation in microbial electrolysis cell (MEC)-based anaerobic digestion for the in-situ biogas upgrading. *Chemical Engineering Journal*, *462*, 142341. https://doi.org/10.1016/j.cej.2023.142341

Tan, G. Y. A., Chen, C. L., Li, L., Ge, L., Wang, L., Razaad, I. M. N., ... Wang, J. Y. (2014). Start a research on biopolymer polyhydroxyalkanoate (PHA): A review. Polymers. *Molecular Diversity Preservation International*. https://doi.org/10.3390/polym6030706.

Tang, C. C., Zhang, B. C., Yao, X. Y., Zhou, A. J., Liu, W., Ren, Y. X., ... & He, Z. W. (2023). Insights into response mechanism of anaerobic digestion of waste activated sludge to particle sizes of zeolite. *Bioresource Technology*, *385*, 129348. https:// doi.org/10.1016/j.biortech.2023.129348.

Tauch, A. & Sandbote, J. (2014). The Prokaryotes, The Family Corynebacteriaceae. Springer, Berlin, Heidelberg.

Tebo, B. M., & Obraztsova, A. Y. (1998). Sulfate-reducing bacterium grows with Cr (VI), U (VI), Mn (IV), and Fe (III) as electron acceptors. *FEMS microbiology letters*, *162*(1), 193-199.

Tec-Campos, D., Posadas, C., Tibocha-Bonilla, J. D., Thiruppathy, D., Glonek, N., Zuñiga, C., ... & Zengler, K. (2023). The genome-scale metabolic model for the purple non-sulfur bacterium *Rhodopseudomonas palustris* Bis A53 accurately predicts phenotypes under chemoheterotrophic, chemoautotrophic, photoheterotrophic, and photoautotrophic growth conditions. *PLOS Computational Biology*, *19*(8), e1011371. https://doi.org/10.1371/ journal.pcbi.1011371

Tharani, D., & Ananthasubramanian, M. (2021). Process intensification in separation and recovery of biogenic volatile fatty acid obtained through acidogenic fermentation of organics-rich substrates. *Chemical Engineering and Processing-Process Intensification*, *169*, 108592. https://doi.org/10.1016/j.cep.2021.108592.

Tonanzi, B., Gallipoli, A., Annesini, M. C., La Penna, C., Gianico, A., & Braguglia, C. M. (2021). Pre-treatments and anaerobic hydrolysis as strategical key steps for resource recovery from sludge: The role of disintegration degree in metals leaching. *Journal of Environmental Chemical Engineering*, *9*(1), 104649. https://doi.org/10.1016/j.jece.2020.104649

Tonanzi, B., Gallipoli, A., Gianico, A., Montecchio, D., Pagliaccia, P., Di Carlo, M., ... & Braguglia, C. M. (2018). Long-term anaerobic digestion of food waste at semi-pilot scale: relationship between microbial community structure and process performances. *Biomass and bioenergy*, *118*, 55-64. https://doi.org/10.1016/j.biombioe.2018.08.001

Trzcinski, A. P., & Stuckey, D. C. (2012). Determination of the hydrolysis constant in the biochemical methane potential test of municipal solid waste. *Environmental Engineering Science*, *29*(9), 848-854. https://doi.org/10.1089/ees.2011.0105.

Tuci, G. A., Valentino, F., Bonato, E., Pavan, P., & Gottardo, M. (2022). Microwave-hydrogen peroxide assisted anaerobic treatment as an effective method for short-chain fatty acids production from tannery sludge. *Processes*, *10*(11), 2167. https://doi.org/10.3390/ pr10112167.

Tuci, G. A., Valentino, F., Parmar, A. C., Pavan, P., & Gottardo, M. (2023). Minimizing tannery sludge in landfilling through a mixed microbial culture approach: Effect of oxidizing pretreatment, temperature and hydraulic retention time on process performances and chromium fate. *Biochemical Engineering Journal*, *200*, 109073. https://doi.org/ 10.1016/j.bej.2023.109073.

Tuci, G. A., Valentino, F., Pavan, P., & Gottardo, M. (2024). Tannery sludge valorization through zeolite-assisted anaerobic process for short-chain fatty acids (SCFAs) production. *Environmental Research*, *246*, 118046. https://doi.org/10.1016/j.envres.2023.118046

Tyagi, V. K., & Lo, S. L. (2011). Application of physico-chemical pretreatment methods to enhance the sludge disintegration and subsequent anaerobic digestion: an up to date review. *Reviews in Environmental Science and Bio/Technology*, *10*, 215-242. https://doi.org/10.1007/s11157-011-9244-9

Uçkun Kıran, E., Trzcinski, A. P., & Liu, Y. (2015). Platform chemical production from food wastes using a biorefinery concept. *Journal of Chemical Technology & Biotechnology*, *90*(8), 1364-1379. https://doi.org/10.1002/jctb.4551

UNIC Italian Tanneries (2021). UNIC Sustainability Report.

UNIC Italian Tanneries (2022). UNIC Sustainability Report.

UNIC Italian Tanneries (2023). UNIC Sustainability Report.

Vaiopoulou, E., & Gikas, P. (2020). Regulations for chromium emissions to the aquatic environment in Europe and elsewhere. *Chemosphere*, *254*, 126876. https:// doi.org/10.1016/j.chemosphere.2020.126876.

Valentino, F., Moretto, G., Gottardo, M., Pavan, P., Bolzonella, D., & Majone, M. (2019). Novel routes for urban bio-waste management: A combined acidic fermentation and anaerobic digestion process for platform chemicals and biogas production. *Journal of cleaner production*, *220*, 368-375. https://doi.org/10.1016/j.jclepro.2019.02.102

Valentino, F., Munarin, G., Biasiolo, M., Cavinato, C., Bolzonella, D., & Pavan, P. (2021). Enhancing volatile fatty acids (VFA) production from food waste in a two-phases pilot-scale anaerobic digestion process. *Journal of Environmental Chemical Engineering*, *9*(5), 106062. https://doi.org/10.1016/j.jece.2021.106062

Varghese, V. K., Poddar, B. J., Shah, M. P., Purohit, H. J., & Khardenavis, A. A. (2022). A comprehensive review on current status and future perspectives of microbial volatile fatty acids production as platform chemicals. *Science of The Total Environment*, *815*, 152500.https://doi.org/10.1016/j.scitotenv.2021.152500.

Vázquez-Fernández, A., Suárez-Ojeda, M. E., & Carrera, J. (2022). Review about bioproduction of Volatile Fatty Acids from wastes and wastewaters: Influence of operating conditions and organic composition of the substrate. *Journal of Environmental Chemical Engineering*, *10*(3), 107917. https://doi.org/10.1016/j.jece.2022.107917

Vidal-Antich, C., Perez-Esteban, N., Astals, S., Peces, M., Mata-Alvarez, J., & Dosta, J. (2021). Assessing the potential of waste activated sludge and food waste co-fermentation for carboxylic acids production. *Science of the Total Environment*, *757*, 143763. https://doi.org/10.1016/j.scitotenv.2020.143763

Viti, C., Pace, A., & Giovannetti, L. (2003). Characterization of Cr (VI)-resistant bacteria isolated from chromium-contaminated soil by tannery activity. *Current microbiology*, *46*, 0001-0005. https://doi.org/10.1007/s00284-002-3800-z

Volker, A. R., Gogerty, D. S., Bartholomay, C., Hennen-Bierwagen, T., Zhu, H., & Bobik, T. A. (2014). Fermentative production of short-chain fatty acids in Escherichia coli. *Microbiology*, *160*(7), 1513-1522. https://doi.org/10.1099/mic.0.078329-0.

Wallen, L. L., & Davis, E. N. (1972). Biopolymers of activated sludge. *Environmental Science & Technology*, *6*(2), 161-164.

Wang, C., Guan, E., Wang, L., Chu, X., Wu, Z., Zhang, J., ... & Xiao, F. S. (2019). Product selectivity controlled by nanoporous environments in zeolite crystals enveloping rhodium nanoparticle catalysts for CO_2 hydrogenation. *Journal of the American Chemical Society*, *141*(21), 8482-8488. https://doi.org/10.1021/JACS.9B01555/SUPPL_FILE/JA9B01555_SI_001.PDF.

Wang, D., Ye, Y., Liu, H., Ma, H., & Zhang, W. (2018). Effect of alkaline precipitation on Cr species of Cr (III)-bearing complexes typically used in the tannery industry. *Chemosphere*, *193*, 42-49. https://doi.org/10.1016/j. chemosphere.2017.11.006.

Wang, J., & Yin, Y. (2022). Biological production of medium-chain carboxylates through chain elongation: an overview. *Biotechnology advances*, *55*, 107882. https://doi.org/10.1016/j.biotechadv.2021.107882

Wang, R., Wang, X., & Zhu, T. (2024). Research progress and application of carbon sequestration in industrial flue gas by microalgae: A review. *Journal of Environmental Sciences*. https://doi.org/10.1016/j.jes.2024.04.018

Wang, X., Zhang, L., Xi, B., Sun, W., Xia, X., Zhu, C., ... & Zhang, Z. (2015). Biogas production improvement and C/N control by natural clinoptilolite addition into anaerobic co-digestion of Phragmites australis, feces and kitchen waste. *Bioresource technology*, *180*, 192-199. https://doi.org/10.1016/j. biortech.2014.12.023.

Woodard & Curran. (2006). Methods for treating wastewaters from industry. *Industrial waste treatment handbook*, 149-334. ISBN 978-0-7506-7963-3.

Worwąg, M., & Kwarciak-Kozłowska, A. (2019). Volatile fatty acid (VFA) yield from sludge anaerobic fermentation through a biotechnological approach. In *Elsevier eBooks* (pp. 681– 703). https://doi.org/10.1016/b978-0-12-815907-1.00029-5

Wu, L., Ning, D., Zhang, B., Li, Y., Zhang, P., Shan, X., ... & Zhou, J. (2019). Global diversity and biogeography of bacterial communities in wastewater treatment plants. *Nature microbiology*, *4*(7), 1183-1195. https://doi.org/10.1038/s41564-019-0426-5

Wu, L., Wei, W., Chen, Z., Shi, X., Wang, D., Chen, X., & Ni, B. (2023). Medium chain fatty acids production from anaerobic fermentation of food wastes: The role of fermentation pH in metabolic pathways. *Chemical Engineering Journal*, *472*, 144824. https://doi.org/10.1016/j.cej.2023.144824

Wystalska, K., & Sobik-Szołtysek, J. (2019). Sludge from tannery industries. In *Industrial and Municipal Sludge* (pp. 31-46). Butterworth-Heinemann. https://doi.org/10.1016/B978-0-12-815907-1.00002-7.

Xiao Q, Wei Y, Wang Y, & Zeng F (2012). Comparison of sludge solubilization by microwave and its combined processes. *China Water & Wastewater,* 28:61–64.

Xiao, K., Yu, Z., Pei, K., Sun, M., Zhu, Y., Liang, S., Hou, H., Liu, B., Hu, J., & Yang, J. (2021). Anaerobic digestion of sludge by different pretreatments: Changes of amino acids and microbial community. *Frontiers of Environmental Science & Engineering*, *16*(2). https://doi.org/10.1007/s11783-021-1458-7

Yin, Y., Chen, Y., & Wang, J. (2021). Co-fermentation of sewage sludge and algae and Fe2+ addition for enhancing hydrogen production. *International Journal of Hydrogen Energy*, *46*(13), 8950-8960. https://doi.org/10.1016/j.ijhydene.2021.01.009

Yu, T. H., Tan, S. I., Yi, Y. C., Xue, C., Ting, W. W., Chang, J. J., & Ng, I. S. (2022). New insight into the codon usage and medium optimization toward stable and high-level 5-aminolevulinic acid production in Escherichia coli. *Biochemical Engineering Journal*, *177*, 108259. https://doi.org/10.1016/j.bej.2021.108259

Yu, Z., He, Z., Tao, X., Zhou, J., Yang, Y., Zhao, M., ... & Li, X. (2016). The shifts of sediment microbial community phylogenetic and functional structures during chromium (VI) reduction. *Ecotoxicology*, *25*, 1759-1770. https://doi.org/10.1007/s10646-016-1719-6

Zeb, A., & Murkovic, M. (2010). Thin-layer chromatographic analysis of carotenoids in plant and animal samples. *JPC–Journal of Planar Chromatography–Modern TLC*, *23*, 94-103. https://doi.org/10.1556/JPC.23.2010.2.1

Zhai, S., Li, M., Xiong, Y., Wang, D., & Fu, S. (2020). Dual resource utilization for tannery sludge: Effects of sludge biochars (BCs) on volatile fatty acids (VFAs) production from sludge anaerobic digestion. *Bioresource technology*, *316*, 123903. https://doi.org/10.1016/j.biortech.2020.123903

Zhang, D., Jiang, H., Chang, J., Sun, J., Tu, W., & Wang, H. (2019a). Effect of thermal hydrolysis pretreatment on volatile fatty acids production in sludge acidification and subsequent polyhydroxyalkanoates production. *Bioresource technology*, *279*, 92-100. https://doi.org/10.1016/j.biortech.2019.01.077

Zhang, R., Chen, L., Niu, Z., Song, S., & Zhao, Y. (2019b). Water stress affects the frequency of Firmicutes, Clostridiales and Lysobacter in rhizosphere soils of greenhouse grape. *Agricultural Water Management*, *226*, 105776. https://doi.org/10.1016/j.agwat.2019.105776

Zhang, L., Tsui, T. H., Loh, K. C., Dai, Y., & Tong, Y. W. (2022). Acidogenic fermentation of organic wastes for production of volatile fatty acids. In *Biomass, Biofuels, Biochemicals* (pp. 343-366). Elsevier. https://doi.org/10.1016/B978-0-323-90633-3.00005-5

Zhao, J., Wu, Q., Tang, Y., Zhou, J., & Guo, H. (2022). Tannery wastewater treatment: conventional and promising processes, an updated 20-year review. *Journal of Leather Science and Engineering*, *4*(1), 10.https://doi.org/10.1186/s42825-022-00082-7.

Zhen, G., Lu, X., Kato, H., Zhao, Y., & Li, Y. Y. (2017). Overview of pretreatment strategies for enhancing sewage sludge disintegration and subsequent anaerobic digestion: Current advances, full-scale application and future perspectives. *Renewable and Sustainable Energy Reviews*, *69*, 559-577. https://doi.org/10.1016/j.rser.2016.11.187.

Zhou, M., Yan, B., Wong, J. W., & Zhang, Y. (2018). Enhanced volatile fatty acids production from anaerobic fermentation of food waste: A mini-review focusing on acidogenic metabolic pathways. *Bioresource technology*, *248*, 68-78.https://doi.org/ 10.1016/j.biortech.2017.06.121.

Zhu, C., Wu, Y., Meng, Z., Yang, Y., Xu, Z., & Ma, H. (2024). A bio-enhanced treatment strategy for tannery wastewater based on progressive activation of microbial agents. *Journal of Water Process Engineering*, *67*, 106087. https://doi.org/10.1016/j.jwpe.2024.106087

Zhu, Y., & Yang, S. T. (2004). Effect of pH on metabolic pathway shift in fermentation of xylose by Clostridium tyrobutyricum. *Journal of Biotechnology*, 110(2), 143–157. https://doi.org/10.1016/j.jbiotec.2004.02.006.

Zigová, J., Šturdík, E., Vandák, D., & Schlosser, Š. (1999). Butyric acid production by Clostridium butyricum with integrated extraction and pertraction. *Process Biochemistry*, 34(8), 835–843. https://doi.org/10.1016/S0032-9592(99)00007-2.

Zorpas, A. A., Vassilis, I., Loizidou, M., & Grigoropoulou, H. (2002). Particle size effects on uptake of heavy metals from sewage sludge compost using natural zeolite clinoptilolite. *Journal of Colloid and Interface Science*, *250*(1), 1-4. https://doi.org/10.1006/ jcis.2002.8246.