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In **Biotechnologies for Sustainable Development and the Environment**

Final Thesis

Co-fermentation of tannery sludge and sunflower seed molasses boosted by *Saccharomyces cerevisiae* bioaugmentation

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1. Introduction

With the 2030 Agenda of the United Nations for Sustainable Development, the European Green New Deal defines a clear strategy for the transition to an economic development model that is aimed not only to profit, but also to social progress and environmental protection.

Nowadays, a more rational and sustainable management of natural resources is a crucial aspect and the essence itself of the circular economy. The aim is to make economic activities more efficient and less impacting on the environment by, for example, increasing the processes' efficiency and reducing waste or operating downstream aiming at recovering and reintroducing into the economic system everything that still has some possible use, instead of directly disposing of it in landfills (UNIC 2020).

In this scenario, the tannery industry has shown its heavy environmental impacts, caused by the operations and the processes performed in the productive/supply chain, where the product is transformed from the raw hides and skins to the final leather (Chiampo *et al.*, 2023). This sector is considered one of the most polluting industrial activities in the world (Manucci *et al.*, 2010).

Leather processing involves some physical, biological, mechanical, and chemical operations (Moktadir *et al.,* 2023), and since the transformation of raw materials into finished products uses water, the main load of pollutants remains in the wastewater (Manucci *et al*., 2010).

The wastewater produced is usually treated in centralised industrial wastewater treatment plants where tannery sludge is produced as waste (Tuci *et al*., 2022). This sludge contains abundant organic matter, pathogenic microorganisms, heavy metal ions and other undesirable constituents. Moreover, due to its high chromium ion content (40-80 g/kg), it is also generally considered hazardous waste (Zhai *et al*., 2020).

Therefore, it is plain that in the leather industry, the disposal of tanning sludge is a major problem; Italy is generally characterised by the strongest limitations on the discharge of treated tannery effluents, if compared to other countries (Giaccherini, 2017), and because of its toxic chemicals and organic compounds, the conventional treatment and disposal of tannery sludge are based mainly on landfilling (Alibardi *et al.,* 2016).New sustainable solutions have been studied recently, using leather solid waste as a potential source, some

valorisation techniques include chrome recovery, anaerobic digestion, compost or vermicompost, gasification for clean energy recovery, co-combustion, and biofuel generation (Moktadir *et al.,* 2023). However, these studies are still limited to laboratory scale trials.

In terms of biological waste valorisation, anaerobic digestion is an established process for the treatment of different kinds of organic waste and it could be a good alternative for the tannery sludge treatment (Tuci *et al.,* 2022), considering that the anaerobic digestion is widely used in sludge treatment due to its low environmental impact and the production of added value products such as short-chain fatty acids (SCFAs), medium-chain fatty acids (MCFAs), and biogas (Wang *et al.*, 2020).

SCFAs are saturated aliphatic acids that contain from two to six carbon atoms. These are substances of great importance, mainly used in the food, pharmaceutical, petrochemical and chemical industries, they can be used, additionally, to generate biopolymers (PHA), bioplastics, and biofuels, among other products (Worwąg *et al.,* 2019). Through chain elongation, which is a set of anaerobic pathways in the fermentation process, short chain volatile fatty acids, such as acetic acid, together with an electron donor such as ethanol, hydrogen or methanol are converted into medium-chain fatty acids (MCFAs), saturated fatty acids characterised by chains with six to twelve carbons (C6-C12) including n-caproic acid, with a greater economic value (Wang *et al.,* 2020).

In order to explore new valorisation routes for this type of waste, this study focuses on an alternative way of achieving chain elongation by investigating the effect of co-fermentation of tannery sludge under different conditions.

1.1 Tannery industry

The Italian leather industry occupies a leading position in the European Union (66% of European turnover) and throughout the World (23% of global turnover) (UNIC 2022).

In this industry, the water, after the hides, is the most important raw material in tanning processes (Alibardi *et al.*, 2016). According to the Italian tannery industry sustainability report of 2022, Italian tanneries use an average of 116,6 litres of water per square metre of leather produced and generate 1.38 kg of waste, one quarter of which is sludge (UNIC 2022).

The tannery wastewater is generated by the tanning processes employed in the leather production where the liquid waste is conveyed to the wastewater treatment plants (WWTP). Here, in order to get a “clear” effluent, the wastewater undergoes physical-chemical primary treatment so the settleable organic and inorganic solids are removed by sedimentation, followed by the elimination of biodegradable dissolved and colloidal organic matter using biological treatment processes. During these operations the main waste produced is the primary and secondary sludge which are then mixed and dewatered with the purpose of reducing the volume of the material and getting the dry matter content required for disposal in landfills (UNIDO, 2011).

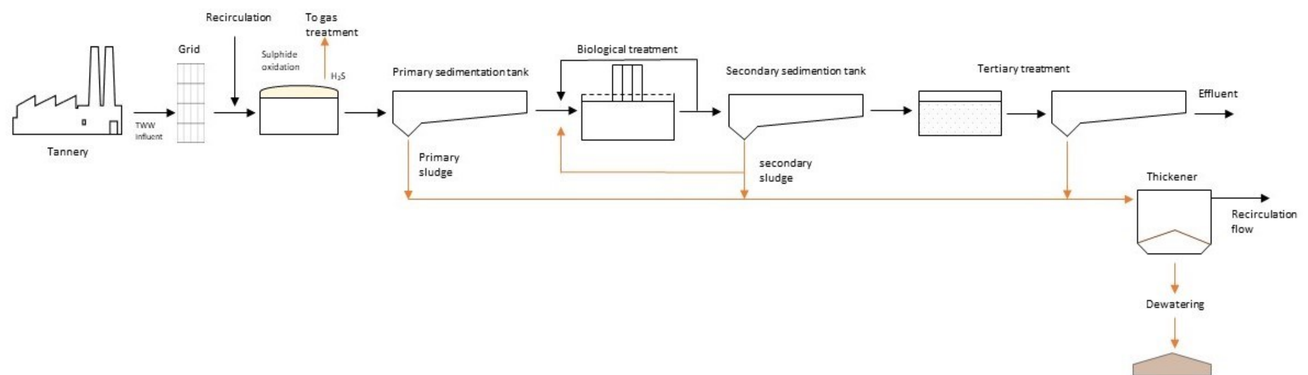


Figure 1: General tannery WWTP scheme.

This sludge, due to its chemical characteristics, is considered as a hazardous waste, especially because of the chromium content. Most leather processing worldwide (about 90%) uses trivalent chromium sulphate as the tanning agent, because of its ability to increase the mechanical strength of the final product. Only about 60% of the chromium added in leather processing is efficiently utilised and the rest is discharged into wastewater (Moktadir *et al*., 2023).

It is reported that the quantity of chromium (Cr(III)) in the tannery sludge is such, that it can be bio accumulated (Moktadir *et al.* 2023), but beyond the environmental problems there are also issues related to the creation of secondary pollutants and high maintenance costs of the tannery sludge treatment (Hashem *et al.,* 2022).

However, in addition to pollutants, this sludge contains a substantial quantity of organic matter that can be reused and recycled to get some valuable components, such as value-added products and bioenergy (Fang *et al*., 2020). Currently, anaerobic digestion is widely

applied for sludge treatment and resource recovery on several organic substrates, however the use of tannery sludge for this technology is still limited and sparce. While there have been recent studies investigating potential applications of tannery sludge, these efforts are predominantly confined to laboratory settings. As a result, the treatment and disposal of tannery sludge still heavily rely on landfilling practices (Alibardi *et al*., 2016).

1.2 Sunflower waste valorization

The sunflower, a primary global oil crop cultivated for the production of edible and biodiesel oil, yields various products and by-products depending on the method of oil extraction employed (De Oliveira Filho & Egea, 2021). Among these, sunflower meal, the principal by-product resulting from seed crushing, (Grasso *et al*., 2020) is currently utilized as feedstuff for ruminants or simply discarded. However, its high nutritional value, widespread availability, and competitive pricing suggest potential applications as feedstock in different industrial processes. Notably, the sunflower by-product possesses a nutrient-dense profile, in fact, it is rich in proteins, lipids, and carbohydrates as well as a modest quantity of polyphenols, minerals and vitamins, making it particularly interesting for incorporation into the chemical, pharmaceutical, and food industries (Náthia-Neves & Alonso, 2021; De Oliveira Filho & Egea, 2021).

The elevated protein content in sunflower meal positions it as a valuable source for extracting vegetable proteins, which have undergone extensive assessments for their utility as food ingredients. Additionally, enzymatic hydrolysis has proven to be an efficient in vitro technique for deriving protein hydrolysates from sunflower meal extracts. These protein hydrolysates have been shown to exhibit significant biological activities beneficial to human health, including antihypertensive properties, as well as anti-inflammatory and immune-modulating effects (De Oliveira Filho & Egea, 2021). The utilization of sunflower for biodiesel production not only yields sunflower meal but also generates crude glycerol as a by-product. The crude glycerol has undergone evaluation as an energy and carbon source in microbial bioconversions aimed at producing different chemicals such as succinic acid, ethanol, citric acid, and polyhydroxyalkanoates (PHA) (Kachrimanidou *et al*., 2013).

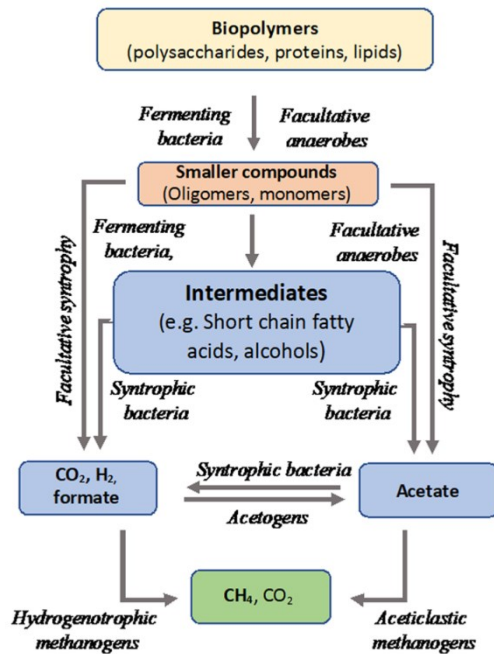
Despite the sunflower by-products' high nutritional value and versatile applications, including foaming, emulsifying, and serving as a polymer in the chemical, pharmacological,

and food industries, their biomass is predominantly limited to animal feeding. The predominant reliance on sunflower by-products for animal feed is primarily attributed to the limited documentation of their utilization in industrial processes, a facet scarcely reported in current literature (Náthia-Neves & Alonso, 2022).

1.3 Anaerobic digestion and fermentation

Anaerobic digestion (AD) is an efficient technology for valuable resources recovery (such as, short-chain fatty acids (SCFAs), hydrogen (H_2) and methane (CH_4)), providing organic waste reduction, sludge stabilisation and biological toxicity decrease (Zhai *et al.,* 2020).
From the metabolic point of view, the AD pathways can be divided into two main routes: the first is represented by the acidogenic fermentation which focuses on breaking down complex organic matter into assimilable compounds, and the second, represented by the methanogenesis pathways where takes place the conversion of these intermediate products into methane (Valentino *et al*., 2021; Vidal-Antich *et al*., 2021). These steps are characterized by different microbial groups and operational conditions, in figure 2 are summarized the stages of the anaerobic digestion and fermentation.

Anaerobic Digestion (AD) **Anaerobic Fermentation (AF)**



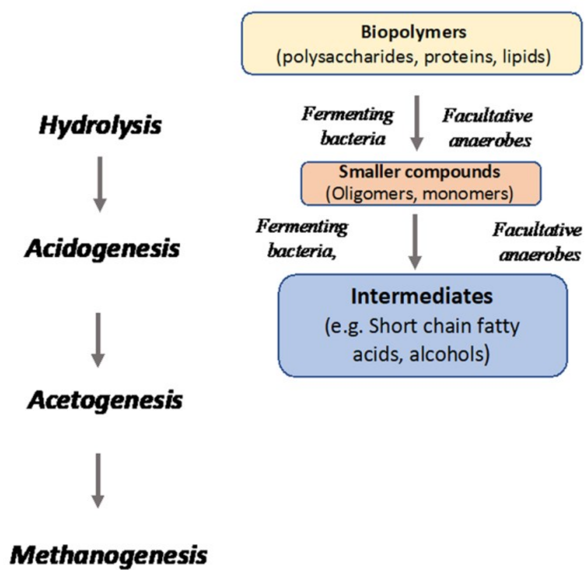


Figure 2: General scheme of steps involved in both AD (with biogas as the final product) and AF (with mainly SCFAs as final products) (Tomás-Pejó et al., 2023).

The first stage of the fermentation process is the hydrolysis, where hydrolytic bacteria are able to secrete extracellular enzymes (e.g., amylases, lipases, or proteases) that can convert polysaccharides into monosaccharides, proteins to amino acids, and fats to polyols and fatty acids (Worwąg *et al*., 2019). This phase is known as a rate-limiting step in the AD of solid organic waste, due to the presence of complex floc structures and hard cell walls. To overcome this problem and to enhance the anaerobic digestion of sludge, various pretreatments, such as thermal, ultrasonic, and acid/alkaline methods, have been applied (Xiao *et al*., 2021).

Through the acidogenesis, also named "dark fermentation", obligate anaerobes bacteria such as *Aerobacter, Clostridium, Escherichia, Pseudomonas, Lactobacterium, Lactobacillus, Micrococcus,* and *Streptococcus*, with their activity, transform low molecular organic compounds mainly into SCFAs (e.g., acetic acid, propionic acid, butyric acid, valeric acid, and caproic acid), alcohols (ethanol and methanol) and gaseous products (CO_2 and H_2). These microorganisms are less susceptible to temperature or pH changes and proliferate much faster than CH_4 bacteria (Worwąg *et al*., 2019).

In the third stage of fermentation, the acetogenesis, ethanol and SCFAs are processed into CO_2, hydrogen, and acetic acid. These transitions involve two groups of anaerobic bacteria of the *Syntrophobacter* and *Syntrophomonas* genuses, which live in symbiosis with the methanogenic bacteria, responsible for the last phase of the digestion process, the methanogenesis. During this step, takes place the conversion of acetic acid, H_2 and CO_2 into methane (CH_4) and CO_2 thanks to obligate anaerobes of the *Methanobacterum, Methanococcus,* and *Methanogenium* genuses (Srisowmeya *et al.,* 2020; Worwąg & Kwarciak-Kozłowska, 2019).

This technology has earned significant attention from researchers as it offers a viable solution for sludge management, including tannery sludge (Moktadir *et al.,* 2023). Moreover, apart from the biogas production, the fermentation products can also be directly used to provide the carbon source needed to sustain other microbially-mediated units, such as biopolymers production (e.g. PHB and PHV), biological nutrient recovery (N, P and S) and chain elongation (Vidal-Antich *et al*., 2021). Thus, the anaerobic digestion can use batch reactors and continuous reactors of one or two stages.

The one-stage anaerobic digestion requires a coordinated metabolism of microbial groups involved in the process, but it is known that the methanogens' generation time is longer than the hydrolytic and acidogenic biomass' and the SCFAs production is faster than the SCFAs conversion rate to CH_4 (Xiong *et al*. 2012; Lozano *et al*., 2009) so the two-stage system allows for the establishment of operational conditions that reduce the start time and microbiota specialization in each reactor, allowing for the most desirable products at each stage to be obtained (Srisowmeya *et al*. 2020; Lozano *et al*., 2009).

1.4 Short chain fatty acids (SCFAs)

Short chain fatty acids (SCFAs) are important intermediates produced in the acidogenesis and acetogenesis step during anaerobic fermentation (Zhou *et al*., 2018).

They are also known by other terms such as volatile fatty acids, and low-weight carboxylic acids.

Generally, SCFAs are water-soluble fatty acids, that can be distilled at atmospheric pressure, some of them are acetic acid, propionic acid, butyric acid, isobutyric acid, valeric

acid, isovaleric acid, and caproic acid which contain between two and six carbon atoms (Varghese *et al.*, 2022).

Their structure contains an acetyl group characterised by the presence of a carbon atom that is double-bonded to oxygen. Due to the electronegativity of oxygen present in the acetyl group, carboxylic acids belong to polar compounds. They have strong hydrophilic properties and despite their high boiling point, these acids can be removed from water through distillation with water steam (Worwąg *et al.,* 2019).

SCFAs produced by the fermentation of organic matter, a renewable carbon source, can be utilised as an alternative to petroleum based SCFAs, and are characterized by a wide range of applications, such as in food, textile, pharmaceutical, leather and plastics industries but also for the production of biodiesel and synthesis of complex polymers (Zhou *et al*., 2018). To make the process more economically sustainable, researchers are now focusing on utilising organic waste for SCFAs synthesis, including agricultural, municipal, and industrial waste streams, where most approaches include mixing more than two substrate, to achieve an optimum nutrient balance and a positive influence on SCFAs production (Varghese *et al*., 2022).

In certain cases, they are also known as medium chain fatty acids (MCFAs) when they have from six to twelve carbon atoms, to simplify, in this thesis the term SCFAs will include the acids from the acetic to caproic.

As reported by M. Zhou and colleagues (2018) in their study, the market prices of SCFAs increase with the increment of carbon numbers, this might be attributed to the wider industrial application of longer-chain fatty acids, and that the production of these is usually more difficult than that of short-chain ones, making, for example, propionate and butyrate more attractive products than acetate according to their market values and applications. Figure 3 shows the chemical properties of SCFAs with some current market data.

Acid	Chemical formula	Molecular mass (g mol^{-1})[a]	Density (g cm^{-3}) at 20°C[a]	Boiling point (°C)[a]	pKa[a]	Average price (USD kg^{-1})[b]	US production in 2016 (MT)[c]
Acetic (HAc)	CH_3COOH	60.052	1.05	117.9	4.76	0.89	1224–2449
Propionic (HPr)	CH_3CH_2COOH	74.079	0.99	141.1	4.88	2.20	11–17
Isobutyric (iHBu)	$(CH_3)_2CHCOOH$	88.106	0.95	154.4	4.84	2.75	–
Butyric (HBu)	$CH_3CH_2CH_2COOH$	88.106	0.96	163.7	4.82	2.55	1.13–2.27
Isovaleric (iHVa)	$(CH_3)_2CHCH_2COOH$	102.133	0.93	176.5	4.77	–	–
Valeric (HVa)	$CH_3(CH_2)_3COOH$	102.133	0.94	186.1	4.84	4.63	0.45–0.91

[a]Physical properties taken from PubChem Compound Database (Information National Center for Biotechnology 2018)
[b]Price data gathered throughout April 2018 from multiple sources (Alibaba 2018)
[c]Domestic production in the US in 2016 (United States Environmental Protection Agency 2016)

Figure 3: SCFAs chemical proprieties and market data (Ramos-Suarez et al., 2021).

It is believed that the improvement of the first step of AD, the hydrolysis, could increase the readily available substrate-carbon for the conversion to SCFAs, which would certainly enhance their production. The methods to enhance the hydrolysis include the optimization of key operational factors such as pH, temperature, and pre-treatment of the substrate before fermentation (Zhou *et al*., 2018).

1.5 Key parameters in the anaerobic fermentation processes of organic waste

As a series of biochemical reactions, anaerobic fermentation can be affected by many environmental factors and operating parameters such as pH, temperature, substrate, and the use of pre-treatments (Fang *et al.*, 2020). So, it is important to maintain the key parameters within the appropriate range for long term operation of AD (Zhang *et al.*, 2014).

1.5.1 pH

The pH is one of the most important factors that significantly influence the efficiency of hydrolysis and acidification (Fang *et al.,* 2020). Previous studies have investigated the optimal pH range for the fermentation steps, but their finding highlight that the type of feedstock determine the ideal range. For example, when food waste is mixed with a high carbohydrates content, the optimal pH range for hydrolysis is between 4 and 6, while a pH

11 is favourable for hydrolysis when an agricultural residue like maize silage is the sole feedstock (L. Wu *et al*., 2023). So consequently, the optimal pH for the following steps of fermentation may be different depending on the type of substrate and the hydrolysis performance.

Many of the bacteria that generate SCFAs do not tolerate an environment that is strongly acidic (pH < 3) or strongly alkaline (pH > 12), actually the fermentative bacteria can perform efficiently in a range of pH between 4.0-8.0, while methanogenic bacteria perform within a pH range of 6.5-7.5 (Srisowmeya *et al*., 2020).

SCFAs are produced generally in a range between 5.0 and 11.0. Wu and colleagues observed that, with primary sludge as substrate, increasing the pH from 4.0 to 6.5 cause an increase on SCFAs production, while keeping a pH 7.5 results to be first a suitable value for the methanogens bacteria and second it led to an increase on hydrolysis efficiency but with lower SCFAs generation (Wu *et al.,* 2009; Xiong *et al*., 2012). More studies using sewage sludge as substrate prove that alkaline conditions only promote the hydrolysis and release of organic matter from the sludge particles into the liquid, while the neutral pH conditions are conducive to the activity of acid-producing microorganisms (Presti *et al*., 2021). In contrast, other research with sludge as substrate observed that alkaline conditions (e.g. pH 10) led to a greater production of SCFAs, up to 3 or 4 times higher than that at pH 5.0 and uncontrolled (Wu *et al.,* 2009; H. Yuan *et al.,* 2006).

The production of SCFAs can vary depending on the pH value (Tuci *et al*., 2023). For instance, the optimal pH range for generating propionic acid during sludge fermentation is typically between 4 and 4.5, while for acetic acid and butyric acid, it falls within the range of 6 to 6.5 (Worwąg *et al.,* 2019). Under alkaline conditions, there is an increase in the production of acetic and propionic acids compared to using acidic or neutral conditions with primary sludge. However, this trend changes when food waste is used as a substrate, where acetic and butyric acids become the primary products of acidogenesis (L. Wu *et al.,* 2023; Yuan *et al*., 2006).

1.5.2 Temperature

Temperature is another key factor to consider. It affects the growth of microorganisms, the activities of enzymes and the hydrolysis of particulate organic matters to soluble

substances. The mesophilic condition could be considered as the most efficient and economical way for SCFAs production since the yield on SCFAs production could be similar to higher temperature, but with lower costs associated (Jiang *et al*., 2013), but studies have shown that increasing the temperature could significantly improve the sludge hydrolysis and acidification. As reported by Rubal and colleagues, temperature has a significant influence on the hydrolysis of proteins, carbohydrates, and lipids; hydrolysis is higher at 35°C than at 25°C, and a higher rate of hydrolysis is observed at 55°C, compared with that at 20°C and 35°C on primary sludge fermentation (Rubal *et al*., 2012). In Worwąg's study the amount of SCFAs obtained from sludge at a temperature of 55°C was up to 40% lower than at 37°C (Worwąg *et al*., 2019).

The temperature could also influence the composition of SCFAs. Researchers on fermentation of food waste found out that acetate and propionate are the most prevalent SCFAs generated at 35C° and 45C°, whereas butyrate was the main product accounting for 81% of all products when the temperature is increased to 55 °C. Different results can be obtained depending on the type of substrate or the absence of inoculation (Jiang *et al.,* 2013).

While various studies have demonstrated advantages under thermophilic conditions, it's important to note that there are also associated disadvantages. One such drawback is the increased sensitivity of the thermophilic process to environmental changes compared to the mesophilic process (Zang *et al.,* 2014).

1.5.3 Total solids (TS) and related organic loading rate (OLR)

Based on the water content of the waste used in the process, the AD has been classified into three types: "wet" when the proportion of total solids (TS) is lower than 10%, "semidry" with a TS ranging between 10 and 15% and "dry" characterised by a TS above 15%, this one mainly applied to the treatment of solid wastes (Liotta *et al.,* 2014; Abbassi-Guendouz *et al.,* 2013)

These last two technologies are attractive because of the less water and energy required for the process, minimal nutrient loss, and the smaller size of the digester required to treat a same amount of organic matter (Abbassi-Guendouz *et al.,* 2013). However, a high initial TS content can limit mass transfer between the substrate and the microorganisms, the

settling properties and it may affect other rheological properties of the medium such as the viscosity, decreasing the microbial activities (Tuci *et al*., 2022).

Indeed, studies have indicated that increasing total solids (TS) concentration can result in a slower rate of hydrolysis and methane production. Additionally, this increase in TS concentration can lead to a higher accumulation of inhibitors such as ammonia (Wang *et al*., 2015; Wang *et al.,* 2020). Wang and colleagues show that with higher TS concentration a higher percentage of butyrate and lower percentage of acetate were achieved in an experiment with food waste under mesophilic conditions (Wang *et al*., 2015). However, studies on this matter remain inconclusive. While some research has reported that high total solids (TS) content could result in higher biogas yields during methane production, others have shown contradictory (Wang *et al.,* 2020).

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 organic loading rate (OLR) (Varghese *et al*., 2022). It is known that a higher OLR means a higher rate of organic matter being introduced into the system, which can lead to higher production rates of SCFAs. Moreover, balancing OLR with other factor like HRT, temperature and pH is essential. As reported by Jiang and colleagues in a research of SCFAs production from food waste, keeping a constant temperature (36°C), pH (6.0) and HRT (5 days), the SCFAs concentration increased as the OLR increased (Jiang *et al.,* 2013).

However, the optimal OLR for SCFAs production can also vary depending on the specific characteristics of the substrate or the microbial community.

1.5.4 Type of substrate

There are several types of wastes that can be used as substrates in acidogenic fermentation such as solid wastes, slurry-like wastes; typical examples are primary sludge from a wastewater treatment plant, food waste, the organic fraction of municipal solid waste and liquid wastes or wastewaters (Vázquez-Fernández *et al*., 2022).

Comparing yields on SCFAs production using different substrates makes sense if the experiments conditions are the same. It is known that good performances are achieved with substrates with a high organic matter and a high availability, especially rich in carbohydrates. Research has found that lipidic substrates are less preferred than

carbohydrates and proteins in fermentation processes for several reasons. Firstly, hydrolysis of lipids tends to be slower compared to carbohydrates and proteins. Secondly, the hydrolysis of lipids produces long-chain fatty acids (LCFAs) and glycerol, which can have inhibitory effects on the fermentation process.The glycerol can be converted to SCFAs but LCFAs could inhibit the metabolism of anaerobic bacteria due their adhesion to the cell walls that can decrease the nutrient transportation (Yin *et al*., 2016; Vázquez-Fernández *et al*., 2022).

In general, the substrates typically used for the fermentation include activated sludge and food waste (Worwąg *et al*., 2019). In agriculture, energy crops and crop residues have gained attention recently due to their abundance, renewable and carbon-based resource even though their low biodegradability due the lignocellulose compounds, makes them especially resistant to degradation processes (Pokój *et al.,* 2015; Nguyen *et al*., 2020; Worwag *et al*., 2019).

Higher concentrations of SCFAs can be obtained with the addition of a co-substrate, where potential toxic compounds could be diluted (Zhou *et al.,* 2018). Moreover, adding other substrates can increase the organic matter content, improve the buffer capacity inhibiting the toxic compounds and it can add a balance of macronutrients, micronutrients, and moisture (Vidal-Antich *et al*., 2021). Thereby anaerobic digestion can treat the organic waste reducing environmental pollution and management costs and also generate bioproducts that can be used as a renewable energy source (Sillero *et al.*, 2022).

1.5.5 Pre-treatment

The hydrolysis in the anaerobic fermentation represents the main rate-limiting step of the whole process. Therefore, it is key to enhance this step, in order to increase the performance of SCFAs production (Zhang *et al.,* 2014). The degradation of the complex polymers such as lipids and proteins take the most time during the anaerobic process and the improvement of hydrolysis through pre-treatments could increase the readily available substrate-carbon for subsequent conversion to SCFAs, which would enhance their production.

Pre-treatment of substrates could improve the generation of soluble chemical oxygen demand (sCOD) from hydrolysis and could influence the yield of SCFAs production. To

accelerate the first step of the anaerobic digestion several approaches were studied, the most common disintegration methods were mechanical grinding, ultrasound, thermal, chemical, and biological (Zhou *et al.,* 2018).

1.6 Chain elongation processes

The chain elongation (CE) is a set of anaerobic pathways where the intermediary products derived from the dark fermentation, for example acetate, ethanol, and CO_2 are converted into short-chain fatty acids and medium-chain fatty acids that can be used directly or as precursors for biofuels and biochemicals (Reddy *et al*., 2020; San Valero *et al*., 2020). This can be implemented as a secondary process where SCFAs, obtained through anaerobic fermentation of organic waste are transformed into acids with a longer chain by microorganisms employing ethanol, hydrogen, or methanol as electron donors (Rogheir *et al.,* 2018).

This is a cyclic process, the reverse βoxidation, where an acetyl-CoA molecule, which is derived from ethanol is added to a carboxylate, elongating its carbon chain length with C_2 at a time (for example the acetate [C_2] is converted to n-butyrate [C_4] and n-butyrate to n-caproate [C_6] and so on) (Spirito *et al.,* 2014).

Acetate and CO_2 are two essential substrates necessary for chain elongation, and both can be produced from organic waste through a biochemical conversion process. Ethanol is also an essential substrate for chain elongation because it can be used to elongate SCFAs into MCFAs, but it can also be converted itself into SCFAs (Rogheir *et al*., 2018). In a circular economy perspective ethanol-containing waste stream are preferred as feedstock, to avoid its addition during the process, reducing the costs and the environmental impacts (Chen *et al*., 2016).

Previous research has shown that chain elongation is influenced by the ratio of ethanol to SCFAs, in fact, in general, adding more ethanol to the mixed culture fermentation enhances the process. However, a very low concentration of ethanol reduces the rates because the substrate is not readily available, while high concentrations of ethanol inhibit the acid producing microorganisms resulting in low biomass conversions. This makes the right concentration of ethanol still unknown (Lonkar *et al*., 2016). In addition to the ethanol

concentration other parameters have been shown to affect the chain elongation process, for example the inorganic carbon concentrations or the presence of yeast extract where, as reported by San Valero and colleagues, in the presence of yeast extract in the medium, the bacteria use the carbon from yeast for growth while ethanol and acids are converted in other acids with a longer chain, on the contrary, with the omission of yeast extract, part of the ethanol and the acids are used for growth by the bacteria (San Valero et al., 2020).
Finally, the CE enables to overcome the limitation of SCFAs extraction faced during primary fermentation processes, because longer chains are more apolar and consequently more hydrophobic, making it easier to separate from the fermentation broth (San Valero et al., 2020; Grootscholten et al., 2013).
Moreover, chain elongation has many advantages for industrial applications, including the possibility to be performed under non-sterile conditions with a mixed microbial culture (Reddy *et al.,* 2018).

1.7 Yeast

Yeasts are eukaryotic microorganisms that live in a wide variety of ecological niches like soil, air or on plants. From a morphological point of view the most common shapes are round, oval, or ellipsoidal and their growth is supported by basic compounds (fermentable sugars, minerals, amino acids…). The reproduction of yeasts is mainly asexual and by budding, which results in a new and genetically identical cell. In industrial fermentation processes, this type of reproduction is recommended to ensure the preservation of the genotype and to maintain stable fermentation behaviour (Maicas, 2020).
During sugar fermentation, yeasts (and some bacteria) break the pyruvate, generated from glucose metabolism, into ethanol and carbon dioxide, with energy (ATP) production (Maicas, 2020). The production of ethanol is aimed at maintaining redox balancing when consuming sugars under anaerobic conditions (Maicas, 2020; Walker & Walker, 2018). Here follows a summary of glycolysis and fermentation by yeast.

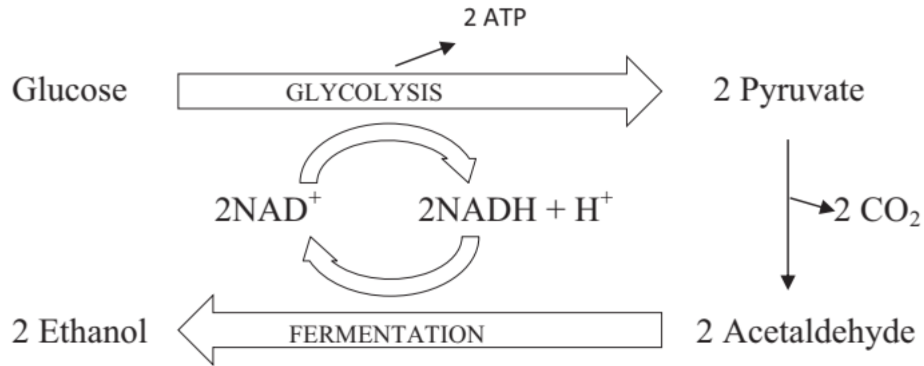


Figure 4: Summary of glycolysis and fermentation in yeast (Walker & Walker,2018)

Saccharomyces cerevisiae is the preferred yeast species for many industrial processes and has been exploited in the production of alcohol since 6000 BC (Jansen *et al.,* 2017). Its use in the fermentation of wines and beers is due to its satisfactory fermentative capacity, rapid growth, and easy adaptation. They tolerate concentrations of SO_2 that normally most non-Saccharomyces yeasts do not survive (Maicas, 2020). This yeast is also responsible for making other fermentation products including baked foods, probiotics, therapeutic proteins, and biopharmaceuticals (Walker & Walker, 2018).

There is a wide diversity of industrial *S. cerevisiae* strains and breeding strategies have been used to improve those strains, allowing the generation of hybrids that outperform the parental strains with improved traits, including stress tolerance, enhanced fermentation performance, and improved processes or quality of the product (Walker & Walker, 2018; Jansen *et al.,* 2017). Direct approaches to introduce new traits into existing strains involve genetic manipulations and adaptive evolution, which can be used to enhance industrially important traits already present in yeast including substrate utilization, ethanol production, and stress tolerance (Walker & Walker, 2018).

Commonly yeast has the characteristics of acid resistance and high metabolic efficiency, which is widely used in the treatment of high-concentration organic wastewater, including the treatment of toxic and refractory pollutant-containing wastewater (Gao *et al.,* 2020); *Saccharomyces cerevisiae* has also a high tolerance to hydrolysate inhibitors and, above all, it is resistant to pH drops, during the consumption of ethanol and consequently the production of acids (Wang *et al.,* 2023; Lian & Zhao, 2014). For these reasons a typical

fermentation can be facilitated by adding supplementary microorganisms with relevant biodegradation capacities (which is known as bioaugmentation) (Jiang *et al*., 2020).

Yeast requires being supplied with the correct nutrients in order to grow and conduct efficient fermentation. In general, biomass or organic waste such as corn grains or molasses, contains high enough levels of proteins and amino acids and readily fermentable sugars such as sucrose, glucose, and fructose but also some metals, like magnesium, iron, calcium, zinc, and potassium which are required for a good growth of the yeast (Suwannarat & Ritchie, 2015; Walker & Walker, 2018).

Lately has been investigated the possibility of ethanol production by yeast from wastewater rich in carbohydrates, so the generated alcohol could be converted to SCFAs or MCFAs (Zang *et al*., 2022).

2. Thesis objective

The aim of this study is to provide an alternative way of achieving chain elongation by investigating the effect of co-fermentation of tannery sludge and a sugar source on SCFAs and MCFAs production, boosted by yeast bioaugmentation. Even though an economic analysis was out of the scope of this thesis, the objective of this work is to provide the first experimental information in the direction of tannery sludge and sunflower seed molasses valorisation into new bioproducts with high economic value. This can be obtained through processes that enhance waste materials with a high organic content that would otherwise be discarded.

In this study two series of batch tests were carried out involving the use of two different strains (strain1 and strain2) of *Saccharomyces cerevisiae* as inoculum and white sugar or sunflower seed molasses as co-substrate to set the production of bio-ethanol, which can be used as final product or as electron donor in chain elongation processes.

The first batch test was conducted using the sunflower seed molasses as substrate and without tannery sludge in order to understand the activity of yeast in a non-sterile environment, which can be influenced by the presence of various contaminants and consequently impact the bioethanol production. While these tests aimed to the ethanol production, the (predicted) presence of SCFAs indicated the diversion of carbon towards alternative pathways so it was also monitored the types and concentrations of SCFAs that could suggest the presence of unwanted contaminants affecting the fermentation process.

The second batch test was carried out adding the tannery sludge as substrate with the objective to see if the ethanol instead of following the traditional fermentative pathways, could be redirected as an electron donor to elongate short-chain fatty acids into valuable medium-chain fatty acids and produce acids through CE processes.

However, the fermentation tests conducted primarily aimed at understanding the microbial processes involved in acidogenic fermentation. Additionally, the study explored the impact of bio-augmentation and co-substrate use, investigating if chain elongation phenomena could be triggered. This exploration enhances acid production by yielding valuable MCFAs, particularly in light of the market cost of caproic acid, which is known to be higher than that of SCFAs.

3. Materials and method

3.1 Substrate

The tannery sludge used for the batch tests was a mixture of primary and secondary sludge procured from a wastewater treatment plant of Montebello Vicentino (Italy). This WWTP treats about 10000 m^3/d of industrial wastewater produced by 23 tannery plants. The tannery wastewaters are subjected to primary treatment and sedimentation then a secondary biological treatment (anoxic/aerobic process) used for biochemical oxygen demand (BOD) and nitrogen removal. The sludge produced as a waste in the end undergoes dehydration reaching a concentration of TS of 83%.

Figure 5: Sample of dried tannery sludge used for the tests.

3.2 Co-substrate

For these experiments were used, as a source of organic matter, common white sugar and sunflower seed molasses, this one collected from Coccitech Srl, an Italian biotechnology company from Treviso.

The sunflower molasses was characterized by 66% of organic compounds and a concentration of TS about 80%.

Figure 6: sample of sunflower seed molasses art room temperature.

3.3 *Saccharomyces cerevisiae*

The yeast used was the *Saccharomyces cerevisiae* in two strains (identified as strain1 and strain2) produced by Renaissance and distributed by Coccitech Srl. These strains were selected aiming to impart specific characteristics to wine, but they result being adaptable, versatile, and resistant to pollutants. Before this research, the yeast was tested in batch tests with the presence of tannery sludge to see if it could survive under the toxic compounds for 30 days.

For the batch tests the yeasts were rehydrated mixing 5g of dry yeast in 50 ml of distilled water and after 15 minutes the yeast mixture was added as inoculum in the bottles.

3.4 Batch tests preparation

The tests were performed in two set, the objective of the first was seeing the fermentation yields using only the yeast and a co-substrate. To explore the handling of the sunflower seed molasses, only this one was used for the mixture for each batch test.

The experiment was carried out using 6 bottles with a capacity of 250 mL, and 150 mL working volume. For the first three bottles (A; B; C) different amounts of sunflower molasses and 10 ml of hydrated yest from strain1 were added, while the others (D; E; F) were prepared in the same way using strain2.

All the tests were placed on a thermal plate set at 50°C, in order to keep the mesophilic condition. The bottles were continuously stirred by means of a magnetic stirrer at 200 rpm. Here follows a summary of the test preparation.

Test	Hydrated yeast (ml)	Sunflower molasses (g)	Tap water (ml)
A1 strain1	10	4	140
B1 strain1	10	8	140
C1 strain1	10	12	140
D2 strain2	10	4	140
E2 strain2	10	8	140
F2 strain2	10	12	140

Table 1: Summary first batch test

For the second batch test, aiming to assess the occurrence of chain elongation, 13 bottles were prepared using 13g of tannery sludge to reach 7% of TS, white sugar or sunflower seed molasses as co-substrate and yeasts. Simultaneously were also prepared blank tests without the yeast to define the co-fermentation yields.

Test	Hydrated yeast (ml)	Sunflower molasses (g)	White sugar (g)	Tap water (ml)	Sludge (g)
Blank test	0	0	0	150	13
A strain1	10	0	7.5	140	13
B strain1	10	0	3.7	140	13
C strain1	10	11	0	140	13
D strain1	10	6	0	140	13
E strain2	10	0	7.5	140	13
F strain2	10	0	3.7	140	13
G strain2	10	11	0	140	13
H strain2	10	6	0	140	13
I	0	11	0	150	13
L	0	6	0	150	13
M	0	0	7.5	150	13
N	0	0	3.7	150	13

Table 2: Summary second batch test

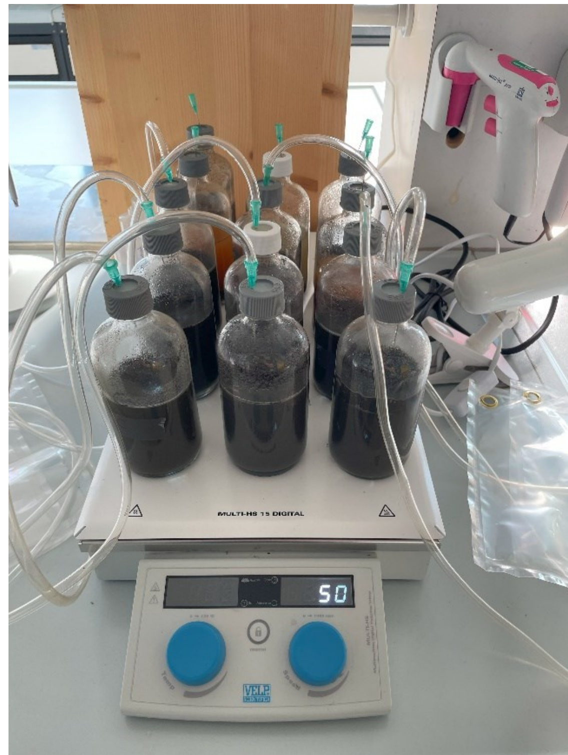


Figure 7: Test bottles from the second batch test

3.5 Batch fermentation test

All the tests were kept in mesophilic conditions using a thermal plate. The bottles were closed with a silicon plug to maintain the anaerobic condition and to be able to withdraw the samples, which was done twice a week to measure the SCFAs production and track the pH.

After the bottles were manually mixed, about 5 ml of samples were taken using a syringe, then, the samples were centrifuged at 4700 rpm for 10 minutes to separate the suspended solids from the mixture and simplify the following filtration which was done with first a 0.45 µm acetate cellulose syringe and after with a 0.22 µm one. Then pH was measured using pH probe.

The expected time to see the results on chain elongation was about twenty days, following otherresearch, so the second tests were stopped after 35 days.

3.6 Analytical methods

3.6.1 SCFAs and alcohols analysis

The SCFAs and alcohols analysis were determined using an Agilent 6890 N gas chromatograph which comes with a flame ionization detector (FID) (T = 250 °C) and an Agilent J&W DB-FFAP fused silica capillary column (15 m length, 0.53 mm diameter, 0.5 mm film). The SCFAs of interest were acetic acid (C2), propionic acid (C3), butyric acid (C4), isobutyric acid (iC4), valeric acid (C5), isovaleric acid (iC5), caproic acid (C6) and isocaproic acid (iC6) while the alcohol analysed with the same procedure were methanol and ethanol. The instrument use hydrogen as carrier and it was programmed with a ramp temperature from 40°C to 160°C, and a post-run phase at 220°C.

The samples for the chromatograph were prepared in Eppendorf with 450 µL of sample, 450 µL of distilled water (dilution 1:2), 100µL phosphoric acid and 100 µL of internal standard.

The more concentrated samples from the first batch test, were diluted more, by adding 600 µL of distilled water (dilution 1:3).

The quantitative analysis of the acids and alcohols was made comparing the peak area in the chromatogram with the one of the internal standard (2-etilbutyrric acid) present in known quantity. The values obtained from the GC analysis were already converted into mgCOD/L while the yield on alcohol and acid production was calculated as follow:

$$Y^{max}Alc: \frac{(gCODAlc/L)}{(gVS_0/L)}$$

$$Y^{max}SCFA: \frac{(gCODscfa/L)}{(gVS_0/L)}$$

Where the $gCOD_{Alc}$ and $gCOD_{SCFA}$ are the values in output from the GC and VS_0 refers to the initial volatile solids content in the substrate.

3.6.2. *Total solids/ Volatile solids*

The total solids (TS) represent the amount of dry residue of a sample, and they are determined after drying it in the oven, then, the sample can be placed in a muffle for the incineration to define volatile solids (TVS) which represents the organic matter.

For the determination of TS of the tannery sludge, first an empty crucible has been weighed in a technical scale, obtaining the value of the tare (W_0), then a representative quantity of sludge has been weighed inside the same crucible obtaining the wet weight (W_1). The sample has been put it inside the oven at 105°C for 48 hours, after drying, the weight obtained represents the dry residue (W_2).

The formula used for the quantification of TS is:

$$TS = \frac{W_2 - W_0}{W_1 - W_0} * 1000 \text{ as } \frac{g_{TS}}{Kg}$$

For the TVS the sample after the stove has been incinerated in the muffle for 24 hours at 550°C. W_3 represent the weight of the crucible after the incineration. So, the TVS calculated are:

$$TVS = \frac{W_2 - W_3}{W_1 - W_0} * 1000 \text{ as } \frac{g_{TVS}}{Kg}$$

The TS has been calculated with the same formula also for the sunflower seed molasses.

3.6.3 *Chemical oxygen demand (COD)*

The chemical oxygen demand is the amount of dissolved oxygen present in a solution that is used to oxidize chemical organic materials.

The COD analysis was carried out using samples of dry sludge and a blank test (with distilled water).

The method consists of an oxidation of organic substances in an acidic environment by potassium dichromate solution at high temperatures. For this analysis 0.02g of powder of dry sludge was transferred into vessel made with Teflon then was added potassium dichromate ($K_2Cr_2O_7$), concentrated sulfuric acid (H_2SO_4), silver sulfate (Ag_2SO_4) and mercury sulphate ($HgSO_4$).

For the digestion, the vessel were placed in a mineralizer, operating in two steps, the first at 175°C and the second at 50°C.

Figure 8: Mineralizer used for the analysis

After this, the potassium dichromate in excess is quantified by titration with ammonium iron sulfate solution (FAS) with ferroin as indicator.
Then the COD was quantified using the equation:

$$COD\ [\frac{mgO_2}{L}] = \frac{(B-C)*\left(V*\frac{N}{T}\right)*8}{W}$$

Where B is the volume of FAS used for blank titration, C is the volume of FAS used for sample titration, N represent the normality of dichromate, V is the volume of $K_2Cr_2O_7$ used, T is the volume of FAS used to quantify the titration, W is the weight of the dry sample and 8 is the weight of the oxygen.

3.6.4 *Soluble chemical oxygen demand (sCOD)*

The soluble chemical oxygen demand (sCOD) represents the soluble organics in the liquid phase available for SCFAs production. This analysis was done with the colorimetric method with a UV/VIS spectrophotometer.

Figure 9: Spectrophotometer used for the sCOD analysis

Some of the samples used for the SCFAs analysis were used, for this analysis, after a dilution (1:100), part of the sample has been transferred into a glass test tube adding an oxidizing solution ($K_2Cr_2O_7$) and a catalyst solution (H_2SO_4). Then the tubes have been placed in an oven settled at 150°C for 2 hours for the digestion before the spectrophotometric analysis. Each analysis was performed in triplicate and the average was reported.

The sCOD was determined using the value of the sCOD concentration obtained and the dilution factor of the sample (D.F.)

$$sCOD\ [\frac{mgO_2}{L}] = sCOD_{mis} * D.F.$$

3.6.5 *TKN*

To determinate the total nitrogen available in the sample, the colorimetric method includes its decomposition into ammoniacal nitrogen that is added to the one already presents in the solution.

So, the TKN is:

$$TKN = N_{org} + N\text{-}NH_3 + N\text{–}NH_4$$

The analysis was carried out with two test on dry sludge. After the shredding the obtained powder of sludge was transferred into two vassel made with Teflon. Then, it was added sulphuric acid (H_2SO_4) and potassium peroxidisulfate ($K_2S_2O_8$) for the acid digestion. The mineralizer works in two steps, after the first one, it was added hydrogen peroxide (H_2O_2) and after the second the solution was filtered with a black ribbon filter and subjected to ammonia steam distillation.

After this, the resulting solution was dilueted (1:10) and the Nessler's reactive was added for the spetrophotometric analysis.

Figure 10: Distiller used for the TKN analysis

The TKN concentration is:

$$\text{TKN}\ \left[\frac{gN}{\text{Kg TS}}\right] = \frac{\text{m}*\text{ABS}*\text{D.F.}*\text{V}}{W}$$

Where m is the slope of the calibration line, ABS is the absorbance obtained after the spectrophotometric analysis, D.F. is the dilution factor right before the Nessler's reactive, V is the volume of the flask used for the distillation, before diluting the sample and W is the weight of the dry sample.

4. Results and discussion

4.1 TS, COD, sCOD and TKN results

Depending on the treated material and the treatment processes, the characteristics of the sludge can be different in terms of solids content, COD, nitrogen and phosphorous content or the presence of hazardous compounds.

For the tannery sludge used in this study the TS was 830 g/Kg while the TVS analysed after the incineration was 550 g/Kg.

The COD and TKN were respectively 628 gO_2/KgTS and 30.7 gN/KgTS.

The sCOD was analysed from samples of the second batch test considering representative the data obtained from the first day, halfway through, and the last day. The results are summarized in the following table:

	sCOD gO_2/L		
	DAY 1	DAY 15	DAY 35
Blank test	8.3 ± 0.4	18.3 ± 0.7	14.9 ± 0.3
A	52.7 ± 0.7	61 ± 2	59
B	40 ± 3	34.9 ± 0.6	45.4 ± 0.2
C	43 ± 3	57.6 ± 0.6	60
D	15 ± 1	48.6 ± 0.4	54.9 ± 0.4
E	61.7 ± 0.8	85.4 ± 0.9	76.7 ± 0.1
F	14 ± 2	38.6 ± 0.5	42.7 ± 0.5
G	63 ± 3	64.7 ± 0.4	64.7 ± 0.7
H	13.8 ± 0.4	14.7 ± 0.1	60.8 ± 0.8
I	64 ± 7	56.4 ± 0.1	51.7 ± 0.9
L	43 ± 3	36.4 ± 0.1	45
M	86.7 ± 0.2	47.4 ± 0.4	67.6 ± 0.3
N	13.7 ± 0.5	10.2 ± 0.2	34.2 ± 0.2

Table 3: sCOD results

Usually, pretreatment methods are used to enhance biosolid hydrolysis and provide the dissolution of the sludge allowing the release of intracellular matter which becomes more accessible to anaerobic microorganisms (Tulun *et al.,* 2019), thus improving COD solubilization and subsequent yields of SCFAs production in the anaerobic fermentation process (Liu *et al*., 2016; Ma *et al*., 2018).

Some of these data reflects the results on alcohols and SCFAs analysed with the gas chromatograph, the higher results (e.g. 85.4 gO_2/L for test E) could be overestimated due the uncontrolled yeast production during the initial period of the test which could indeed lead to higher concentrations of ethanol and potentially other compounds, impacting the accuracy of gas chromatography and sCOD results. Diluting the sample before analysis can help bring the concentrations within the linear range of the detector and improve accuracy so a dilution 1:100 has been chosen for all the samples.
However, most of them are promising results since the data considered accurate (which reflects the sum of $gCOD_{Alc}/L$ and $gCOD_{SCFA}/L$) shows higher results than 34.1 g O2/L obtained by Bonato E. in a recent study on tannery sludge (Bonato E., 2022).
The result of the TS analysis made with the sunflower seed molasses was 803 g/Kg while the VS was hypothesized for each dose utilized for the tests considering a 95% of inert materials within:

Sunflower seed molasses (g)	Volatile Solids (g/L)
4	20.3
6	30.4
8	40.5
11	55.7
12	60.8

Table 4: VS (g/L) hypothesized for dose of sunflower seed molasses.

4.2 Fermentation and alcohol production (First batch test)

The molasses is an agro-industrial by-product that approximately contains 50% of sugar and about 50% of organic and inorganic compounds, including water (Lin & Tanaka, 2006). It is also known that molasses, if not pre-treated, is contaminated with wild microorganisms, which interfere with the fermentation process by consuming sugar and reducing ethanol production (Pandey *et al*., 2022). The high total solids (TS) content of

sunflower seed molasses suggests that its concentration of sugars is higher than that of sugarcane or beet molasses. Therefore, the subsequent study focuses on its contamination levels and evaluates the performance of yeast in utilizing this raw material for ethanol production during fermentation. The batch tests were prepared using the sunflower seed molasses in different concentrations with the two strains of *Saccharomyces cerevisiae* and were kept in anaerobic conditions for 26 days without pH control.

Figure 11 shows the trend on alcohols production for each test in relation to the ethanol production in order to see the yeast's efficiency to ferment sugars into ethanol.

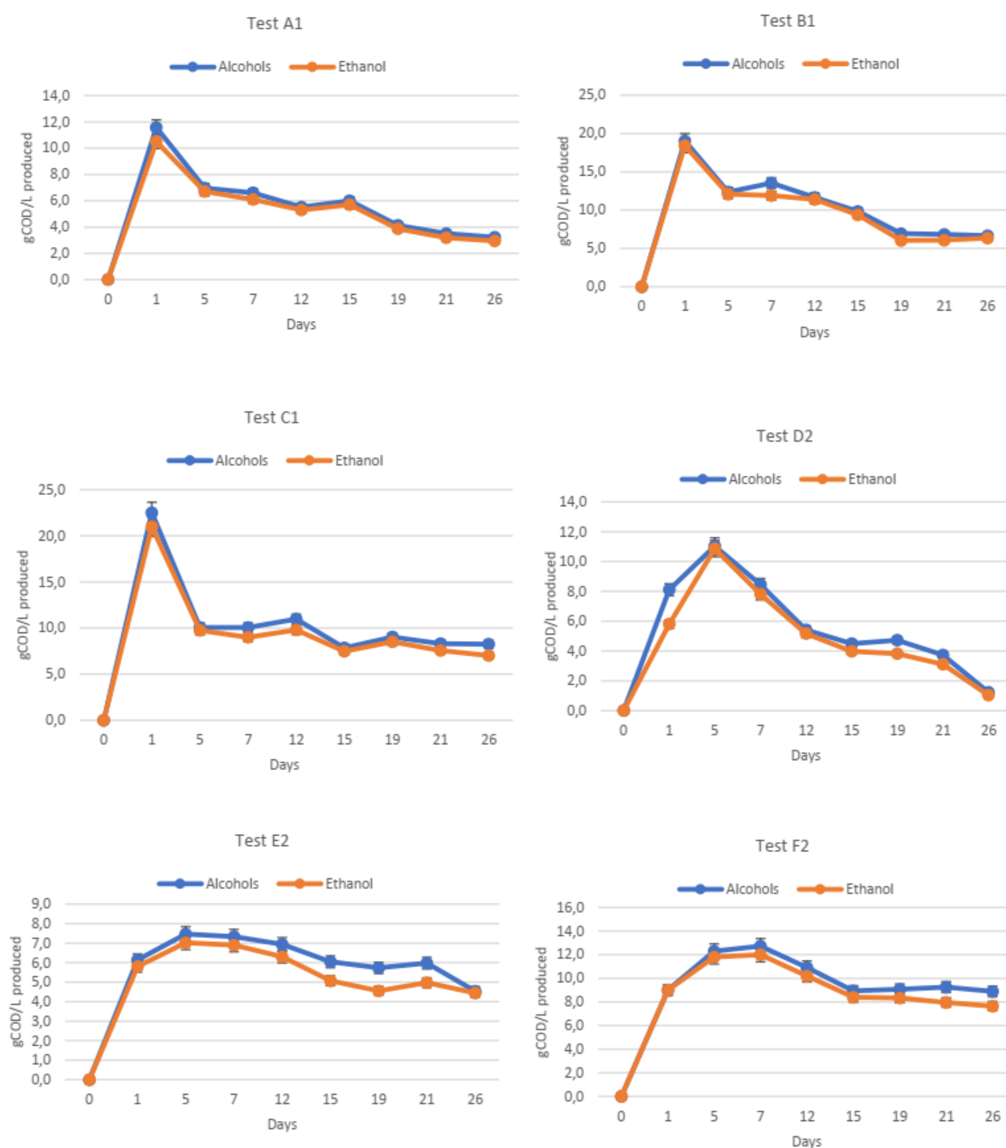


Figure 11 Alcohols and the relative portion of ethanol produced for each batch test. (Srain1: A1=4g; B1=8g; C1=12g; Strain2: D2=4g; E2=8g; F2=12g)

According to the graphs (Figure 11), the impact of interfering microorganisms on methanol production is minimal, and the predominant contribution to total alcohol production comes from the ethanol produced by yeast.

As observed in the graphs where peaks in alcohol production are evident, there is a consistent portion of ethanol produced. Regarding the tests with strain 1 (tests A1, B1, C1), the maximum concentration of alcohol was achieved, measuring at 11.6, 19.0, and 22.5 gCOD/L, corresponding to 10.5, 18.4, and 21.0 gCOD/L of ethanol, respectively. Similarly, for strain 2 (tests D2, E2, F2), the higher concentrations of alcohol at 11.0, 7.5, and 12.7 gCOD/L correspond to 10.9, 7.0, and 12.0 gCOD/L of ethanol.

This suggests that, for practical purposes, the value of methanol can be disregarded due to its insignificant contribution in comparison to ethanol.

Moreover, comparing the ethanol production between the two strains it can be observed that, under the same conditions, strain1 reached values of ethanol higher than strain2 when the concentration of the substrate was at 8g and 12g. This indicates that strain1 performs better in ethanol production under the established conditions, a trend that can also be observed by examining the fermentation yield (Figure 12) calculated in $gCOD_{Alc}/gVS_0$.

With strains 1, the three concentrations of molasses resulted in different peaks of alcohol yield, although the trend is nearly parallel. In the figure, it is evident that after day 0, each of the three batches reached a peak corresponding to 0.57, 0.47, and 0.37 $gCOD_{Alc}/gVS_0$, equivalent to 4g, 8g, and 12g of molasses. After one day, alcohol production occurred with amounts of 11.6, 19.0, and 22.5 gCOD/L, respectively, this was followed by a halving of the alcohol production yield curve and a subsequent slow and constant decline. On the last day of sampling, the alcohol produced was 3.2, 6.7 and 8.3 gCOD/L respectively for the test A1 with 4 g of molasses, B1 with 8g and C1 with 12g.

The behaviour of strain2 with the three concentrations of molasses resulted to be different. As shown in figure 12, with less molasses (4g), the yield peaked after two days with 0.54 $gCOD_{Alc}/gVS_0$, which corresponds to 11.0 gCOD/L of alcohol, followed by a decline until 1.2 gCOD/L produced on the last day. This appeared to be the most favourable option for strain2, as with the other concentrations of molasses, no peak was reached, and the fermentation yield hardly exceeded 0.2 $gCOD_{Alc}/gVS_0$.

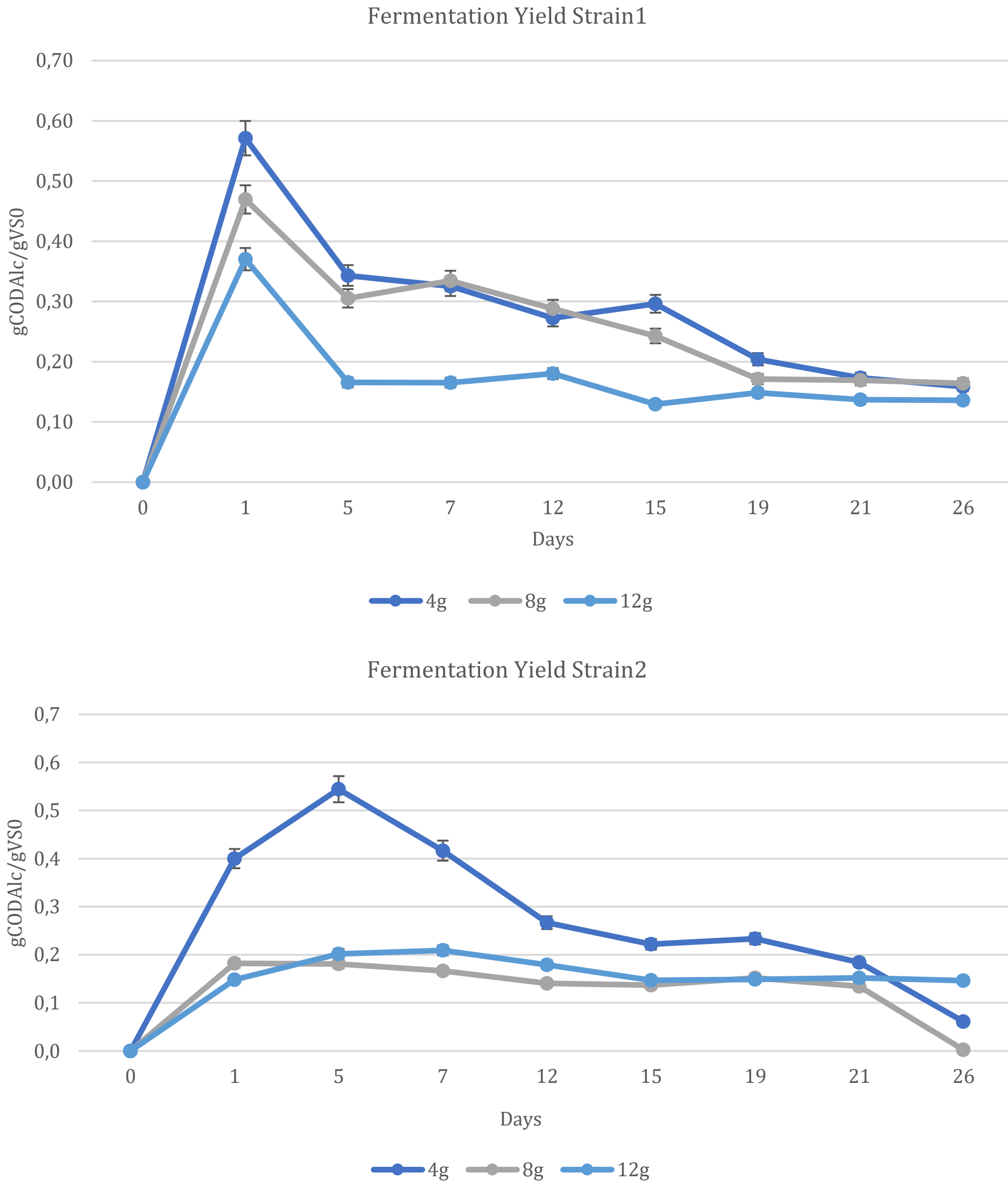


Figure 12: Alcoholic fermentation yields in strain1 and strain2 using different concentration of molasses.

It was hypothesized that high concentration of molasses may hinder the activity of the yeast due the high content of sugar, and it can be also related to the presence of inhibitory factors that probably exist in molasses and may reduce the Saccharomyces' performance (Takeshige & Ouchi, 1995). This is in line with the study by Tang and colleagues where the tests with less diluted molasses have shown a growth inhibition of bacteria but also of yeast (Tang *et al.*, 2010).

This pattern can be explained in general by the presence of inhibitors, such as the fermentative bacteria inside the molasses that, over time, consume the ethanol produced by *Saccharomyces cerevisiae* for their growth and acids production. For this reason, the tests also included the data on SCFAs produced. The following table collect the maximum yield on alcohol production and SCFAs production for the two strains with the three concentrations of molasses.

Molasses (g)	Molasses (gVS/L)	Strain1		Strain2	
		Y^{max}_{Alc}	Y^{max}_{SCFA}	Y^{max}_{Alc}	Y^{max}_{SCFA}
4	20,3	0.57 ± 0.04	0.31 ± 0.04	0.54 ± 0.04	0.28 ± 0.03
8	40,5	0.47 ± 0.03	0.20 ± 0.01	0.18 ± 0.01	0.18 ± 0.02
12	60,8	0.37 ± 0.03	0.13 ± 0.01	0.21 ± 0.02	0.14 ± 0.02

Table 5 : Maximum yields results on SCFAs and Alcohols productions.

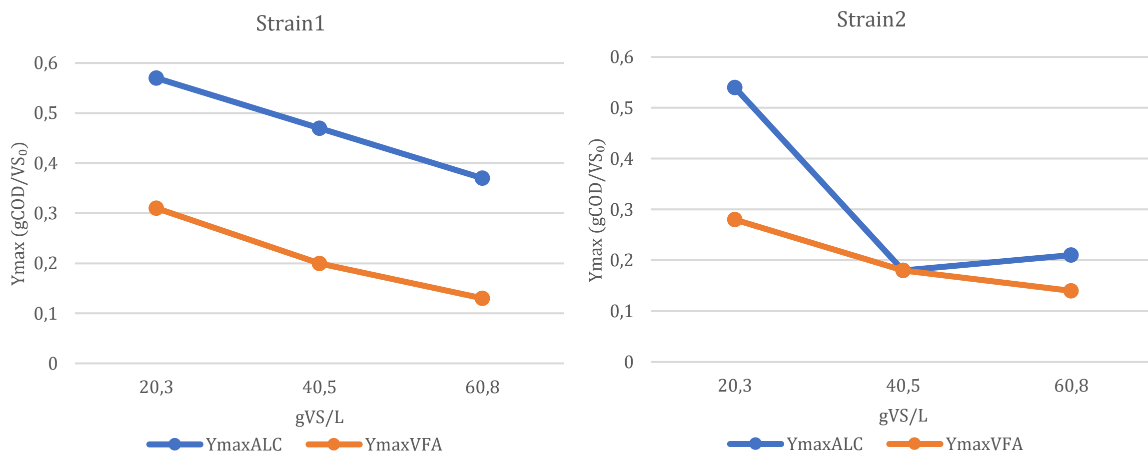


Figure 13: Maximum yields on SCFAs and Alcohols production in strain1 and strain2.

How it can be seen in the graphs (Figure 13), for both strains of yeast, the maximum yield on alcohol production is higher than the yield on SCFAs for almost all the concentrations of

molasses used, which means that there is a percentage of contamination in the substrate but not enough to inhibit completely the alcohol production.

With strain1 the contamination is linear, so when increasing the molasses concentration, the yields of alcohol and SCFAs production decline, and so does the yeast and bacteria activity. On the other hand, with strain 2, it was observed that using less molasses resulted in similar yields between the two strains, with alcohol yields of 0.57 and 0.54 $gCOD_{Alc}/gVS_0$ and SCFAs yields of 0.31 and 0.28 $gCOD_{SCFA}/gVS_0$, respectively. However, increasing the amount of this substrate could lead to contamination issues. For instance, with 40.5 and 60.8 gVS/L of molasses, the maximum yields reached were 0.2 and 0.13 $gCOD_{Alc}/gVS_0$ for alcohols and 0.18 and 0.14 $gCOD_{SCFA}/gVS_0$ for SCFAs. This indicates that, particularly with strain 2, lower dosages are more advisable to minimize contamination levels and yeast inhibition. The main acid detected in all tests were the acetic acid with 5-5.5 gCOD/L, butyric with 2-3 gCOD/L and traces of propionic acid (<0.7 gCOD/L).

The following figures depict the distribution of alcohol production percentages (expressed as the sum of ethanol and methanol) and SCFAs production over time, with 100% representing the total of alcohols and SCFAs present in the samples.

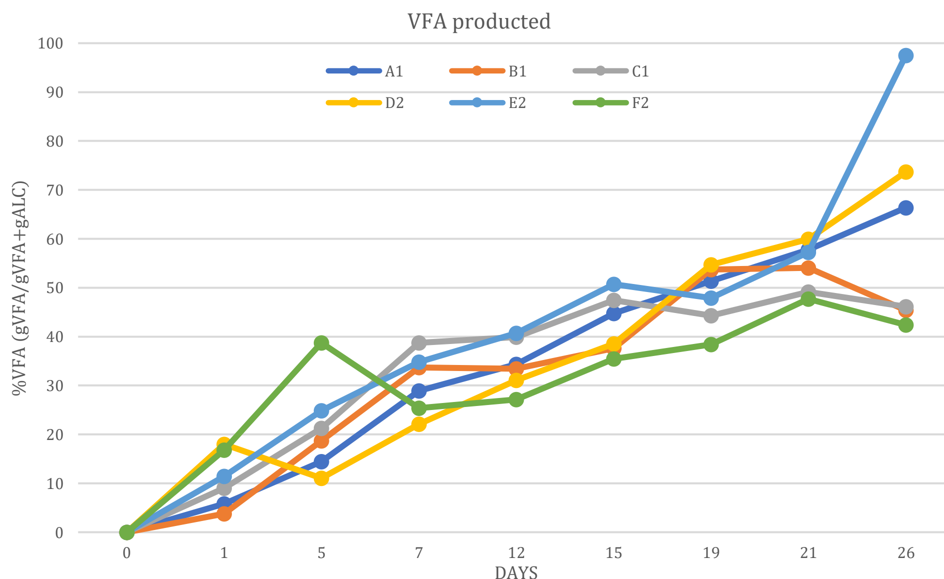


Figure 14: Percentages on VFAs production for strain1 and strain2 with different molasses concentrations (Srain1: A1=4g; B1=8g; C1=12g; Strain2: D2=4g; E2=8g; F2=12g).

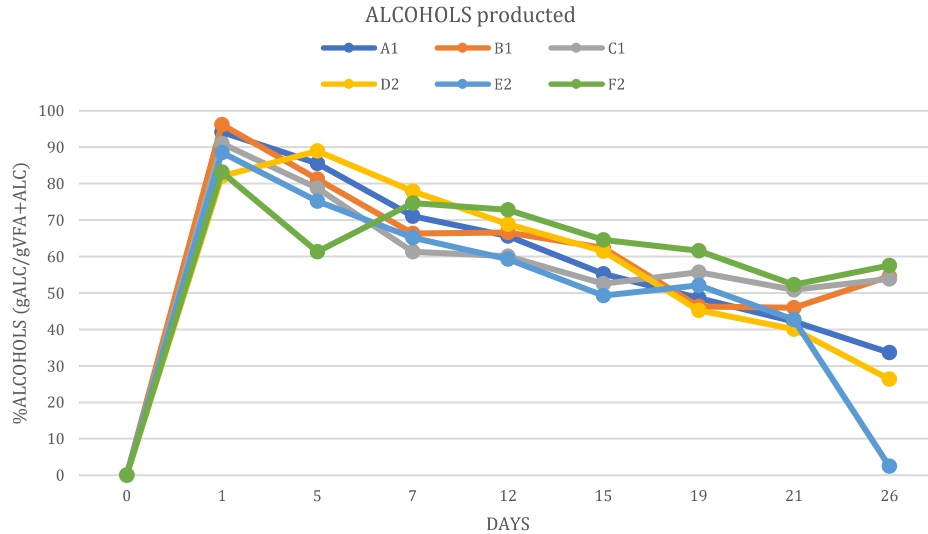


Figure 15 : Percentages on Alcohols production for strain1 and strain2 with different molasses concentrations (Srain1: A1=4g; B1=8g; C1=12g; Strain2: D2=4g; E2=8g; F2=12g).

These graphs illustrate that regardless of the concentration of molasses used in the tests, in the first day the production of alcohols is leading (ranging from 82% to 96%). However, as fermentation progresses, the activity of fermentative bacteria becomes dominant, leading to an increase in the percentage of SCFAs over time. In the end of this trial, the percentage of alcohol produced are over the 50% for the higher concentration of molasses in both strains, while the sample with strain2 and 8g of molasses collapsed with the 2% of alcohols produced.

Considering the lower molasses concentration, the percentage of alcohol produced in both strains remains relatively consistent, ranging between 26% and 34%. This suggests that, in terms of balancing the amount of molasses used and the risk of contamination, the optimal compromise for maximizing yeast's alcohol production lies with the lower quantity of this substrate.

Research on bioethanol production reports that the optimal conditions for molasses fermentation (from sugar refinery) are characterized by a pH of 4.5 and a temperature of 30° (Zentou *et al*., 2017). In this study the pH was uncontrolled and ranged from 6 to 5 in the end, when SCFAs were produced. In addiction the temperature was lower than the one suggested, but the aim of these tests was only to provide information on the molasses' contamination and what might happen if it is used in a co-fermentation.

4.3 Tannery sludge fermentation and chain elongation (Second batch test)

4.3.1 Blank test and co-fermentation tests

The acidogenesis' performance was evaluated through the concentration of SCFAs produced ($gCOD_{SCFA}/L$). The tests were kept in anaerobic and mesophilic conditions for 35 days.

The fermentation was conducted without pH control due the buffering capacity of the tannery sludge already investigated by Tuci and colleagues (Tuci *et al.,* 2022). Thus, the pH remained between 6.0 and 7.7 with a slight lowering towards the end of the tests, parallel to the acids production.

Most of these tests show the best rate of SCFAs production after the second week of trial, beyond which there was a general decline in the production, especially of the acids with longer chain.

In the blank test, made with water and tannery sludge, the absence of yeast explains the lack of ethanol. However, the SCFAs production in this test reached 11,9 $gCOD_{SCFA}/L$ after 3 days, which is a higher result compared to other studies with waste activated sludge (WAS) or sewage sludge that reported a production of 7.0 $gCOD_{SCFA}/L$ and 10.0 $gCOD_{SCFA}/L$ respectively (Zhou *et al*., 2013; Niero *et al*., 2021). Moreover, the peak was reached after 15 days with 19.27 $gCOD_{SCFA}/L$ produced, which is a promising result compared to a similar batch study on tannery sludge where the production of SCFAs was around 9.5-10.25 $gCOD_{SCFA}/L$ (Tuci *et al.,* 2022).

To enhance the digestion efficiency of the sludge it can be combined with carbon-rich substrates (Rughoonundun *et al*., 2012). Tests I, L, M, N were performed to assess the impact of a co-substrate addition to the tannery sludge. These tests, confirmed that the incorporation of sugar or sunflower seed molasses can improve the fermentation activity, as shown in figure 16.

Comparing the two substrates the best performance was reached by using sugar in the lower quantity (test N) where after 15 days were produced 38.5 $gCOD_{SCFA}/L$, almost twice as much as the test with only tannery sludge. On the other hand, test I and L with 11g and 6g of sunflower seed molasses produced lower amount of SCFAs, respectively 20.6 $gCOD_{SCFA}/L$ and 16.9 $gCOD_{SCFA}/L$ but it can be seen as a great result compared to a similar

study with activated sludge and corn stover (another agricultural residue), performed in mesophilic condition, which reached only 11.9 $gCOD_{SCFA}/L$ (A. Zhou *et al.*, 2013). In addition, these results agree with another study where sewage sludge was co-digested with sugar beet molasses, confirming that higher quantities of molasses can increase the concentration of SCFAs (Kalemba & Barbusiński, 2017).

The fermentation yield for each test was calculated considering the $mgCOD_{SCFA}$ presents in the sample and gVS_0 of the substrate. In correspondence with the peak of SCFAs produced in the blank test (19.27 $gCOD_{SCFA}/L$), the yield was 0.35 $gCOD_{SCFA}/gVS_0$ which is close to the result obtained by Morgan-Sagastume and colleagues (0.33 $gCOD_{SCFA}/gVS_0$) in a study with WAS in mesophilic conditions (Morgan-Sagastume *et al.*, 2015).

The difference in the yields between the tests with the co-substrates is not obvious, as both conditions are characterized by good results, better than the blank test, reaching yields of 0.40-0.45 $gCOD_{SCFA}/gVS_0$ between the tenth and the twenty-second day of trial. The highest value was achieved by test N using 3.7g of sugar as co-substrate where the yield reached 0.5 $gCOD_{SCFA}/gVS_0$ after 15 days.

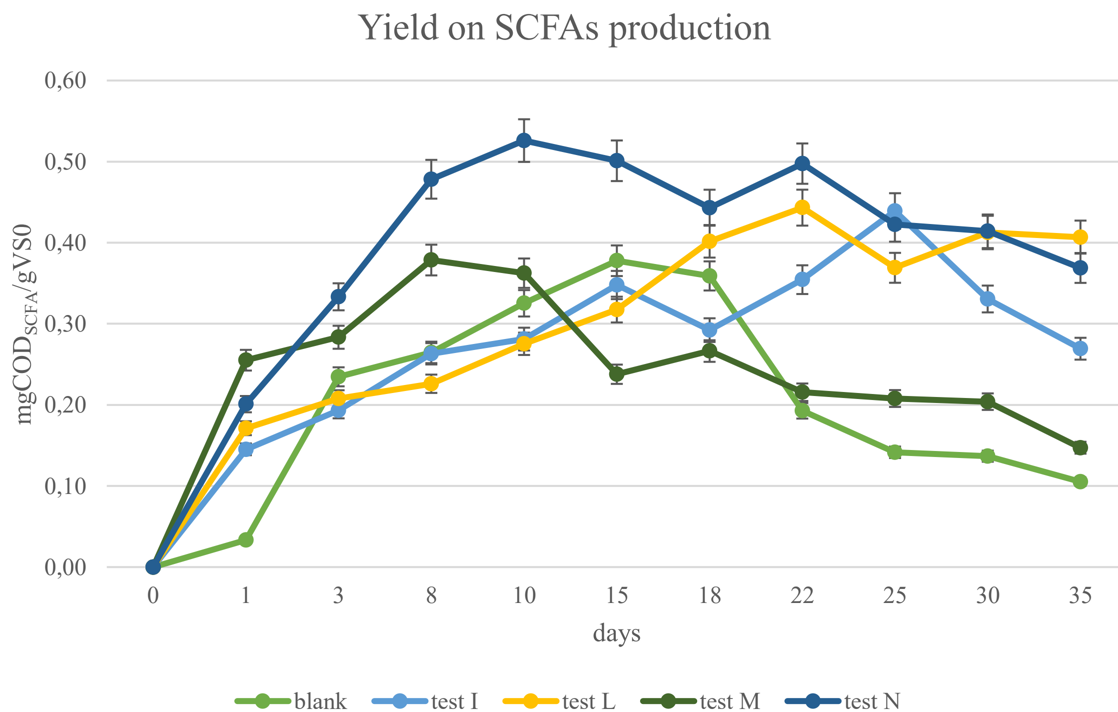


Figure 16 : Yield on SCFAs production with the Blank test, I, L, M, N

Just as the overall production varies, the composition of SCFAs differs between these tests as well. As expected, given absence of the yeast in all the tests, the alcohol production resulted minimal and so did the production of acids with longer chain. The blank test (Figure 17) leads to a high production of acetic acid (always dominant over the other acids produced). After two weeks the acetic acid was at 63% and in the end was at 53%, this is in line with the study published by Tuci and colleagues using tannery sludge in mesophilic conditions, in which the percentage of acetic acid was 37%- 41% representing the majority of the produced acids (Tuci *et al*., 2022).

All the tests with the supplement of a co-substrate show a predominance of the butyric acid over the acetic followed by the propionic acid and isobutyric (Figure 17). This can be discordant with other researchers of co-fermentation of sludge and agricultural residues (A. Zhou *et al.,* 2013; Kim *et al*.,2012; Rughoonundun *et al.*, 2012) which see a dominance of acetic acid followed by the propionic. Although, as reported by Alibardi and colleagues in a study, it can be confirmed that the fermentation products depend on the origin and composition of the organic waste, showing a correlation between the concentration of butyric acid and the carbohydrate, and protein contents (Alibardi *et al*., 2016a). Greater attention was given to test I, where small amounts of caproic acid (2% with respect to the total of SCFAs produced) were detected, although no significant concentrations of ethanol were found due to the absence of yeast. However, it could indicate that a chain elongation process had been initiated, even if the conditions were likely unfavourable for further progression.

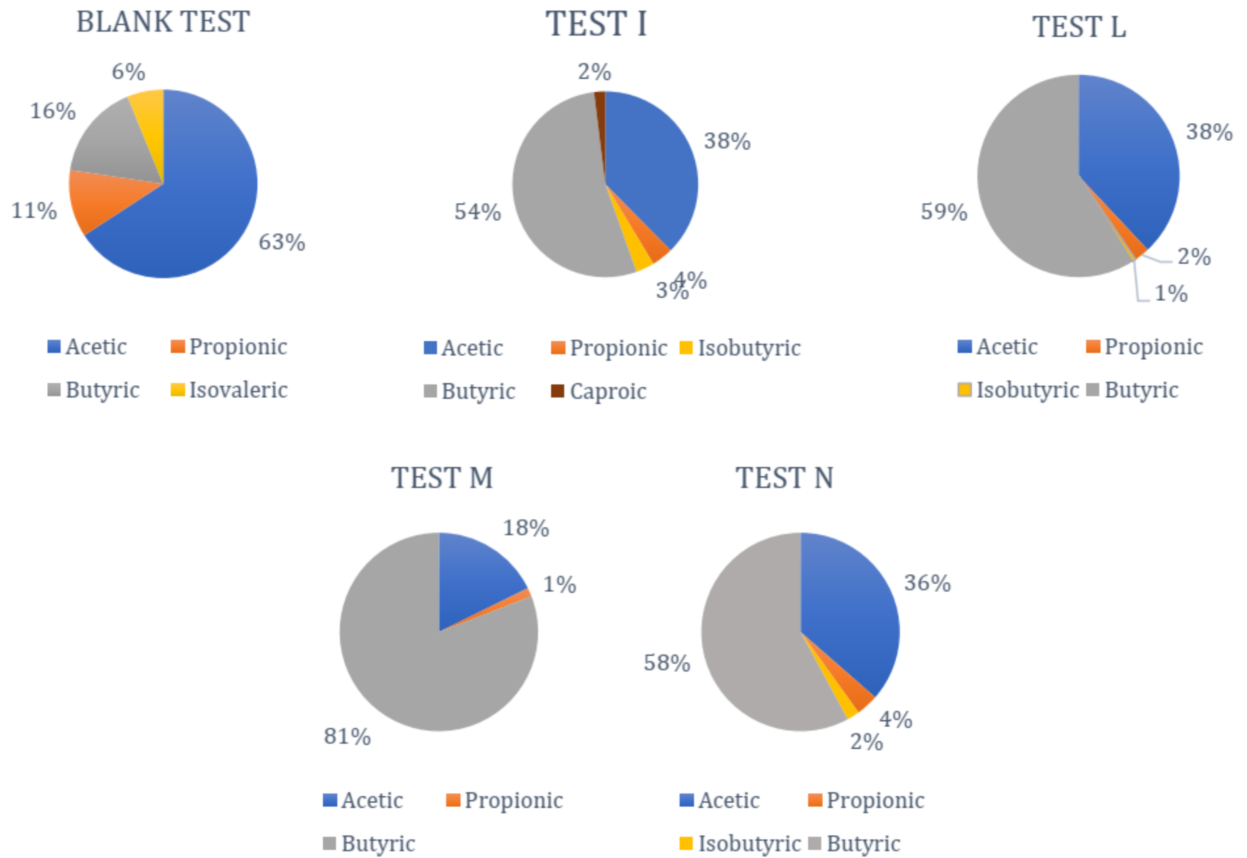


Figure 17: SCFAs production in the Blank test, test I (with sunflower seed molasses), test L (with sunflower seed molasses), test M (with sugar) and test N (with sugar).

4.3.2 Tests with strain1

Tests A, B, C, D were made using strain1 of *Saccharomyces cerevisiae* with the co-substrates.

Different quantity of co-substrate influenced as expected the ethanol production and consequently the chain elongation process.

The tests A and B, carried out with white sugar, show two different trends of acids production. The test with 7.5g of sugar (A), demonstrates a light increase in SCFAs produced until a peak is reached, corresponding to 23.5 $gCOD_{SCFA}/L$, on the eighteenth day, followed by a clear decrease until less than 10 $gCOD_{SCFA}/L$ of acids produced. Instead, in test B with 3.7g of sugar, SCFAs production is more sustained from the beginning, reaching a peak of 34.5 $gCOD_{SCFA}/L$ after 15 days, and then slowly decreasing but still maintaining the production over 20 $gCOD_{SCFA}/L$.

In parallel with the growth of the acids, the concentration of ethanol in test A undergoes variations, reaching its maximum with 38.7 gCOD/L, but it remains twice as much as the SCFAs produced, except on day 18 where the acetic acid reached its peak, while the concentration of ethanol was 20.9 gCOD/L.

In contrast, in test B it is shown that from day 8, where the test reached the highest value of ethanol (20,7 gCOD/L), there is a subsequent decrease in its concentration in conjunction with an increase in SCFAs production, suggesting that the ethanol was employed for SCFAs production and chain elongation.

These tests suggested that that higher quantity of sugar (A) causes an excess of ethanol production that inhibits the fermentative bacteria and the chain elongation process (Figure 18).

On the other hand, test B highlights that lower sugar quantity can enhance the fermentative bacteria activity, while also allowing the ethanol production by the yeast but in lower concentration than in test A, thus reducing its inhibitory action. Moreover, the produced ethanol can be converted into acids in the chain elongation process, which is in line with the study reported by Lonkar and colleagues for their research on the optimum alcohol concentration for chain elongation (Lonkar *et al.*, 2016).

So, it can be confirmed that higher sugar content did not allow for a parallel increase in SCFAs production, thus the fermentation yield in test A is lower than in test B, with the highest values being 0.23 $gCOD_{SCFA}/gVS_0$ after 18 days for the former and 0.45 $gCOD_{SCFA}/gVS_0$ after 15 days for the latter.

In Figure 18 is represented the trend of the SCFAs and alcohol production in these tests. It is clear that the excess of ethanol in test A, has prevented a high production of SCFAs, but also the elongation of the acids, in fact, the acetic acid prevails in all the sample taken, followed by the butyric, propionic and isovaleric with a percentage of 37-73%, 19-22%, 3-13% and 3-5% respectively. Only after a month the conditions appeared to be suitable for the caproic acid production, but with a lower amount respect to the acetic.

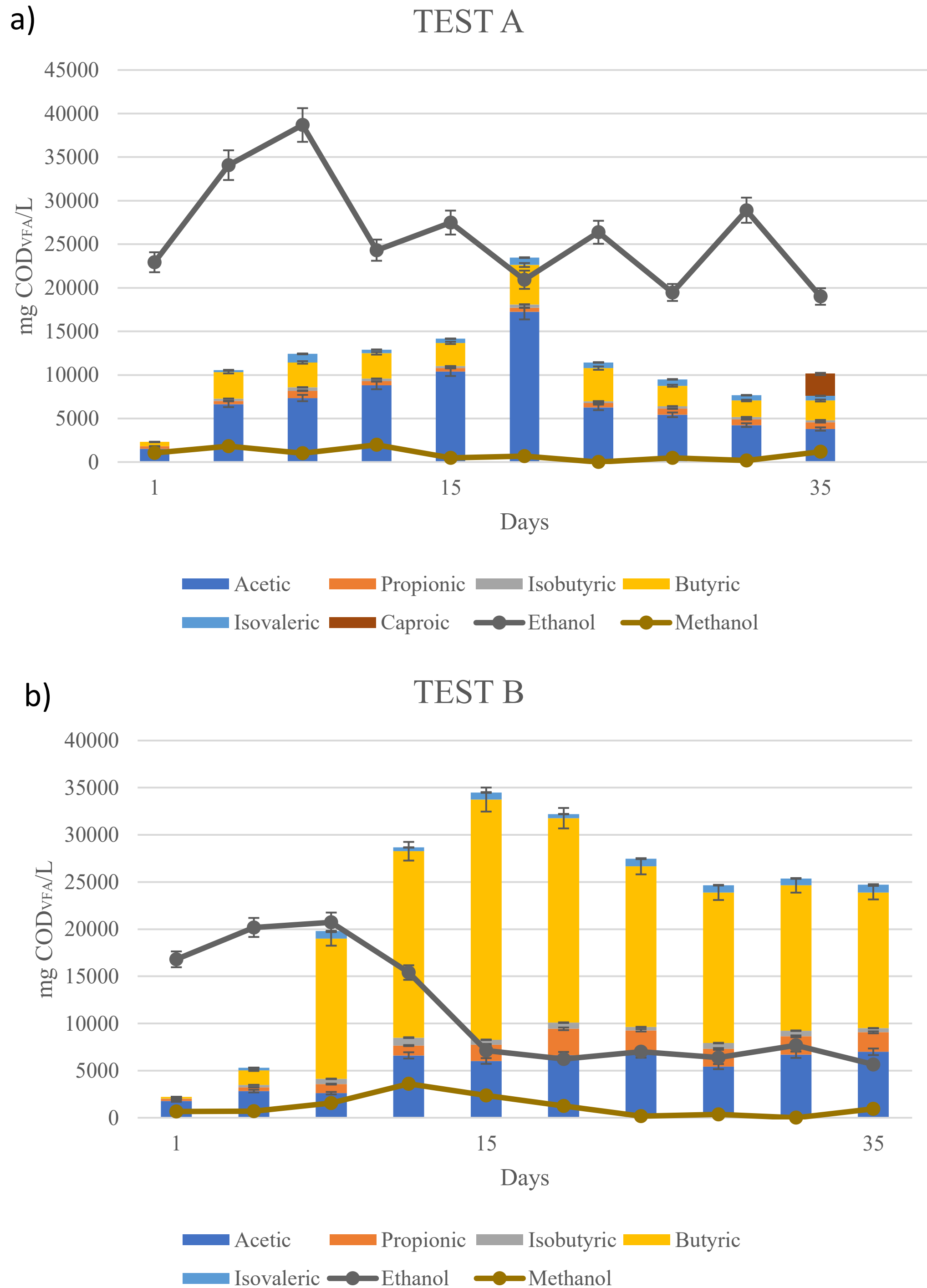


Figure 18: SCFAs and alcohols production in tests A (a) and B (b).

In test B the chain elongation process here leads mainly to butyric acid, which reaches a peak after 15 days where it touched 25,4 gCOD/L which means the 74% of the acid production in that sample, followed by acetic acid (15%), propionic acid (5%), isobutyric and isovaleric (2%), a trend that is kept constant until the end of the trial.

Even though the process of chain elongation was probably started, it didn't lead to the production of acids with longer chains.

Better results were obtained with the use of sunflower seed molasses as co-substrate as tests C and D show in general a more sustained SCFAs production. The best performance is reached with test C, the one with the higher quantity of sunflower seed molasses. The results from both tests (Figure 19) demonstrate a pattern of ethanol production followed by its subsequent consumption, accompanied by the production of SCFAs.

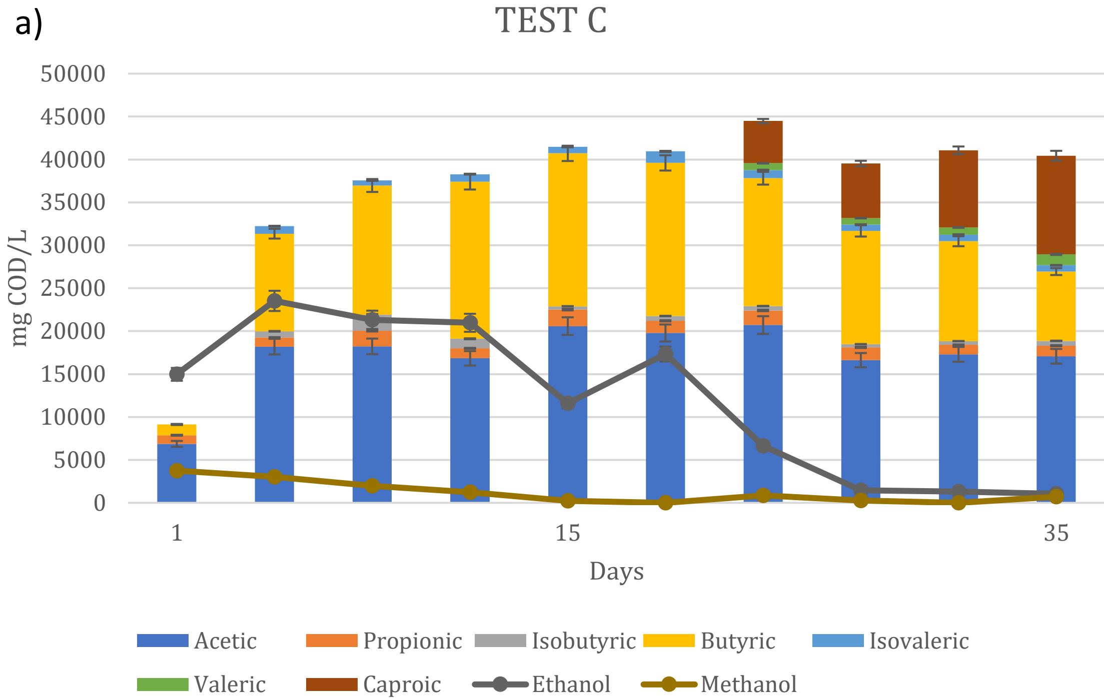
However, in test C the gradual consumption of the ethanol leads to caproic acid production from the third week of test, highlighting the effectiveness of the chain elongation process. The ethanol in this test reaches 20 gCOD/L, which is lower than test A and it is not enough to inhibit the fermentation and chain elongation.

In this test the acids production is over 35.0 $gCOD_{SCFA}$/L for most of the samples, the SCFAs production rapidly increases after 3 days and then slowly reaches 44.5 $gCOD_{SCFA}$/L on day 22, when caproic acid appears as well. In parallel to the appearance of the caproic acid the amount of ethanol decreases until 1.0 gCOD/L on the last day of sampling.

Therefore, these tests demonstrate that a low quantity of molasses (6g in test D) is not enough to reach a supported production of ethanol and thus allow chain elongation, this also impacted the SCFAs production, which reach the maximum at 31.6 $gCOD_{SCFA}$/L.

In test D the trend of SCFAs production is similar to that of test B, with approximately 25 $gCOD_{SCFA}$/L, and also the composition of the acids results comparable. In test D the rapid ethanol consumption is followed by a high production of butyric acid which represents the 65% - 70% of the produced acids in all the samples taken. However, the chain elongation was not sustained, as the caproic acid is always low in quantities, in fact, it does not exceed the 5% of the total acids produced, which is not enough to hypothetically be separated from the fermentation broth.

The composition of the acids produced in tests C is different. As shown in figure 19, the consumption of ethanol and some of the butyric and acetic acid from day 18 leads to the appearance of the caproic acid. Indeed, the concentration of acetic acid ranged from 75% in the first day to 42% in the last while the ethanol values decreased from 23.5 gCOD/L to 1.0 gCOD/L. The highest value of the caproic acid is reached in the last day where it represents the 28 % of the acids produced with 11.5 gCOD/L.



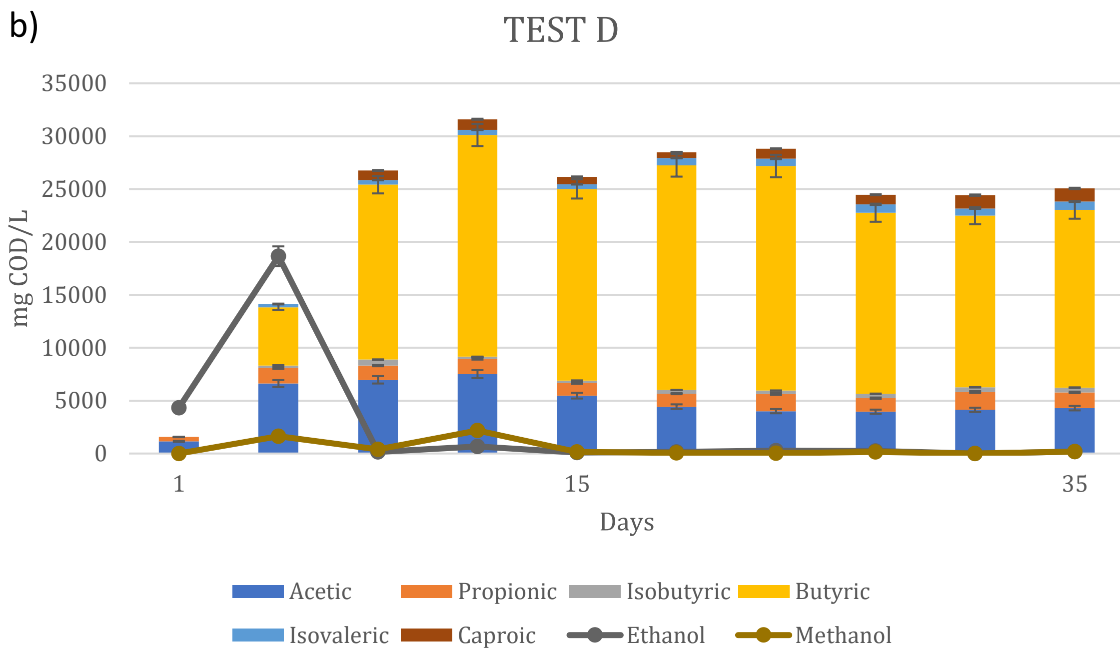


Figure 19: SCFAs and alcohol production in tests C (a) and D (b)

4.3.3 Tests with strain2

As for the tests with strain1, these tests were supported by the addition of the same quantities of sugar and sunflower seed molasses.

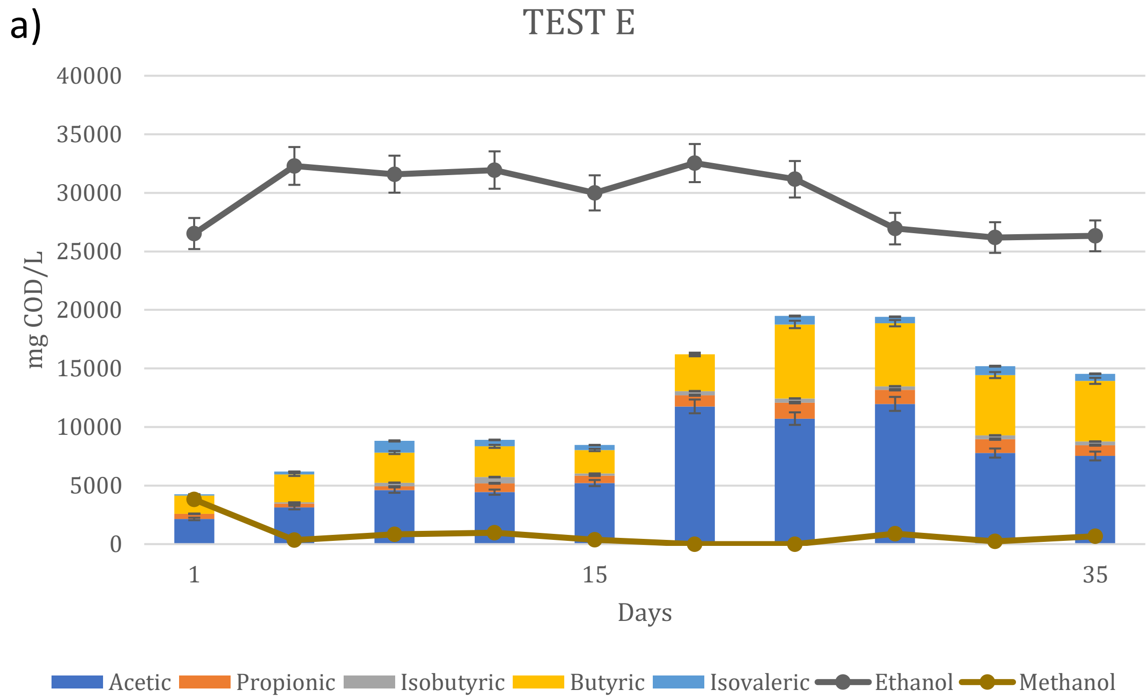
Test E (with 7.5g of sugar) confirms what previously reported, that a high quantity of sugar leads to an excessive ethanol production compromising the chain elongation process, in

fact, in this test, the production of ethanol reached 32.3 gCOD/L on day 3, five times the acids produced in that sample.

From the third week a decrease in ethanol is noted, related to its consumption to obtain butyric acid and other SCFAs, consequently there is a general increase of the SCFAs produced but still lower compared to the amount of ethanol produced during this run. In this test the highest value of SCFAs produced is 19.5 $gCOD_{SCFA}/L$ on day 22, close to the one obtained in test A with the same conditions, and much lower than test F, carried out with less sugar. In the test F, with 3.7g of sugar, less ethanol production enables the chain elongation with the production of the caproic acid. Figure 20 shows the rapid ethanol consumption in the first week of the test, which lowers from 15.0 gCOD/L to 1.2 gCOD/L at the end of the test, in parallel with the appearance of a high amount of caproic acid (21.0 gCOD/L after 8 days). The amount of acids produced in this test reaches the maximum with 36.7 $gCOD_{SCFA}/L$ after the first week. Afterwards, a decrease of SCFAs production marks also a reduction of the caproic acids which still remains over 8.3 gCOD/L, more than a third of the total acids produced. That suggests that this strain of *Saccharomyces cerevisiae* works better than strain1in the same conditions, because in test B, as discussed before, the ethanol consumption only leaded to a high butyric acid production.

The fermentation yield here reflects this test's performance reaching 0.48 $gCOD_{SCFA}/gVS_o$ in test F which is approximately twice that of test E that yielded 19.5 $gCOD_{SCFA}/gVS_o$.

The composition of SCFAs in test E can be compared to the one presented in test A (with strain1), showing a dominance of acetic acid and butyric acid with a percentage of 51%-62% and 24%-36% respectively, followed by the presence of propionic acid (6%-10%) and isovaleric acid (3%-5%).



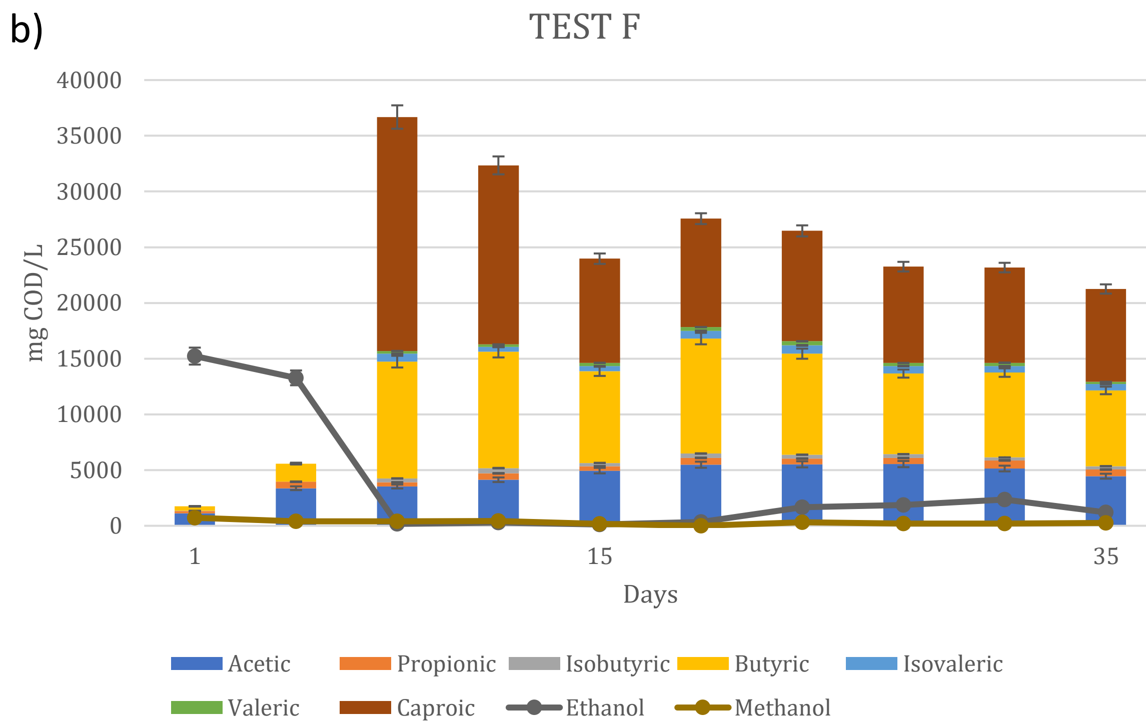


Figure 20: SCFAs and alcohols production in tests E (a) and F (b)

More interesting is the composition of SCFAs of test F where the main acid produced is the caproic acid, representing around 40% of the total acids for most of the samples, followed

by acetic acid (21%), butyric acid (32-34%) and lower amount of propionic, isovaleric and valeric acid (<3%).

Finally, the batch tests with sunflower seed molasses as co-substrate (G and H) prove, like tests C and D, that 6g are not enough to carry out the chain elongation process. Whereas the best results were obtained in test G using 11g of molasses, where the decrease of ethanol led to a higher production of caproic acid with respect to test C under the same conditions.

Figure 21 shows the ethanol consumption in test G correlated to the increase in SCFAs production, and particularly the caproic acid. From 32.2 gCOD/L the concentration of ethanol declines until 3.6 gCOD/L while the amount of SCFAs produced reaches the peak with 43.02 $gCOD_{SCFA}/L$ after twenty days. In parallel with the growth of the quantity of SCFAs, the value of caproic acid has also increased. In this test the caproic acid reaches a concentration between 17.2 gCOD/L and 15.4 gCOD/L from the tenth to the eighteenth day. This trend is similar to the one already reported in test C, but the presence of the caproic acid in this test is more dominant., which are high concentrations compared to the other test. After that, there is a slight decrease, until a concentration of 7.3 gCOD/L, a value similar to the one reached in the last sample in test F.

Test H shows a similar amount of SCFAs produced, where the fluctuating consumption of ethanol ranges from 21.1 gCOD/L to 2.8 gCOD/L, leading to an increase in SCFAs concentration with a peak of 39.4 $gCOD_{SCFA}/L$ on day 18.

The maximum fermentation yields are similar between these two tests, the higher was reached by test H with 0.46 $gCOD_{SCFA}/gVS_0$ despite the one achieved by test G wich was 39.4 $gCOD_{SCFA}/gVS_0$ on day 18.

Comparing test H to test D, here the production of acids is higher, but it leads to a majority of acetic acid, while in test D with a greater ethanol consumption butyric acid prevails. Therefore, in test H probably the ethanol produced was used by the fermentative bacteria for their growth more than in the chain elongation process.

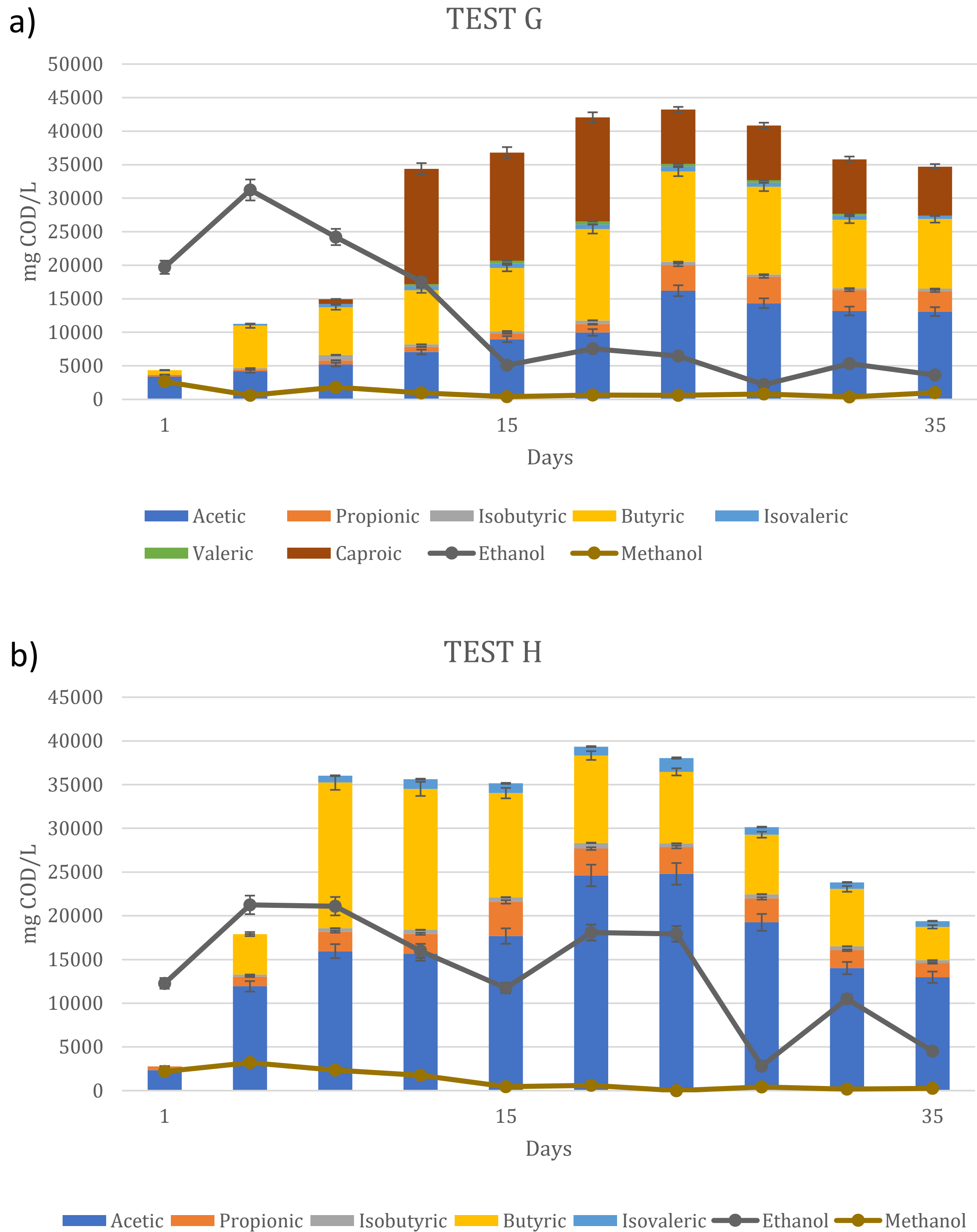


Figure 21: SCFAs and alcohol production in tests G (a) and H (b)

The composition of SCFAs in the test G is more similar to test F than test C operated in the same condition, probably due the characteristics of this strain of *Saccharomyces cerevisiae*. The amount of acetic acid increased over time, but its percentage of the total decreased from 78% to 38% on the last day. Instead, in correspondence to the increase in butyric acid

its percentage increased from 15% to 30%. The third most abundant acid is the caproic acid that ranged from 45% after 15 days to 21% of the total acids produced on day 35. Followed by propionic acid (7%-9%) and traces of valeric, isovaleric and isobutyric (<2%).

In test H the distribution of SCFAs it's dominated by the acetic acid which ranged from 84% to 50%, followed by butyric (20%-38%) and propionic (11%-8%), which is a similar composition to the blank test but the amount of acids produced in test H is higher, which confirms the increased efficiency with the addition of a co-substrate.

These results confirm that, as already proved in Lonkar's article, very low ethanol concentrations reduce chain elongation rates, although high concentrations inhibit the acid producing microorganisms (Lonkar *et al*., 2016). It must exist an optimal ratio between the gCOD/L of alcohols and the gCOD/L of SCFAs to obtain chain elongation which is however not reported in the literature. From these tests it can be assumed that with the conditions settled for these tests, a concentration of ethanol exceeding 30 gCOD/L could inhibit the fermentation bacteria and the chain elongation process for both strains of *Saccharomyces cerevisiae,* while better results are reached with 15-20 gCOD/L.

Moreover, these tests show that the use of sunflower seed molasses as the co-substrate is efficient with both strains, as it allowed for an enhancement of the acid production without inhibiting the fermentation process. With the appropriate amount of molasses (here assumed to be 11g), in the test boosted by *Saccharomyces cerevisiae*, the chain elongation process leads to caproic acid production with high enough percentages to allow its separation from the fermentation mix.

The appearance of the caproic acid and the amount of SCFAs produced can validate that a suitable microbial culture can enhance the stability and efficiency of the anaerobic digestion of this substrate even better than operating with thermophilic condition or with a pre-treated sludge (Tuci *et al.,* 2022).

Most studies on bio-augmentation primarily focus on methane production, with less attention given to SCFAs, consequently, there is limited literature available for comparison with these results. However, the study conducted by Reddy and colleagues confirms this study's findings, namely that bio-augmentation allows for the highest SCFAs production than any other experimental conditions (Reddy *et al*., 2018).

In the study carried out by Reddy and colleagues, the acetic acid concentration showed an increment for the first twenty days from 5.18 gCOD/L to 6.53 gCOD/L followed by

decrement to 4.10 gCOD/L and then increased again to 6.7 gCOD/L (Reddy *et al*., 2018). The duration of this study was significantly longer, and its primary focus was on the production of medium-chain fatty acids. However, a similar fluctuation in the abundance of acetic acid was observed across most of these tests. This finding confirms that in the presence of a bio-augmented culture, acetic acid is continuously produced and utilized for the generation of other compounds (Jiang et al., 2020).

The best solutions in terms on chain elongation resulted to be tests F and G with strain1 of *S. cerevisiae* and 3.7g of sugar and 11g of molasses as co-substrates since the caproic acid was more abundant, with 21.0 gCOD/L and 17.2 gCOD/L. These results are in line with those from by Reddy and colleagues who obtained around 17.9 gCOD/L of caproic acid with bioaugmented culture and leachate as substrate.

Table 6 : Summary of the main average data obtained from the first batch trial with tannery sludge.

Test	Final pH	Alcohols	SCFAs	Alcohols		Main SCFAs			Yield Alcohols	Yield SCFAs
				Methanol	Ethanol	Acetic	Propionic	Butyric		
		gCOD/L	gCOD/L	gCOD/L	gCOD/L	gCOD/L	gCOD/L	gCOD/L	$(gCOD_{Alc}/(gVS_0)$	$(gCOD_{SCFA}/(gVS_0)$
A1	5.5 ± 0.2	5.9 ± 0.6	3.5 ± 0.1	0.43 ± 0.09	5.5 ± 0.2	2.7 ± 0.1	0.21 ± 0.06	0.6 ± 0.1	0.29 ± 0.02	0.17 ± 0.03
B1	6.1 ± 0.3	10.9 ± 0.1	5.5 ± 0.2	0.64 ± 0.07	10.2 ± 0.3	2.9 ± 0.3	0.45 ± 0.07	2.1 ± 0.2	0.27 ± 0.02	0.14 ± 0.03
C1	6.3 ± 0.4	10.9 ± 0.1	6.0 ± 0.7	0.91 ± 0.08	10.0 ± 0.2	3.0 ± 0.3	0.46 ± 0.09	2.4 ± 0.2	0.18 ± 0.03	0.09 ± 0.02
D2	5.7 ± 0.4	5.9 ± 0.2	2.8 ± 0.6	0.7 ± 0.1	5.2 ± 0.4	1.5 ± 0.4	0.27 ± 0.08	1.2 ± 0.1	0.29 ± 0.03	0.16 ± 0.04
E2	5.6 ± 0.4	5.5 ± 0.2	4.1 ± 0.4	0.6 ± 0.1	5.6 ± 0.4	2.6 ± 0.2	0.27 ± 0.08	1.1 ± 0.1	0.14 ± 0.04	0.10 ± 0.03
F2	5.8 ± 0.1	10.1 ± 0.3	5.4 ± 0.6	0.72 ± 0.08	9.4 ± 0.2	3.5 ± 0.2	0.46 ± 0.09	1.3 ± 0.1	0.17 ± 0.04	0.09 ± 0.02

Table 7 : Summary of the main average data obtained from the second batch trial with tannery sludge (not shown, data of isovaleric, valeric and isocaproic acids are <0.9 gCOD/L).

Test	Final pH	Alcohols gCOD/L	VFAs gCOD/L	Alcohols		Main SCFAs and caproic acid					Yield	
				Methanol gCOD/L	Ethanol gCOD/L	Acetic gCOD/L	Propionic gCOD/L	Isobutyric gCOD/L	Butyric gCOD/L	Caproic gCOD/L	Yield Alcohol ($gCOD_{Alc}/(gVS_0)$)	Yield SCFAs ($gCOD_{SCFA}/(gVS_0)$)
BLANK	7.5	0.20 ± 0.01	11.1 ± 0.1	0.2 ± 0.01	0	6.7 ± 0.3	1.4 ± 0.4	0.4 ± 0.2	1.7 ± 0.3	0	0.20 ± 0.02	0.09 ± 0.03
A	7.0	27.1 ± 0.1	11.5 ± 0.3	0.9 ± 0.1	26.0 ± 0.6	7.2 ± 0.2	0.6 ± 0.2	0.3 ± 0.2	2.7 ± 0.3	0	0.27 ± 0.01	0.11 ± 0.02
B	7.0	12.5 ± 0.5	22.5 ± 0.2	1.2 ± 0.1	11.3 ± 0.5	5.2 ± 0.5	1.6 ± 0.2	0.5 ± 0.1	14.6 ± 0.4	0	0.16 ± 0.04	0.29 ± 0.01
C	6.9	13.2 ± 0.3	36.5 ± 0.2	1.2 ± 0.1	12.0 ± 0.4	17.2 ± 0.5	1.4 ± 0.1	0.7 ± 0.2	12.9 ± 0.4	3.2 ± 0.4	0.12 ± 0.05	0.30 ± 0.05
D	7.1	2.9 ± 0.4	23.1 ± 0.4	0.4 ± 0.3	2.5 ± 0.6	4.9 ± 0.3	1.3 ± 0.1	0.3 ± 0.2	15.4 ± 0.6	0.7 ± 0.3	0.03 ± 0.01	0.27 ± 0.05
E	7.0	30.4 ± 0.4	12.1 ± 0.4	0.8 ± 0.1	29.6 ± 0.3	6.9 ± 0.3	0.8 ± 0.2	0.3 ± 0.1	3.6 ± 0.3	0	0.30 ± 0.03	0.12 ± 0.02
F	7.0	3.9 ± 0.2	22.2 ± 0.2	0.3 ± 0.1	3.7 ± 0.4	4.3 ± 0.2	0.5 ± 0.4	0.3 ± 0.2	7.2 ± 0.5	9.2 ± 0.5	0.05 ± 0.04	0.28 ± 0.02
G	7.1	13.3 ± 0.2	29.8 ± 0.5	1.0 ± 0.3	12.3 ± 0.3	9.6 ± 0.1	1.8 ± 0.3	0.4 ± 0.2	9.2 ± 0.5	8.1 ± 0.4	0.12 ± 0.04	0.27 ± 0.03
H	6.9	14.8 ± 0.6	27.8 ± 0.4	1.1 ± 0.4	13.6 ± 0.4	15.9 ± 0.2	2.3 ± 0.5	0.4 ± 0.3	8.5 ± 0.3	0	0.17 ± 0.03	0.33 ± 0.05
I	6.7	3.7 ± 0.5	31.1 ± 0.4	2.6 ± 0.2	1.2 ± 0.4	10.8 ± 0.5	0.9 ± 0.4	0.9 ± 0.1	17.8 ± 0.1	0.5 ± 0.2	0.03 ± 0.01	0.28 ± 0.02
L	7.0	1.0 ± 0.1	26.3 ± 0.1	0.8 ± 0.4	0.3 ± 0.5	9.5 ± 0.6	0.9 ± 0.2	0.2 ± 0.1	15.5 ± 0.7	0	0.01 ± 0.01	0.30 ± 0.01
M	5.8	2.9 ± 0.1	24.6 ± 0.1	2.4 ± 0.5	0.5 ± 0.2	7.4 ± 0.3	0.6 ± 0.3	0	16.6 ± 0.7	0	0.03 ± 0.03	0.25 ± 0.03
N	6.5	1.5 ± 0.1	30.6 ± 0.3	0.9 ± 0.3	0.6 ± 0.3	11.6 ± 0.1	0.8 ± 0.3	0.4 ± 0.2	17.9 ± 0.8	0	0.03 ± 0.04	0.39 ± 0.04

5. Conclusions

In a perspective of innovation and sustainable waste valorisation to produce valuable organic compounds, this study aimed to provide insights into an innovative approach for chain elongation, utilizing tannery sludge and a sugar source through co-fermentation techniques, with a specific emphasis on enhancing SCFAs and MCFAs production by employing yeast bioaugmentation.

With the first tests, the aim was to assess the level of sunflower seed molasses contamination by bacteria on pure yeast strain. This allowed to observe how the production trend of alcohols, and particularly the ethanol, by yeast was affected by the presence of contaminants. The production of SCFAs starting from the first week highlighted the richness of the molasses and enabled the continuation of subsequent tests aimed at achieving SCFAs production, and overall, the chain elongation process.

Therefore, the second batch tests performed emphasized the SCFAs production achieved through the supplementation of a co-substrate to the tannery sludge. The co-fermentation tests reached higher yields compared to those obtained solely from the tannery sludge, which already showed promising results. The anaerobic fermentation of the tests involving tannery sludge, along with sunflower seed molasses or sugar as co-substrates, yielded with an average of 24-31gCODSCFA/L. This output was predominantly characterized by acetic acid and butyric acid.

The use of yeast bioaugmentation affected the SCFAs production positively. An abundant production of acids, including peaks exceeding 40 $gCOD_{SCFA}$/L, were reached after 18 days and a substantial part of caproic acid was produced.

The use of strain2 of *Saccharomyces cerevisiae* in tests F and G has evidently played a role in the observed outcomes. Specifically, reaching peaks of 21.0 gCOD/L for test F and 17.2 gCOD/L for test G indicates the effectiveness of this particular strain combined with a co-substrate, in enhancing SCFA production.

These findings highlighted the importance of maintaining an optimal ethanol concentration to facilitate chain elongation without negatively impacting the SCFA-producing microorganisms involved in the process. It was hypothesized that using lower amount of white sugar (3.7g) can enhance the fermentative bacteria activity, while also allowing the

ethanol production by the yeast. Alternatively, the use of sunflower seed molasses had success with the higher amount (11g) which enhanced the activity of the fermentative bacteria without the yeast's inhibition.

Achieving a balance in ethanol levels appears crucial for the success of the chain elongation process, thus more research on the use of the sunflower seed molasses must be done considering its importance as a waste material, unlike white sugar.

These promising results achieved from these batch tests open up future possibilities for further studies and applications in the field of chain elongation, including the use of immobilized yeast in a separate bio-ethanol production tests to furnish the electron donor amenable in following fermentation processes where chain-elongation can occur. This approach can offer advantages in terms of improved control over substrate feeding and potentially increased MCFAs productivity.

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