Brewery spent grain cascade process: protein recovery and improved downstream fermentation for simultaneous volatile fatty acid production and recovery

A thesis submitted to the National University of Ireland as fulfilment of the requirements for the degree of Doctor of Philosophy

by

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Declaration of authorship

I, Juan Castilla-Archilla, declare that this thesis has been generated by me and presents my original work and results unless stated otherwise. This work was not been used to obtain any other degree at the National University of Ireland, or elsewhere.

Juan Castilla-Archilla

For my wife, Mónica, who decided to share this journey of life with me. My daughter, Alejandra, I hope to be able to teach you all the good things I can learn from this life.

For my parents, Juan and Amalia, who are still teaching me to work hard and never give up without forgetting the important things of life. My sister, Ana, I have the luck to be sharing this life with you since my first memory.

&

For my grandparents, Miguel and Ana, who witnessed how I started my PhD and I'm sure you are proud of me. Your kindness and love fill the heart of everyone who was lucky to be around you. My grandmother Rosa, who was unique and strong despite the difficulties of life.

I could not have become who I am without any of you

"Lo mejor será que bailemos" "¿y que nos juzguen por locos sr conejo?"
"¿usted conoce algún cuerdo feliz?" – Bailemos pues

Alone, we go faster. Together, we go further

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Publication	Author contribution
Chapter 2: Castilla-Archilla J., O'Flaherty V., Lens P. N. L. (2019) "Biorefineries: Industrial Innovation and Tendencies" In: J.-R. Bastidas-Oyanedel, J. S. Schmidt, (Eds.), Biorefinery: Integrated Sustainable Processes for Biomass Conversion to Biomaterials, Biofuels, and Fertilizers, Springer, Switzerland, pp 3-35	**Castilla-Archilla J.**: conceptualization, design, literature revision, writing - original draft and manuscript. **O'Flaherty V.**: writing - review & editing. **Lens P. N. L.**: funding acquisition, supervision, writing - review & editing
Chapter 3: Castilla-Archilla J., Thorn C., Pau S., Lens P. N. L. (2022) "Screening for suitable mixed microbial consortia from anaerobic sludge and animal dungs for biodegradation of brewery spent grain" Submitted to Biomass and Bioenergy (major revisions) **The work in this chapter formed part of a collaboration where any microbial analysis work (lab work, bioinformatics and data representation) were completed by Dr Camilla Thorn*	**Castilla-Archilla J.**: conceptualization, methodology, investigation, interpretation, writing – original draft. **Thorn C.**: methodology, interpretation, writing – review & editing. **Pau S.**: investigation. **Lens P. N. L.**: funding acquisition, methodology, supervision, writing - review & editing.
Chapter 4: Castilla-Archilla, J., Papirio, S., and Lens, P. N. L. (2021) "Two step process for volatile fatty acid production from brewery spent grain: hydrolysis and direct acidogenic fermentation using anaerobic granular sludge" Process Biochemistry 100, 272–283. doi: 10.1016/j.procbio.2020.10.011	**Castilla-Archilla J.**: conceptualization, methodology, investigation, interpretation, writing - original draft. **Papirio S.**: methodology, validation, supervision, writing - review & editing. **Lens P. N. L.**: funding acquisition, methodology, supervision, writing - review & editing.
Chapter 5: Castillla-Archilla J., Heiberger J., Mills S., Hilbig J., Collins G., Lens P. N. L. (2021) "Continuous Volatile Fatty Acid Production From Acid Brewery Spent Grain Leachate in Expanded Granular Sludge Bed Reactors" Frontiers in Sustainable Food Systems 5, 129. DOI=10.3389/fsufs.2021.664944	**Castilla-Archilla J.:** conceptualization, methodology, investigation, interpretation and writing – original draft and manuscript. **Heiberger J.**: investigation and interpretation. **Mills S.**: methodology, interpretation and writing - draft review. **Hilbig J.**: investigation. Collins G.: methodology and writing - supervision. **Lens P. N. L.**: Funding acquisition, Methodology, Supervision, Writing - review & editing.

Abstract

This PhD thesis aimed to develop sustainable volatile fatty acid (VFA) production using brewery spent grain (BSG) as lignocellulosic feedstock, with an overall production in Ireland above 170,000 tonnes. VFAs were produced by using partially inhibited anaerobic digestion (AD) to avoid the decomposition of fermented compounds into biogas. AD is the conversion of organic matter to biogas by four different steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis. VFAs are produced during the acidogenesis and acetogenesis step. Thus, the control of the operational conditions to avoid methanogenesis leads to the accumulation of VFAs. Firstly, two different approaches were followed for VFA production: i) the simultaneous hydrolysis and fermentation, and ii) a two-step process using a pretreatment hydrolysis to obtain a liquid rich in carbohydrates for downstream fermentation as a second step. A second part was developed to recover additional added value compounds (other than VFAs, i.e. protein) from the BSG during the hydrolysis step, and optimisation of the downstream process by the simultaneous production and recovery of the VFAs.

In the first experimental chapter (***Chapter 3***), different inocula were tested for their ability to degrade BSG. The highest solids degradation was achieved by the inocula which also presented the highest gas production. On the other hand, the inocula that were able to accumulate fermented compounds presented an inefficient solid degradation despite presenting a large hydrolytic community. This led to the hypothesis that possible inhibition of the hydrolysis process was caused by the accumulation of the VFAs. Further study of the AD process of lignocellulosic materials for chemical production is required to determine this possible inhibitory effect if methane is not the end product.

The second experimental chapter of this thesis covered a two-step process, first the hydrolysis pretreatment of BSG, and second, the fermentation of the liquid hydrolysate. This approach relied on the production of a mixed liquor of simple carbohydrates that could be easily fermented by microbial communities, as the hydrolysis step is not required. Different combinations of sulfuric acid and BSG concentrations were tested for the release of carbohydrates into the liquor fraction. The optimal conditions corresponded to 7 % BSG w/w (dry matter), 1.5 % of sulfuric acid v/v at 121 °C for 20 min.

The efficiency of this combination resulted in the extraction of 0.44 g of carbohydrates per g of BSG. The obtained hydrolysate was used for the acidogenic fermentation in batch reactors under different pH conditions (***Chapter 4***). The liquid hydrolysate was directly used without detoxification or chemical addition. However, it was diluted to a concentration of 15 g/L and the pH was increased up to the operational pH (7.0 and not further control, 4.5, 5.0, 6.0 and 8.0). The highest VFA concentration was achieved at pH 6.0, which corresponded to 17.6 (± 0.67) gCOD/L and VFAs contributed 93.0 % of the sCOD. However, the highest VFA production rate was obtained at pH 5.0 which corresponded to 0.79 gCOD/(L·h). The next step was the continuous production of VFAs using a high rate reactor to achieve a large volumetric production (***Chapter 5***). The highest achieved VFA production corresponded to 120.4 (± 15.0) mmol/L, which contributed approximately 83.4 (± 5.9) % of the soluble COD. However, VFA production was unstable as the concentration of acetic acid, propionic acid, butyric acid and ethanol fluctuated as major compounds. This fluctuation could be the result of metabolic fluxes caused by the stress responses of fermentative microorganisms to the continuous accumulation of fermented compounds. Thus, the simultaneous production and recovery of the VFA could contribute to alleviate such effects and increase the VFA selectivity.

Chapter 6 and ***Chapter 7*** of this PhD thesis developed an optimization during the hydrolysis step and the acidogenic fermentation to improve any future applications for the potential scale up of the two-step process. As BSG retains a considerable pool of high value compounds, a cascade process was developed by combining a chemical pretreatment with enzymatic hydrolysis (***Chapter 6***). The best combinations led to a liquid fraction rich in carbohydrates up to 39.9, 52.4 and 44.6 g/L of total carbohydrates. Besides the liquid fraction, two different solid fractions were obtained with great potential for food industrial applications due to the content of proteins and water retention capacity.

Finally, further optimisation for the VFA production and recovery was developed by coupling an electrochemical cell unit to the recycling line of an upflow anaerobic sludge bed reactor (UASB). In this case glucose was used as the sole carbon source. The glucose concentration was increased from 15 to

60 g/L. The highest VFA production achieved was above 30 g/L of VFAs. The electrochemical cell was able to enhance the VFA concentration up to 4 fold during the recovery process, although this was when operating at lower glucose concentrations. The UASB-electrochemical cell system showed additional advantages, such as the reduction of alkaline demand and favoured the production of caproic acid with higher value than acetic and butyric acids. The process requires further optimisation as the electrochemical cell was under dimensioned. Nevertheless, the process showed a high potential for the recovery of the VFAs.

In summary, this PhD thesis demonstrated the potential for using BSG as a feedstock in a biorefinery. The combination of different chemical and enzymatic treatments during initial processing allowed for the recovery of proteins and the release of high concentrations of carbohydrates into the liquid fraction. During downstream processing, this carbohydrate rich fraction led to a high concentration of VFAs, which in combination of low pH operational conditions, led to minimal methane production. Finally, a novel electrochemical cell was implemented, which demonstrated efficient VFA recovery and decreased the requirement for chemical (base) addition.

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Chapter 1

General introduction

Keywords: Biorefinery; circular economy; biomas disposal; biomass feedstock

1.1 General introduction

Industrial advancement during the last century has led to a more comfortable way of life. However, this has often been at the expense of the environment (Yang et al., 2018). At the beginning of the 20th century, water and fossil fuels were considered an unlimited resource and not enough attention was paid to waste treatment. This attitude resulted in the increase of the greenhouse gas emissions and deterioration of natural habitats, such as the vast plastic accumulation in the Pacific and Atlantic oceans. Fortunately, in recent years, sustainability has become more prominent, driven by heightened environmental awareness and decreasing reservoirs of fossil fuels and natural resources. For example, in Ireland the solid wastes, which were previously sent to landfills have been diverted to new waste disposal facilities such as incinerators (Davies, 2008; EPA, 2019). However, many of these wastes could be used to produce compounds with high added value. The next milestone in sustainability must be achieved by replacing fossil fuel based industries with wastes, which act as renewable biomass.

The biorefinery concept has emerged as an alternative to traditional petroleum refineries, where renewable by-products and wastes are used for production of high value compounds (***Figure 1.1***). In a biorefinery, renewable biomass is separated and fractionated into a range of products such as chemicals, biomaterials, or biofuels. Wastes are starting to be considered valuable entities and many researchers are focusing on acquiring a deep understanding of advanced processes to obtain high value products from different biowastes, not only from waste treatment (Karthikeyan et al., 2017). An example of the evolution from waste treatment to waste valorisation is the shift from aerobic to anaerobic digestion. In the past, aerobic digestion was the main process for wastewater treatment which results in the production of large volumes of activated sludge, which requires further downstream processing. Anaerobic digestion allows the conversion of organic matter into biogas, CO_2 and CH_4 with much less sludge production (Chetty and Pillay, 2015), making wastewater a resource for biogas production.

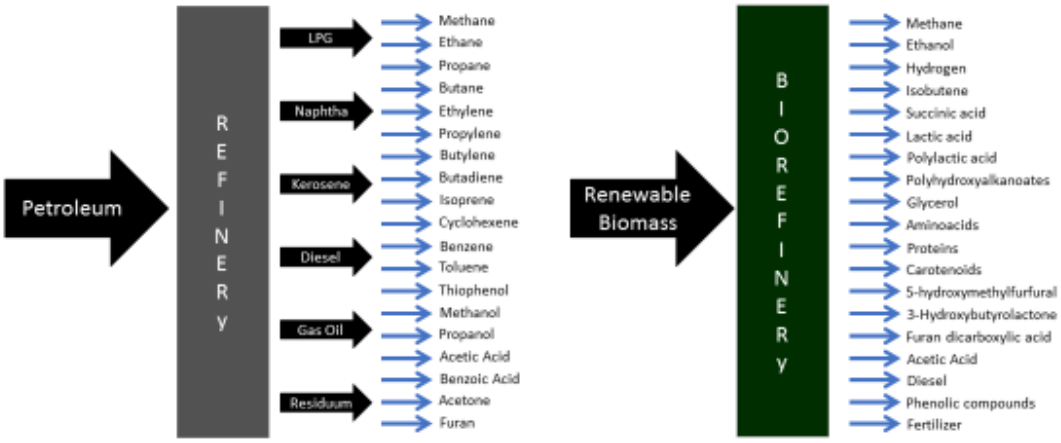

Figure 1.1 *Range of products and chemicals that are obtained in a traditional petroleum-based refinery and in a biorefinery*

The partial or total inhibition of methane production during anaerobic fermentation can lead to accumulation of some intermediate compounds such as hydrogen, lactate, or volatile fatty acids (VFAs) (Rosa et al., 2014; Cisneros-Perez et al., 2017). Different strategies have been followed at lab scale to promote hydrogen production, coupled to simultaneous accumulation of VFAs (Garcia-Aguirre et al., 2017). VFAs are short chain fatty acids (C2 to C6) with a broad range of industrial applications, however their current production relies on fossil fuel sources (Strazzera et al., 2018). The main problem with VFA production from renewable sources is the diluted concentration of VFAs in the fermented broth. This relegates VFA production as an intermediate step for production of other compounds that are easier to recover, e.g. polyhydroxyalkanoates (PHA) or lipids for biodiesel production (Albuquerque et al., 2011; Patel et al., 2018). Nevertheless, recent improvements in extraction systems may allow efficient extraction of VFAs as the final product, especially if a high VFA concentration can be obtained (Zhang and Angelidaki, 2015; Saboe et al., 2018; Wainaina et al., 2019). Thus, this enables the uncoupling the VFA production from fossil fuels and provides a trade opportunity in countries that rely on importing gas and oil. However, further investigations must be carried out to develop this process.

Conventional wastewater cannot be used to achieve a higher VFA concentration due to the low organic matter content. In addition, wastewater often contains inhibitory compounds for VFA production. For example, the raw dairy wastewater typically contains a large amount of fats that are inhibitory for microbial degradation (Chetty and Pillay, 2015). Thus, the current system for dairy wastewater treatment includes the removal of the fats prior to the anaerobic process for methane production, which decreases the organic content below 5 g/L (Chetty and Pillay, 2015; Trego et al., 2021), which is not sufficient for VFA production and recovery. Therefore it is necessary to find an alternative to traditional wastewater treatment. For example, solid by-products or a waste with low or no value that could be used as a source of carbohydrates. However, if this solid by-product/waste requires a pretreatment, the feasibility of the process could be compromised by a higher cost to obtain the final product than the value of the product itself.

The first milestone of the present work is to find an industrial solid waste, or low value by-product, as feedstock. The characteristics of this by-product/waste must meet some criteria such as large availability. It would also be beneficial if it contained additional compounds of interests other than carbohydrates, which could be recovered by a sustainable fractionation of the biomass in a biorefinery approach. For example, the hydrolysis of the solid feedstock by a combination of different physical, chemical and biological processes to obtain a liquid hydrolysate rich in carbohydrates with the simultaneous recovery of additional added value compounds (e.g. proteins and fibers). The recovery

of these added value compounds can partially counterbalance the costs of the hydrolysis process, such as energy use or enzymes.

1.2 Biorefinery definition

The biological processes for conversion of raw materiasl into a final product have been largely exploited throughout history, e.g. the fermentation of milk to yogurt or barley to beer. Despite the widespread application of these different practices, it was not until the 17th century that the microorganisms responsible for these processes were observed and identified. This led to the development of more sophisticated technologies, with industrial microbiology taking off on a large scale for the production of pharmaceutical compounds, e.g. antibiotics, hormones and steroids. These innovations led to the start of the biotechnology industry, which relies on microbial activity to obtain high value products at industrial level. Nowadays, this sector plays a key role in sustainability and reducing reliance on fossil fuels.

1.2.1 Plant refinery session

The use of renewable biomass for chemical and energy production started at the end of the 19th century. However, this was rapidly displaced by the use of petrol, before increasing again at the beginning of the 1980s, when crops were suggested as renewable feedstock for the production of fuels and organic chemicals (Levy et al., 1981). This definition was expanded upon in the biomass refinery concept (Koukios 1985). The approach was adopted and further developed by the Plant Refinery Session in the early 90s, emphasizing the differences between the biofuel production facility and the biorefinery facility (Galletti, 1991). The biorefinery facility, beside energy, would produce industrial intermediates with a wide range of chemicals and materials (Wyman and Goodman, 1993).

1.2.2 International energy agency, working group bioenergy

Currently, the Bioenergy working group of the International Energy Agency (IEA) developed the biorefinery concept as the sustainable processing of biomass into a spectrum of marketable products (food, feed, materials and chemical) and energy (fuels, power and heat) (IEA Bioenergy, 2009). The IEA classified biorefineries based on four features: i) platforms, ii) products, iii) feedstocks, and iv) processes. Platforms were considered the principle feature as they are the link between raw and final product, and they can be used to link different biorefinery concepts (Cherubini et al., 2009). Regarding the feedstock, there were two main parts: i) dedicated crops and ii) residues, subsequently dividing each of them into different categories (Cherubini et al., 2010).

1.2.3 Green biorefinery

Additionally, the term of green biorefinery was introduced by Morais and Bogel-Lukasik (2013), based on the twelve principles of the green chemistry. These twelve principles were designed by

Anastas and Warner (1998) to tackle and reduce pollution in the chemical industry. They considered alternative processes and the use of less polluting materials to make a more environmental and sustainable system. For instance, the term “green” was established at the end of the 1980s to define the sustainable development for economic progress as Green Economy (Allen and Clouth, 2012). The use of this term has evolved in the last decade, related with a lower carbon dependency and more environmental friendly practices (Barbier, 2010).

1.2.4 Circular economy

The largest greenhouse gas emissions from material management are related with the extraction, processing and production stages (PACE, 2020). The development of the biorefinery should go hand-in-hand with the switch from the linear economy to the circular economy. In other words, moving away from the take-make-use-dispose practise (Lieder and Rashid 2016) and closing the loop, turning wastes into resources (Deselnicu et al. 2018). However, approximately only 9 % of the world operates in a circular economy system (PACE, 2020). To promote the circular economy, biorefineries could become a technological hub for collection, treatment and production of value added compounds from current wastes. Thus, reinforcing industrial sustainability and moving away from the use of fossil fuels (***Figure 1.2***)

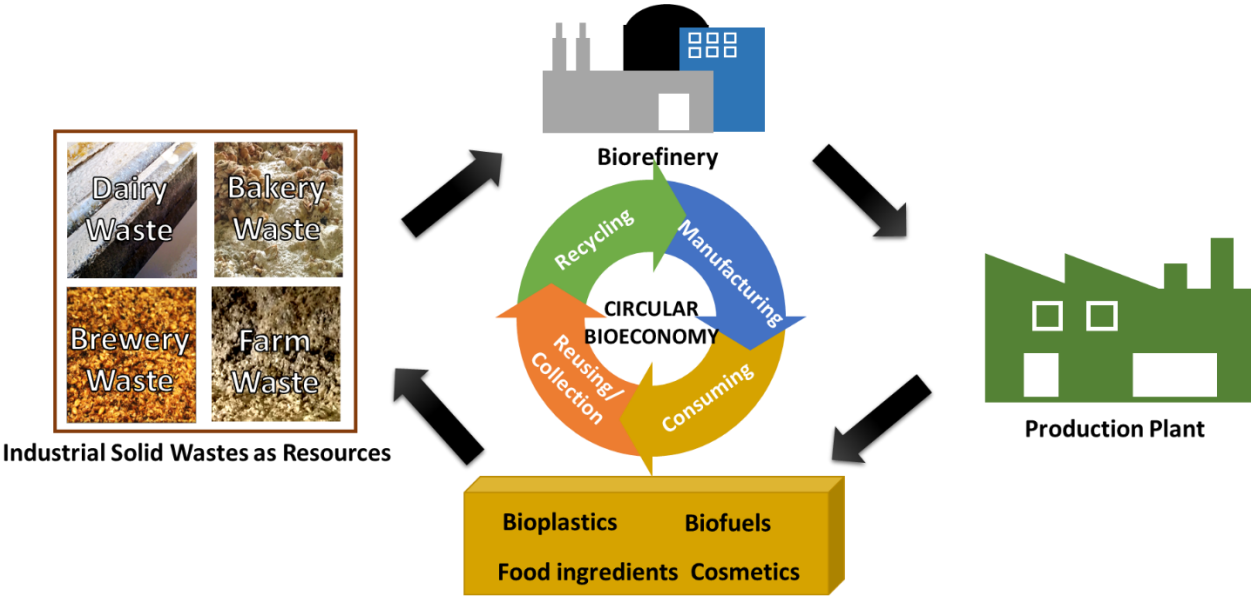

Figure 1.2 *Biorefinery plant closing the loop for the circular bioeconomy approach. Solid wastes are broken down using chemical, physical and biological processes, up to single molecules known as chemical building blocks. The chemical building blocks are combined or used as final products for the production plants*

There are alternative definitions for the biorefinery concept and classification. Some of them have been summarized in ***Table 1.1***. However, based on the aforementioned statements, the present work has accepted the definition and classification of biorefineries given by the IEA working group Bioenergy. Although an important amendment must be made for further improvement of

sustainability. The feedstock should not be split in dedicated crops and residues, instead it should be classified based on its composition and the different fractions that can be extracted for downstream valorisation. For example, the amount of proteins, lipids, cellulose, hemicellulose or the phenolic content.

Table 1.1 *Biorefinery and biorefining definition from different authors broadly used in the actual industry*

Author	Year	Definition
Koukios	**1985**	*A biomass refinery is an integrated complex of industrial processes that separate the major components of bioresources and convert them to fuels, food, feed, fiber, fertilizer and chemicals*
Wyman and Goodman	**1993**	*A biorefinery could produce a wide range of chemicals and materials through microbial conversion of renewable resources*
Kamm et al.	**2008**	*Biorefining involves the transfer of efficiency and logic from the fossil-based chemistry and substance conversion industry as well as energy production to the biomass industry*
Biopol	**2008**	*Biorefinery is the sustainable processing of biomass into a spectrum of marketable products*
Biorefinery Euroview	**2008**	*Biorefineries are integrated bio-based industries, using a variety of different technologies to produce chemicals, biofuels, food and feed ingredients, biomaterials and power from biomass raw materials*
IEA Bioenergy Task 42	**2009**	*Biorefining is the sustainable processing of biomass into a spectrum of bio-based products and energy*
Morais and Bogel-Lukasik	**2013**	*Green biorefinery is based on the ideal processes that use a non-polluting material as a feedstock, there is no production of any contaminant and solvents are avoided*

1.3 Potential feedstock for an Irish biorefinery

Many different wastes have been broadly studied for the development of the biorefinery approach, from industrial to household wastes. Even though food wastes from households, restaurants or similar settings represent a continuous source of organic feedstock, their wide heterogeneous and changing composition could affect the stability of the biorefinery processes (Cristobal, 2019). On the other hand, some of the agricultural and food processing wastes present a more homogeneous fraction, but these products could be more affected by seasonality (Fava et al., 2015; Cristobal et al., 2019). Thus, the present work was looking for an industrial waste produced throughout the year, with consistent characteristics. Another key factor is the composition of the material, which should contain high value compounds to facilitate a more straight forward valorisation in a shorter term. This section aims to

evaluate the different industrial sectors that contribute to the categories in the EPA Waste Classification that could be used as source of renewable feedstock for a biorefinery (EPA 2019).

1.3.1 Industrial sectors and current disposal of the biomass

In Ireland, the production of solid wastes in 2016 was above 47 million tonnes (Eurostat, 2020). In 2018, the Environmental Protection Agency (EPA) updated the EPA Waste Classification which includes a total of 843 different types of wastes, split into 10 different categories with several subdivisions based on their characteristics (EPA 2019). At least 37 of these categories could be easily used as a renewable biorefinery feedstock. Most of these wastes are sent for incineration or co-incineration (1,177,175 tonnes), landfilling (570,000 tonnes), composting and biostabilization (602,700 tonnes); and gas production via anaerobic digestion (110,000 tonnes). The number of landfills in Ireland has decreased in the last decade at the same time that the number of incineration and co-incineration plants have increased (EPA 2020). This was caused by a change in waste management practices in the mid 1990s due to the requirement for more sustainability and lower solid waste production (Davies 2008). Despite this improvement in waste management, industrialisation in the last decade has resulted in the increase of the greenhouse gas emissions in Ireland (Jensen, 2021). Thus, not only waste management must be improved, but also the valorisation of current wastes as alternative to fossil fuels.

The goal of this PhD thesis is to find a well-established industry in Ireland that generates a stream of solid wastes or low value by-products, in enough quantity to be used for chemical and energy production. Some of the most important industrial sectors from the EPA classification (2019) that could be used as a source of feedstock for biorefinery purposes can be summarised in three key sectors:

1. **Meat and livestock:** beef, pig meat, sheep meat and poultry
2. **Prepared food:** chocolate confectionary, frozen prepared-food, cooked meats and meat ingredients, and bakery products and snacks
3. **Beverages:** whiskey, cream liqueur, beer and cider

Industries in these fields such as dairy, brewing or meat industries have a large contribution to the Irish economy and have increased their exports and value over the last decade (***Figure 1.3***).

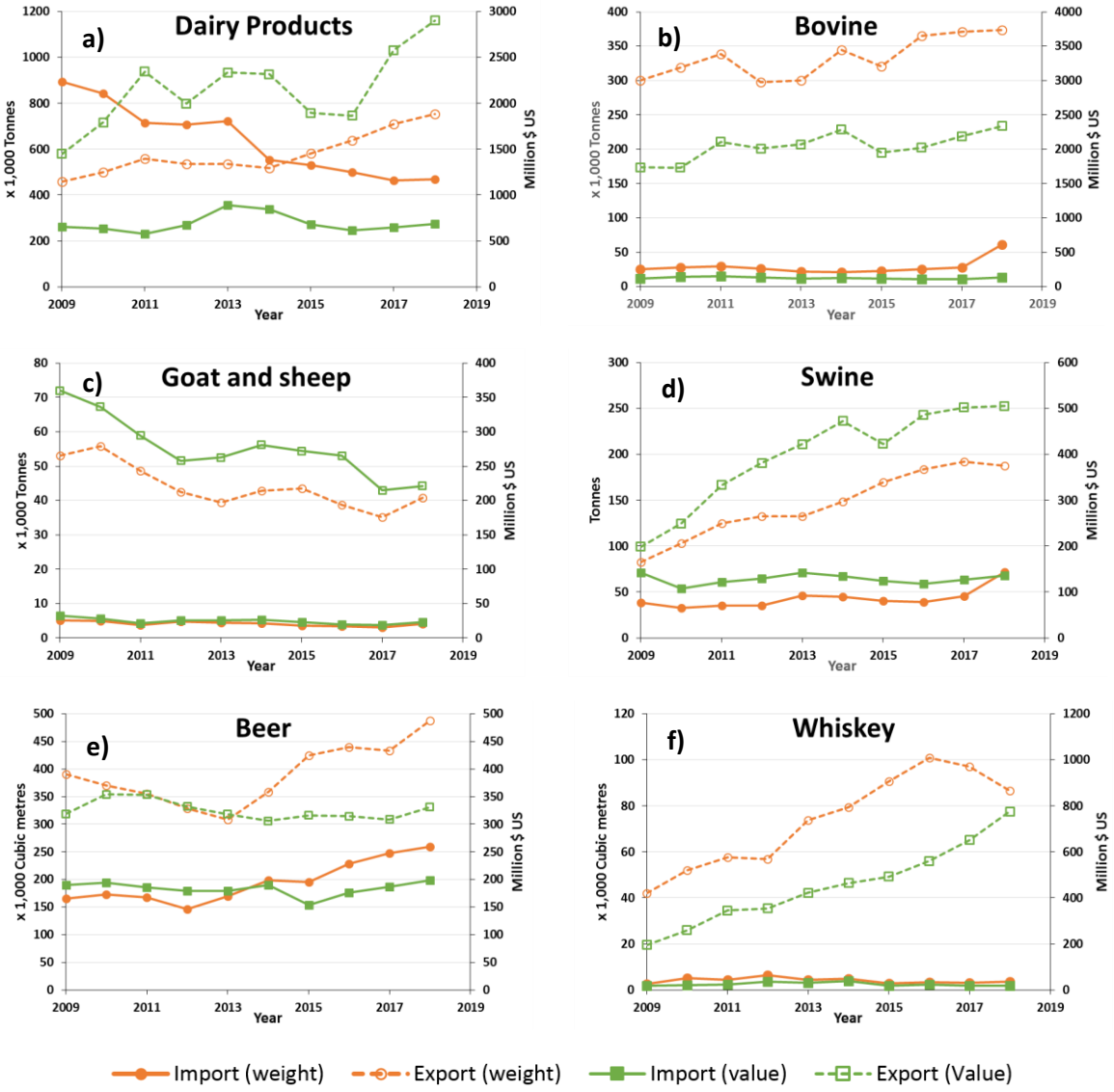

Figure 1.3 *Main exports from the Irish industry; processing food (a), livestock (b, c and d), and beverage (e and f)*

Most solid wastes from the different industries are mixed and treated equally, for example non-hazardous organic material from the dairy, brewery or food confectionary industries (EPA, 2020). This results in a loss of opportunities for the current wastes that contain valuable compounds. Some of these products could even be considered as a by-product instead of wastes, with uses in the chemical, cosmetic, or food industries. Some examples are the production of proteins using dairy wastes, potato wastes or brewery wastes.

1.3.2 Biomass selection for this PhD thesis

During the development of this PhD thesis, some different industrial waste samples were collected for their valorisation. This includes rumen fluid, known as belly grass, which is a mix of the content from the four stomachs of cows, this material was sourced from a slaughterhouse in County Mayo (Ireland). The non-used dough was collected from an industrial pizza confectioning plant in County

Longford (Ireland). However, because of the large content of oil and a discontinuous production this waste was discarded to be studied in this PhD, despite of being rich in carbohydrates. Brewery spent grain was collected from a plant located in County Galway (Ireland). Brewery spent grain was selected as the feedstock for this PhD thesis. This was decided based on the large availability and constant composition of the brewery spent grain throughout the year.

The main goal of this PhD thesis was **i)** the fractionation and processing of brewery spent grain into different streams, including a liquid fraction rich in carbohydrate and two solid streams rich in lignin and proteins and **ii)** the production and simultaneous recovery of the VFAs from the carbohydrate rich liquid fraction.

1.4 Thesis scope

The core of this PhD thesis is the sustainable production and recovery of VFAs from brewery spent grain. Thus, contributing to shifting the view of anaerobic digestion from a waste treatment system to a more sophisticated tool for chemical and energy production. However, the conditions mentioned above, such as the requirement of a liquid hydrolysate rich in carbohydrates, will have a cost that the production of the VFAs as the sole final product could not compensate. Implementing the biorefinery approach using brewery spent grain as feedstock could counterbalance the cost of the initial processing. The production of a different range of compounds such as proteins, lignin, VFAs, hydrogen, carbon dioxide and methane could also make up some of the costs.

Chapter 2 is a review of some examples of bioerefineries using lignocellulosic feedstock. In addition, Chapter 2 provides an overview of the anaerobic digestion process and how this can be used for volatile fatty acid production, including current approaches for the use and recovery of volatile fatty acids. Two different approaches for VFA production are reviewed, i) the simultaneous degradation and fermentation process, and ii) a two-step process focus on hydrolysis and downstream fermentation of the liquid hydrolysate. As well, the current treatments for lignocellulosic materials as feedstock to provide added value compounds is overviewed. Finally, the main composition and characteristics of the brewery spent grain with current use and valorisation are presented.

Chapter 3 describes simultaneous hydrolysis and fermentation of brewery spent grain as a lignocellulosic feedstock using sacrificial tests. Different sources of inocula were used to find a suitable microbial consortium for chemical and energy production. This includes industrial sludge, cow rumen fluid, and animal faeces.

Chapter 4 describes a two-step process for the production of the volatile fatty acids. First, a thermal diluted sulfuric acid hydrolysis was carried by a combination of different acid and brewery spent grain

concentrations. The liquor produced was fermented using anaerobic granular sludge under different pH values in a batch reactor system. Finally, this approach was chosen for further development along the thesis due to the content of high value compounds in BSG to be recovered during the first step.

Chapter 5 followed up the batch approach developed during Chapter 4 for the VFA production. The pretreatment conditions developed at chapter 4 were used to obtain the liquid hydrolysate. In this case, the fermentation was carried out using a continuous expanded granular sludge bed reactor. The system was operated at pH 5.90 (± 0.05) at different organic loading rates and inhibition of methanogenesis was achieved using specific operational conditions.

Chapter 6 focused on optimising the hydrolysis of brewery spent grain using a cascade process for the high-value product recovery, lignin valorisation, and simultaneous release of the carbohydrates into the liquid fraction. First, a combination of different parameters (acid, temperature and time) for the pretreatment was assessed, followed by enzymatic hydrolysis. The release of carbohydrates, and the effect of the pretreatment on the protein and lignin extraction were evaluated.

Chapter 7 aimed to enhance the production of the volatile fatty acids using a synthetic high strength feedstock with carbohydrate concentrations up to 60 g/L. A similar system than in Chapter 5 was used, however in this case an electrochemical cell was used for the recovery of the volatile fatty acids, alleviating their possible toxic effect to the bioreactor biomass.

Chapter 8 summarises the principle conclusions of this work, including a diagram with the whole proposed process for the brewery spent grain biorefining. This could be used as foundation for scaling up the process and practical implication of a biorefinery for production of proteins, lignin, volatile fatty acids and biogas.

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Chapter 2

Review and statement of the thesis

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2.1 Chemical platform

The development of the biorefinery concept mirrors that of the petrochemical refinery, as the initial goal of petrochemical processes was to produce fuels but it progressed to include the production of many chemical and pharmaceutical products (Jones 2006). Similarly, the objective of the biorefinery is to develop a new biochemical platform which relies on renewable biomass rather than fossil fuels (Werpy et al., 2004). The ability to utilise renewable biomass, including wastes, to produce chemical compounds would reduce reliance on fossil fuels and at the same time provide a commercial opportunity for countries without their own fossil fuel resources.

In 2004 the U.S Department of Energy developed a list of basic compounds which could replace fossil fuels. This list, known as the chemical building blocks, identified 12 of the most promising chemicals from over 300 molecules. These twelve compounds include: aspartic acid, succinic acid, glucaric acid, itaconic acid, 3-Hydroxybutyrolactone, sorbitol, 2,5 – Furand dicarboxylic acid, 3-Hydroxypropionic acid, glutamic acid, levulonic acid, glycerol and xylitol (Werpy et al., 2004). In 2010 additional criteria were used to update the list, focused on the industrial development of the compounds and excluding molecules with low extend for new derivatives or with low research activities. Newly added compounds included, lactic acid, as precursor of PLA; and ethanol, a precursor of chemical compounds such as ethylene (Bozell and Petersen, 2010). Similarly, the project E4tech analysed the industrial market in UK to identify the most notable chemicals based on the market attractiveness and industrial uses (E4tech, 2017). They identified 30 compounds, with lactic acid the most attractive to be produced in the UK.

A lot of effort and investment has been put into the production of these compounds. However only some successful cases achieved industrial level production. For example, 3-hydroxipropionic acid has been widely investigated, but no further announcement of industrial production has been made (Novozymes 2014; Sims 2012). In other cases, the technological process belongs to a single company or the technology is not mature enough. For example, 1,3–propanediol and bio-esobuteno, developed by Dupont & Lyle Bioproducts and Global Bioenergies (DuPont, 2018). Some other processes have been scaled to industrial levels and are being used by different companies including the production of bioethanol, lactic and polylactic acid, succinic acid, and biogas.

2.1.1 Ethanol production

Bio-ethanol production from crops has only increased since the year 2000, despite the initial process being developed at the end of the 19th century. This is because cheap, abundant petroleum took over in the 20th century. The current change back to renewable fuels was triggered by three main

reasons: i) decrease in fossil fuel sources, ii) dependency on third countries; and iii) increasing air pollution.

2.1.1.1 Market development in USA

The petrol embargo in the summer of 1973 led to the battle for energy security and the development of the Solar Energy Research Institute (SERI), which was operational by 1977 (NREL, 2002). At the time, increasing pollution problems were addressed by some states by enforcing the use of more oxygenated compounds in gasoline to improve combustion and reduce contamination (Vinuesa et al., 2003). In the 1990s, the use of ethanol rather than fossil fuel products was promoted by a tax exemption (Duffield et al., 2015).

In 1991, the SERI was given National Laboratory Status and its name was changed to the National Renewable Energy Laboratory (NREL) to integrate all renewable energy technologies. This research centre implemented the Alternative Fuels Utilization Programme, which involved different stakeholders (i.e. government agencies, industry, universities, and coalitions), to develop competitive renewable energy technologies at commercial level (Bailey and Colucci 1997). As a result, from 1980 to 2000 ethanol production cost dropped down from $ 1,056.69 to 371.00 per cubic meter (NREL, 2002).

Finally, further progress was made in the beginning of the 21st century, when California enforced the use of bioethanol instead of fossil fuels. This was followed by other states, which made a large contribution for the development of the bioethanol industry (Duffield et al., 2015). Nowadays, the USA is the biggest producer of bioethanol with more than half of the worldwide production using different renewable sources, such as corn, sorghum, sugar wastes, beverage wastes, wheat straw and lignocellulosic materials (RFA, 2018).

2.1.1.2 Valorisation of agricultural surplus: Bazancourt-Pomacle ethanol and biorefinery

At the beginning of the 20th Century, the Bazancourt-Pomacle biorefinery site was developed and included a dedicated crop field for sugar beet, with a distillery plant. In 1950, the distillery was converted into a sugar mill factory and it was converted into a cooperative, which eventually evolved with other sites to emerge as Cristal Union. At the end of the 1980s the overproduction of sugar beet resulted in the development of a research centre for its alternative use and valorisation. At the beginning of the 1990s, a factory for production of starch and glucose was added and the Abro-Industrie Recherches et Développements (ARD) research centre was developed for further valorisation of the sugar beet, wheat cereal and alfalfa (Schieb et al., 2014). As a result of the French Governments industrial policy for competiveness clusters in 2005, the Cristanol plant was developed

for production of ethanol from sugar beet and cereal on the site in 2007. In addition, the Futurol project opened a pilot facility for production of ethanol using lignocellulosic materials as feedstock.

Currently, the site of the Bazancourt-Pomacle has evolved into a biorefinery hub using wheat, sugar beet and alfalfa, for production of sugar, starch, ethanol, liquid CO_2, surfactant and cosmetic ingredients. The current capacity of the Bazancourt-Pomacle facility is above 235,000 cubic meters per year (Schieb et al., 2014). In addition, through the financial support of local authorities the plant developed a research centre within multiple colleges and universities. This allowed for the development of biotechnological processes on industrial scales, such as the Bioamber facility plant (Schieb et al., 2014; Stadler and Chauvet, 2018). This resulted in the construction of a demo platform for additional projects, such as the development of isobutene by Global Bioenergies (Global Bioenergies, 2015).

2.1.1.3 Disseminated ethanol production from current wastes: St1 Biofuels

The Finnish company St1 Biofuels Oy, currently owned by St1, started as a joint venture in 2006 between St1 and VTT Technical Research Centre of Finland. This project was a cooperation between different stakeholders, with companies from different industrial clusters and sectors of innovation, partially supported by the Finnish Government (Mäkinen and Leppälahti 2009). First, the Etanolix technology was developed for production of ethanol using different wastes, such as bakery and sweet wastes. Afterwards, additional technologies were developed using lignocellulosic material and municipal and retail wastes, such as Cellunolix and Bionolix, respectively. Ethanol production started in 2007 with small plants in areas close to the sites where wastes were produced. These plants produced ethanol at a final concentration of 85 %. This diluted ethanol was subsequently transported to a central hub for the hydration process, resulting in a final ethanol concentration of 99.8 %.

This design, of small production sites close to the original waste production avoided transporting large amounts of the raw solid material and reduced transport costs. Thereafter, bigger additional plants were developed within different industries to use their current wastes as the main sources, offering other advantages than only production of bioethanol (Novozymes, 2015). For example, one of the plants is located beside an enzyme production plant owned by DuPont, which allows the use of enzymes for the process, reducing costs. Another plant was built at the side of the Hartwall brewery, for simultaneous production of animal feed as co-product. Further plants were developed using municipal and retail wastes, coupled with biogas, electricity and fertilizer production.

2.1.1.4 Problems and failures during scaling-up

Although the production of ethanol from lignocellulosic materials has been possible at industrial scale, some plants have failed as their processes were economically unfeasible due to different factors.

The first commercial-scale cellulosic ethanol production facility was developed in 2012 in Italy with the Proesa technology by Beta Renewables using giant reed as feedstock (Nickel, 2014). This technology was exported to countries, including USA, China and Brazil. Nevertheless, after a couple of years the company announced its shutdown for economic reasons (Patel et al., 2019). There were several problems related to the source of feedstock and mechanical issues during the up-stream process when using technologies typically applied to other lignocellulosic feedstock. For example, the higher difficulty of treating corn stove instead of wood chips (Slupska and Bushong 2019), or cane leaves rich in silica that caused greater damage than wheat straw during the pretreatment step (Marques 2018). This failure was attributed to a lack of expertise in agronomic and agricultural engineering specifically relating to transport, storage and pretreatment of the biomass source (Dale 2018).

GranBio (Brazil) implemented the Beta Renewables technology for ethanol production using sugarcane as lignocellulosic feedstock. The technology was supposed to hydrolyse the material within 19 hours, however it took between 48 to 90 hours (Marques, 2018). This, and other problems related with the plant production made it unfeasible at industrial scales. Subsequently, GranBio has developed their own yeast stream to improve the process (GranBio, 2015) and acquired different licenses fpr technology which improve process efficiency (GranBio, 2020); in combination with the use of a more efficient hybrid sugarcane (GranBio, 2018).

In 2009, POET developed a pilot plant as part of the Liberty Project for ethanol production using corn cob as feedstock (POET, 2009). The plant was expected to be operational by 2011, however difficulties related to the pretreatment process held ethanol production up until 2017 (Kennedy, 2017), increasing the cost of the plant and the delaying commercialization of the technology (Slupska and Bushong, 2019). Eventually by 2019 as a results of a loss of competitiveness for cellulosic biofuels with the decrease of the volume obligations in USA, POET-DSM announced that the production of ethanol would be paused. Then, all the efforts was shifted towards research and development for the improvement of the technology (POET, 2009).

The production of ethanol from microalgae was a trending topic at the beginning of the 21st Century. Many companies developed different technologies, such as engineered microorganisms, for the production of the ethanol (Ziolkiwska and Jadwiga, 2014; de Farias Silva and Bertucco, 2016). These approaches promised to achieve much higher quantities and yields than the current ethanol production technologies can produce (Khanra et al., 2018). Moreover, during upscaling to industrial plants, the production and the recovery yield were much lower than expected, making the process economically unfavourable (Lane, 2015). As a result, many of these companies closed or shifted to

conversion of ethanol or biofuels to more valuable compounds such as cosmetic supplements, nutraceuticals, pigments, human and animal feed (Wesoff, 2017).

2.1.2 Bioplastics

Nowadays the term of bioplastic is poorly defined, but in general it represents plastics originating from renewable sources which are fully biodegradable (Li, K., and Juliane 2009). Different studies have investigated bioplastic production, and identified some of the best candidates that fit this description such as PLA and polyhydroxyalkanoates (PHA) (Albuquerque et al., 2007; Braunegg et al., 1998; Shen et al., 2009; Siracusa and Ingrao, 2016). These bioplastics are both polyesters with potential for use in biomedical and food packing, which are synthesized through the fermentation of renewable resources by microorganisms.

Industrial production of PHA started in 2010 with an estimated production of 50,000 tons of commercial grade PHA named Mirel, developed by Metabolix and Archer Daniels Midland Company under the name Telles. However, in 2012 the closure of the plant was announced due to uncertainty around the projected capital and production (DiGregorio, 2009; Laird, 2012; Metabolix 2012). Although some other attempts have been announced (Laird, 2018; Bio-on 2015a), no further industrial production of PHA has been done so far.

The commercial production of PLA is well developed, with several markets including, chemical production for cosmetics, or automotive industry or the food industry. A joint venture between Cargill and Dow started the production of PLA, using corn as a feedstock in 2001 under the trade name NatureWorks (Larson, 2010). Subsequently, Cargill acquired whole control of the company and changed the name to NatureWorks, doubling the production capability to 140,000 tonnes per year.

Sulzer Chemtech opened a pilot plant in Switzerland in 2011. The total production capacity of this plant was 1,000 tonnes of PLA. This plant was equipped with their own technology which relied in a new cost effective polymerization process (Corbion, 2008). The aim of the plant was to show customers the polymerization process on a one-to-one basis before acquiring this technology. Indeed, one of the developments of this company led to an improvement in the heat resistance of the current bioplastics and make them more competitive in price, employing a cost-efficient polymerization process using lactide monomers to produce high-quality PLA. This new PLA product endures temperatures of up to 180°C, making it possible to use this material in the automotive, electronic and textile industries. This technology was implemented by NatureWorks in 2013 to upgrade the quality of the product and the process up to 150,000 tons/year (***Figure 2.1***) (NatureWorks, 2012).

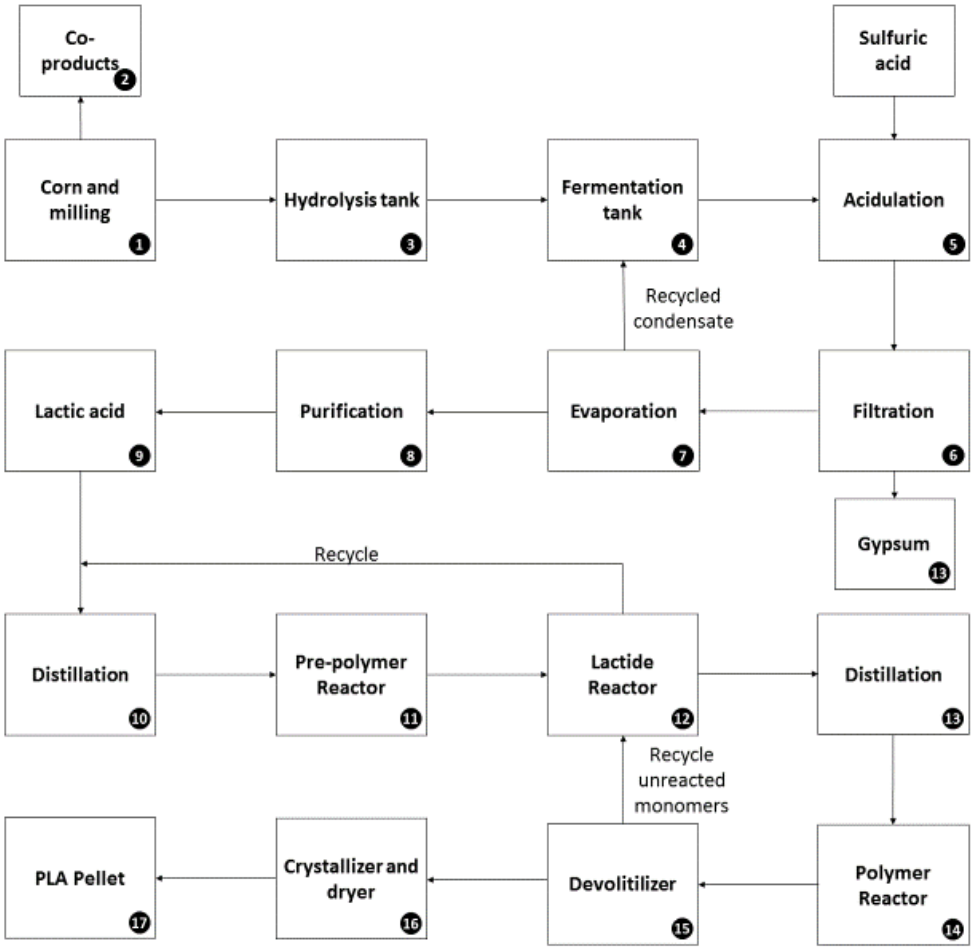

Figure 2.1 Diagram of polylactic acid (PLA) production at the Natureworks plant using Ingeo technology (adapted from: Vink et al., 2010; Erwin and Steve, 2015)

In 2007, a new competitor joined the PLA market, known as Futerro, a joint venture between Total Petrochemicals and Galactic, the former being a producer of lactic acid. They developed a production plant in Belgium in 2010 with a production capacity of 2,000 tonnes/year (Laird, 2011). This joint venture ended in 2016, when Galactic took full ownership of Futerro. At the same time, Total Petrochemicals started a new joint venture with Corbion to develop a new production plant in Thailand. This plant owned by Total Carbion PLA also uses Sulzer's polymerization technology and Corbion's technology. Initially, they developed a pilot plant with a capacity of 1,000 tonnes per year for further optimization prior scaling up to industrial level (Corbion, 2008). Finally, a plant producing 75,000 tonnes of PLA per year was opened in 2019 (Morão and de Bie, 2019).

Since the break-up of the joint venture, Futerro developed the Loopla technology. This technology allows the reconversion of used PLA into lactic acid and subsequent production into new PLA material (Laird, 2011). In 2018, Futerrro, Sulzer and TechnicFmc developed the PLAnet^{TM} initiative. The aim of the company is to develop, commercialize and support the integral development of PLA facilities of

up to 100,000 tonnes per year (Sulzer, 2019). This includes the production of lactic acid from biomass, purification of lactide and polymerization to obtain PLA.

2.1.3 Succinic acid

DNG Green Technology was formed in 2008 as a spin off from Diversified Natural Products. The new company retained all intellectual property, join ventures, employees and contracts related with to succinic acid production (Runge, 2014). In the same year, they developed a joint venture named Bio-Amber with Abro-Industrie Recherches et Développements (ARD), which resulted in the development and construction of the BioDémo platform in Bazancourt-Pomacle, beside the sugar mill owned by Cristal Union and the ethanol production plant mentioned in section (***2.1.1.2***) (Runge, 2014; Stadler and Chauvet 2018). This plant initially produced 2,000 cubic tonnes of succinic acid using modified yeast which was developed by the U.S. DOE under the Alternative Feedstocks Program (Ebert, 2007). This bacteria achieved the theoretical maximum conversion of glucose into succinic acid at lab scale, however its implementation at industrial scale resulted in a lower conversion (Carlson et al., 2016). Some other additional problems were related with the cost of base chemicals to prevent decreasing of the pH and resultant salt precipitation during the recovery phase (Hoeven, 2016). These issues were solved at the pilot facility by changing the bacteria into a yeast strain developed by Cargill, operating at a lower pH that also simplified the downstream process (Rokem, 2020). Before finishing the development of the pilot facility, in 2010, DNP Green Technology acquired the whole control of the joint venture, changing its name to Bioamber Inc (Runge, 2014).

Bioamber established a new joint venture with Mitsue & Co in 2011 to develop an industrial plant for succinic acid production in Sarnia (Canada) with an initial capacity of 17,000 tonnes, and potential of up to 35,000 tonnes. Beside succinic acid, the plant aimed for the production of 23,000 tonnes of 1,4-butanediol per year. This joint venture planned the construction of another plant in Thailand by 2012 and a third one in North America or Brazil (Jagger, 2011). However, by 2016 another company, Mater-Biotech (Italy), claimed to open the first commercial biobased 1,4-butanedioal plant in 2016 (Novamont, 2016). In 2017 after Bioamber showed the increasing number of sales, the company bought all the shares to Mitsue and gained the whole control of the company (Songer, 2017). In 2018 Bioamber had liquidity constraints and finally declared bankruptcy (Guzman, 2018). Finally the plant was acquired by LCY Biosciences, who has completed recently the preparations of the plant to resume the production of succinic acid (LCYGroup, 2020).

2.1.4 Biogas production from complex/mixed wastes

Biogas production is traditionally just a tool for waste processing and cost reduction, instead of fuel optimization. For example, in the dairy industry the driving force of using anaerobic instead of aerobic

technologies was to avoid the aeration system and a significant reduction of the biomass production that must be treated downstream (Chetty and Pillay, 2015). However, the number of plants in Europe that are producing biomethane as renewable gas has increased in the period 2018 – 2020 from 483 to 729 plants (EBA, 2020). The countries with the largest number of biomethane plants are Germany, France and the United Kingdom, corresponding to 232, 131 and 80 plants, respectively. In contrast, only one plant in Ireland is producing renewable biogas (EBA, 2020).

An Irish company, Green generation uses wastes from different industries, including prepared industrial food waste, such as dough; lignocellulosic material; animal fats and pig and cow slurry for the production of biogas. This biogas is upgraded within the plant, which allows its injection to the gas grid since 2020 (EBA, 2020). The remaining fraction after the anaerobic digestion is used as fertilizer. Another example of biogas production is Dairygold, a large dairy company in Mitchelstown (Ireland) that reuses the biogas produced in their own plant. This company has built the largest above-ground reactor for treatment of dairy wastewater. Besides their own wastes, this reactor allows the treatment of many wastewaters from industries in the surrounding area.

Incorporating the upcycling process of the biogas to obtain higher additional value compounds could increment the competitiveness of the biorefineries by increasing the range of final products (Hagman et al., 2018). For example, the CO_2 after biogas upgrading, and the liquid fraction after the anaerobic digestion could be used for microalgae production (Jebali et al., 2018). Furthermore, existing biogas plants have been proposed to become a core element of biorefineries (Andersen et al., 2018). Indeed, the anaerobic digestion process could be tuning for the production of chemical compounds if methane production is inhibited.

2.2 Anaerobic digestion

Anaerobic digestion is the process whereby raw material is converted to methane and carbon dioxide by microorganisms in absence of oxygen. These microorganisms co-operate and interact to break down complex organic polymers into smaller molecules, and finally to biogas, mostly composed of methane and CO_2 (Azman et al., 2015). This process mainly consists of four different steps: i) hydrolysis, ii) acidogenesis, iii) acetogenesis and iv) methanogenesis (***Figure 2.2***) (Schink, 1997; Monlau et al., 2011; Kleerebezem et al., 2015).

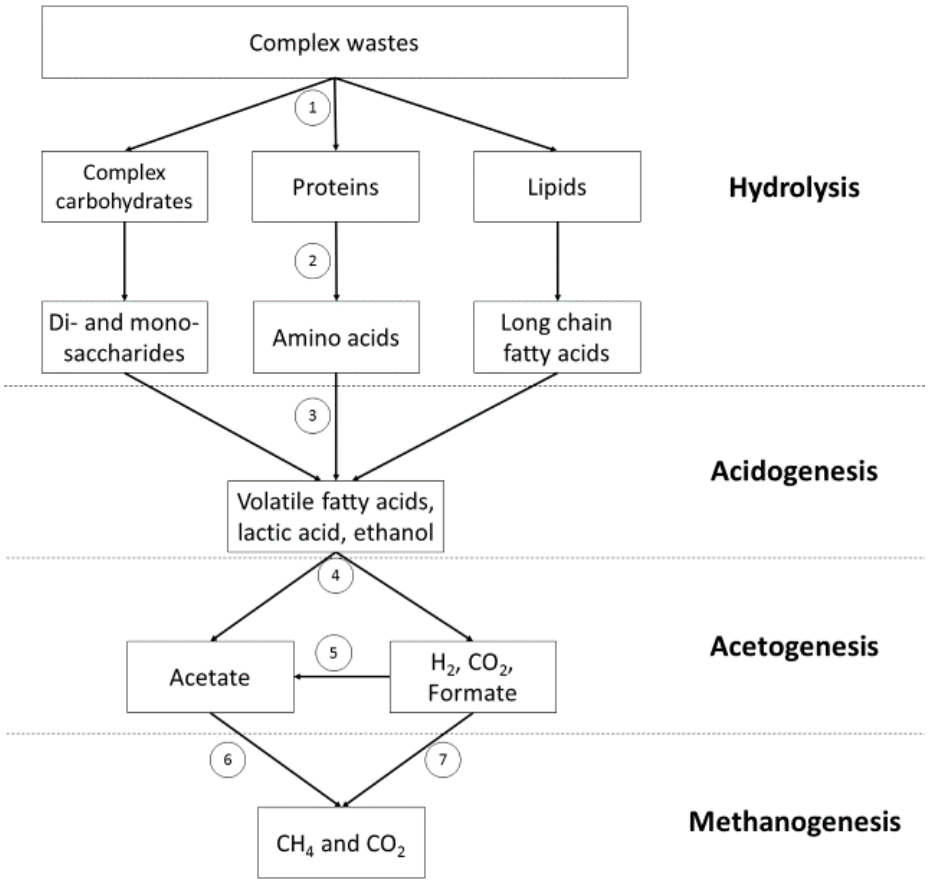

Figure 2.2 Anaerobic carbon degradation of complex wastes until CH_4 and CO_2. 1: hydrolytic microorganisms, 2: primary fermenting bacteria, 3: primary fermenting bacteria, 4: secondary-fermenting (syntrophic) bacteria, 5: homoacetogenic bacteria: 6: acetate-cleaving methanogens; 7: hydrogen-oxidizing methanogens (adapted from: Schink, 1997; Kleerebezem et al., 2015)

2.2.1 Hydrolysis

Organic material is decomposed into soluble molecules by hydrolytic microorganisms, such as hydrolytic bacteria, fungi or cellulose-degrading bacteria (Liang et al., 2014). For example, the degradation of proteins to amino acids, lipids to long chain fatty acids, or cellulose and hemicellulose to disaccharides and monosaccharides, e.g. cellobiose, maltose, glucose, xylose or arabinose. However, the accumulation of some of these hydrolysed compounds, such as humic acids or reducing sugars, could inhibit hydrolysis (Andrić et al., 2009; Azman et al., 2015).

2.2.2 Acidogenesis

The decomposition and fermentation of soluble organic substances to short chain compounds is known as acidogenesis. These compounds include lactic acid, ethanol or volatile fatty acids (VFAs) (Nzeteu et al., 2018; Teixeira et al., 2020). The production of the different middle compounds is influenced by the starting substrate. Thus, a change in the substrate composition could shift the production of intermediate compounds. In addition, operational conditions and other factors alter

process functions and could trigger the accumulation of the middle fermented compound (Itoh et al., 2012). If these compounds exceed the inhibitory threshold concentration, it could affect the next steps of the anaerobic digestion process.

2.2.3 Acetogenesis

Bacteria further degrade intermediate compounds to acetate and hydrogen, which are used by methanogenic archaea in the final step. Methanogens can only use acetate, hydrogen, carbon dioxide and one carbon compounds, such as formic acid, to produce methane and carbon dioxide (Schink, 1997). However, if hydrogen is not consumed and the hydrogen partial pressure increases in the system, acetate production becomes thermodynamically unfavourable (Siriwongrungson et al., 2007). Thus, there is an obligate syntrophic relationship between the acetogens and methanogens, whereby methanogens keep hydrogen levels low allowing acetate production to proceed (Schink, 1997; Conrad, 1999).

2.2.4 Methanogenesis

Acetoclastic methanogens use the acetate and hydrogenotrophic methanogens use carbon dioxide and hydrogen for the methane production (Schmidt and Ahring, 1991). Therefore, hydrogenotrophic methanogens are in competition with the homoacetogens for hydrogen availability (Kotsyurbenko et al., 2001). Under normal circumstances, methanogenic hydrogen oxidation is favoured as it is energy favourable (Schink, 1997). Although, there are some situations where homoacetogens are more active, for example, in slightly acidic conditions or at lower temperatures (Schink, 1997, Kotsyurbenko et al., 2001).

Some other situations could result in reduced or completely inhibited methane production. For example, low pH, increased organic loading rate or increased ammonium concentration (Shi et al., 2017; Infantes et al., 2011). This could lead to accumulation of fermented compounds instead of methane, for example, succinic acid, lactic acid or volatile fatty acids. Lactic and succinic acid are currently produced at industrial scale from renewable sources, however in the case of the volatile fatty acids their production relies on fossil sources.

2.3 Volatile fatty acids

Volatile fatty acids are short chain fatty acids (C2 to C6) with a large range of applications and their current industrial production relies on fossil fuel sources (Strazzera et al., 2018). VFAs can be used for the synthesis of a broad range of different chemicals at industrial level (Kim et al., 2018). Many studies have focused on the simultaneous production of VFAs and hydrogen (Garcia-Aguirre et al. 2017; Zhu and Yang 2004; Jiang et al. 2009), however most of these consider hydrogen as the main product and are not focused on optimization of VFA production (Infantes et al., 2011; Kargi et al., 2012; Ghimire et

al., 2015). For this last process focused on hydrogen, many researchers use the term of dark fermentation (Cisneros-Perez, 2017). Other researchers focusing on the production of VFAs use the term of acidogenic fermentation (Ramos-Suarez et al., 2021).

The main problem for VFA production at large scales is that their recovery is difficult at the low concentrations obtained during the anaerobic fermentation (Kim et al., 2018), meaning that VFAs are often considered intermediate compounds instead of a final product. Consequently, the main use of the VFAs to date has been for the production of other chemicals, which are easier to recover such biopolymers, lipids or larger carboxylic compounds (Albuquerque et al., 2011; Agler et al., 2014). There are different approaches for VFA production. The accumulation of some chemicals in the broth could inhibit the last steps of the anaerobic fermentation process, for example the increase of the ammonia concentration which led to the collapse of the methane production (Shi et al., 2017). Some operational conditions can be manipulated to promote VFA production and inhibit methanogenesis such as, temperature, pH and organic loading rate (Kargi, Eren, and Ozmihci 2012). Biomass pretreatment is also often used to inhibit methanogens, for example heat pretreatment, pH shocks or wash-out (Cisneros-Perez et al., 2017, Pugazhendhi *et al.*, 2017). However, biomass pretreatment can also have a negative impact on homoacetogens, which could decrease the range of the VFA produced (Zhang *et al.*, 2007; Larriba *et al.*, 2016; Rodríguez-Valderrama *et al.*, 2019; Menezes and Silva, 2019; Pugazhendhi *et al.*, 2017).

2.3.1 Production approach

The use of some operational conditions or accumulation of inhibitors could change the final product of the AD process from methane to VFAs (Agler *et al.*, 2013; Lu *et al.*, 2015; Chapleur et al., 2016). However, VFAs can have a self-inhibitory effect if their concentration in the broth is high (Kaur et al., 2020). Therefore, the approach followed during this thesis to prevent methane production was the use of a high organic loading rate that triggered VFA accumulation. This was achieved by using a lignocellulosic biomass as a feedstock to obtain a high concentration of organic compounds. However, the increase of concentration of the VFAs could affect the hydrolysis, acidogenesis and acetogenesis (Siegert and Banks, 2005; Jankowska et al., 2017). Indeed the accumulation of the VFAs or other fermented compounds can trigger the metabolic fluxes resulting in the shift of different fermented compounds over time (Ciranna *et al.*, 2014; Regueira *et al.*, 2018; Saady, 2013), which should be studied for the stability of the process. Also, a similar effect for shifting the end products has been reported when combining and changing different types of wastes overtime (van Aarle et al., 2015).

pH and temperature are key in the production of the VFAs. For example, operating the system under thermophilic conditions improve hydrogen production, meanwhile changing the temperature

to mesophilic conditions resulted in a decrease of hydrogen and increase of the VFA production (Kargi et al., 2011). Lowering temperature was previously demonstrated to shift the primary fermentation product from acetic to butyric acid (Infantes et al., 2011). Operating the system at higher pH resulted in a decrease of butyric acid concentration (Uke and Stentiford, 2013), and an increase in propionic acid production instead of acetic acid (Infantes et al., 2011).

To understand and evaluate the effect of the VFA accumulation and process fermentation, two different approaches were investigated during the development of this thesis. The first was a focus on the simultaneous hydrolysis and acidogenic fermentation of lignocellulosic feedstock (***Chapter 3***). The second approach was based on a two-step process, first hydrolysis was carried out to obtain liquid hydrolysate rich in carbohydrates (***Chapter 4***) then, this liquid hydrolysate was used for VFA production (***Chapter 5***) and further studies was carried by the simultaneous VFA production and recovery (***Chapter 7***).

2.3.1.1 Simultaneous hydrolysis and acidogenesis

The simultaneous degradation and fermentation for VFA production involves the feasibility of treating the feedstock in a single step, resulting in a lower energy demand and the minimal control requirements for simultaneous hydrolysis and production of the fermented compounds. In this case, a long hydraulic retention time (HRT) (10 to 49 days) is required in order to degrade the solid fraction (Li et al., 2014, Nzeteu et al., 2018; Texeira et al., 2020).

Besides physical and chemical treatment, other techniques used to improve anaerobic digestion of lignocellulosic feedstock include, codigestion (Ngan et al., 2020), and bioaugmentation with hydrolytic microorganisms (Martin-Ryals, et al., 2015). Codigestion is widely applied for methane production from complex feedstock, for example the use of lignocellulose or feedstock with a high lipid content as the sole carbon source could result in an unbalance in the anaerobic digestion process (Ngan et al., 2020). This approach is used at industrial level for the degradation of complex mixtures of feedstock (Angelidaki and Ellegaard, 2003). Nevertheless, some lignocellulosic feedstock is still too recalcitrant for microorganisms to break down and additional pretreatment is required (Singh and Kumar, 2019). As an alternative, bioaugmentation can be used to improve the native hydrolytic community for degradation of cellulose and hemicellulose by introducing microorganisms with a better hydrolytic activity (Haruta et al., 2002; Aydin et al., 2017; Tsapekos et al., 2017). This has been successfully used in batch reactors (Peng et al., 2014; Baba et al., 2017). However, some difficulties can occur in continuous reactors when the introduced microorganisms do no develop or stabilised population over time (Tsapekos et al., 2017; Li et al., 2020).

Some of the most common reactors for simultaneous hydrolysis and fermentation are semi-continuous stirred tank reactors (Li et al., 2014), or leaching bed reactors (Cysneiros et al., 2012; Uke and Stentiford, 2013; Li et al., 2017; Nzeteu et al., 2018; Texeira et al., 2020). In all of them, one of the major problems is the accumulation of the VFAs. This, could affect the hydrolysis of even simple molecules such as glucose (Siegert and Banks, 2005). Leaching bed reactors have been shown to be effective when operating with food waste as feedstock (Want et al., 2014; Nzeteu et al., 2018; Texeira et al., 2020). However, food waste is readily degradable and it is possible to obtain a larger amount of VFAs then when using more complex wastes, such as cellulosic feedstock (Cysneiros et al., 2012; Li et al., 2017; Texeira et al., 2020). Therefore, the screening for a suitable source of inocula with an active hydrolytic activity could improve the performance for the biodegradation of lignocellulose and simultaneous production of VFAs.

2.3.1.2 Two-step process

The main advantages of using a two-step process are a higher control of the operational conditions and a much lower hydraulic retention time. For simultaneous hydrolysis and acidogenesis the total time could be up to 49 days in a two-step system, whereas the liquid fermentation could be carried within hours. The most typical reactor configuration for the production of VFAs in the liquid phase are continuous stirring tank reactor (CSTR) (Bengtsson et al., 2008; Shen et al., 2014; Palomo-Briones et al., 2017), up-flow anaerobic sludge bed (UASB) reactors (Mota et al., 2018; Rodriguez-Valderrama et al., 2019), fixed bed reactors (Zhang et al., 2008; Pugazhendhi et al., 2017; Gorgec and Karapinar, 2019), packed bed reactors (Domingos et al., 2017) and expanded granular sludge bed (EGSB) reactors (de Menezes and Silva, 2019).

Two-step processes have been largely applied for hydrogen production, with volatile fatty acids considered as a by-product. Some studies have even used the resultant VFAs for the production of methane and did not consider the recovery or use of VFAs for any upgrading process (Nkongndem et al., 2015). As well, some of these studies have been more focusing on hydrogen production rather than VFAs production, pretreating the biomass in order to decrease not only the methanogens, but also homoacetogens that could consume the hydrogen in favour of VFA production (Carrillo-Reyes et al., 2012, Antonopoulou et al., 2016, Carrillo-Reyes et al., 2016).

Reactor configuration and the aggregation state of the mixed microbial culture, for example granular or flocculent sludge, have a strong influence on the microbial selection pressure by the operational conditions of the system (Cisneros-Pérez et al., 2015; Atasoy et al., 2019). For example, using the same feedstock and same pretreatment, an anaerobic fluidized-bed reactor with an up-flow velocity of 13 m/h led to a higher hydrogen and lower VFA production than an EGSB reactor an up-

flow velocity of 3 m/h (Cisneros-Pérez et al., 2015; Cisneros-Pérez et al., 2017). In order to increase hydrogen yield and volumetric production, short HRTs have been beneficial and resulted in butyric acid as the major product due to the wash-out of homoacetogens (Pugazhendhi et al., 2017; Gorgec and Karapinar, 2019; Rodriguez-Valderrama et al., 2019; De Menezes and Silva, 2019). Indeed, the use of larger HRTs can result in a more complex mixture of VFAs than a short HRT (Zhang et al., 2007). Even though inoculum pretreatment has a great impact on the initial hydrogen and VFA production, the operational parameters of the reactor have a more extended influence on VFA production (Cisneros-Pérez et al., 2015).

2.3.1.3 Volatile fatty acid recovery

Many different technologies have been evaluated for the recovery of VFAs, including liquid-liquid extraction (Reyhanitash et al., 2016; Rocha et al., 2017; Saboe et al., 2018), filtration (Zacharof and Lovitt, 2015), electrodialysis (Jones et al., 2015; Scoma et al., 2016; Tao et al., 2016; Bak et al., 2019), and microbial electrochemical cells (Zhang and Angelidaki, 2015). In addition, the emergence of new membranes in the market is attractive as their cost is decreasing and they are able to achieve a higher selectivity towards VFAs (Cohen-Tanugi et al., 2016).

The use of different compounds as extractants, additives such as amines, esters or organo-phosphourus compound, in the diluent during the liquid-liquid VFA recovery can enhance the process efficiency by up to 85 % (Kaur et al., 2020). The recovery of a larger broth of VFAs that contains caproic acid would result more efficient when using organic solvents due to the increasing alkyl length of the VFA (Rocha et al., 2017). Therefore, promoting the elongation of short volatile fatty acids into longer VFAs, for example acetic or butyric to caproic acid, could be beneficial to achieve a higher recovery efficiency. A two-step process would allow the recovery of the VFAs from the aqueous fraction using heavy solvents and extracts, followed by distillation of the VFA containing fraction (Saboe et al., 2018). Some parameters that affect the equilibrium between the VFAs in the aqueous and the organic liquid fraction are pH, acid concentration and ratio between permeate and retentate (Rocha et al., 2017; Saboe et al., 2018). An alternative method would be to promote the elongation for caproic acid production, which is easier to recover (Xu et al., 2020). The increase of the caproic acid concentration above 10 g/L in its undissociated form would result in the formation of a second hydrophobic phase allowing the easier recovery of the caproic acid (Xu et al., 2015).

Electrodyalysis combines a membrane separation system with electrochemical interaction to separate cations and anions by their electrical potential differences (Bak et al., 2019). Baroi et al. (2015) recovered butyric acid, which contained a low concentrations of acetic acid, using a pure culture in reverse electro enhanced dialysis technology, followed by the distillation of the butyric and

acetic acid using octanol. This purification step represents 35 % of the total operation costs (Baroi et al., 2017). Some important parameters for VFA recovery using electrodyalysis are the applied voltage, the volumetric ratio of the broth that contains VFAs, and the liquid fraction for the VFA recovery (Bak et al., 2019). Similar approaches have been used for lactic acid recovery and purification (González et al., 2008; Komesu et al., 2017). Zhang and Angelidaki (2015) used a microbial electrodialysis cell, by combining electrodialysis with microbial electrochemical systems. Thus, the required potential could be obtained by the oxidation of the organic matter and a lower electricity input was thus required. An additional advantages of this system was the production of hydrogen and alkali, which could contribute to the neutralization of the pH during VFA production and decreasing chemical requirements for the process (Zhang and Angelidaki, 2015).

2.4 Lignocellulosic feedstock

Lignocellulose represents the most abundant biomass which could act as a source of carbohydrates for bio-industrial purposes. However, due to its complex and recalcitrant structure, where cellulose and hemicellulose are interwoven within lignin, microorganisms cannot degrade and use it (***Figure 2.3***) (Ayyachamy et al., 2013). Cellulose is a linear semi-crystalline biopolymer formed by D-glucose units, hemicellulose is a heterogeneous polysaccharide formed by xylose, arabinose (pentoses), glucose, galactose and mannose (hexose); while lignin is an aromatic biopolymer (Ravindran and Jaiswal, 2016). Properties and the ratio of cellulose, hemicellulose and lignin vary between different types of lignocellulosic biomass (Roy et al., 2020).

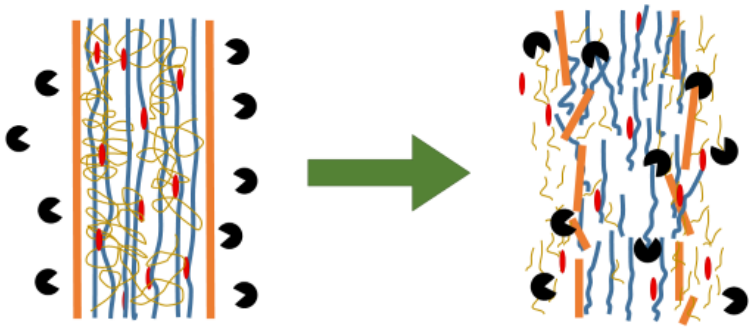

Figure 2.3 *Lignocellulose structure before and after pretreatment to break down the lignin and cellulose crystallinity to facilitate the degradation of the microorganisms for production of simple carbohydrates (adapted from Mosier et al., 2005).* ᗧ *enzymes,* ■ *lignin,* *cellulose,* *hemicellulose, and* ⬮ *proteins*

Only a small group of microorganisms have the ability to break down the lignin (Sanchez, 2009). Thus, to make this material susceptible for microbial degradation it is important to break the lignin outer layer and crystalline structure of the cellulose (Mosier et al., 2005; López-Linares et al., 2020). Physical processing of the material does not largely contribute to improve enzymatic digestibility and is quite limited when compared with chemical pretreatment methods (Niemi et al., 2012; Niemi et al.,

2013). The use of acid method is quite extended, with sulfuric acid used the most (Wagner et al., 2021). However, this approach could also convert the carbohydrates into compounds which inhibit downstream fermentation processes, such as furfural or 5- hydroxymethylfurfural (Yoon et al., 2014; Tizazu and Moholkar, 2017). Alkali hydrolysis has also been studied as pretreatment for the breakdown of the lignocellulose (Ravindran et al., 2018) or organosolvent pretreatment targeting lignin degradation, having both a more limited effect on cellulose degradation than acid pretreatment (Patel et al., 2018; Oliva et al., 2020). Additionally, the use of ionic liquids is increasing as an alternative to traditional organic solvents due to lower vapour pressure and being more environmental friendly (Liu and Chen, 2006). Ionic liquids are organics salts composed of organic cations and an organic or inorganic anion (Karimi et al., 2013). Based on the nature of the ionic liquid, this could dissolve the lignin structure to facilitate enzymatic hydrolysis (Ma et al., 2016). In addition, the ionic liquid could be recycled for sequential pretreatments without losing its potential (Xu et al., 2017).

The different combined parameters for the chemical hydrolysis (temperature, time and acid concentration) can be integrated in a single value known as combined severity factor. This factor is based on the Arrhenius equation, but it fits the multiphasic behaviour for lignocellulose degradation with an initial high rate of lignin loss of pseudo-first-order, followed by the bulk-phase delignification, and a very slow final dissolution (Chum et al., 1990). This factor was widely evaluated for the effectiveness of carbohydrate production (Carvalheiro et al., 2004), or as pretreatment to enhance the enzymatic hydrolysis (Zoulikha et al., 2015).

One parameter used to determine the degradation of the lignocellulose structure is the water retention capacity. This represents the ratio between the amount of water retained and the weight of the solid fraction, which is affected by the composition of the material, the particle size and pore size (Robertson et al., 2000; Raghavendra et al., 2004, Saw et al., 2008; Lara-Vazquez et al., 2014). Based on the severity of the pretreatment and the change of the composition, the ability of the lignocellulosic material for water retention could increase or decrease. For example, dissolving fats in the lignocellulosic matrix increases the water retention capacity (Raghavendra et al., 2004), whereas the destruction of fibre results in a loss of water retention capacity (Célino et al., 2014). Therefore, the hydration dynamic can contribute to understanding the effectiveness of the applied treatment (Sanchez et al., 2019) and could predict the status of the lignocellulosic feedstock prior to enzyme addition.

Many studies focused on different pretreatment methods for the recovery of high-value compounds, such as fibres, proteins or phenolic compounds from lignocellulosic material, instead of carbohydrates (Martínez et al., 2012; Valadez-Carmon et al., 2018). The valorisation of current

lignocellulose residues into more valuable products would bring new opportunities for industrial development in the biorefinery field. For example, the combination of mechanical homogenization, thermal hydrolysis and chemical precipitation for recovery and purification of pectin using cocoa waste as feedstock (Muñoz-Almagro et al, 2019). This biorefinery approach using cocoa wastes would use a series of different steps to extract polyphenols, antioxidant compounds and use the remaining fraction for chemical and biogas production (Martínez et al. 2012; Valadez-Carmona et al. 2018; Handojo et al., 2019). Some examples are the recovery of high value compounds such as pectin and phenolic compounds from pomegranate peels with production of bioethanol using the remaining solid fraction (Talekar et al., 2018), or the recovery of different compounds from citrus peel wastes such as essential oils, pectin, succinic acid and fertilizer (Marin et al., 2007; Patsalou et al., 2020).

Certainly, some of these products have a higher value than carbohydrates and their recovery should be targeted during the production of carbohydrates. For example, as explained in section ***2.1.1.4*** of the current chapter, the use of microalgae for carbohydrate and ethanol production was considered a failure, however, shifting ethanol to higher value compounds made the process feasible (Wesoff, 2017). Thus, hydrolysis should be focused on the fractionation and recovery of the different high value compounds from lignocellulosic feedstock and resultant residues can be used for carbohydrate or biogas production. This approach is known as cascade process, which becomes an important part of the biorefinery approach developed during the current PhD thesis. Brewery spent grain (BSG) is a by-product produced in large volumes, with low or no value from beer preparation with a stable composition, rich in proteins, carbohydrates and lignin, with a constant supply. This makes brewery spent grain an optimal candidate for a biorefinery using a cascade process for recovery of the different high valuable compounds.

2.5 Brewery spent grain

The basic ingredients for beer production are water, barley, hops and yeast. Barley is processed by briefly submerging it in a tank with water and placing it in a germination vessel to promote the natural release of some of the carbohydrates, such as the starch contained in the endosperm (***Figure 2.4***). Before germination is complete, the grains are dried to a final water content between 4 – 5 % to avoid the consumption of the carbohydrates available inside the grain. This is known as malted barley and is then ground, mixed with water and boiled to obtain a sweet liquor (Arendt and Zannini, 2013). This sweet liquid, known as the wort, is recovered for downstream processing, and the residual solid fraction left with none or low value is known as brewery spent grain. The latter is the most abundant solid waste from the brewery industry with, approximately, around 15 – 20 kg of BSG produced per hectolitre of beer (Mussatto *et al.*, 2006). Just in Ireland, in 2019, the production of beer was above

8.2 million cubic meters (IBA, 2020). Thus, over 170,000 tonnes of this brewery spent grain was produced.

Brewery spent grain has a dry weight content of around 23 – 31 % of proteins, 19 – 26 % lignin, 17 - 22 % hemicellulose and 25 – 31 % cellulose (Mussatto *et al.*, 2006; Vieira *et al*. 2014; Rommi *et al*. 2018). Some compounds of brewery spent grain have shown prebiotic and immunomodulatory activity. The antioxidant and phenolic content strongly depends on the variety of barley and the malting processes during the brewery process (Moreira et al., 2013).

The recovery of carbohydrates from brewery spent grain has been largely investigated to use them for production of different chemicals, including, for production of bioethanol (Wilkinson et al., 2014; Liguori et al., 2015; Rojas-Chamorro et al., 2020), biobutanol (Plaza et al., 2017; Giacobble et al., 2019; López-Linares et al., 2020), lactic acid (Mussatto et al., 2007; Pejin et al., 2018) or lipid (Patel et al., 2018). Some other researchers have focused on the recovery of bioactive compounds (Cermeño et al. 2019), phenolic extracts (McCarthy et al., 2012; Stefanello et al., 2018; Connolly et al., 2019) or protein extraction (Niemi et al., 2013; Connolly et al., 2013; Qin et al., 2018). However, there are few studies that evaluate the simultaneous recovery of carbohydrates and other fractions such as lignin or proteins (Niemi et al., 2013; Rommi et al., 2018). The protein and carbohydrate fractions are closely related, as the latter behave as barrier for enzymatic and mild chemical protein extraction (Niemi et al., 2013). The husk, composed mainly of cellulose with a small fraction of lignin, and the pericarp act as protective cover for the grain (Jaeger et al., 2021). Inside, the grain the aleurone that surrounds the endosperm costumes of a protein rich fraction (***Figure 2.4***). As well, additional protein are interwoven with hemicellulose forming additional cell walls such as the scutellum (Arendt and Zannini, 2013).

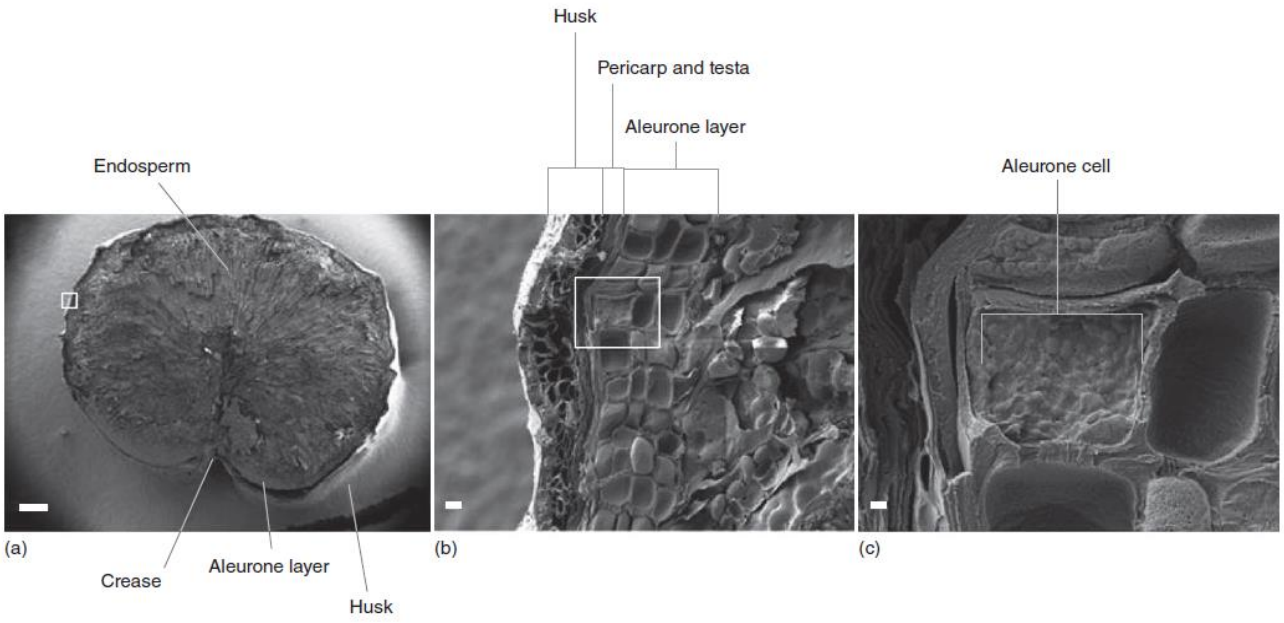

Figure 2.4 *Structure of the barley kernel a) sectioned transversely, b) detailed husk, and c) starchy endosperm (Arendt and Zannini, 2013). Permission for using the image from Elsevier*

The recovery of different fractions from brewery spent grain, including a protein rich solid fraction, a phenolic rich solid fraction and a carbohydrate rich liquid fraction could contribute to the development of a biorefinery. Furthermore, if a cascade process could be carried out within the brewery, even the protein and phenolic fraction could be used as biospecialities for food ingredients, or as source of human and animal dietary products and prebiotic compounds (Cermeño et al., 2019). As the mixed carbohydrates in remaining liquid fraction does not have high value, this can be used in a downstream fermentation for production of chemicals, such as the volatile fatty acids.

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Chapter 3

Biodegradation of brewery spent grain by mixed microbial cultures from anaerobic sludge and animal dungs

A modified version of this chapter has been submitted as:

> **Castilla-Archilla J.**, Thorn C., Pau S., Lens P. N. L. (2022) "Screening for suitable mixed microbial consortia from anaerobic sludge and animal dungs for biodegradation of brewery spent grain" Submitted to Biomass and Bioenergy (major revisions)

Keywords: Lignocellulose; brewery spent grain; mixed microbial culture; hydrolysis; anaerobic digestion; volatile fatty acids

Abstract

Brewery spent grain is a lignocellulosic waste and is a valuable resource from a biorefinery perspective. Bioaugmentation with specialised inocula can be a viable means of improving the degradation of this recalcitrant feedstock. This study used a biodegradation activity test to screen six different sources of microbiota for chemical and energy production from brewery spent grain. The inocula used were anaerobic granular sludge as i) intact granules and ii) crushed granules, as well as animal digestive fluids iii) bovine rumen fluid, and animal manures iv) giraffe manure, v) rhino manure, and vi) tiger manure. Despite the herbivores faeces presenting the largest hydrolytic community, the best solids destruction was achieved using anaerobic granular sludge, intact or crushed, i.e. 64.8 (± 11.9) and 63.4 (± 3.7) % VS destruction. Granular sludge also resulted in the highest gas production, followed by the giraffe manure. Contrastingly, the highest volatile fatty acid accumulation was achieved using rhino and tiger manure, i.e. 3.86 (± 0.55) and 3.20 (± 0.74) gCOD/L, respectively. Interestingly, the poorest solids destruction was with the inocula that accumulated the largest amount of fermented compounds. Further research is required to determine the inhibitors of the hydrolysis and degradation of brewery spent grain.

3.1 Introduction

The finite availability of fossil fuels in conjunction with increasing concern into environmental sustainability and guardianship have led to the development of the biorefinery concept. This is the sustainable process of transforming renewable biomass, through a combination of physical, chemical and biological processes, into a spectrum of products and energy equivalent to fossil fuel-based products (Biorefinery Euroview, 2009; Biopol, 2009). Anaerobic digestion (AD) is one the most extensively applied methods for the treatment of organic solid wastes for sustainable energy production. The process of AD, whereby raw material is finally converted into methane and carbon dioxide, can be defined by four sequential steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis. While historically the desired end product of AD was methane for energy production, more recently it has been adapted as a means of producing higher value intermediary compounds, such as succinate, short chain fatty acids and ethanol (Infantes et al., 2011; Garcia-Aguirre et al., 2017). This can be achieved through the partial inhibition of some of the final steps of AD, leading to the accumulation of intermediate compounds (Shi et al., 2017; Zhang et al., 2018), which are chemical building blocks (Bozell et al., 2010; Castilla-Archilla et al., 2019).

Hemicellulosic and lignocellulosic wastes represent promising feedstock sources for such AD biorefineries due to their abundance and availability (Lynd et al., 2015). Industrial lignocellulosic waste, with low or no value, can provide a constant supply of renewable biomass. A potential source of such a feedstock is from the brewery industry, whose most abundant solid waste is brewery spent grain (BSG). This is a solid stream rich in carbohydrates, lignin, proteins and lipids (Mussatto et al., 2006; Vieira et al., 2014; Rommi et al., 2018) where the average production corresponds to 15-20 kg of BSG produced per hectolitre of beer (Mussatto et al., 2006). In Ireland in 2019, more than 8.2 million cubic meters of beer were produced (IBA, 2020), thus over 170,000 tonnes of this material was produced. However, the use of lignocellulosic material in AD is not well implemented when compared with other more easily biodegradable feedstocks. This is a consequence of the complex structure of lignocellulosic feedstocks, which renders them difficult for microbes to degrade rapidly, therefore resulting in low rates of solid destruction. Additionally, this process can be inhibited by compounds produced during AD, such as humic acids, phenolic compounds or volatile fatty acids (VFAs) (Fernandes et al., 2015; Chapleur et al., 2016; Azman et al., 2017).

The direct AD of some lignocellulosic materials has been studied but the resulting methane production and solid destruction were inefficient (Singh et al., 2019). To improve this, different approaches have been tested, including co-digestion, chemical addition and more recently bioaugmentation (Tsapekos et al., 2017; Wall et al., 2018). The purpose of bioaugmentation is to enhance the native hydrolytic community capable of degrading cellulose and hemicellulose by

introducing more performant hydrolytic microorganisms (Angelidaki et al., 2000; Haruta et al., 2002; Li et al., 2020). This has been applied as a strategy in batch systems where improved overall methane production demonstrated the benefits of bioaugmentation using pure cultures (Peng et al., 2014) and mixed cultures from rumen fluid (Baba et al., 2017). However, when operating systems in continuous mode, microorganisms typically had to be periodically inoculated into the reactor as their positive effect diminished over time (Martin-Ryals et al., 2015; Tsapekos et al., 2017; Jiang et al., 2020). In the work of Li et al. (2020) however, the benefits following bioaugmentation with *Bathyarchaeota* enriched culture took up to four months to stabilise in a continuous digester, resulting in significant positive effects on the biomethane production from cow manure digester. These remained, although slightly decreased, once bioaugmentation stopped as the supplemented microorganisms became a stable part of the microbial community over time (Li et al., 2020). Indeed, the use of mixed microbial cultures was shown to have a higher robustness to become adapted on process performance and more resilient for lignocellulose degradation (Rahman et al., 2002; Liu et al., 2018; Li et al., 2020). However, the continuous addition of any pure culture, cellulolytic bioculture or mixed microbial culture to promote the bioaugmentation along time would result in additional costs. Therefore, the use of an inoculum that contains a stable hydrolytic activity in its native composition could result in an advantage.

The goal of this chapter was to evaluate and compare different inocula during AD to identify the most suitable consortia for the degradation of BSG. For this purpose, a range of inocula were selected where a different microbial composition was expected as a result of their origin and the content of intestinal track of animals that feed on lignocellulosic materials. These included anaerobic granular sludge from an industrial dairy wastewater treatment facility. Both intact and crushed sludge were tested, where we hypothesised crushed sludge might perform better due to enhanced substrate availability for all microbes compared to those in intact granules. Bovine rumen fluid from cow was used as the microbial consortia present were expected to have strong hydrolytic activity. In addition, faeces from various zoo animals were used, namely giraffe, rhinoceros and tiger. These animals were chosen to represent a ruminant, a monogastric herbivore, and a carnivore as a control with a differentiated microbial community than the herbivore animals. We hypothesised these would serve as candidate sources of microbiota capable of hydrolysing the complex biomass while allowing for the simultaneous production of valuable compounds and/or energy. To assess this, we measured the rate of biomass degradation, the production of fermented compounds (alcohols, VFAs and lactic acid) and biogas. In an effort to elucidate the microbial consortia contributing to the production of these various compounds, the prokaryotic microbial community from the various inocula was characterised using 16S rRNA profiling at the start of the trial and once most biodegradation had occurred (day 21).

3.2 Material and methods

3.2.1 Feedstock

Fresh BSG was supplied by a local lager brewery in Galway (Ireland), and once collected, the BSG was frozen in individual bags. Total solids (TS) and volatile solids (VS) concentrations were determined prior to freezing. Before use, frozen BSG was defrosted overnight at 12°C.

3.2.2 Source of inoculum

Six different sources of inocula were tested. Granular sludge was collected from an up-flow anaerobic sludge bed reactor treating dairy wastewater for methane production at ambient temperature (Kilconnell, Co. Galway, Ireland) and kept at 4°C until further use. This sludge was used for two of the inoculum sources: i) granular (intact) sludge and ii) as crushed sludge (granules were crushed using a Homogenizer Safe-Seal 30 mL, GPE Scientific, UK).

The cow rumen fluid consisted of a composite sample of the rumen contents from the four different stomachs of three different cows removed during meat processing at a slaughterhouse (Ballyhaunis, Co. Mayo, Ireland). The solid fraction of the rumen fluid was retained, TS and VS were calculated, and the sample was stored 15 °C for 24 hours until use.

Three different animal manures (giraffe, rhinoceros and tiger) were obtained from Dublin Zoo (Ireland). The giraffe was selected as a rumen animal, which diet is based on alfalfa, supplemented with leaves from browsing and maintenance pellets. Rhinoceros was chosen as monogastric herbivore, therefore hindgut fermenter fed mainly alfalfa, hay and haylage, as well as a concentrate of proteins. Tiger was chosen as a control with an expected lower lignocellulosic hydrolytic activity as the tiger's diet was carnivorous, fed principally with horse and pig meat. Faeces were collected and returned to the lab where they were blended using a Waring 2-Speed Laboratory blender (USA) at the highest speed. Water was added during the blending of the different inocula to ensure proper mixing, the different amount of water for each of the inoculum was subtracted later to the final water addition during the experimental set up.

3.2.3 Biodegradation activity test

Activity tests were carried out in sacrificial bottles, where each bottle (HDPE plastic) had a total volume of 500 mL and a working volume of 400 mL, adapted from Nzeteu (2016). Bottles were sealed with a rubber stopper, perforated gas tight with a needle to allow attachment of a gas bag (***Figure 3.1***). Sodium bicarbonate was used as a buffer at a concentration of 10 g/L. Bottles were fed with BSG at a concentration of 6 g VS/L with an inoculum to substrate ratio of 1:4 (1.5 g VS/L of inoculum).

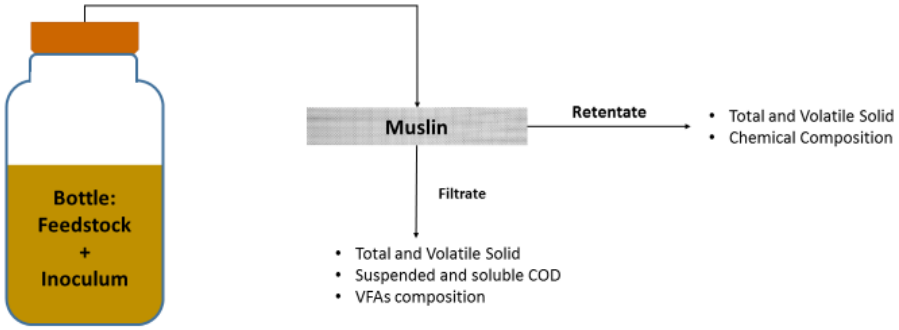

Figure 3.1 *Experimental bottle and sampling for the biodegradation activity test. A muslin was used to split the liquid and solid fraction.*

Experimental bottles were set up using the different inocula and brewery spent grain as feedstock, with the addition of the buffer to the final working volume. Control bottles were also set up consisting of inocula and buffer in the absence of feedstock to determine the contribution of organic compounds from the endogenous material present in the inocula. The inoculum was first added into all the bottles with a fraction of the buffer. The bottles were sparged with nitrogen, and placed under incubation at 37°C for 24 hours prior to feeding for acclimation of the inocula and further degradation of any organic carbon residues therein. Following acclimatization, BSG was added to the experimental bottles, and all bottles were subsequently filled with the buffer to reach the total volume required. All bottles were once again sparged with nitrogen and placed in the incubator (Innova 44) at 37 °C and 100 rpm. This was considered time 0 of the experiment. The gas bags were attached to the bottles on day 30 and were changed every day of sampling.

Destructive sampling was performed on days 0, 1, 2, 4, 7, 10, 14, 21 and 30, where five bottles were taken down at each of these day (three experimental bottles containing inoculum and substrate and two controls containing only inoculum). This was repeated for each inoculum (n=6), thus 45 sacrificial bottles were sampled on each of the 9 days. For the sampling, the content of each bottle was fractionated into leachate and retentate using muslin cloth (***Figure 1***). TS and VS were calculated for both fractions, and a small fraction of each was flash frozen using liquid nitrogen and stored at -80°C for microbial community analysis. The filtrate was used for quantification of soluble chemical oxygen demand (sCOD), VFAs composition, lactic acid, ethanol, total protein and ammonia.

3.2.4 Chemical analysis

TS and VS were quantified out using standard methods (APHA, 2012). The sCOD was determined using the Reagecon 0 to 1,500 mg/L range kit (Reagecon, Ireland) where 2 mL of sample was added to the vials which were then placed at 150 °C for 2 hours. Vials were cooled and the absorbance was measured using a DR2500 spectrophotometer (Hach, Germany). The biogas was collected in a gas

sampling bags (Tedlar SCV) and gas volumes were determined by water displacement and corrected to standard conditions (0 °C and 1 atm). Biogas composition (H_2, CO_2 and CH_4) was analysed using gas chromatography (GC; AAgilent 7890A, USA) equipped with a glass column and a flame ionisation detector. The carrier gas was argon and the flow rate was 24 mL/min. VFA analysis was performed using a Varian 450-GC equipped with CombiPal autosampler and a flame ionisation detector, following Nzeteu *et al.* (2018). Lactic acid, ethanol, proteins and ammonia were analysed using a discrete nutrient analyser Gallery Plus® (ThermoFisher, Finland) based on a colorimetric assay (O'Dell. 1996; ISO 15923-1, C, 2013).

3.2.5 Calculations

The VS fraction, gas production, VFAs and sCOD for each sample day were calculated by subtracting the average value of control bottles from the average in the experimental bottles.

The VS destruction represents the percentage between the amount of BSG consumed at each point since the beginning of the trial and the amount of VS of BSG at the beginning of the trial (***Equation 1***):

$$\%\, VS_{Destruction} = \frac{VS_0 - VS_P}{VS_0} \cdot 100 \qquad \textbf{\textit{Equation 1}}$$

Where $VS_{Destruction}$: amount of BSG consumed, VS: volatile solids amount of BSG, 0: initial point of the trial, P: sample point

The acidification yield was calculated based on the total contribution of the individual VFAs together in terms of g COD/L, divided by the overall sCOD (***Equation 2***):

$$Acidification\ Yield = \frac{Total\ VFA\ concentration}{Soluble\ COD} \cdot 100\ \ [gCOD/100\ gCOD_{Soluble}] \quad \textbf{\textit{Equation 2}}$$

The gas production rate was calculated using the difference between the cumulative gas before and after the log phase production per amount of inoculum (g of VS) (***Equation 3***):

$$GPR = \frac{V_f - V_i}{t_f - t_i} \cdot \frac{1}{VS} \quad [\frac{mL}{(day \cdot g\ VS_{inoculum})}] \quad \textbf{\textit{Equation 3}}$$

Where V: total volume, t: time in days of the log phase, $VS_{inoculum}$: amount of inoculum in terms of VS, f: end of the log phase, i: initial of the log phase.

The different rates corresponding to VS destruction, gas and VFA production were calculated using the linear phases from the graphs obtained during the trials, if possible. The number of days corresponding to the different linear phases have been given with the rate and the R-squared value for each of the rates along the experiments. For example, the methane production rate of the giraffe manure was calculated using the linear phase between days 7 to 21 with $R^2 = 0.99$ (***Figure 2D***) or in the

case of the crushed granular sludge the initial VS destruction rate was calculated only between the beginning of the trial and day 1 as no trend over time was detected.

3.2.6 Nucleic acid extractions and sequencing analysis

DNA and RNA were co-extracted from 0.75g (fresh weight) of the retentate (***Figure 1***), from sample days 0 and 21 of each of the three biological replicates from the 6 inoculum types (n=36 extractions). Further details can be found in the supplementary material of the thesis (***Supplementary information Chapter 3***).

3.3 Results

These degradation tests aim to evaluate the capability of each one of the different inoculum to degrade the BSG into valuable compounds. The initial pH for all the different conditions was above 8.00, in all the cases the pH decreased achieving the lowest value within the first week and increased after (***Table 3.1***).

Table 3.1 *pH value for all the different conditions at the beginning, lowest value achieved and end of the trial*

	Day 0	Lowest pH		Day 30
		Day	pH	
BMP - A	8.67 ± 0.03	2	7.55 ± 0.05	8.02 ± 0.15
BMP - B	8.36 ± 0.03	4	7.35 ± 0.04	8.02 ± 0.03
BMP - C	8.36 ± 0.01	4	7.01 ± 0.04	8.07 ± 0.03
BMP - D	8.26 ± 0.03	2	7.24 ± 0.04	7.56 ± 0.17
BMP - E	8.36 ± 0.01	2	7.18 ± 0.04	7.65 ± 0.29
BMP - F	8.42 ± 0.04	7	7.16 ± 0.04	8.02 ± 0.03

3.3.1 Biodegradation performance

3.3.1.1 Granular sludge

The VS destruction achieved with granular sludge followed a linear trend until day 4 (***Figure 3.2a***), with a destruction rate of -0.456 g VS_{BSG}/(L·day·g $VS_{Inoculum}$) (R^2 0.99) (***Table 3.2***). After this, the destruction rate decreased and by the last two sampling points (day 21 and 30), the VS destruction was practically non-existing.

The highest VFAs concentration achieved was on day 2, with 0.32 (± 0.07) g COD/L, while the total sCOD peaked on day 7 at 1.91 g COD/L (***Figure 3.2a***). The acidification yield during this period represented less than 25% of the overall sCOD. For the last two sampling points of the experiment, VFAs were no longer detected and the sCOD was almost depleted by the end of the trial (days 21 and 30).

In the case of the gas production, methane was the major product with a final concentration of 47.7 (± 19.9) % (***Figure 3.3a***). Two different production rates were evident: the first from day 0 to day 4, and the second from day 4 to day 14. The first production rate corresponded to 85.43 ml CH_4 /(day·g $VS_{Inoculum}$) (R^2 0.99) and the second rate was 26.06 ml CH_4 /(day·g$VS_{Inoculum}$) (R^2 0.98) (***Table 3.2***). After day 21, there was no further gas production.

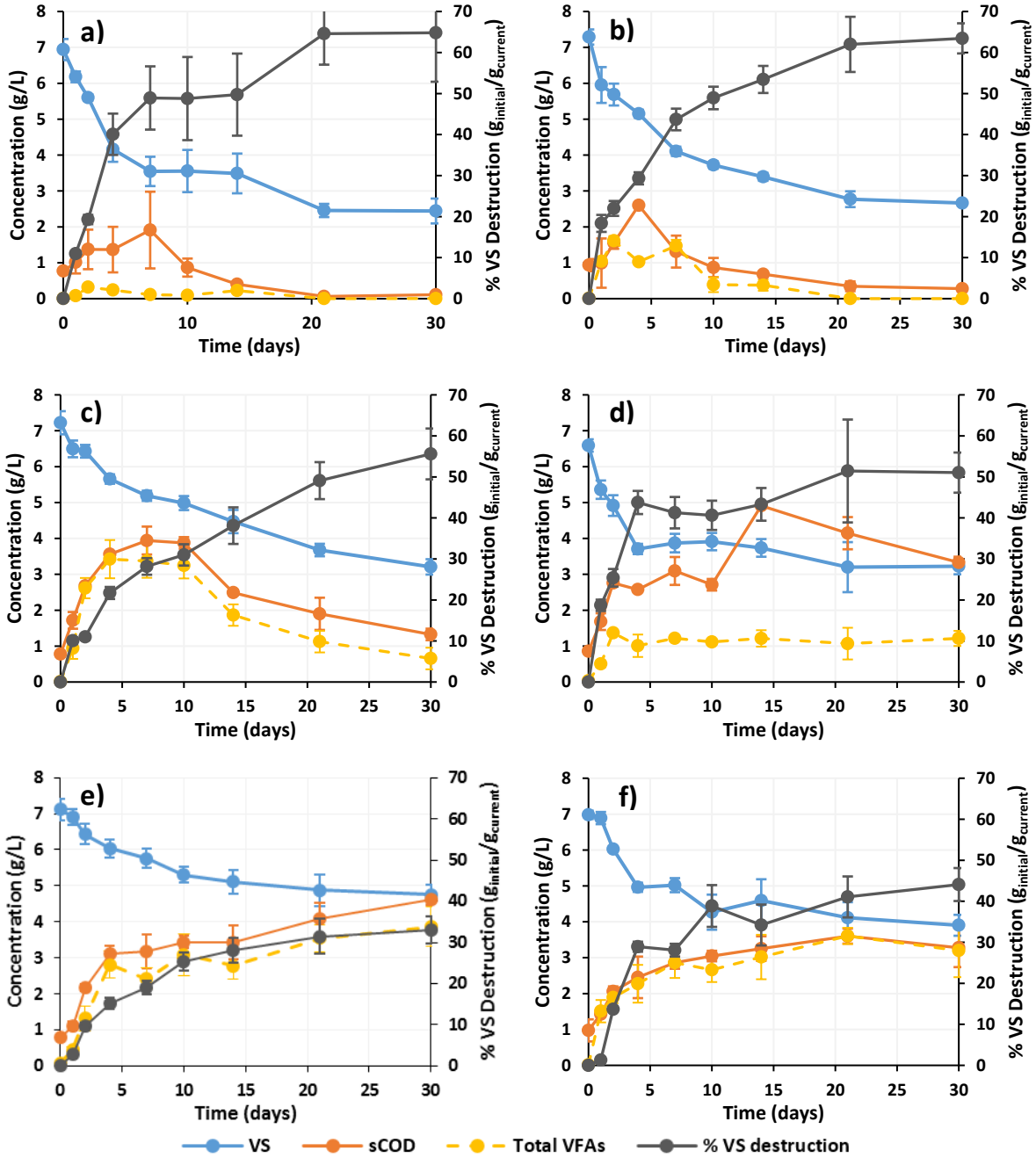

Figure 3.2 *Solid destruction, soluble chemical oxygen demand and concentration of volatile fatty acids for each of the experiments. a) Granular sludge, b) Crushed granular sludge, c) Giraffe manure, d) rumen fluid, e) rhino manure, and f) tiger manure*

3.3.1.2 Crushed granular sludge

For the crushed granular sludge, four different rates of VS destruction were observed (***Table 3.2b***). In the first 24 hours, the solid destruction rate was the fastest, -0.889 g VS_{BSG}/(L·day·g $VS_{Inoculum}$) (***Table 3.2***), which corresponded to a VS destruction of 18.34 (± 2.07) %. After the initial day, the VS destruction rate decreased and remained constant until day 7 with a rate of -0.205 g VS_{BSG}/(L·day·g $VS_{Inoculum}$) (R^2 0.99). The third rate from day 7 to day 21 was -0.062 gVS_{BSG}/(L·day·g$VS_{Inoculum}$) (R^2 0.99). Between the last two points of this trial the destruction rate was -0.019 gVS_{BSG}/(L·day·g$VS_{Inoculum}$), with a final VS destruction of 63.45 (± 3.67) %.

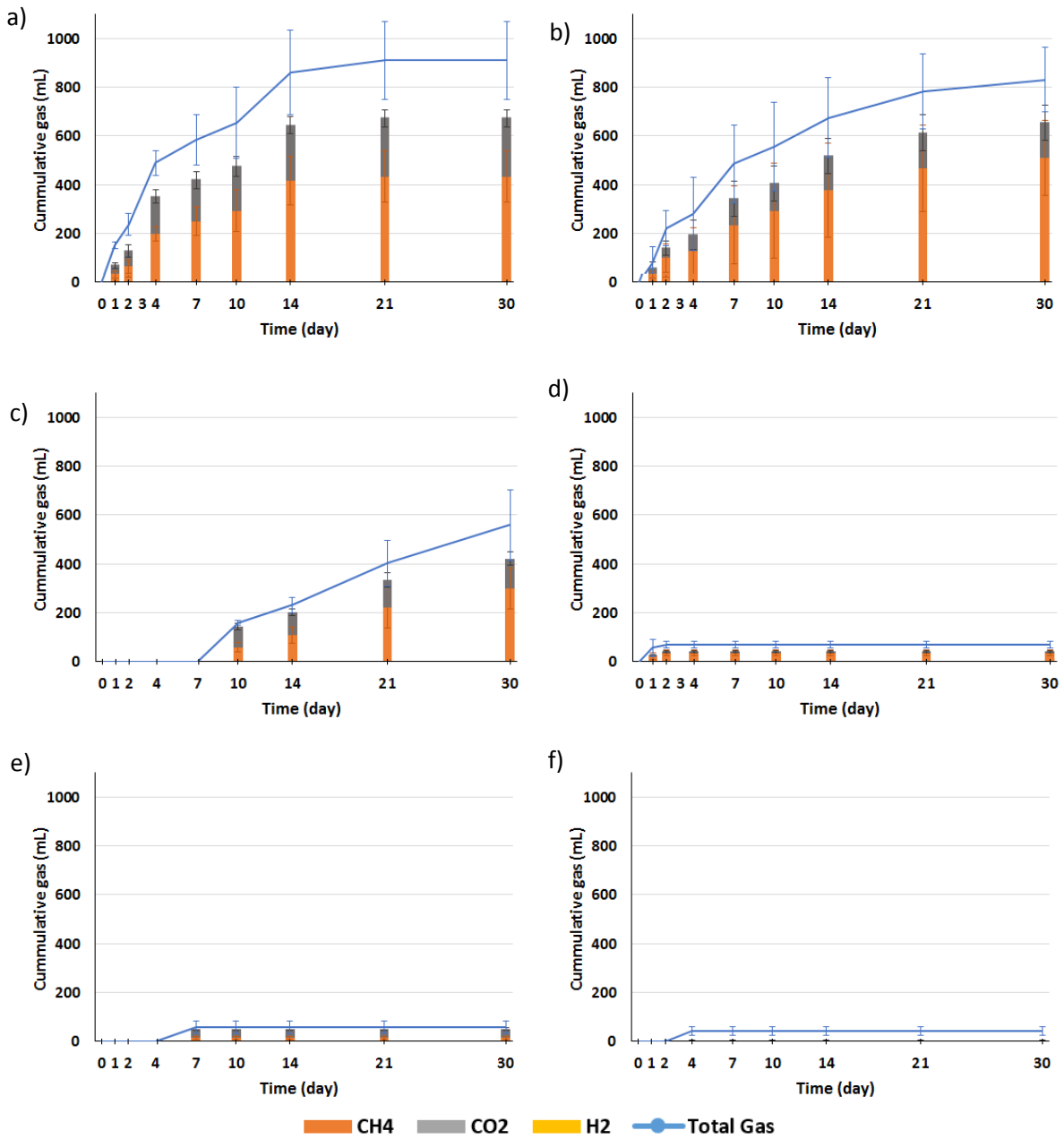

Figure 3.3 *Gas production and its composition for each of the experiments on BSG degradation during the incubation of BSG with different inocula. a) Granular sludge, b) Crushed granular sludge, c) Giraffe manure, d) rumen fluid, e) rhino manure and f) tiger manure*

The highest sCOD concentration achieved with this inoculum was on day 4, with a concentration of 2.60 (± 0.08) g COD/L (***Figure 3.2c***). By the next sample point, day 7, the sCOD concentration had halved and continued to decrease slightly over the course of the experiment to a final concentration of 0.38 (± 0.07) g COD/L. The VFA content followed a similar trend to the sCOD (***Figure 3.2b***). From day 1 to day 7 the VFA concentration was almost stable, fluctuating around 1.29 (± 0.26) g COD/L. After this point the VFA concentration decreased until being completely consumed by day 21.

The initial gas production rate was constant until day 7 at 44.47 ml CH_4/(day·g$VS_{Inoculum}$) (R^2 0.96) (***Table 3.2***). After this day the gas production rate decreased slightly from day 7 to day 21, corresponding to 14.26 ml CH_4/(day·g$VS_{Inoculum}$) (R^2 0.98). In the case of the crushed granular sludge there was production of gas until the last day of the trial, where the production rate was 3.56 ml CH_4/(day·g$VS_{Inoculum}$).

3.3.1.3 Giraffe manure

Using giraffe manure, an initial VS destruction (day 0 - day 2) of 10.99 (± 0.79) % was achieved, with a destruction rate of -0.364 VS $_{BSG}$/(L·day·g $VS_{Inoculum}$) (R^2 0.94) (***Table 2***). From day 4 until day 21 this rate decreased and was almost constant at -0.114 g VS_{BSG}/(L·day·g $VS_{Inoculum}$) (R^2 0.99). The final VS destruction rate between the two last points corresponded to -0.052 g VS_{BSG}/(L·day·g $VS_{Inoculum}$), where the final VS destruction at the end of the trial was 55.60 (± 6.20) % (***Figure 3.2c***).

Table 3.2 *Main parameters for the solid biodegradation activity test for each of the different conditions*

	Initial Rate Destruction gVS_{BSG}/(L·day·g $VS_{Inoculum}$)	**Final % VS destruction**	**sCOD $_{final}$** g/L	**Acidification Level, final** g COD/g sCOD	**Final Accumulative Gas** mL
Granular sludge	-0.456	64.8 ± 11.9	0.11 ± 0.07	0	916.0 ± 241.1
Crushed granular sludge	-0.889	63.4 ± 3.7	0.28 ± 0.02	0	831.4 ± 132.9
Giraffe manure	-0.364	55.6 ± 6.2	1.33 ± 0.16	0.49 ± 0.28	786.0 ± 155.1
Rumen fluid	-1.232	47.6 ± 6.7	3.33 ± 0.55	0.36 ± 0.12	58.7 ± 24.8
Rhino manure	-0.280	33.1 ± 4.23	4.62 ± 0.16	0.84 ± 0.15	50.3 ± 26.2
Tiger manure	-0.090	44.1 ± 4.0	3.28 ± 0.54	0.98 ± 0.38	45.3 ± 18.8

The sCOD and VFAs began increasing immediately, and on days 2 and 4 over 96 % of the sCOD was attributed to VFAs (***Figure 3.2c***). After day 4, the ratio between VFAs and sCOD decreased, and the VFA concentration from day 4 until day 10 fluctuated around 3.34 (± 0.63) g COD/L. This concentration begun to decrease, reaching a final VFA concentration of 0.65 (± 0.30) g COD/L by day 30. The VFA profile consisted mainly of acetic, propionic and butyric acid. Acetic acid was the major compound at the start of the trial and accumulated until day 4, after which it decreased and by day 10, propionic acid was the major compound (***Figure 3.4c***). The butyric acid concentration peaked on day 7, then

decreased and was completely consumed by day 21. On day 30, there were still small concentrations of acetic (0.17 ± 0.05 g COD/L) and propionic (0.41 ± 0.15 g COD/L) acid.

Biogas production from giraffe manure was absent until day 7 and the first gas sample could be collected at day 10. Between these two points, CO_2 was the major gas produced. However, its production rate significantly decreased after this point to 2.72 ml CO_2/(day·g $VS_{inoculum}$) (R^2 0.96) until the end of the trial (***Figure 3.3c***). The methane production was constant from day 7 to day 21, with a rate of 21.88 ml CH_4/(day·g $VS_{inoculum}$) (R^2 0.99) (***Figure 3.3c***). From day 21 to 30, the methane production rate halved to just 12.08 ml CH_4/(day·g $VS_{inoculum}$).

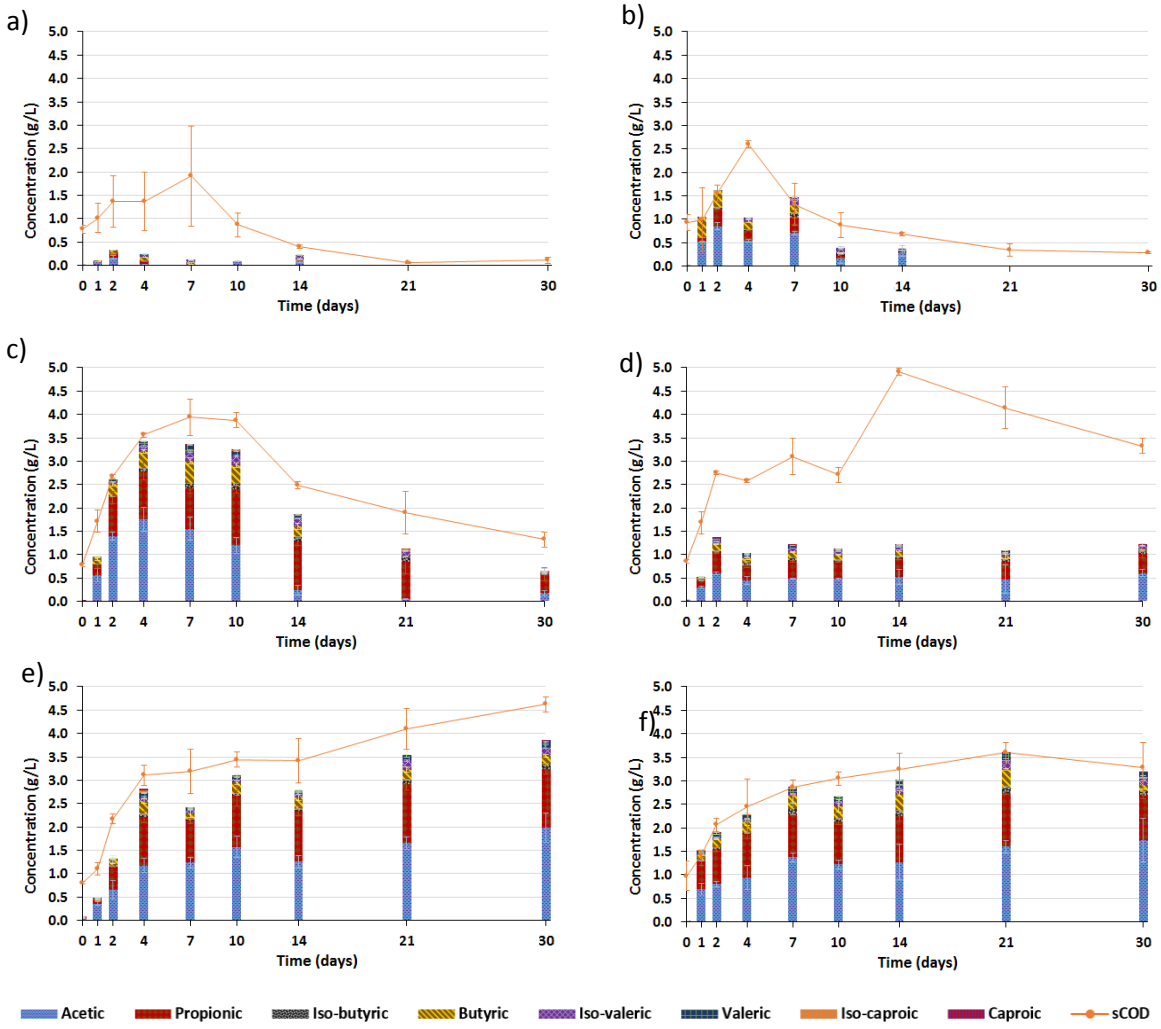

Figure 3.4 *Volatile fatty acid profile and total soluble chemical oxygen demand. a) Granular sludge, b) Crushed granular sludge, c) Giraffe manure, d) rumen fluid, e) rhino manure, and f) tiger manure*

3.3.1.4 Rumen fluid

In the first 24 hours, rumen fluid allowed a VS destruction rate of -1.232 g VS_{BSG}/(L·day·g $VS_{Inoculum}$), with a VS destruction of 18.69 (± 1.38) % (***Table 3.2***). By day 4 the VS destruction reached 43.79 (± 2.82) %. Thereon, until the end of the trial, the VS destruction was quite poor, where the VS at the end of the trial (day 30) was 51.52 (± 4.89) % (***Figure 3.2d***).

VFA production peaked on day 4 with a total concentration of 1.37 (± 0.05) g COD/L (***Figure 3.2d***). This was almost constant throughout the experiment, where the average VFA concentration from day 2 until the end of the trial was 1.21 (± 0.21)/g COD L. A similar trend was observed in sCOD concentrations during the early stages of the trial (day 0 – 7). However, the highest concentration of sCOD, 4.91 (± 0.82) g COD/L was achieved on day 14 (***Figure 3.2d***). Thereafter, the sCOD concentration decreased continually until day 30 to a final concentration of 2.71 (± 0.16) g COD/L. The total gas production was less than 100 mL over the whole trial, and this was produced between days 1 and 4 (***Figure 3.3d***).

3.3.1.5 Rhinoceros manure

By day 4, the VS destruction associated with rhinoceros manure as inoculum was 14.51 (± 1.27) % (***Figure 3.2f***), which corresponded to a rate of -0.280 g VS_{BSG}/(L·day·g $VS_{Inoculum}$) (R^2 0.97) (**Table 3.2**). This rate decreased as the trial proceeded, with two distinct rates: from day 4 to 10 and from day 10 to 21, with a VS destruction rate of 0.122 g VS_{BSG}/(L·day·g $VS_{Inoculum}$) (R^2 0.98) and 0.039 g VS_{BSG}/(L·day·g $VS_{Inoculum}$) (R^2 0.97), respectively. The final VS destruction achieved with this inoculum was 27.80 (± 2.78) % (***Figure 3.2e***).

The VFA profile consisted mainly of acetic, propionic and butyric acid (***Figure 3.4e***). Acetic acid was the major compound comprising around 50 % of the total VFAs after the second day. Propionic acid ranged between 35 – 40 %, and butyric acid between 5 – 10 %. The sCOD increased most rapidly from day 0 to day 4, when it reached a concentration of 3.11 (± 0.25) g COD/L (***Figure 3.2e***). The sCOD continued increasing, but at a much lower rate, until day 30 when it reached its highest concentration 4.62 (± 0.52) g COD/L. After day 4 the average contribution of the VFAs to the total sCOD was fairly constant (***Figure 3.2e***), with an acidification level of 84.58 (± 5.07) %.

The total gas production with this inoculum was 63.0 (± 26.6) mL, which was produced between days 4 and 7 in all the replicates (***Figure 3.2e***). This gas composition was mainly CO_2 and a small fraction of CH_4, 34.0 ± 5.5 mL and 16.5 ± 5.2 mL, respectively.

3.3.1.6 Tiger manure

Virtually no VS destruction occurred in the first 24 hours when incubating the BSG with tiger manure inoculum (***Figure 3.2f***). From day 1 to day 4 the VS destruction increased from 1.29 (± 0.50) % to 28.93 (± 1.22) %, where the VS destruction rate was -0.627 g VS_{BSG}/(L·day·g $VS_{Inoculum}$) (R^2 0.98) (***Table 3.2***). From day 7 until the end of the trial the VS destruction slowed down, and by the end of the trial the VS destruction corresponded to 44.08 (± 4.02) %.

The VFA concentration increased throughout the experiment, with the highest concentration detected on day 21 at 3.61 (± 0.74) g COD/L (***Figure 3.2f***). The VFA profile was similar over the 30 days, where acetic acid was the main compound, followed by propionic acid and then by butyric acid, which represented, respectively, 54.5, 29.7 and 4.6 %. Valeric acid comprised approximately 4.5 % and iso-valeric acid, detected after the day 14, close to 5 % (***Figure 3.4f***). A small amount of gas was detected by day 4 (42.2 (± 18.8) mL) with CH_4 and CO_2 below the detection limit (***Figure 3.3f***).

3.3.2 Microbial community analysis of the inocula

As the aim of this chapter was to find the best microbial community of hydrolysing BSG, data were mined for prokaryotic genera that are typically associated with enhanced abilities to degrade cellulosic biomass (***Figure 3.5***). We searched for the genera listed by Liang *et al.* (2014) as cellulase producers, in their research isolating and screening cellulolytic bacteria. The common trend to all samples was an increase in these genera with time, where in some samples virtually none were detected on day 0. These included both granular sludge types and counter-intuitively, rumen fluid. Tiger manure, with its large contribution from various *Clostridium*, contained the most members of potentially cellulolytic bacterial genera.

Further details regarding the (**S3.2.1 Comparison of the microbial composition of the inocula**, (**S3.2.2 Presence vs activity of OTUs in each inocula (Day 21)** and (**S3.2.3 Differences in microbial community structure and relationships with biodegradation data** can be found in the supplementary information at the end of the thesis.

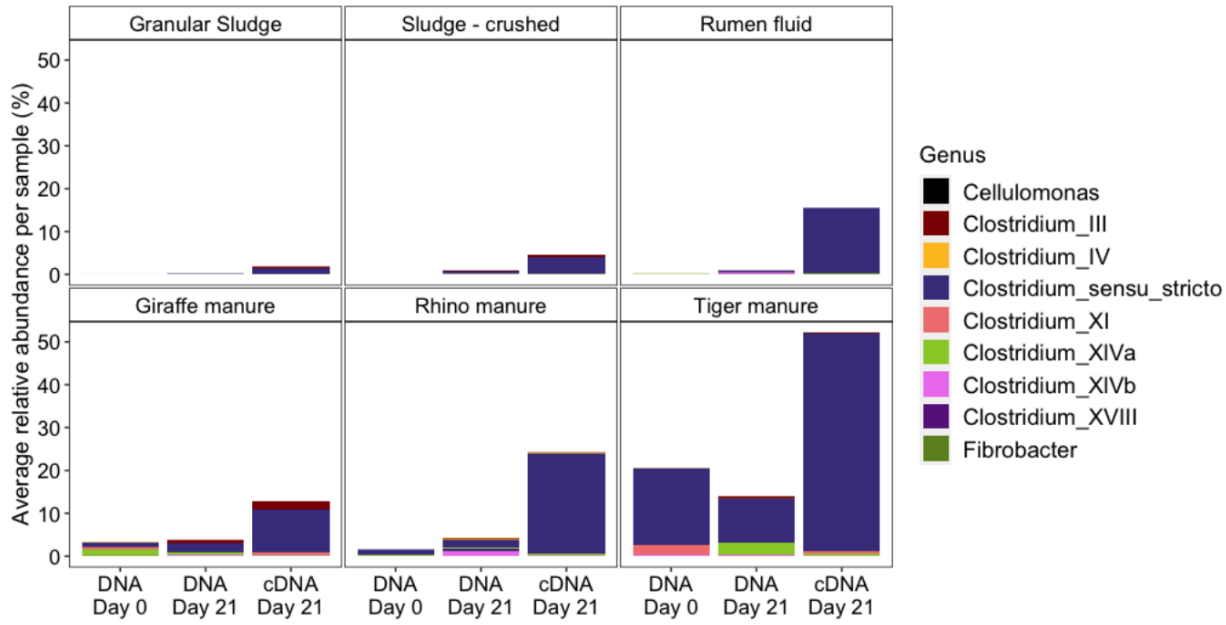

Figure 3.5 *Relative abundances of bacterial genera associated with cellulose degradation, as detected in each of our inocula at the different sample times. Bars represent the average relative abundance detected in biological replicates (n=3)*

The majority of the archaeal community comprised of methanogens (around 87%), with unknown archaea contributing to 10% and *Thermoplasmatales* the final 3%. The sludge samples contained the largest methanogenic consortia, where archaea contributed more than 22% of these communities at the start of the experiment (***Table 3***). Archaea were present in rumen fluid at just 3% relative abundance and in all other samples at less than 1%, where they were virtually absent from tiger manure (0.02% of initial community). While relative numbers of archaea dropped significantly in sludge samples, down to 10% or below by day 21, they contributed to more than a third of the active (cDNA) population. In contrast, archaea increased in number in all samples of animal origin, but most notably in giraffe manure where they increased from 0.8% to 9% relative abundance. In both rumen fluid and tiger manure, archaea were present (DNA) in higher numbers than they were active (cDNA) on day 21, while the giraffe and rhinoceros manure followed the opposite trend (higher abundance at cDNA than DNA level) (***Figure 3.5***).

Table 3.3 *Relative abundances of archaea in the DNA fraction (days 0 and 21) and in the cDNA fraction (day 21) in the 6 inocula used to degrade brewery spent grain*

Inoculum	Relative abundances of archae (%)		
	DNA		cDNA
	Day 0	Day 21	Day 21
Granular sludge	21.94	6.14	35.65
Crushed granular sludge	27.65	10.43	38.85
Giraffe manure	0.78	9.04	13.05
Rumen fluid	2.99	4.99	2.22
Rhino manure	0.17	1.38	2.31
Tiger manure	0.02	0.28	0.07

3.4 Discussion

Six diverse inocula were assessed for their potential to effectively use a relatively recalcitrant (lignocellulosic) material, BSG, as the feedstock. Their performance can be compared firstly based on the destruction of the feedstock, as well as the rate of production of intermediates and end products, where methane will be discussed first as VFA accumulation is dependent on this.

3.4.1 Volatile solids destruction

The highest final VS destructions, of over 60%, were achieved using granular and crushed granular sludge. These were followed (in decreasing efficiency) by giraffe manure (55%), rumen fluid (50%), tiger manure (45%) and rhinoceros manure (33%). The microbial community data were mined for genera associated with cellulolytic activity, the two best performing inocula (sludge) had the fewest of these members, with their total absence detected on day 0. Indeed, it was the tiger manure where

the most abundant of these species were detected, perhaps not surprising as the *Clostridiales* were a dominant clade in this dung type, as is frequently seen in other carnivorous animals (Menke et al., 2014; An et al., 2017). This could explain how this inoculum was able to achieve a VS destruction of 10% better than that of manure from the herbivorous rhinoceros. While the genera searched for were not exhaustive, it appears that the higher VS destruction rates achieved with granular sludge samples could not be explained by the presence of more cellulolytic bacteria.

Rumen fluid resulted in the highest initial VS destruction rates, as expected due to the presence of hydrolytic bacteria and high enzymatic content typical in this sample type (Baba et al., 2017; Deng *et al.*, 2017). However, after the second day the VS fraction remained constant and its destruction only increased at the end of the trial. On the other hand, the sCOD increased from day 10 to day 14 and decreased slowly (***Figure 3.2d***). This could be related to the solubilisation of suspended solids that contributed to the increased sCOD, such as humic acids, which have been shown to inhibit the hydrolytic enzymes responsible for lignocellulosic degradation (Andric et al., 2009).

Another evident trend in the granular sludge samples with the best VS destruction, was their enhanced methane production (***Figure 3.3a***) and lack of VFA (***Figure 3.2a***) accumulation when compared to the other inocula.

Further details for relation of the microbial community and volatile solids destruction can be found in the supplementary material (***S3.3.1 Volatile solids destruction***).

3.4.2 Gas production

The six different inocula used in the present work can be divided in two different main groups based on their biogas production. The group responsible for a high production of gas consisted of granular sludge, crushed granular sludge and giraffe manure. The group presenting poor gas production, all below 100 mL after the 30 days of the experiments, included rumen fluid, rhinoceros manure and tiger manure.

The inocula group capable of high gas production could be subdivided based on two different behaviours: i) inocula that started to produce gas immediately, namely granular and crushed granular sludge; and ii) inocula that produced gas only from day 7, namely giraffe manure. The granular sludge was from an anaerobic reactor for methane production, thus high methanogenic activity was expected and indeed biogas production was quite fast with hydrolysis as the rate limiting step, with VFA accumulation close to zero.

Between the two sludge samples, the only difference was the integrity of the granule structure in the granular sludge inoculum versus its absence in the crushed sludge. The destruction of the granular

structure was performed in an effort to provide the enzymes and hydrolytic bacteria a greater accessibility to colonise and attack the complex cellulose and hemicellulose structures. However, in contrast the granular structure allows soluble compounds to be rapidly converted into methane due to the close proximity of the different microbial groups involved (Trego et al., 2020), where complex molecules released from the hydrolysis of the BSG are converted to VFAs in the external layers of the granules and these are taken up by methanogens in the internal layers by diffusional transport (MacLeod et al., 1990; Gonzalez-Gil et al., 2001). This explains the low VFA and sCOD concentration with this inoculum (***Figure 3.2a***). Conversely, with crushed granular sludge, the intermediate compounds were produced and excreted directly in the medium, thus promoting the slightly lower gas production rate seen in the crushed sludge variant (***Figure 2D***). In addition, the structure of granular sludge has been shown to protect methanogens from toxic compounds and hostile environments (Show et al., 2020), a benefit that is lost upon crushing. Similar results were obtained by Schmidt and Aching (1991) when working with intact and disintegrated granules, where the specific methanogenic activity of hydrogenotrophic methanogens (fed with H_2 and CO_2) increased following disintegration, while the activity of acetoclastic methanogens (fed acetate) decreased following granule disruption. They determined this adverse effect was because the methanogens were no longer able to metabolize acetate (Schmidt and Aching, 1991). However, perhaps therein, as well as in our study, lower gas production could be attributed to the fact that flocculants are more sensitive to environmental factors compared to granules with a higher tolerance to toxic compounds in the media (Fang et al., 2000). Indeed in our samples, while relatively more methanogenic archaea were detected on day 21 in samples seeded with crushed than with intact granules, the proportion of active members was notably higher in the granular sludge (DNA: cDNA of 1 : 5.8) than in crushed sludge (DNA:cDNA of 1 : 3.7). A lower proportion of transcriptionally active methanogens in the crushed granules could suggest that conditions were less optimal than those with intact granules. VFA accumulation could have contributed to such a toxic effect on the methanogens, thereby lowering the number of active members and therefore methane production rates (Chapleur et al., 2016). This would explain why biogas production was ongoing in the crushed granular sludge until the end of the trial when compared with the intact granular sludge from which no gas was collected on day 30.

Despite the fact that cumulative gas production from giraffe manure at the end of the trial was lower than in the two sludge samples (***Table 3.2***), gas production in the former was still ongoing and VFAs and sCOD remained (***Figure 3.2c*** & ***Figure 3.3c***). This suggests further methane production could have occurred upon prolonged incubation. Inoculation with giraffe manure resulted in poor initial rates of gas production (***Figure 3.3c***), which could be a result of the low abundance of archaea in this sample on day 0 (***Table 3***). Methanogens are typically associated with slow doubling times, in the range

of four to fourteen hours with some even taking 12 days (Jabłoński et al., 2015), thus any increase in abundance would likely be slow. Indeed, by day 21 the relative abundance of archaea in this giraffe manure had increased the most, by more than an order of magnitude where, in contrast, numbers in both sludge inocula decreased (**Table 3.3**).

In the case of the other three 'low gas producing' inocula, biogas was detected at only one sample point for both tiger (day 4) and rhinoceros faeces (day 7), the two inocula with the lowest relative abundances of archaea on both days 0 and 21. With rumen fluid as the inoculum, gas production was only measurable on the first two days of the trial, in which both methane and carbon dioxide were detected. The relative abundances of archaea initially detected in rumen fluid samples were the highest of the four animal-origin inocula. While the relative abundance of this clade increased slightly with time (**Table 3.3**), their relative abundance in the active (cDNA) fraction from the same samples was 50% lower than that in the DNA fraction. This suggests some inhibition of transcriptional activity, indicating conditions were sub-optimal for the methanogenic archaea within these samples.

3.4.3 Volatile fatty acid production

As VFAs are typically consumed in AD systems by acetoclastic methanogens, it is no surprise that the three inocula that saw the highest methane production accumulated the lowest VFA concentrations. Contrastingly, the best performing inoculum in terms of total VFAs produced by the end of the trial was rhino manure, which presented the lowest methane production, and the lowest solid destruction. This was closely matched by the accumulation seen in tiger manure samples, where also minimal methanogenesis was detected. Significant concentrations of VFAs also accumulated when using giraffe manure, however this only lasted until day 4, after which VFAs started to be consumed (***Figure 3.4c***). This coincided likely with the increase in methanogenic archaea detected with this inoculum by day 21 (**Table 3.3**).

In samples seeded with rumen fluid, VFA concentrations constituted around 35% of the total sCOD, and the constituents of the remaining fraction of the sCOD are still not clear. It was possible to determine this fraction was not composed of proteins or lactic acid and neither were any alcohol groups detected. More analysis need to be done to identify these soluble compounds, which currently we hypothesise are long chain fatty acids and/or humic acids, produced during the hydrolysis of the BSG (Ueno et al., 2001; Azman et al., 2017).

3.4.4 Potential inhibition

A number of studies have demonstrated the inhibitory effects of different compounds (i.e. humic acids, phenolic compounds or VFAs) and their threshold concentrations at each of the four steps of AD (Siegert et al., 2005; Fernandes et al., 2015; Chapleur et al., 2016). These studies demonstrated

that the most sensitive step is hydrolysis, followed by methanogenesis, and acetogenesis and then acidogenesis. Thus, the accumulation of the VFAs in the present study could have hindered the VS degradation (***Figure 3.2***).

Chapleur *et al*. (2016) studied the inhibitory effect of phenol on the degradation of lignocellulosic materials. One of the first effects, even at a low concentration, was the accumulation of acetic and propionic acid in the system. Acetic acid took 10 days to be consumed and propionic acid 20 days, thus resulting in a lower biogas production rate (Chapleur *et al*., 2016). This trend was similar to the present study when giraffe manure was used as an inoculum, where until day 7 only hydrolysis was seen, in the absence of both acidogenesis and methanogenesis (***Figure 3.2c***). In our case, however, the toxic compounds would likely have been the VFAs. However, by day 10 there was gas production and VFAs started to be consumed sequentially starting with butyric, then acetic and finally propionic acid. This behaviour would be related to the acclimatization period of the methanogens to the new environmental conditions in the biodegradation activity test and the toxic effect of the VFAs, with concentrations up to 3.86 g/L (***Figure 2***). A similar adaptation period of the inoculum to fermented compounds was observed by Haruta *et al.* (2002) when working with rice straw.

In terms of VFA toxicity during AD, Siegert and Banks (2005) studied their toxic effect on methane production using lignocellulosic material as a feedstock. The increase in VFA concentration initially inhibited cellulolytic activity, then hydrolysis of glucose, methane fermentation and finally, when the VFA concentrations reached 8 times higher than those at the start, acidogenesis and acetogenesis were affected (Siegert and Banks, 2005). The accumulation of fermented compounds perhaps contributed to the lower gas production rates in some of our samples, and to the longer lag phase in gas production from giraffe manure.

Similar observations of inhibition have been reported when studying the effect of fermented compounds and chemicals, such as humic acid, fulvic acid, long chain fatty acids or phenol on the hydrolysis of lignocellulosic and hemicellulosic materials (Hobson et al., 1997: Fernandes et al., 2015; Chapleur et al., 2016). Thus, perhaps it was one of the unidentified intermediates constituting the remaining 65% of the sCOD not attributable to VFAs which was particularly inhibitory in rumen fluid seeded samples. It was assumed that both ruminant samples (giraffe manure and cow bovine rumen fluid) would be well adapted to the BSG feedstock. However, this proved not to be the case for rumen fluid (***Figure 3.2d***). Perhaps this inoculum was more sensitive to changing conditions, whereas microbes originating from manure had already been pre-exposed to sub-optimal conditions (for example low temperature and oxygen) and were able to adjust better. In all other inocula tested except rumen

fluid, VFAs contributed to approximately 90% of the total sCOD for the majority of the experiments (***Figure 3.4***).

The granular sludge, crushed or not, was from an industrial methane production reactor treating dairy wastewater after fat removal, which is an easier biodegradable material compared to BSG. Yet despite this, these two inocula performed well under the conditions tested. Li *et al*. (2020) achieved the highest degradation of a lignocellulosic feedstock with the inocula containing the highest enrichment of cellulolytic bacteria, which differs with the present study as granular sludge presented the lowest cellolytic bacteria content (***Figure 3.5***). The VS destruction by the rumen fluid and manure from herbivorous animals was expected to be faster, as their diet relies on a high capacity to degrade fibres. Among these inocula of herbivore origin, the giraffe manure presented the highest solid destruction and it was the only one capable of consuming VFAs and producing methane in high amounts. This could indicate a relationship between VFA accumulation and a poorer solid destruction rate, thus explaining why the granular sludge was more effective at degrading the lignocellulosic feedstock, due to a higher active methane production which proved advantageous due to the consequence that it lowered the VFA concentrations. Similarly, Jiang *et al*. (2020) obtained optimal AD performance when increasing the organic loading rate using food waste during the bioaugmentation that enhanced the population of acetoclastic methanogens.

A similar relationship between VFAs and hydrolytic activity was determined by Jankowska *et al.* (2017) during VFA production from maize silage, among other compounds. One of their findings was the association between the increase in VFA concentration and the reduction in hydrolysis yield. Sasaki *et al.* (2010) optimised methane production from rice straw by submerging carbon fibre textiles, with the aim of retaining and increasing the number of microorganisms. However, they determined that this increase in microorganisms was not enough to fully explain the higher decomposition of the rice straw. In the reactor containing carbon fibre textiles, methane production increased more than 3.5 fold, while the reactor without fibres showed an accumulation of VFAs. This VFA accumulation was not considered by the authors, but could have played a key role in the low biodegradation of rice straw. The toxic effect of accumulated VFAs, could have played a role in decreasing the solid destruction from 57.2 to 30.5 % with and without the carbon fiber textiles (Sasaki *et al.,* 2010). In the present study, the conversion of VFAs into methane when using granular sludge and giraffe manure would have alleviated this possible toxic effect and improved the solid degradation. Contrastingly in samples with little methane production, the hydrolytic activity may have been negatively affected by the accumulation of VFAs. However, further research must be done to validate this hypothesis. Furthermore, the VS degradation of lignocellulosic feedstock by herbivore inocula could potentially be

improved, for example by the continuous recovery of the VFAs using membrane processes that would lower the toxicity in the media (Saboe et al., 2018).

3.5 Conclusions

This study has shown the influence of using different inocula for the production of different chemical compounds from the same feedstock. The best solid destruction was attained using granular sludge, although in relation of the ratio of solid destruction to accumulated fermented compounds, the best performance was from the rhino manure inoculum. An increasing amount of research is focusing on the use of AD to produce intermediate fermented compounds. The hydrolysis efficiency and the accumulation of fermented compounds should be studied to determine if there is any inhibitory effect or deficiency at this level. A deeper understanding of the anaerobic digestion process of lignocellulosic materials for chemical and material production is required as the role of VFAs in the solid degradation is not clear. If methane is not the end product, the relationship between the accumulation of intermediate products, such as VFAs, and a lower hydrolysis yield must be established. A microbial community resistant to high concentrations of fermented compounds should be established, or these fermented compounds should be recovered from the medium to alleviate their toxic effect. Another valuable avenue of research could be into the combination of different inocula that show cellulosic activity and acidogenic activity in order to maximise hydrolysis and VFA accumulation rates.

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Chapter 4

Thermal diluted acid hydrolysis of brewery spent grain and downstream hydrolysate acidogenic fermentation

A modified version of this chapter has been submitted as:

> **Castilla-Archilla, J.**, Papirio, S., and Lens, P. N. L. (2021) "Two step process for volatile fatty acid production from brewery spent grain: hydrolysis and direct acidogenic fermentation using anaerobic granular sludge" Process Biochemistry 100, 272–283. doi: 10.1016/j.procbio.2020.10.011

Keywords: brewery spent grain; pretreatment; thermal diluted acid hydrolysis; carbohydrates; acidogenic fermentation; volatile fatty acids

Abstract

Brewery spent grain (BSG) is an industrial waste stream with high potential for biorefining purposes due to its high content in carbohydrates, along with proteins and lignin. This work evaluated the production of volatile fatty acids (VFAs) by a two step process using BSG as renewable feedstock. Initially, a thermal (121 °C) diluted acid (sulfuric) hydrolysis of BSG at for 20 minutes was carried out in order to obtain a liquid hydrolysate rich in carbohydrates. Eighteen different combinations in terms of total solids (TS) of BSG (4, 7 and 10 % w/w) and sulfuric acid (0.0, 0.5, 1.0, 1.5, 2.0 and 3.0 % v/v) were used to perform the BSG hydrolysis. The combination using 7.0 % TS of BSG and 1.5 % of sulfuric acid was the most efficient in terms of total carbohydrate recovery (i.e. 0.44 grams of total carbohydrates per gram of TS recovered) without necessarily extra dosing sulfuric acid. Subsequently, an acidogenic batch fermentation using anaerobic granular sludge for 72 hours was performed on the BSG hydrolysate, which was fermented under five different pH conditions (uncontrolled pH from an initial pH 7.0, and constant pH controlled at 4.5, 5.0, 6.0 and 8.0). The highest VFAs concentration was obtained at pH 6.0 and reached 16.89 (± 1.33) g COD/L.

4.1 Introduction

The use of waste as feedstock for biorefineries is increasing with the aim to promote the valorisation of low-value residues and pursue the zero waste perspective (Carmona-Cabello et al., 2018). In the last century, the treatment of organic waste streams has shifted from aerobic to anaerobic bioprocesses, which require a lower energy consumption and allow the simultaneous treatment of waste and production of energy. Anaerobic digestion is one of the sustainable processes driving the transition from fossil fuels to modern, renewable energy sources. This process mainly consists of four different steps: i) hydrolysis, ii) acidogenesis, iii) acetogenesis and iv) methanogenesis (Monlau et al., 2011). The partial or total inhibition of methanogenesis can shift biomethane production to some of the intermediate compounds as final products, such as lactate (Itoh et al., 2012), hydrogen (Rosa et al., 2014; Cisneros-Pérez et al., 2017) or volatile fatty acids (VFAs) (Garcia-Aguirre et al., 2017). Some strategies to prevent methanogenesis and trigger VFA accumulation include the increase of the organic loading rate (Veeravalli et al., 2014) or the ammonium concentration (Shi et al., 2017; Zhao et al., 2018; Bengtsson et al., 2008; Infantes et al., 2011).

VFAs are short chain fatty acids (C_2 to C_6) with a broad range of industrial application and their current production relies on fossil sources (Strazzera et al., 2018). VFAs can be produced from renewable biomass through anaerobic fermentation, but their recovery is still a bottleneck in this technological development. This is especially the case for low strength wastewaters, which yield only diluted VFA concentrations. Consequently, this process is till now mainly used as intermediate step to obtain compounds that are easier to recover, e.g. polyhydroxyalkanoates (PHA) or lipids for biodiesel production (Albuquerque et al., 2011; Patel et al., 2018). Nevertheless, recent improvements in the extraction systems may allow to efficiently extract the VFAs as the final products, especially if high VFA concentrations can be obtained (Wainaina et al., 2019; Saboe et al., 2018). Thus, VFA production depends less on fossil sources and provides a trade opportunity in countries that rely on importing gas and oil. However, some parameters of the acidogenesis phase, such as the VFA concentration and the operating pH must be further investigated.

One of the most important factors affecting the yield and stability of a bioprocess is the consistent composition and availability of the feedstock throughout the year (Ravindram et al., 2016; Fava et al., 2015). Lignocellulosic materials (LMs) represent the most abundant organic feedstocks for bio-industrial purposes, with low or no costs, in comparison to the more expensive energy crops. However, the main issue when using LMs in fermentation processes is associated with their complex structure, where cellulose and hemicellulose are interwoven within lignin (Ayyachamy et al., 2013), being recalcitrant to microbial degradation in the absence of a pretreatment (Mancini et al., 2016). Many studies have focused on different pretreatment methods to make LMs more accessible to

microorganisms and thus release an elevated amount of high-value molecules, such as sugars, proteins or phenolic compounds. Some examples of these pretreatments are enzymatic hydrolysis (Niemi et al., 2012), diluted acid treatment (Carvalheiro et al., 2004) and steam explosion (Silveira et al., 2018). With regard to chemical pretreatments, the hydrolysis efficiency of lignocellulosic wastes depends on the combination of different pretreatment parameters such as temperature, time and acid concentration. This combination of parameters can be captured in a single value, known as the severity factor (Carvalheiro et al., 2004; Zoulikha et al., 2015; Fockink et al., 2018).

Many agro-food industries could provide a constant supply of LMs to be used for either energy production (Mancini et al., 2016; Dishman et al., 2017) or the recovery of high value compounds (Rocha-Martín et al., 2017). One source of sustainable biomass for biorefinery platforms is the brewery sector. The basic ingredients of beer are water, barley, hops and yeast, and the most abundant waste stream deriving from the brewing industry is the malted barley obtained post boiling. This latter mentioned solid waste is known as brewery spent grain (BSG), and approximately 15 – 20 kg is produced per hectolitre of beer. Conventionally, BSG is used for animal feeding or burnt after drying. Rarely, BSG has been used for environmental applications, such as a co-substrate in anaerobic digesters aimed at methane production (28). Recovery of valuable compounds, including lignin (Niemi et al., 2012), polyphenols (Stefanello et al., 2018), prebiotics (Moreira et al., 2013), proteins (Connolly et al., 2013), and total carbohydrates (TC), mainly glucose, xylose and arabinose (Rommi et al., 2018; Ravindran et al., 2018; Outeiriño et al., 2019), from BSG has only recently gained interest.

The consistent composition and year-round availability make BSG a suitable renewable feedstock for VFA production. To enable this, a combination of different pretreatment methods and fermentation technologies is required, and the feasibility to combine them must also be evaluated. As pretreatment method for BSG, a thermal diluted sulfuric acid pretreatment was used in this study, as similar processes showed to be reliable methods used at the industrial scale, only requiring a single reaction step and a short time for the hydrolysis of the material. Therefore, we preliminarily focused on a first step on the hydrolysis of BSG using different sulfuric acid and BSG concentrations, in order to obtain a liquid hydrolysate rich in easily fermentable carbohydrates. The pretreatment conditions (i.e. low acid concentration, short reaction time of 20 minutes, and a temperature of 121°C) allowed the solubilisation of complex carbohydrates without the production of toxic compounds (e.g. furfural, hydroxymethylfurfural, formic acid, levulinic acid or total phenolic compounds) that inhibit subsequent fermentation (Carvalheiro et al., 2004; Djioleu and Carrier, 2016). Subsequently, as a second step, two stirred tank reactors inoculated with anaerobic granular sludge were run in batch mode for the production of VFAs from the obtained carbohydrate-rich liquid hydrolysate. It should be noted that between step one and two, only pH neutralization was done after the thermal diluted

sulfuric acid step and no further treatment was carried to remove possible inhibitory compounds co-released during the BSG hydrolysis. The VFA accumulation during acetogenic fermentation was promoted by the operating parameters initial organic concentration and pH. Different pH values were used to run the batch acetogenic fermenting reactors and evaluate the TC consumption, highest VFA production rate, total VFA concentration and VFA composition.

4.2 Material and methods

4.2.1 Feedstock and inoculum

BSG was supplied by a local brewery in Galway (Ireland) for the production of lager beer. Once collected, the BSG was mixed and frozen in individual bags of 3 kg each at -20 °C. Prior to the hydrolysis step, the BSG was defrosted overnight at 15°C.

As inoculum, an anaerobic granular sludge collected from an UASB reactor treating dairy wastewater for methane production (Kilconnell, Ireland) at ambient temperature was used. The total (TSS) and volatile (VSS) suspended solids concentrations of the inoculum were 72.8 and 59.1 mg/g of wet inoculum, respectively. No pretreatment was applied to the inoculum.

4.2.2 Diluted acid hydrolysis of brewery spent grain

After defrosting, the BSG was mixed with distilled water and blended using a laboratory Waring 2-Speed Laboratory blender (USA) for 1 minute to break down the external structure of the grain and facilitate hydrolysis. Three different total solids (TS) concentrations of BSG (4, 7 and 10 % w/w) were used for the hydrolysis of BSG and the extraction of easily fermentable carbohydrates. The blended mixtures were placed in 500 mL Pyrex bottles with a working volume of 400 mL.

Sulfuric acid (95 % purity and 1.83 g/mL density), supplied from Fisher Chemical (UK), was used to perform diluted acid hydrolysis of BSG at 121 °C for 20 minutes in an autoclave (Sanyo Labo, Japan) as described by Carvalheiro et al. (2004) and Djioleu and Carrier (2016). This cost-efficient method allows the hydrolysis of carbohydrates and proteins, while lignin remains as a precipitate (Ravindran et al., 2018). Sulfuric acid was added at six different concentrations (0.0, 0.5, 1.0, 1.5, 2.0 and 3.0 % v/v), resulting in a total of 18 different combinations in terms of sulfuric acid and TS concentration of BSG. Each combination was run in triplicate. The severity factor was calculated as proposed by Carvalheiro et al. (2004) and Fockink et al. (2018) for the selected combinations to be used as feedstock for the batch acetogenic fermentation. Once hydrolysis was completed, the bottles were placed on ice to stop the reaction, and the hydrolysate was centrifuged at 5000 rpm for 30 minutes (Beckman Coulter, U.S.A.; JS-5.3 Rotor) to recover the supernatant.

4.2.3 Batch fermentation

As substrate for the acidogenic fermentation, the liquid hydrolysate obtained from the diluted acid hydrolysis performed at 7 % (w/v) of TS of BSG and 1.5 % of sulfuric acid (v/v) was used, as this pretreatment condition resulted in the highest extraction efficiency in terms of g TC/g TS (***Table 4.1***). The fermentation system consisted of two duplicate stirred tank reactors running as twin reactors operated at 37 °C. The reactors were made of a section of acrylic tube (inner diameter 15 cm) sealed at both sides with acrylic discs, thus creating a total volume of 2.1 L. A port in the side of the reactors allowed sampling, while a pH probe, gas outlet and tubing for NaOH addition were inserted through the top of the reactor (***Figure 1***). The reactors were operated in batch mode with a working volume of 1.75 L each. In both reactors, the hydrolysate was mixed with distilled water to give a total initial carbohydrate concentration of 16 g COD/L. The reactors were seeded with 3.0 g/L VSS of granular sludge and sparged for 3 minutes with nitrogen gas (N_2) to remove oxygen from the medium. To maintain homogeneous conditions in each reactor, a magnetic stirrer (Stuart UC151, UK) was used and operated at 1,500 rpm.

Table 4.1 *Operating conditions used for the hydrolysis of brewery spent grain as well as the composition of carbohydrates extracted and efficiency of the hydrolysis process expressed as percentage of the total carbohydrates recovered per gram of total solids used*

H_2SO_4 %	TS (BSG) %	Cellobiose (g/L)	Glucose (g/L)	Xylose (g/L)	Arabinose (g/L)	Efficiency (% gTC/gTS)
0	7	1.13 (± 0.13)	1.23 (± 0.83)	0.00 (± 0.00)	0.00 (± 0.00)	8.18 (± 1.3)
0	10	1.55 (± 0.25)	0.75 (± 0.75)	0.00 (± 0.00)	0.00 (± 0.00)	7.32(± 1.4)
1	7	0.70 (± 0.70)	10.05 (± 0.95)	10.40 (± 1.00)	11.10 (± 0.50)	40.35 (± 1.9)
1	10	2.20 (± 0.35)	12.50 (± 4.15)	14.10 (± 7.40)	14.50 (± 7.25)	34.7 (± 0.5)
1.5	7	0.00 (± 0.00)	12.80 (± 1.70)	12.05 (± 0.85)	11.80 (± 0.10)	43.8 (± 1.1)
1.5	10	2.00 (± 0.10)	16.70 (± 0.70)	15.65 (± 0.05)	12.75 (± 0.15)	36.6 (± 0.4)
3	7	0.00 (± 0.00)	11.32 (± 0.04)	6.58 (± 0.09)	5.96 (± 0.01)	44.6 (± 2.7)
3	10	0.60 (± 0.05)	20.74 (± 1.49)	19.08 (± 2.21)	8.68 (± 1.49)	42.5 (± 0.5)

VFA production was analysed under both uncontrolled and controlled pH conditions over a period of 72 h. The experimental run at uncontrolled pH started from an adjusted pH of 7.0 (± 0.01), and no further correction was performed while the process was ongoing, with the aim determining the lowest tolerable pH by the system. In the experiments run at controlled pH, four experimental runs were performed at different pH, where the pH was maintained at either 4.5, 5.0, 6.0 or 8.0 (± 0.05 units) throughout. This was achieved using a pH controller (Alpha 100; Thermo Scientific, Singapore) connected to a peristaltic pump (Masterflex 323, UK) for the addition of a 3 M NaOH solution.

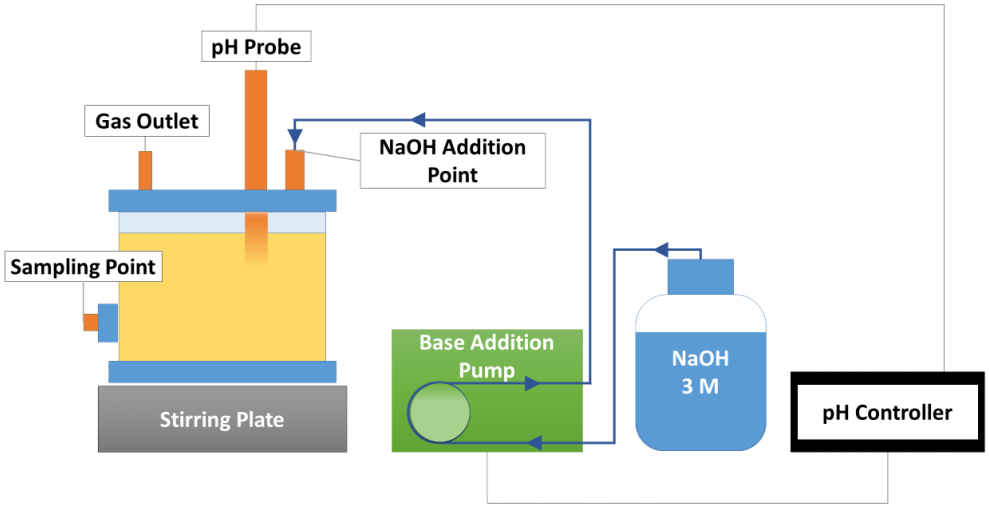

Figure 4.1 *Schematic experimental set up of the stirred tank reactors operated in batch mode for VFA production usng the hydrolysate of brewery spent grain as feedstock. The stirring plates, pH controller and probes as well as the dosing of the NaOH solution are shown*

The reactor operation was monitored by analysing TC, monosaccharides, VFAs, lactic acid, ethanol, soluble chemical oxygen demand (sCOD), TSS and VSS. Sulfate, proteins and ammonium were analysed at the beginning and the end of the batch incubations.

4.2.4 Physico-chemical analysis

The sCOD was analysed using a commercial kit (Reagecon, Ireland) with a range from 0 to 1,500 mg COD/L. 2 mL of sample was added to the vial and digested at 150°C for 2 hours, and the absorbance was measured with a DR2500 spectrophotometer (Hach, Germany). Protein analysis was performed using the Lowry method (1951). TC were analysed by the Dubois method (1956). Glucose, xylose, arabinose and cellobiose were measured using a 1260 Infinity II liquid chromatograph (Agilent, Germany) equipped with a Hi Plex H 7.7 x 300 mm and 8 µm (p/n PL1170-6830) column (Agilent, UK) kept at 60 °C and an RI detector at 55 °C. The mobile phase was sulfuric acid with a concentration of 0.005 M and a flow rate of 0.7 mL/min. Lactic acid and ethanol were analysed by liquid chromatography using a Prominence LC-20A Series HPLC (Shimadzu, Japan) with a Rezex ROA Organic Acid H+ column (Phenomenex, USA) heated at 40 °C and an SPD-20A UV detector set at 220 nm (Dreschke et al., 2019). As the mobile phase, a 0.0065 mM sulfuric acid solution was used at a flow rate of 0.6 mL/min.

VFA analysis was performed with a 450-GC gas chromatograph (Varian, USA) equipped with a CombiPAL autosampler, a flame ionisation detector and a FFAP BP21 capillary column (SGE Analytical Science, Australia) of 30 m length, 0.25 mm internal diameter and a 0.25 µm film. Helium was used as carrier gas with a flow rate of 1 mL/min. The oven temperature was risen from 60 (10 s) to 110 °C (20

s) at a rate of 30 °C/min, and from 110 to 200 °C at a rate of 10 °C/min. The temperatures of the injector and detector were 250 and 300 °C, respectively.

Ammonium and sulfate analyses were performed using a Gallery Plus® discrete nutrient analyser (ThermoFisher, Finland) based on a colorimetric assay (O'Dell et al., 1996; ISO 15923-1, 2013). TSS and VSS concentrations were measured every 24 hours, following the procedure reported in standard methods (Apha, 2012).

4.2.5 Calculations

The carbohydrates release efficiency during the thermal diluted sulfuric acid hydrolysis was calculated taking into account the total concentration of carbohydrates extracted, the amount of liquid recovered, as well as the TS concentration and volume of sulfuric acid used.

For the acidogenic batch fermentation, to evaluate the effect of the operating pH, TC consumption and VFA production rates were calculated using the exponential phase after the initial acclimatization period from each of the replicate reactors. The rates were determined using 6 – 9 experimental points, always accepting an R-squared value above 0.89.

The calculation of the total carbohydrate consumption rate is reported in ***Equation 1***:

$$-r_{TC} = \frac{[Y]_i - [Y]_f}{t_i - t_f} \qquad [{}^{gCOD}/_{(L \cdot h)}] \qquad \textit{Equation 1}$$

where $-r_{TC}$: total carbohydrate consumption rate, Y: total carbohydrate concentration, t: time of the log phase for TC consumption, i: at the beginning of log phase, f: at the end of the log phase.

The total VFA production rate was determined as reported in ***Equation 2***:

$$r_A = \frac{[A]_i - [A]_f}{t_i - t_f} \qquad [{}^{gCOD}/_{(L \cdot h)}] \qquad \textit{Equation 2}$$

where r_A: total VFA production rate, A: total VFA concentration, t: time of the log phase for VFA production, i: at the beginning of the log phase, f: at the end of the log phase.

At each pH, the acidification level was determined (Equation 3) as previously reported by Bengtsson et al. (2008) by calculating the percentage of the total amount of VFAs (in terms of g COD/L) above the total sCOD (which includes the VFAs):

$$Acidification\ Level = \frac{Total\ VFA\ concentration}{Soluble\ COD} \qquad [{}^{gCOD}/_{g\ sCOD}] \qquad \textit{Equation 3}$$

This percentage was used to evaluate the optimal pH at the end of each log phase as the highest amount of VFAs in terms of COD divided by the overall sCOD. The TC depletion (Equation 4) and the sCOD consumption (***Equation 5***) were calculated considering the initial and final points of the log phase

for VFA production. The extent of sulfate reduction was calculated using the initial and final points of each experiment (***Equation 6***).

$$Total\ carbohydrates\ depletion = \frac{[Y]_i - [Y]_f}{[Y]_i} \quad [{}^{g\ consumed}/_{g\ beginning}] \qquad \textbf{Equation 4}$$

$$sCOD_{consumption} = \frac{[sCOD]_i - [sCOD]_f}{[sCOD]_i} \quad [{}^{g\ consumed}/_{g\ beginning}] \qquad \textbf{Equation 5}$$

where Y: total carbohydrate concentration, t: time of log phase for VFAs production, i: at the beginning of the log phase, f: at the end of the log phase.

$$Sulphate\ reduction = \frac{[S]_0 - [S]_e}{t_0 - t_e} \quad [{}^{g\ consumed}/_{g\ beginning}] \qquad \textbf{Equation 6}$$

where S: sulfate concentration, t: time of log phase for VFAs production, 0: beginning of the trial, e: end of the trial.

4.3 Results

4.3.1 Efficiency of diluted acid hydrolysis of brewery spent grain

After acid hydrolysis, for the TC obtained from the 18 different pretreatment combinations; the lowest amount of carbohydrates recovered was in the absence of sulfuric acid, ranging between 2.5 – 8.5 g/L with increasing TS (***Figure 4.2a***). The highest TC concentration was achieved using 10 % TS and 3 % sulfuric acid (v/v). Standardising the extraction efficiencies by the TS concentration used, all the different conditions resulted in a similar ratio of 2.96 (± 0.20) g TC per gram of TS. The concentration of TC extracted increased linearly from 0.0 to 1.0 % v/v of sulfuric acid. At sulfuric acid concentration concentrations from 1.5 % upwards, the efficiency between the TC extracted and the volume of sulfuric acid used was lower. The composition of the carbohydrates recovered was mainly characterized in terms of glucose, xylose and arabinose (***Table 4.1***). The monosaccharides were analysed in the samples that were considered for the extraction at the fermentation step (7 and 10 % TS of BSG at 1.0 and 1.5 % of sulfuric acid), as well as for lowest and highest sulfuric acid concentration (***Table 4.1***). The presence of cellobiose was detected in all the samples for 10 % TS of BSG, and in the case of 7 % TS for 0.0 and 1.0 % of sulfuric acid.

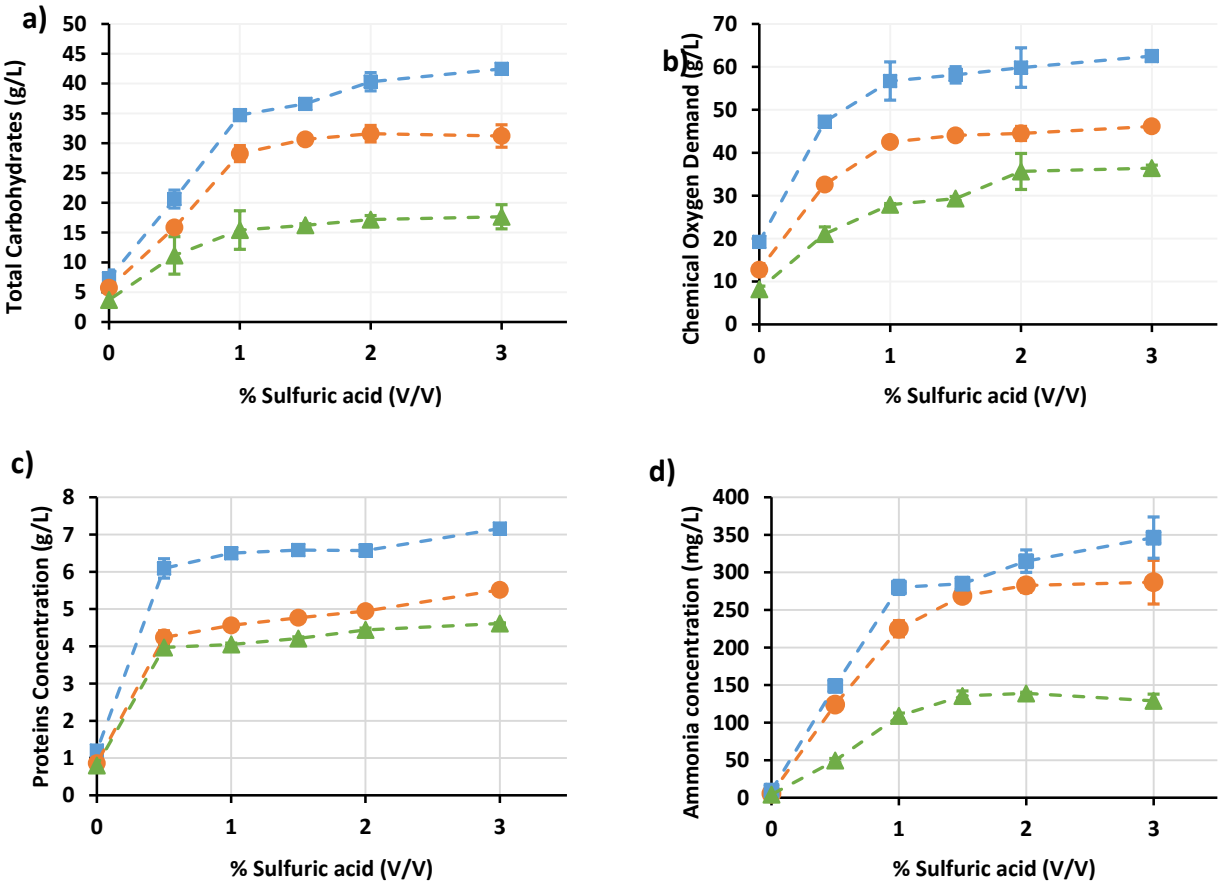

Figure 4.2 Influence of the sulfuric acid concentration on a) release of total carbohydrates, b) chemical oxygen demand (COD), c) proteins and d) ammonium, during the diluted acid hydrolysis of brewery spent grain at 121°C for 20 minutes. Three different total solids concentrations of brewery spent grain (10% TS 7% TS 4% TS) were used

For protein extraction, increasing the sulfuric acid concentration from 0.0 to 0.5 % (v/v) resulted in the highest release of proteins (***Figure 4.2c***). After this point, the protein extraction efficiency decreased. Similarly, the extraction of ammonium was the most efficient at sulfuric acid concentrations between 0.0 to 1.0 % (v/v) (***Figure 4.2d***).

All combinations of sulfuric acid at 10 % TS of BSG and the combinations of 7 % TS of BSG at 1.0 % of sulfuric acid concentration or lower, resulted in an uncompleted hydrolysis, as indicated by the presence of cellobiose (***Table 4.1***). Taking into account the concentration of all the compounds extracted during the hydrolysis, the conditions operated at 1.5 % of sulfuric acid led to the highest overall efficiency, although this did not result in the highest total carbohydrate concentration. Furthermore, a 7 % TS concentration of BSG was considered the optimal (***Table 4.2***).

Table 4.2 Total carbohydrates efficiency recovery, gram of total carbohydrates per gram of brewery spent grain used (dried weight), for the thermal diluted acid hydrolysis of brewery spent grain carried out at 121°C for 20 minutes under different sulphuric acid and brewery spent grain concentrations

% TS BSG (w/w) / % H2SO4 (v/v)	4 %	7 %	10 %
0.0	0.09 (± 0.03)	0.08 (± 0.01)	0.07 (± 0.01)
0.5	0.25 (± 0.05)	0.23 (± 0.01)	0.21 (± 0.01)
1.0	0.39 (± 0.03)	0.40 (± 0.02)	0.35 (± 0.01)
1.5	0.41 (± 0.01)	0.44 (± 0.01)	0.37 (± 0.02)
2.0	0.43 (± 0.02)	0.45 (± 0.02)	0.40 (± 0.01)
3.0	0.42 (± 0.05)	0.45 (± 0.03)	0.42 (± 0.01)

4.3.2 Acidogenic fermentation of brewery spent grain hydrolysate

4.3.2.1 Initial conditions

The pH of the liquid fraction obtained after BSG hydrolysis at 7 % TS and 1.5 % sulfuric acid was 0.93 (± 0.06). The pH was adjusted by the addition of NaOH before starting acidogenic fermentation in batch reactors. The average initial conditions for the acidogenic fermentation in all experiments were sCOD 23.1 (± 2.1) g /L, TC 16.9 (± 2.2) g COD/L, proteins 3.35 (± 0.4) g/L, ammonium 194.9 (± 7.0) mg/L and sulfate 14.7 (± 1.8) g/L (Table 2).

4.3.2.2 Acidogenic fermentation at uncontrolled pH

In the experiment operated without pH control, the pH quickly decreased from 7.00 (± 0.01) to 6.66 (± 0.03) in 2.5 hours, reaching a final value of 4.08 (± 0.05) at the end of the experiment (***Figure 4.3***). The total carbohydrate concentration decreased at a constant rate of 0.24 (± 0.13) g COD/(L·h) to a concentration of 7.32 (± 1.93) g COD/L after 36 hours, when a pH of 4.19 (± 0.18) was observed. Subsequently, carbohydrate consumption almost ceased with a final total carbohydrate consumption efficiency of 63.7 (± 4.4) % (***Equation 4***). With regards to monosaccharides, glucose was completely consumed, while the arabinose and xylose concentration remained constant (***Table 4.3***).

Table 4.3 *Glucose, xylose and arabinose concentration during the acidogenic fermentation along the batch experiment of the brewery spent grain hydrolysate at uncontrolled pH*

Time (hour)	0	13	22	30	46	60	71
Glucose (g/L)	5.81 (± 0.19)	4.08 (± 0.63	2.63 (± 0.71)	0 (± 0)	0 (± 0)	0 (± 0)	0 (± 0)
Xylose (g/L)	5.53 (± 0.20)	5.46 (± 0.12)	4.60 (± 0.55)	5.22 (± 0.38)	4.95 (± 0.41	5.03 (± 0.28)	4.86 (± 0.41)
Arabinose (g/L)	2.75 (± 0.10)	2.35 (± 0.36)	2.67 (± 0.08)	2.55 (± 0.12)	2.52 (± 0.01)	2.55 (± 0.05)	2.45 (± 0.03)
pH	6.98 (± 0.01)	5.11 (± 0.23)	4.59 (± 0.18)	4.44 (± 0.17)	4.11 (± 0.07)	4.10 (± 0.06)	4.08 (± 0.05)

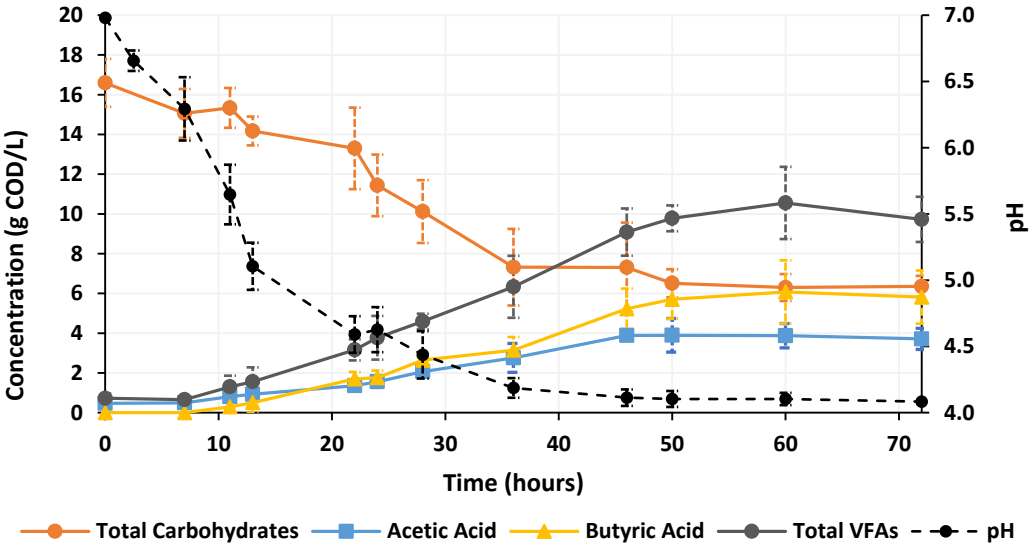

Figure 4.3 Profiles of total carbohydrates consumption, acetic and butyric acid production, and pH in the batch reactors fed with the brewery spent grain hydrolysate and operated at uncontrolled pH and 37°C

In terms of VFAs produced, acetic acid production started after approximately 7 hours, with a production rate of 0.06 (± 0.02) g COD/(L·h). Butyric acid was produced after 11 hours with a production rate of 0.15 (± 0.02) g COD/(L·h), i.e. higher than that observed for acetic acid. The acidification level in terms of g COD of VFAs per 100 g of sCOD was 44.0 (± 7.0) %, with acetic and butyric acids as the major constituents representing 95.5 % of the total sCOD (**Figure 4.4**). Lactic acid production started after 11 hours, but its concentration only reached a peak of 1.43 (± 0.43) g COD/L, i.e. 36.8 (± 12.1) and 24.6 (± 8.0) % lower than, respectively, the acetic and butyric acid concentration.

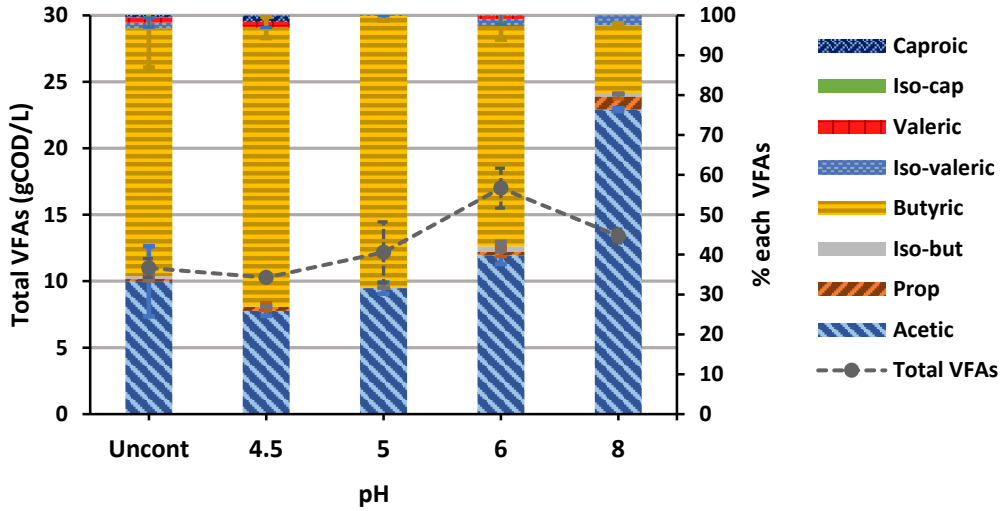

Figure 4.4 Total volatile fatty acids production and percentage of each VFA over total VFAs after the exponential phase during the acidogenic fermentation *under different pH conditions*

4.3.2.3 Acidogenic fermentation at controlled pH (4.50, 5.0, 6.0 and 8.0)

When keeping the pH constant and above 4.5 throughout the experiment, the percentage of TC consumption exceeded 90 % and was similar for each condition (**Figure 4.5*a***). The TC consumption rate increased at higher operating pH (**Figure 4.5*b***). Glucose was completely consumed after approximately 44 hours when working at pH 4.5, and only 24 hours were required to completely remove glucose when increasing the pH up to 8.0. After glucose was consumed, the degradation of xylose and arabinose started (data not shown).

The total amount of VFAs produced at each of the pH conditions tested (4.5, 5.0, 6.0 and 8.0) is shown in ***Figure 5***. The acetic and butyric acid content was the most significant fraction of the sCOD (***Figure 5***), ranging between 95.5 and 99.8 %. At pH 4.5 and 5.0, the VFA concentration remained stable from 29 to 35 hours and from 11 to 23 hours, prior to resume production until a final concentration of VFAs of 11.0 (± 0.04) and 12.2 (± 2.3) g COD/L, respectively (***Figure 4.6a***). In the case of pH 6.0 and 8.0, the intermediate phase with a stable VFA concentration was not observed. Furthermore, a delay of the VFA production compared to carbohydrate consumption was observed (***Figure 4.6a*** & **Figure 4.7**). The acetic acid production rate increased when operating the process at a higher pH. The highest production rate for butyric acid was 0.52 (± 0.04) g COD/(L·h) achieved at pH 5.0 (**Figure 4.5*b***).

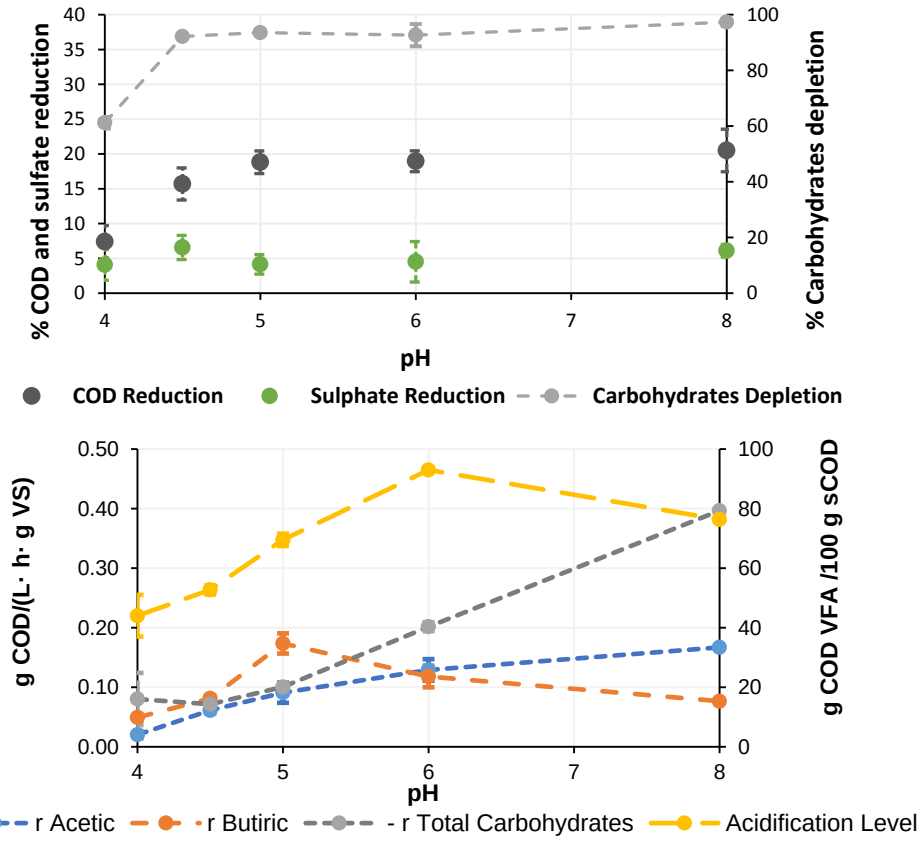

Figure 4.5 *a) Total carbohydrates depleted, reduction of soluble COD and sulphate obtained during the acidogenic fermentation of hydrolysate from brewery spent grain under different pHs in batch reactors as well as b) production rate of the butyric and acetic acid, total carbohydrates consumption rate and acidification level*

Lactic acid was produced under all conditions tested. Lactic acid production started earlier than VFA production at pH 4.5 and reached the highest concentration of 3.87 (± 0.76) g COD/L at 49 h and decreasing at the end of the trial to 2.07 (± 0.72) g COD/L. At pH 6.0, the production of lactic acid started simultaneously with VFAs, with lactic acid reaching the highest concentration of 1.08 (± 0.30) g COD/L at 17.5 (± 4.7) h, before being completely consumed after 49 h. At pH 8.0, lactic acid reached a peak of 0.62 (± 0.02) g COD/L after 18.5 h (***Figure 4.6b***).

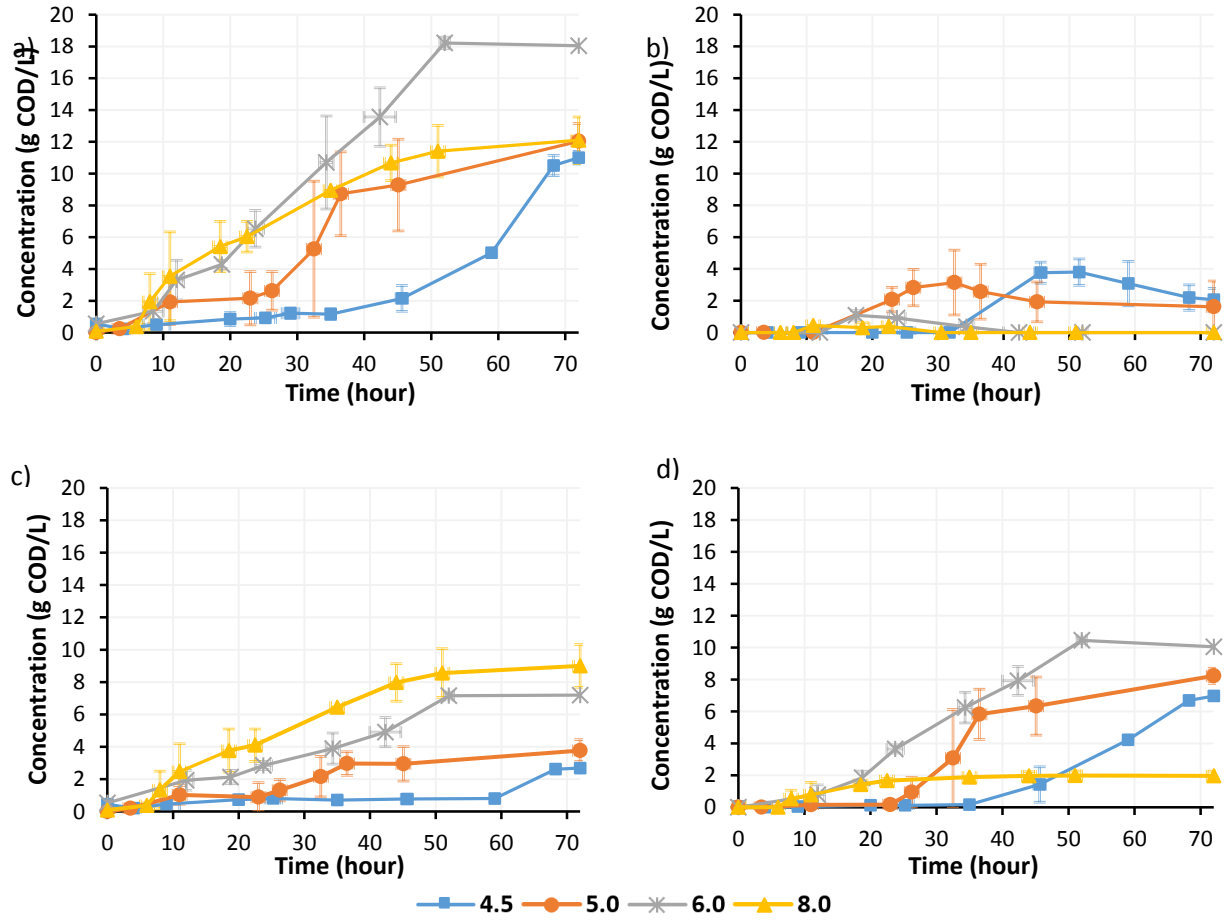

Figure 4.6 *Fermented compound production during the acidogenic fermentation of brewery spent grain hydrolysate in a batch-operated stirred tank reactor at 37 °C under different pH values. a) Total volatile fatty acids, b) lactic acid, c) acetic acid and d) butyric acid*

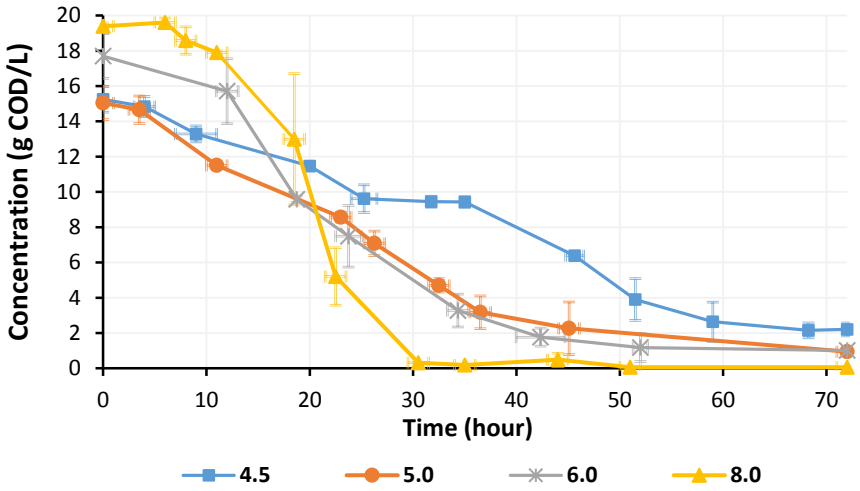

Figure 4.7 Total carbohydrates consumption at different pH (4.5, 5.0, 6.0 and 8.0) during acidogenic fermentation of brewery spent grain hydrolysate in two batch-operated stirred tank reactors at 37 °C

4.3.2.4 Nitrogen compounds, sulfate and soluble chemical oxygen demand

The average initial proteins and ammonium concentrations for all the combinations corresponded, respectively, to 3.4 (± 0.4) g/L and 194.9 (± 7.0) mg/L. The final protein concentration was 1.7 (± 0.3) and 0.9 (± 0.2) g/L in the case of uncontrolled pH and pH 8.0, respectively. This led to a lower increase of the ammonium concentration in the case of uncontrolled pH with 273.1 (± 7.7) mg/L, and a higher increment at pH 8.0 up to 468.5 (± 6.5) mg/L.

The COD consumption and sulfate reduction percentage at the end of the acidogenic fermentation were investigated for each of the pH conditions tested (**Figure 4.5*a***). The lowest COD consumption of approximately 7.4 (± 2.3) % was obtained at uncontrolled pH, while the highest COD consumption of 18.9 (± 0.1) and 20.5 (± 3.1) % (Equation 5) was observed at pH 6 and 8, respectively (**Figure 4.5*a***). The sCOD/sulfate ratio in the reactors at the beginning of the experiments was 1.41 (± 0.10) and 1.28 (± 0.10) at the end of the experiments. The average initial and final sulfate concentrations in both reactors were 15.84 (±2.20) g/L and 14.99 (± 2.50) g/L, respectively, under all the conditions investigated. This implies an average sulfate reduction efficiency of 5.1 (± 1.8) % (**Figure 4.5*a***). The ethanol concentration constantly remained below the detection limit at all pH values investigated.

4.4 Discussion

4.4.1 Operational conditions during the acidogenic fermentation

This study shows for the first time the feasibility of producing high concentrations of VFA from BSG by combining a diluted acid hydrolysis of the BSG in step one with a direct acidogenic fermentation of the BSG hydrolysate using untreated anaerobic granular sludge. Despite the anaerobic granular sludge originated from an industrial reactor aimed at methane production, the operating conditions adopted in this study resulted in VFA accumulation rather than methanogenesis as no VFA consumption was

observed after reaching the maximum VFA concentration (***Figure 4.6***). This supports previous research, which showed that a low pH and a high initial organic loading act as inhibitors of methanogenesis (Shi et al., 2017; Infantes et al., 2011; Görke et al., 2008; Xie et al., 2014). The highest acidification level was achieved at pH 6.0, with VFAs representing 93.0 % (± 2.1%) of the soluble COD (17.6 (± 0.67) g/L) (***Table 4.4***). This is an improvement of the acidification yield when compared to systems using real wastewater as feedstock under similar optimised operational conditions (***Table 4.4***).

The VFA profile obtained here was similar to those observed in other studies. In the work of Domingos et al. (2014) using lactose as source of carbohydrate at the same pH and temperature, butyric acid was the major VFA produced (Domingos et al., 2014), although a lower TC consumption and VFAs production rate (0.14 and 0.07 g COD/(L·day), respectively) were observed compared to this study (***Figure 4.5b)***. Moreover, the sludge used by Domingos et al. (2014) was an acclimated anaerobic acidogenic consortium from a continuous reactor for VFAs production. As in the present work no methane inhibitors or pretreatment of the granular sludge was applied, the observed high TC consumption rate (***Figure 4.5b)*** can be attributed to acidogenesis and acetogenesis of the monosaccharides (e.g. glucose, arabinose and xylose; **Table 4.1**) and protein released from the BSG during the acid hydrolysis step. Similarly, suppression of the methanogenesis when operating under similar conditions using sugarcane molasses as feedstock resulted in butyric acid as the major end product, but with a lower acidification level (Silva et al., 2013).

Table 4.4 Average physical-chemical composition of different feedstocks for batch acidogenic fermentation aimed at volatile fatty acid production. The percentage of the main acids (i.e. Acetic, Propionic and Butyric) as well as the acidification level in each study is provided

Feedstock	sCOD (g/L)	TC (g COD/L)	pH	Temperature (°C)	VFAs (gCOD/L)	(Ac:Prop:But) (% of VFAs)	Acidification Level	Reference
Cassava alcohol wastewater	32.1	10.1	9.0 – 5.7	35	24.3	(37:20:40)	71.3	Xie et al. (2014)
Cheese whey	-	16.5	6.0	37	19.2	(15:17:55)	N.G.	Domingos et al., (2017)
Sugarcanne molasses	8.1	N.G.	6.0	37	3.0	(53:09:26)	42.1	Silva et al., (2013)
Glycerol	10.0	N.G.	10 (initial)	35	6.7	(08:91:00)	89.9	Shen et al. (2014)
Cheese whey	3.0	N.G.	10.0 → 7.0	35.0	1.1	(07:34:49)	54.7	Atasoy et al. (2020)
BSG hydrolysate	23.1	16.9	6.0	37.0	16.9	(40:01:55)	93.0	This study

*N.G.: data not given

Interestingly, hydrolysates produced by alkaline fermentation (pH 8 in this chapter) give similar acidogenic fermentation end products (***Figure 5)***. Xie et al. (2014) operated a (high-strength wastewater fed) system by correcting the pH up to 9.0 every 12 hours obtaining butyric acid as major compound, which can be a result of pH fluctuating between 9.0 and 5.7. Nevertheless, when the system was run at constant pH 8.0 and above, butyric acid production was lower due to the increased pH used increased. Likewise, Shen et al. (2014) used glycerol as feedstock at an initial pH of 10.0, resulting mostly in the production of propionic acid with an acidification level of 89.9 %. This results was similar to that obtained in our work, where operating the system at pH 8.0 decreased the butyric acid and increased the propionic acid concentration (***Figure 4.4***). In contrast, Atasoy et al. (2020) reported the production of solely butyric acid as the major compound at all alkaline pH values investigated, with cheese whey as feedstock and using granular sludge from an UASB reactor treating municipal wastewater.

4.4.2 Total carbohydrates and monosaccharides consumption

Under uncontrolled pH, when the pH achieved the final value of 4.08 (± 0.05), only glucose was completely consumed (***Table 4.4***). On the other hand, more than 90 % of the TC was consumed in all experiments with a controlled pH (***Figure 4.5a***). The lowest pH achieved presumably led to process termination without arabinose and xylose consumption. Casey et al. (2010) similarly investigated the effect of pH and acetic acid on the co-fermentation of glucose and xylose, where low pH and increasing acetic acid concentrations led to an inhibition of the xylose consumption, while glucose consumption was unaffected. These results are in agreement with those obtained in the present study, where at the lowest pH only glucose was consumed. The accumulation of free acids likely led to product inhibition (e.g. acetate inhibition), which hindered the fermentation of xylose and arabinose.

The complete depletion of glucose under all pH values and the absent (at uncontrolled pH) (***Figure 4.7***) or delayed (at pH 4.5 or higher) consumption of xylose and arabinose (data not shown) can also be explained by a mechanism of carbon catabolite repression (Temudo et al., 2009) or the need for an extra ATP that microorganisms require for the uptake of xylose and arabinose (Zhou et al., 2018; Yao and Shimizu, 2013). Indeed, microorganisms start to consume pentoses (e.g. xylose and arabinose) only when the hexoses (e.g. glucose) are depleted (Zhang et al., 2018).

4.4.3 Carbon compounds conversion

The acidification level (i.e. the ratio between the total VFAs and sCOD) increased from 44.0 (± 7.0) % at uncontrolled pH to 93.0 (± 2.1) % at pH 6.0, whereas it decreased to 76.4 (± 0.6) % at pH 8.0 (***Figure 4.5b***). The highest VFA concentration was also obtained at pH 6.0. This increase in the acidification level from the lowest pH up to pH 6.0 can be explained by a minor concentration of the

undissociated form of VFAs, which has a strong inhibitory effect on the microorganisms at low pH (Green et al., 2007). The relation of the acids between the dissociated and undissociated form is given by the pH and the dissociation constant (Rumble et al., 2017), which is 4.76 and 4.82 for acetic and butyric acid, respectively (Khan et al., 2019). In this case, the VFA production would result in a self-inhibitory effect at higher free acid concentrations, which can be obtained by either increasing the total VFA concentration or decreasing the pH (Green, 2007). Therefore, to achieve the same inhibitory effect a lower VFA concentration would be required when decreasing the pH. This would explain the lower acidification level at pH 4.5 and 5.0 (***Figure 4.5b***), the incomplete sugar consumption and the lactic acid accumulation (**Figure 4.6*b* & Figure 4.7**). To overcome an excessive concentration of undissociated acids, the pH can be increased inducing a higher demand for alkali. Another strategy could be the use of continuous-flow systems, which withdraw the VFAs from the reactor mixed liquor (Yan et al., 2019; Aydin et al., 2018). For instance, bioreactor configurations with an immersed membrane system (Wainaina et al., 2019), or with a recirculation line coupled with a membrane contactor (Wainaina et al., 2019; Aydin et al., 2018) can allow maintenance of a low free acid concentration and can thus work at a lower feed pH or a higher TC load, meanwhile keeping the same acidification level.

The trend for the VFA production was constant with a final stable concentration until the end of the trial, indicating a low extent of the methanogenesis or even its inhibition at the last stage of the batch experiments when a decrease in VFAs was not observed after reaching the maximum VFA concentration (***Figure 4.6***). This could explain also the higher VFA production rate at pH 5.0 compared to the uncontrolled pH or at pH 6.0 (***Figure 4.5b***). The highest VFA production rate was at pH 5.0, where butyric acid peaked; meanwhile the acetic acid production rate increased along with the operating pH (***Figure 4.5b***). This shows that a lower pH is more favourable for the production of butyric acid.

Under all the acidic pH conditions investigated, butyric acid was the most produced VFA in terms of COD, followed by acetic acid. pH 8.0 resulted in a lower butyric acid production (***Figure 5***). The higher build-up of butyric acid and the absence of ethanol production has been related with the production of hydrogen gas (Zhang et al., 2013). Even though hydrogen was not measured in this study, a high hydrogen pressure likely led to the high butyric acid concentration (Zhang et al., 2013; Rodríguez et al., 2006).

Regarding the production of lactic acid, a similar trend was reported by Domingos et al. (2013) using lactose as the feedstock, which was converted into lactic acid after five days without VFA production. In the present work, at low pH (4.5 and 5.0), lactic acid was produced before the VFAs

(***Figure 4.6***), while lactic acid and VFA production occurred simultaneously at higher pH (6.0 and 8.0), although a lower lactic acid concentration was observed (***Figure 4.6b***).

The spectrum of fermentation products obtained can be explained by the use of the anaerobic granular sludge as inoculum. These granules harbour a highly diverse microbial community that results in a complex different combinations of pathways (Temudo et al., 2009; Owusu-Agyeman et al., 2018). These different pathways are influenced by the operational conditions (e.g. different pH and higher organic loading concentration), with pyruvate as the intermediate compound between the monosaccharides and the different spectrum of products obtained during the fermentation process, e.g. lactic acid, acetic acid, butyric acid and propionic acid (Atasoy et al., 2020; Casey et al., 2010; Zhou et al., 2018). The adaptation period of the microorganisms resulted in a delay for the VFA production, which was longer as lowering the pH (***Figure 4.6***). However, the TC concentration was partially consumed before the lactic acid or VFA production. This could have resulted in the accumulation of pyruvate, which can increase the intracellular NADH. Thus, in combination with a possible decreased intracellular pH, it would have promoted the activation of lactate dehydrogenase resulting in the conversion of pyruvate into lactic acid (Collet et al., 2009; Gheshlaghi et al., 2009). In addition, the longer lag phase for VFA production resulted in a higher accumulation of lactic acid at pH 4.5 and 5.0 (***Figure 4.6a***). Upon prolonged batch incubation, however, the microorganisms eventually consume the lactic acid for VFA production (Seeliger et al., 2002), as also observed in this study (***Figure 4.6a & b***)

4.4.4 Conversion of nitrogen and sulfur compounds

The consumption of the proteins during the acidogenic fermentation led to an increment of the ammonium concentration in the fermented liquor. Although the ammonium concentration has a low influence on the anaerobic digestion process, the strongest inhibition effect is due to the free ammonium (FA), which is a function of the total ammonium concentration and the pH through the Anthonisen equation (Anthonisen et al., 1976). FA has been reported to hinder the different steps of the anaerobic digestion when its concentration is increased, the methanogenesis being the most sensitive step. An FA concentration above 30 mg/L starts to inhibit methanogenesis, being almost completely inhibited at a concentration close to 60 mg/L (Shi et al., 2017). In contrast, the acidogenesis is negatively affected only when above 300 mg/L of FA (Zhao et al., 2018). During this work, when operating the system under acidic conditions the FA was much lower (between 0.0 and 0.7 mg/L at pH 4.5 and 6.0, and 65.2 mg/L at pH 8.0), which was below the inhibitory threshold for VFA production.

Sulfate removal was only observed to a limited extent (i.e. 5 %) and was similar at all pH values studied (***Figure 4.5a***). This probably occurred through a partial sulfate reduction towards sulfide production. However, as the hydrogen sulfide concentration was below the detection limit (data not

shown), this suggests that the sulfate reducing bacteria (SRB) were unlikely to be active or were outcompeted by the acidogens populations. Sulfate reducers can use simple organic electron donors such as ethanol and lactate (Papirio et al., 2013). However, during the degradation of carbohydrates to VFAs and lactic acid by acidogens, SRB were likely not favoured kinetically to use these simple compounds at the low pH in the 72 h of the batch experiment.

4.4.5 Hydrolysis efficiency

The thermal diluted acid hydrolysis at 121 °C for 20 minutes allowed the release of glucose, xylose and arabinose from BSG (***Table 4.1*****Table 4.2**). The use of 1.5 % and 2.0 % sulfuric acid (v/v) with 7 % of TS (w/w) resulted in the most efficient combination in terms of TC per g of TS, 0.44 g and 0.45 TC/g BSG, respectively (***Table 4.2***). As their efficiencies were similar, the lower sulfuric acid concentration was used to progress forward, with a severity factor corresponding to 1.09 under these conditions. In the case of 10 % TS, the presence of cellobiose indicated (***Table 4.1***) the occurrence of an incomplete hydrolysis, while conversely in the case of 7 % no cellobiose was detected.

The extraction efficiency was similar for both TC and sCOD and increased linearly when the sulfuric acid concentration was increased from 0.0 to 1.0 % (v/v) (***Figure 4.2***). After this point, the efficiency in terms of carbohydrates release and sulfuric acid used decreased, resulting in a lower improvement of the carbohydrates extracted from 1 to 3 %. The highest recovery of monosaccharides in the present work was achieved at 1.5 % of sulfuric acid and decreased using 3 % sulfuric acid at 7 % BSG (w/w). For the combinations with 10 % of BSG (w/w), glucose and xylose increased for all the sulfuric acid increments used, meanwhile the arabinose concentration decrease in the case of 3 % sulfuric acid (***Table 4.1***). Even if this combination resulted in the highest TC concentration, the loss of arabinose and the incomplete hydrolysis of the BSG, as indicated by the presence of cellobiose, decreased the efficiency of the TC extracted per amount of BSG used (***Table 4.2***). As a consequence, not only should the severity factor be used to calculate the efficiency of the hydrolysis, but also the TS concentration of the lignocellulosic biomass.

In the last years, studies focusing on the use of BSG as feedstock have proposed different strategies for the storage and initial treatment of the BSG. Few researchers used the BSG as received (Moreira et al., 2013; Kemppainen et al., 2016) and others dried or milled the BSG into different sizes (Patel et al., 2018; Ravindran et al., 2016; Rojas-Chamorro et al., 2020; Plaza et al., 2017). These physical changes are not considered as a pretreatment. However, Niemi et al. (2012) determined their effect during the hydrolysis. In the case of wet milled samples, a more homogeneous slurry was produced. Dried samples have been reported to form aggregations and more quickly sediment after submersion in water, being this related with the hornification of the BSG (Niemi et al., 2012).

The hydrolysis of BSG for recovery of carbohydrates has been studied using a single or two step-process (

Table 4.5). Carvalheiro et al. (2004) studied the hydrolysis of BSG using a single step, a thermal diluted acid hydrolysis, achieving a high xylose and arabinose recovery at a low severity factor and maximum glucose extraction under a higher severity factor (1.94). These observations are slightly different than those of the present work as a higher glucose extraction was obtained (

Table 4.5) using a lower severity factor (1.09). This could be explained by how Carvalheiro et al. (2004) stored the BSG: washing and drying remove some of the remaining sugars from the brewery process and hornify some of the carbohydrates. Therefore, the use of the raw BSG not only reduces the energy consumption, but also increases the TC recovery.

In studies using a two step hydrolytic pretreatment, some have mainly focused on glucose recovery, using the first step for the enrichment of cellulose in the solid fraction for the next extraction, without quantifying the sugars extracted (mainly xylose and arabinose). Ravindram et al. (2016) carried out different pretreatments followed by enzymatic hydrolysis, with the microwave assisted alkali hydrolysis being the most effective, followed by diluted sulfuric acid. However, the overall efficiency was much lower as only the TC from the second extraction were quantified (

***Table* 4.5**). Similarly, Patel et al. (2018) followed a method for cellulose concentration using an organosolvent pretreatment achieving a TC concentration of 71.6 g/L, mainly made up of glucose and xylose.

Table 4.5 *Different studies for the hydrolysis of the brewery spent grain using different process in one or two combined steps for the recovery of monosaccharides*

BSG Characterisation			1st extraction	TC	Glu	Xyl	Gal	Ara	2nd extraction	TC	Carbohydrates Recovery	Efficiency recovery	Reference
TC (%)	Proteins (%)	Lignin (%)		g/L	g/L	g/L	g/L	g/L		g/L	g TC/ g BSG	g TC recovered/ 100 g TC raw BSG	
55.1	12.54	13.7	Organosolv pretreatment (10 % BSG w/w; 60 % ethanol; 1 % H_2SO_4 wt dry BSG; 175 °C, 60 minutes)	N.G.	N.G.	N.G.	N.G.	N.G.	Enzymatic hydrolysis (10 % TS; 50 °C; 27 h)	71.6	NG	NG	Patel et al., (2018)
43.5	23.3	19.4	Wet milling (95 % particle size < 40 μm) + Enzymatic extraction (3 % TS; 50 °C; 5 h)	6.7	2.8	2.1	0.28	1.12	None	-	0.15	0.35	Niemi et al., (2012)
47.8	23.5	22.8	TDAH (8 % BSG w/w; 3 % H_2SO_4 V/V; 130 °C; 15 min)	43.5	3.99	26.74	-	12.77	None	-	NG	NG	Carvalheiro et al., (2004)
46.2	-	-	Microwave assisted alkali (1 % BSG w/v, 0.5 % NaOH (w/v); 400 W; 60 s)	N.G.	N.G.	N.G.	N.G.	N.G.	Enzymatic hydrolysis (10 % TS; 50 °C; 120 h)	18.6	0.23	0.50	Ravindram et al., (2018)
43.5	22.6	4.1#	Steam explosion	-	5.69	8.23	-	3.84	Enzymatic hydrolysis (20 % TS)	N.G.	0.26	0.62	Kemppainen et al., (2016)
45.6	23.1	12.5	TDAH (11.11 % BSG; 1 % H_2SO_4 wt TS; 130 °C; 26 min)	43.4	9.44	21.68	2.31	11.08	Enzymatic hydrolysis (5 % TS ; 50 °C; 24 h)	14.4 (72.04*)	0.48	0.94	Rojas-Chamorro et al., (2020)
46.0	NG	NG	TDAH (15 % BSG w/w; 1 % H_2SO_4 wt dry BSG; 121 °C; 30 min)	47.0	20.0	18.4		8.6	Enzymatic hydrolysis (10 % TS; 50 °C; 48 h)	32.9	0.43	0.95	Plaza et al., (2017)
ND	ND	ND	TDAH (7 % BSG w/w; 1.5 % H2SO4 v/v; 121 °C; 20 min)	32.0	12.8	12.05	-	11.8	None	-	0.44	ND	This study

*ND: not determined

*NG: data not given

#: acid insoluble lignin

In the pretreatments where the overall carbohydrates extracted are considered, the obtained efficiency values are seen to be higher. Rojas-Chamorro et al. (2020) and Plaza et al. (2017) used a two step process, with the first step being a thermal diluted acid hydrolysis (similar to the first step applied in this study). Both studies used a lower sulfuric concentration and a higher concentration of BSG than those used in this study, resulting in a lower efficiency recovery and a higher TC concentration compared to this study. However, the final overall efficiency of the two step hydrolysis process recovered almost all the initial carbohydrates from the BSG (Rojas-Chamorro; Plaza et al., 2017).

Besides carbohydrates, the use of sulfuric acid as a pretreatment agent allowed the release of high-value compounds such as lignin, proteins and soluble fibres from BSG (***Figure 4.2***). Rommi et al. (2018) compared different pretreatment combinations (i.e. raw BSG, steam explosion, alkaline and acid extraction) for lignin and protein recovery, based on a previous work focused on carbohydrates extraction from BSG (Rojas-Chamorro et al., 2020). They observed that the solubilisation of different organic compounds like lignin, proteins and polysaccharides strongly depended on the pretreatment combination. For example, steam explosion and hydrolysis increased lignin solubilisation, while protease was required to improve protein extraction (Rommi et al., 2018). Similarly, the diluted acid hydrolysis and neutralization used in the present study could be developed to recover some of the proteins extracted using the lowest solubility point of proteins (i.e. pH 3 – 5) (Liu et al., 2018; Guimaraes et al., 2012). This was, however, out of the scope of the present study, although the recovery of a broader range of end compounds beyond VFAs would be beneficial for the overall process.

4.5 Conclusions

The feasibility of VFA production from BSG was demonstrated in this study, reinforcing the change in the point of view of BSG from a solid waste into a valuable resource. First, a mixed liquor rich in carbohydrates with 0.44 g of carbohydrates per gram of total solids of BSG was obtained using a single step of a thermal diluted sulfuric acid hydrolysis (1.5 % sulfuric acid v/v). Then, the BSG hydrolysate was used for VFA production during carbohydrate fermentation by an anaerobic granular sludge. At the different pH values tested, the contribution of VFAs to the soluble COD at pH 6.0 was approximately 93.0 (± 2.1) %. Therefore BSG has a great potential as a raw feedstock for VFA production. Further research is required for the development of the process at a larger scale and in a continuous-flow mode.

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Chapter 5

Continuous volatile fatty acid production from brewery spent grain hydrolysate using an expanded granular sludge bed reactor

A modified version of this chapter has been submitted as:

> **Castillla-Archilla J.**, Heiberger J., Mills S., Hilbig J., Collins G., Lens P. N. L. (2021) "Continuous Volatile Fatty Acid Production From Acid Brewery Spent Grain Leachate in Expanded Granular Sludge Bed Reactors" Frontiers in Sustainable Food Systems 5, 129. DOI=10.3389/fsufs.2021.664944

Keywords: brewery spent grain; pretreatment; thermal diluted acid hydrolysis; carbohydrates; acidogenic fermentation; volatile fatty acids

Abstract

The production of volatile fatty acids (VFAs) in expanded granular sludge bed (EGSB) reactors using liquid hydrolysate from thermal diluted acid hydrolysis of brewery spent grain was evaluated. Partial inhibition of the anaerobic digestion process to induce VFA accumulation was achieved by applying a high organic loading rate (from 15.3 to 46.0 gCOD/(L·day)), and using a feed with an inlet concentration of 15 g/L total carbohydrates. Two EGSB reactors were operated under identical conditions, both inoculated with the same granular sludge. However, granular sludge in one reactor (R1) was subsequently disaggregated to flocculent sludge by a pH shock, whereas granules remained intact in the other reactor (R2). The hydraulic retention time (HRT) of both reactors was decreased from 36 to 24, 18 and 12 hours. The main fermented compounds were acetic acid, butyric acid, propionic acid and ethanol. Despite fluctuations between these products, their total concentration was quite stable throughout the trial at about 134.2 (± 27.8) and 141.1 (± 21.7) mmol/L respectively, for R1 and R2. Methane was detected at the beginning of the trial, and following some periods of instability in the granular sludge reactor (R2). The hydrogen yield increased as the HRT decreased. The highest VFA production was achieved in the granular sludge reactor at a 24 h HRT, corresponding to 120.4 (± 15.0) mmol/L of VFAs. This corresponded to an acidification level of 83.4 (± 5.9) g COD of VFA per total gram of soluble COD.

5.1 Introduction

Anaerobic digestion (AD) has been widely implemented as a method of biological wastewater treatment, where mixed microbial communities convert organic matter to biogas (CH_4 and CO_2). However, in the last decade there has been a growing interest in hydrogen production via dark fermentation, facilitated by the partial inhibition of the anaerobic digestion process (Cisneros-Pérez *et al.*, 2015). During the acidogenesis step of AD, hydrogen and carbon dioxide are produced, along with fermented products such as ethanol and/or volatile fatty acids (VFAs). To improve this process and maximize hydrogen production, different pre-treatments have been applied to the inoculum and during reactor start up, including heat treatment (Cisneros-Pérez *et al.*, 2015; Carrillo-Reyes *et al.*, 2016), wash-out of methanogens (Cisneros-Pérez *et al.*, 2015; Carrillo-Reyes *et al.*, 2016) or pH shocks (Kan, 2013). The alteration of different operational conditions, such as pH or organic loading rate, can however, change the metabolic activity microorganisms. Different reactor configurations can also influence microbial community composition by altering selection pressures such as solids retention time, resulting in the wash out of microorganisms with slower growth rates, e.g. methanogens, or enriching faster growing organisms, e.g. acidogens (Lim *et al*., 2020; Etchebehere *et al.*, 2016).

The VFAs produced simultaneously with hydrogen are valuable chemicals, currently obtained from fossil sources. Their potential production from renewable sources has not yet been developed at industrial scale. The development of hydrogen production from wastewater into more sophisticated technologies for VFA production could allow VFAs to be produced independently of fossil sources. One of the difficulties is, however, to maintain consistent VFA production, despite shifts in microbial community composition and changes in bioreactor performance (Cisneros-Pérez *et al.*, 2017). The accumulation of organic compounds in the reactor mixed liquor can alter reaction kinetics in the microbial metabolic pathways of acidogenesis and methanogenesis (Ciranna *et al.*, 2014; Pugazhendhi *et al.*, 2017; Menezes and Silva, 2019). This can activate or inactivate the consumption of hydrogen by different microorganisms, resulting in unstable hydrogen and VFA production (Saady, 2013; Regueira *et al.*, 2020). In addition, the main problem with VFA production from wastewater is that their recovery is difficult and energy demanding, due to low VFA concentrations and complex composition of the bioreactor mixed liquor (Reyhanitash *et al.*, 2016). The current state of-the-art for VFA extraction enables their recovery when operating at a low pH and with VFA concentrations exceeding 200 – 500 mmol/L (Rocha *et al.*, 2017; Reyhanitash *et al*., 2017; Saboe *et al.*, 2018).

Many wastewaters are low or medium strength and do not contain high carbohydrate concentrations, making them unsuitable for VFA production and recovery. Various reactor configurations have been optimised to use such wastewaters for hydrogen production via dark fermentation, without focusing on VFA production. Some of these reactors were operated with a

synthetic feed concentration of about 60 g/L total carbohydrates (TC) (Zhang *et al.*, 2008), and an organic loading rate (OLR) of up to 240 g TC/(L·d) (Zhang *et al.*, 2008; Menezes and Silva, 2019). However, waste streams with a higher TC concentration would be required for targeted VFA production, such as those originating from the agroindustry (Menezes and Silva, 2019). The agriculture and food industry could, therefore, become a supplier of renewable feedstock for VFA production. These wastes, mostly lignocellulosic materials, are abundant and can be obtained with low or no cost. For example, the brewing industry produces high amounts of brewery spent grain (BSG), which is a solid, lignocellulosic material rich in carbohydrates, proteins and lignin. After the hydrolysis process, most of these carbohydrates are present in the liquid hydrolysate at concentrations up to 30 – 70 g/L of TC (Patel et al., 2017). This liquid hydrolysate can be used for VFA production without any further treatment for production of VFAs as demonstrated in ***Chapter 4***.

The aim of this work was to study the production of VFAs from BSG by two different sludge types, in a continuous high rate EGSB system with a long HRT (36 to 12 hours) and high OLR (10 to 30 gTC/(L·d)). Two continuous EGSB systems were used, seeded with the same granular sludge. After seeding, the granular sludge in one reactor was disaggregated by providing a pH shock. This allowed to study the effects of the microbial selection pressure on the resulting flocculent sludge. The effectiveness of this strategy for targeted VFA production was determined by monitoring subsequent sludge development and measuring VFA and hydrogen production in both reactors.

5.2 Material and methods

5.2.1 Feedstock and inoculum

The hydrolysate from the hydrolysis pretreatment developed in ***Chapter 4 w***as used for the continuous VFA production. Briefly, BSG at 7% TS concentration (w/w) was mixed with 1.5 % sulfuric acid (v/v), and autoclaved at 121 °C for 20 minutes. Two modifications were carried out in the present work, the remaining solids following hydrolysis were washed with water of an equal volume to the extracted hydrolysate, obtaining a second liquid with a TC concentration between 6 – 8 g/L. This extract was used to dilute the initial feedstock to a final concentration of 15 g/L of TC. In addition, the hydrolysate was centrifuged after neutralization to remove any further precipitate in the liquid fraction. Thus, the feed was mainly composed of 6.5 (± 1.4) g/L glucose, 5.5 (± 0.4) g/L xylose, 2.8 (± 1.0) g/L arabinose and 2.1 (± 0.4) g/L proteins.

Once the feedstock was prepared, it was placed in a fridge at 4 °C and connected to the inlet of the EGSB reactors. The feedstock was continuously stirred using a magnetic stirrer (Stuart UC151, UK) operated at 1,500 rpm. Initially, the feedstock was replaced every 3 days. However, after the first two weeks of operation it was noticed that the TC concentration in the feedstock tank decreased slightly

three days after preparation. The feedstock was, subsequently, replaced every two days and the bottle was closed with a rubber bung to prevent contamination. A needle with a 25 µm filter connected was used to pierce the bung and prevent vacuum formation. Consequently, no further degradation of TC was noticed in the feedstock.

Same granular sludge was used to seed the reactors. To study the selection pressure of the EGSB reactor system, two biomass types derived from the same UASB inoculum were studied: flocculent and granular sludge. Both reactors were seeded with the same granular sludge at an inoculum concentration of 1.5 g/L of volatile suspend solids (VSS). Granules in Reactor 1 (R1) were disaggregated into flocculent sludge by increasing the pH from 5.9 to 10.5 for 12 hours on day 21, similarly to Kan (2013). In contrast, Reactor 2 (R2) was operated with intact granular sludge during the whole experiment.

Due to a decrease in VFA production in R1 beginning on day 67, the reactor was reseeded with fresh granular sludge on day 81, with the same initial concentration of 1.5 g/L VSS. The fresh granular sludge was added without emptying the reactor, the flocculent sludge already present inside of the reactor was not removed.

5.2.2 Experimental set-up

Two EGSB reactors (total volume of 2.27 L; working volume of 1.78 L) were operated under the same conditions (***Figure 5.1***). The feedstock was introduced from the bottom of the reactor using a pump (Master-Flex L/S, USA). To keep the up-flow velocity constant, a recycling line was connected from the fourth port of the reactor (70 cm from the bottom) to a connection tube placed below the feed inlet. Reactor mixed liquor was circulated through this line by a pump (Watson-Marlow 323, UK). A gas meter (Omega, USA) was connected to the outlet gas line, with a port for sampling purposes. A Cole-Parmer 300 (China) pH controller, connected to a Master-Flex pump, was used for sodium hydroxide addition (3 M), maintaining the pH at 5.90 (± 0.05). The pH probe of the pH controller was placed in the third port from the bottom of the reactor (40 cm) and the NaOH addition tube was connected to the recycling line of the reactor. The temperature was kept at 37 °C using a water bath (Grant Instruments TC120).

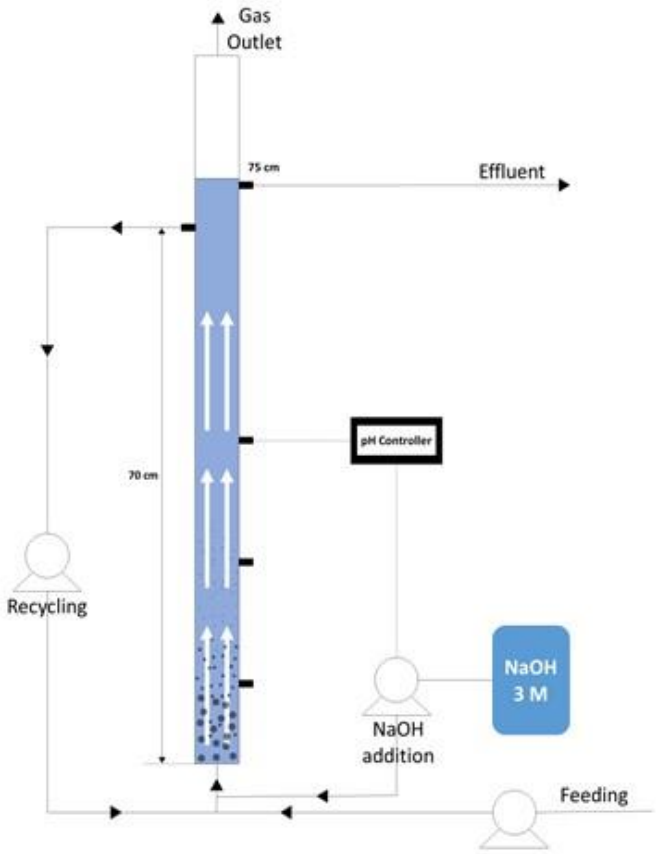

Figure 5.1 *Schematic set up of the EGSB reactor used for the continuous production of VFAs. Pump for the influent supply, recycling pump and base addition (3M NaOH) connected to a pH controller are shown*

5.2.3 Experimental design

Initially, during the first 12 hours of operation, the reactors were operated in batch mode, with a feedstock concentration of 10 g/L TC to allow the acclimatization of the anaerobic granular sludge. After the acclimatization period both reactors were fed with a constant TC concentration of approximately 15 g/L. R1 was started in advance, and 15 days later, Reactor 2 (R2) was started-up in a similar way. R1 was operated for 108 days and R2 was operated for 93 days. The trial was divided into four different phases in which the HRT was gradually decreased from 36 hours (Phase I) to 12 hours (Phase IV), as indicated in Table 1. All the changes in the reactor, HRT and up-flow velocity were carried out simultaneously in both reactors.

The initial up-flow velocity was 6 m/h, which was decreased to 3.0 m/h, at day 29 for R1 and day 14 for R2. A further reduction from 3.0 to 2.0 m/h in R1 (flocculent sludge) and from 3.0 to 2.5 m/h in R2 (granular sludge) was done on days 81 and 66, respectively.

Table 5.1 *Hydraulic retention time and organic loading rate for each of the different phases during the continuous acidogenic fermentation of brewery spent grain hydrolysate using in an expanded granular sludge bed reactor*

		Phase I	Phase II	Phase III	Phase IV
HRT (h)		36	24	18	12
OLR (gTC/(L·d))		10	15	20	30
Time (days)	**R1**	0 → 37	37 → 67	67 → 94	94 → 108
	R2	0 → 22	22 → 52	52 → 79	79 → 93

*Note that R1 was started-up 15 days prior to R2. Changes in HRT and OLR were done on the same operational day

5.2.4 Analytical methods

Total suspend solids (TSS) and VSS were sampled twice weekly from the effluent and the recycling ports at 70 and 75 cm height, respectively, in order to determine any possible wash-out and biomass flotation within the reactor due to the settleability of the biomass. Samples for chemical analysis were centrifuged at 9,000 rpm for 5 minutes and the supernatant was filtered using a syringe filter with 0.22 µm pore size. The samples were then frozen for further analysis. TC analysis was performed by the Dubois method (Dubois et al., 1956). Ammonia, sulfate and total proteins were analysed by using a discrete nutrient analyser (Gallery Plus, ThermoFisher) as described by Castilla-Archilla *et al*. (2020). The sCOD was analysed using a continuous Seal Analytical AA3 flow autoanalyser (Norderstedt, Germany) (SEAL, 2019).

VFAs, glucose, xylose, arabinose, lactose and ethanol were measured using a 1260 Infinity II liquid chromatograph (Agilent, Germany) equipped with a Hi Plex H 7.7 x 300 mm and 8 µm (p/n PL1170-6830) column (Agilent, UK) kept at 60 °C and an RI detector at 55 °C. The mobile phase was sulfuric acid with a concentration of 5 mM and a flow rate of 0.7 mL/min (Castilla-Archilla et al., 2020). The composition of the gas in terms of H_2, CH_4 and $C0_2$ was analysed by gas chromatography (7890B, Agilant, USA) as reported by Oliva *et al.* (2020).

5.2.5 Calculations

All gas was converted to standard conditions (1 atm, 273 K). The gas production rate was calculated using the fraction for each of the gases multiplied by the total gas accumulated between two points and divided by the time elapsed (***Equation 1***) (Mota *et al.*, 2018):

$$r_G = \frac{(V_{T,n} \cdot Y_{G,n}) - (V_{T,n-1} \cdot Y_{G,n-1})}{(t_n - t_{n-1})} \qquad (mL/day) \qquad \textit{Equation 1}$$

where: $V_{T,n}$, $V_{T,n-1}$ are total gas produced at time n and n-1; $Y_{G,n}$, $Y_{G,n-1}$: fraction of the gas at time n and n-1; t_n, t_{n-1}: time at point n and previous point.

The volumetric gas production rate (VGPR) was calculated by dividing the gas rate production by the working volume of the reactor (***Equation 2***):

$$VGPR = \frac{r_G}{V_W} \qquad \left(^{mL}/_{day \cdot L}\right) \qquad \textit{Equation 2}$$

where V_w is the working volume of the reactor

The hydrogen yield (HY) was considered as the amount of hydrogen (mmol) per amount of the TC consumed (mmol) (Fernández, Villaseñor and Infantes, 2011). The molecular weight of the TC was calculated based on the contribution of each of the monosaccharides (***Equation 3***):

$$HY = \frac{\frac{r_{H_2}}{22.4}}{\frac{([TC]_i - [TC]_e) \cdot Q_f}{P.m.(TC)}} \qquad \left(^{mmol\ H_2}/_{mmol\ TC_{consumed}}\right) \qquad \textit{Equation 3}$$

Where, r_{H2} is the hydrogen production rate (mL/day), [TC] the total carbohydrates concentration, Q_f the feeding flow, P.m. the molecular weight for the total carbohydrates, i: the inlet of the reactor and e: effluent of the reactor. This was used to compare how much of the TC was used for hydrogen production in each of the phases.

5.3 Results

5.3.1 Total carbohydrates fermentation during the acidogenic fermentation

The main fermented compounds obtained in both reactors were acetic acid, butyric acid, propionic acid and ethanol, their concentrations fluctuated in both reactors during the different phases (**Figure 5.2*a***). In both EGSB systems, propionic acid concentrations decreased when a lower HRT was applied. In R1, acetic acid was the major compound, followed by ethanol, propionic and butyric acid during Phase 1. After disaggregation of the granular sludge by increasing the pH, the butyric acid concentration increased and the ethanol and propionic acid concentrations decreased. A similar trend was observed during Phase II until day 50, when butyric acid was the major fermentation product and propionic acid concentrations were much lower than in the previous phase (**Figure 5.2*a***).

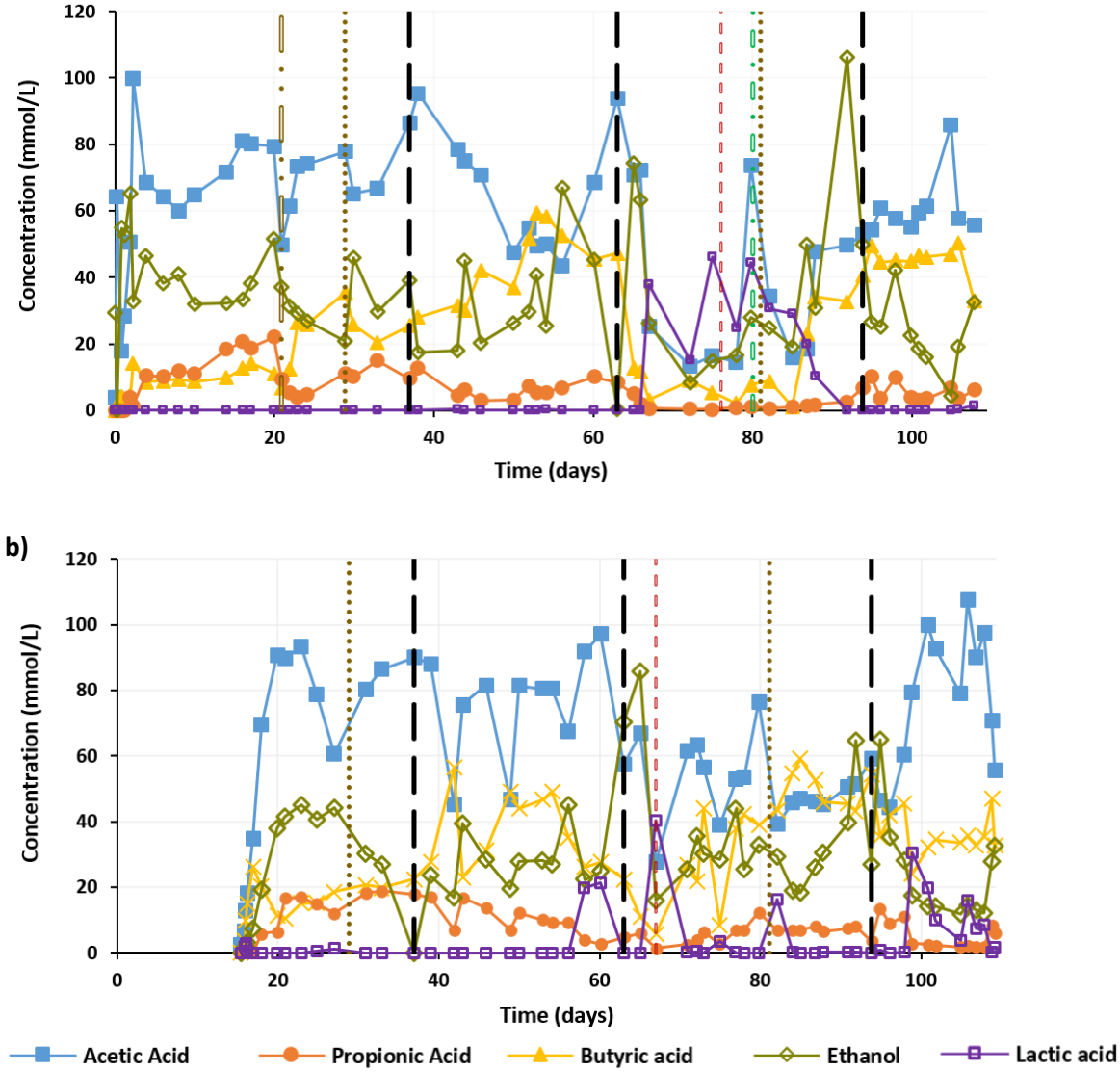

Figure 5.2 *Production of main fermentation compounds along the trial for the continuous acidogenesis of brewery spent grain hydrolysate in an expanded granular sludge bed reactor.* ***a)*** *Reactor 1 (flocculent sludge) and* ***b)*** *Reactor 2 (granular sludge).* — · *flocculation of the granular biomass;* ····· *change in up-flow velocity;* — – *change in HRT;* – – ◦ *biomass addition. Note that R2 started 15 days after R1, as an independent system. The changes for the operational conditions were applied simultaneously for both reactors*

Lactic acid was produced in R1 during Phase III, and during periods of instability in R2. In R1, when the OLR was increased on day 66 up to 20 g TC/(L·d) (Phase III), lactic acid became the major product replacing all the other compounds (**Figure 5.2*a***).). On day 80, R1 was reseeded with the same fresh granular sludge and lactic acid was still the major compound until day 86. Subsequently, lactic acid concentrations decreased and were depleted by day 92. At the same time, the concentration of acetic acid, butyric acid and ethanol started to increase 5 days after the reseeding of the reactor. Ethanol achieved its maximum concentration during this phase. By phase IV, butyric and acetic acid became the major fermentation products (**Figure 5.2*a***).

In R2, the trend was quite similar during each phase, with acetic acid as the major compound throughout its operation (**Figure 5.2*b***). During Phase III, the pH of R2 dropped to 4.85 on day 52 due

to a problem with the NaOH addition pump. As a result of this instabiltity, the lactic acid concentration increased, while the butyric acid and ethanol concentrations decreased (**Figure 5.2*b***). Lactic acid was detected on some other days, however no pH instabilities or operational problems corresponded with these peaks. During Phase-III the acetic acid concentration increased until the upflow velocity was reduced from 3.0 to 2.0 m/h on day 66, resulting in a decrease in acetic concentration from 76.5 to 39.2 mmol/L (**Figure 5.2*b***). Acetic acid then fluctuated with butyric acid and ethanol as the major compound, throughout Phase -lll. Acetic acid was once again major compound in Phase IV (**Figure 5.2*b***).

Both reactors performed similarly in terms of the TC reduction, which exceeded 98 % during all phases. The total amount of ethanol, acetic acid, butyric acid and propionic acid remained almost constant throughout the trial (**Figure 5.3**), except when lactic acid became the major product, during periods of instability such as the beginning of Phase III in R1 until five days after the reactor was reseded (days 63 to 85) (**Figure 5.2**a). During this period, when lactic acid was the major product, the combined concentration of ethanol, acetic acid, butyric acid and propionic acid decreased from day 67 to day 87 (**Figure 5.3**). In addition, both reactors presented similar fluctuations between the main fermentation products.

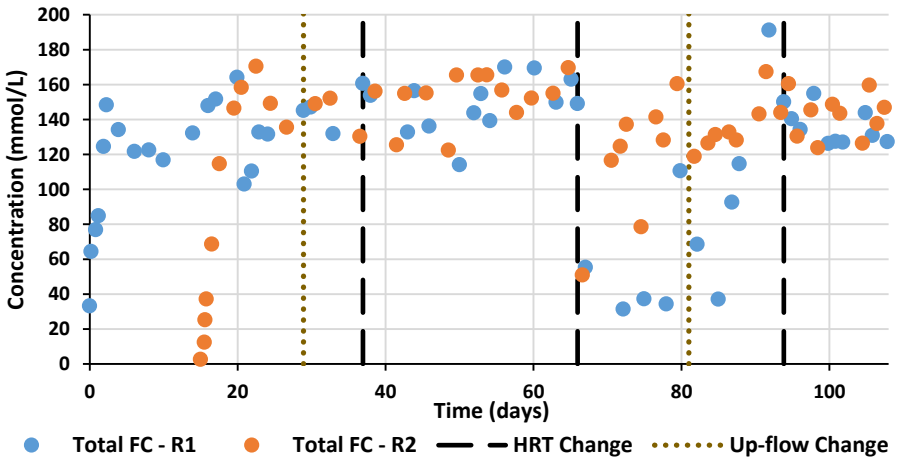

Figure 5.3 *Total concentration of the fermentation products (acetic acid, propionic, butyric acid and ethanol) for R1 and R2 during the continuous acidogenic fermentation of brewery spent grain hydrolysate in expanded granular sludge bed reactors. Note that R2 started 15 days after R1. The changes for the operational conditions were applied simultaneously for both reactors (see**Table 5.1**)*

5.3.2 Biomass behaviour

5.3.2.1 Flocculent sludge (R1)

R1 was initially operated for three weeks with intact granular sludge before granules were disaggregated by increasing the pH on day 21 for 12 hours. During these initial three weeks, some changes in the biomass characteristics were observed. The granules, which were initially black, compact and spherical, began to peel (***Figure 5.4a***), resulting in the formation of grey flocculent sludge. By day 9, the sludge bed had three different stratified layers despite the upflow velocity in the reactor

being 6 m/h (***Figure 5.4b***). The bottom layer was composed of black granular sludge similar in appearance to the seed sludge. The top layer was mostly grey-brown flocculent sludge, with some aggregates. The intermediate layer consisted of black granules coated with flocculent biomass, similar to that of the top layer. By day 15, further disaggregation of the new grey-brown aggregates in the top layer occurred and the black granules in the middle layer were no longer covered by the grey flocculent sludge (***Figure 5.4c***), resulting in the wash-out of some of this biomass. Initially, the VSS concentration in the recycling and effluent lines was lower than 2 g VSS/L. After natural disaggregation of the new aggregates, this increased to above 6.0 g VSS/L on day 18 (***Figure 5.4***).

Figure 5.4 *Granular sludge development in an EGSB reactor aimed at acidogenesis of brewery spent grain hydrolysate. Fig. 4A – Fig. 4D corresponds to Reactor 1 (R1) and Fig. 4E – Fig. 4H corresponds to Reactor 2 (R2).* ***a)*** *R1 day 0, the fresh granular sludge (inoculum);* ***b)*** *R1 day 9, three different layers in the sludge bed;* ***c)*** *R1 day 19, the granular sludge and the new flocculent sludge prior pH disaggregation;* ***d)*** *R1 day 30, flocculent sludge after the pH disaggregation.* ***e)*** *R2 day 4, granular sludge and new flocs;* ***f)*** *R2 day 13, three layers within the sludge bed;* ***G)*** *R2 day 16, granular sludge and flocs after natural disaggregation; and* ***H)*** *R2 day 40, two different layers in the sludge bed with granular sludge at the bottom and flocculent sludge on top*

To fully disaggregate the remaining granules in R1, the pH was increased to 10.5 for 12 hours on day 21. Subsequently, all granules were disintegrated and only flocculent sludge was present in R1 (***Figure 5.4d***). On day 29, the up-flow velocity was decreased from 6 to 3 m/h, which resulted in a reduction in the VSS content in the recycling and effluent lines (***Figure 5.5a***). When the HRT was further shortened from 36 to 24 h (Phase II), the VSS concentration in the effluent ports increased, to reach its highest concentration of over8 g VSS/L. Beyond this point the VSS concentration decreased, with the lowest values in Phase III-a (0.87 (± 0.03) and 0.86 (± 0.11) g VSS/L for the recycling and effluent ports, respectively). On day 80 due to low VFA production, R1 was reseeded with granular sludge. The up-flow velocity was decreased on day 81 to 2 m/h. The VSS during the end of this Phase III-b was around 4.0 and 3.6 g VSS/L in the recycling and effluent lines. Finally, during Phase IV the VSS in both sampling ports decreased to a similar value of 2.6 g VSS/L (***Figure 5.5a***).

5.3.2.2 Intact granular sludge

R2 was seeded with the same granular sludge, which developed in a similar manner to R1 for the first 15 days of operation, i.e. the granular sludge peeled and flocculent sludge of a lighter colour appeared in the upper part of the sludge bed (***Figure 5.4e***). This flocculent sludge started to form aggregates and cover some of the original black granular sludge, which resulted in three layers in the bed of the reactor: i) black granules at the bottom, ii) black granular sludge covered by the flocculent sludge in the middle section, and iii) new aggregates and granules of grey-brown colour formed at the top section of the bed sludge (***Figure 5.4f***). The VSS concentration in the recycling line increased from 0.67 (± 0.01) g VSS/L on day 0 to 2.44 (± 0.01) g VSS/L on day 13.5 (***Figure 5.5***). In order to prevent disaggregation of the newly formed aggregates due to the shear forces, the up-flow velocity was changed from 6 to 3 m/h. Nevertheless, the biomass followed a similar trend to R1, and on day 15 the new grey flocculent sludge coating the black granules, as well as the new aggregates and granules with a lighter colour were naturally disaggregated (***Figure 5.4g***). This resulted in an increase in the VSS concentration in the effluent and to a greater extent, the recycle line (***Figure 5.5b***).

For the remainder of the trial, the bottom of the sludge bed in R2 was composed of mainly black granules. The top section was composed of flocculent sludge and the middle section of a mixture of both (***Figure 4-H***). The flocculent sludge bed section expanded through Phase I and by Phase II reached the recycle line. However, the VSS content in the effluent was higher during Phase I, with the highest concentration corresponding to 9.30 (± 0.85) g VSS/L. When the HRT was decreased to 18 h the VSS content in both the effluent and recycle lines decreased until the end of the trial (***Figure 5.5***).

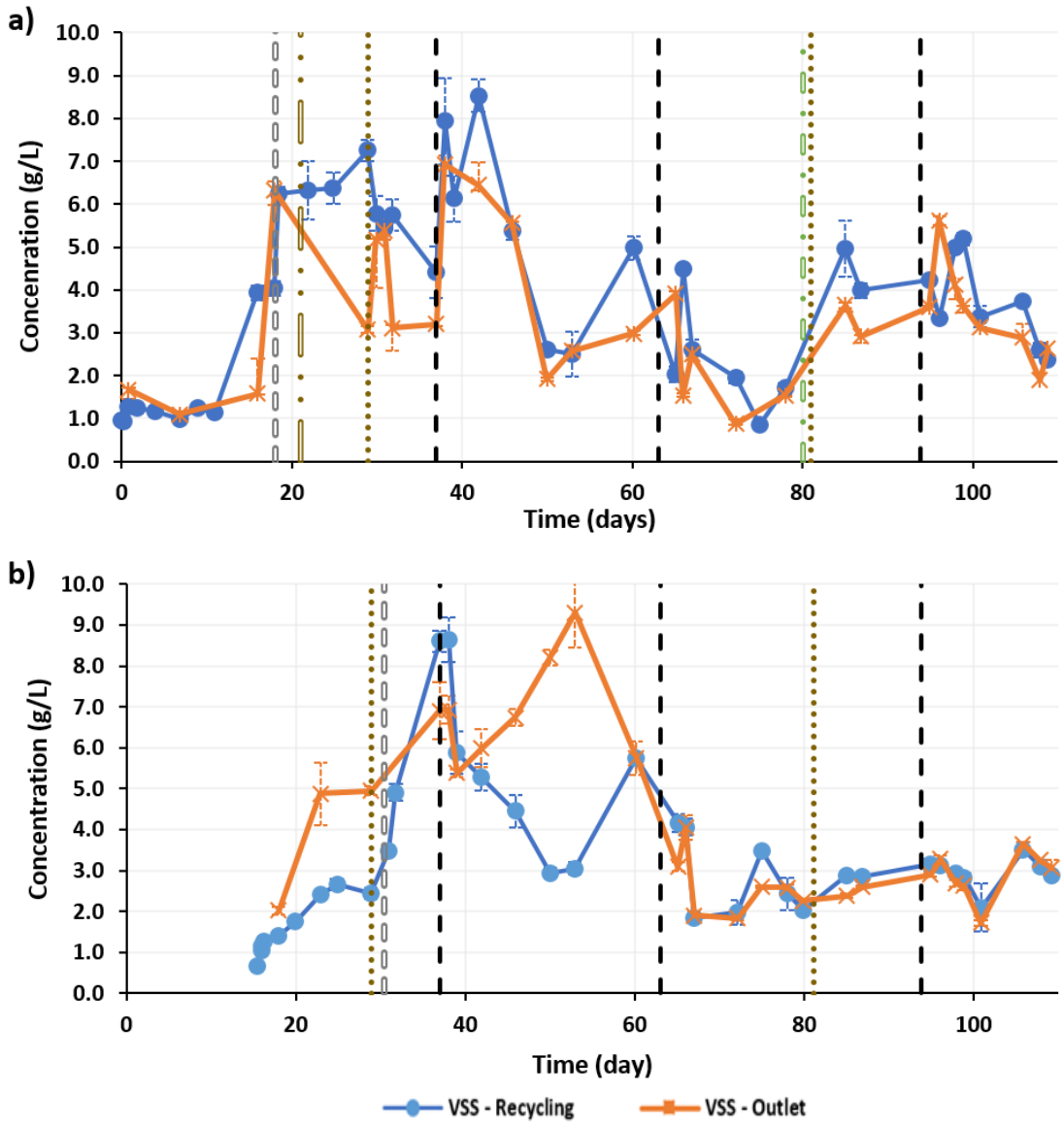

Figure 5.5 *Volatile suspended solids in the recycling and effluent sampling ports during the continuous acidogenic fermentation using brewery spent grain liquid hydrolysate in an expanded granular sludge bed reactor throughout the trial for A) Reactor 1 (flocculent sludge), and B) Reactor 2 (granular sludge) (natural disaggregation of the new grey biomass; flocculation of the granular biomass by pH increase; up-flow changes; HRT changes; biomass addition). Note that R2 started 15 days after R1. The changes for the operational conditions were applied simultaneously for both reactors*

5.3.3 Gas production

H_2 and CO_2 were the major components of the biogas (**Figure 5.6**). At the beginning of the trial, methane was detected in both reactors until day 21 in R1 and day 19 in R2 (**Figure 5.6**). Methane was also detected in R2 from day 55 to 57, albeit below quantification level as the signal during GC analysis was below the lower standard value (5%). Following the reseeding of R1 on day 81, methane was detected between days 88 and 90 at a concentration of 7 %. Prior to this, H_2 was the major compound of the total biogas and no methane was detected. For all other gas phase samples in both reactors methane was below than the detection limit.

Generally, in R1, the VGPR increased when operating at a lower HRT (***Figure 5.5a***). Although during Phase III the VGPR decreased until the reactor was reseeded, after which, the VGPR increased. In R2, the gas production was more stable for Phase I than Phase III, when the VGPR increased with decreasing HRT (***Figure 5.5b***). The highest VGPR and HY were achieved during Phase III before reducing the up-flow velocity from 3. 0 to 2.5 m/h. By Phase IV, the VGPR and HY decreased further until the end of the trial (***Figure 5.5b***).

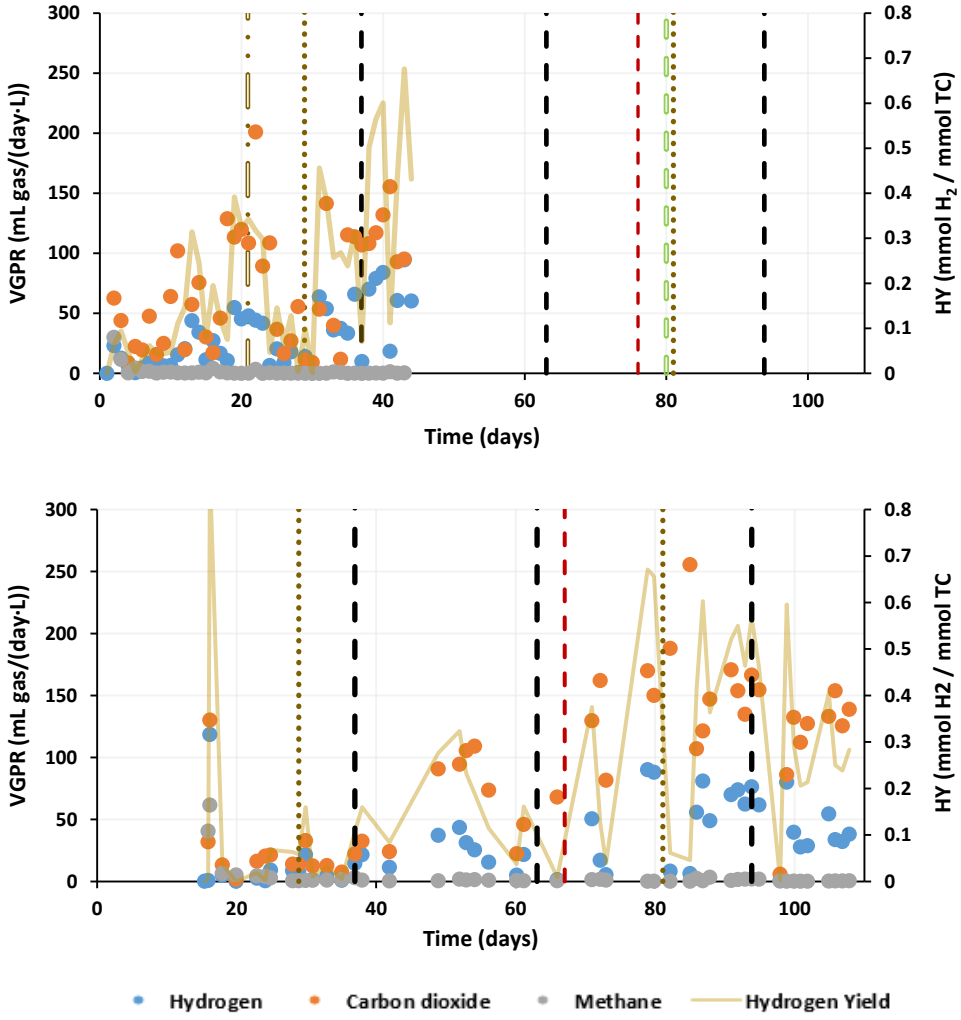

Figure 5.6 *Volumetric gas production and hydrogen yield during the continuous acidogenic fermentation of brewery spent grain liquid hydrolysate in an expanded granular sludge bed reactor for A) Reactor 1 (flocculent sludge) and B) Reactor 2 (granular sludge). VGPR: Volumetric Gas Production Rate, HY: Hydrogen Yield. Note that R2 started 15 days after R1. The changes for the operational conditions were applied simultaneously for both reactors*

5.3.4 Sulfate and nitrogen compounds

The highest sulfate effluent concentration occurred during Phase I in both reactors (***Figure 5.7***). In R1, the sulfate concentration decreased during Phase II and the sulfate removal efficiency was 50.9 %. When the HRT was decreased to 18 h, the sulfate concentration increased. The final sulfate removal efficiency was 29.5 % at the end of the trial (HRT = 12). For R2, the sulfate concentration decreased at

the end of Phase I and during Phase II. The lowest sulfate concentration was achieved during Phase III, with a sulfate removal efficiency corresponding to 29.1 %.

The protein concentration in R1 during Phase I fluctuated between 0.4 and 0.6 g/L, increasing up to 1.8 g/L during the disaggregation of the granular sludge (***Figure 5.7b***). During Phase II, the protein concentration slightly increased, with a protein removal efficiency of 72.5 (± 4.7) %. During Phase III-a, the protein removal efficiency decreased to between 36.5 and 17.6 %, before increasing again after reseeding. On the other hand, the ammonium concentration during this phase decreased and did not increase again for the rest of the trial. In R2, the protein concentration was stable in the effluent from day 2.5 until approximately day 41, with average protein concentrations of 0.51 (± 0.06) g/L. On day 43 the protein removal efficiency was lower with a higher effluent concentration (***Figure 5.7b***), along with the production of lactic acid as a result of a period of instability. The ammonium concentration was only stable until day 30 (***Figure 5.7b***). On day 33, the ammonium concentration started to increase to its highest concentration (0.44 g/L) on day 41 (***Figure 5.6b***).

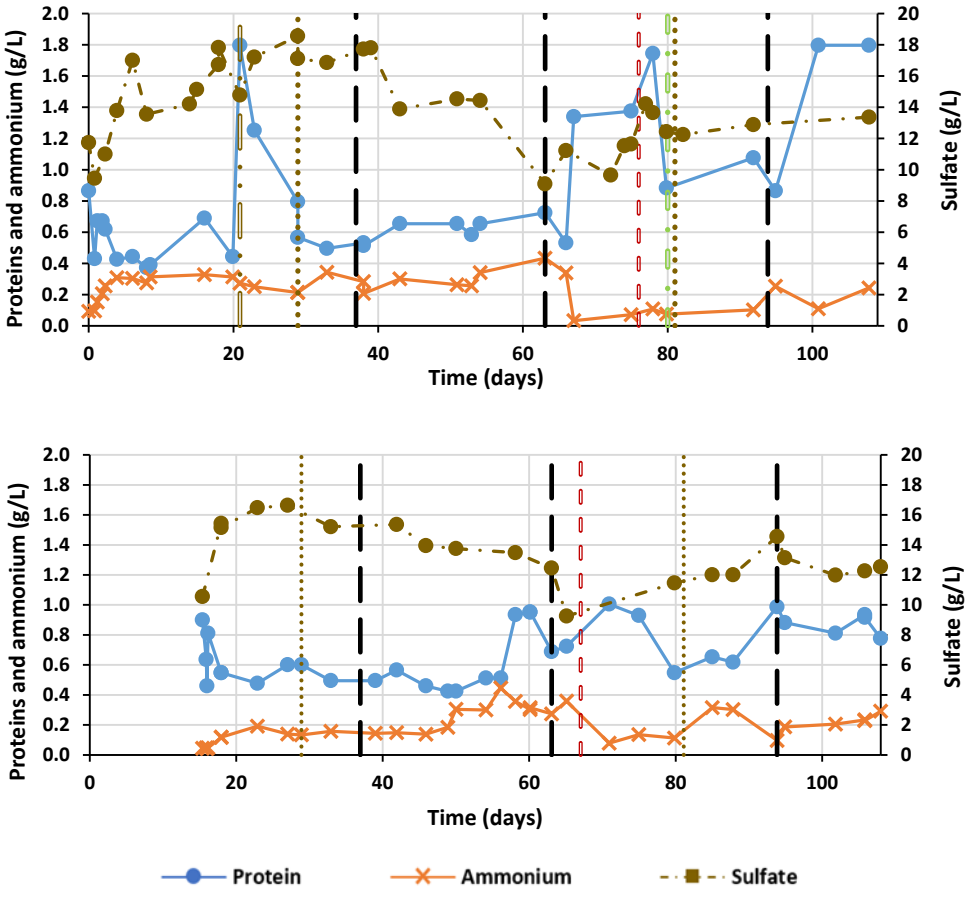

Figure 5.7 *Sulfate, protein and ammonium concentration during the continuous acidogenic fermentation using brewery spent grain liquid hydrolysate in and expanded granular sludge bed reactor along the trial in A) Reactor 1 (flocculent sludge) and B) Reactor 2 (granular sludge) (— · flocculation of the granular biomass; ····· up-flow changes; — – HRT changes; ▭ ▭ ◦ biomass addition). Note that R2 started 15 days after R1. The changes for the operational conditions were applied simultaneously for both reactors*

5.4 Discussion

5.4.1 Conversion of carbon compounds

This study demonstrated the production of VFAs using granular and flocculent anaerobic sludge, without pre-treatment, from thermal acid hydrolysate of brewery spent grain. The use of granular sludge (R2) resulted in stable VFA production, with the highest production, 120.4 (± 15.0) mmol/L (total VFAs), at a HRT of 24 hours. On the other hand, the flocculent sludge reactor (R1) had poorer performance, as operating the system at a HRT of 18 h suppressed VFA production.

Many studies have focused on the production of hydrogen as the main product of dark fermentation, without optimisation for VFA production. In addition, many report the average VFA value and not the continuous VFA production (Pugazhendhi et al., 2017; Rodriguez-Valderrama et al., 2019; De Menezes and Silva, 2019). In all these studies, and the present study, a decrease in HRT resulted in higher butyric acid production and lower total VFA production. Pugazhendhi et al. (2017) obtained the highest VFA production, a mixture of acetic, butyric and propionic acid, at a HRT of 3 hours using glucose as feedstock in a fixed bed reactor (**Table 5.2**). On the other hand, the highest HY occurred at a 1.5 hours HRT with an increase in the butyric acid concentration and a decrease in overall VFA production (Pugazhendhi *et al.*, 2017). Furthermore, in the present study, operating the system at longer HRT (36 hours) resulted in a higher yield in terms of VFA production as a function of TC consumed: 1.28 (± 0.13) and 1.38 (± 0.13) mmol VFA/mmol TC, respectively, R1 and R2.

Table 5.2 *Optimal conditions for volatile fatty acids production in different high rate reactors*

Type of reactor	HRT (h)	pH	Temperature (°C)	Feedstock	TC (g/L)	HAc (mmol/L)	HBu (mmol/L)	HProp (mmol/L)	HVa (mmol/L)	HCa (mmol/L)	EtOH (mmol/L)	Lactic Acid (mmol/L)	Sugar Reduction (%)	Total VFAs (mmol/L)	References
UASB	24.0	5 - 6	21.0	Sucrose	31.0	27.5	55.5	0.3	-	-	10.2	-	93.5	83.3	Rodriguez-Valderrama et al., 2019
UASB	4.8	2.7	30.0	Sucrose	4.5	7.8	1.1	1.1	0.4		-	3.6	80.3	10.0	Mota et al., 2018
UASB	4.0	4	37.0	Glucose	10.0	25.0	11.8	0.0	-	5.5	5.8	-	99.5	36.8	Zhang et al, 2007
EGSB	2.0	3.76 - 4.51	30.0	Sucrose	15.0	25.0	46.5	5.4	0.0	0.0	0.0	0.0	79.0	76.9	de Menezes and Silva, 2019
Fixed bed reactor	3.0	5.5	37.0	Glucose	15.0	20.0	43.1	27.0	-	-	-	15.6	87.3	90.1	Pugazhendhi et al., 2017
EGSB	24.0	5.85 - 6.0	37.0	Glucose, Xylose and Arabinose	15.0	74.4	36.6	9.5	0.0	0.0	31.1	3.4	98.7	120.5	This study

- **Not specified**

Similar results to the present study were obtained by De Menezes and Silva (2019), when working at the same TC concentration (15 g/L) (**Table 5.2**). Furthermore, in that study, a lower TC inlet concentration led to a larger range of VFAs (De Menezes and Silva, 2019). Moreover, in the present study, the range of the VFAs increased as the propionic acid concentration increased at a longer HRT (***Figure 2***). Zhang et al. (2007) obtained a more complex mixture of fermented compounds (e.g. ethanol, acetic, propionic, butyric and caproic acid) when using a 4 hour HRT versus 1 hour. At the lower HRT, the total amount of VFAs decreased and the butyric acid concentration increased, meanwhile the other compounds diminished (Zhang et al., 2007).

Rodriguez-Valderrama et al. (2019) operated a reactor at 21 °C under different HRTs (4, 12 and 24 hours), but kept the OLR constant by varying the COD inlet concentration. The authors pre-treated the biomass by rinsing with sterilized isotonic solution and heating at 90 °C for 1 h to inhibit methanogens and improve the hydrogen production. The major fermented compounds produced were acetic acid and butyric acid, representing more than 93 % of the total fermented compounds under all conditions investigated. Nevertheless, the total amount of VFAs reached by Rodriguez-Valderrama et al. (2019) was lower than in the present work (**Table 5.2**).

Regardless of the biomass type, either flocculent or granular, the total fermented compounds were similar for both reactors, in contrast to previous studies using granular and flocculent sludge (Atasoy *et al.*, 2019). Atasoy et al. (2019) used granular and flocculent sludge from different processing stages of a municipal wastewater treatment plant. The similar total VFA concentrations for both biomass types in the present study could be as a result of using the same inoculum for both reactors.

Kan (2013) used different biomass pretreatment methods to enhance hydrogen production, including, heat treatment which suppressed the activity of some homoacetogens and base pre-treatment (pH 10.0), which led to a larger range of VFAs produced. Many studies pre-treated the inoculum to enhance the hydrogen production (Zhang *et al.*, 2007; Larriba *et al.*, 2016; Rodríguez-Valderrama *et al.*, 2019; Menezes and Silva, 2019; Pugazhendhi *et al.*, 2017), however the highest VFA production they achieved was lower than in the present study (**Table 5.2**). In addition, any studies that reported VFA production resulted in similar fluctuations for the individual fermented compounds (Cisneros-Pérez *et al.*, 2017; Muñoz-Páez *et al.*, 2020; Mota *et al.*, 2018). These fluctuations could be the result of metabolic fluxes caused by stress responses of fermentative organisms to the continuous accumulation of fermented compounds (Ciranna *et al.*, 2014; Regueira *et al.*, 2018; Saady, 2013). Therefore, to avoid this fluctuation and allow targeted production of desired products, further research on the metabolic pathways of fermentation is required, e.g. through metabolic flux modelling (Regueira *et al.*, 2018; Regueira *et al.*, 2020).

Almost all previous studies (**Table 5.2**) created a high OLR by decreasing the HRT, whereas only a few have tried to operate the system at high OLR by increasing the total carbohydrate. This high TC approach was followed by Zhang *et al.* (2008), who operated a reactor with HRTs ranging from 0.25 to 3 hours and glucose concentrations from 5 to 120 g/L. Combinations of HRTs of 1.5 hours with 60 g/L and 2 h with 80 g/L led to a glucose consumption exceeding 80 and 90%, respectively. Meanwhile, the optimal conditions for gas production were at HRTs of 0.25, 0.5 and 0.75 h. The longer HRT could have been beneficial for the accumulation of a high concentration of fermented compounds rather than hydrogen production, however the production of the VFAs was not reported by Zhang *et al.* (2008).

Aside from the periods of instability, with lactic acid production, the VGPR throughout the trial increased as the HRT was reduced. This is in line with other studies that demonstrated increased hydrogen concentrations and HY at low HRT (Menezes and Silva, 2019; Cisneros-Pérez *et al.*, 2017; Zhang *et al.*, 2007). In addition, in these and the present study, propionic acid concentrations decreased. The decrease in HRT resulted in metabolites remaining inside of the system for a shorter period, which contributed to an increase in HY but a decrease in the concentration of compounds originating from hydrogen scavengers, such as propionic acid (Cord-Ruwisch *et al*., 1988; Saady, 2013). Thus, the uptake of hydrogen or intermediate metabolites by the scavengers was slower than the hydrogen production rate, resulting in a higher hydrogen release. Therefore, the use of a longer HRT is beneficial for the VFA production. Likewise, the decrease of the sulfate concentration would be related to a higher activity of the sulfate reducing bacteria, which convert sulfate into hydrogen sulfide (Papirio et al., 2013). The presence of propionic acid at the largest HRT in both systems could have resulted in a low sulfate reduction efficiency (Bertolino *et al.*, 2012). Moreover, as sulfate reducing bacteria are hydrogen scavengers, the increase of the hydrogen availability in the media could have contributed to their development.

5.4.2 Performance and selection pressure of the flocculent (R1) and whole granular (R2) sludge reactors

The anaerobic granular sludge used as inoculum was from an industrial reactor operated for methane production (Castilla-Archilla *et al*., 2020). Thus, some methane production was expected. The operational conditions showed, nevertheless, to be adequate to suppress methane production with no need for biomass pre-treatment. Methane was detected in both reactors from the beginning of the trial until day 21 and 29 in R1 and R2, respectively (***Figure 6***). On the other hand, the highest VFA concentrations were obtained on day 3 and 4, in R1 and R2 respectively. High concentrations of VFAs with an acidic pH in the liquor can trigger methane inhibition, resulting in VFA accumulation (Agler *et al.*, 2013; Lu *et al.*, 2015). This could explain the decrease in volumetric methane production rate after

72 hours (**Figure 5.6**), when total VFA concentrations reached 115.56 and 108.5 mmol/L, in R1 and R2, respectively. The remaining methane production could be a result of accumulation and subsequent release of gas inside of the granules (Wang and Liang, 2016).

During Phase III-a, R1 started to produce lactic acid and the total VFA and ethanol concentrations decreased from day 67 to day 80, when the average total VFA concentration was 23.04 (± 4.60) mmol/L (**Figure 5.2**). Despite the low VFA concentration no methane was detected during this period, which could have been a result of the wash-out of methanogens from the reactor due to the flocculent nature of the sludge and the low growth rates of the methanogens (Lim *et al.*, 2020). A similar outcome was reported by Palomo-Briones et al. (2017) when using flocculent sludge in a continuous stirred tank reactor operated at a long HRT (24 h). This enriched the biomass for lactic acid production by causing washout of acidogenic microorganisms (Palomo-Briones *et al.*, 2017). Indeed, the wash-out strategy is effective in enriching hydrogen producing bacteria, when using flocculent sludge without additional treatment (Carrillo-Reyes *et al.*, 2016). de Menezes and Silva (2019) used thermally treated blended granular sludge and obtained lactic acid as the major product, which was favoured by increasing the inlet carbohydrates concentration coupled to longer HRTs (Menezes and Silva, 2019). Approximately one week after reseeding, methane was detected on day 88 with a simultaneous increase in the VFA concentration (***Figure 5.2a*** & ***Figure 5.6a***). This could be explained by a lag phase for the fresh granular sludge to acclimatise to the conditions in the reactor, with a pH close to 6.0 and high concentrations of lactic acid. In addition, as the flocculent sludge prior reseeding was left in the reactor, this sludge was adapted to the medium. Thus, initially the flocculent sludge would have had a higher carbohydrate uptake, favouring lactic acid production, whereas the granular sludge would have produced VFAs and methane. Once the VFA concentration increased, methane production ceased again, probably because the inhibitory threshold concentration for methanogens was reached again (Agler *et al.*, 2013).

During periods of instability in R2, methane was detected at low concentrations in the biogas (< 5 %). This could be as a result of the reduction of the VFA concentrations in the reactor mixed liquor during the production of lactic acid. Therefore, the decrease of the VFA concentration observed on day 51 would have allowed the methanogens to become active again, accounting for the methane production observed on day 55. The role of the selection pressure in the present study would have been different for each of the reactors based on the sludge type, flocculent (R1) and granular (R2). The development of flocculent sludge in R1 led to wash-out, not only of methanogens but also of other bacteria beneficial for hydrogen and VFA production present at Phase I and II. However, during Phase III the operational conditions promoted lactic acid production over VFAs (***Figure 2-A***). Granulation protects the microbial community within the granules from inhibitors present in the media and

improves biomass retention in reactors (Lu *et al.*, 2015). Therefore, the granular sludge in R2 may have protected some of the methanogens, which became active again when the conditions of the reactor mixed liquor were favourable. This could explain the methane production inside of the reactor when the VFA concentrations were low enough to promote methanogenesis. Additionally, it may be possible that some bacteria within the granules were still growing, and were continually replacing other bacteria which were sloughed off the granule surface and washed out. Therefore, the granular sludge could have acted as a carrier material (Menezes and Silva, 2019) and source of bacteria to replace the washed-out fraction. The use of the anaerobic granular sludge without pretreatment can be more beneficial for the production of VFAs. Especially as the operational conditions demonstrated to prevent the production of methane (***Figure 5.6***).

5.4.3 Aggregation and disaggregation of the biomass

In both systems the biomass initially had a similar behaviour, where new biomass with a grey colour developed (***Figure 5.4***). Similar changes of colour have been related with the development of granular biomass for hydrogen production (Fang *et al*., 2002). Thus, this may have been the result of the growth and development of acidogenic and hydrogen producing organisms favoured by the operational conditions in the reactor, e.g. lower pH, higher temperature and higher organic content. Despite reducing the upflow velocity in R2 to prevent disaggregation, the newly formed grey aggregates in the stratified sludge bed (***Figure 5.4f***), and the grey coating on the original black granules broke apart. A similar effect was reported by Muñoz-Páez *et al.* (2020) when using agave bagasse hydrolysate for hydrogen production. Initially, their reactor was fed using glucose and xylose, once agave hydrolysate was used as feedstock, the granules started to break down (Muñoz-Páez *et al.*, 2020). The authors attributed this to the spatial distribution of microbes in the granules and the change of the substrate.

In the present study, between the formation and disaggregation of the new granules there was no change in any of the operational conditions (i.e. HRT, pH or change of substrate). Additionally, no significant changes in the appearance of the black granular sludge in the bottom layer were observed. Therefore, this could be a result of the development of a new, predominantly acidogenic microbial community on the surface of some of the seed biomass in the middle of the sludge bed and in the new, flocculent biomass on top of the sludge bed. Acidogenic organisms often grow as filamentous outgrowths on the surface of methanogenic granules and as flocculent biomass in the reactor liquor (Arcand et al., 1994; Batstone et al., 2004; Guiot et al., 1992; McHugh et al., 2003; McAteer et al., 2020). It may be the case that the flocculent biomass in the present study consisted of acidogenic organisms, which used the outer layers of the methanogenic granules for attachment until some conditions changed and the flocculent sludge became loose. For example, the loss or change in extracellular polymeric substances production which contribute to the adhesion of the biomass (Lu *et*

al., 2015). However, more studies on the microbial community composition, EPS type and role of the acidifiers in aggregation are required to determine the changes in biomass composition and their role in stability improvement.

5.5 Conclusion

This study demonstrated the feasibility for the production of VFAs using a high rate system with BSG liquid hydrolysate as feedstock. No pretreatment to the biomass was required as the operational conditions minimized or suppressed methanogenesis, with the production and accumulation of VFAs. The use of granular sludge resulted in more stable VFA production compared to flocculent sludge, which produced lactic acid when the HRT was 18 hours. Operating the system at 24 hours HRT allowed a maximum VFA production of 120.4 (± 15.0) mmol/L, which contributed approximately 83.4% (± 5.9) of the soluble COD. Further research is required to optimise this process and convert it from simply a wastewater treatment processes into a combined wastewater treatment-chemical production bioprocesses.

5.6 References

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Chapter 6

Brewery spent grain cascade process for recovery of carbohydrates, proteins and lignin

Keywords: lignocellulose; brewery spent grain; cascade process; pretreatment; thermal diluted acid hydrolysis; severity factor; enzymatic hydrolysis; water retention capacity

Abstract

Brewery spent grain (BSG) contains a large fraction of proteins, lignin and carbohydrates. Some of these compounds have a high value in the food and pharmaceutical industry. This chapter investigated a cascade process by combining a chemical pretreatment step followed by enzymatic hydrolysis. First, a response surface methodology was used to optimise diluted hydrochloric acid hydrolysis with different combinations of temperature, time and acid concentration. Three different combinations were identified based on i) the highest protein content in the solid fraction after the pretreatment (C1: 0.49 % acid at 87.7 °C for 92.7 min), ii) the highest liquid recovery (C2: 0.80 % acid at 121.0 °C for 142 min), and iii) the lowest acid concentration (C3: 0.10 % of acid, 104.0 °C for 70 min). In addition, a fourth condition was added based on the lowest water retention capacity (C4: 0.20 % of acid, 121.0 °C for 20 min). The enzymatic hydrolysis for the four different conditions was carried out for 24 hours to further enhance the release of the carbohydrates. The efficiency of the enzymatic hydrolysis was evaluated in presence and absence of a large concentration of reducing carbohydrates, this was carried out centrifuging the slurry after pretreatment, recovering and washing the solid prior to adding the enzyme; in the second case the enzymatic cocktail was added straight after the chemical pretreatment. No effect was observed, and even hydrolysing the whole slurry resulted in a higher release of carbohydrates. The overall carbohydrate recovery efficiency in the liquid fraction for C1, C2, C3 and C4 corresponded to 399.1 ± 26.1, 535.8 ± 28.7, 257.0 ± 11.5 and 446.3 ± 81.1 mg carbohydrate per dry gram of BSG. The solid fractions for C1, C2 and C4 were evaluated using a larger volume. For each of them two different solid fractions were obtained, a fine solid and a coarse solid fraction. The highest protein content in the fine solid was for C4, meanwhile in the case of the coarse solid fraction the highest proteins content corresponded to C1. Further analysis is required to determine the potential of these solid fractions as food supplement.

6.1 Introduction

Growing populations have resulted in increased food demand. Thus, alternative sources of food are required to mitigate this demand. For example, lignocellulose is the largest solid biomass that could be valorised as an alternative source of fibre, proteins and nutraceutical compounds (Contreras et al., 2019). The recovery of high value compounds from existing lignocellulosic waste streams represent an opportunity for revalorisation through decomposition and fractionation of dietary fiber, protein or phenolic acids (Martinez et al., 2012; Laguna et al., 2018). This process could be coupled with carbohydrate extraction for chemical and fuel production (Celiktas et al., 2014).

Brewery spent grain (BSG) is a lignocellulosic by-product of beer production with a large amount of protein, phenolic compounds and fibre that have shown prebiotic and nutraceutical activities (Vieira *et al*. 2014; Rommi *et al*. 2018; Cermeño et al., 2019). In the beer production process, barley is germinated to activate natural enzymes and initiate degradation of the grain. Then, this malted barley is milled and suspended in water. To promote further hydrolysis the temperature is slowly increased until the denaturalization of the enzymes present (Mussatto et al., 2006). As a result, the easiest biodegradable compounds are solubilised and the most recalcitrant compounds are concentrated in the remaining solid. For example, more than 98% and 92% reductions in the starch and glucan content, respectively, meanwhile arabinoxylan is concentrated almost 3.5 times and the protein fraction above 2.5 times (Celus et al., 2006). The recovery and use of these complex compounds has been investigated previously. For example, recovery of the carbohydrate fraction for chemical and energy production (Wilkinson et al., 2014; Plaza et al., 2017; Rojas-Chamorro et al., 2020), or the recovery of bioactive compounds such as protein or phenolic compounds (McCarthy et al., 2012; Cermeño et al., 2019; Connolly et al., 2019). However, few studies evaluated the simultaneous recovery of the carbohydrates or other fractions such as lignin or proteins (Niemi et al., 2013; Rommi et al., 2018).

BSG consists of the husk, the pericarp and the seed coat which are mainly composed of interwoven cellulose, hemicellulose and lignin, with a small fraction of proteins and lipids (Mussatto et al., 2006; Lynch et al., 2016; Jaeger et al., 2021). These fractions act as a barrier for enzymatic and mild chemical hydrolysis during protein extraction (Niemi et al., 2013; Ibbet et al., 2019). The most common pretreatment for the release of these proteins is alkaline pretreatment, which solubilises proteins with low degradation of the lignocellulose fraction (Connoly et al., 2019; Wilkinson et al., 2014). Many studies have focused on the optimization of carbohydrate solubilisation from BSG using enzymatic hydrolysis (Niemi et al. 2012). These optimisations usually include an initial chemical hydrolysis with a combination of different temperature, time and acid concentration that degrade not only cellulose and hemicellulose but also proteins (Plaza et al. 2017, Patel et al. 2018; Ravindran et al. 2018; Rojas-Chamorro et al. 2020). This combination of time, temperature and acid concentration during the

pretreatment can be integrated into a single value known as severity factor (Carvalheiro et al. 2004). Increasing this factor results in better carbohydrate solubilisation, but also partially degrades solubilised proteins into peptides (Kemppainen et al., 2016).

The current chapter aims to investigate the minimum severity factor for the breakdown of the lignocellulose wall that protects the protein fraction in BSG using different combinations of i) temperature, ii) acid concentration, and iii) time as pretreatment step prior to enzymatic hydrolysis. The effect of these combinations is evaluated in terms of final carbohydrate efficiency release after the enzymatic hydrolysis, as well as the recovery and quality of the protein and lignin solid fractions. First, a diluted acid hydrolysis was optimised before enzymatic hydrolysis. After acid pretreatment and enzymatic hydrolysis, the carbohydrate and protein release were evaluated using acid and alkaline conditions in short volume. The best hydrolysis results were carried out using a larger volume for the fractionation of the liquid and the solid streams. The carbohydrate, protein and ammonium concentration were analysed in the liquid fraction; the total solid, water retention capacity (WRC), liquid/solid ratio and protein fraction were analysed in the solid fractions.

6.2 Material and methods

6.2.1 Feedstock

BSG used for the production of Indian Pale Ale was kindly supplied by a local brewery in Galway (Ireland), which differed from the BSG used in ***Chapter 4*** and ***Chapter 5***, which was from lager production. The BSG was collected from the plant after the wort production. This was mixed and frozen in individual bags of 500 g each at -20 °C within 3 hours. Prior to the hydrolysis step, the BSG bag was defrosted overnight at 15 °C.

6.2.2 Hydrolysis process

6.2.2.1 Sulfuric and hydrochloric acid comparison

The BSG was blended using distilled water in a Waring 2-Speed laboratory blender (USA) at the highest speed for 1 minute. The first set of experiments tested the effectiveness of thermal diluted acid hydrolysis using hydrochloric or sulfuric acid at different concentrations (0.00, 0.05, 0.10, 0.25, 0.5, 1.00 and 1.50 % v/v). Sulfuric acid (95 % purity and 1.83 g/mL density) and hydrochloric acid (37 %) were supplied from Fisher Chemical (UK). A solid concentration of 10 % (w/w) BSG was used and hydrolysis was done in an autoclave (Sanyo Labo, Japan) at 121 °C for 20 minutes as described previously (Carvalheiro et al., 2004; Djioleu and Carrier, 2016). Liquid samples obtained after hydrolysis were analysed for monosaccharides and disaccharides (cellobiose, maltose, glucose, xylose and arabinose), total protein and ammonia content. Based on these experiments, hydrochloric acid was chosen as a pretreatment for the subsequent steps of this work.

6.2.2.2 Pretreatment optimization

A Response Surface Methodology (RSM) was developed using Minitab v18. This statistical tool was used to optimise different combinations of temperature, acid concentration and time in order to optimise the maximum carbohydrate solubilisation, liquid recovery and protein content in the solid residue. The maximum and minimum value for the optimization were introduced to the software to generate the different combinations (45 combinations), based on three different values for each of the three different parameters (***Table 6.1***). This optimization was done prior to enzyme addition using Duran bottles (250 mL) with a working volume of 100 mL. Incubations at temperatures higher than 100 °C were carried out in an autoclave (Sanyo Labo, Japan) and incubations below 100 °C were carried out in a thermostatic incubator (Grant, UK).

Table 6.1 *Parameters used in the Response Surface Methodology for the optimization of the thermal diluted hydrochloric acid hydrolysis as pretreatment prior to enzyme addition*

Acid concentration (% v/v)	Temperature (°C)	Time (min)
0.10	60.0	20
0.55	90.5	100
1.00	121.0	180

6.2.2.3 Enzymatic hydrolysis

The three different combinations obtained during the RSM trial (**Table 6.2*: Condition 1, 2 and 3***) plus an additional condition based on the results of the lowest water retention capacity of the initial experiment (**Table 6.2*: Condition 4***) were used as pretreatments to test enzymatic hydrolysis. The enzymatic hydrolysis for each of the conditions was carried out using Depol 40L (kindly provided by Biocatalysts, UK) with an enzymatic load of 15 cellulose units/g of BSG (TS). To evaluate the possible inhibition of the enzymes in the presence of reducing sugars and inhibitor compounds released during the pretreatment, two different approaches were tested for the efficiency of the enzymatic hydrolysis. The first approach involved adding the enzymes to the whole slurry (WS) obtained after pretreatment. The second was by centrifuging the WS, recovering the pellet and re-suspending it in the same amount of liquid that was recovered during the centrifugation. The enzymatic hydrolysis was carried out in 250 mL Duran bottles in an incubator at 50 °C. To ensure the correct homogenization the bottles were mixed agitated using 100 rpm using the thermostatic incubator (Grant, UK). The pH was initially corrected to optimal conditions for the enzymatic hydrolysis as suggested by the manufacturer (pH 5.0). The total length of the enzymatic hydrolysis was 24 hours. All tests were performed in replicates (n = 6). Samples were taken at 0, 3, 6, 12, 18 and 24 hours by removing an aliquot using aseptic

techniques and sterilised implements. Samples were then boiled for 5 minutes. After the enzymatic hydrolysis was completed, the solubilisation of carbohydrates and proteins was evaluated under acidic (pH 1.0) and alkaline (pH 8.0) conditions. This was done by subdividing the six replicates in half (n = 3 for each pH). The pH was corrected and bottles were left overnight at 4 °C. The following morning, the liquid fraction was recovered by centrifugation (Beckman Coulter, Allegra X-30R Centrifuge (Germany)) and filtered using filter paper (Sigma-Aldrich, Grade 542, 150 mm diameter) to remove any remaining solid particles. The efficiency of the process was based on the carbohydrates recovered by gram of BSG used (TS), also the ratio between the liquid and solid fraction was calculated to determine the efficiency of the hydrolysis in terms of liquid recovered.

Table 6.2 *Parameters used for pretreatment prior the enzymatic hydrolysis of the brewery spent grain*

Condition	Acid concentration (% v/v)	Temperature (°C)	Time (min)
C1	0.49	87.7	92
C2	0.80	121.0	142
C3	0.10	104.0	70
C4	0.20	121.0	20

6.2.2.4 Solid and liquid fractionation

Conditions 1, 2 and 4 (***Table 6.2***) were selected for further experimentation on solid and liquid fractionation using a larger volume. Enzymatic hydrolysis was carried out for 24, 6 and 24 hours for C1, C2 and C4, respectively, using a working volume of 800 mL in a 1000 mL Duran bottle under the same conditions. Following hydrolysis, the solid fraction and an aliquot of the liquid fraction were stored for further analysis. The process used for the fractionation of these hydrolysis experiments is summarised in ***Figure 6.1***. Briefly, a fraction of the WS was taken before and after the enzymatic hydrolysis. After the enzymatic hydrolysis the pH was corrected to 1.0, and left overnight at 4 °C. The following day the slurry was filtered using a 100 µm sieve, which was selected after sieves with different pore size (250, 100 and 25 µm) were tested. Two different fractions were obtained: a coarse solid (CS) fraction with particles larger than 100 µm and a liquid fraction containing fine solids smaller than 100 µm. The liquid fraction was centrifuged and the fine solid (FS) fraction was recovered and frozen for further analysis. The acid liquid fraction was neutralized and filtered using paper filter (Sigma-Aldrich, Grade 542, 150 mm diameter). The CS were re-suspended with distilled water using the same volume as the liquid with fine particles. Then, a sample was taken and the pH was increased to 8.0. The bottle was left overnight at 4 °C, and the slurry was sieved using a 25 µm sieve.

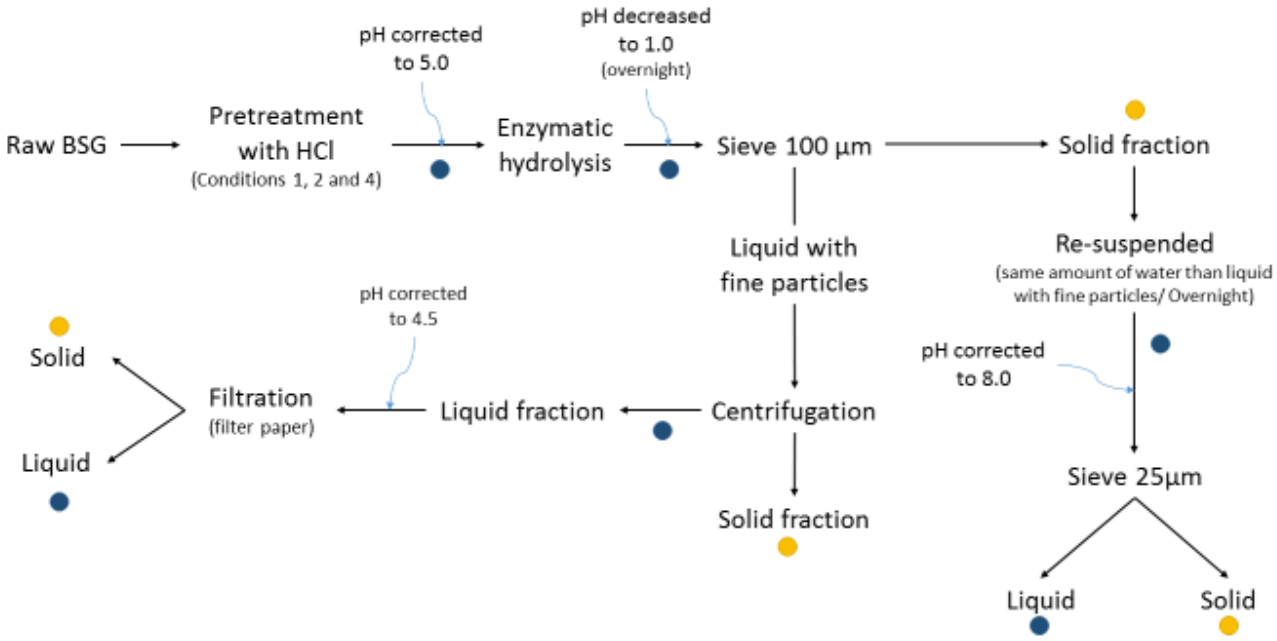


Figure 6.1 *Process diagram for the hydrolysis of brewery spent grain and the fractionation and treatment of the different streams. Three different pretreatment conditions were used before enzymatic hydrolysis. The blue dots represent sampling of liquid (●), and the yellow dots represent the samples taken from the solid fraction (●). There was no solid fraction after neutralization of the acid liquid, which has been represented with the yellow shape circle (○)*

6.2.3 Physico-chemical analysis

The protein content in the solid fraction was determined by the Kjeldahl method with a correction factor of 5.50 as a more accurate approximation for BSG (Mariotti et al., 2019). The characterisation of the solid samples in terms of hemicellulose, cellulose and lignin content was carried out by Celignis Limited (Limerick, Ireland). In addition, for the raw BSG the starch content was analysed as well.

Samples for chemical analysis were centrifuged at 9,000 rpm for 5 minutes and the supernatant was filtered using a syringe filter with 0.22 µm pore size. The samples were then frozen for further analysis. Protein and ammonia were analysed using a discrete nutrient analyser (Gallery Plus, ThermoFisher) as described in ***Chapter 4***. Total suspend solids (TSS) and volatile suspend solids (VSS) were measured following the standard method (APHA, 2012).

Maltose, cellobiose, glucose, xylose and arabinose were measured using a 1260 Infinity II liquid chromatograph (Agilent, Germany) equipped with a Hi Plex H 7.7 x 300 mm and 8 µm (p/n PL1170-6830) column (Agilent, UK) kept at 60 °C and an RI detector at 55 °C. The mobile phase was sulfuric acid at a concentration of 5 mM and a flow rate of 0.7 mL/min.

6.2.4 Calculation

The efficiency of hydrolysis was calculated based on the total carbohydrates in the liquid fraction after the neutralization of the acid liquid as explained in ***Chapter 4***. The severity factor for each of the combinations during the pretreatment step was calculated as described in ***Chapter 4***.

The WRC was calculated following Robertson et al. (2000). This represents the amount of water retained by the solid fraction after centrifugation as it is reported in ***Equation 6.1***:

$$WRC = \frac{Residue\ fresh\ weight - residue\ dry\ weight}{Residue\ dry\ weight}\ (g/g) \qquad \textbf{Equation 6.1}$$

6.3 Results

6.3.1 Comparison using hydrochloric and sulfuric acid

The hydrochloric acid performed better in terms of carbohydrates and proteins release from BSG (**Figure 6.2** 1***a*** ***& 1b***). The increase of the pentoses (xylose and arabinose) and hexose (glucose) were similar using 0.25 % hydrochloric acid. Almost the maximum xylose and arabinose concentration was achieved at a 1.0 % acid concentration. On the other hand, the glucose concentration was still increasing from 1.0 to 1.5 % (**Figure 6.2** ***1a***). The release of carbohydrates using sulfuric acid was slower than with hydrochloric acid (**Figure 6.2** ***2a***). For solubilised proteins and ammonia, the highest concentrations and rate were also achieved with hydrochloric acid (**Figure 6.2** ***2b***).

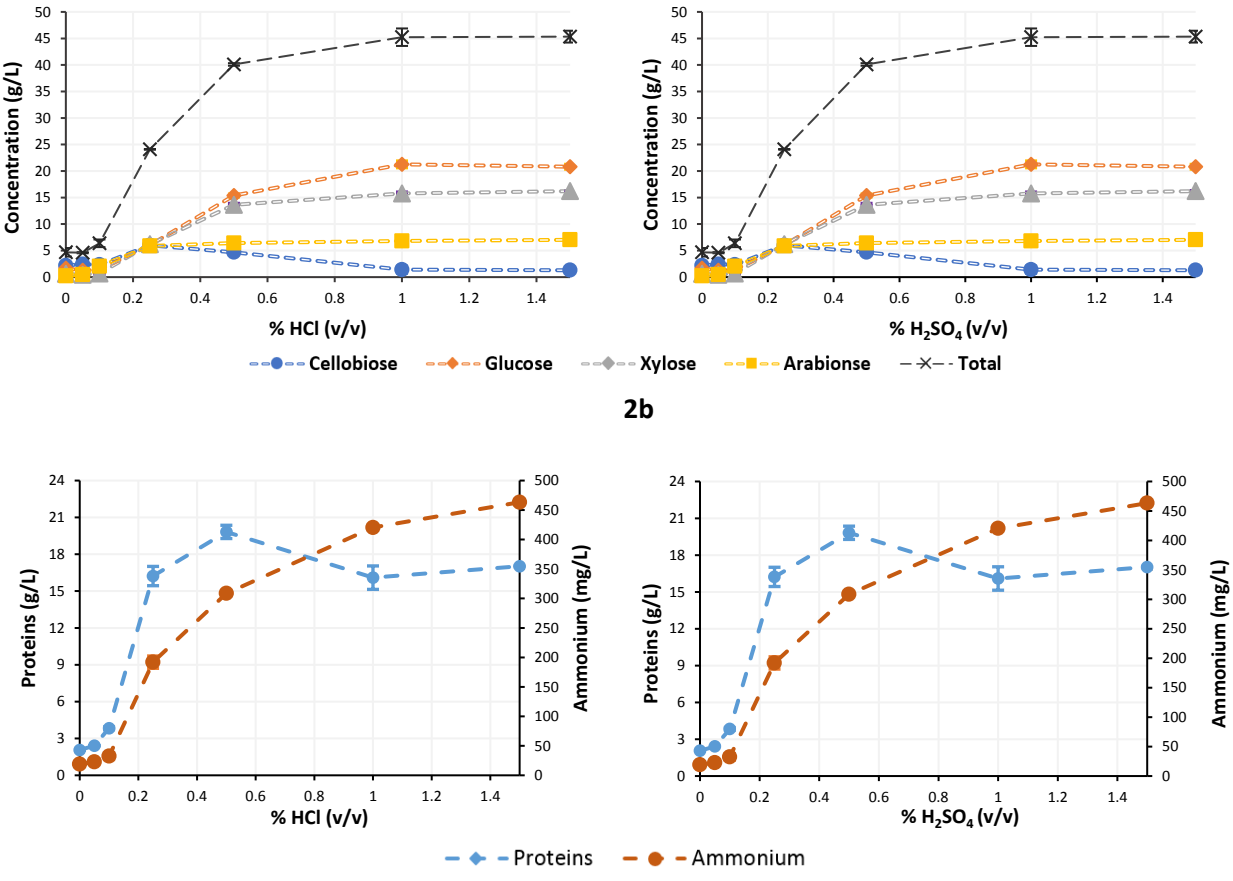


Figure 6.2 *Compound characterisation in the filtered liquid fraction (20 µL) of the hydrolysis of brewery spent grain 10 % TS (w/w) under different acid concentrations, hydrochloric (**1**) or sulfuric (**2**), in the autoclave at 121 °C for 20 min.* ***a)*** *Carbohydrates characterisation, and* ***b)*** *proteins and ammonium concentration*

The WRC in the sulfuric acid pretreatment increased from 0 to 0.10 % v/v and achieved the lowest WRC with an acid concentration of 0.50 %. For the hydrochloric acid, this increase of the WRC was not as clear as with sulfuric acid, achieving the lowest WRC at 0.25 % hydrochloric acid (**Figure 6.3*a***). In both treatments, the WRC followed a similar trend where the WRC increased with a higher rate for the sulfuric acid. In terms of the water recovery, ***Figure 6.3b*** shows the amount of liquid fraction recovered from the initial 100 mL. In both cases the highest increase of the liquid fraction recovered was from 0.05 to 0.25 % acid. The liquid fraction recovered was higher with hydrochloric than with sulfuric acid (**Figure 6.3*b***). However, the liquid from the hydrochloric acid hydrolysis was lower when increasing the acid concentration from 0.25 to 1.5 %, whereas the liquid recovered from sulfuric acid was almost constant with concentrations ranging from 0.5 to 1.5 % (**Figure 6.3*b***).

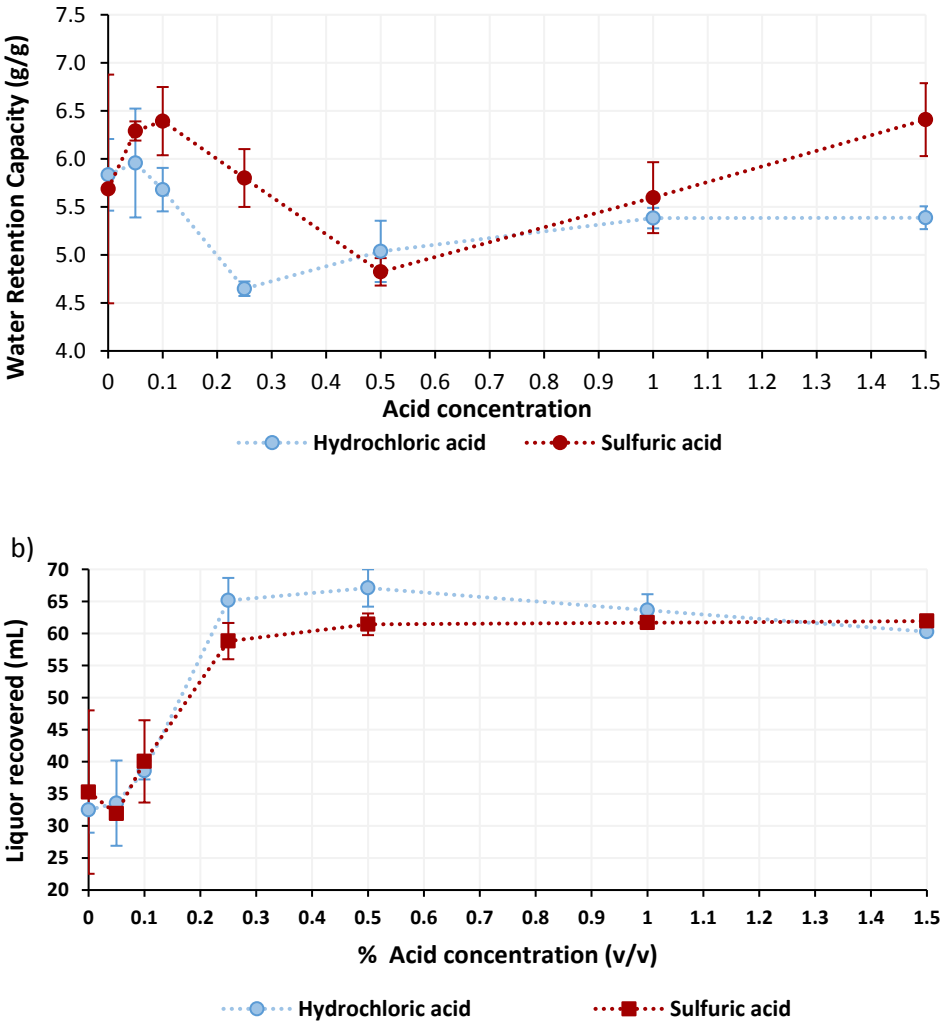


Figure 6.3 a) *Water retention capacity (gram of water per gram of total solids), and* ***b)*** *liquor recovered from 100 mL during the hydrolysis of brewery spent grain with 10 % TS (w/w) carried in the autoclave at 121 °C for 20 min with sulfuric and hydrochloric acid under different concentrations*

Some changes were observed in the colour and some properties of the BSG structure when the acid concentration was increased, such as the WRC and the liquid recovered (***Figure 6.3***). Hydrochloric acid

concentrations of 0.10 %, or less, resulted in a liquid fraction with a milky colour (***Figure 6.4***) and a white layer was observed on the top of these samples. When the hydrochloric acid concentration was increased from 0.10 to 0.25 %, the liquid fraction turned to a golden/brown colour. The white layer then changed to a darker colour. There were some floating particles after centrifugation, with a larger amount observed upon increasing the acid concentration.

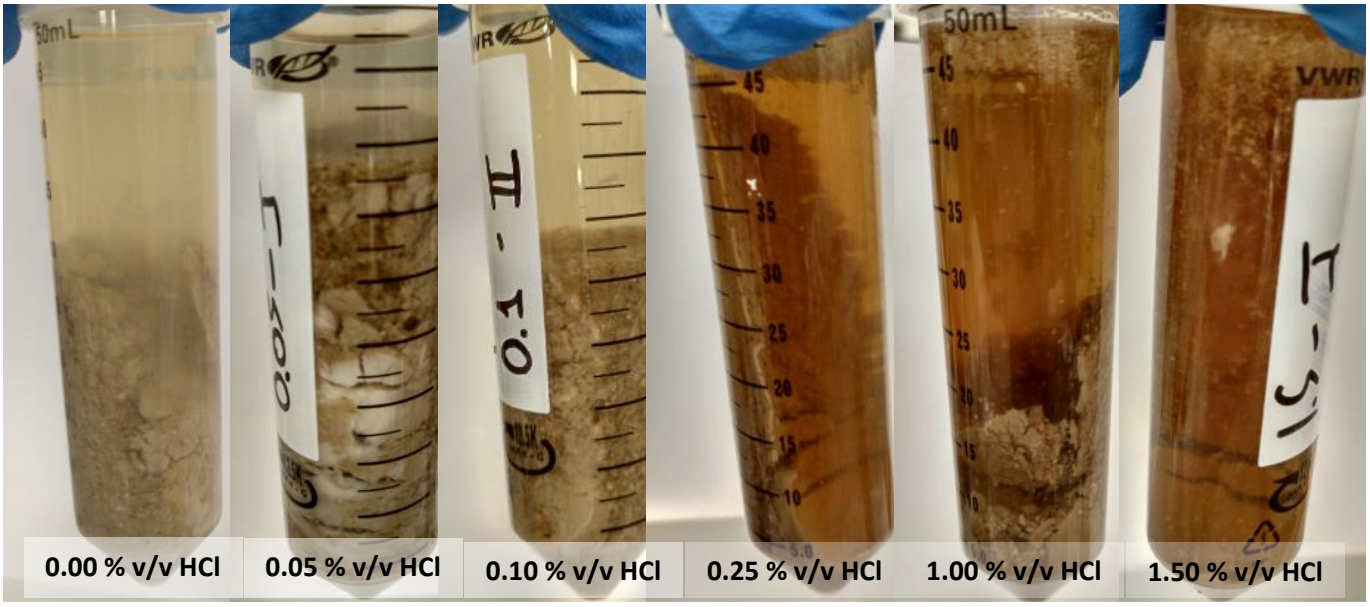


Figure 6.4 *Tubes after centrifugation of the whole slurry for each of the different thermal diluted hydrochloric acid pretreatments of brewery spent grain at different acid concentration carried at 121 °C for 20 minutes*

6.3.2 Response surface methodology

The RSM was used to evaluate the effect of combinations of acid concentration, temperature and time on the diluted acid hydrolysis pretreatment. To generate these combinations, the highest and lowest parameters were fixed, and the software used three different values for each parameter (***Table 6.1***) to generate 45 different combinations. The main parameters evaluated by the response surface methodology included the total protein content in the solid fraction, total carbohydrates, monosaccharide concentration, pentose concentration (arabinose and xylose) and solid weight.

As lignocellulose is a protective barrier for proteins (Ibbet et al., 2019), the conditions with the maximum protein content in the pellet was selected as an indicator for when this barrier started to break down. This corresponded to a combination of 0.49 % acid at 87.7 °C for 92.7 min, which was used to generate Condition 1 (C1), and which had a severity factor of 0.70. The second condition was related with the WRC of the BSG, which was based on the lowest amount of solid obtained. The resulting combination corresponding to Condition 2 (C2) was 0.80 % acid at 121.0 °C for 142 min with a severity factor of 1.87. The third condition was designed by fixing the acid concentration at the

lowest level tested, giving Condition 3 (C3) a combination of 0.10 % of acid, 104.0 °C for 70 min. Condition 4 (C4) was added based on the WRC during the initial experiment when comparing hydrochloric acid with sulfuric acid (***Figure 6.3a***). Thus, Condition 4 was carried out using 0.20 % of acid, 121.0 °C for 20 min.

6.3.3 Small enzymatic hydrolysis

The enzymatic hydrolysis for the four different conditions was carried out for 24 hours to test additional degradation of the complex carbohydrates by the enzymes, using the WS and the re-suspended pellet (RP). ***Figure 6.5*** shows the carbohydrates concentration during the enzymatic hydrolysis. The biggest increases in total carbohydrates were in condition C1 and C4 for the WS, corresponding to 23.10 (± 5.11) and 24.91 (± 4.64) g/L, respectively. In addition, in the case of the WS for C1 and C4 there was maltose present at the beginning of the enzymatic hydrolysis. The maltose was fully consumed in C1 after 12 hours, in C4 the maltose concentration increased during the beginning of the enzymatic hydrolysis, with the highest concentration achieved after 9 hours, and the concentration slightly decreased at the end of the hydrolysis (***Table 6.3***). Maltose was not present in any of the other samples. In C2, the WS and the RP hydrolysis resulted in a similar carbohydrate increment, 14.31 (± 1.24) g/L and 13.56 (± 2.33) g/L. C2 differed from the rest of the conditions as more carbohydrates were released from the RP with an increase of 5.17 (± 1.69) g/L, whereas the increase of the WS was 3.74 (± 2.86) g/L. This increase in carbohydrate concentrations was mainly by cellobiose, as all the other type of carbohydrates kept a similar concentration until the end of the hydrolysis step (***Table 6.3***).

Table 6.3 *Increase in carbohydrate concentration after 24 hours of enzymatic hydrolysis for four different pretreatment conditions*

Cond.	Whole Slurry					Resuspended Pellet				
	Cellobiose g/L	Maltose g/L	Glucose g/L	Xylose g/L	Arabinose g/L	Cellobiose g/L	Maltose g/L	Glucose g/L	Xylose g/L	Arabinose g/L
1	5.05 ± 0.81	-2.45 ± 0.49	12.56 ± 1.34	7.45 ± 0.71	0.50 ± 1.75	1.77 ± 0.37	0.00 ± 0.00	8.93 ± 1.29	5.50 ± 0.65	0.07 ± 0.14
2	2.67 ± 0.63	0.00 ± 0.00	0.71 ± 0.90	0.36 ± 0.81	0.00 ± 0.52	3.47 ± 0.20	0.00 ± 0.00	1.18 ± 0.67	0.31 ± 0.52	0.22 ± 0.30
3	-1.90 ± 0.28	0.00 ± 0.00	11.96 ± 0.66	3.32 ± 0.21	0.95 ± 0.09	-2.63 ± 1.13	0.00 ± 0.00	11.96 ± 0.64	3.28 ± 29	0.95 ± 0.28
4	3.65 ± 0.83	-0.41 ± 0.42	11.71 ± 1.28	9.03 ± 0.92	0.93 ± 1.20	1.55 ± 0.93	-0.02 ± 0.03	6.68 ± 1.40	5.30 ± 1.64	0.77 ± 1.98

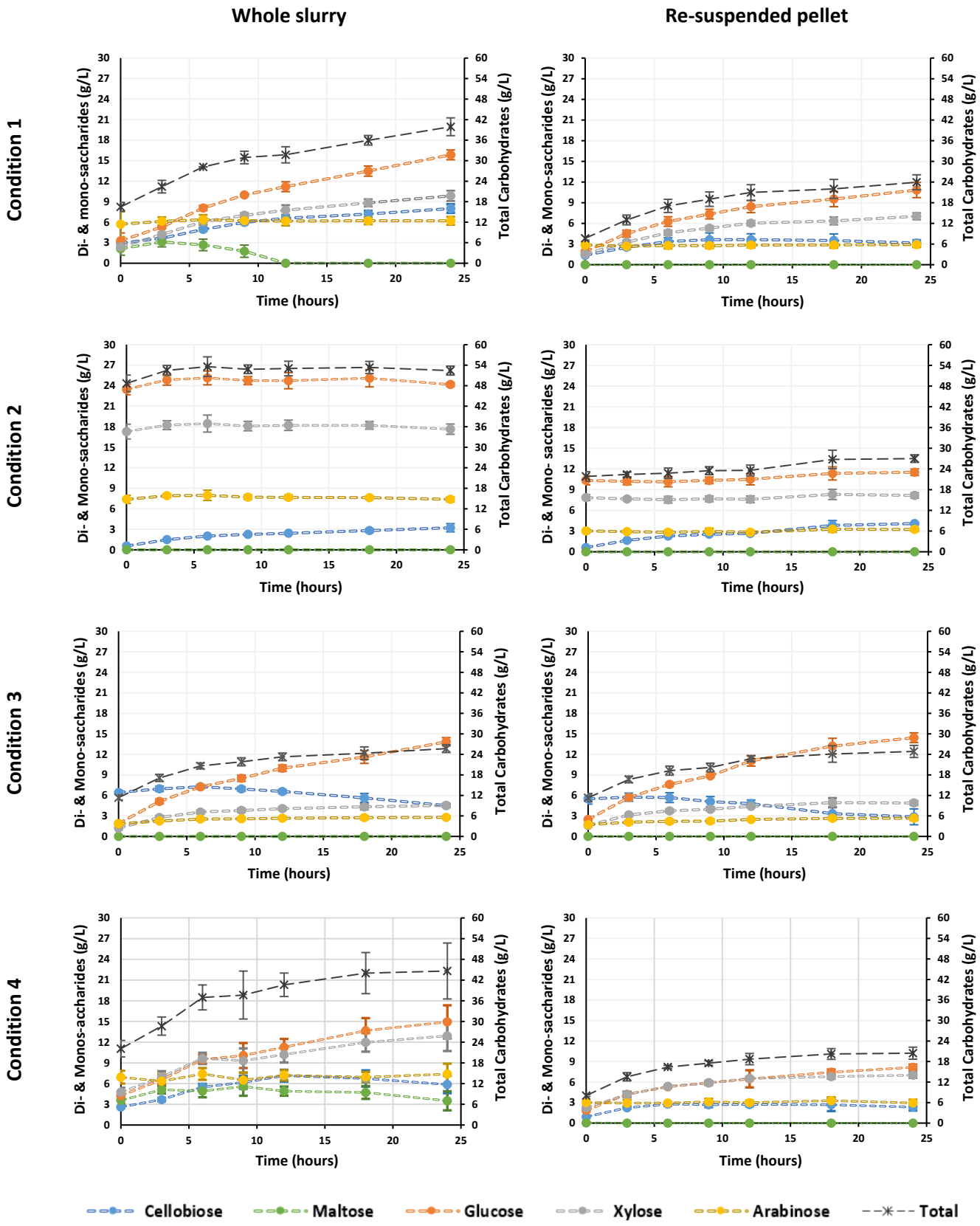


Figure 6.5 *Carbohydrate release during the enzymatic hydrolysis for the four different pretreatments.* ***Left****) Whole slurry and* ***right****) resuspended solid*

For conditions C1, C2 and C3, the cellobiose concentration increased along the enzymatic hydrolysis, whereas the cellobiose concentration only decreased in the case of C3 (***Table 6.3***). For all of the different enzymatic hydrolysis conditions tested, the lowest monosaccharide released during the enzymatic hydrolysis corresponded to arabinose (***Table 6.3***). The largest increase in xylose concentration was in C2 and C4, slightly lower compered to when using RP and the highest release for the WS at C4 (***Table 6.3***). C1 and C4 had a similar efficiency during the enzymatic hydrolysis, although the overall efficiency was higher for C4 as the initial concentration of the carbohydrates was higher at time 0 for C4 than for C1 (***Figure 6.5***).

For each of the hydrolysates, the liquid recovered as supernatant during the centrifugation and the total amount of solid in the pellet were used to calculate the ratio between the liquid and solid fraction. This was used to understand the extent of the hydrolysis for the lignocellulose degradation. The lowest ratio between the liquid and solid fraction was obtained for C3, which had the lowest severity factor (***Table 6.4***). As well, the influence of the pH above this liquid ratio was studied at acid and alkaline pH, respectively, 1.0 and 8.0. In all the cases where the enzymatic hydrolysis was carried out using the RP, the ratio liquid/solid was higher for the lowest pH. On the other hand, only C2 had a higher ratio under acid conditions after the WS enzymatic hydrolysis, as conditions C1, C3 and C4 presented a larger liquid fraction at alkaline conditions. For C2 centrifuging the slurry under acidic conditions resulted in an increase in the volume of supernatant recovered, meanwhile the alkaline conditions resulted in a lower ratio between the liquid and solid phase.

Table 6.4 *pH after thermal diluted hydrochloric acid pretreatment and ratio between liquid and solid fraction of the slurry after the acid hydrolysis, whole slurry left overnight at pH 1.0 and pH 8.0, and resuspended solid left overnight at pH 1.0 or pH 8.0*

	pH	Condition 1	Condition 2	Condition 3	Condition 4
After acid hydrolysis	*pH:*	*1.00 ± 0.03*	*0.81 ± 0.04*	*2.94 ± 0.10*	*1.71 ± 0.02*
	Ratio:	1.38 ± 0.30	1.64 ± 0.08	0.33 ± 0.07	1.66 ± 0.19
Whole slurry	1.0	1.33 ± 0.30	2.24 ± 0.07	0.48 ± 0.10	2.31 ± 0.09
	8.0	1.63 ± 0.25	1.36 ± 0.25	0.51 ± 0.06	2.42 ± 0.27
Resuspended pellet	1.0	2.24 ± 0.07	1.76 ± 0.15	0.20 ± 0.05	2.61 ± 0.43
	8.0	1.36 ± 0.25	1.13 ± 0.03	0.16 ± 0.02	2.03 ± 0.10

Despite of using the same pretreatment and enzymatic hydrolysis condition, the change of the pH overnight before centrifuging resulted in changes in colour. The acid pH corresponded to a golden colour, meanwhile the alkaline conditions resulted in a dark brown colour (***Figure 6.6***).

Figure 6.6 *Recovered supernatant after enzymatic hydrolysis of pretreated brewery spent grain using 0.80 % of HCl (v/v) at 121.0 °C for 142 minutes with an initial volume of 150 mL of water. The slurry before centrifuging was left overnight at 4.0 °C a pH: 1.0 for the left bottle (golden colour), and 8.0 for the right bottle (dark brown colour)*

6.3.4 Large enzymatic hydrolysis

Larger enzymatic hydrolysis experiments (800 mL) were carried out in triplicate to evaluate the amount and composition for the liquid and the solid fraction for C1, C2 and C4. The process depicted in ***Figure 6.1*** was used for these hydrolysis experiments. The process was initially designed following the previous step by the centrifugation of the slurry instead of using the 100 µm sieve to recover the liquid and solid fraction. However, during this process there were two main issues: i) some of the solids were floating in the liquid and when using the paper filter this was clogged and no liquid was passing through the paper; and ii) when centrifuging it was possible to observe two different layers of solid material in the centrifuge bottle (***Figure 6.7***). The texture of the upper layer was similar to mud made by fine particles, and in the bottom layer it was possible to observe the remaining fractions from the grain such as the husk (***Figure 6.7e & f***). First, for C2 and C4 the final slurry was passed through the sieves from the biggest to the smallest size (250, 100 and 25 µm). Some of the biggest particles passed through the 250 µm sieve, and the main fraction with large solid particles was retained in the 100 µm sieve, in the case of the 25 µm sieve nothing was retained. Thus, it was decided to use a 100 µm sieve before centrifuging to split the two layers and recover the two fractions independently. Once the acid liquid with FS was centrifuged, it was possible to recover a pellet with those FS (***Figure 6.7h***). Additionally, the colour of the fine particles in the upper layer changed based on the diluted acid pretreatment applied. Fine particles were white for the lowest severity factor in C2 and C3 (***Figure 6.7c***), but got darker as the severity factor increased (***Figure 6.7b & h***).

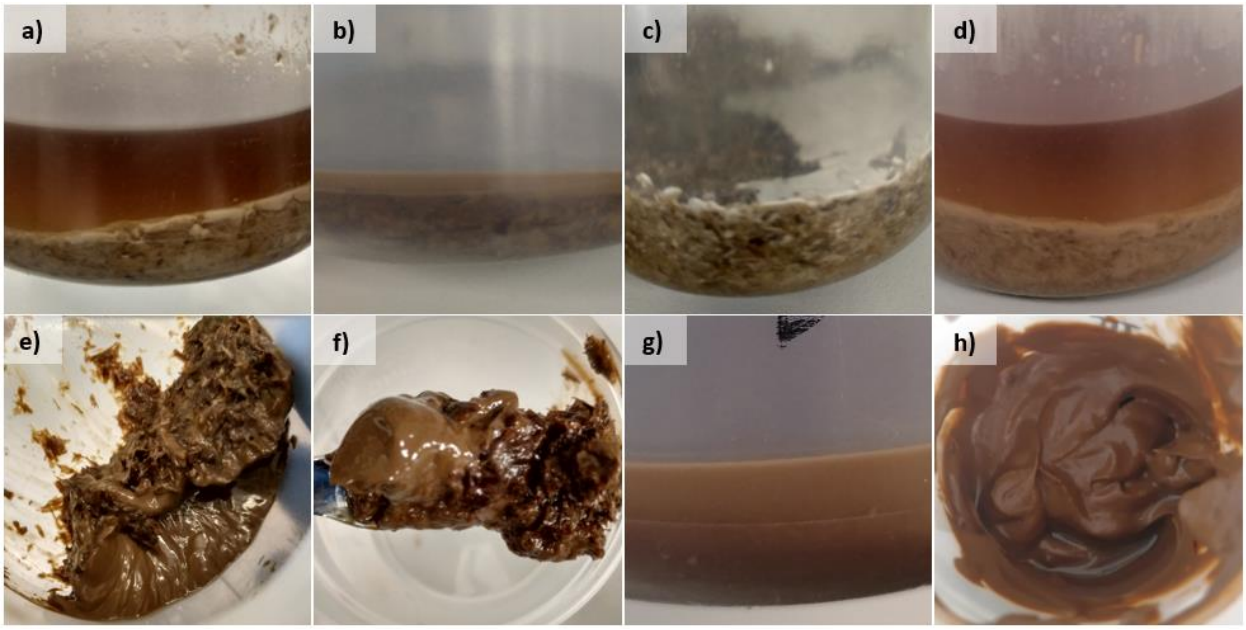


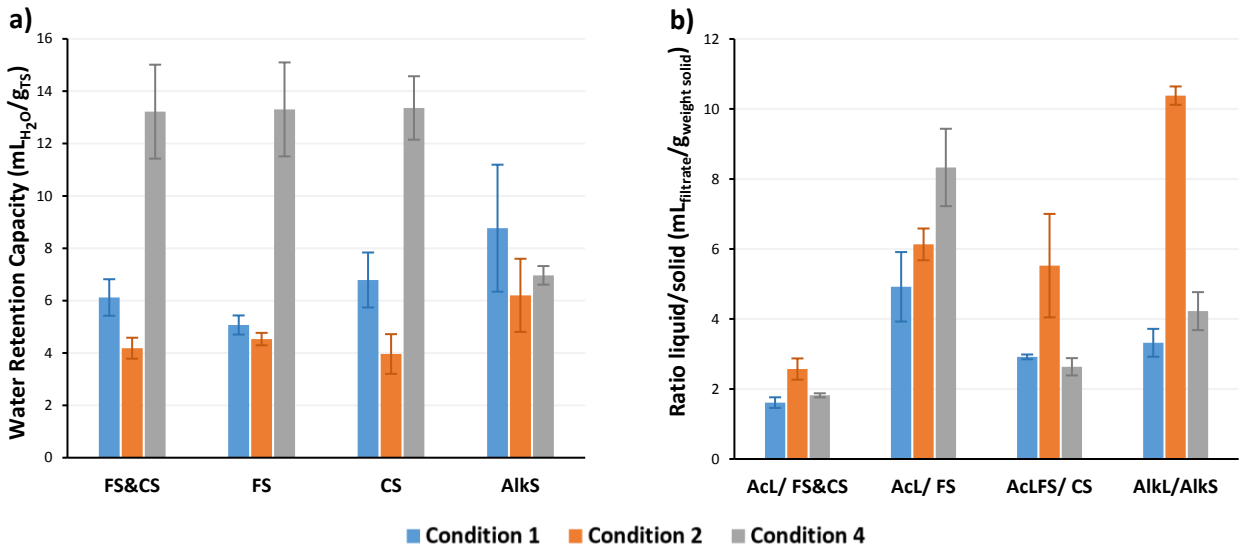
Figure 6.7 *Centrifuged slurry after the diluted hydrochloric acid pretreatment followed by the enzymatic hydrolysis, corresponding to* ***a)*** *C1,* ***b)*** *C2,* ***c)*** *C3 and* ***d)*** *C4. Picture* ***e)*** *shows the centrifuged bottle with the mixed slurry,* ***f)*** *shows on the left part of the picture the upper layer with the fine solid and on the right the bigger particles with the husk and other fraction of the grains. Picture* ***g)*** *is the solid precipitated after the centrifugation of the acid solid with fine particles. Picture* ***h)*** *shows the collected fraction of the fine particles after centrifugation*

The pH of the recovered acid liquid fraction after centrifugation was then increased up to 4.5 to precipitate some of the proteins and any other compounds that was solubilised under extreme acid conditions. However, in the case of C2, with the highest severity factor, after neutralization and filtration of 559.7 (± 23.6) mL, only 4.6 (± 1.7) g of solid were collected. When neutralizing the acid liquid obtained using other conditions with a lower severity factor C1 and C4, no solids were precipitated.

As the protein recovery is done mainly in the literature under alkaline conditions (Connolly et al., 2019; He et al., 2021), the CS fraction was re-suspended and the pH was increased to 8.0. The liquid and solid fractions were analysed before and after the pH increase. In C1, the resulting protein content in the liquid fraction increased over 50 % from the re-suspended to the alkaline liquid. In C2, the protein concentration in the liquid fraction almost doubled before and after changing the pH to alkaline conditions (***Table 6.5***). For C4, no major changes in the liquid were found before and after this pH increase. Under the alkaline conditions, the WRC was higher in the case of C1 and C2, decreasing in the case of C4.

When the CS fraction for C2 was re-suspended and the pH increased to 8.0, this resulted in a loss of more than half of the previous weight before alkaline resuspension. The initial solid amount used for this alkaline wash corresponded to 128.8 (± 28.2) g, after increasing the pH to 8.0, leaving it

overnight at 4 °C, only 52.3 (± 3.0) g of solids were retained in a 25 µm sieve. The liquid ratio for this case was greater than for C1 and C4 (***Figure 6.8***).



Where:

FS: fine solids **CS:** coarse solids **AcLFS:** Acid liquid with fine solids

AlkL: alkaline liquid **AlkS:** alkaline solids

Figure 6.8 a) *Water retention capacity for the different solid fractions after the thermal acid diluted and enzymatic hydrolysis of brewery spent grain (10 % w/w) under different conditions (C1, C2 an C3).* ***b)*** *Ratio between the liquor and solid fraction after each of the separation steps along the process*

Table 6.5 and ***Table 6.6*** show the summary of the main parameters measured during the large hydrolysis for the liquid and solid fraction, respectively. The largest amount of proteins and ammonium in the liquid fraction corresponded to C2, with the highest severity factor. Correspondingly, all the solid fractions of C2 presented the lowest protein content. The protein concentration in the liquid fraction in C4 was slightly higher than in C1. The WRC for C1 was slightly different between the FSs and CSs, with the highest value for the alkaline solids. C2 had the lowest WRC for all the different solid fractions, meanwhile C4 presented the highest value among the conditions for the fine and course solids with similar values (***Figure 6.8a***). Additionally, due to the different sampling of the slurry, solid and liquid fractions, the total amount slightly varied for the different conditions. For example, in C4 extra samples were taken for the solids. To compare the different conditions between them, the ratio between the amount of solids and liquid was calculated for each of the separation steps, i.e. sieve at 100 µm, centrifugation, sieve at 25 µm, as well the overall solid extracted from the slurry (coarse plus fine solids) and the acid liquid fraction (***Table 6.5b***).

Table 6.5 *Liquid characterisation for each of the fractions produced during the fractionation and different streams for brewery spent grain cascade process*

Liquid fraction	Pre-treated liquid			Hydrolysed slurry			Acid liquid		
	C1	C2	C4	C4	C4	C4	C1	C2	C3
Amount (mL)	NA	NA	NA	NA	NA	NA	496.3 ± 25.1	560.0 ± 23.8	486.7 ± 20.8
Total Protein (g/L)	7.6 ± 0.5	19.0 ± 0.2	9.2 ± 0.3	8.1 ± 0.4	17.5 ± 0.3	9.6 ± 0.6	8.1 ± 0.5		10.3 ± 0.5
Ammonium (mg/L)	124.7 ± 5.9	190.1 ± 1.3	130.3 ± 9.2	115.8 ± 6.2	148.1 ± 4.5	124.4 ± 8.0	115.8 ± 7.7		127.6 ± 5.6
Liquid fraction	**Final**			**Re-suspended liquid**			**Alkaline liquid**		
	C1	C2	C4	C1	C2	C4	C1	C2	C3
Amount (mL)				680.33	636.50	659.72	510.3 ± 30.1	542.0 ± 45.3	501.7 ± 27.5
Total Protein (g/L)	7.7 ± 0.5	16.4 ± 0.3	9.0 ± 0.1	1.4 ± 0.2	2.4 ± 0.6	2.6 ± 0.3	2.0 ± 0.4	4.6 ± 0.5	2.6 ± 0.4
Ammonium (mg/L)	112.4 ± 8.7	162.4 ± 4.1	123.1 ± 9.1	34.5 ± 3.5	60.8 ± 13.4	35.8 ± 5.3	26.1 ± 2.9	62.5 ± 2.0	35.0 ± 5.5

Table 6.6 *Solid fraction characterisation during the fractionation and different streams for brewery spent grain cascade process*

	Fine solids			Coarse solids			Alkaline solids		
	C1	C2	C4	C1	C2	C4	C1	C2	C4
Amount (g)	103.1 ± 17.0	91.6 ± 7.4	59.4 ± 10.6	205.7 ± 4.1 *181 ± 2.6	128.8 ± 28.2 *105.7 ± 18.4	208.1 ± 8.4 *185.1 ± 6.2	154.5 ± 10.9	52.2 ± 3.0	119.6 ± 10.9
TS (%)	16.5 ± 1.0	18.1 ± 0.8	7.1 ± 0.9	13.0 ± 1.7	20.5 ± 3.4	7.0 ± 0.6	10.6 ± 2.34	14.2 ± 2.7	12.6 ± 0.5
Proteins (%)	26.2 ± 2.2	18.1 ± 0.8	27.3 ± 0.5	27.7 ± 3.0	11.5 ± 0.8	15.7 ± 1.2	14.0 ± 0.3	8.7 ± 0.3	12.6 ± 0.6
WRC (Gh_2O/ Gts)	5.1 ± 0.4	4.5 ± 0.2	13.3 ± 1.8	6.8 ± 1.0	4.0 ± 0.8	13.4 ± 1.2	8.8 ± 2.4	6.2 ± 1.4	7.0 ± 0.4
Ratio liquid/solid (Ml/g)	4.9 ± 1.0	6.1 ± 0.5	8.3 ± 1.1	2.9 ± 0.1	5.5 ± 1.5	2.6 ± 0.25	3.3 ± 0.4	10.4 ± 0.3	4.2 ± 0.5

6.4 Discussion

6.4.1 Effect of pretreatment

The first part of this work compared the use of sulfuric or hydrochloric acid for the thermal diluted acid hydrolysis of the BSG. Sulfuric acid is the most commonly used acid for pretreatment of lignocellulosic material (Yoon et al., 2014; Zoulikha et al., 2015; Plaza et al., 2017; Tizazu and Moholkar, 2017; Rojas-Chamorro et al., 2020). However, hydrochloric acid was tested here as it is a food grade quality chemical and target chemical compounds for this study are potentially for use in the food or cosmetics industry. The use of hydrochloric acid resulted in a more efficient release of carbohydrate and protein, which is in agreement with previous studies (White et al., 2008). The hydrochloric ions have been suggested to be responsible for disrupting hydrogen bonds in lignocellulose (Wilkinson et al., 2014).

When the acid concentration was increased from 0.10 to 0.25 % (v/v), the colour of the solids and the liquid phase changed (***Figure 6.4***). The darker colour in the solid fraction could be a result of the production of humins (Patil and Lund, 2011). However, aside from C2 the severity factor was not high enough to promote the kinetic reaction when using 1.0 % hydrochloric acid (v/v) or less. A similar colour change was observed during the fractionation of the hydrolysates after enzymatic hydrolysis using larger volumes (***Figure 6.7***). In all these cases, the colour change in the liquid could be a result of the release of polyphenols or other compounds from the matrix of the BSG. In addition, the release of these compounds could be related to the darkening of the solid fraction due to the Maillard reaction resulting in formation of melanoids, a coloured polymeric fraction with important antioxidant and probiotic activities (Patrignani and Gonzalez-Forte, 2021). This causes a reduction of the crystallinity and a loss of the proteinic structure, which facilitates the binding of phenolic compounds to the carbohydrate oligomers (Hellebois et al., 2021; Patrignani and Gonzalez-Forte, 2021). Alternatively, the phenolic compounds and the proteins could interact to develop phenolic-protein conjugates with antioxidant and antimicrobial activity (Liu et al., 2019), making them a good resource for the pharmaceutical or food industry (Buitimea-Cantua et al., 2018). Some of these reactions also take place during the malting and mashing processes in the brewery (Celus et al., 2006). Thus, the use of mild thermal diluted acid pretreatment could create a similar relative structure to that which is formed during the roasting of barley. For example, at higher severity factors the colour of the liquid and FS turned from white to light-brownish, and finally dark brown (***Figure 6.4*** and ***Figure 6.7***).

6.4.2 Enzymatic hydrolysis

The combination of parameters for pretreatment was optimised using RSM. This was to identify different conditions that could lead to the most efficient enzymatic hydrolysis. To do this, different parameters were chosen as indicators for the efficiency of the hydrolysis. For example, the reduction of the hemicellulose was followed by the release of the xylose and arabinose. However, as no degradation of pentose was detected under any of the conditions, the resulting combination was with the maximum parameters given to the software. In the same way, the optimal combination for glucose or total carbohydrate release corresponded to the combination with the highest acid concentration, temperature and time used during this part of the trial (1.00 % v/v of acid, 121 °C and 180 min). These conditions had a severity factor of 2.07, higher than when using the 1.5 % hydrochloric acid at 121.0 °C for 20 min. Furthermore, increasing then the acid concentration from 1.0 to 1.5 % resulted in a very small increase of the amount of carbohydrates released (**Figure 6.2**). Thus, these parameters were discarded as pretreatment to evaluate the degradation of the lignocellulose structure during the enzymatic hydrolysis.

For all the pretreatment combinations, the enzymatic hydrolysis was carried using the WS or the RP. The highest carbohydrate release during the enzymatic hydrolysis was achieved using the WS for C1 and C4, which was approximately 40 and 80 % higher than RS. This could be explained by the wash-out of some solubilised or suspended carbohydrates in the liquid fraction, such as colloids of small fractions of hemicellulose or starch (Hubbe and Rojas, 2008; Wilkinson et al., 2016; He et al., 2019; Wagner et al., 2021). The results of the present work differ from some previous publications that attribute the lower efficiency of the enzymatic hydrolysis to the release of carbohydrates and inhibitory compounds in the broth when using thermal acid hydrolysis (Yoon et al., 2014; Plaza et al., 2017; Wagner et al., 2021). However, these publications carried out the hydrolysis using a higher acid concentration. For example, using 2% sulfuric acid (v/v) for 15 min at 121 °C as a pretreatment showed the lowest glucose extraction during enzymatic hydrolysis, extracting only 37.26 mg of glucose per dry gram of BSG (Wagner et al., 2021). For WS in the present work, this efficiency in C1 and C4 corresponded to 125.61 and 106.87 mg glucose per dry gram of BSG, respectively. Plaza et al. (2017) carried out a pretreatment of BSG by decreasing the pH to 1.0 using sulfuric acid at 121 °C for 20 min, tested after the enzymatic hydrolysis using the WS and RP. They obtained a higher extraction efficiency for glucose using the RP, meanwhile the WS resulted in a higher release of pentose (xylose and arabinose) (Plaza et al., 2017).

In C2 the enzymatic hydrolysis efficiency decreased for the WS and RP. As C2 had the highest SF, the poor enzymatic hydrolysis performance may have been related with the presence of inhibitory compounds such as 5-hydroxymethilfurfural, levulonic acid or humins (Patil and Lund, 2011; Yoon et al., 2014; Plaza et al., 2017). Tizazu and Moholkar (2017) studied the degradation of monosaccharides and production of inhibitory compounds using 33 % (w/v) sugarcane bagasse at 2 % sulfuric acid (v/v) with different temperatures (100 to 130 °C) and time (0 to 120 min). In their work, even without degradation of xylose, glucose or arabinose, there was a small production of inhibitory compounds (Tizazu and Moholkar, 2017). In the case of C2 during the present work, the solid concentration and acid concentration were much lower and as acid has the most significant effect during the pretreatment hydrolysis (Zoulikha et al., 2015), the production of inhibitory compounds would have been lower. Besides, the efficiency of the enzymatic hydrolysis did not improve for RS which would have had some of these compounds washed away and the total carbohydrates concentration at the beginning of the enzymatic hydrolysis was smaller than the WS for C1 and C2 (***Figure 6.5***). The other possibility for the limited enzymatic hydrolysis in C2 could be related with the complete degradation of hemicellulose, resulting in an increase of the cellulose content giving rise to interactions between microfibrils and lignin, thus limiting the enzyme accessibility (Wagner et al., 2021). Even more, if the solid contains a larger lignin fraction, this could be adsorbed by the enzymes in non-productive binding

having a higher adsorption capacity when operating at lower pH (Pareek et al., 2013; Rahikainen et al., 2013). This effect increased when the enzymatic hydrolysis is carried out at 50 °C (Mou et al., 2021) with the higher reduction of the efficiency activity for xylanase and cellulase, whereas ß-glucosidase was the less affected enzyme (Berlin et al., 2006). This could explain the limited enzymatic efficiency for C2 with mainly only cellobiose released.

Based on the results for the xylose and monosaccharides release during the enzymatic hydrolysis for the different conditions, the pretreatments C1, C3 and C4 were effective (***Table 6.3***). However, the most efficient hydrolysis performance was under C1 and C4 with a release of 234.4 (± 39.3) and 225.04 (± 81.7) mg carbohydrates per dry gram of BSG. Only C2 showed a poor enzymatic hydrolysis in WS and RS. Although the efficiency of C2 pretreatment corresponded to 486.9 (± 24.2) mg carbohydrate per dry gram of BSG, the slight release of glucose and cellobiose after 6 hours of enzymatic hydrolysis increased the efficiency up to 535.8 (± 28.7) mg carbohydrate per dry gram of BSG. For all the other conditions, the enzymatic hydrolysis of WS performed better than RP (***Figure 6.5***). C3 was considered non-effective as the hydrolysis efficiency (***Table 6.3***), carbohydrate concentration and the liquid recovered were lower than the other conditions. Only between 13.95 and 33.6 % of the total water content in the slurry was recovered as supernatant after centrifugation. It has been reported that the chemical hydrolysis is largely increased when the pH is below 2.0 (Wilkinson and Cook, 2014), which could explain the low efficiency of C3. For these reasons, and in view of the main objective to produce a liquid fraction rich in carbohydrates, C3 was not tested at larger volume.

6.4.3 Solid fraction

For all the different pretreatment conditions it was possible to observe two layers when centrifuging, the FS on top and the CS on the bottom fraction (***Figure 6.4*** and ***Figure 6.7***). These two different layers with fine and course solids were also observed previously by Ibbett et al. (2019), who were able to obtain a FS with a protein concentration up to 44.9 (± 1.4) % by an improved milling process during the absence of chemical or enzymatic hydrolysis (Ibbett et al., 2019) (this concentration was corrected to the factor 5.50 used for the Kjeldahl method (Mariotti et al., 2008)).

Despite the higher concentration of the acid used during the pretreatment step in C1 than in C4, the use of a lower temperature could have led in the uncomplete degradation of the hemicellulose. Thus, the proteins can interact with the arabynoxylans, or some other hydrolysed oligomer residuals, which led in the aggregation of the proteins in the CS fraction (Hellebois et al., 2021), which would explain the largest protein content in the CS for C2 (***Figure 6.8***). Similarly, the higher temperature of the pretreatment in C2 than in C4 would have resulted in a larger degradation of the pectin-rich fractions, and set the crystallinity in a new stable conformation where proteinic structures are

significantly reduced (Ouhida et al., 2002; Patrignani and Gonzalez-Forte, 2021). As well, this would have had a great impact on the degradation of hemicellulose which protects proteins inside of the BSG. Thus, increasing the severity factor resulted in a higher glucose and protein concentration in the liquid fraction, as [C2] > [C4] > [C1] (***Figure 6.8***).

The lower acid concentration and time in C4 than C1 could have resulted in a higher release of the proteins linked with the lignocellulose fraction, but the reduction in hydrolysis time would have prevented the aggregation of the proteins with the CSs. For example, in C1 a lower pectin degradation could have bound a larger amount of proteins (Ouhida et al., 2002), increasing the protein content in the CS. Meanwhile in the case of C2 as the severity factor was higher, the hemicellulose was fully degraded, explaining the lower content of proteins and limited enzymatic hydrolysis (Hellebois et al., 2021; Wagner et al., 2021). In the case of C2, not only the hemicellulose would have been degraded, but also some fraction of the proteins into peptides. When operating with a higher acid concentration, this degradation of the proteins to peptide is much larger (Kemppainen et al., 2016). In ***Chapter 4***, the diluted acid hydrolysis was carried out using a lower concentration of BSG (7 % instead of 10 % w/w), and 1.5 % instead of 0.80 % sulfuric acid (v/v). Therefore, in ***Chapter 4*** it would have been possible that a larger fraction of the proteins was degraded to peptides and became soluble at a pH lower than 1.0. When the acid liquid hydrolysate was extracted and then neutralised, the protein content corresponded to 32.6 % of proteins above the total solids, although the non-volatile solid fraction corresponded the 47 % of the total solids due to the large amount of acid and base used.

The resuspension of the CS in alkaline liquid was intended to recover further compounds that could be solubilised under alkaline pH. This is the most common approach for protein recovery. The largest solubilisation of solids when CS were re-suspended at alkaline pH corresponded to C2, which enhanced the protein concentration in the liquid fraction 2 fold. Hellebois et al. (2021) studied this effect determining that the heat pretreatment led to significantly higher active glutelin network chains. Similarly, the higher SF in C2 compared with C4 would have resulted in a higher cellulose degradation and lignin enrichment. The hydrophobicity of proteins increases when the pH decreases (Berlin et al., 2006; Mou et al., 2021), which, with the electrostatic force would have contributed to the protein absorption on the lignin by hydrogen bonds and other non-covalent forces (Pareek et al., 2013; Rahikainen et al., 2013). Therefore, it would have been possible that at pH 1.0, the proteins and other oligomers interacted to form aggregates with a size larger than 100 µm, which would have been disintegrated by increasing the pH from 1.0 to 8.0. As well, this loss of hydrophobicity by increasing the pH could explain the higher WRC in C2 for the alkaline solid fraction.

The WRC has a key role in the access of microorganisms and enzymes to degrade lignocellulose (Raghavendra et al., 2004; Jeihanipour et al., 2010; Guo et al., 2018; Oliva et al., 2020). However, the absorbed water in C3 was higher than for the other conditions (***Figure 6.4***), whereas the enzymatic hydrolysis efficiency was limited with a lower amount of carbohydrate released (***Table 6.3***). This effect is related with the initial breaking down of the crystallinity structure of the cellulose (Mosier et al., 2005; López-Linares et al., 2020). Thus, a low degradation of this structure would have resulted in the higher WRC, as more water was absorbed. Further degradation of the cellulose and hemicellulose structure would have resulted in the solubilisation of these structures decreasing the WRC and increasing the liquor recovered from the hydrolysate. This was easily observed in the initial thermal diluted acid hydrolysis when comparing hydrochloric and sulfuric acid (***Figure 6.3***). For the sulfuric acid, the increase of the acid concentration up to 0.10 % (v/v) resulted in a higher WRC but without carbohydrate degradation. When the acid concentration was increased to 0.25 and 0.50 % the WRC decreased and the recovered liquid was higher (***Figure 6.2***). This behaviour corresponded as well with the degradation of glucose and the highest degradation of arabinose and xylose. The different solid fractions obtained could be valorised as food supplement. For example, the WRC has been related with nutritional properties (Raghavendra et al., 2004; MartÍnez et al., 2012). In the case of the FS for C4, the WRC observed (***Figure 6.8***) was higher than many of the current sources of fibres from the food industry (Raghavendra et al., 2004).

6.5 Conclusion

A cascade process approach using BSG was followed to obtain a liquid hydrolysate rich in carbohydrates and the production of two different solid streams (fine and coarse solids). The use of a mild pretreatment and the enzymatic hydrolysis of the WS resulted in a higher carbohydrate efficiency recovery for C1, C2 and C4, corresponding to 399.1 (± 26.1), 535.8 (± 28.7), 257.0 (± 11.5) and 446.3 (± 81.1) mg carbohydrate per dry gram of BSG respectively. The WRC of the solids is equal to or higher than some of the current sources of fibres for the food industry. The quality of proteins enriched in the FSs should be evaluated. As well, the possible enrichment of the solid fractions with phenolic compounds or lignin was related with the change of colour by production of melanoids or phenolic-protein conjugates. The combination of these products could result in a food supplement with a high potential in antioxidant and prebiotic activity. Finally, the liquid hydrolysate rich in carbohydrates is an ideal candidate for the production of the VFAs.

6.6 References

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Chapter 7

Continuous volatile fatty acid production and recovery at high organic concentration using an upflow granular sludge bed reactor

Keywords: glucose; expanded granular sludge bed; high strength feedstock; organic loading rate; acidogenesis; volatile fatty acids; electrochemical cell

Abstract

A novel approach for volatile fatty acid production and recovery was developed by coupling an electrochemical cell to the recycling line of an expanded granular sludge bed reactor. The reactor was fed with glucose (from 15 g/L up to 60 g/L, pH 5.7 at 37 °C) to improve the VFA production as no hydrolysis step is required. A synthetic broth was initially used to characterise the VFA rate transfer in the electrochemical cell. Different voltages were applied (1.5, 2.0, 2.5, 3.0 and 3.5 V) between the cathode and the anode chamber. The lowest voltages (1.5 and 2.0) were ineffective as the current dropped to 0 by day 2. In the case of the higher voltages, the best recovery rate was at 3.5 V. In the first phase, 15 g/L of glucose was used to feed the UASB reactor with a 36 h hydraulic retention time (HRT). For the next phase the HRT was reduced to 24 h, and this was kept constant throughout the trial. The coupling of the electrochemical cell to the UASB reactor was done when the feed glucose concentration corresponded to 15 of g/L. This resulted in the removal of the VFAs from a total concentration of 6.72 (± 1.16) g/L to 3.65 (± 0.45) g/L. As well, the sodium hydroxide consumption decreased by about 49 %. The concentration of VFAs in the anode chamber was enhanced up to 4 fold in comparison with the VFA concentration in the fermentation broth. The electrochemical cell was under dimensioned for the reactor size, thus when the OLR was increased above 30 g/(L·day) the flow of fresh water continuously added to the anode chamber was increased in order to avoid any possible saturation of the VFAs. This led to dilution of the VFA recovery solution. The highest concentration of VFAs in the reactor corresponded to 32 g/L and was achieved when using 60 g/L of glucose as feedstock. Interestingly, the largest caproic acid production achieved was 6.01 g/L when the system operated with a glucose feeding of 30 g/L.

7.1 Introduction

In the last decades the production of hydrogen from organic wastes is gaining more attention, often coupled to the simultaneous production of volatile fatty acids (VFAs) as by-products. These are valuable chemicals, currently obtained from fossil fuel sources but which could be produced from renewable feedstock. However, renewable VFA production has not yet been developed at industrial level (Strazzera et al., 2018). The main bottleneck is the rather low concentration of VFAs obtained in the acidogenic bioreactor fermentation broth, which makes their recovery difficult. Currently, extraction systems are capable of recovering VFAs in systems that operate at a pH lower than 5.0 or with VFA concentrations in the range of 20 – 50 gl/L (Saboe et al., 2018; Wainaina et al., 2019). However, such high concentrations in the reactor could lead to the accumulation of the VFAs in undissociated forms, which are toxic for the microorganisms (Kaur et al., 2020). Thus, some research focuses on the chain elongation of VFAs for production of e.g. caproic acid, which is easier to recover (Spirito et al., 2014; Xu et al., 2020). Or on the use of VFAs as intermediate compound for production of other chemicals, such a biopolymers, e.g. polyhydroxyalkanoates (Albuquerque et al., 2011).

An alternative process to recover VFAs as final product is the electrodialysis system. This combines a membrane separation system for the VFA recovery using an electrochemical cell. The recovery of VFAs is driven by the different voltages applied between the cathode and the anode (Bak et al., 2019). The protons are reduced at the cathode, and the VFAs migrate to the anode through the anion exchange membrane (AEM) (Zhang and Angelidaki, 2015). Despite obtaining a higher VFA concentration, the downstream processing for the purification step constitutes up to 35 % of the total costs (Baroi et al., 2017). One additional advantage of this approach is the production of OH^-, which contributes to neutralizing the pH and decreasing the chemical cost for maintaining a stable pH (Zhang and Angelidaki, 2015; Xu et al., 2015).

The aim of this chapter is to achieve high levels of VFA production by using a synthetic high strength feedstock with carbohydrate concentrations from 15 to 60 g/L. The feedstock was fermented at low retention time with anaerobic granular sludge to produce VFAs under acidogenic conditions using an upflow anaerobic sludge bed reactor (UASB). In ***Chapter 5***, operating the same system at pH 6.0 and with lower carbohydrate concentrations (15 g/L) prevented methane production achieving a high acidification yield. The initial carbohydrate concentration was increased stepwise, while keeping a constant hydraulic retention time. Thus, increasing the organic content and the VFA concentration within the reactor. Simultaneously, an electrochemical cell (EC) was coupled to the recycling line for the recovery and concentration of VFAs from the fermentation broth, simultaneously alleviating their possible toxic effect.

7.2 Material and methods

7.2.1 Feedstock

7.2.1.1 Electrochemical cell characterisation with synthetic broth

The electrochemical cells were tested using a synthetic feedstock, consisting of acetate, propionate, butyrate and ethanol, using the optimal VFA production obtained in ***Chapter 4*** (6.79 g/L of sodium acetate, 1.34 g/L of sodium propionate, 5.14 g/L of sodium butyrate and 1.67 g/L of ethanol). As well 300 mg/L of ammonium chloride, 300 mg/L of potassium phosphate and 7.5 g/L of sodium bicarbonate were used to increase the conductivity of the synthetic broth. The pH of this solution was corrected to 6.0. For the anode, a solution of 7.15 g/L (50 mM) sodium sulfate was used for the recovery of the VFAs.

7.2.1.2 Acidogenic fermentation

The continuous reactor was fed with a synthetic feedstock with glucose as main carbon source based on the recipe of Zhang et al. (2008), the main difference was that 10 g/L of sodium bicarbonate was added and a higher concentration of proteins was used. Peptone from wheat was used as proteic substrate (Sigma Aldrich), with a ratio of 208 mg of peptone per g of glucose, similar to the ratio obtained in the hydrolysate in Condition 4, ***Chapter 6***. The glucose concentration in the feedstock was increased during each phase from 15 to 30, 45 and 60 g/L. Every time the feedstock was prepared, it was sparged for 5 minutes with nitrogen gas to remove oxygen. The bottle was closed and a gas bag with nitrogen was attached to prevent the formation of vacuum inside of the bottle. The bottle was place in a fridge at 4 °C. The bottle was continuously stirred to homogenize the feedstock.

7.2.2 Inoculum

The acidogenic fermentation reactor was inoculated with granular sludge from an up-flow sludge blanket reactor at the Faxe Wastewater Treatment Plant (Lindegårdsvej, Denmark). This system treats wastewater from a brewery and sweet confectionary plant at 35 °C, with an average COD concentration of 3.9 g COD/L. The inoculum total suspend solids (TSS) and the volatile suspended solids (VSS) corresponded to 8.69 (± 0.09) and 7.71 (± 0.07) g per 100 g of wet granular sludge, respectively. The reactors were seeded with 1.5 g/L of VSS.

7.2.3 Experimental design and operation

7.2.3.1 Electrochemical cell characterisation with synthetic broth

Each of the electrochemical cells were constructed as previously described by Yang et al. (2022), with the only difference being the use of an anion exchange membrane (AEM). Briefly, two pieces of polycarbonate plastic chambers (5 x 5 x 8 cm) were bolted together, separated by an AEM (AMI 7001, Membrane International, Ringwood, NJ, USA). The AEM membrane was submerged for 24 hours in

sodium hydroxide with a concentration of 50 g/L. Each of the chambers had a total volume of 200 mL, and a working volume of 180 mL. The cathode electrode was an alloy mesh (4 x 4 cm) made of titanium coated with IrO_2 (4 x 4 x 0.1 cm) (Magneto Special Anodes B.V., Netherlands). The anode electrode was a titanium woven wire mesh (4 x 4 x 0.015 cm) (William Gregor Limited, UK), this electrode was coated with 0.5 mg Pt/cm^2 (20 % Pt/C, JM) and Nafion (5%, Dupont). The electrical connections were done as described by Zhang and Angelidaki (2015). An external resistance of 10 Ω was used in the external circuit to record the current.

Different voltage potentials (Vellemean, DC lab switching mode power supply) were applied between the cathode and the anode to study the VFA recovery rate. These voltages corresponded to 1.5 V, 2.5 V, 3.0 V and 3.5 V. The conductivity, pH and VFA concentration were determined for the cathode and the anode chambers. The cathode section was continuously stirred using a magnetic stirrer to improve the homogenization of VFAs inside of the chamber.

7.2.3.2 Acidogenic fermentation and volatile acid recovery

Two twin up-flow anaerobic sludge BED (UASB) reactors were used for the acidogenic fermentation (total volume 1.69 L, working volume 1.06 L). A recycling line was used to keep upflow velocity constant (2.5 m/h) using a peristaltic pump (Watson-Marlow 323, UK) (***Figure 7.1***). The feedstock was introduced from the bottom of the reactor using a pump (Longer Pump BT100-2J, China). The outlet gas line was connected to an automated water displacement gas meter with a 100 mL compartment (Angelidaki et al., 1991). A loop in the effluent line was introduced to prevent the gas escape through the effluent line due to the backpressure generated by the water displacement gas meter. The pH of the reactor was controlled by a pH regulator and dispenser (ProSystem Aqua, Spain) for sodium hydroxide addition, maintaining the pH at 5.70. The temperature was kept at 37 °C using a water bath. To avoid the wash-out of the biomass, a filter was added after the recycling line and before the effluent. The filter was made by adding a titanium mesh with 1 mm pore size in a sectional tube with an internal diameter of 4.8 cm (***Figure 7.2***).

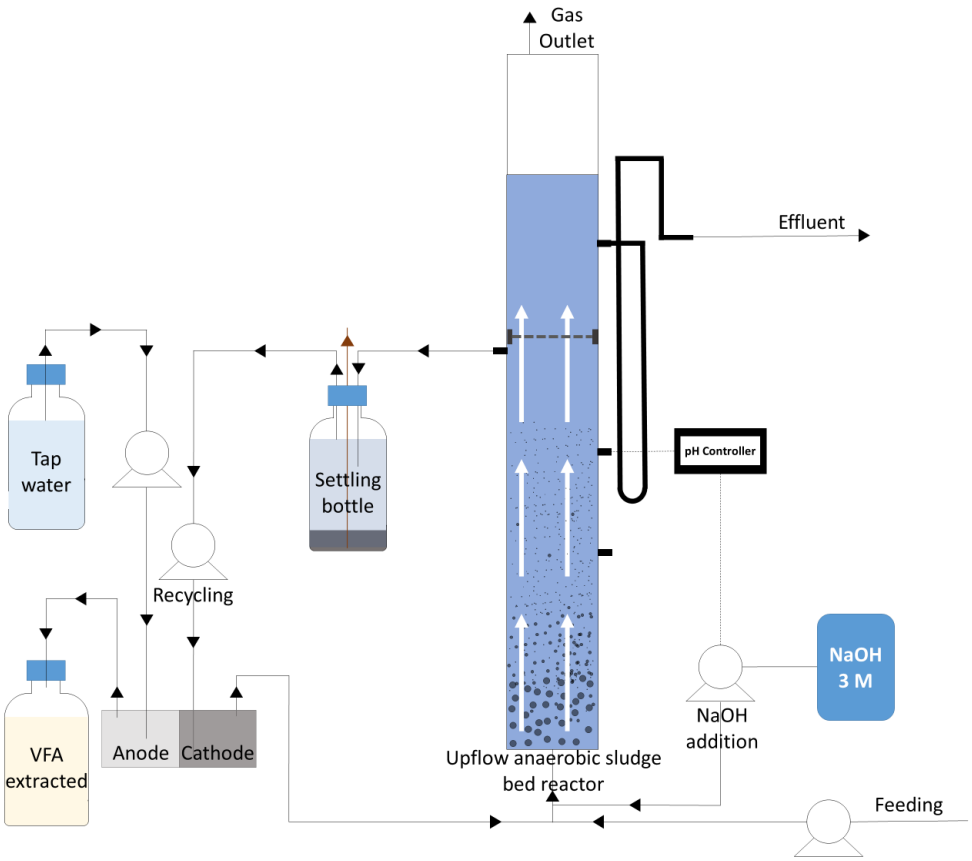

Figure 7.1 *Schematic set up of the volatile fatty acid production and recovery system. The recycling line of the upflow anaerobic sludge bed reactor is coupled to the cathode of an electrochemical cell for the volatile fatty acid recovery*

Figure 7.2 *Sectional tube with a titanium mesh with a pore size of 1 mm, and placement inside of the upflow anaerobic sludge bed reactor*

Once the granular sludge was placed in the reactors, these were filled up with feedstock at a concentration of 10 g/L glucose. Then, the reactors were sparged with nitrogen gas for 5 minutes to remove oxygen from the medium. The system was then run for 24 hours in batch mode to allow sludge acclimatization. After this period, for Phase I, the feedstock was spupplied at an initial hydraulic

retention time (HRT) of 36 hours and a glucose concentration of 15 g/L. For the second phase, the HRT was decreased and kept for the rest of the trial to 24 h. At the third phase, the electrochemical cell was connected to the fermentation reactor by connecting the recycling line to the cathode. During the initial 9 days the tap water added in the anode was not removed. After this period, a pump was connected to continuously add fresh tap water to the system with a HRT of 5 days in the anode chamber. A further decrease of the HRT from 5 to 2 days on day 67, corresponding to Phase IV. The glucose concentration was increased stepwise from 15 g/L in Phase III up to 60 g/L in the final phase of the trial (***Table 7.1***).

Table 7.1 *Hydraulic retention time and glucose feeding concentration for each of the phases, with and without the electrochemical cell coupled to the recycling line of the reactor. HRT: hydraulic retention time; EC: electrochemical cell*

Phase	HRT (h)	[Glucose] (g/L)	EC	Time (days)
I	36	15	Off	0 → 19
II	24	15	Off	19 → 47
III	24	15	On	47→ 59
IV	24	30	On	59 → 83
V	24	45	On	83 → 101
VI	24	60	On	101 → 114

In addition, during the development of the trial, further adaptions were carried in the set-up. To avoid the accumulation of the biomass inside the anode chamber, a 250 mL Duran bottle was added in the recycling line before the EC as a settling bottle during Phase III. The sludge was recovered from the bottle once per week and reintroduced to the reactor. During Phase VI, a Duran bottle (250 mL) was added in the gas outlet, before the gas counter to avoid any possible clogging of the gas meter.

7.2.4 Analytical methods

Total suspended solids (TSS) and volatile suspend solids (VSS) were determined using the standard method (APHA, 2012). The gas composition (H_2, CO_2 and CH_4) was determined by gas chromatography (Trace 1310 GC-TCD, Thermo Fisher, Denmark) as described by Yang et al. (2022). Ethanol, acetic acid, propionic acid, butyric acid, valeric acid and caproic acid were measured by gas chromatography (HP 5890 series II) with nitrogen as carrier gas, equipped with a fused silica capillary column (Yang et al., 2022). Lactic acid and glucose were measured using High Performance Liquid Chromatography (Agilent) as described by Tsapekos et al. (2020).

7.2.5 Calculations

The recovery rate for each of the VFAs was calculated by the slope obtained in the increase concentration of the cathode chamber during the EC characterisation. The current density was calculated as previously described by Zhang and Angelidaki (2012). Briefly, the current intensity was obtained by dividing the measured voltage across the external resistor by the resistance. Then, the current intensity was devided by the surface area of the anode electrode to obtain the current density. The yield gas production was calculated as described in ***Chapter 5***.

7.3 Results

7.3.1 Electrochemical cell characterisation with synthetic broth

Different voltages were applied between the anode and cathode chambers for approximately one week at each of the voltages. The lowest voltages (1.5 and 2.0 V) started with a similar current. However, this current dropped to almost 0 by day 2 (***Figure 7.3***). At 2.5 V the current slightly decreased initially, becoming more constant after 1.2 days with an average current of 6.54 (± 1.17) mA/m^2. A similar trend was observed at 3.0 V when initially the current decreased, although the current for 3.0 V was almost double than that for 2.5 V. Application of 3.5 V resulted in a larger increase in the current, more than five times higher than 2.5 V, and almost three times higher than at 3.0 V (**Figure 7.3**).

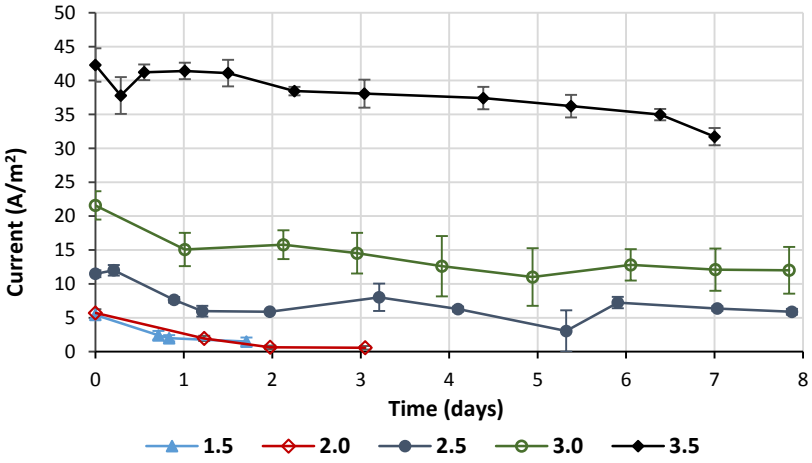

Figure 7.3 *Current corresponding to each of the different voltages applied between the cathode and the anode chamber using the same volatile fatty acid synthetic broth*

When the voltage applied was 3.5 V, the liquid level in the anode started to increase and decrease in the cathode (***Figure 7.2***). In EC1, the cathode chamber was completely empty by day 7 and in EC2 the cathode chamber was completely empty by day 8.

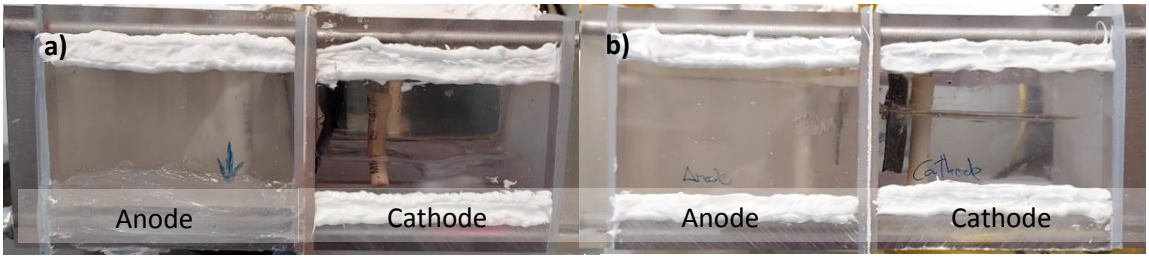

Figure 7.4 *Electrochemical cells after 6 days applying a voltage between the cathode and the anode of 3.5 V, a) EC1 and b) EC2*

Table 7.2 summarises the recovery rate and final recovery for the VFAs in the anode chamber for the synthetic broth. ***Figure 7.5*** shows the profile for the different parameters measured along the trial. Two different rates in terms of current and acid recovery were detected when operating the system at 2.5 V (***Table 7.2***). Under this voltage, the acetic acid concentration increased during the first day up to 582.9 (± 24.9) mg/L, with the higher concentration on day 7 corresponding to 1,419.56 (± 24.9) mg/L (***Figure 7.5c***). As well, this behaviour was detected for propionic and butyric acid (***Figure 7.5 g*** & ***i***). The concentration of acetic acid was similar for 3.0 and 3.5 V, although at the last point the acid concentration decreased. This decrease at the end of the trial using 3.0 V took also place for the butyric and propionic acid concentrations.

Table 7.2 *Average current, recovery rate and final acid recovery in the anode chamber of a synthetic mixture broth for recovery of acetic, propionic and butyric acid under different voltages*

	Current	Acetic acid		Propionic acid		Butyric acid	
		Rate	Recovery	Rate	Recovery	Rate	Recovery
	(A/m^2)	(mg/L/day)	(100g /g)	(mg/L/day)	(100g /g)	(mg/L/day)	(100g /g)
2.5 V 1)	10.38 ± 2.0	489.7 ± 33.0	22.0 ± 2.4	58.2 ± 38.1	20.0 ± 2.3	313.0 ± 52.6	18.8 ± 2.7
2)	6.5 ± 1.2	142.0 ± 32.5	67.9 ± 14.1	23.1 ± 6.6	60.2 ± 13.3	117.9 ± 43.4	53.5 ± 13.1
3.0 V	12.84 ± 3.52	590.2 ± 80.9	62.7 ± 15.1	73.4 ± 7.0	54.1 ± 11.7	277.4 ± 24.0	53.5 ± 12.3
3.5 V	37.83 ± 3.32	1034.5 ± 25.8	83.5 ± 6.0	96.7	77.7 ± 43.2	407.3 ± 62.5	74.0 ± 13.2

The conductivity in the anode chamber was similar for all conditions tested, regardless of the voltage applied (***Figure 7.5***). Whereas in the cathode this largely increased as when using a higher voltage. For example, by day 7 the difference in conductivity between the cathode and the anode chamber when applying 3.5 V corresponded to 47.5 (± 2.0) mS/cm (***Figure 7.5 a***&***b***). Similarly, using 3.5 V reached quickly the lowest pH in the anode chamber, and the highest pH for the cathode (***Figure 7.5 c***&***d***). Although at the end of this condition the pH increased.

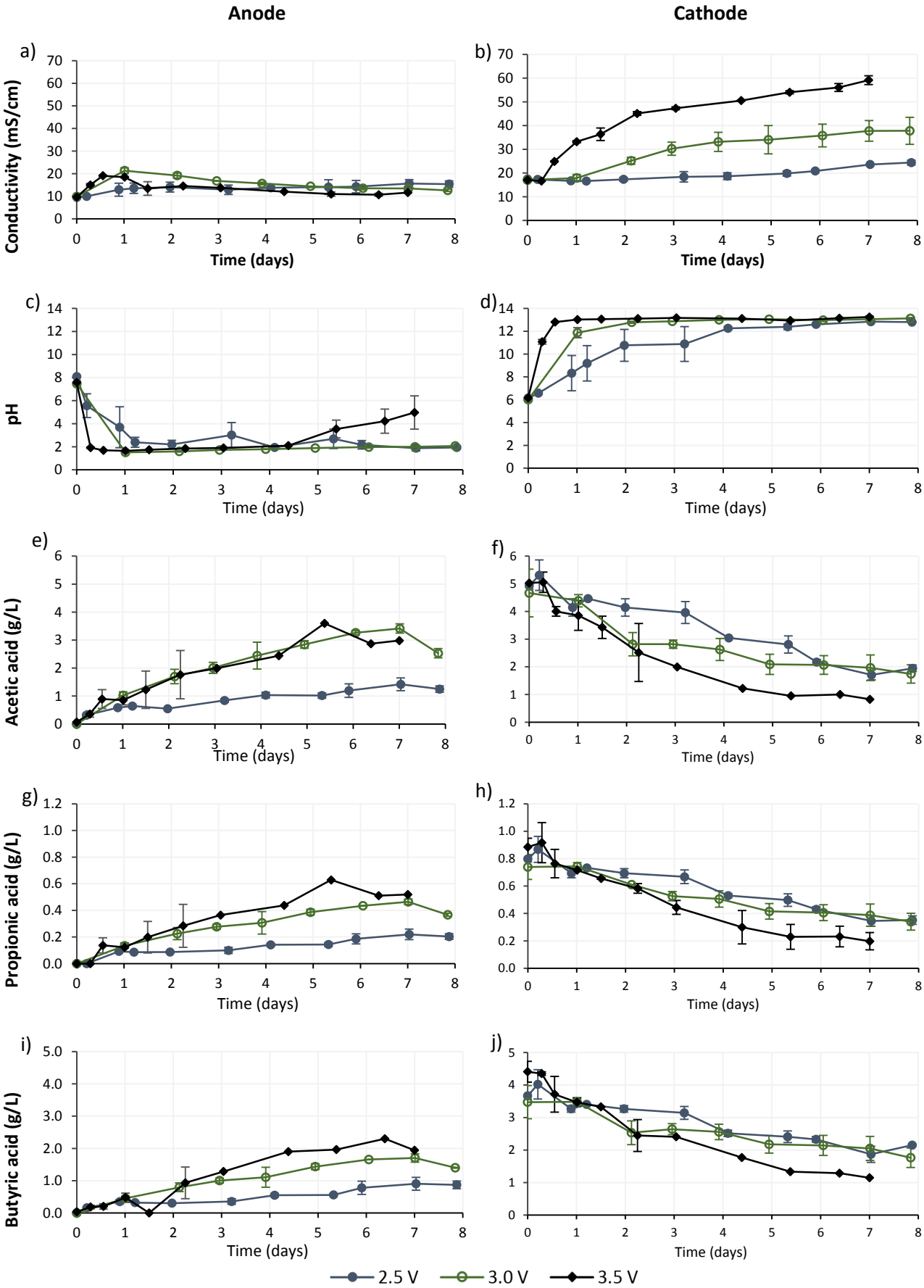

Figure 7.5 *Conductivity, pH and acid concentration along the recovery of acetic, propionic and butyric acid under different voltages applied between the anode and cathode chamber of the electrochemical cell*

The increase of the current and the recovery rate during the VFA recovery from the synthetic broth did not follow a linear trend (***Figure 7.6***). The increase of the current by almost three times, from 13.0 (± 3.6) to 38.5 (± 2.7) A/m^2, resulted in a lower increase for the recovery rate of the VFAs (***Figure 7.6***).

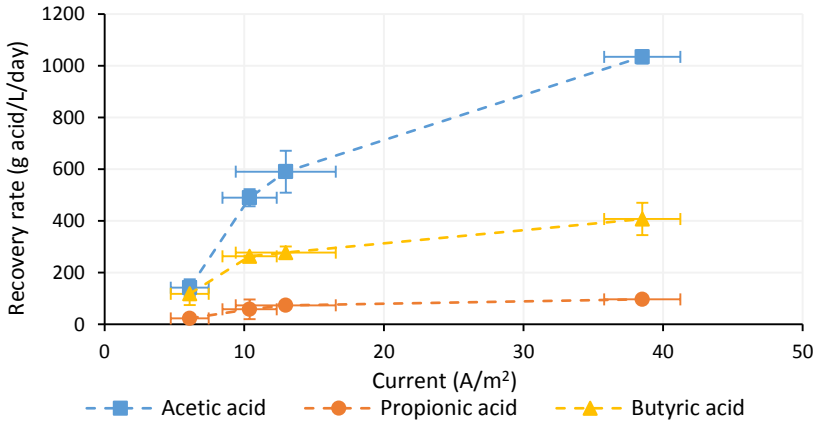

Figure 7.6 *Recovery rate of a synthetic broth (acetic, 4.89 g/L; propionic, 1.02 g/L; and butyric acid, 4.11) using an electrochemical cell and different voltages. The different currents were calculated based on the stability periods for the different voltages (2.5, 3.0 and 3.5 V)*

7.3.2 Continuous volatile fatty acid production and recovery

7.3.2.1 Reactor performance

A short batch period (1 day) was used to acclimatize the inoculum to the new operational conditions. In both reactors, butyric acid was the major acid produced during this period, followed by acetic acid. Once the reactors were operated as continuous system, acetic surpassed the butyric acid concentration and there was production of low concentrations of ethanol, propionic acid, valeric acid and caproic acid. Between days 9 – 10, the caproic acid concentration started to be produced, becoming even the major compound in R1 at the end of Phase I. Ethanol was produced during the batch adaptation period of R1, being fully consumed by day 2. After day 4, ethanol was again detected in the fermentation broth, with a continuous low production along the trial. In R2, the caproic acid concentrations were similar to the butyric acid concentration, whereas acetic acid was the major fermented compound. The ethanol production in R2 took place on day 5. As well, caproic acid was produced during Phase I for both systems, with caproic acid being produced along the trial (***Table 7.3***).

Each of the ECs were connected to each UASB reactor on day 47, starting then Phase III. This resulted in a decrease of the total VFA concentration in both systems and in the sodium hydroxide consumption (***Table 7.3***). Both reactors had a lower acetic and butyric acid concentrations, whereas caproic acid was the major compound in the fermentation broth in R1. The major different between the two UASB reactors was the concentration of these compounds, in R1 the concentration of the three acids was similar, in R2 acetic acid was the major fermented compound followed by butyric and caproic acid. Nevertheless, the total VFA concentration was similar for both reactors. During Phase III,

there was some turbidity in the anode chamber of EC1, which was a result of a tear in the membrane. EC1 was disconnected for 12 hours and the membrane was replaced on day 55. At Phase IV, the glucose concentration in the feedstock was increased to 30 g/L. Similar to Phase III, the major compounds in R1 were butyric and caproic acids. By day 78 the total VFA concentration in R1 had decreased by half, from 20.14 to 9.14 g/L. Acetic acid was the major product in R2 at the beginning of the phase, although by day 67.0 butyric acid became the major fermentation product and by the end of phase IV, acetic and caproic acid had a similar concentration. The EC2 membrane was broken on day 73, thus the EC2 was disconnected for 12 hours and connected again on day 74.

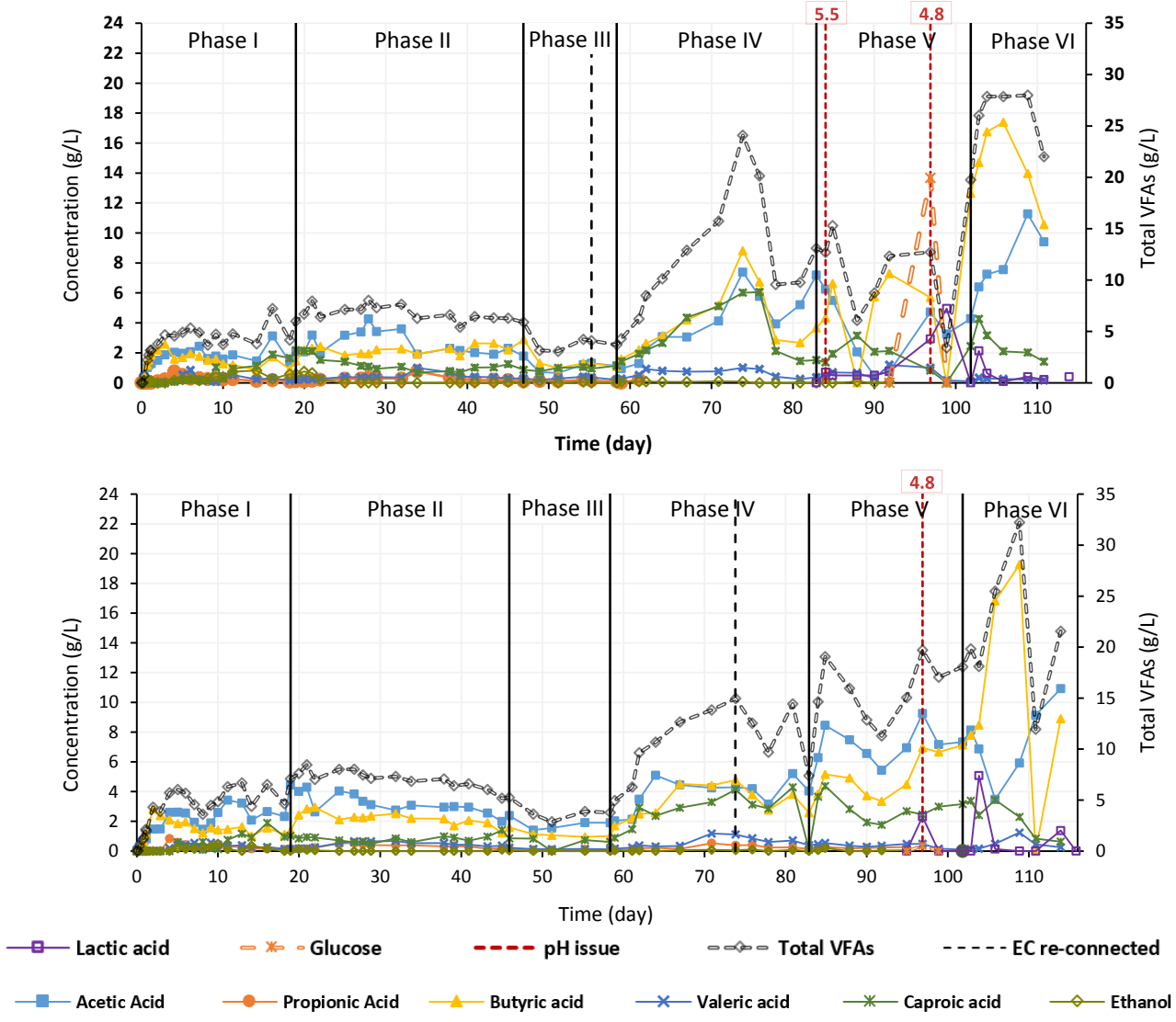

Figure 7.7 *Reactor performance for the fermentation of the glucose for the volatile fatty acid production using an expanded granular sludge bed reactor with an electrochemical cell coupled to the recycling line since Phase III. a) System 1 and b) System 2*

In Phase V, there were two instability periods in R1 due to a decrease of pH. The first time, the pH decrease to 5.5, resulting in a lower production of VFAs with lactic acid production, which was not detected previously. After this short period, butyric acid became the major fermentation compound.

However, on day 97 the pH dropped to 4.8. This resulted in incomplete consumption of the glucose, as its concentration reached 13.68 g/L. On day 99, the total VFA concentration was 3.41 g/L, and the lactic acid concentration increased to 4.96 g/L. The profile of the fermented compounds in R2 was similar to the previous phases, acetic acid was the major compound followed by butyric and caproic acid. Also, the pH dropped in R2 on day 97, with some lactic acid production (2.32 g/L) and a small accumulation of glucose (0.34 g/L). After this decrease of pH, the profile of VFAs was similar and no further lactic acid was detected during this phase. In Phase VI, the glucose concentration in the feedstock was increased up to 60 g/L, meanwhile the HRT was kept at 24 h. In both systems butyric acid peaked at the beginning of this phase, whereas acetic acid increased along the phase (***Figure 7.7***).

Table 7.3 *Main average parameters during the simultaneous production and recovery of the volatile fatty acids using an expanded granular sludge bed reactor coupled with an electrochemical cell*

		Acetic acid (g/L)	Butyric acid (g/L)	Caproic acid (g/L)	Total VFAs (g/L)	NaOH Consumption (g/day)	CO_2 production yield (mMol / mmol glucose consumed)	CH_4 production yield (mMol / mmol glucose consumed)
Reactor 1	*Phase I*	1.93 ± 0.46	1.60 ± 0.45	0.77 ± 0.77	4.72 ± 1.10	1.60 ± 1.02	0.07 ± 0.02	0.03 ± 0.01
	Phase II	2.59 ± 0.75	2.19 ± 0.35	1.24 ± 0.36	6.72 ± 1.16	3.95 ± 1.46	0.87 ± 0.31	0.33 ± 0.12
	Phase III	1.00 ± 0.26	1.19 ± 0.14	0.98 ± 0.07	3.65 ± 0.45	1.79 ± 0.79	0.95 ± 0.11	0.29 ± 0.05
	Phase IV	4.02 ± 2.08	3.97 ± 2.06	3.17 ± 1.76	12.22 ± 5.62	6.00 ± 2.68	0.64 ± 0.10	0.25 ± 0.07
	Phase V	3.41 ± 2.02	5.29 ± 3.83	1.74 ± 1.18	11.38 ± 6.49	14.51 ± 3.36	0.92 ± 0.29	0.16 ± 0.06
	Phase VI	8.37 ± 1.75	15.70 ± 1.40	2.59 ± 1.15	26.35 ± 12.63	39.89 ± 6.92	0.52 ± 0.39	0.05 ± 0.04
Reactor 2	*Phase I*	2.52 ± 0.76	1.66 ± 0.37	0.74 ± 0.48	5.40 ± 1.07	1.63 ± 0.61	0.42 ± 0.22	0.17 ± 0.11
	Phase II	3.13 ± 0.64	2.17 ± 0.43	0.83 ± 0.22	6.96 ± 0.92	3.91 ± 1.01	0.85 ± 0.32	0.39 ± 0.15
	Phase III	1.69 ± 0.22	1.04 ± 0.07	0.55 ± 0.32	3.54 ± 0.40	2.10 ± 0.45	0.50 ± 0.40	0.30 ± 0.18
	Phase IV	3.85 ± 1.01	3.24 ± 1.00	2.58 ± 1.23	10.64 ± 3.23	7.41 ± 0.37	1.11 ± 0.35	0.44 ± 0.20
	Phase V	7.21 ± 1.07	5.13 ± 1.37	2.87 ± 0.77	15.96 ± 2.65	12.56 ± 1.46	1.03 ± 0.53	0.29 ± 0.12
	Phase VI	7.41 ± 2.38	12.25 ± 4.81	2.17 ± 1.10	21.52 ± 6.28	30.55 ± 4.56	1.01 ± 0.16	0.23 ± 0.12

Hydrogen was detected under the quantification limit throughout the trial (< 5 % hydrogen). In R1, the carbon dioxide yield was higher than the methane yield throughout the trial (***Figure 7.8***)). During Phase I, almost no gas was produced. Phase II and III resulted in a stable gas production where the carbon dioxide yield fluctuated between 1.51 and 0.49 mM carbon dioxide/mM glucose. This carbon dioxide yield was stable in R1 for Phases III, IV and V, with an average concentration of 0.85 (± 0.28) mM carbon dioxide produced per mM of glucose consumed. The carbon dioxide yield decreased in

Phase VI to 0.12 mM carbon dioxide/mM glucose. The methane yield in R1 was constant from Phase II to Phase IV, with an average concentration of 0.30 (± 0.11) mM methane/mM glucose. The methane yield of R1 during Phase VI was to 0.14 (± 0.04) mM methane/mM glucose.

The gas production in R2 was larger and with more variability as carbon dioxide and methane fluctuated throughout the trial (***Figure 7.8***). The average carbon dioxide yield in R2 from Phase II to V corresponded to 0.90 (± 0.28) mM carbon dioxide/mM glucose, which decreased in Phase VI. In Phase III methane became the major gas produced in R2 instead of carbon dioxide for a short period of time. In the case of the methane yield, a similar trend was followed in R2 as in R1, with an average yield for Phases II, III and IV about 0.39 (± 0.18) mM methane/mM glucose, corresponding to the yield during Phase VI of 0.29 (± 0.12) mM methane/mM glucose.

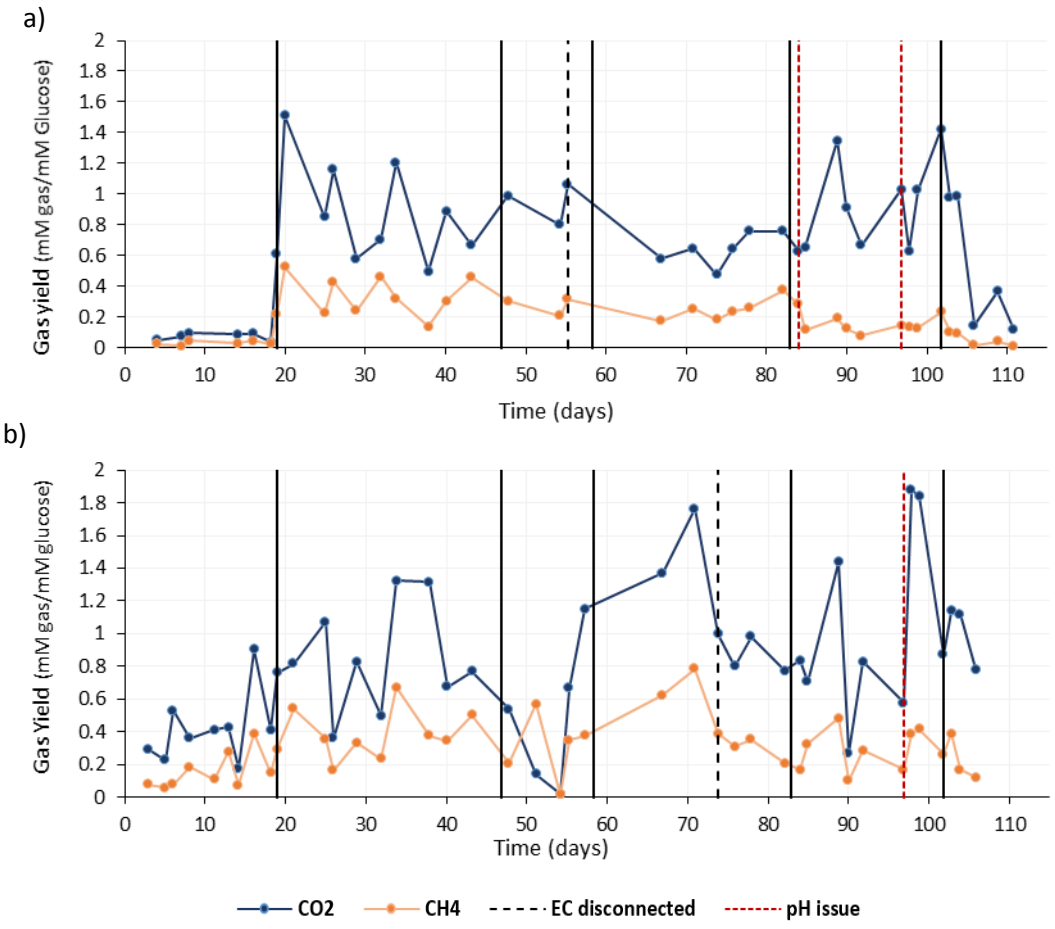

Figure 7.8 *Carbon dioxide and methane production yield (mM of gas per mM of glucose consumed) of the glucose fed UASB reactors. a) R1 and b) R2*

7.3.2.2 Electrochemical cell

Each of the cathode chambers of the EC was connected to the recycling line of the UASB reactor on day 47.8. The anode chamber was filled with tap water. The conductivity in EC1 increased after 3 days from 0.95 to 19.60 mS/cm. The maximum current achieved was 19.72 A/m^2, which decreased to 11.00 and 8.20 A/m^2 before the AEM was replaced on day 55. Acetic, butyric and caproic acid rapidly

increased, this concentration six days after connecting EC1 amounted to 4.07, 5.01 and 2.42 g/L for, respectively, acetic, butyric and caproic acid. Propionic and valeric acid were also detected at 0.48 and 1.03 g/L, respectively. The AEM was replaced and the EC1 connected again on day 55. After fresh water was continuously added to the anode chamber the VFA concentration increased slower (***Figure 7.9a***). The conductivity in the anode chamber in EC2 achieved quickly 11.81 mS/cm on day 50.8, from this day until the fresh water was continuously added to the anode chamber (day 56.8), the conductivity was almost constant corresponding to 10.35 (± 0.68) A/m^2. The maximum VFA concentration was achieved on day 56.8, with the primary compound being acetic acid, followed by butyric and caproic acid at concentrations of 10.98 g/L, 5.10 g/L and 3.04 g/L, respectively. Small amounts of valeric and propionic acid were produced at 0.68 and 0.47 g/L, respectively.

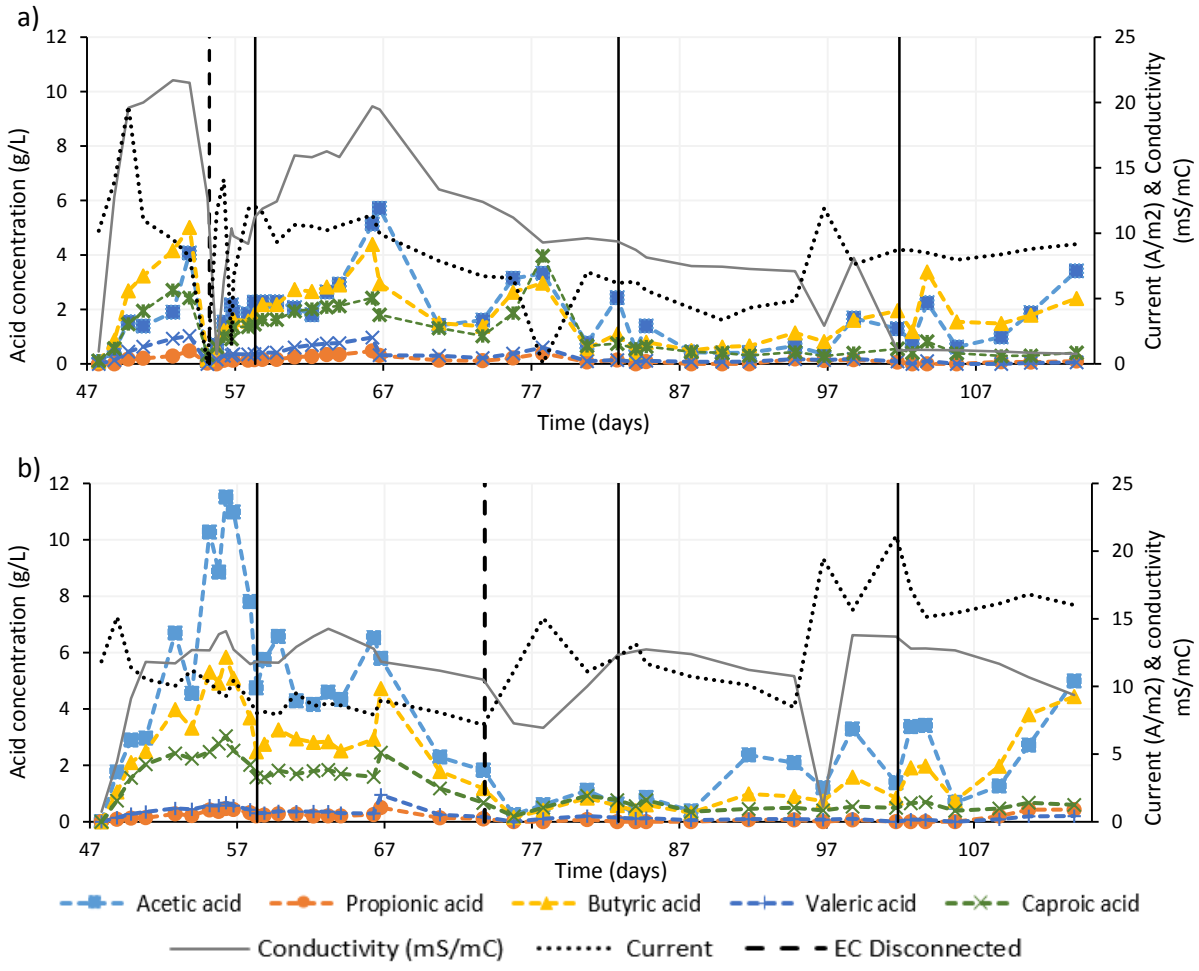

Figure 7.9 *Electrochemical cell performance for the recovery of the volatile fatty acids from the fermentation broth a) EC1 and b) EC2*

During Phase IV, in R1, the acetic acid concentration decreased for the first five days of Phase IV to 1.8 g/L, after which, the concentration increased to 5.7 g/L on day 67. Butyric and caproic acid achieved the highest concentration before decreasing the HRT at 4.39 and 2.07 g/L respectively. On day 78, caproic acid became the major acid in the anode chamber, and the current almost ceased. After this day all the VFAs, and acetic and butyric acid concentrations slightly increased by the end of

Phase IV. In the case of EC2, the highest concentration was also achieved before reducing the HRT. Acetic acid was the major compound with a concentration of 6.53 g/L, followed by butyric and propionic acid that reached 4.73 and 2.45 g/L, respectively. Then, the AEM was replaced in EC2 and fresh tap water was added to the anode chamber. The concentration of the acetic, butyric and propionic acid slightly increased in the anode chamber by the end of Phase IV.

Phase V resulted in the lowest VFA accumulation in the anode chamber. The VFA concentration in EC1 was almost constant until day 94.8 when the butyric acid concentration started to increase, followed by acetic acid (***Figure 7.9a***). In EC2 acetic acid was the major acid, and the butyric acid concentration was lower. In both EC units the current largely increased at the same time that the conductivity almost dropped to 2.92 for EC1, and 1.11 mS/cm for EC2 on day 97. This corresponded with the lowest acidification period in both reactors. In the final Phase VI, the current was almost constant for EC1 and EC2, with EC2 higher than EC1 (***Figure 7.9***). On the other hand, the conductivity was similar and decreasing slightly for both systems.

7.4 Discussion

7.4.1 Fermentation performance

The use of a simple carbohydrate (glucose) as the substrate for the fermentation step was proposed in order to obtain a fast conversion of the carbohydrates into VFAs. The glucose fed UASB system gave high concentrations of VFAs as a hydrolytic step was not required. This represents an advantage over more complex carbohydrates such as cellulose, where hydrolysis is the limiting step for VFA production (*Sieger and Bank, 2005*). Other systems to achieve a similar VFA concentration required a longer HRT, from 10 to 49 days (Li et al., 2014; Nzeteu et al., 2018; Xu et al., 2020). On the other hand, some systems work with a high volumetric production by using a lower concentration of easy biodegradable carbohydrates (up to 15 g/L) and lower HRT than the present study, achieving high volumetric production of total VFAs up to 72.0 and 56.0 g/(L·day), with a lower concentration (6 – 7 g/L) than in the present study (Pugazhendhi et al., 2017; de Menezes and Silva, 2019). The main VFA produced during those studies were acetic and butyric acid in low concentration, i.e. 1.50 g acetic acid/L and 4.10 g butyric acid/L (de Menezes and Silva, 2019); and, 1.20 g acetic acid/L and 3.80 g butyric acid/L (Pugazhendhi et al., 2017). These concentrations are lower than the VFA concentration achieved in the present work. Moreover, there was no production of caproic acid in these studies.

The loop in the effluent line was added as the gas started to leave the reactor through the effluent line instead of the outlet gas line when the water column reached 40 mL. Thus, the loop has slightly increased the gas pressure inside of the reactor. Caproic acid is produced by chain elongation that is favoured by high hydrogen pressures, using lactic acid or ethanol as electron donors and butyric acid

as electron acceptors (Spirito et al., 2014; Nzeteu et al., 2018; Baleeiro et al., 2021). At the end of Phase I, caproic acid started to increase in both systems, however during Phase II its concentration decreased, indicating that the increase in OLR may have resulted in a decrease in the caproic acid concentration, with larger amounts of acetic and butyric acid produced. This is in agreement with previous studies where increased OLRs resulted in a higher production of acetic and butyric acid at the expense of caproic acid (Pugazhendhi et al., 2017; de Menezes and Silva, 2019). In Phase III, the concentration of acetic and butyric acid decreased in the fermentation broth due to the connection of the EC. In Phase IV, the OLR was increased from 15 to 30 g glucose/L/day and the caproic acid concentration increased in both systems. The highest caproic acid concentration was achieved during this phase in R1 (6.04 ± 0.02 g/L). In the subsequent phases, increasing the OLR resulted in a lower production of caproic acid and higher production of butyric and acetic acid. It is possible that the increase in OLR inhibited the elongation process for the caproic production, or caproic acid production was restricted by the absence of hydrogen. The increase of the OLR resulted in a larger amount of carbon dioxide produced, the increase of the carbon dioxide in the total gas fraction would have resulted in a higher activity of the methanogens (Xu et al., 2021). As well, the carbon dioxide could drag the hydrogen in the liquid phase decreasing the hydrogen partial pressure in the liquid. The EC would have alleviated this effect by increasing the partial hydrogen pressure in the liquid fraction, favouring the chain elongation and preventing the methanogenesis.

In both systems, there was methane present throughout the trial despite the rather low pH (5.7). Acetoclastic and hydrogenotrophic methanogens can be active at pH's as low as 5.5, or even, in the case of hydrogenotrophs, as low as 4.8 (Baleeiro et al., 2021). Hydrogen levels in both systems were minimal, even though hydrogen was produced in the cathode chamber. Thus, all hydrogen was consumed for methane production or chain elongation. Chain elongation could explain the higher caproic acid production and lower gas yield in R1 than R2. A higher hydrogen pressure may have triggered the production of butyric acid rather than acetic acid (Saady, 2013). At the same time, if this partial pressure of hydrogen cannot be reduced by methanogens, acetic acid production would be inhibited (Siriwongrungson et al., 2007). The higher conversion yield of the glucose to hydrogen and carbon dioxide in R2 (***Table 7.3***) could be explained by the total VFA production and its profile. The total VFA concentration in the fermentation broth for R1 and R2 was similar at the beginning of the trial, but R1 presented a higher VFA concentration from Phase III onwards (***Table 7.3***). In the case of Phase V, the decrease of VFA during this phase can be attributed to an inefficient glucose consumption and lactic acid production. For the butyric and caproic acid concentration, these achieved a higher average concentration in R1 than R2. Meanwhile acetic acid was the major compound in R2. Acetic acid production is related with a larger gas production (Saady et al., 2013), meanwhile caproic acid

production is made via gas scavenging (Spirito et al., 2014). Therefore, the higher acetic acid and lower caproic production in R2 resulted in a larger amount of gas with a higher conversion of glucose to carbon dioxide and methane (***Figure 7.8***).

Further optimisation during the fermentation could be done by decreasing the pH to reduce methanogenesis. As well, lowering the temperature or increase the pressure of the headspace can be considered to increase the hydrogen solubility. Finally, using a real liquid hydrolysate as the liquid fraction rich in carbohydrates obtained in Chapter 6 should be carried out to evaluate the stability and efficiency of the process.

7.4.2 Volatile fatty acids

The increase in the current during the VFA recovery from the synthetic broth did not follow a linear trend (***Figure 7.6***). The increase of the recovery rate was less effective when increasing the current after 10 A/m^2. The highest voltage (3.5 V) led to the highest conductivity difference between the cathode and the anode chamber (***Figure 7.5a&b***). This could have led to a high osmotic pressure which triggered the migration of water from the cathode to the anode (***Figure 7.4***). Acetic acid presented the highest recovery rate at all voltages applied. Furthermore, the increase of the current resulted in a larger increase of the recovery rate for acetic acid than in the case of the butyric or propionic acid (***Figure 7.6***). This could be as a result of the shorter chain of acetic acid and a higher concentration in the synthetic broth. In the case of butyric acid, the higher recovery rate would have been as a result of the larger butyric concentration than that of propionic acid. However, further optimization of the EC is required to determine the optimal current and recovery rate for combinations of different currents with different VFA concentrations.

It was expected that acetic acid would be the main fermentation product in the anode side as this presented the highest recovery rate during the EC characterisation. However, during some periods when the butyric acid concentration was slightly higher in the fermentation broth, butyric acid was the major extracted compound in the anode chamber, i.e. in R1 on days 48 to 54 , from days 60 to 69 and from days 87 to day 110.7 (***Figure 7.9a***). In the case of the anode chamber in EC2, butyric acid only became the highest extracted compound during Phase VI, when the difference between butyric and acetic acid in the broth was above 13 g/L. The main difference between the two EC systems was the current, which was higher in EC2. Working with similar concentrations of acetic and butyric acid and a low current (6.1 ± 1.4 A/m^2) during the EC characterisation resulted in similar recovery rates of 141.95 (± 32.45) g acetic acid/L/day and 117.9 (± 43.4) g butyric acid/L/day. This difference increased when using a higher current (***Figure 7.6***). Thus, in the case of EC1 the lower current in the system led to a higher effect on the recovery rate driven by the acid concentration in the fermentation broth.

The largest caproic acid concentration in the anode chamber for EC1 was in Phase IV, where caproic acid was the major fermented compound in R1 with a concentration of about 3.96 g/L on day 77.8 (***Figure 7.9a***). On the previous days in R1, caproic acid achieved the highest concentration in the fermentation broth (6.04 ± 0.02 g/L). During this period in Phase IV the current amounted to 7.1 (± 0.6) A/m^2 and the HRT of the anode was 3 days. However, a higher recovery of caproic acid took place in EC1 and EC2 during Phase III. The caproic acid concentration in the anode chamber of EC1 was 2.5 times higher than the concentration inside the fermentation broth, with the EC current corresponding to 9.6 (± 1.1) A/m^2 and the HRT of the anode was 5 days. During this period, the current in EC2 was similar (9.8 ± 0.5 A/m^2). However, even with a lower caproic acid concentration in the fermentation broth (0.7 ± 0.1 g/L), the concentration in the anode chamber was higher in EC2 than EC1 (3.8 times higher than in the fermentation broth). This corresponded with the period where EC2 achieved the highest VFA recovery. This represents a higher recovery using EC, than a similar system used by Xu et al. (2020), who were able to increase the acetic, butyric and caproic acid concentration by 1.76, 1.26 and 1.51 times. The only difference between their system and the present work was the cathode: they used a titanium mesh (Xu et al., 2020), whereas in the present study the titanium mesh was coated with IrO_2. In addition, Xu et al. (2020) tested an electrodialysis/phase separator cell and a pertraction system (membrane-based liquid-liquid extraction). The main aim of their work was to reach a concentration of undissociated caproic acid higher than 10.9 g/L that resulted in a second liquid phase (Xu et al., 2020). A lot of work has been done for chain elongation and the recovery of caproic acid, with petraction systems as the most common recovery system, where the driving force for the extraction is mainly due to the pH gradient (Xu et al., 2021). The advantage of the system in the present chapter is the high VFA concentration obtained in the UASB reactor, enhancing the driving force for the VFA extraction.

The efficieny of VFA recovery using liquid-liquid extraction systems increases exponentially when the pH is lower than the pKa (Saboe et al., 2018; Kaur et al., 2020). The EC coupled to the recycling line of the EGSB reactor allowed the concentration of VFAs in the anode chamber and consequent reduction of the pH. This prevents the contamination of the chamber, but the major advantage is the use of an organic phase for the recovery and fractionation of VFAs by liquid-liquid extraction. Indeed, this technique has been broadly considered for the recovery of VFAs (Spirito et al., 2014; Saboe et al., 2018; Kaur et al., 2020). However, as the last approach relies on the undissociated form of the acids and the hydrophobicity of the molecules, the VFA with lower numbers of carbons result in a low recovery efficiency as the alkyl length of the VFA is the shortest, i.e. acetic acid (Rocha et al., 2017). In the present study the anode chamber had a pH ranging between 1.2 and 1.8, thus, VFAs would be in their undissociated form largely enhancing the efficiency for the VFA extraction using an organic phase

(Kaur et al., 2020). For example, using the highest VFA concentration achieved in the anode chamber during the present study, with the graphs for the equilibrium concentration of VFAs in the organic phase at pH 3.0 from Saboe et al. (2018), the estimated concentration of VFAs in the organic fraction would be about 40.4, 50.98 and 39.10 g/L for acetic, butyric and caproic acid, respectively.

The coupled UASB-EC system showed promising results, as good recovery of the VFAs from the fermentation broth, decrease of the alkaline demand and the production of hydrogen was beneficial for chain elongation. However, the EC was not able to recover the largest fraction of VFAs from the fermentation broth, which is likely related with an under dimensioning of the unit. Thus, further optimisation is required to optimise the VFA recovery process. For this, a deeper characterisation by combining different currents and acid concentrations is required to determine the optimal combination for the recovery. As well, a deeper understanding of the relation between the current and the conductivity to determine the possible saturation of the system. For example, if the conductivity is still constant but voltage decreases, as happened at the beginning of the trial using the lowest voltages (1.5 and 2.0 V). Also, this effect was observed during the simultaneous production and recovery of the VFAs, when on day 88 the current decreased to 0.04 A/m^2 when the highest caproic acid was achieved. Then, the current increased in the subsequent days by the continuous addition of fresh water in the anode chamber that lowered the VFA concentration. Thus, it is necessary to determine any possible saturation effect related to the VFA concentration in the anode chamber and the required HRT to avoid such a problem.

7.5 Conclusion

This study was designed as a proof of concept to demonstrate the potential for VFA recovery by increasing their concentration in the fermentation broth with a larger volumetric production rate and recovery by coupling an EC to the recycling line of a UASB reactor. This was possible by using a high rate reactor with good biomass retention and a high feedstock concentration of a simple carbohydrate (glucose). The major fermented compounds were acetic acid, butyric acid and caproic acid. The EC was able to concentrate the VFAs up to 4.6 times in the anode chamber. The system shows some promising advantages as it allows VFA production under optimal conditions and the recovery of VFAs in their easier to recover (undissociated) form. At the same time, the EC contributed to increases in the hydrogen partial pressure and decreases in alkaline requirement.

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Chapter 8

General discussion and future perspectives

8.1 Key findings

The pillar for the design of this thesis was the development of a biorefinery in Ireland using a common industrial by-product. Brewery spent grain was selected due to its composition and as an abundant feedstock in Ireland with low value, with a production above 170,000 tons in 2019 (***Chapter 2***). The main objective was the production of the volatile fatty acids using brewery spent grain as a feedstock. The initial approach for simultaneous hydrolysis and acidogenic fermentation for volatile fatty acid production evolved to a more complex process in view of the high valuable compounds that are contained in the brewery spent grain, including the carbohydrates, proteins and lignin. ***Figure 8.1*** gives the main findings of this PhD thesis and the connections between the different chapters of the thesis.

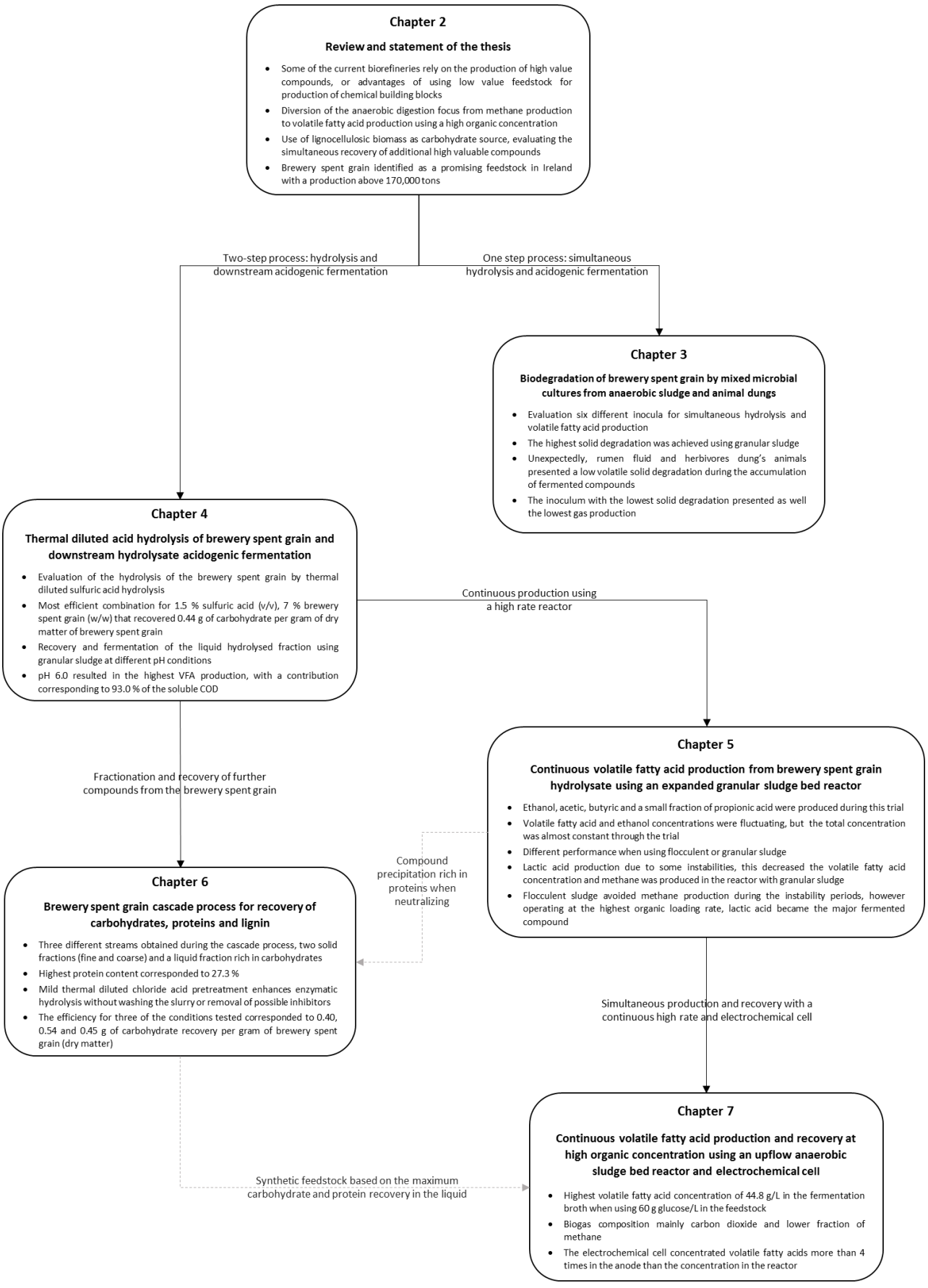

Figure 8.1 *Key findings from the different chapters, development and relation between chapters of this PhD thesis*

8.2 Core findings and future recommendations

During the introduction of this thesis (***Chapter 1***) it is was mentioned that the current definition of a biorefinery should exclude the classification "Crop biorefinery" and "Residue biorefinery". The residues should be characterised by their composition and the required pretreatment for their valorisation. Then, the biorefinery should be classified by the process technology and the platform. For example, in the case of the BSG the process technology would involve chemical and biochemical processing to obtain three different streams, each of them aiming for different platforms: i) carbohydrate platform, ii) protein platform and iii) lignin platform. On the other hand, some of the current biorefineries for production of biogas or ethanol rely on the fees that companies pay for the disposal of the wastes. However, as the valorisation of wastes and the closing of the circular economy is improving, these wastes will be regarded as valuable resources instead of nuisance material for disposal.

The strength of the biorefinery should be focused on the production of a different range of final products during the processing of the source used as feedstock. This could give extra capacities to the biorefinery plant for diverting the process based on the market demand to increase the profits of the plant. Such biorefineries should operate as a hub centre to treat products with similar initial characteristics, increasing their production capacity and achieving the minimum volume required for the feasibility of the plant. For example, the approach for the protein recovery as applied in ***Chapter 6*** would have led in a liquid fraction rich in carbohydrates. These proteins could be used in the food industry, meanwhile the liquid could be used inside of a carbohydrate platform in combination with other liquors with similar composition. However, more work is required at lab scale to test the combination of different waste streams. The different technologies should be carefully evaluated for each of the different feedstock and wastes, as highlighted in ***Chapter 2***. Some of the unsuccessful industrial cases were related with using a technology developed for a similar feedstock that did not perform as well as expected. For instance, this was the case of unexpected difficulties when treating corn stove instead of wood chips (Slupska and Bushong, 2019).

The first approach followed during the experimental work of this PhD thesis was the simultaneous hydrolysis and fermentation of the brewery spent grain in ***Chapter 3***. As BSG is a lignocellulosic material, not all microorganisms are able to degrade it. Thus, the chapter focused on evaluating six different inocula with a microbial consortium potentially capable to degrade and accumulate fermented compounds. The solid degradation by the rumen fluid and manure from herbivore animals were expected to achieve the higher performance. Surprisingly, rumen fluid presented the highest initial destruction rate, but it almost ceased after 2 days. The highest solid degradation and gas

production was achieved by granular sludge, which presented the lowest hydrolytic activity. In addition, giraffe manure presented an unusual trend in comparison with the other sludge, as the solid degradation was still ongoing by the end of the trial after 30 days. In this case, the giraffe manure initially had VFA accumulation, however by day 10 these were being consumed. This improvement for the solid degradation during the middle of the trial could be related to an improvement in the efficiency for the methanogenization of the fermented compounds. Thus, one of the finding of this chapter was the association between the increase in the VFA concentration and the decrease of the solids degradation. The hypothesis developed during ***Chapter 3*** is that VFAs affect or even completely inhibit the hydrolytic degradation of the lignocellulosic feedstock. Therefore, further studies to determine the type of inhibition is important. Biodegradation activity tests should be carried out using granular sludge, with and without chemical inhibition of the methanogenesis, under different initial VFA concentrations. Further research is required the obtained the results to calculate the kinetic and stoichiometric parameters of the lignocellulose feedstock degradation. For example, the Lineweaver-Burk plot can be used to obtain the maximum rate and the Michaelis constant to evaluate any possible inhibition of the lignocellulose by VFAs.

The second approach was based on a two-step process developed during ***Chapter 4***. First, the hydrolysis of the BSG, and the recovery of the liquid fraction for the acidogenic fermentation was investigated using batch stirring tank reactors. The efficiency of the hydrolysis was determined by the ratio of the total amount of the carbohydrates recovered in the liquid fraction per gram of BSG dry matter. The most efficient was using 7 % of BSG (w/w) with a 1.5 % of sulfuric acid concentration (v/v). Increasing the concentration of BSG resulted in a lower carbohydrate efficiency recovery. Similarly, a higher amount of sulfuric acid did not result in a better extraction as well. The remaining solid was optimised in a later part of the PhD thesis, which was carried out with some changes during ***Chapter 6***. The recovered liquid from the hydrolysis was then fermented at different pH values using batch reactors. The fastest production rate was achieved at pH 5.0, although the highest acidification yield achieved at pH 6.0. Operating the system at a pH lower than 4.5 resulted in an inefficient consumption of the carbohydrates as the pentose sugars were not consumed (arabinose and xylose).

The thermal diluted sulfuric acid hydrolysis and downstream acidogenic fermentation of the brewery spent grain showed a high efficiency during the batch experiments. Therefore, the next step was the continuous fermentation of the liquid hydrolysate. This was carried out in ***Chapter 5*** using a continuous high rate system. During this work and the previous trial (***Chapter 5*** and ***Chapter 4***) no pretreatment of the biomass was carried out as the operational conditions were expected to inhibit the methanogenesis. In the previous batch experiments (***Chapter 4***), gas production and gas composition were not analysed. In the case of the continuous production, two twin reactors were

running, one of them with the intact granular sludge, and in the other, the granules were disaggregated to compare the operational pressure conditions. The best performance was using granular sludge, in this case methane production was reductant, and was only detected during some instability periods when VFA concentrations decreased. Even, the produced liquid hydrolysate in ***Chapter 4*** and ***Chapter 6*** contains a mixture of hexose (glucose) and pentose (xylose and arabinose), which was fully consumed as demonstrated in ***Chapter 4*** and ***Chapter 5***.

During the preparation of the feedstock, when using the batch reactors in ***Chapter 4*** the liquid fraction after the thermal diluted acid hydrolysis was neutralised inside the reactors. However, in the continuous VFA production, the liquid hydrolysate was neutralised in a bottle. This neutralisation led into the precipitation of a solid fraction. It was hypothesised that this fraction could be the precipitation of some organic compounds, such as proteins and salts formed by the sulfuric acid during the hydrolysis and the sodium hydroxide during the neutralisation step. The sample had a high content of inorganic matter, 52.1 (± 5.6) % of non-volatile solids, which was a result of using the sulfuric acid and sodium hydroxide during the hydrolysis and neutralisation steps (***Chapter 5***). In addition, a sample of this solid was used to determine its protein content, which corresponded to 37 %. Thus, above 75 % of the volatile solids corresponded to proteins. A deeper study of the hydrolysis process was subsequently developed during ***Chapter 6*** to enhance the protein recovery at the same time of the carbohydrate release.

The holistic processing of the BSG was tackled by a cascade process in ***Chapter 6***. The main goal of this chapter, beside the optimal carbohydrate release, was to understand how the different pretreatment combinations affect the composition of the different extracted fractions. In this case, enzymes were used for further improve the hydrolysis. To target higher value markets, such as food, cosmetics or pharmaceuticals, hydrochloric acid was used instead of sulfuric acid and the enzymatic cocktail was food quality grade. Different combinations of time, acid and BSG concentrations were applied. To determine the extent of the hydrolysis, additional techniques could be used instead of the lignocellulosic characterisation by the common degradation of the material by subsequent chemical hydrolysis, for example nuclear magnetic resonance spectroscopy (NMR) or fourier-transform infrared spectroscopy (FTIR) (Bevilacqua et al., 2017; Guo et al., 2018). This could be used to determine the structure and the crystallinity of the BSG in a faster and easier way. Additionally, it was not possible to try a BSG concentration higher than 10 % as the blender was not able to manage it. The physical disruption of the BSG resulted in a higher water capacity adsorption, thus the liquid in the mixture decreased, making it impossible to blend the BSG. This happened during ***Chapter 4*** and ***Chapter 6***. Some other physical treatments for wet material should be considered in order to increase the BSG fraction, which would be as well evaluated for the release of the protein, lignin and carbohydrate fractions.

During this work, three different conditions achieved a large release of the carbohydrates. Although in one of the conditions, the carbohydrates were mainly released during the thermal diluted hydrochloric acid pretreatment (Condition 2). In the case of the conditions where enzymatic hydrolysis enhanced the release of the carbohydrates (Conditions 1 and 4), only one concentration of enzymes was evaluated. Further work should be done to determine the optimal dose of enzymes in order to avoid an overuse that would lead in an unnecessary extra cost.

The final stage of this PhD thesis was the continuous production and recovery of the VFAs. A novel process using an electrochemical cell directly coupled to the recirculation of the expanded granular sludge bed reactor was tested in ***Chapter 7***. This was a proof of concept to demonstrate the feasibility of the process. The electrochemical cell was able to increase the VFA concentration in the anode chamber more than 4 times than the current concentration in the fermentation broth. In addition, the electrolysis process resulted in a lower sodium hydroxide consumption and the production of hydrogen. Even, the hydrogen was completely consumed for chain elongation and methane production. Nevertheless, further studies are required as the electrochemical cell was under dimensioned, which would not only enhance the VFA recovery but also the chain elongation. Further optimisation of the process could be done by evaluating the decrease of the pH that would reduce the alkaline consumption and could also reduce the methane production. Using a lower temperature in the reactor, i.e. 30, 25 or 20 °C, could increase the solubility of the hydrogen.

8.3 Future developments

8.3.1 Brewery spent grain biorefinery

The obtained results are encouraging as foundation for the scaling up process and practical implication of a biorefinery using BSG as feedstock. The BSG could be used for the production of two solid fractions of food grade quality, VFAs and syngas production (***Figure 8.2***), and the remaining liquid fraction as potential feedstock for biogas and fertilizer production. This process is be divided in **i)** upstream process and **ii)** downstream process:

i) The upstream process would be focus on the production of the three different streams: the coarse and fine solids, and the liquid fraction rich in carbohydrates. The fine solids have a texture similar to dough, their content on lignin and possible antioxidant and probiotic activity must be analysed. As well, the organoleptic properties should be determined as food additive, or even for use in the pharmaceutical or cosmetic industry. The coarse solids are composed by the remaining husk and other larger fractions of the BSG. In the same way, the characterisation of the coarse solids must be carried out to target some of the different industrial markets, with a lower market value, for instance, as animal food supplement.

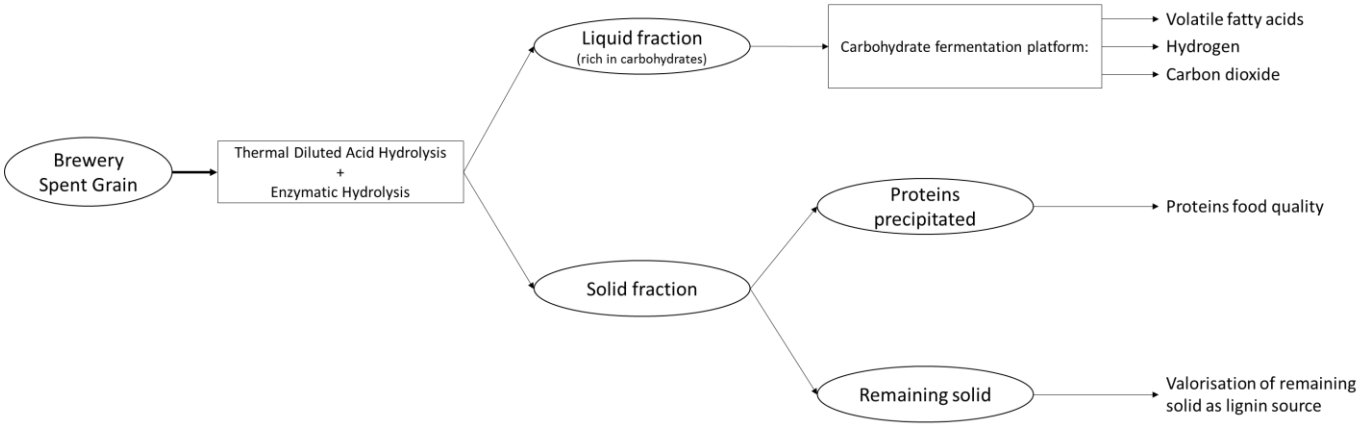

Figure 8.2 Proposed diagram for a brewery spent grain biorefinery developed under the current PhD thesis. The brewery spent grain is chemically pretreated prior enzymatic hydrolysis, after two different solid fractions are obtained. The liquid fraction is used in a downstream process for volatile fatty acid production, with biogas as by-product

ii) The downstream process is focused on the use of the liquid fraction for production of high valuable products. This liquid is rich in carbohydrates consisting in a mix of pentoses and hexoses. Therefore, this liquid could be integrated inside of a biorefinery carbohydrate platform, even combining with other liquids with similar characteristics. The current PhD thesis proposed the use of this easily biodegradable carbohydrates for the production of the VFAs. Thus, during the downstream fermentation process the main final product would be the VFAs, gas (CO_2 and H_2) as by-product and remaining liquid for biogas (CH_4 and CO_2) production or/and as a fertilizer.

8.3.2 Simultaneous volatile fatty acid production and recovery

The next step for the simultaneous volatile fatty acid production and recovery is the fractionation of each of the individual VFAs as a final product, i.e. acetic acid, butyric acid and caproic acid. The system developed during this PhD thesis using a higher rate reactor (expanded granular sludge bed reactor) coupled with the electrochemical cell to increase the VFA concentration could be integrated with a recovery system. For example, the liquid-liquid recovery and distillation system proposed by Saboe et al. (2018) could achieve high extraction yields of the VFAs from the cathode chamber due to the low pH and the quality of the phase, only anions can cross the anion exchange membrane (***Figure 8.3***). The organic phase used would be recovered by distillation and the individual VFAs recovered as final products. The systems should be evaluated in terms of energy consumption, for example the electricity consumed by the electrochemical cell and the energy in the distillation column. On the other hand, there are some parameters that could counterbalance and underpin the feasibility of the process, for example the lower required alkaline chemical addition, the production of a higher value compound (caproic favoured above acetic), and the remaining organic fraction for biogas production that could power the distillation unit. All these parameters would be used to evaluate the economic feasibility of a pilot plant for the production and fractionation of each of the VFAs as final products.

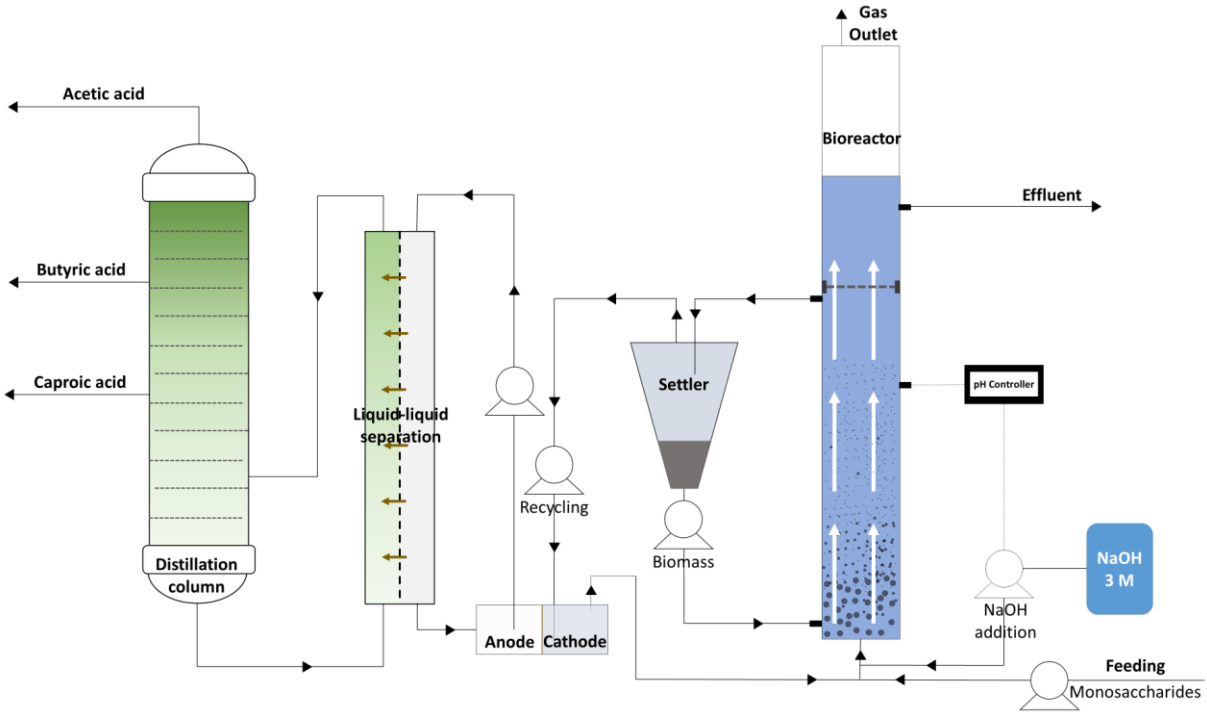

Figure 8.3 *Continuous system for simultaneous production, recovery and fractionation of volatile fatty acids. The easily biodegradable carbohydrates from brewery spent grain (**Chapter 6**) are fed into the expanded granular sludge bed reactor (**Chapter 5**). The recycling line is connected to a settler to decrease and return the solid particles and biomass into the reactor, the clearer liquid is coupled within an electrochemical cell for recovery of the volatile fatty acids at the anode chamber (**Chapter 5**). The anode, with low pH, is connected in a closed loop with a liquid-liquid recovery system using an organic phase to extract the volatile fatty acids. Finally, the organic phase is boiled in a distillation column for the fractionation of each of the individual volatile fatty acids and the organic phase is recovered. The unit for simultaneous volatile fatty acid production and recovery, bioreactor and electrochemical cell, has been developed during this PhD thesis. The liquid-liquid separation and the distillation column has been adapted from Saboe et al. (2018)*

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Supplementary information Chapter 3

S3.1 Methods

S3.1.1 Nucleic acid extractions

DNA and RNA were co-extracted from 0.75g (fresh weight) of the retentate (***Figure 1***), from sample days 0 and 21 of each of the three biological replicates from the 6 inoculum types (n=36 extractions) using the CTAB-phenol chloroform method of Griffiths *et al*. (2000). The resulting nucleic acid pellet was eluted in nuclease free water. To investigate the active fraction present on day 21, an aliquot of eluted pellet was DNase treated with Turbo DNase (Ambion) following the manufacturer's instructions to isolate the RNA. PCR with universal prokaryotic primers 515F/806R (Caporaso et al., 2012) was used to verify total removal of genomic DNA, checking for the absence of PCR product (at a series of dilutions). The generation of cDNA was performed using SuperScript® III Reverse Transcriptase (Invitrogen), following the manufacturer's recommended guidelines, on a normalised concentration of RNA (10 ng μL^{-1}). DNA was normalised to 50 ng μL^{-1} and samples were shipped on dry ice to the RTL sequencing facility (Lubbock, Texas, US). Amplicon library preparation was performed using the Parada *et al*. (2016) version of primers 515f/806r targeting the archaeal/bacterial V4 16S rRNA region, followed by addition of sequencing adapters and dual-index barcodes, using a limited number of PCR cycles as per the Illumina protocol (Illumina, 2013). Paired-end sequencing was performed using the Illumina MiSeq platform with 2 x 300bp V3 kit chemistry to a minimum depth of 10K.

S3.1.2 Sequence analysis

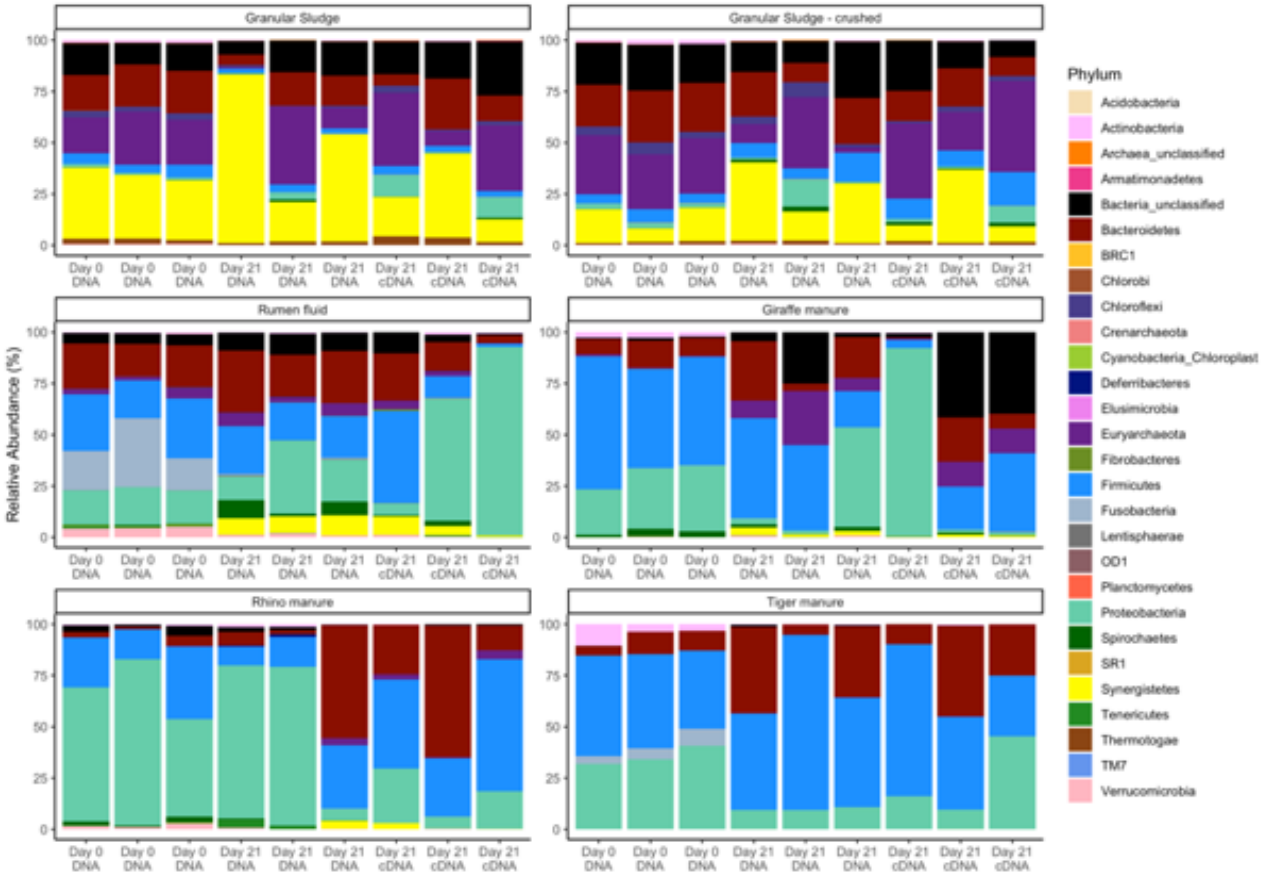
Mothur (version v.1.44.3) was used to process of paired-end 16S rRNA reads (Schloss et al., 2009) following the pipeline detailed in Kozich *et al*. (2013). The SILVA database (v132), trimmed to the V4 region, was used for alignment of sequences and the Ribosomal Database (v.9) was used for classification. Samples contained an average of 19,200 reads after quality filtering. The dataset was normalised for subsequent analyses by subsampling to the lowest number of sequences. Distance matrices were created in Mothur using the Theta Yue Clayton metric for the three subsets of data (DNA day 0; DNA day 21 and cDNA day 21) which were subsequently ordinated using nMDS. Clustering of samples as a function of inoculum source was tested for statistical significance using analysis of molecular variance, and Spearman correlations of experimental variables with nMDS axes were tested using the corr.axes function in Mothur. Differentially abundant OTUs were detected with LEfSe analysis (Segata et al., 2011). Data visualisation was performed in R (R Core Team, 2019) using the ggplot2 package (Wickham, 2011). Statistically significant differences in means as a function of inoculum were tested for using the non-parametric Kruskal-Wallis test. Where statistical differences were seen ($p < 0.01$), a post-hoc test was performed using Fisher's Least Significant Difference (LSD)

and these statistical tests were performed using the agricolae package (de Mendiburu and Yaseen, 2011).

S3.2 Microbial community analyses of the inocula

S3.2.1 Comparison of the microbial composition of the inocula

Initial prokaryotic microbial populations within the manure samples (DNA day 0) were dominated by relatively few phyla, namely the *Firmicutes, Proteobacteria* and *Bacteroidetes*, and this was particularly the case for the tiger manure (***Supplementary Figure 1***). The same phyla were present in rumen fluid samples, in addition to a significant contribution from the *Fusobacteria*. As expected, no large differences were seen between crushed and un-crushed granular sludge, where both differed considerably from the inocula of animal origin, with dominant phyla from the *Synergistetes, Euryarchaea* and *Bacteroidetes*. Additionally, a higher proportion of unclassified bacteria were present within these sludge samples.



Supplementary Figure 1 *Relative abundances of OTUs assigned to the level of phylum, in each of the 6 inocula used for BSG degradation. Nine bars are shown for each inocula, where the first three are DNA day 0 samples, and subsequent three from DNA day 21 and the final three from cDNA day 21 samples*

By day 21 in most samples, except tiger manure, a similar trend of increased numbers of dominant phyla at the DNA level was observed, alongside more variability between triplicate samples. Rumen

fluid, giraffe manure and rhinoceros manure experienced an increase in abundance of *Synergistetes* and *Euryarchaeota* which initially (day 0) were present in very low numbers in these samples. The dominant phyla present at the cDNA level followed similar trends to those in their DNA counterparts on day 21.

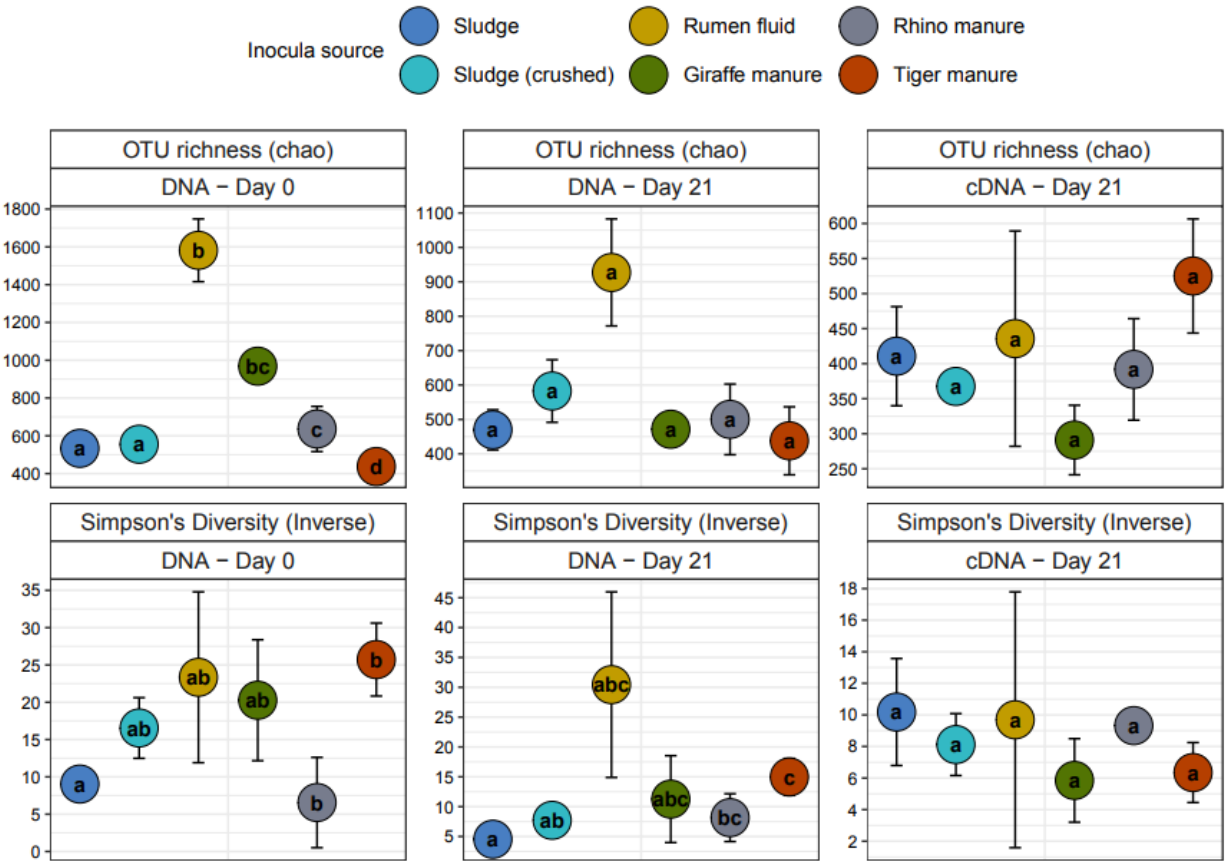
Alpha diversity analysis included sample coverage, which indicated sufficient coverage (> 97%) for all samples except giraffe manure on day 0 (96%) and rumen fluid at the DNA level on both days 0 (93%) and day 21 (96%) (***Supplementary Table 1***). The number of OTUs observed in these samples clearly explained this trend, where rumen fluid contained almost triple, and giraffe manure double, the amount of OTUs detected in other samples (***Supplementary Figure 2***). The rhinoceros manure samples were significantly richer ($p < 0.05$) than the sludge and tiger manure samples. The tiger manure that was the least rich ($p < 0.05$) of all inocula with only 300 OTUs observed.

Supplementary Table 4 *Alpha diversity indices for the six inocula degrading BSG on days 0 (DNA level) and 21 (DNA and cDNA level) data shown is the average of three biological replicates*

Day	Fraction	Inocula	Percent Coverage (Good's)	Observed OTUs	Estimated OTUs (Chao)	Simpson's Evenness	Simpson's Diversity (Inverse)
0	DNA	GS	97.7	340.8	533.7	0.03	9.0
		GS crushed	97.6	363.1	555.4	0.05	16.6
		Rumen fluid	92.6	948.2	1581.7	0.02	23.3
		Giraffe manure	95.7	602.9	968.4	0.03	20.3
		Rhino manure	97.2	384.3	636.1	0.02	6.6
		Tiger manure	98.2	299.2	437.4	0.09	25.7
21	DNA	GS	97.8	312.1	469.8	0.01	4.5
		GS crushed	97.4	346.7	582.6	0.02	7.7
		Rumen fluid	95.9	569.0	927.1	0.05	30.4
		Giraffe manure	98.1	298.8	471.4	0.04	11.3
		Rhino manure	97.8	306.3	500.2	0.03	8.2
		Tiger manure	98.2	276.1	437.9	0.05	15.0
	cDNA	GS	98.3	272.9	410.7	0.04	10.2
		GS crushed	98.5	231.8	367.5	0.03	8.1
		Rumen fluid	98.1	264.3	435.7	0.03	9.7
		Giraffe manure	98.8	184.1	291.0	0.03	5.8
		Rhino manure	98.3	250.9	391.8	0.04	9.3
		Tiger manure	97.7	283.6	525.1	0.02	6.3

While a similar trend in richness was still present on day 21, differences between inocula were no longer statistically significant. From day 0 to 21, a reduction in the number of OTUs detected was seen in the rumen fluid and giraffe manures samples, both of which fell by more than 40%. Rhinoceros manure also saw a reduction, albeit much smaller while granular sludge and manure samples

remained similar or fell very slightly. There were no statistically significant differences between the number of OTUs detected in the active (cDNA) fraction of the six inocula on day 21, and with the exception of tiger manure, fewer OTUs were detected in the cDNA than the DNA fraction. Patterns in richness between inocula generally translated into similar patterns in diversity, except for tiger manure where a higher evenness (more equitably distributed OTUs) resulted in the highest diversity index for these samples on day 0.

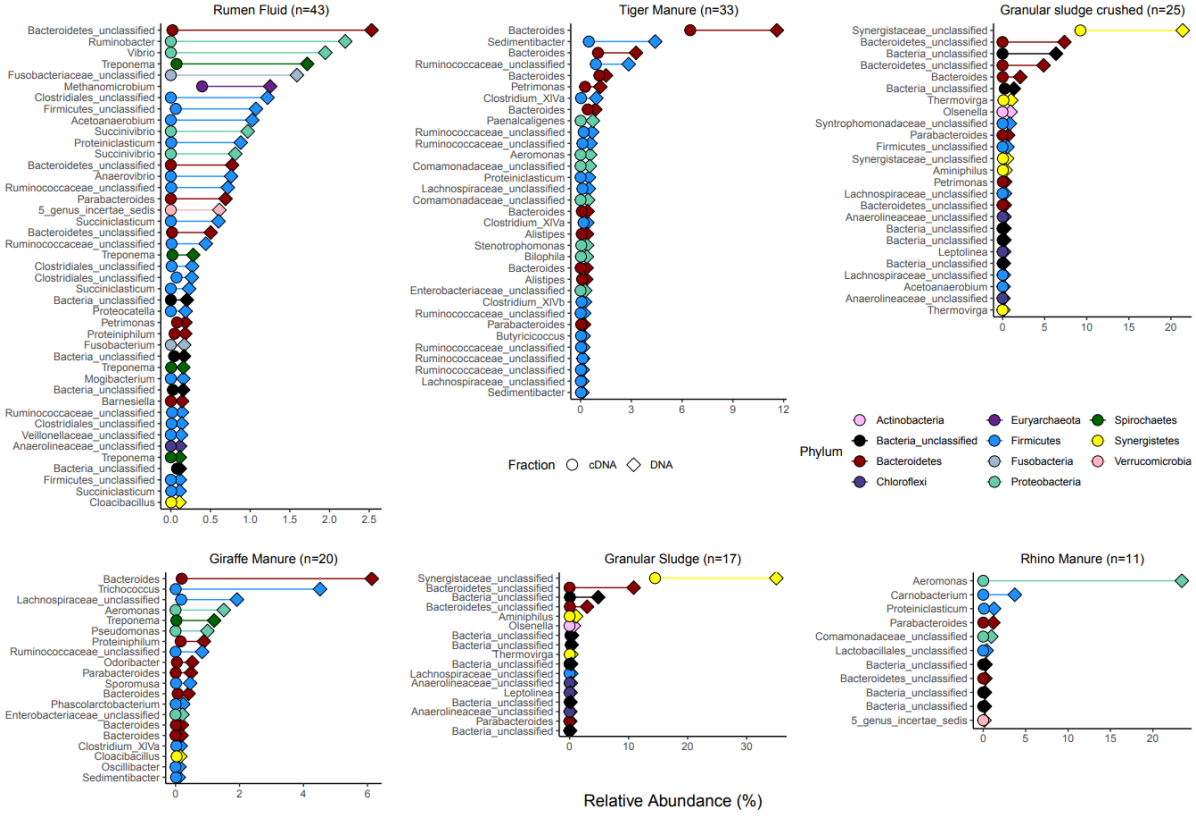


Supplementary Figure 2 Alpha diversity indices of observed OTU richness and Simpson's diversity of BSG degradation by different inocula. Points represent the mean of each inoculum in the three datasets (DNA day 0 and 21 and cDNA day 21) and error bars show standard error of the mean (n=3). Letters within the points illustrate statistical differences as a function of inoculant for each day or fraction, where shared letters denote no difference ($p > 0.05$), and unshared letters denote a statistical difference within a group ($p < 0.05$)

S3.2.2 Presence vs activity of OTUs in each inocula (Day 21)

A differential abundance analysis was performed to detect OTUs that were present (DNA) in significantly higher numbers than they were active (cDNA) with the intention of determining OTUs were perhaps under non-optimum conditions. Rumen fluid samples had more than double the number of lower activity OTUs than the other inocula on day 21, with 100 OTUs present in higher abundance at the DNA level. Rhinoceros manure meanwhile had the fewest number of lower activity OTUs (n=18), while giraffe (38 OTUs) and tiger (51 OTUs) were similar. Lower activity OTUs were

present in crushed granular sludge (40 OTUs) than in its uncrushed counterpart (28 OTUs). Upon investigating the more abundant of these OTUs (> 0.1% at DNA level) (***Supplementary Figure 3***), both rumen fluid and tiger manure samples showed a trend of OTUs from the *Firmicutes* that were most often under-represented in the active fraction, a number of which were unclassified *Ruminococcaceae* and *Lachnospiraceae*. A number of OTUs from the genera *Bacteroides* were identified as lower in activity, particularly so in the giraffe and tiger manure samples. Only one OTU from the methanogenic archaea was identified as significantly lower in abundance in the active (cDNA) fraction: *Methanomicrobium* in the rumen fluid sample.

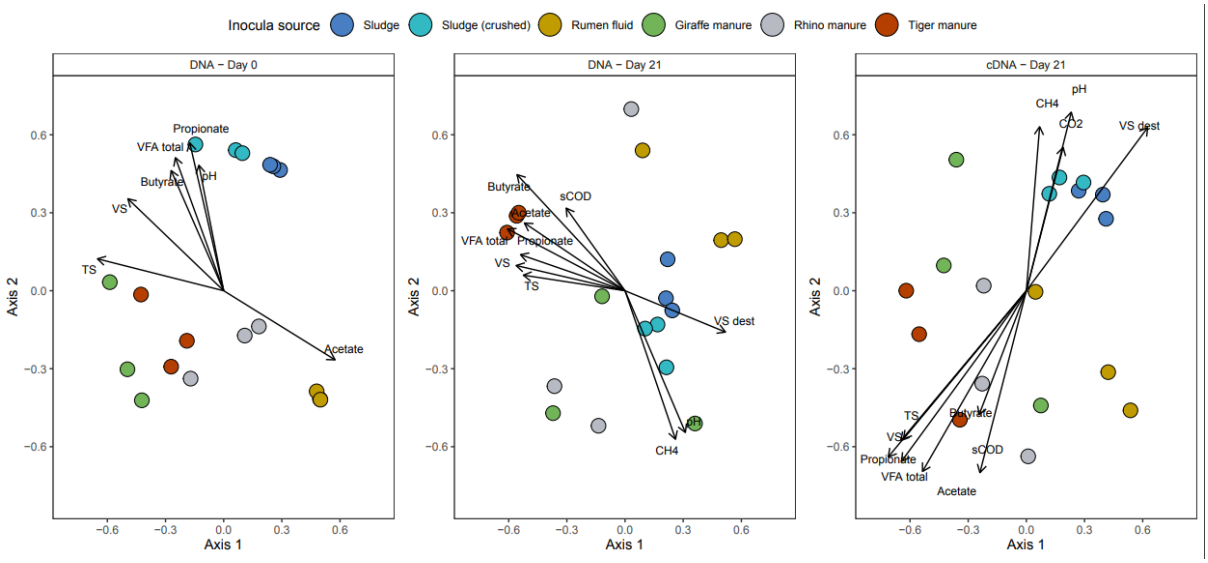


Supplementary Figure 3 *OTUs that were significantly more abundant in the DNA (present) and cDNA (active) fractions from the same samples on day 21 of the trial on BSG degradation with different inocula, as determined using LEfSe analysis. OTUs are labelled by their genera level assignment and coloured by their phylum level assignments. Points depict the average relative abundance in the three biological replicates, with abundance at the DNA level denoted with diamonds and at the cDNA level with circles*

S3.2.3 Differences in microbial community structure and relationships with biodegradation data

Ordination of samples was performed using nMDS of distance matrices made from the three data sub-sets (DNA day 0; DNA day 21 and cDNA day 21), where the stress index was acceptable for all three sub-sets (stress < 0.17). In all 3 datasets, grouping of samples was seen as function of inoculum origin ($p < 0.01$; AMOVA). As expected, over time the variability between biological replicates increased (more spread on DNA day 21 than day 0) except for tiger manure, and the active community

(cDNA) was more variable than the community in the DNA fraction on the same day 21. On day 0, the experimental conditions had the most effect upon the structuring of the granular sludge samples, namely pH and VFA concentrations, except acetate which had a strong effect on the rumen fluid microbial structure (***Supplementary Figure 4***). On day 21, the structure of the microbial community in both the present (DNA) and active (cDNA) fractions of the tiger manure samples were correlated with the concentration of intermediates, namely VFAs and sCOD. Meanwhile methane volume and pH correlated with both granular sludge type communities, in both DNA and cDNA fractions, as well as with select giraffe manure samples in the DNA fraction.



Supplementary Figure 4 *nMDS of DNA sample dissimilarity as calculated with the Yue & Clayton metric. Clustering of samples was tested for statistical significance by analysis of molecular variance (AMOVA). Arrows represent Spearman's rank correlations of experimental variables with a significant impact (p,0.05) on structuring the communities in each of the three datasets*

S3.3 Discussion

S3.3.1 Volatile solids destruction

An evident difference in the microbial community between the sludge samples and animal dungs was the presence of highly abundant OTUs assigned to the phylum of *Synergistetes*. While a relatively recent characterised phylum, members of this clade are often detected in anaerobic environments, and indeed comprise a member of the core microbiome of sludge AD systems proposed by Rivière *et al.* (2009). The *Synergistetes* are most often found in anaerobic systems that are rich in amino acids [46], such as in digesters treating protein-rich dairy wastewater from which our granular sludge was sourced. The majority of *Synergistetes* are unable to use carbohydrates (Jumas-Bilak and Marchanding, 2014), and instead obtain energy by their efficient degradation of amino acids, from which they produce short chain fatty acids and sulfate, which can then be used by methanogens and sulfate reducers (Vartoukian et al., 2007). The high protein content typically seen with this feedstock,

around 27% dry weight (Mathias et al., 2015), would therefore make it an ideal substrate for this phylum and could explain the increase in its relative abundance seen in all inocula, except tiger manure from which it was absent.

S3.3 References

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